Capillary isoelectric focusing on the Agilent Capillary Electrophoresis system

Technical Note

Introduction

This publication describes the setup of a capillary isoelectric focusing (cIEF) method to run on the Agilent Capillary Electrophoresis (CE) system. With this method proteins and peptides can be separated according to differences in their isoelectric points (pI). The pI of a protein is an important characteristic and cIEF can be used to either determine a protein's pI or to perform separations based on pI differences[1]. Separations based on pI are used in protein analysis for identification, purity assessment, degradation checking, microheterogeneity analysis, clinical diagnosis, and the analysis of complex protein mixtures. The cIEF method described here is a two step method. First, the capillary is filled with a mixture of protein sample and ampholytes which is then focused by applying a high field strength (V/cm). The ampholytes form a pH gradient in the capillary and the proteins are focused into sharp zones at the pH region which corresponds to their pI. In the second step the contents of the capillary are mobilized past the detector by applying pressure to the inlet vial while maintaining the field strength. Focused peaks are monitored by the detector and appear in order from basic to acidic proteins. TEMED is used as a spacer between the outlet end of the capillary and the detection point so that focusing of the proteins takes place between the capillary inlet and detection point. This enables the detection of very basic proteins. As a starting point for pI determination, we recommend a broad range of ampholytes covering pI values from 3 to 10 (figure 1).

Agilent Technologies

Isoelectric focusing

During the focusing process in cIEF, the protein sample is concentrated many times, with the additional benefit that very low protein concentrations can be detected using UV detection alone.

A disadvantage of the cIEF process is that the protein sample is concentrated to such a degree that the protein precipitates; a protein's solubility can decrease at high concentrations. It is recommended that Triton X-100 (reduced) be included in the sample ampholyte mixture in order to increase protein solubility and to prevent capillary clogging. The risk of protein precipitation is highest at the detection window because this area cannot be thermostatted. The higher temperature in this area may increase protein unfolding which can also cause precipitation. The addition of Triton X-100 impedes this process and ensures more stable runs with this cIEF method.

Hydroxyethylcellulose (HEC) is also included in the protein/ ampholyte mixture at a concentration of 0.4 %. This increases the viscosity of the sample ampholyte mixture and may also increase solubility of proteins. The higher viscosity slows down the speed of mobilization in the second step of the method which improves reproducibility (figure 2) and allows the use of variable mobilization pressures. For example, by decreasing the mobilization pressure from 50 to 25 mbar, the pI resolution can be significantly improved, however, with a longer run time. The increased viscosity also helps to improve peak shape due to decreased diffusion of the proteins.

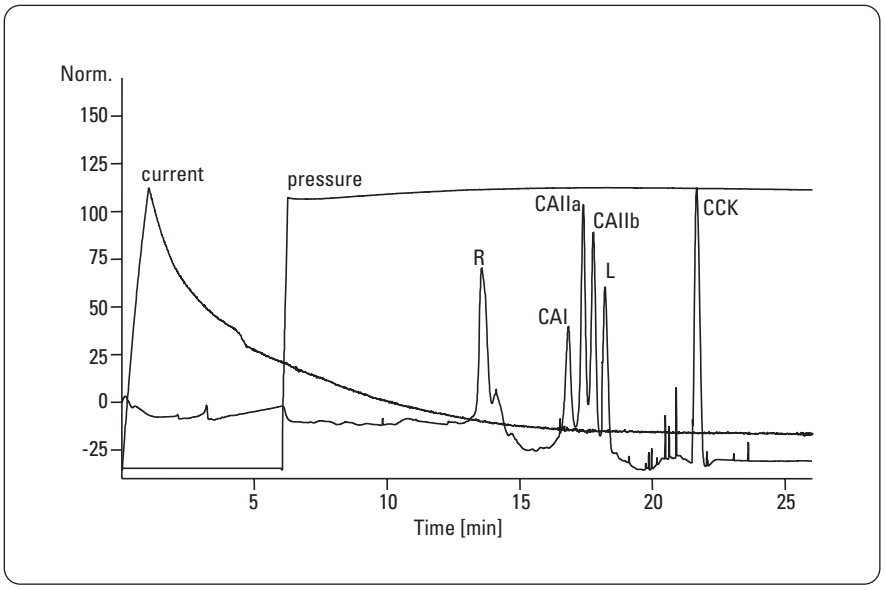

Figure 1
Separation of six proteins: ribonuclease A (R), carbonicanhydrase I (CAI), carbonicanhydrase IIa (CAIIa), carbonicanhydrase IIb (CAIIb), lactoglogulin (L) and CCK flanking peptide. Current and pressure: 50 mbar mobilization pressure.

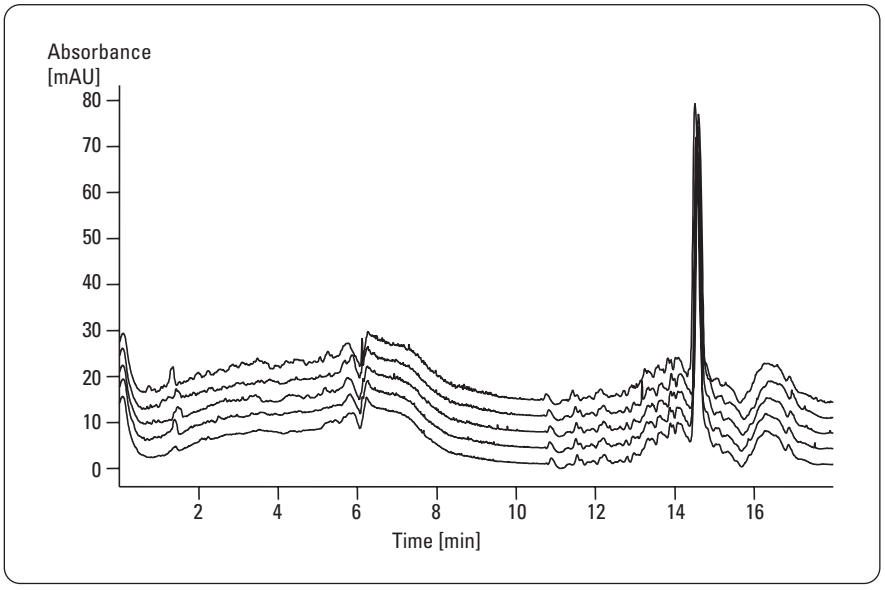

Figure 2
Reproducibility of 5 runs lactoglobulin (0.5 g/L) with % RSD of 0.23 for migration time and % RSD of 1.3 for peak area.

pI determination

The pI of the protein of interest can easily be determined by constructing a calibration plot of pI against migration time of standard proteins (figure 3) or synthetic pI markers. The Agilent ChemStation provides a special type of calibration for cIEF, pI calibration, which can be used to determine the pI of unknown proteins.

Protein quantitation

The cIEF method also offers the possibility to quantitate focused proteins. Figure 4 shows the linearity of the peak area of carbonic anhydrase over a broad range of concentrations. The reproducibility of quantitation was demonstrated in figure 2, where five lactoglobulin runs showed a RSD of 1.3 % for peak area.

Stability

Stability of the cIEF separation was observed for over 200 consecutive runs using standard proteins (see legend of figure 1). However, it should be appreciated that stability depends on the specific properties of the sample. Important factors, such as protein solubility and ionic strength of the sample matrix can vary from sample to sample and can negatively impact the stability of the method.

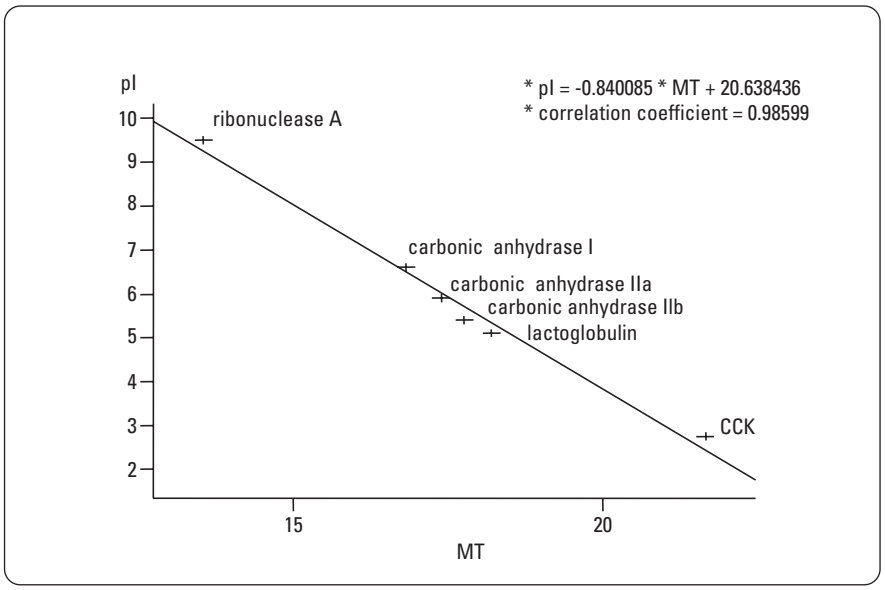

Figure 3
Calibration plot of the protein separation from figure 1.

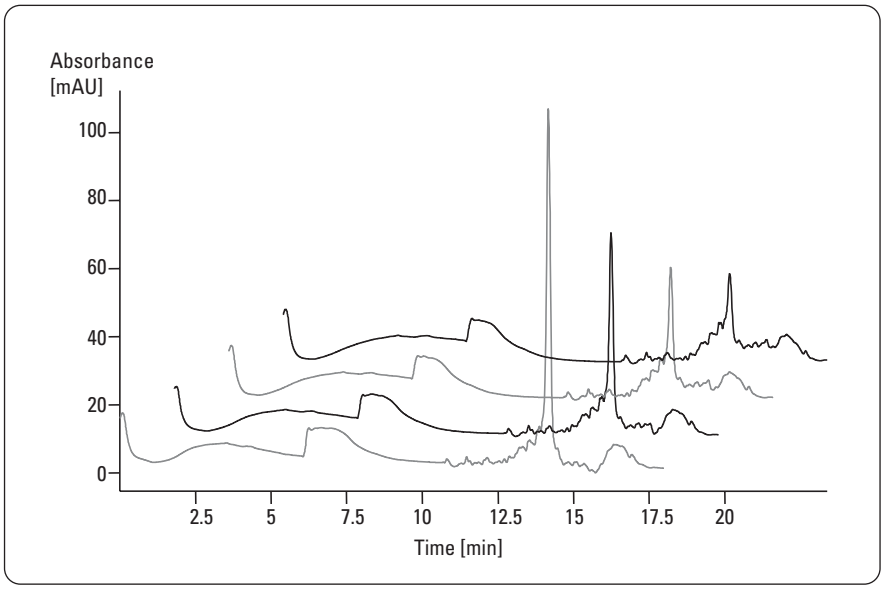

Figure 4
Four concentrations of carbonic anhydrase IIa (500, 250, 125 and 62.5 mg/L) showing linearity of peak area (r^2 = 0.9998) for protein quantification.

Sample considerations

The two major issues in cIEF are high salt concentration and high protein concentrations of the sample. The final protein concentration, which can be injected in the sample/ampholyte mixture, depends on the solubility of protein components and the sensitivity requirements. We recommend using a protein concentration range from 0.5 to 2.5 mg/mL. However, some proteins, for example, immunoglobins, membrane proteins and very hydrophobic or high molecular weight proteins may precipitate even at these low concentrations. CIEF is a concentrating method where a focusing peak increases up to 400 fold in concentration. For such proteins it is highly recommended to start with very diluted protein concentrations to prevent irreversible capillary clogging.

Another important point concerning sample preparation is excessive ionic strength introduced by the sample matrix. The process of isoelectric focusing can be negatively effected by a high concentration of salt or ionic detergents. This can result in the need for increased focusing time and peak broadening during mobilization. Therefore, samples with salt concentrations above 50 mM should be desalted prior to analysis by means of dilution, dialysis, gel- or ultra-filtration (figure 5).

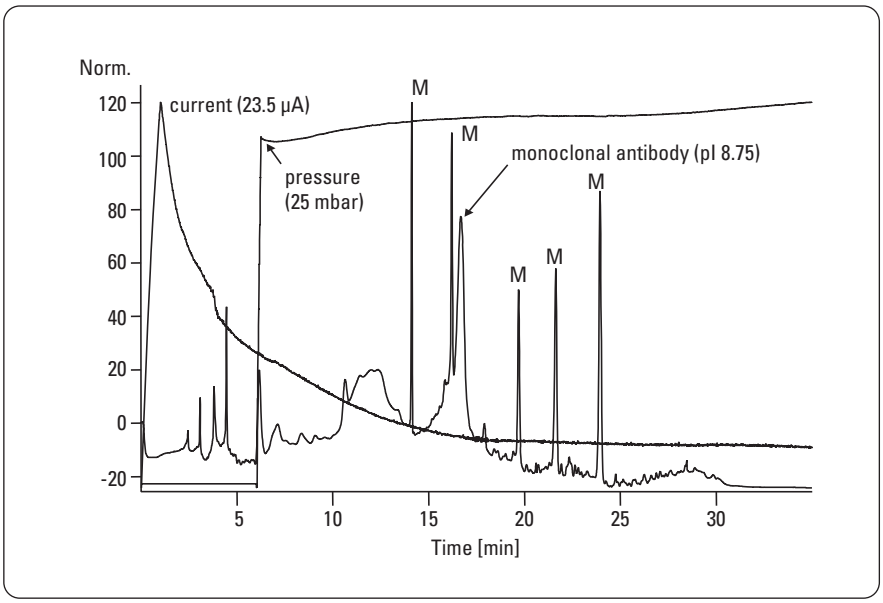

Figure 5
Example for pI determination of an immunoglobin. Synthetic markers (M, BioMark pI 5.3-10.4) were mixed with 0.3 g/L recombinant monoclonal antibody after ultra-filtration. Mobilization pressure was 25 mbar.

Method for capillary isoelectric focusing (cIEF)

HPCE mode
cIEF using PVA coated capillary (the 64.5 cm x 50 µm id capillary which is supplied should be cut to 48.5 cm total length, providingan effective length of 40 cm.)

Home values

Lift offset:	4
Cassette temperature:	25 °C
Inlet home vial:	4 (acidic)
Outlet home vial:	5 (basic)

Conditioning
Serial processing
No replenishment

Preconditioning: use table

1. flush:	3 minutes
I:	2 blank mixture
O:	1 waste

Post conditioning: use table

1. flush:	4 minutes
I:	3 phosphoric acid (10 mM)
O:	1 waste

Injection
Use table

1. flush:	2.00 minutes
I:	Inject vial
O:	1 waste

Electric

Switch electric:	on
Polarity :	positive
Voltage:	0 kV
Power:	system limit
Current:	150 µA
Lower alarm limit of current:	1 µA

Time table

Store voltage:	no
Store current:	yes
Store power:	no
Store pressure:	yes
Store temperature:	no
Stop time:	35 minutes
Post time:	off

Time table

0.0	voltage 0 kV
1.0	voltage 30 kV
6.0	pressure 0 mbar
6.25	pressure 50 mbar

DAD signals

A:	yes 214, 5 nm, ref off
B:	yes 280, 20 nm, ref off
C:	yes 280, 10 nm, ref 290,5 nm
Spectrum:	all in peak
Range:	190 to 600 nm
Threshold:	5 mAU

DAD settings

Stop time as HPCE	35 minutes
Post time:	off
Time table:	DAD is empty
Peak width:	> 0.05 minutes
Response time:	1.0 seconds
Prerun autobalance:	on
Postrun autobalance:	off

cIEF method protocol

1. Cut the 50-µm PVA capillary to 48.5 cm total length and install in cassette and insert into instrument

2. Prepare the "hydroxyethylcellulose-water solution"

a) Fill a bottle with 35 mL CE water and add a stir bar.
b) Place bottle on a heatable magnetic stir plate and heat it to 70°C.
c) Weigh out 1.6 g of hydroxyethylcellulose
d) Add cellulose to the stirring water (add slowly so that no solid clumps are formed).
e) Stir this solution at 70 °C for approximately one hour.
f) Let the dissolved hydroxyethylcellulose come to room temperature and make up to 40 mL with CE water before use.
The hydroxyethylcellulose-water solution can be stored for several days at 2-8 °C.
g) Centrifuge 500 µL of this solution at 14000 g for 10 minutes before use in step 3.

3. Prepare the "ampholyte mixture"

In a 1-mL Eppendorf tube combine and vortex:
• 200 µL ampholyte
• 20 µL TEMED
• 20 µL Triton X-100
• 400 µL hydroxyethylcellulose-water solution (centrifuged 4.0 % stock)
The ampholyte mixture can be stored for several days at 2-8 °C. The mixture is a turbid emulsion and must be vortexed prior to use.

4. Prepare the sample

a) Add up to 84 µL of a 0.5 to 2.5 mg/mL solution of a protein into an Eppendorf tube.
b) Make up with CE water to a volume of 84 µL and gently mix.
c) Add an aliquot of 16 µL of the prepared ampholyte mixture and mix it gently by pippeting up and down several times.
d) Vortex for 10 seconds and centrifuge at 14000 g for 10 minutes.
e) Remove the centrifugate to a sample microvial and place this into the sample carousel.

5. Prepare catholyte (22.5 mM NaOH, 0.4 % HEC)

This is prepared from 0.1 M NaOH and the hydroxyethylcellulose-water solution.
a) Fill a 100 mL graduated flask with 50 mL CE water.
b) Aliquot 22.5 mL of the 0.1 M NaOH solution into a 100 mL graduated flask.
c) Add 10 mL of the hydroxyethylcellulose-water solution.
d) Make up to the 100 mL mark with CE water.
e) Add stir bar and stir for 20 minutes.
f) Filter this solution (0.45 µm) before use.

6. Prepare anolyte (3.3 mM H_3PO_4, 0.4 % HEC)

This is prepared from 0.1 N phosphoric acid and the hydroxyethylcellulose-water solution.
a) Fill a 100 mL graduated flask with 50 mL CE water.
b) Add 10 mL of the 0.1 M H_3PO_4 solution into a 100 mL graduated flask.
c) Add 10 mL of the hydroxyethylcellulose-water solution.
d) Make up to the 100mL mark with CE water.
e) Add stir bar and stir for 20 minutes.
It is important to filter this solution (0.45 µm) before use.

7. Prepare acidic wash (3.3 mM H_3PO_4)

This is prepared from the supplied 0.1 N phosphoric acid.
a) Fill a 100 mL graduated flask with 50 mL CE water.
b) Add 10 mL of the 0.1 N H_3PO_4 solution into a 100 mL graduated flask.
c) Make up to the 100 mL mark with CE water.

8. Prepare blank mix

a) Add 840 µL of water into an Eppendorf tube.
b) Add a aliquot of 160 µL of the prepared ampholyte mixture and vortex.
c) Vortex for 10 seconds and centrifuge at 14000 g for 10 minutes or filter through 0.45 µm.

9. Vial positions

Anolyte 4
Catholyte 5
Acidic wash 3
Blank mix 2
Waste (100 µL water) 1

PVA capillary treatment

Prior to first use, equilibrate the PVA capillary by flushing it for 10 minutes with acidic wash solution. We further recommend running one to three injections of a blank mix (step 8 on previous page) for capillary equilibration.

Cleaning procedure

After use and for long term storage we recommend the following cleaning procedure for the PVA capillary: Flush the PVA capillary for 10 minutes with CE-water and then 10 minutes with air from a capped empty vial. This can easily be set up as a short method which can be added to the end of a sequence.

Conclusion

This Techical Note shows a setup for running a cIEF method on an Agilent CE system. The two step format of the method and the use of PVA coated capillaries facilitated a stable and reproducible performance. The application of a wide range ampholyte mixture enabled pI determination and quantitation of proteins and peptides in pI range from 3 to 10.

Description	Recommended supplier	Quantity	Part number
Ultra pure water for CE	Agilent Technologies	1 (500 mL)	5062-8578
0.1 N NaOH	Agilent Technologies	1 (250 mL)	5062-8575
0.1 N Phosphoric acid	Agilent Technologies	1 (250 mL)	5062-8577
PVA coated capillary			
50 µm x 64.5cm (56 cm effective)	Agilent Technologies	1 (2 p/pk)	G1600-61219
Hydroxyethylcellulose	Merck	1 (100 g)	822068
TEMED *(BioChemika)*	Fluka	1 (10 mL)	87689
Triton X-100 reduced	Sigma	1 (5 g)	X100RS-5G
Servalyt 3-10	Serva	1 (10 mL)	42940.01

Table 1
Parts required to run cIEF on the Agilent Capillary Electrophoresis system.

Notes:
Catholyte and analyte can be used for about five runs before replacement. Replenishment can not be used for catholyte and analyte, due to their high viscosity. To check for method or capillary performance we recommend running a standard protein (for example, carbonic anhydrase 0.5 mg/mL) or synthetic pI markers after sample runs.

References

1.
T. Wehr, R. Rodriguez-Diaz and M. Zhu, "Capillary Electrophoresis of Proteins", *Chromatographic Science Series, Marcel Dekker Inc., New York, Vol. 80,* **1999.**

www.agilent.com/chem/ce

Published November 1, 2008
Publication Number 5989-9852EN

Agilent Technologies