UNIVERSITY OF PÉCS

Doctoral School of Chemistry

Dynamics of Isoelectric Focusing

Ph.D. Thesis

Dr. Anna Takácsi-Nagy

Supervisors:

Dr. Ferenc Kilár, Dr. Wolfgang Thormann

Professors

PÉCS, 2017

1 Introduction

Isoelectric focusing (IEF) is recognised as a powerful analytical separation technique that can be applied to separate amphoteric components based on their unique isoelectric points (p*I*). It has become established as a valuable addition to the tools of many researchers engaged in analytical or preparative procedures. This increased demand for the technique engenders a need for suitable calculations in its analysis. The emergence of computers has brought renewed interest in simulations and model programs, which increase our understanding of many areas of separation science research. Before any laboratory work commences, computer simulations allow exploration of the behaviour of a system in an articulated way. This imitation of a real-world process, such the prediction of separation dynamics, formation and stability of the pH gradient, electrophoretic mobilisation, and focusing behaviour of amphoteric compounds shows good qualitative agreement with the practical experiments published in the literature (Thormann and Mosher 1988; Mosher, A., *et al.* 1992; Mosher, Thormann, *et al.* 1989; Mosher, Dewey, *et al.* 1989; Thormann *et al.* 1986).

Simulations are suitable for surveying the entire separation process step by step, and for optimising separation conditions such as the injection protocol, the properties of carrier ampholytes or the electrolyte systems to be applied.

Injection protocol

In usual IEF configurations performed in capillary, sample components and carrier ampholytes are introduced into the separation channel as mixtures. A further development, the sequential injection protocol of Kilár *et al.* (Kilár *et al.* 1998) offers efficient separation of amphoteric compounds that have p*I*s outside the range of the established pH gradient. The separate introduction mode of the ampholyte and sample components into the electrophoretic space, *i.e.* the sandwich (ampholytes/sample/ampholytes) or half-sandwich (ampholyte/sample or sample/ampholyte) sampling strategies, gives unique advantages for analysis and has advantages for MS detection (Páger *et al.* 2011).

Ampholytes, impurities and electrolytes

In natural gradients as described by Svensson (Svensson 1961), an electrical current passes through a mixture of ampholytes establishing the focusing gradient. These natural pH gradients are formed by well-defined amphoteric electrolytes (ampholytes) with high buffering capacity, good conductivity. Carrier ampholytes should also be small molecules having little or no optical

absorbance at wavelengths where proteins typically absorb (Berkelman 2005). The absence of chemical and biological reaction with the separand or a very low non-specific binding of dyes and other molecules is also a criterion (Haglund 1971; Rilbe 1973; Righetti 1983; David Garfin and Ahuja 2005). The applied ampholytes are usually present in aqueous solution, thus molecules of water are always present in the IEF system. Molecules of pure water can be considered as biprotic ampholyte molecules having two intrinsic pK values both equal to 7.0 (Righetti 2005), but when applied as a carrier ampholyte, they do not carry significant current, thus the transference number approaches zero. The current carried by hydrogen and hydroxyl ions is negligible (Shimao 1981). In the absence of sufficient carrier ampholytes, neutralisation of the carried ions of water occurs in the neutral point of the pH gradient (Svensson 1962; Kolin 1970). This process is responsible for the accumulation of a pure water zone.

Focusing configurations are sensitive to even very small concentrations of impurities or salts added in electrolytes or in the system (Fidler *et al.* 1985). They can greatly alter the migration rates of components and cause a pH shift towards the end of the column and even distortion. In the presence of carbonate salts at alkaline pH, it is expected that the pH, conductivity, and ionic strength in the system will be affected (Persat *et al.* 2009; Mikkonen *et al.* 2015). Carbon dioxide dissolved in the terminating electrolyte has an impact on conventional IEF and this effect is often used to provoke endosmosis (Delincée and Radola 1978) and so achieve mobilisation (Hjertén and Zhu 1985).

The formation and stability of the pH gradient is also dependent on the anolyte and catholyte used in an IEF system. The composition and physical chemical parameters of electrolytes play a significant role in CE performance. The ionic strength and pH of electrode solutions has an influence both on sensitivity and on efficiency. In general, non-volatile acids and bases are used as anolytes and catholytes, respectively. In most cases, solutions of phosphoric acid and sodium hydroxide have been found to be suitable.

2 Aims of the study

The aim of this work is to study certain processes involved in capillary isoelectric focusing with the help of computer modelling. Computer simulations are important because some parts of the separation processes are not clearly visible during the experimental procedure. The following aims were pursued concerning capillary isoelectric focusing systems:

- To investigate the impact of different sampling strategies and initial sample distribution on the isoelectric focusing process.
- To explore the effect of the pH of the anolyte and the catholyte on the selectivity and speed of the isoelectric focusing.
- To investigate the impact of different electrode solution pairs on the dynamics of separation.
- To point out the conditions that lead to the formation and prevention of the pure water zone during focusing.
- To study certain impurities and their impact on the ionic strength, conductivity and pH of electrophoretic systems.

3 Materials and Methods

3.1 Computer simulation program

The modelling of IEF was possible with the model versions linked to the names of Bier and co-workers (Bier *et al.* 1983; Saville and Palusinski 1986; Palusinski *et al.* 1986) and Mosher and Thormann advanced their model GENTRANS (generalised model for transient electrophoretic processes) based on this early simulation program (Thormann *et al.* 1986; Mosher, Thormann, *et al.* 1989; Sounart *et al.* 2005; Thormann *et al.* 2010). It permits the handling of proteins and peptides (Mosher, Gebauer, *et al.* 1992), biprotic ampholytes, monovalent weak acids and bases, and monovalent strong acids and bases. It calculates *in situ* electroosmotic flow from wall titration data (Mosher *et al.* 1995; Thormann *et al.* 1998) and allows the use of plug flow (Thormann *et al.* 1993). The program has been extended for application of 300 components with 20000 as the maximum number of segments. Inputs required include the length of the separation space and its segmentation, the initial distribution of each component, the pK and mobility values that describe each compound, the input data for electroosmosis, the current

density and the amount of electrophoresis time. The model outputs concentration, pH, ionic strength, flow distributions, and conductivity values for each inserted component, and at the end of the simulation process the net electroosmotic flow and current density as functions of time are also available. Simulations can be executed with fixed or variable boundary conditions at the column ends, to obstruct or allow free transport of buffer and sample compounds in and out of the separation space, respectively. This simulation tool also encompasses the selection of data smoothing, which removes negative concentrations caused by numerical oscillations and thereby allows simulations to be executed at a smaller number of segments.

The presented model describes in detail the principles of electroneutrality of solutions, conservation of mass, and charge and various reaction equilibria (Okhonin *et al.* 2004; Andreev and Lisin 1993; Mosher, Dewey, *et al.* 1989). This one-dimensional simulation tool encompasses second order centred numerical schemes with a uniform grid and it does not deal with capillary wall adsorption; the only attention is on the longitudinal distribution of analytes.

3.2 Input conditions and execution of simulations

The described simulation program, GENTRANS was used in this work. Simulations were performed in a 10 cm electrophoresis column divided into 10000 segments of equal length (x = 10 μm). At the beginning of the separation, 2 cm (from 3% to 23% or 40% to 60% of the capillary length) of the total column was occupied by carrier ampholyte and sample components, at the anodic capillary end or in the centre of the capillary. If not stated otherwise, 101 hypothetical biprotic carrier ampholytes were used to establish broad (eight-pH-unit) and narrow (two-pH-unit) gradients between the anolyte (10 mM phosphoric acid) and catholyte (20 mM sodium hydroxide). Simulations were made with pH 3.00-11.00; 5.00-7.00; 7.00-9.00; 4.80-6.80; 7.20-9.20; 5.20-7.20; 6.80-8.80; 4.00-8.00; 6.00-8.00 gradients and some variations with added extra components. For each ampholyte, ΔpK was 2.5; the ionic mobility was 2.5×10^{-8} m^2/Vs. If not stated otherwise, the initial concentration of carrier ampholytes was 200 μM. The difference between the p*I* values varied between 0.02 (configuration with 101 and 141 carrier ampholytes), 0.04 (arrangement with 101 amphoteric electrolytes in 4.00-8.00 pH gradient) and 0.08 (setup with 101 carrier ampholytes in 3.00-11.00 pH gradient). Sample components occupied 2 % (0.2 cm) of the total column length and were introduced in between the two ampholyte zones (sandwich sampling), as a short zone within the carrier ampholytes, at the anodic or cathodic end of the ampholyte zone, or at the anodic or at the cathodic side of

the ampholyte zone (half sandwich). Simulations were made also with samples mixed with carrier ampholytes. A small quantity of chloride ions (2.711 mM) was added to the sample components, since such a counter component is typical for IEF separations and some selected simulations were performed with a small amount of carbonic acid (1 mM) in the catholyte to represent the uptake of atmospheric carbon dioxide at alkaline pH.
Simulations for 40, 25 or 10 minutes electrophoresis were performed at a constant voltage of 1000 V or at a constant current density of 100 or 200 A/m^2 and with a constant cathodic EOF of 100 µm/s or zero EOF. The initial boundary widths were overall 0.001% and an open cathodic column end allowed free transport of mass into and out of the separation space. Employed boundary conditions were constant at the anodic column end (Thormann *et al.* 2007). Data smoothing as described by Mosher *et al*. (Mosher *et al.* 2011) was used. Employing 10000 segments and personal computers featuring Intel Pentium G 870 3.1 GHz and G 2130 3.2 GHz, the CPU time were about 54 and 88 hours. For making plots, data were imported into the SigmaPlot Scientific Graphing Software Windows Version 11.0 (SPSS, Chicago, IL, USA).
Input data of analytes and electrolytes used for simulation runs are presented in Table 1.

In one kind of simulation, two-pH-unit gradients with stepwise increasing concentration sequence of added carrier ampholytes on the anodic side 2.31, 2.88, 3.60, 4.50, 5.63, 7.04, 8.80, 11.0, 13.7, 17.2, 21.5, 26.8, 33.6, 41.9, 52.4, 65.5, 81.9, 102, 128 and 160 µM were analysed. The opposite arrangement of edge components (from 160 to 2.31 µM) was used on the cathodic side.

For cIEF-MS coupling, the use of a volatile electrode solution is required, thus the second type of simulation was executed with 10 mM formic acid and 20 mM ammonium hydroxide and compared with the simulation which contained 10 mM phosphoric acid and 20 mM sodium hydroxide as anolyte and catholyte, respectively.

In the third type of simulation 100 mM ammonium hydroxide (pH: 5.0), 50 mM formic acid (pH: 11.0) and their pH-adjusted variations were applied. The desired pH values of catholyte (pH: 10.8; 10.3; 9.8; 8.9; 8.1) and anolyte (pH: 5.0; 2.5) were achieved by titrating 50 mM formic acid with 100 mM ammonium hydroxide and 100 mM ammonium hydroxide with 50 mM formic acid, respectively.

Table 1. Input parameters of sample components and electrolytes used in simulations[a)]

Compound	pKa_1	pKa_2	Mobility ($x10^{-8}$ m^2/Vs)	Initial concentration[b)] (μM)		
				anolyte	sample	catholyte
p*I* 5.3 dye	3.70	6.90	2.0		463	
p*I* 6.4 dye	4.68	8.12	2.0		398	
p*I* 6.6 dye	5.10	8.10	2.0		520	
p*I* 7.2 dye	5.70	8.70	2.0		368	
p*I* 7.9 dye	6.81	8.99	2.0		329	
p*I* 8.6 dye	7.70	9.50	2.0		281	
p*I* 10.4 dye	9.50	11.30	2.0		352	
H_3PO_4 [c)]	2.00	-	3.67	10,000		
HCOOH	3.75	-	5.66	10,000		
H_2CO_3	6.35	10.33	4.61; 7.18			1000
Cl^-	-	-	7.91		2711	
Na^+	-	-	5.19			20,000
NH_4^+	9.25	-	7.62			20,000
H^+			36.27			
OH^-			19.87			

a) pK and mobility values were taken from the Simul5 data base (http://web.natur.cuni.cz/gas/).

b) For all 7 dyes the concentrations correspond to 143 μg/mL. Dyes are injected as hydrochlorides.

c) Phosphoric acid was treated as monovalent weak acid as it was employed in a low pH environment only.

4 Results and Discussion

4.1 Computer simulation of different sampling strategies for capillary isoelectric focusing

Various sample introduction schemes and isoelectric focusing of seven analytes and 101 carrier ampholytes with a uniform p*I* distribution ($\Delta pI = 0.08$) forming a pH gradient of 3.00–11.00 between a 20 mM catholyte (NaOH) and 10 mM anolyte (phosphoric acid) were investigated using computer modelling. Simulations were performed at a constant voltage of 1000 V and a constant cathodic endosmotic flow of 100 µm/s.

In the sampling strategy where the analytes and the carrier ampholytes were applied in a homogenous mixture, a transient double-peak approach to equilibrium is predicted. In all of the other cases, where the samples were placed as a short zone within the initial ampholyte zone, sandwiched (in absence of carrier ampholytes) between zones of carrier ampholytes, or introduced before or after the initial carrier ampholyte zone, the separation of the analytes is observed to be much faster than the separation of the carrier ampholytes. When sample injection occurs at the anodic end or at the anodic side as a short zone in the presence or absence of carrier components, the separation procedure is considered as a cationic process. An anionic process is expected in the opposite case, where samples are placed as a short zone at the cathodic side or cathodic end of the ampholyte zone. A mixed process occurs when analytes are positioned as a short zone in the presence or absence of the ampholytes in the centre of the initial arrangement of carrier ampholytes. The character of the process depends on the environment (pH) and the properties (p*I*) of the molecule ion. Simulations undertaken with gradients that commenced with p*I* 7 carrier component, samples with $pI > 7$ behave as cations and $pI < 7$ analytes behave as anions. The separation process of the seven analytes is completely different to the double-peak approach observed where separation commences with a uniform mixture of samples and carrier ampholytes. Separation occurs in a transient environment formed by the concomitant accumulation of acidic and depletion of basic carrier ampholytes, thereby forming a pH gradient between approximately pH 3 and pH 7.

The developed pH and electric field patterns are very similar for the four sampling methods. However, sample injection as a short zone within the ampholyte area has an impact on the peak height and concentration of the carrier ampholytes.

Although the simulation operates with 101 carrier components, sandwich injection produces a gap with a relatively low number of carrier ampholytes in this region, which can be considered as a hot spot in the electric field pattern and has a deleterious effect. This phenomenon occurs as a result of the lower concentration of ampholytes in the initial fluid element. This region is initially occupied by the sample and the normal density of ampholytes cannot be established there. Therefore, the properties of the region are dependent on the sample matrix; the pH gradient becomes flatter. This phenomenon, called “memory effect”, is unique to the sandwich sampling strategy (Takácsi-Nagy *et al.* 2012; Thormann and Kilár 2013). In a real focusing system, there are more components present in this hiatus because of the number of originally applied ampholytes.

4.2 The impact of the water zone on separation

The character and the impact of the water zone on separation in the presence of seven analytes and using two-pH-unit gradients with end components having p*I* values at, below or above the neutral point (pH 7) was studied. In this set of simulations, 10 mM phosphoric acid 20 mM sodium hydroxide served as the catholyte and anolyte, respectively. Six simulation configurations with two-pH-unit gradients (5.00–7.00, 7.00–9.00, 4.80–6.80, 6.80–8.80, 5.20–7.20 and 7.20–9.20) were studied using a constant current density of 100 A/m^2 and constant electroosmotic flow.

Simulation data reveal that the zone of pure water develops during focusing when the pH gradient established by the carrier ampholytes does not cover the neutral region, when it ends at pH 7.00 or when it begins at pH 7.00. In the case of a gradient terminating below or at pH 7.00, the evolution of the water zone is visible on the cathodic side of the pH range. A water zone on the opposite, anodic side is expected when the applied pH gradient begins at or above pH 7.00. No water zone is predicted when “good” carrier components cover the neutral region. Moreover, water zones are also formed in the presence of sample components that migrate outside of the focusing gradient. These components form ITP zones that migrate behind the leading components of the electrode solutions, namely the cation of the catholyte (components with p$I > 7$) or the anion of the anolyte (p$I < 7$).

Two 7.00–9.00 pH configurations with different applied current densities were chosen and analysed. At the higher current density (200 A/m^2), the separation is at a more advanced stage because the doubled amount of Coulombs were flown through the column, compared to 100 A/m^2. This effect is clearly seen not only in the length of the formed water zone, but also in the number of edge components migrating from the cathodic end of the gradient (nine and four components form the isotachophoretic decay structure in the cathodic part of the gradient). The region of the initial sample zone (gap) that was placed between zones of carrier ampholyte is more pronounced in the case of the higher current density. This gap is characterized with a smaller carrier ampholyte concentration and a shallower pH gradient (Takácsi-Nagy *et al.* 2012).

Furthermore, the current density has an impact on the shape and peak heights of analytes and overall components focused during the simulation. We expect the concentrations of all amphoteric compounds to be elevated and the peaks to be sharper with less overlap. However, the increased current has no effect on the plateau concentrations of the ITP zones, but their boundaries become sharper. These characteristic phenomena of ITP are well known (Mao *et al.* 2000; Mosher and Thormann 2002; Thormann and Mosher 2006; Thormann *et al.* 2007).

Configurations with added edge components were studied using computer simulations. Other carrier, “edge” components with continuously decreasing concentrations on both sides of the main carriers were applied in order to mimic commercial mixtures of ampholytes, which are generally used for capillary isoelectric focusing. It was shown that the presence of additional carrier ampholytes in small amounts prevents the formation of the water zone and reduces the conductance gap, as they cover the neutral region. The added compounds act as isotachophoretic spacers between the focusing gradient and the electrolyte solutions or the ITP zones of amphoteric sample components with p*I* values outside the pH gradient or other ions.

It is important to note that the total number of carrier ampholytes (main gradient components and edge components) used in this simulation is still much lower than the number of carriers in experimental studies using commercial ampholytes. Still, this approximation reflects the reality of commercial narrow range carrier ampholytes and gradients commencing or ending at pH 7.00, and they can be used in cIEF without any additional or edge components to bridge the electrolytic gap and hot spot.

The effect of atmospheric carbon dioxide on isoelectric focusing was studied in another set of simulations. Configurations comprising the pH ranges 5.00–7.00 and 7.00–9.00 were resimulated with 1 mM carbonic acid added into the 20 mM sodium hydroxide catholyte.

It was found that carbon dioxide dissolved and converted to carbonic acid in catholyte at high pH might have an impact on the established zone structures. The composition of the acidic agent is dependent on the pH environment — we find carbonate ions in the catholyte, hydrogen carbonate behind the phosphoric acid, or both, as shown in Table 1. Simulation predictions illustrate the presence of carbonic acid throughout all zones between the catholyte and anolyte and the formation of a zone of increasing length behind the anolyte. Furthermore, the added carbonic acid in the system acts as a counter ion, mobilising the carrier ampholyte components on the cathodic side, and preventing the formation of pure water zone. This process is very similar to the process of electrophoretic mobilisation, where anions are added to the catholyte (Thormann and Mosher 2008), which can be considered as a contributor to cathodic drift. The formed ITP zone of the p*I* 7.00 carrier ampholyte reaches a relatively high concentration (1.286 mM), which also contains carbonic acid at a concentration of 2.32 µM, and therefore its pH becomes 6.9937 and its conductivity is 0.3276 mS/m. The carbonic acid concentrations in the anionic ITP structure formed by the p*I* 5.00 to 5.04 carriers are between 0.106 and 0.114 mM and no significant impact on the pH or conductivity is predicted for this side of pH gradient. This behaviour is comparable to the situation in anionic ITP at alkaline pH (Thormann and Mosher 2006; Thormann *et al.* 2007).

The amount of the acidic component in the catholyte has an obvious effect on the concentration of carbonic acid in the focusing part and in the ITP zones. A concentration of less than 1 mM carbonic acid in the system besides the catholyte produces lower amounts of acid in the overall pattern and occurs in shorter zones behind the anolyte. Conversely, carbonic acid applied above 1 mM results in a higher concentration in the focusing part and ITP zones, and produces a larger region behind the anolyte.

However, the number of carrier components taking part in the formation of the ITP structure becomes reduced in the presence of carbonic acid in the IEF system. Still, carbonic acid has no impact on the migration rate of the anolyte boundary. These effects are in good agreement with the experiences of Hirokawa *et al*. in studying anionic ITP at high pH (Hirokawa *et al.* 1991).

4.3 The impact of the electrolyte properties on separation

In the previous simulations, phosphoric acid and sodium hydroxide solutions were used as the anolyte and catholyte, respectively. To ensure compatibility with coupling of cIEF to MS detection, volatile electrolytes are needed. The comparison of the previous setup with phosphoric acid and sodium hydroxide and the simulation using the volatile electrode solutions formic acid and ammonium hydroxide is presented.

Alteration of the electrode solutions had no essential effect on the focusing part of the separation. Both the migration rates of the ITP boundaries and the plateau concentrations of the migrating ITP zones are dependent on the properties of the electrolytes used in the separations (Takácsi-Nagy *et al.* 2017). There is no significant difference between the focusing part of the two simulations or the migration of the sample components. Five analytes (p*I* 5.3, 6.4, 6.6, 7.2, and 7.9) focus within the pH range and samples with p*I* 8.6 and 10.4 behave as expected and migrate anionically outside the 4.00–8.00 pH gradient, which is in agreement with previous findings (Thormann and Kilár 2013).

Further simulations were executed in order to examine the effect of pH-adjusted electrolytes in electrophoretic systems. To study the electrophoretic behaviour and impact of pH-adjusted electrolytes on separation, focusing gradients comprising two-pH-unit ampholytes were placed in the centre of the separation column (40–60%) and EOF input was reduced to zero.

Samples sandwiched between 100 mM ammonium hydroxide and 50 mM formic acid, which serve as the catholyte and anolyte, respectively, reach their isoelectric positions, and good performance in isoelectric focusing is predicted. The IEF separation mechanism and the distribution of the analytes behave very similarly to previous simulations comprising 7.00–9.00 pH gradients. Data obtained from simulations with pH-adjusted electrolytes show that gradual titration of the catholyte with the anolyte results in a migration change of all components in the gradient. When the solution of ammonium hydroxide is titrated with the anolyte, formate ions migrate from the catholyte through the entire separation space into the anolyte, where they accumulate, similar to the carbonate ions added to the catholyte. All carrier and sample components possess a partial net positive charge that forces that they migrate electrophoretically towards the cathode under the influence of the applied electric field. The nature of the separation is rather isotachophoretic than isoelectric and the shape of the analytes

becomes rather broad, as previously described (Chartogne *et al.* 2002). This phenomenon is more pronounced with increasing amounts of formic acid in the catholyte.

Although the data presented describe a cathodic electrophoretic mobilisation, all conclusions reached are valid also for mobilisation towards the anode. The same migration mechanism is predicted when the anolyte contains the ions of the catholyte — the analytes become negatively charged and migrate as isotachophoretic zones towards the anode. This could be interpreted as a chemical mobilisation, where electroneutrality inside the capillary is not achieved because of the presence of formate ions in the catholyte. This is in good agreement with previous studies (Hjertén *et al.* 1987; Thormann and Mosher 2006; Thormann *et al.* 2007). If both electrolytes are altered, the character of the ITP migration of the analyte zones is bidirectional and their shape becomes more expanded.

Both the migration rates of the ITP boundaries and the plateau concentrations of the migrating ITP zones are dependent on the properties of the electrolytes used in the separation (Takácsi-Nagy *et al.* 2017).

5 Thesis points

1. Comparing sampling strategies, when the sample is introduced as a short zone within or adjacent to the carrier ampholytes, the migration of analytes is faster than the migration of carrier ampholytes. This separation and focusing is predicted to proceed as a cathodic, anodic, or mixed process. After the initial separation, analytes continue to separate and reach their focusing locations. This is completely different from the double-peak approach to equilibrium observed in the case where samples and ampholytes are applied as a homogenous mixture.
2. Sequential injection setups offer new possibilities in the application of ampholyte zones that do not cover the p*I*s of the analytes. Simulations performed with various injection modes suggest that sample placed at the anodic side or at the anodic end of the initial pH gradient are the favourable configurations for capillary isoelectric focusing.
3. Sandwich sampling results in a gap in the focusing column, as the concentration of the carrier ampholytes will be relatively lowered where the sample occupies the initial position of carrier ampholytes. As a result of this phenomenon, the pH gradient is flatter, and the region is likely to represent a conductance gap (hot spot) that could have deleterious effects. This is completely different to the other injection methods.
4. Characteristics of narrow, two-pH-unit gradients ending or beginning around pH 7.00 were studied, allowing the evaluation of the zone of pure water. A water zone formed on the cathodic side of the gradient is expected when the pH range ends below or at pH 7.00, whereas a water zone on the anionic side is observed when gradient begins at or above pH 7.00. No water zone is expected when the components of the established gradient cover the neutral zone. This case is very similar to the simulations using added “edge” carrier components with gradually decreasing concentrations at the edges of the pH range, which mimic commercially available ampholyte mixtures used for cIEF, having no sharp discontinuities at the gradient edges.
5. The length of the water zone is dependent on the applied current density and the duration of power application. It increases as the amount of Coulombs applied increases and as the power application is prolonged. Furthermore, impurities, such as atmospheric carbon dioxide converted to carbonic acid in the catholyte or other salts, influence the evolution of the pure water zone. Although carbonic acid has no significant effect on the conductivity or pH profiles, amphoteric sample components of p*I* 7.00 and below become electrophoretically mobilised towards the cathode.

6. Different electrode solutions, acids and bases, used as anolytes and catholytes, respectively have no significant impact on the focusing part of the separation, but the isotachophoretic structures and plateau concentrations on either side of the pH gradient are dependent on the properties of the electrolytes. Simulation also revealed that p*I* 7.00 ampholyte and carrier components with p*I* values below 7.00 on the cathodic side become electrophoretically mobilised and form isotachophoretic zones that migrate towards the cathode.
   Gradually altering the pH of the catholyte by adding formic acid induces an electrophoretic migration of all components towards the catholyte; the shapes of analytes become broader, and the gradient more compressed. Migration of all compounds in the opposite direction is expected when the anolyte is titrated with ammonium hydroxide. Analytes with bidirectional migration and enlarged gradients are observed when both electrolytes are pH-adjusted.

6 List of Publications

Publications related to the thesis.

I. **Takácsi-Nagy, Anna**, Kilár, Ferenc, Páger, Csilla, Mosher R.A, Thormann, Wolfgang: Sampling strategies for capillary isoelectric focusing with electroosmotic zone mobilization assessed by high-resolution dynamic computer simulation. Electrophoresis 2012; 33(6):970-980. doi: 10.1002/elps.201100525 IF: 3.303 (2012)

II. **Takácsi-Nagy, Anna**, Kilár, Ferenc, Thormann, Wolfgang: Modeling of formation and prevention of a pure water zone in capillary isoelectric focusing with narrow pH range carrier ampholytes. Electrophoresis 2017; 38 (5):677-88. doi: 10.1002/elps.201600314 IF: 2.482 (2017)

III. Páger, Csilla; Vargová, Andrea; **Takácsi-Nagy, Anna**; Dörnyei, Ágnes; Kilár, Ferenc Effect of electrolyte pH on CIEF with narrow pH range ampholytes. Electrophoresis 2012, 33, 3269-3275. doi: 10.1002/elps.201200175 IF: 3.303 (2012)

Abstracts and Poster presentations related to the thesis

1. Kilár, Ferenc, Páger, Csilla, **Takácsi-Nagy, Anna**, Thormann, Wolfgang: Kapilláris izoelektromos fókuszálás tömegspektrometriával kapcsolva – modell-számítások. Elválasztástudományi Vándorgyűlés 2008, 5-7 November 2008, Sárvár, Hungary, Abstract Book, p. 68
2. **Takácsi-Nagy, Anna**, Páger, Csilla, Kilár, Ferenc, Thormann, Wolfgang: Advances of computer modelling in capillary isoelectric focusing. CECE 2010 7th International Interdisciplinary Meeting on Bioanalysis, 14-17 October 2010, Pécs, Hungary, Abstract Book, p.63, P28
3. **Takácsi-Nagy Anna**, Páger Csilla, Wolfgang Thormann, Kilár Ferenc: A kapilláris izoelektromos fókuszálás új gyakorlatának elméleti megközelítése, Elválasztástudományi Vándorgyűlés 2010, 10-12 November 2010, Tapolca, Hungary, P-39
4. Kilár Ferenc, Páger Csilla, Dörnyei Ágnes, Vargová Andrea, **Takácsi-Nagy Anna**, Thormann Wolfgang: Isoelectric focusing coupled to mass spectrometry for bioanalysis, CECE 2011 - 8th International Interdisciplinary Meeting on Bioanalysis, 3-4 November 2011, Brno, Czech Republic, Abstract Book, p. 28 (ISBN: 978-80-904959-0-6)
5. Kilár, Ferenc, Páger, Csilla, Dörnyei, Ágnes, A. Vargová, **Takácsi-Nagy, Anna**, Thormann, Wolfgang: The strength and future of isoelectric focusing in bioanalysis. 27th International Symposium on Microscale Bioseparations and Analyses MSB 2012, 12-15 February 2012, Geneva, Switzerland, KN37
6. **Takácsi-Nagy, Anna**, Thormann, Wolfgang, Kilár, Ferenc: Modeling of sampling strategies for capillary isoelectric. 30th International Symposium on Microscale Bioseparations and Analyses MSB 2014, April 27 – May 1 2014, Pécs, Hungary, P70
7. **Takácsi-Nagy, Anna,** Kilár, Ferenc, Thormann, Wolfgang: Modeling of the development of a pure water zone in capillary isoelectric focusing. 10th Balaton Symposium on High-Performance Separation Methods, 2-4 September 2015, Siófok, Hungary, P55

Lectures related to the thesis

1. **Takácsi-Nagy, Anna,** Kilár, Ferenc, Páger, Csilla, Dörnyei, Ágnes, Thormann, Wolfgang: Capillary isoelectric focusing coupled to mass spectrometry. CECE 2009 6th International Interdisciplinary Meeting on Bioanalysis, 6-7 November 2009, Pécs, Hungary, Abstract Book, p.36, L18
2. Páger, Csilla, **Takácsi-Nagy, Anna**, Thormann, Wolfgang, Kilár, Ferenc: Novel methodology to couple isoelectric focusing with mass spectrometry – experimental and theoretical advances. 25th International Symposium on Microscale BioSeparations MSB 2010, 21-25 March 2010, Prague, Czech Republic, Abstract Book, p.32, L13
3. **Takácsi-Nagy, Anna,** Páger, Csilla, Kilár, Ferenc, Thormann, Wolfgang: XXXIII. Kémia Előadói Napok, 25-27 October 2010, Szeged, Hungary
4. Kilár, Ferenc, Páger, Csilla, Vargová Andrea, **Takácsi-Nagy, Anna**, Thormann, Wolfgang: Capillary isoelectric focusing combined with MS detection. HPLC 2011 36th International Symposium on High Performance Liquid Phase Separations and Related Techniques, 19-23 June 2011, Budapest, Hungary, L16 (ISBN: 978-963-89335-0-8)
5. Kilár, Ferenc, Páger, Csilla, Dörnyei, Ágnes, A. Vargová, **Takácsi-Nagy, Anna**, Thormann, Wolfgang: Isoelectric Focusing Coupled to Mass Spectrometric Detection. 18th International Conference on Chemistry, 22 – 25 November 2012, Băile Felix, Romania
6. Kilár, Ferenc, Páger, Csilla, **Takácsi-Nagy, Anna**, Thormann, Wolfgang: Practical Insights of Capillary Isoelectric Focusing Coupled to Mass Spectrometry. 14th International Symposium and Summer School on Bioanalysis, 28 June – 6 July 2014, Bratislava – Smolenice, Slovakia, L25

Diploma Thesis

Takácsi-Nagy Anna: Új elválasztástechnikai módszerek a gyógyszeranalízisben, gyakorlati és elméleti modell-kísérletek (2010)

Presentations and posters not related to the thesis

Gálicza, Judit, **Takácsi-Nagy, Anna**, Fisher-Fodor, Éva, Soriţău, Olga, Andrea Vargová, Beáta Ábrahám, Ferenc Kilár, Szabolcs Lányi: Internalization and Cytotoxicity Assays of Transferrin Complexes on Stem cell-like Tumour Glioblastoma Cells. 17th International Conference on Chemistry, 3-6 November 2011, Cluj-Napoca, Romania