UNIVERSITY OF PÉCS
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Novel capillary isoelectric focusing
with mass spectrometry

PhD Thesis

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1 Introduction

The capillary isoelectric focusing (CIEF) is a high-resolution electrophoretic technique for separation and analysis of proteins and peptides based on their isoelectric points (pI's) in a pH gradient. This method has been applied successfully for determination of pI's of the amphoteric molecules and analysis of the complex biological samples (physiological liquids and tissues). Nowadays the range of application of this technique is continuously increasing, since the efficiency of the separation can be increased with coupling CIEF to different chromatography techniques and mass spectrometry.

The two-dimensional polyacrilamide gel electrophoresis is the most commonly used method to separate proteins. Proteins are separated according to their isoelectric point along a pH gradient by isoelectric focusing in the first dimension, and according to their size with SDS-PAGE in the second dimension. Despite the selectivity and sensitivity of two-dimensional gel electrophoresis, this technique is difficult to use routinely because it requires many time-consuming manual operating steps (casting of gels, preparation of samples, running of gels, and staining of gels) for protein separation and detection and the quantitative accuracy of the method is also poor. Therefore, in recent years the significantly developing capillary isoelectric focusing-mass spectrometry (CIEF-MS) tries to substitute the above-mentioned method and becomes an alternative approach for protein analysis. The use of CIEF in conjunction with MS provides a high resolution separation technique coupled with a sensitive detection device. The advantage of the CIEF-MS methods is the speed, automation and sensitivity. But there are some difficulties as well, e.g. the presence of carrier ampholytes causes suppression of protein ion intensities.

The capillary isoelectric focusing with the sequential injection of carrier ampholytes and sample offers new possibilities for the elaboration of an effective CIEF-MS connection. During the experiments we have searched for such experimental condition that results charged state compounds in presence of low concentration of ampholyte. These circumstances support the establishment of a more effective MS detection. Since the focusing step after the sequential injection of the ampholytes and the analytes include a very complex process, a computer simulation of this isoelectric focusing will be necessary to understand these processes.
2 Aims

In this study the capillary isoelectric focusing preceded with the sequential injection protocol was studied and new methods were developed after modifying the experimental conditions. The main goal was to evolve an effective capillary isoelectric focusing-mass spectrometry coupling.

The detailed aims of this study were the followings:

- To replace the anolyte and catholyte solutions of the capillary isoelectric focusing method with volatile electrolyte solutions, in order to get a method compatible with mass spectrometric detection.
- To determine the effect of quality (pH range), length and number of ampholyte zone(s) on the capillary isoelectric focusing separation applying sequential injection protocol.
- To model the different injection protocols in order to reveal the dynamic of the separations using a broad pH range ampholyte.
- To modify the composition (pH) of the electrolyte solutions in order to set such experimental conditions where certain components are able to migrate out of the pH gradient.
- To develop a CIEF-MS method with sequential injection setup for separation of a protein mixture.
3 Materials and methods

Eight amphoteric analytes with different isoelectric points (4-amino-2-hydroxybenzoic acid (pI 2.7), 2-amino-5-chlorobenzoic acid (pI 3.0), 2-aminobenzoic acid (pI 3.5), 2-chloro-4-(4-morpholinylmethyl)-6-nitrophenol (pI 5.3), 2-chloro-4-[(4-methyl-1-piperazinyl)methyl]-6-nitrophenol (pI 6.4), 2-(4-morpholinylmethyl)-4-nitrophenol (pI 6.6), 2,6-bis-(4-morpholinylmethyl)-4-nitrophenol (pI 7.9) and 4-nitro-2,6-bis-(1-piperidinylmethyl)-phenol (pI 10.4)) were applied as sample components in the experiments. The protein sample contained lysozyme, cytochrome C, myoglobin and β-lactoglobulin A.

3.1 Capillary isoelectric focusing conditions

A HP 3D model was used for the isoelectric focusing experiments. A part of the separations was performed in 50 µm i.d. uncoated fused-silica capillaries with total length between 49-81.3 cm. The proteins separation was carried out in 124 cm (effective length to the UV detector 20.5 cm) x 50 µm i.d. PVA (polyvinyl alcohol) coated capillary. The field strength varied between 200 and 300 V/cm. Formic acid and ammonium hydroxide with different pH values and concentrations or these mixture solutions were used as anolyte and catholyte, respectively. The carrier ampholytes and the sample were injected in three consecutive steps applying 50 mbar (at the anodic end of the capillary), by varying the type and the lengths of the ampholyte zones. The displacement of the ampholytes and the sample towards the cathode is due to the electroendosmosis in the uncoated capillary. In the PVA coated capillary, after two or ten minutes focusing, the mobilization of the isoelectric pattern to cathodic side was performed under a 50 mbar pressure, while maintaining focusing voltage was applied. The UV detection was carried out by on-column photometric measurements in case of analytes with pIs 2.7, 3.0 and 3.5 at 200 and 250 nm, in case of analytes with pIs 5.3, 6.4, 6.6, 7.9 and 10.4 at 200, 250 and 280 nm, in case of proteins at 200 and 280 nm.

3.2 MS conditions

The CE system was coupled to an Agilent LC/MSD Trap XCT Plus MS instrument equipped with an ion trap analyser and controlled with the Agilent LC/MSD Trap software 5.3. CE-MS coupling was carried out using a triaxial electrospray interface. The sheath liquid (1:1 mixture of methanol and water) was delivered at a flow rate of 10 µL/min by an Agilent
1100 Series isocratic pump equipped with a degasser and a 1:100 splitter. During the measurements of the eight amphoteric analytes with different isoelectric points, the mass spectrometer was used in the negative ion mode for the 50-1000 m/z mass range. Every five scans were averaged for data acquisition. The capillary voltage was 3000 V. Nitrogen was used as a drying gas at 250°C (flow rate, 4 L/min), and as a nebulizer gas (12.5 psi).

During the measurements of protein samples, the mass spectrometer was used in the positive ion mode for the 300-3000 m/z mass range. Fifteen scans were averaged for data acquisition. The capillary voltage was 3000 V. The pressure of the nebulizer gas was set to 10 psi. The temperature of the drying gas was set to 325°C (flow rate 5 L/min). The sheath liquid was a mixture of MeOH:H2O (4:1, v/v) containing 1% HCOOH. It was delivered at a flow rate of 5 µL/min.

### 3.3 Computer simulation

The dynamic computer simulations were performed with GENTRANS. For making plots, simulation data were imported into SigmaPlot Scientific Graphing Software version 10. Seven amphoteric sample components (pI=5.3, 6.4, 6.6, 7.2, 7.9, 8.6 and 10.4) were used in the experiments. A 10 cm focusing space (capillary) divided into 4000 segments of equal length and a constant voltage of 1000 V were employed. 101 hypothetical biprotic carrier ampholytes were used to establish a pH gradient between anode and cathode. Their pI values uniformly span the range 3.0-11.0 (ΔpI = 0.08). For each ampholyte, ΔpK was 2.5, the ionic mobility was 2.5 x 10⁻⁸ m²V⁻¹s⁻¹ and the initial concentration was 0.0002 mol/dm³. Initially, the overall sample and carrier ampholyte zones occupied 20 % of total column length. 0.01 mol/dm³ phosphoric acid and 0.02 mol/dm³ NaOH served as anolyte and catholyte, respectively.

### 4 Results and discussion

The capillary isoelectric focusing method preceded with the sequential injection protocol (i.e., the analytes are inserted between two ampholytes zones by separate injections) was modified to be compatible with mass spectrometric detection. The non-volatile electrolyte solutions were replaced by solutions of the volatile formic acid and ammonium hydroxide. Moreover the dynamic coating of the capillary with methylcellulose was also
neglected; therefore the EOF was not controlled in our experiments. It was found that both the concentration and the pH of the electrolyte solutions influenced the pH gradient evolved during the focusing.

4.1 Effects of quality, length and number of ampholyte zone(s) on focusing

The main advantage of the sequential injection protocol is that ampholytes, which do not cover the pI's of the sample components, can be used for separation.

Applying UV detection, we can visualize the position of the ampholyte components (at 200 nm) and the sample components (at 280 nm). Although the sample components having pI values outside the pH range of the applied ampholytes migrated toward the edges of the ampholyte zone but they did not leave the pH gradient even if the quality of the ampholytes was varied. Since the focusing and mobilization of the amphoteric compounds (towards the detector) take place simultaneously in uncoated capillary, we assumed that at the time of the detection the analytes had not reached their “final position” yet, where the pH was equal to their isoelectric points. Therefore probably a transitional state was detected. The pH of the anolyte or catholyte solution also influences the direction of the analyte migration at the edge of the ampholyte zone, since the pH determines the charge of the compounds. However the compound can stop and remain at the edge of the ampholyte zone if the charge of the compound in the electrolyte solution does not ensure the further migration of the compound away from the ampholyte zone.

Efficient separation of the analytes (except those with pIs 6.4 and 6.6) was observed when only one ampholyte zone was applied either before or after the sample mixture. Introducing an analyte into the capillary after a broad pH range ampholyte solution, the analyte is in contact with the anolyte solution (where the pH is smaller than the analyte’s pI), and it becomes cation and migrates toward the cathode, i.e. it gets into the ampholyte zone. The compounds migrate in the ampholyte zone until they find their “final positions”, where the pH is equal to their isoelectric points and their charges become zero. The separation occurs according to their isoelectric points. Applying reverse injection protocol, the sample compounds are in contact with the catholyte solution (where the pH is bigger than their pI) and therefore they become anion and migrate toward the anode, i.e. they get into the ampholyte zone. Applying ampholytes with narrow pH range before or after the sample zone in the sequential injection, the separation of the analytes was not expected, however it still occurred. It could be explained by that the ampholyte mixture might contain some – probably
minor – amounts of components with pI's outside the indicated pH range, and those ampholyte components are responsible for the separation.

For a correct comparison of the results with MS and UV detection we applied the same effective (detection) length (69 cm) and the same field strength in all experiments. The shorter separation time in the experiments with MS detection could be a result of the flow of the sheath liquid (and the nebulizer gas), which may cause a siphoning effect. The other phenomenon, i.e., the broadening of the peaks in the MS isoelectropherograms, however, was probably due to the substantial dilution of the peak zones by the sheath liquid, which sometimes could cause lower resolution, as well. Despite of these disadvantages of the sheath flow and nebulizer gas, the detection and identification of the analytes was easily obtained by their known MS spectra i.e., using the extracted ion electropherograms.

The efficiency of MS detection in the CIEF-MS experiments is remarkably influenced by the chemical environment of the analyte. In our setup, where the focusing takes place in the presence of electroosmosis after the sequential injection of the ampholytes and samples, the formation of the pH gradient is more complex. The ampholytes and the sample components migrate in the presence of electroosmosis but their migration velocities are continuously changing until all substances find their “final position” in the moving zone. This “final position” of components having pI's outside the pH range of the carrier ampholytes will result that sample components remain in charged state. Upon the “completed focusing” these components form a queue at the (moving) border between the ampholytes and the catholyte or anolyte zones. Those compounds, which are at the edge of the ampholyte zone, are in charged state, which contributes to a more effective MS detection. On the other hand, when the concentration of the ampholytes is not high, the suppression effect on the ionization is also diminished, causing a better MS detection efficiency.

Finally we can conclude (from the experiments with applying different ampholyte solutions and injection protocols) that the sequential injection setup in capillary isoelectric focusing experiments can be efficiently combined with mass spectrometry detection. This CIEF-MS method gave a fast separation of amphoteric compounds (within 10 minutes) in uncoated fused-silica capillary. The successful experiments of using only one zone (before or after the sample zone) offer a new possibility for the sequential injection protocol. Since the focusing step after the sequential injection of the ampholytes and the analytes include a very complex process, i.e., the positioning (migration) of the ampholyte components and the sample components from different zones, while everything is moving by the endosmotic flow,
certainly a detailed modelling of this isoelectrofocusing will be necessary to understand these processes.

### 4.2 Simulation of different injection protocols

During high-resolution computer simulation, the effect of various injection protocols on the separation was studied in uncoated capillary using hypothetical biprotic carrier ampholytes with pH range 3-11. Electroosmosis developed in the capillary is transporting the entire liquid (the pH gradient) towards the cathode. Seven different sampling strategies (injection protocols) were compared, namely the application of the sample mixed with the carrier ampholytes (as is customarily done), as a pulse within the initial carrier ampholyte zone (as a pulse at the anodic end, at the cathodic end or somewhere in between the two ends of the initial ampholyte zone), sandwiched in absence of carrier ampholytes between zones of carrier ampholytes or before or after the carrier ampholytes.

With sampling as a short zone in absence or presence of carrier ampholytes in the applied sample, separation and focusing of analytes is shown to proceed as a cationic process (sample applied at anodic end or anodic side of carrier ampholytes), an anionic process (sample applied at cathodic end or cathodic side of carrier ampholytes), or a mixed process (sample applied within carrier ampholyte zone or between two zones of carrier ampholytes). Separation occurs like in zone electrophoresis with the exception that separation is taking place in a non-uniform environment which changes with time (because of the gradual forming of pH gradient). This is different from the double peak approach to equilibrium which takes place when analytes and carrier ampholytes are applied as a homogenous mixture. In these cases, separation of the analytes is predicted to be much faster than the separation of the carrier components. Thus, after the initial separation, analytes continue to separate further and eventually reach their focusing locations at which their net charge is vanishing.

Simulation data reveal that the concentration of the carrier ampholytes within the fluid element initially occupied by the sample will be lower compared to the other parts of the gradient applying sample application between two zones of carrier ampholytes (sample does not contain ampholyte, sandwich sampling). As a consequence thereof, the properties of this region are sample matrix dependent, the pH gradient is flatter and the region is likely to represent a conductance gap (hot spot) which could have deleterious effects.

Simulation results demonstrated that the application of the sample in a short zone could be advantageous in CIEF experiments using uncoated capillary.
In order to apply the CIEF with sequential injection protocol more efficiently for the separation of amphoteric substances and to understand the processes occurring during the separation we are planning further simulations with narrow pH range ampholytes.

4.3 Effect of anolyte and catholyte composition on focusing

In the CIEF-MS experiments, if the components could migrate out of the ampholyte zone (i.e. they could migrate in an ampholyte-free environment, in charged state), that would diminish the undesirable ion suppression effects of the ampholytes and thus a more efficient mass spectrometry (MS) detection would be possible.

With decreasing the pH of the catholyte below the pI of an analyte (so in the catholyte this compound becomes cation and migrates towards the cathode), or increasing the pH of the anolyte above the pI of an analyte (so in the anolyte this compound becomes anion and migrates towards the anode), the analytes having isoelectric points outside the pH range of the ampholytes should migrate outside the ampholyte zone after the focusing process.

In this study three, narrow-range ampholyte solutions from different sources were applied and the sample mixture contained five analytes (some of them having pI's falling out of the ampholytes’ pH ranges) and we investigated the effect of electrolyte (anolyte or catholyte) pH on resolution of focusing. It was found, however, that the change in the composition (pH and concentration of certain ions) of both the anolyte and catholyte solutions had significant influence on other parameters in the focusing process, as well. Major influences of the pH adjustment were observed in the following factors: 

i.) length of the ampholyte zone;  
ii.) analysis time;  
iii.) migration properties of the analytes in correlation with their pI's;  
iv.) resolution of the analytes;  
v.) peak shape (efficiency of the focusing);  
vi.) ionization states of the analytes (changing of the absorption spectrum).

Applying two ampholyte solutions from different sources with the same pH range (7–9 pH), although we found analogy in the separations, the position of the analyte with pI 10.4 in the pH gradient was different using the same experimental conditions. In case of the BioLyte ampholyte the analyte with pI 10.4 was always accompanied by ampholytes during the migration (irrespectively the electrolyte combination used); meanwhile with Ampholine pH 7–9 this compound mostly appeared at the edges of the ampholyte zone or in the catholyte solution. This means that components with pI's outside the indicated limiting pH value (pI >9) can be present in the BioLyte ampholyte mixture. The pI's of these ampholyte compounds are around 10.4.
By applying catholytes with pH lower than pH=10, we observed that upon the isoelectric focusing separation the pI 10.4 dye migrated before the front of the ampholyte zones under certain experimental conditions (in the case of Ampholine pH 7–9: pH_\text{anolyte} = 6.0, 6.9 or 7.5 and pH_\text{catholyte} = 9.9 or 9.1; in the case of Servalyt pH 6–8: pH_\text{anolyte} = 5.0, 5.9 or 6.5 and pH_\text{catholyte} = 8.9). It is in accordance with the expectation that the pI 10.4 dye migrates positively charged in catholyte solution at pH 9.9 or 9.1, and there is no ampholyte components present with pI's above 10 in the Ampholine pH 7-9 mixture. Since in the runs with Servalyt pH 6-8 the analyte with pI 10.4 migrated out from the pH gradient (ampholyte zone) less time than in the case of Ampholine pH 7-9, therefore the Servalyt pH 6-8 ampholyte mixture could contain some components with pI's between 9 and 10.4. Meanwhile, we previously mentioned that the BioLyte pH 7-9 ampholyte mixture contained components with pI's around pH 10.4, which did not allow the analyte with pI 10.4 to “leave” the pH gradient. At the anodic end of the ampholyte zones, we could never detect that the analyte with pI 5.3 migrated outside the ampholyte zone, not even in the cases where the pHs of the anolytes were higher than pH=5.3. The explanation of this phenomenon (i.e. analytes still migrate together with ampholytes) should be attributed to the composition of the ampholyte mixtures. It means that components having pI's below the lower pH limit of the mixture indicated by the manufacturer can be present in the ampholyte mixtures (e.g. compounds with pI's around 5.3).

The comigration and the change in the migration order of the components (analytes with pI's 6.6 and 6.4) reflect a strong influence of the electrolyte composition on the focusing phenomenon and at the same time on the charge-state of the compounds.

With altering the pHs of the electrolyte solutions it was revealed that the commercially available ampholytes always contained components with pI's outside the indicated pH range. Despite of this unexpected composition of the ampholyte mixtures, it was possible to obtain such experimental conditions (with using appropriate electrolytes, especially setting the catholyte’s pH below the pI of the analyte(s)), where the component migrated outside the ampholyte zone in a charged state. This is advantageous when other detection methods, e.g. mass spectrometry are used in combination with isoelectric focusing.

### 4.4 CIEF-MS separation of proteins

One of the biggest challenges in the CE analysis of the proteins is the elimination of the irreversible interaction between the macromolecules and the capillary inner wall. The
adsorption onto the capillary wall may lead to peak broadening and decrease of the separation efficiency. Applying coated capillary we can prevent and reduce the interaction between proteins and the capillary wall.

The CIEF-MS analysis of the proteins (lysozyme, cytochrome C, myoglobin and β-lactoglobulin A) was performed in a PVA (polyvinyl alcohol) coated capillary. As there is practically no EOF in this capillary, therefore the mobilization of the pH gradient towards the detection point is accomplished by pressure after the focusing step in the CIEF experiments. A classical requirement of the CIEF-MS analyses is the reduction of the concentration of ampholytes to evade the suppression of the ionisation of the sample. At the same time, the low concentration of ampholytes influences the efficiency of the focusing. Therefore we did not decrease the concentration of ampholytes below 1%.

With varying the pHs of the electrolyte solutions, we found that only the compound having pI higher than the pH range of the ampholyte migrated outside the pH gradient. Therefore we applied ampholytes with lower pH range (4-6) beside the pH 7-9 ampholytes in order to see some proteins leaving the ampholyte zone. Applying ampholyte with pH range 7-9, the lysozyme (pI=11.1) and the cytochrome C (pI=10.2) appeared at the edge of the ampholyte zone, but not in the catholyte. Using ampholyte with pH range 4-6, we increased the time of the focusing step, but the proteins did not leave the ampholyte zone, only the total time of the separation increased. Applying ampholyte with pH range 4-6, we made a comparison between two injection setups, where either two or only one ampholyte zone was applied next to the sample zone; and it revealed that the proteins could be separated using both injection protocols. Although the lysozyme did not migrate outside the pH gradient, the mass spectrum of the lysozyme contained signals corresponding to ampholyte compounds in a very small amount. It means that the lysozyme reached the MS detector almost in an ampholyte-free environment.

During CIEF-MS analysis of the proteins with the sequential injection setup (applying low ampholyte concentration and appropriate experimental conditions) analytes having pIs outside the pH range of the carrier ampholytes will migrate in charged state (either in the ampholyte zone or out of it) and this promotes more efficient mass spectrometry detection of proteins.
5 Conclusions

The capillary isoelectric focusing method preceded with the sequential injection protocol was successfully combined with an MS instrument equipped with an ion trap analyser. During the systematic modification of the experimental conditions influencing the efficiency of the separation, we found novel methods for the more efficient separation and more effective MS detection of the amphoteric molecules.

Theses of the work:

• The electrolyte solutions of the capillary isoelectric focusing with sequential injection setup were replaced by volatile anolyte (formic acid) and catholyte (ammonium hydroxide) solutions. After these modifications the method can be connected to mass spectrometry detection. It was found that, both the concentration and the pH of the electrolyte solutions influenced the properties of the pH gradient evolved, i.e. the efficiency of the focusing.

• Efficient separations were achieved when only one ampholyte zone was injected (either before or after the sample mixture). Thereby a new injection protocol was introduced.

• The developed CIEF-MS method gave a fast separation of amphoteric compounds. The nature of the electrospray interface (sheath liquid, nebulizer gas) diminished the efficiency of the separation, but the identification of the almost unresolved peaks was easily obtained by MS when the MS spectra of the substances had been known.

• During the simulations of different injection protocols, it was established that the injection of the sample as a short zone (before or after the initial carrier ampholyte zone) could be a favourable configuration for the capillary isoelectric focusing using uncoated capillary (in the presence of the electroosmotic mobilization). The simulations helped to understand the complex processes taking place during the focusing.

• By altering the composition (pH) of the electrolyte solutions, we accomplished that a compound having pI outside the pH range of the ampholyte could migrate outside the ampholyte zone.

• An effective CIEF-MS method was developed for the separation of a protein mixture.
Publications, posters

Publications related to the thesis

Cs. Páger, Á. Dörnyei, F. Kilár: Sequential injection setup for capillary isoelectric focusing combined with MS detection
Electrophoresis 2011, 32, 1875–1884     IF: 3,303

A. Takácsi-Nagy, F. Kilár, Cs. Páger, R. A. Mosher, W. Thormann: Sampling strategies for capillary isoelectric focusing with electroosmotic zone mobilization assessed by high-resolution dynamic computer simulation

Cs. Páger, A. Vargová, A. Takácsi-Nagy, Á. Dörnyei, F. Kilár: Effect of electrolyte pH on capillary isoelectric focusing with narrow pH range ampholytes
Electrophoresis (submitted for publication)     IF: 3,303 (2011)

Presentations and posters related to the thesis


Cs. Páger, A. Vargová, F. Kilár: The effect of pH of the background electrolytes on CIEF applying sequential injection protocol. HPLC 2011 36th International Symposium on High-

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Performance Liquid Phase Separations and Related Techniques, 19-23 June 2011, Budapest, Hungary, P1-G-146-Tu


**Publications not related to the thesis**

A. Gáspár, **Cs. Páger**: Capillary electrophoretic determination of mercury compounds in different matrixes. Chromatographia, 2002, 56, 115-120  
**IF: 1.23**

**IF: 1.325**

**IF: 3.743**

**IF: 4.531**

**IF: 0.879 (2011)**

**Presentations and posters not related to the thesis**


