PRINCIPLES AND APPLICATIONS OF CAPILLARY ISOELECTRIC FOCUSING

Primer

Agilent Technologies

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FORWARD BY PROFESSOR PIER GIORGIO RIGHETTI

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It was in 1960 when a humble infantryman, Harry Svensson-Rilbe, a trooper formerly associated with Tiselius, started shaking the foundations of the Institute of Biochemistry in Uppsala, by reporting on the possibility of separating such amphoteric macromolecules as proteins into a non-isocratic field, a most unorthodox electrophoretic protocol calling for a pH gradient in between the electrodic reservoirs. Heresy, pure heresy, of course, and nobody paid much attention to it. Having been amply described already in 1960-1961, how comes that isoelectric focusing (IEF) went largely unnoticed for just about a decade? Well Harry was a free-lance scientist in a Stockholm Hospital and had only, as an adept scholar, a student of medicine, Olof Vesterberg, not so skilled in organic chemistry. So, although the theory was fully outlined, the practice lacked the much-needed infantry for covering and surveying the grounds, i.e. the pH gradient along the migration path, so as to ensure its temporal stability (a physical nonsense, considering that the proton is the most mobile species ever reported in an electric field). One needed not just a few amphoteric ions, able to reach steady-state conditions along the separation space, but a whole army of them, extending, like the Chinese Great Wall, in between anode and cathode. Well, it turned out that Svensson's pupil, had been moonlighting and pouring over textbooks of organic chemistry, and surfaced with a remarkable synthesis of the much wanted "carrier ampholytes": a chaotic synthesis, to be sure, as chaotic as a medical student would possibly devise. Can chaos generate order? Apparently yes, if the order is established by a strong force field, a steep voltage gradient forcing this army of amphoteres to join arms in an orderly fashion, assuming a (quasi) Gaussian distribution about their respective isoelectric point (pI) values. Everything under control, then? Well, with such a superb technique, one of the few ones able to counteract entropy, trying to dissipate peaks via diffusion in the surrounding medium, by producing sharply focused zones, no matter how carelessly the experiment had been carried out, one would think yes, all quiet on the western front! Svensson and Vesterberg were indeed convinced that a breakthrough had been achieved, and they approached LKB Produkter AB, a Stockholm company (Bromma, Sweden), with a proposal for commercial production. As a safety measure, the two pilgrims were sent to the Mecca of Separation Science, Uppsala, to consult with the high priest of electrophoresis, Arne Tiselius. Poor Arne, who had

been witnessing the steady corrosion of his moving boundary method, scolded them, infuriated at the notion that they would dare to bring forward a technique surely bound for failure, considering that macro-ions, on their approach to pI values, would likely aggregate and precipitate. Notwithstanding this negative report, H. Haglund (a former pupil of Tiselius too) managed to squeeze out of his reluctant company (LKB Produkter AB) 60,000 SKr to produce both the carrier ampholytes and a vertical density gradient column for preparative purification of proteins. It seems only yesterday that these trials were launched; today, 40,000 published scientific reports later, these brave authors offer to the readers an updated easy introduction to the IEF technique, meanwhile miniaturized in a capillary format. I can only congratulate them for a very good job.

Pier Giorgio Righetti
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FORWARD BY PROFESSOR WOLFGANG THORMANN

Wolfgang Thormann

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Isoelectric focusing is a high-resolution electrophoretic technique for separation and analysis of amphoteric compounds, mainly proteins and peptides, in a pH gradient that increases from anode to cathode. In the past 30 years, considerable effort has been applied to developing capillary isoelectric focusing, which is as instrumental form of this separation technique, by utilizing capillaries or microchannels as focusing columns and commercial carrier ampholytes as separation medium. Carrier ampholytes are added to the sample and establish upon power application the required pH gradient such that amphoteric analytes can be sorted based on their isoelectric points. Separations can be conducted in stationary or flowing solutions. When capillary isoelectric focusing is performed in quiescent solution, either an array or scanning detector has to be employed, or the sample pattern must be mobilized either during or following focusing such that proteins can be detected as they pass an on-column absorbance or fluorescence detector that is mounted towards one column end or as they are swept into the ionization source of a mass spectrometry detector that is placed at the column end. In the same time period, dynamic computer simulation of isoelectric focusing provided insight into the fundamentals and complexity of carrier ampholyte based isoelectric focusing, including focusing and separation of amphoteric sample components, pH gradient formation, shifts and stability, electrophoretic mobilization, and impact of buffer flow on analyte detection. These efforts led to the understanding of the processes involved in isoelectric focusing and to the development of improved experimental procedures. Using coated capillaries, various capillary isoelectric focusing protocols comprising mobilization by application of flow or electrophoretic mobilization were commercialized and are being successfully used for analysis of charge heterogeneity in monoclonal antibodies and other proteins as well as for the determination of isoelectric points of proteins.

This primer provides a simple way to learn the basics of capillary isoelectric focusing and provides selected references with which the reader can obtain further insights into this fascinating analytical technique.

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INTRODUCTION

In the past 20-25 years, isoelectric focusing (IEF) has become an indispensable bioanalytical measurement method in research, development and manufacturing quality control of a new generation of therapeutics, namely biopharmaceuticals[1]. More recently, with phasing out of intellectual property rights on original biopharmaceuticals and simultaneous emergence of biogenerics (biosimilars or follow-on-biologics), the method has become of crucial importance in determining their similarity with originator biopharmaceuticals.

With the advent of capillary electrophoresis, IEF has moved away from flat gel plates to capillaries. Capillary isoelectric focusing (CIEF) provides automation, repeatable detection and quantification of recombinant proteins, easy documentation and archiving of analysis results, and thereby meets requirements of regulatory authorities. However, the practical execution of IEF in the capillary format requires a good understanding of electrophoretic movement of the analytes and a lot of practical experience. This primer does not intend to provide a comprehensive understanding of the fundamentals of IEF and CIEF but aims to give the interested reader a good start into the usage of CIEF on the Agilent 7100 capillary electrophoresis system. The practical work described here is largely based on a master's thesis by Theresa Kristl done at the University of Salzburg, Austria, under supervision of Assoc. Prof. Dr. Hanno Stutz in the Division of Chemistry and Bioanalytics, Department of Molecular Biology.

ABOUT THE AUTHORS

Theresa Kristl studied molecular biology that encompassed a joint study program at the University of Salzburg and the University of Linz, both Austria. In March 2011 she started her master's thesis "Application of Capillary Isoelectric Focusing for the Characterization of Proteins / Allergens — Optimization, Validation and Comparison of Different Strategies" supervised by Assoc. Prof. Dr. Hanno Stutz as part of a research sponsorship agreement with Agilent Technologies, Waldbronn, Germany. In June 2012 she started her Ph.D. thesis under the supervision of Univ.-Prof. Dr. Christian G. Huber, University of Salzburg. Her master's thesis was awarded by the Austrian Society of Chemistry (GOECH).

Hanno Stutz is currently Assoc. Prof. at the Division of Chemistry and Bioanalytics, Department of Molecular Biology, University of Salzburg, Austria. He studied biology, with his master's and Ph.D. theses performed on analytical topics, addressing trace analyses of pesticides in water with capillary zone electrophoresis (CZE) and cyclodextrin-mediated micellar electrokinetic chromatography (CD-MEKC) with offline preconcentration by solid phase extraction. His postdoctoral fellowship was spent at the Institute for Reference Materials and Measurements (IRMM) within the Joint Research Centre (JRC) of the European Commission (Geel, Belgium) where his research focus was on the characterization of metal-binding proteins with CZE-UV and CZE-ESI-MS with preceding in-capillary preconcentration. After his return to the University of Salzburg, he complemented his fields of research by CIEF, affinity CIEF, capillary wall coating strategies for preventing protein adsorption, CZE-ESI-TOF MS, method validation, statistics and quality assurance for proteins and biopharmaceuticals. He was awarded the Appreciation Award of the Austrian Ministry for Science and Traffic 1998, the Science Award of the Austrian Society of Chemistry (GOECH) in 2005, and the ASAC Feigl Award of the Austrian Society of Analytical Chemistry (ASAC) in 2009. He is board member of the GOECH and ASAC.

Christian Wenz is R&D scientist at Agilent Technologies, Waldbronn, Germany. He studied biochemistry at the University of Hannover, Germany, and completed his Ph.D. thesis in the group of Prof. Alfred Pingoud, Department of Biochemistry, Justus-Liebig-University, Giessen, Germany, on the structure and function of proteins that interact with nucleic acids. After a postdoctoral fellowship at the Swiss Institute for Experimental

Cancer Research (ISREC) in Epalinges, Switzerland, he joined Agilent Technologies in 2002. During the last ten years, Christian was involved in instrument and application development for automated electrophoresis systems, for example, the Agilent 3100 OFFGEL fractionator and the Agilent 2100 bioanalyzer. Since 2009 he has worked with the Agilent 7100 capillary electrophoresis system. He is the author of several Application Notes and Technical Literature published by Agilent Technologies for this instrument.

Gerard Rozing recently retired from Agilent Technologies, Waldbronn, Germany. He finished undergraduate studies in chemical engineering and organic chemistry in 1971 at the University of Amsterdam. He obtained his Ph.D. in organic chemistry early 1977 from the University of Amsterdam, which was followed by a post-doctoral stay at the State University of Ghent, Belgium as a NATO Science Fellow till early 1978. Further post-doctoral studies in chromatography and analytical chemistry followed at the University of Amsterdam in the group of Prof. Hans Poppe. In 1979 Gerard joined Hewlett-Packard, Waldbronn, Germany as an R&D Chemist working on stationary phase development and HPLC System qualification. Gerard held positions as R&D project leader and later R&D section manager. In 2000 Gerard became the University Relations and External Collaborations Manager and held that position till retirement on September 1, 2012. Gerard became the first Research Fellow in the Chemical Analysis Group of Agilent Technologies in 2006. Gerard is a member of the MSB Strategic Planning Committee and of the editor advisory board of Electrophoresis and Journal of Separation Science. He now serves as a guest scientist at the Free University of Brussels and at the University of Amsterdam.

Symbols	
v_e	electrophoretic velocity
μ_e	electrophoretic mobility

Abbreviations	
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CIEF	capillary isoelectric focusing
CZE	capillary zone electrophoresis
2D-GE	two-dimensional gel electrophoresis
EOF	electroosmotic flow
id	inside diameter
IEF	isoelectric focusing
IPG	immobilized pH gradient
ITP	isotachophoresis
MEKC	micellar electrokinetic chromatography
pI	isoelectric point
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

ELECTROPHORESIS

1.1 Principles of Electrophoresis

In electrophoresis, separation is based on the differential speed of movement of charged species in an electric field. The charged species are molecules with strongly or weakly ionized groups, or inorganic ions such as sodium (Na^+), magnesium (Mg^{2+}) or lithium (Li^+), which are separated in an electrolytic solution. Within the electric field, the charged species move towards the electrode of the opposite charge. The velocity at which a charged species travels, called electrophoretic velocity, v_e, depends in particular on its mass / charge ratio as well as on the electric field strength and the electrolyte properties. Normalizing for the electric field yields the electrophoretic mobility, μ_e.

Electrophoresis was discovered in the early nineteenth century. Mathematical equations describing the movement of ions in an electric field were developed by Kohlrausch[2] and others, and led to practical implementation by Tiselius[3] in the 1930s. However, his approach of moving boundary electrophoresis in free solution, was not able to separate compounds with small differences in mass / charge ratio. Convection, caused by the high currents involved, resulted in band distortion. So rather than using free solutions, later methods used solid or gel matrices on a flat plate that inhibited convection and facilitated separation of compounds into discrete and stable bands or zones. Tiselius[4] named such methods "zone electrophoresis".

This method quickly gained popularity because gel matrices were readily available (agarose) or could be prepared simply (polyacrylamide). The method was manual but inexpensive and easily handled by laboratory technicians. A plethora of methods labeled as slab-gel electrophoresis has been described, delivering molecular size and charge information of peptides, proteins and nucleic acids[5], for example, SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) that provides an estimation of protein molecular weight. Laboratory equipment manufacturers offered the required chemicals and supplies for do-it-yourself slab-gel electrophoresis. Later, precast gels became available and facilitated practical work. Pulsed-field approaches delivered more selectivity in the separation of compounds with similar mass / charge ratio or nucleic acids with high molecular weight[6]. Combination of electrophoretic separations has led to very high resolution two-dimensional gel electrophoresis separation methods (2D-GE)[7].

To counteract the disastrous effects of convection caused by Joule heating, Hjertén[8] realized that a capillary tube might be a better approach. For practical reasons, a tube diameter of 3 mm was chosen, which mandated the capillary to spin along its axis in order to obtain a homogeneous temperature distribution in the capillary. Mikkers et al.[9] adapted an isotachophoresis (ITP) system to use 0.2 mm inside diameter (id) PTFE tubes for capillary electrophoresis, which was more practical and brought improvement. However, it was not until 1981 when Jorgenson and Lukacs in their seminal paper demonstrated ultrahigh efficiency of free solution electrophoretic separation in narrow bore (50–75 µm id) fused-silica capillaries[10]. The name capillary zone electrophoresis (CZE) became the general designation of the technique. Soon, different varieties of slab-gel electrophoretic separation methodologies were established in capillary format. Some were propagated from slab-gel electrophoresis such as capillary gel electrophoresis (CGE). New methods of capillary electrophoresis were conceived from liquid chromatography such as micellar electrokinetic chromatography (MEKC) or capillary electrochromatography (CEC). Isotachophoresis (ITP) was already executed in capillary format and readily ported onto the narrow id capillary format used for CZE, in particular for providing sample preconcentration.

1.1.1 Electroosmotic Flow

One characteristic of electrophoresis in fused-silica capillaries is the phenomenon of electroosmotic flow (EOF). In a fused-silica capillary filled with an electrolyte solution an electrical double layer is formed on the inner capillary surface. As silanol groups in silica are slightly acidic, the inner surface is negatively charged when the electrolyte solution has a pH > 3. These charges are balanced by ions of opposite charge from the electrolyte solution. Since there must be electroneutrality over the cross section of the capillary, there is a slight excess of positive ions close to the inner wall of the fused-silica capillary. When an electric field is applied to the electrolyte solution along the capillary axis, the excess of cations close to the wall, which are mobile, are drawn to the electrode of opposite charge dragging along their solvation shell of water. Consequently a net flow of the liquid phase, EOF, in the capillary occurs. The reversal process, that is the generation of a potential by flow of an electrolyte solution through a capillary, is called streaming potential.

1.2 Principles of Isoelectric Focusing

As explained above, electrophoresis separation is based on the differential rate of migration of charged species along the direction of the electric field. While traversing the separation channel, the analyte ions form zones according to their mass / charge ratio. These zones undergo broadening due to molecular diffusion and slow mass transfer when convection or interactions with the capillary surface occur.

In isoelectric focusing, a counteracting force is present which upon broadening, drives the charged analytes back into their zones. This force originates in the amphoteric nature of both the analyte molecules and the components of the separation matrix.

Amphoteric molecules contain ionizable acidic and basic groups and can take a net positive or negative charge, depending on the pH of the molecule's environment. Each amphoteric molecule becomes neutral at the pH where positive and negative charges in the molecule are balanced. This pH is called the isoelectric point or pI. Proteins and peptides contain multiple ionizable carboxylic acid and amino groups and are by nature amphoteric molecules. Their pI depends on their particular amino acid composition, but also on the three-dimensional structure, that is, conformation, which effects the molecular environment and thus the ionization constants of individual amino acids within the protein. Figure 1.1 illustrates the amphoteric behavior of a model protein with a pI of 5.5.
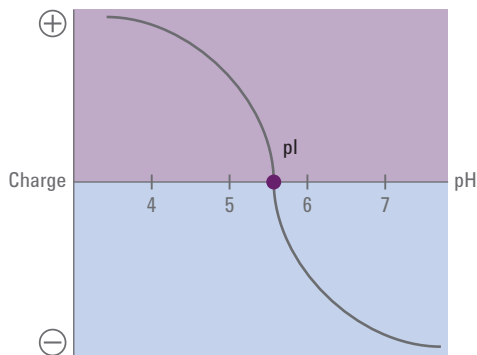
Figure 1.1 Amphoteric behavior of a model protein with a pI of 5.5. (adapted from Lottspeich, F., Bioanalytik, 2006, 2).

In a constant pH environment, the net charge and therefore the v_e of the molecule is constant and unidirectional. In a changing pH environment, for example, a pH gradient from low to high pH, the charge of an amphoteric molecule changes. As a consequence the magnitude and direction of its v_e change.

In a typical example of IEF, an electrolyte solution with low pH (the anolyte) is in contact with an electrode, which is selected as the anode.

At the opposite side a counter electrode is in contact with an electrolyte solution of high pH (the catholyte) which serves as the cathode. Between the electrodes a mixture of amphoteric compounds, called carrier ampholytes, is placed. Carrier ampholytes are aliphatic oligo-amino oligo-carboxylic acid molecules of varying length and branching. They can be prepared by Michael reaction of unsaturated carboxylic acid with polyethylene amine[11]. Therefore, carrier ampholytes are amphoteric molecules themselves containing both ionizable acidic and basic functional groups and have a defined pI. Carrier ampholytes have average molecular weights ranging from approximately 200 to 1000[12]. Figure 1.2 shows a typical structure of a carrier ampholyte[13].
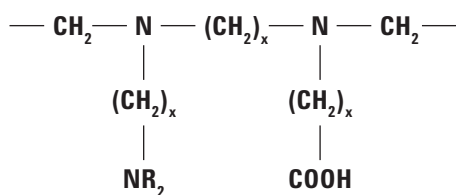
$$— CH_2 — N — (CH_2)_x — N — CH_2 —$$
$$| \qquad\qquad |$$
$$(CH_2)_x \qquad (CH_2)_x$$
$$| \qquad\qquad |$$
$$NR_2 \qquad\quad COOH$$

Figure 1.2 Chemical structure of a model carrier ampholyte[13]

In practice, a mixture of carrier ampholytes covering a range of pIs is used. Although IEF may take place in a free solution containing ampholytes, in common practice the carrier ampholytes are mixed with an anti-convective polymer such as polyacrylamide. The mixture is placed on a supporting plate of glass or plastic, hence the designation slab-gel electrophoresis.

At the beginning of the run, this mixture has a uniform pH, which equals the average pI of all carrier ampholytes. On application of electric field, current starts to flow causing the ampholytes to move according to their net charge. At the same time, hydronium ions start to move towards the cathode and hydroxide ions towards the anode. The most acidic carrier ampholyte travels furthest towards the anode before it acquires a zero net charge and the movement stops. A less acidic ampholyte neutralizes earlier, migrates less and remains adjacent to the more acidic ampholyte. On the other side, the most basic ampholytes travels farthest towards the cathode before becoming neutralized. Hence, a pH gradient is established with the lowest pH at the side of the anode and the highest pH close to the cathode.

Once the gel plate is prepared, samples containing unknown proteins and proteins with known pI (called pI markers) are applied to small wells in

the middle of the plate and the electric field is applied. The proteins start moving according to their charge and become neutralized at the location where the local pH matches their pI. Once the current has plateaued, the electric field is switched off. The zones can be visualized with dyes or stains.

Additives may be required to enhance solubility of proteins (at their pI, solubility of proteins is at minimum while the focusing process renders the protein in the zone at its highest concentration) or to avoid protein aggregation[14].

Although the methodology has proven to be very versatile, it is labor-intensive and time-consuming. Handling of toxic chemicals and experience in practical execution are required. The method has low reproducibility, shows cathodic drift (loss of proteins at high pH on the cathodic side) and is not quantitative. However, accurate information can be obtained about the pI of unknown proteins by comparison of their position within the pH gradient relative to markers of known pI. This has been proven to be invaluable in combination with other protein characterization methods.

A significant improvement was achieved with the introduction of immobilized pH gradient (IPG) gels by Bjellqvist et al.[15]. In this case, carrier ampholyte molecules are covalently linked to the matrix that is used as an anti-convective medium. The carrier ampholytes (now called immobilines) contain a polymerizable vinyl group which enables crosslinking of the carrier ampholyte to the polyacrylamide matrix. Immobilines with differing pIs are used to obtain a pH gradient.

In using an immobilized pH gradient, a number of the above mentioned practical disadvantages in working with carrier ampholyte generated pH gradients are offset. IPGs are offered as rehydratable gels on polyester sheets (typically 25 by 11 cm) by many suppliers. Appropriate devices for holding the gels and providing electric power are available from GE Healthcare Life Sciences and several other manufacturers. Such instruments also provide cooling of the gel plate and aid to apply the sample. IPG gels appear to be well suited for 2D-GE where IPG-strips (5–10 mm width) are used in the first dimension[16].

With the Agilent 3100 OFFGEL fractionator, IPG strips are used to sort a protein or peptide mixture according to their individual pI while allowing recovery of the proteins and peptides from solution. Other systems using slab-gel IEF for fractionation of proteins and peptides have been introduced by Invitrogen and Protein Simple.

Nevertheless, IPG-IEF remains labor intensive, difficult to automate, delivers poor quantification and prohibits electronic archiving.

CAPILLARY ISOELECTRIC FOCUSING

Besides on a flat surface, IEF in a gel has also been executed in a tube. Relatively wide diameter PTFE or glass capillaries (0.5–2 mm id) have been used[17]. Nevertheless, executing IEF in a tube has been as laborious as slab-gel IEF regarding the preparation of the separation matrix. Only low voltages can be used so that thermal gradients in the gel are minimized. In spite of the low voltage, extensive cooling is required and separation takes overnight.

Hjertén and Zhu[18] were the first to realize, that very narrow inside diameter capillaries, such as used in capillary electrophoresis, would be much better suited to dissipate the heat generated by the current, would allow a much higher electric field to be applied and thus accelerate the separation. Glass capillaries with 0.2 mm id were applied in their work. Since fused-silica capillaries have become the de facto standard in CZE, meanwhile also in CIEF fused-silica capillaries of 50 and 75 μm id are used exclusively. Although IEF can be done in a capillary, or a flat conduit, without a supporting matrix, it has become common practice to use a gel to assist in suppressing the electroosmotic flow and reduce diffusion.

Nowadays, CIEF can be performed on standard equipment for capillary electrophoresis such as the Agilent 7100 capillary electrophoresis system. CIEF is one mode of operation in the arsenal of electrophoretic separation methods available on commercial CE instruments. The Agilent 7100 CE system facilitates the usage of fused-silica capillaries, delivers low pH and high pH electrolytes (that is, anolyte and catholyte), and conducts the electric field, the mobilization of the pH gradient, UV-Visible detection, and automation. In the next sections, the Agilent 7100 CE system was used for experiments unless otherwise stated.

2.1 Two-step capillary isoelectric focusing

There are a number of implications in working with capillaries for CIEF. Unlike slab-gel IEF or IPG gel electrophoresis, in CIEF sample proteins, standards (that is, pI markers), carrier ampholytes, a polymer and optional additives are premixed and filled together in the fused-silica capillary as illustrated in Figure 2.1.

Figure 2.1 A mixture of carrier ampholytes and sample proteins is filled into the separation capillary. The grey color indicates premixing.

After connecting one end of the filled capillary with the low pH electrolyte (anolyte) and the other end with the high pH electrolyte (catholyte), the electric field is applied (Figure 2.2). Hydronium ions from the anolyte vial will move towards the cathode while hydroxide ions move simultaneously from the catholyte vial towards the anode. During this process the proteins and the ampholytes become sorted in the capillary from low to high pI.
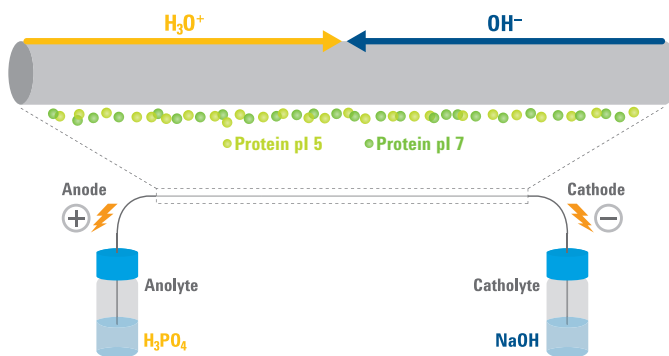

Figure 2.2 Schematic of creation of the primary pH gradient by anolyte (diluted ortho-phosphoric acid) and catholyte (diluted sodium hydroxide solution) after applying voltage by migration of OH$^-$ and H$_3$O$^+$ ions.

It is imperative to understand that the processes depicted in Figure 2.2, Figure 2.3 and Figure 2.4 take place simultaneously and not successively.

At the beginning of the focusing process a high current is observed, which continuously decreases over several minutes until the current reaches a minimum. The reason for this characteristic course of the current is related to the formation of the pH gradient. The analytes and carrier ampholytes initially possess a net charge, which decreases upon neutralization by H$_3$O$^+$ and OH$^-$ during the focusing process. The initial current is carried by OH$^-$ and H$_3$O$^+$, and their counter-ions, for example, sodium and

phosphate, charged carrier ampholytes (when forming the natural pH gradient), and analytes. If the focusing process finishes, the current becomes extremely low (mostly 10 % of the initial current value), since the analytes and carrier ampholytes lose their net charge. Eventually, equilibrium is achieved in which carrier ampholytes and the amphoteric sample molecules (proteins, peptides) are lined up from low to high pH (Figure 2.3 and Figure 2.4).

To be detected and isolated, the analytes must be moved past the point of detection. This separate process follows immediately after the focusing step and is described in a following paragraph. Since the mobilization is a detached step, this approach is called two-step CIEF.
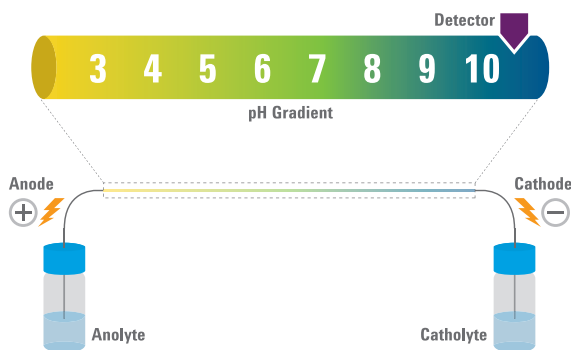

Figure 2.3 Formation and stabilization of natural pH gradient by carrier ampholytes. Both formation of the pH gradient and focusing of analytes occur simultaneously during the focusing step, but are depicted separately.
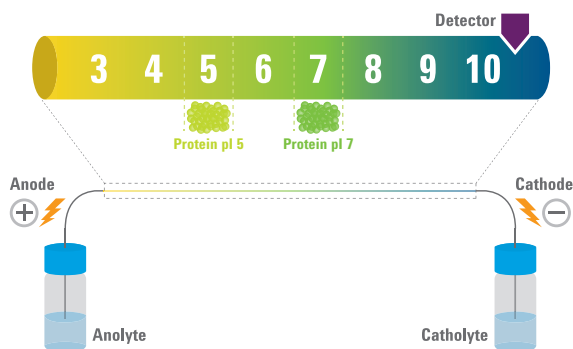
Figure 2.4 During the focusing step the model's proteins with pI 5 and pI 7 approach their focusing zone from both sides of the capillary. Finally, both proteins with pI 5 and pI 7 are focused in a narrow band at the pH = pI region, respectively.

2.2 Single-step capillary isoelectric focusing

In single-step CIEF, the focusing of proteins and ampholytes takes place in the presence of a residual EOF within the fused-silica capillary[19, 20]. In this case, protein zones become focused while being driven towards the point of detection by the EOF. If the EOF velocity is lower than the velocity of the focusing zones, the entire train of proteins can reach equilibrium before emerging at the detector end[21, 22].

In practice, the entire capillary is first filled with the catholyte. The protein sample and the carrier ampholytes are mixed and introduced at the inlet (anodic) capillary end. The zone length is 10–15 percent of the effective capillary length (to the point of detection). After application of the electric field, two processes occur in parallel:

A. The longitudinal pH gradient is formed with simultaneous separation and focusing of proteins in the sample.

B. With the presence of a low EOF, the entire zone of focused proteins and ampholytes is displaced towards the cathode and will pass the point of detection. Basic proteins reach the detector prior to neutral and acidic compounds.
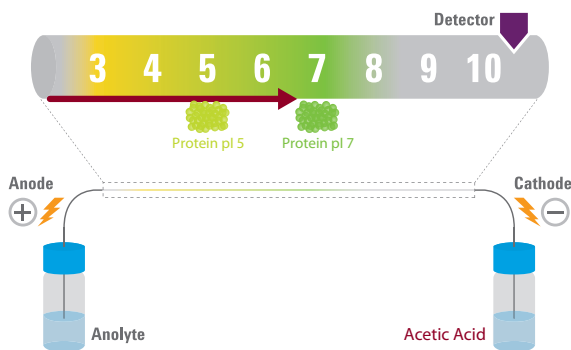
Figure 2.5 An illustration of the single-step CIEF process (modified according to reference 22 with permission).

Though generally believed to be a simple approach, in reality single-step CIEF is not. The residual EOF in particular needs to be balanced against the velocity of the carrier ampholyte or analyte zones to ensure complete focusing of proteins prior to the arrival of focused proteins at the point of detection. If this is not assured, artifacts such as double or multiple peaks might occur. Therefore, coated capillaries are still needed. Alternatively, a hydrophilic polymer such as hydroxymethyl cellulose is added, which deactivates the inner surface of the fused-silica capillary.

Uncoated fused-silica capillaries have been used for single-step CIEF. But in order to allow basic proteins to reach equilibrium at their high pI, a strong base such as tetramethylethylenediamine (TEMED) is added, which is sorted directly in front of the most basic analyte or ampholyte zone at the cathodic side. Schwer has critically reviewed this approach[23]. This primer focuses on two-step CIEF.

PRACTICAL ASPECTS OF CIEF

3.1 Electroosmotic flow suppression

It is obvious that during the two-step CIEF process, which is an equilibrium process, a hydraulic flow (EOF) present, may impede the complete sorting of proteins according to their pI prior to their passage of the point of detection. There are several approaches to suppress the EOF. Neutral high molecular weight polymers such as hydroxypropylmethyl cellulose[24] or polyacrylamide, which are added to the carrier ampholyte or sample mixture and the separation electrolyte, suppress the EOF by adhering to the fused-silica capillary surface and increase the viscosity of the separation medium to reduce broadening of focused zones by molecular diffusion. Capillaries with a permanent linear polyacrylamide coating on the capillary inner wall have proven to be very useful and are available commercially. However, since such coatings are exposed to extreme high and low pH, concerns existed about the stability of the coatings. Nowadays, capillary coatings apparently do not suffer from these limitations to a large extent (see Chapter 4, Applications of CIEF).

3.2 Detection in two-step CIEF

The point of detection in a CE instrument is placed towards the outlet end of the separation capillary. Once the protein or peptide mixture has been sorted according to their pI in the CIEF process, the individual analyte zones are distributed along the capillary from lowest to highest pI and preferably before the point of detection. Thus, the analyte zones have to be moved toward the capillary outlet in order to pass the point of detection. Two approaches have preferably become established for this purpose: hydrodynamic or pressure mobilization, and chemical mobilization.

During pressure mobilization, a slight gas pressure is applied to the anolyte vial on the inlet side[15]. Therefore, the content of the fused-silica capillary is pushed forward and every zone will be driven to the point of detection and beyond to the outlet side of the capillary. During this process, application of the electric field is continued to conserve the zone width. Nevertheless, since there is pressure-driven flow in the capillary, some broadening of the focused analyte zones may occur.

In chemical mobilization, the anolyte or catholyte is replaced by another electrolyte solution with high ionic strength or different pH. For example, by using sodium chloride on the cathodic side, chloride ions start to move into the capillary, replace the hydroxide ions and disrupt the pH gradient[25].

The sample analytes and carrier ampholytes acquire a net charge and start to move towards the outlet thereby passing the point of detection.

Alternatively, a solution of a weak acid can be used to replace the catholyte (Figure 3.1). The acetate ions from the chemical mobilizer (acetic acid) enter the capillary from the cathodic side, whereas the hydronium ions from the anolyte (H_3PO_4) enter the capillary from the anodic side. The pH gradient is disrupted by acetic acid and carrier ampholytes, and analytes are provided with a net positive charge, which leads to their mobilization towards the cathode, that is, the capillary side where the detection point is situated. The principle of the chemical mobilization using acetic acid is demonstrated in Figure 3.1. It is important to mention that proteins with pI < 4 cannot be mobilized with acetic acid. For more acidic proteins, a stronger acid should be used in the catholyte.
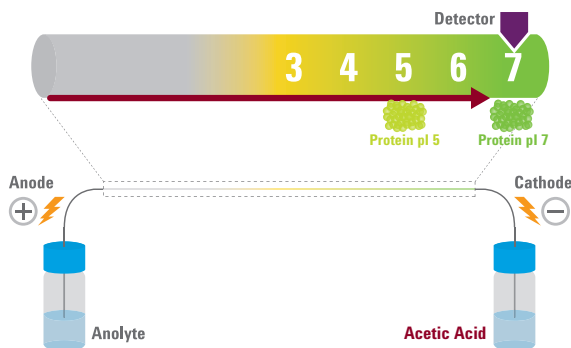
Figure 3.1 Principle of chemical mobilization using acetic acid as chemical mobilizer. The pH gradient is disrupted by acetic acid and the analytes get positively charged which mobilizes them towards the detector.

At this point, an important alternative instrumental implementation of CIEF needs to be mentioned. Wu and Pawlyszin[26] introduced "whole capillary image detection" by means of a charge-coupled device camera as a way to avoid the necessity to move the capillary contents beyond the point of detection. This approach has been commercialized by Convergent Biosciences (now part of ProteinSimple, Santa Clara, CA, USA) and became known as the iCE280 [27]. This method is designated as imaged CIEF in contrast to classical CIEF described above.

This primer introduces CIEF on a standard CE system such as the Agilent 7100 capillary electrophoresis system. As a standard system, the Agilent 7100 CE system is a multi-method and multi-functional system that

facilitates execution of various capillary electrophoresis separation modes[28]. In contrast, the ProteinSimple iCE280 is a dedicated instrument optimally suited for CIEF, but as the only methodology. A potential user must make a well-considered choice which system to use: classical CIEF or imaged CIEF.

In the next sections practical details of execution of classical CIEF are explained. The phenomena described here are to some extent specific to CIEF.

3.3 Stability of the pH gradient

A prime prerequisite for repeatable and reproducible CIEF separations is the stability of the pH gradient. The following experimental factors influence gradient stability:

- Concentration of anolyte and catholyte
- Carrier ampholyte
- Anodic and cathodic drift

Since electrolysis processes take place in the anolyte and catholyte reservoirs, the conductivity and pH of the electrolyte solution may change over time[29]. This effect can be counteracted by using a relative high concentration of ortho-phosphoric acid for the low pH electrolyte and of sodium hydroxide on the high pH side. It is a good practice to exchange the vials with electrolyte solutions after 2–5 runs.

For commercial reasons, carrier ampholytes with different chemical structures and ionizable groups have been introduced and marketed under trade names such as Ampholine, Pharmalyte, (both from Amersham Pharmacia Biotech, Uppsala, Sweden), Servalyte (Fluka, Buchs, Switzerland), and Bio-Lyte (BioRad, Hercules, CA, USA). These are formulated to cover a particular pH range and will be composed of different numbers and type of isoforms[30, 31]. In practice, a user may want to mix different pH ranges to have a better resolution in a particular pH range. The Salzburg group selected Pharmalytes that were best suited in their practical work, substantiating the results obtained by others[30,31].

Another phenomenon is the loss of carrier ampholytes from the separation capillary, which is called anodic and cathodic drift. It is actually caused by another electrophoresis effect called isotachophoresis in which the cation in the catholyte solution drives the positively-charged ampholyte out of the capillary on the cathodic side. This effect can be counteracted by using a higher concentration of the catholyte. But more recently it has become common practice to use so-called spacers in the starting mixture[32].

3.4 Use of spacers

As explained above, the acidic and alkaline carrier ampholytes may be lost in the anolyte and catholyte vials respectively, which reduces the resolution of very acidic and basic analytes by a compression of the pH gradient in their respective focusing region. In addition, since the complete capillary is filled with the solution of carrier ampholytes and sample, proteins focused behind the detector will not be detected during the mobilization step.

A special electrolyte solution has to be added to the sample, which is able to block electrophoretically the capillary segment between the catholyte and the detector. As a result, proteins will not be focused behind the detector. To avoid loss of acidic carrier ampholytes, a similar electrophoretic lock is required on the anodic side. As a consequence of addition of these electrolytes, the pH gradient is compressed because only a reduced capillary length is available for the formation of the pH gradient. This may lead to a reduction in resolution[14]. These electrolytes are called spacers and are added to the sample prior to a run.
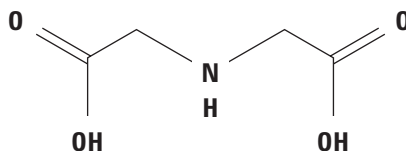
Figure 3.2 Chemical structure of iminodiacetic acid anodic spacer.
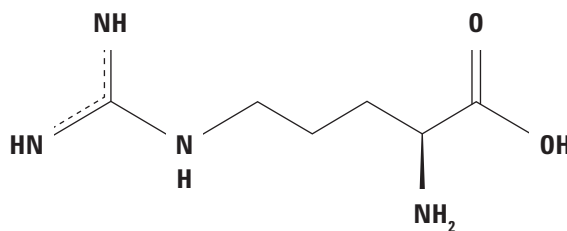
Figure 3.3 Chemical structure of arginine cathodic spacer.

The anodic spacer will be a molecule with a pI value above the pH of the anolyte solution but still below pH 3 which nominally refers to the most acidic carrier ampholyte contained in the wide pH range carrier ampholyte mixture. Accordingly, the cathodic spacer molecule should have a pI below the pH of the catholyte but above pH 10 corresponding to the most alkaline carrier ampholyte contained in the wide pH range carrier ampholyte mixture.

Spacer compounds should also be good carrier ampholytes to keep their position at the respective end of the pH gradient and efficiently act as electrophoretic blocking compounds. Iminodiacetic acid with a pI of 2.2 and L-arginine with a pI of 10.7 fulfill these requirements and represent good carrier ampholytes. Both compounds have successfully been applied as spacer compounds previously[33]. The chemical structures of iminodiacetic acid and L-arginine are given in Figure 3.2 and Figure 3.3 respectively. The anodic and cathodic spacer solutions act as sacrificial ampholytes, because they protect the carrier ampholytes from leaving the capillary. The localization of the anodic and cathodic spacer solutions is illustrated in Figure 3.4. The concentration of the spacer solutions has to be adjusted to the respective separation challenge. High cathodic and / or anodic spacer concentrations lead to a compression of the pH gradient, which has a negative influence on resolution. If too low spacer concentrations are selected, the pH gradient disposable for CIEF is more extended, which is favorable for separation but bears the danger of carrier ampholyte loss. In particular, a low cathodic spacer concentration additionally affects the detection of alkaline proteins focused beyond to the detection window. Thus, it is crucial to test different spacer combinations and concentrations to combine optimized resolution with temporal stabilization of the pH gradient and detection of all relevant analytes.
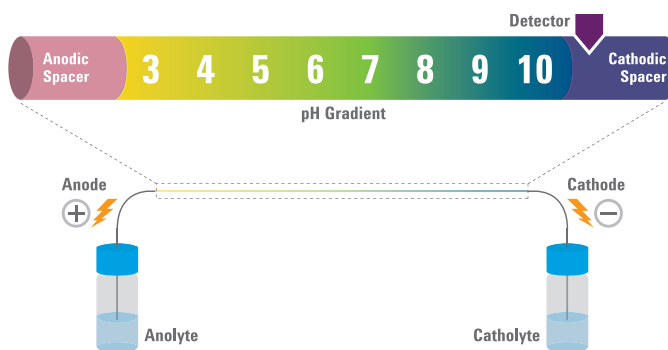
Figure 3.4 Localization of anodic and cathodic spacer solutions within the separation capillary after the establishment of the pH gradient during the focusing step.

3.5 pI markers

After the focusing of proteins during the CIEF process, the assessment of the pI of a particular zone remains a critical step[34]. Although linearity of the pH gradient may be assumed, without a reference or calibration the estimation of pI will be largely inaccurate. It has become common practice to use pI markers in the sample (or in a separate run or parallel lane in

case of slab gel IEF) to establish the pI of the unknown protein through interpolation. For a reduction of interpolation errors these reference compounds or pI markers should flank the target protein as closely as possible.

Peptides are preferably used nowadays as pI markers because they are structurally homogeneous, stable molecules. The pI range can be covered by varying the type and number of amino acids in the peptide. An example of commonly used pI markers is given in Table 3.1.

Peptide	pI value
Trp-Tyr-Lys-Lys	9.99
Trp-Tyr-Tyr-Tyr-Lys-Lys	9.50
Trp-Glu-His-Arg	7.00
Trp-Glu-His	5.52
Trp-Asp-Asp-Arg	4.05

Table 3.1 List of commercial peptidic pI markers (from AB SCIEX) covering a pI range between 4.1 and 10.0. Sequences refer to synthetic peptides proposed by Shimura et al.[35] for pI calculations in CIEF.

3.6 Precipitation and aggregation of focused proteins

While focusing in a narrow zone, the local concentration of each protein increases. At the same time, the protein's charge is neutralized. As a consequence the solubility of the protein is reduced. Precipitation and / or aggregation of proteins in the focused zone may or will occur. Precipitation shows as extremely narrow peaks or spikes, randomly distributed within the trace from run to run. Precipitation or aggregate formation affect the actual focusing process. If protein aggregates or precipitates cannot be flushed from the capillary, they might block it. Also, the current might become unstable and eventually drop to zero in the case of protein aggregation or precipitation.

Additives may be used in the sample mixture, which improve the solubility of the protein particularly at its isoelectric point to avoid precipitation. Different solubilizers have been described and tested such as glycerol, ethylene and propylene glycol as well as zwitter ionic and neutral detergents. Urea is also a frequently applied solubilizer in IEF[36]. Urea solutions have to be prepared freshly since urea is known to degrade to ammonia and isocyanate[37] which can carbamylate N-terminal and ϵ-amino groups and changes the net charge of proteins inducing structural heterogeneity[38].

3.7 Incomplete focusing

Particular attention has to be given to a complete focusing of all analytes before they pass the detector. Since analytes migrate from both capillary ends towards their individual focusing zone, two signals might be detected for one analyte species if focusing is incomplete. This might be erroneously interpreted as coexisting isoforms or an impurity[39]. The course of the current during analyte focusing might provide a rough estimation of the achieved progress in focusing. With reaching the minimum current, at least carrier ampholytes have taken their final position. In case of doubt, the focusing time can be extended to compare whether peak profiles coincide (focusing complete) or still change, indicating incomplete focusing in the latter case. However, users have to be aware that an extended focusing time inherently includes the risk for an increased propensity of protein aggregation or precipitation in the capillary due to the enhanced contact of proteins with (nearly) zero net charge in highly concentrated zones. Moreover, this head-to-head migration is also the reason for an occurrence of peaks prior to the initialization of the mobilization step. This is related to a passage of analytes from the distal part of the capillary, that is, behind the detector, on their way to their focusing zone[32].

3.8 Ionic strength of protein samples in CIEF

Protein samples of low ionic strength are preferred in CIEF. Protein samples dissolved in high concentration electrolytes and buffers cause peak currents in the initial phase of focusing which might damage coated capillaries and denature proteins by in-capillary temperature increase due to Joule heating. In case of biological samples, desalting is recommended prior to CIEF. However, low salt contents have been shown to improve resolution compared to pure water in some instances[40].

4 APPLICATIONS OF CIEF

The essential requirement for separation of compounds by isoelectric focusing is their amphoteric nature as explained previously. That is, the compounds have protonated groups that can be deprotonated such as amino groups and vice versa such as carboxylic acid groups. Hence, prime candidates for separation by CIEF are proteins and peptides. Nevertheless, also small molecules may be amphoteric for which CIEF can be used for pI and pK$_a$ measurement[41].

With the introduction of the capillary format for IEF and the availability of CIEF on capillary electrophoresis instrumentation, CIEF became an important analytical technique in the late eighties and early nineties during the early phases of development and manufacturing of recombinant proteins. The main purpose of CIEF was and remains the determination of the pI of the purified recombinant protein and / or assessment of the charge heterogeneity of the particular protein.

This heterogeneity is a consequence of the cellular machinery producing the recombinant protein. On the other hand it may be a consequence of sheer structural degradation. It is easily realized, for example, how deamidation of an asparagine or glutamine amino acid residue in the protein changes the pI of the particular protein. Since there is one more ionizable group in the molecule, this renders it to be a different molecule. For high molecular weight proteins such as biotherapeutics with many asparagines or glutamines the number of structural changes by posttranslational modification may grow rapidly.

Meanwhile, for many of the first generation of recombinant proteins used for therapeutic purposes, patent protection has expired or will expire soon. Cost-effective copies of these proteins (biosimilars) such as erythropoietin (EPO) are entering the pharmaceutical market. Biosimilars however exhibit the same high molecular complexity as the original protein. They also may be manufactured by another biological route and are therefore quite sensitive to posttranslational modifications during the manufacturing processes. When recombinant proteins or biosimilars are used for purposes other than intended such as in sports doping, forensic labs need to be able to analyze them.

Monoclonal antibodies are a particular class of proteins and are used in cancer treatment. They are prepared by another route than recombinant

proteins but which is equally complex. Monoclonal antibodies have a molecular weight around 150,000. By their biological function, they show a large structural variety in particular regarding glycosylation. Obviously these molecules may undergo posttransitional modification during manufacturing and degradation during storage, affecting their efficacy and safety when used in treatment. About 40–50 % of biotherapeutics nowadays are monoclonal antibodies[42].

Especially in proteomics, the pI of an unknown protein is of paramount importance in its identification from databases like ExPASy or SwissProt. Together with structural information from peptides, knowing the pI will be very helpful in identification of the unknown protein.

In contrast to slab gel isoelectric focusing, where calibration of the pH scale is obtained from measurement of the distance traveled by each protein or peptide, in CIEF a calibration with pI markers is required to establish the pI of an unknown protein or peptide. Righetti has provided an excellent overview of pI determination[34, 43]. In an unequivocal way this author provides a keen insight into the benefits and pitfalls of pI determination with CIEF. Particularly the assumption of linearity of the pH gradient needs to be treated with utmost care. In clinical analysis CIEF has become a method of choice for the determination of hemoglobin charge variants as an indicator for the severity of diabetes[44].

4.1 Recommended methods

The following sections give practical guidelines for performing CIEF and outline model methods for the screening of samples for proteins of roughly known or even unknown pI. These methods do not claim to achieve best resolution for the individual target samples of the reader, but are intended as a starting point and for subsequent refinement of the CIEF method. Depending on the actual sample composition and concentration of analytes, users are encouraged to consider, for example:

- Changes in the sample composition
- Composition and concentration of carrier ampholytes
- Mixtures of wide and narrow pH range carrier ampholytes
- Modification of capillary length
- Spacer concentrations
- Focusing time

4.2 Reagents, capillaries and consumables

The equipment and chemicals listed in this chapter can be ordered from the vendors given in Table 4.1. For full details of ordering information, please refer to the respective websites. Table 4.2 lists ordering details for Agilent consumables. Table 4.3 lists the vendor and product information for non-Agilent consumables.

Vendor	Web site
Agilent Technologies, Inc., Santa Clara, CA, USA	www.agilent.com
AppliChem GmbH, Darmstadt, Germany	www.applichem.com
AB SCIEX, Framingham, MA, USA	www.absciex.com
GE Healthcare, Waukesha, WI, USA	www.gehealthcare.com
Merck GmbH, Darmstadt, Germany	www.merck.com
Metabion International AG, Martinsried, Germany	www.metabion.com
Sigma Aldrich Co. LLC., St. Louis, MO, USA	www.sigmaaldrich.com

4.1 Websites of vendors for reagents, capillaries and consumables.

Component	Agilent order number
Alignment interface for standard capillaries 50 µm id (color code: green)	G1600-60210
µSIL-FC capillaries (coated with a 0.075 mm thick film of fluorocarbon polymer)	194-8111
Rat anti-DYKDDDDK monoclonal antibody	200477-21
260 nm band pass detector filter assembly	G7100-62700
280 nm high pass detector filter assembly	G7100-68750
Vial crimp / snap, polypropylene (1 mL)	5182-0567
Snap caps, polyurethane	5181-1512
Sample vial, polypropylene (100 µL)	9301-0978

Table 4.2 Ordering details for Agilent reagents, capillaries and consumables.

Component	Vendor	Order number
Sodium hydroxide solution (NaOH) 1.000 mol / L	AppliChem	011002006
CIEF capillary: neutral coated capillary	AB SCIEX	477441
CIEFgel	AB SCIEX	477497
3 pI Markers 7.0; 5.5; 4.1 from the CIEF peptide marker Kit	AB SCIEX	A58481
Ribonuclease A (RnA), pI 9.68 & Carbonic Anhydrase II (CA II), pI 5.91 from Beckman CIEF Kit	AB SCIEX	477490
Pharmalyte pH 3-10 (PL 3-10) for IEF, 0.36 meq / mL pH	GE Healthcare	17045601
Pharmalyte pH 5-6 (PL 5-6) for IEF, 0.36 meq / mL pH	GE Healthcare	17056401
Pharmalyte pH 5-8 (PL 5-8) for IEF, 0.36 meq / mL pH	GE Healthcare	17045301
Ortho-phosphoric acid (H_3PO_4): min. w=85 %	Merck	K42661473
Acetic acid, 100 %	Merck	1000632500
Synthetic peptide 1 (SP 1, pI 4.72), purity > 95 %	Metabion	Synthesized on demand
Synthetic peptide 2 (SP 2, pI 4.51), purity > 95 %	Metabion	Synthesized on demand
Methyl cellulose, viscosity 1,500 cP, 2 % in H_2O(20 °C)	Sigma Aldrich	M0387
Urea, purity ≥98 %	Sigma Aldrich	U6504
L-Arginine: $C_6H_{14}N_4O_2$; ≥99,5 % (NT)	Sigma Aldrich	100930559
Iminodiacetic acid: $C_4H_7NO_4$; 98 %	Sigma Aldrich	2055554
Albumin, from chicken egg white (ovalbumin): lyophilized powder ≥ 98 %	Sigma Aldrich	1000946409
Myoglobin, from horse heart, minimum 90 %, (PhastGel)	Sigma Aldrich	3097050
Fluorescent IEF-Marker pI 7.2	Sigma Aldrich	89951
Fluorescent IEF-Marker pI 7.6	Sigma Aldrich	89952
Carbonic Anhydrase II (CA II), pI 5.4	Sigma Aldrich	2325766

Table 4.3 Ordering details for non-Agilent reagents, capillaries and consumables.

The following items need to be provided by the user:

- Lab water purification system providing ultrapure (Type I) water
- analytical balance
- laboratory balance
- spatula
- pipettes with micro pipette tips, 1–10 µL, 10–100 µL, 100–1000 µL
- vortexer
- volumetric flasks
- centrifuge
- safe-lock tubes, 1.5 mL, 500 µL

4.3 Experimental

4.3.1 Installing a capillary in the CE cartridge

An AB SCIEX neutral coated capillary or Agilent µSIL-FC capillary, both with 50 µm id, can be used for the CIEF measurements.

1. Cut the capillary to the appropriate length immediately prior to installation. For subsequent method examples a total capillary length of 32–33 cm is applied.

2. Insert the capillary and align with the alignment interface. Check the correct alignment of the preburned capillary window by holding against the light or by using a magnifying glass.

3. Insert the alignment interface in the cartridge and close the cartridge.

4. Before installing the cartridge in the Agilent 7100 CE system, ensure a detector filter assembly has been inserted. Use either the 260 nm band pass filter assembly (G7100-6200) or the newer 280 nm high pass filter assembly (G7100-68750).

4.3.2 Handling and storing capillaries

Handle the AB SCIEX neutral coated capillary according to the manufacturer's instructions. Avoid allowing the capillary to dry out. When not in use for several hours or overnight, store the capillary in a refrigerator at 4–8 °C with the capillary ends immersed in ultrapure water. When not in use for longer periods, fill the capillary with CIEF gel.

The Agilent µSIL-FC capillary is stable from pH 2.5 to 10.0 and can withstand short flushes with 0.1 mol / L NaOH to remove contaminants. When not in use, flush the capillary with water and methanol, and then dry. Store in dry state at room temperature.

4.3.3 Conditioning of AB SCIEX neutral coated capillaries

1. Before using a new AB SCIEX neutral coated capillary, flush the capillary with 10 mmol / L ortho-phosphoric acid at 900 mbar for 4 minutes, then flush with ultrapure water at 900 mbar for 15 minutes.

2. Before the first run of each day, flush the capillary with ultrapure water at 900 mbar for 15 minutes.

4.3.4 Conditioning of Agilent µSIL-FC capillaries

1. Before using a new Agilent µSIL-FC capillary, flush the capillary with 350 mmol / L acetic acid for 5 minutes at high pressure (3.5 bar), followed by ultrapure water for 2 minutes and then 0.5 % methyl cellulose for 5 minutes.

2. Repeat the flushing sequence in step 1 once every day and after cleaning the capillary (step 3).

3. After every sixth sample injection, flush the capillary with 0.1 mol / L NaOH for 2 minutes at 1 bar, followed by water for 30 minutes, and then condition the capillary as in step 1.

4.3.5 Preparing anolyte and catholyte working solutions

	CIEF gel / MilliQ	H_3PO_4 / NaOH	Final concentration
Anolyte	818 µL CIEF gel	82 µL 1.00 mol / L H_3PO_4	91.1 mmol / L
Catholyte	883 µL MilliQ	17 µL 1.00 mol / L NaOH	18.9 mmol / L

Table 4.4 Preparation of anolyte and catholyte working solutions for pressure mobilization in 2 mL Eppendorf tubes.

	CIEF gel / MilliQ	H_3PO_4 / NaOH	Final concentration
Anolyte	720 µL CIEF gel or MilliQ	180 µL 1.00 mol / L H_3PO_4	200 mmol / L
Catholyte	630 µL MilliQ	270 µL 1.00 mol / L NaOH	300 mmol / L

Table 4.5 Preparation of anolyte and catholyte working solutions for chemical mobilization in 2 mL Eppendorf tubes.

1. Mix the anolyte and catholyte working solutions thoroughly by vortexer.

2. Centrifuge the working solutions as well as the respective samples at 14,000 rpm and 20 °C for 5 minutes to remove air bubbles and particulate matter.

3. Transfer 600 µL of anolyte and catholyte working solutions from the top of the Eppendorf tubes into buffer vials from Agilent.

Always prepare anolyte and catholyte working solutions freshly. Both working solutions can be used for 2—4 runs depending on the analysis time.

4.3.6 Preparing spacer solutions

1. Prepare a stock solution of 500 mmol / L L-arginine in ultrapure water as cathodic spacer.

2. Prepare a stock solution of 200 mmol / L iminodiacetic acid in ultrapure water as anodic spacer.

4.3.7 Preparing the chemical mobilizer

1. Prepare a solution of 22 or 350 mmol / L acetic acid in ultrapure water as chemical CIEF mobilizer.

4.3.8 Preparing a 1 % methyl cellulose solution

1. Heat 15 mL ultrapure water to about 80 °C.

2. Add 0.4 g methyl cellulose powder slowly under stirring into the hot water and continue stirring for 5 more minutes.

3. Remove the solution from the heater, add ice-cold water to a final volume of 40 mL and mix.

4. Cool the solution to −20 °C and mix every 30 minutes until frozen.

5. Store mixture overnight at 4−8 °C.

4.3.9 Preparing a 0.6 % methyl cellulose solution containing 3 M urea

1. Add 1.8 g urea to 6 mL of the 1 % methyl cellulose solution prepared in section 4.3.8.

2. Add ultrapure water to a final volume of about 9 mL.

3. Mix until the urea is completely dissolved.

4. Add ultrapure water to a final volume of 10 mL.

5. Filter solution.

4.3.10 Preparing a 0.5 % methyl cellulose solution

1. Dilute a volume of the 1 % methyl cellulose solution prepared in section 4.3.8 with the same volume of ultrapure water.

4.3.11 Parameter settings

Table 4.6 shows settings for a general CIEF method, whereby special settings for pressure and chemical mobilization are indicated. Table 4.7 shows a separate set of parameter settings for antibody analysis in the pH range 5−8.

Capillary Electrophoresis			
Home values	Cassette temperature	20.0 °C	
	Inlet home vial	6 (anolyte)	Vial locations are exemplary only
	Outlet home vial	7 (catholyte)	
Replenishment	No replenishment used		
Preconditioning	Flush	120 s	Inlet: anolyte
			Outlet: waste
	Flush	240 s	Inlet: ultrapure water
			Outlet: waste
Postconditioning	No postconditioning used		
Electric	Electric	On	
	Polarity	Positive	
	Voltage	15.0 kV	Pressure mobilization
		25.0 kV	Chemical mobilization
	Current	20 µA	
	Power	6.0 W	
	Low Current Alarm Limit	Off	
Injection	Flush	200 s	Inlet: sample
			Outlet: waste
			Overfill of capillary
	Load Inlet Vial	ultrapure water	Wash step
	Load Outlet Vial	ultrapure water	Wash step
	Wait		
Time Entries	Stop time	45.0 min	For pressure mobilization
		60.0 min	For chemical mobilization
	Post time	Off	
Timetable	For pressure mobilization		
	0.1 min	Change voltage	15 kV
	5.9 min	Change voltage	15 kV
	6.0 min	Change voltage	21 kV
	6.0 min	Change pressure	Internal pressure: 48 mbar
	For chemical mobilization		
	0.1 min	Change voltage	25 kV
	14.9 min	Change voltage	25 kV
	15.0 min	Change voltage	30 kV
	15.0 min	Change outlet vial	Outlet vial: acetic acid

Table 4.6 Settings for a general CIEF method, indicating special settings for pressure and chemical mobilization (continued overpage).

Diode Array Detector			
Settings	Stop time	As CE: 45 min (for pressure mobilization)	
		As CE: 60 min (for chemical mobilization)	
	Post time	Off	
	Response time	2.0 s	
	Peak width	0.10 min	
	Prerun autobalance	On	
	Postrun autobalance	Off	
Spectra	All		
Signals	Store	Signal / Bandwidth [nm]	
	A: Yes	270 / 4.0	
	Reference	Off	
Instrument Curves (Store Data)			
Settings	Collect voltage	Yes	
	Collect current	Yes	
	Collect power	No	
	Collect pressure	Yes	
	Collect temperature	No	

Table 4.6 Settings for a general CIEF method, indicating special settings for pressure and chemical mobilization (continued from previous page).

Capillary Electrophoresis			
Home values	Cassette temperature	20.0 °C	
	Inlet home vial	6 (anolyte)	Vial locations are exemplary only
	Outlet home vial	7 (catholyte)	
Replenishment	No replenishment used		
Preconditioning	High pressure flush 3.5 bar	180 s	Inlet: 4.3 mol / L urea
			Outlet: waste
	High pressure flush 3.5 bar	120 s	Inlet: ultrapure water
			Outlet: waste
Postconditioning	High pressure flush 3.5 bar	120 s	Inlet: ultrapure water
			Outlet: waste
Electric	Electric	On	
	Polarity	Positive	
	Voltage	0 kV	
	Current	20 µA	
	Power	6.0 W	
	Low current alarm limit	Off	
Injection	High pressure flush 2.0 bar	100 s	Inlet: sample
			Outlet: waste
			Overfill of capillary
	Load inlet vial	ultrapure water	Wash step
	Load outlet vial	ultrapure water	Wash step
	Wait		
Time Entries	Stop time	30 min	
	Post time	Off	
Timetable	0.17 min	Change voltage	25 kV
	5.00 min	Change voltage	25 kV
	5.01 min	Change voltage	0 kV
	5.02 min	Change outlet vial	Outlet vial: acetic acid
	5.03 min	Change voltage	30 kV
Diode Array Detector			
Settings	Stop time	As CE: 30.0 min	
	Post time	Off	
	Response time	2.0 s	
	Peak width	> 0.05 min	
	Prerun autobalance	On	
	Postrun autobalance	Off	
Spectra	None		

Table 4.7 Settings for the CIEF separation of antibodies in the pI range from 5 to 8 with chemical mobilization (with acetic acid). The high pressure flush is applied to the Inlet in all cases (continued overpage).

Diode Array Detector		
Signals	Store	Signal / Bandwidth [nm]
	A: Yes	280 / 20
	Reference	550 / 100
	B: Yes	280 / 20
	Reference	Off
Instrument Curves (Store Data)		
Settings	Collect voltage	Yes
	Collect current	Yes
	Collect power	No
	Collect pressure	Yes
	Collect temperature	No

Table 4.7 Settings for the CIEF separation of antibodies in the pI range from 5 to 8 with chemical mobilization (with acetic acid). The high pressure flush is applied to the Inlet in all cases (continued from previous page).

4.4 CIEF method examples

4.4.1 CIEF separation of model proteins and peptides covering a pl range from 9.68 to 4.51
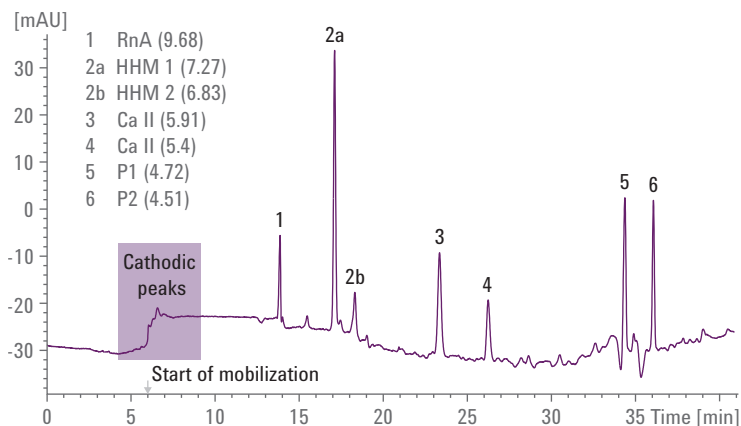

Figure 4.1 CIEF separation of model proteins and peptides covering a pl range from 9.68 to 4.51 (pressure mobilization).

Conditions	
Carrier ampholytes	0.8 % (w / w) PL (3-10)
	0.5 % (w / w) PL (5-6)
Gel	CIEF gel (AB SCIEX)
Spacers	21.4 mM L-arginine
	0.9 mM iminodiacetic acid
pl markers	0.43 mg / mL ribonuclease A (RnA)
	38 μg / mL horse heart myoglobin (HHM)
	36 μg / mL carbonic anhydrase II (Ca II, 5.91)
	45 μg / mL carbonic anhydrase II (Ca II, 5.4)
	10.2 μg / mL P1
	5.4 μg / mL P2
Anolyte	90.9 mM H_3PO_4 with CIEF gel
Catholyte	19.2 mM NaOH
Injection	Flush 200 s with sample
Focusing	6 min, +15.0 kV
Mobilization	48.0 mbar, +21.0 kV
Capillary	23.7 cm, 50 μm id (eCAP, AB SCIEX)
Temperature	20.0 °C
Detection	270 nm, 4 nm bandwidth (without reference)
	260 nm band pass detector filter assembly installed
Scan rate	2.5 Hz

4.4.2 CIEF separation of model proteins and peptides covering a pI range from 7.27 to 4.51
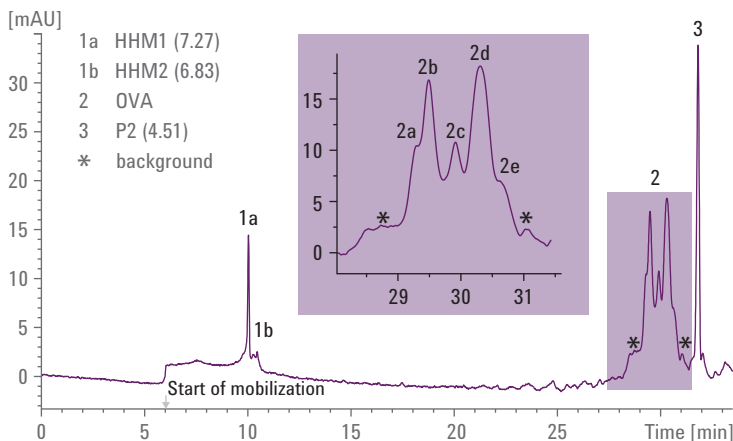

Figure 4.2 CIEF Separation of Model Proteins and Peptides Covering a pI range from 7.27 to 4.51 (pressure mobilization)

Conditions	
Carrier ampholytes	0.8 % (w / w) PL (3-10)
	0.5 % (w / w) PL (5-6)
Gel	CIEF gel (AB SCIEX)
pI marker	38 µg / mL horse heart myoglobin (HHM)
	5.4 µg / mL P2
Analyte	0.52 mg / mL ovalbumin (OVA)
Anolyte	90.9 mM H_3PO_4 with cIEF gel
Catholyte	19.2 mM NaOH
Injection	Flush 200 s with sample
Focusing	6 min, +15.0 kV
Mobilization	48.0 mbar, +21.0 kV
Capillary	23.7 cm, 50 µm id (eCAP, AB SCIEX)
Temperature	20.0 °C
Detection	270 nm, 4 nm bandwidth (without reference)
	260 nm band pass detector filter assembly installed
Scan rate	2.5 Hz

4.4.3 CIEF separation of model proteins and peptides covering a pI range from 7.0 to 4.1
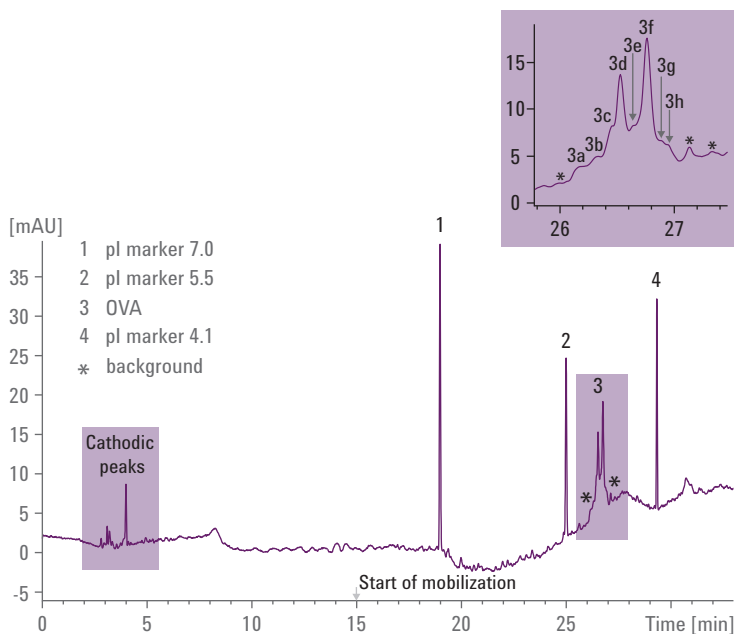

Figure 4.3 CIEF separation of model proteins and peptides covering a pI range from 7.0 to 4.1 (chemical mobilization with acetic acid).

Conditions	
Carrier ampholytes	1.8 % (w / w) PL (3-10)
	0.3 % (w / w) PL (5-6)
Gel	CIEF gel (AB SCIEX)
Spacers	10.7 mM L-arginine
	1.8 mM iminodiacetic acid
pI markers	Peptidic markers with pI 4.1, 5.5 and 7.0 (Beckman Coulter)
Analyte	0.36 mg / mL ovalbumin (OVA)
Anolyte	200 mM H_3PO_4 with CIEF gel
Catholyte	300 mM NaOH
Injection	Flush 200 s with sample
Focusing	15 min, +25.0 kV
Mobilization	Replace catholyte with 22 mM acetic acid, apply +30.0 kV
Capillary	23.9 cm, 50 µm id (eCAP, AB SCIEX)
Temperature	20 °C
Detection	270 nm, 4 nm bandwidth (without reference)
	260 nm band pass detector filter assembly installed
Scan rate	2.5 Hz

4.4.4 CIEF separation of monoclonal antibodies and peptides covering a pl range from 5 to 8
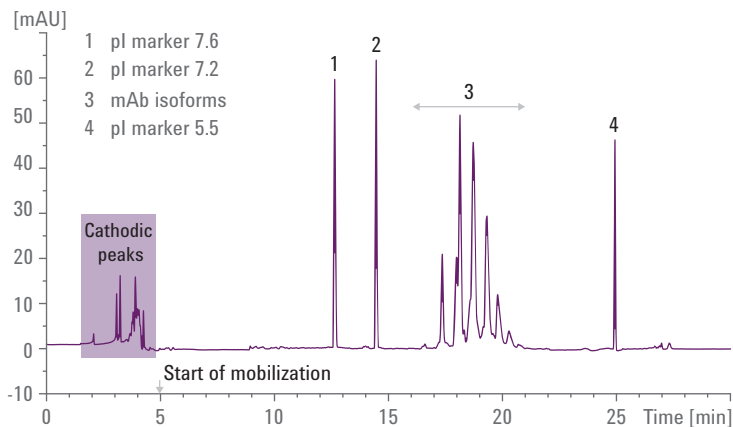

Figure 4.4 CIEF separation of monoclonal antibodies and peptides covering a pl range from 5 to 8 (chemical mobilization with acetic acid).

Conditions	
Carrier ampholytes	2.5 % (w / w) PL (5-8)
Gel	0.5 % methyl cellulose with 2.5 M urea
Spacers	19 mM L-arginine; 8.4 mM iminodiacetic acid
pl markers	Peptidic marker with pl 5.5, 7.2 and 7.6 (Sigma-Aldrich & AB SCIEX)
Analyte	0.092 mg / mL desalted rat anti-DYKDDDDK monoclonal antibody
Anolyte	200 mM H_3PO_4
Catholyte	300 mM NaOH
Injection	High pressure flush at 2 bar for 100 s with sample
Focusing	5 min, +25.0 kV
Mobilization	Replace catholyte with 350 mM acetic acid, apply +30.0 kV
Capillary	24.5 cm, 50 µm id (Agilent µSIL-FC)
Temperature	20 °C
Detection	280 nm, 20 nm bandwidth with reference 550 nm, 100 nm bandwidth 280 nm high pass detector filter assembly installed
Scan rate	5 Hz

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