A Holistic Approach to Understanding cIEF

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INTRODUCTION

There are multiple applications for cIEF in the biotechnology industry. These include as determination of protein charge heterogeneity, stability of formulations, determination of lot consistency, and purity assessment. This poster discusses critical variables to consider for cIEF separation and describes optimization of these variables for maximum resolution and reproducibility.

EXPERIMENTAL SET-UP

All cIEF separations were carried out using PA 800 Series capillary electrophoresis system (SCIEX, Fullerton, CA), each equipped with a UV detector and 280 nm filter. Separations were performed using neutral capillaries (SCIEX, P/N 477441, 50 µm i.d., 375 µm o.d., 30.2/20.0 cm long) and carried out at 20˚ C, unless otherwise indicated.

VARIABLES IN cIEF

Mobilization Technique

In cIEF, detection is achieved by mobilizing the pH gradient across the capillary window using a variety of strategies including chemical and pressure mobilization. Chemical mobilization offers higher peak efficiency and resolution than pressure mobilization due to the absence of laminar flow (Figure 1).

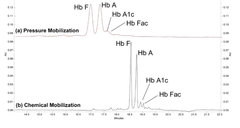

Figure 1. Separation of hemoglobins A and F by cIEF using (a) pressure and (b) chemical mobilization. Sample: 100 µL of cIEF gel (Beckman Coulter, P/N 477497), 2.0 µL of hemoglobin AF, 6.0 µL of Pharmalyte* 5-8 carrier ampholytes (GE Healthcare, P/N 17-0453-01), 9.0 µL of 0.5 M arginine (Arg) and 4.0 µL of 0.2 M iminodiacetic acid (IDA). Anolyte: 200 mM phosphoric acid. Catholyte: 300 mM sodium hydroxide. Focusing: 7.5 min at 25 kV. Pressure mobilization: 0.7 psi at 30 kV for 20 min. Chemical mobilization: 25 min at 30 kV using 350 mM acetic acid at cathodic side.

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Aperture in Capillary Cartridge

The detection aperture has an effect on the measurement of peak width. Peak efficiency and resolution increase with decreasing peak width (Figure 2).

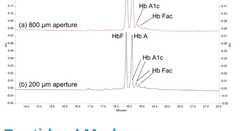

Figure 2. Separation of hemoglobins A and F by cIEF using chemical mobilization and different apertures: (a) 800 and (b) 200 µm. Experimental conditions are as described in the caption for Figure 1.

Peptide pI Markers

The use of synthetic oligopeptides as pI markers increases the precision and accuracy of the pI determination since they are not complicated with post-translational modifications as proteins can be. The peptides used in this work are listed on Table 1. In a previous work [Shimura et al, Electrophoresis 21, 603 (2000)], 8.40a and 8.40b peptide markers could not be separated. However, we were able to resolve them by cIEF (Figure 3).

pI Marker	Amino Acid Sequence
99.9	H-Trp-Tyr-Lys-Lys-OH
9.50	H-Trp-Tyr-Tyr-Lys-Lys-OH
8.40 a	H-Trp-Glu-Tyr-Tyr-Lys-Lys-OH
8.40 b	H-Trp-Tyr-Lys-OH
7.00	H-Trp-Glu-His-Arg-OH
6.66	H-Trp-Glu-His-His-OH
5.52	H-Trp-Glu-His-OH
4.05	H-Trp-Asp-Asp-Arg-OH
3.4	H-Trp-Asp-Asp-Asp-OH

Table 1. Amino acid sequence and pI values of peptides used as pI markers.

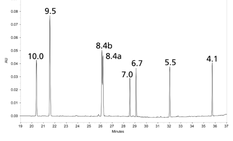

Figure 3. Separation of synthetic peptide pI markers by cIEF. Sample: 100 µL of 3 M urea-cIEF Gel, 2.0 µL of each peptide (1.25 mM), 12.0 µL of Pharmalyte 3-10 carrier ampholytes, 20 µL of 0.5 M Arg and 2.0 µL of 0.2 M IDA. Focusing was 15 min at 25 kV. Chemical mobilization was 20 min at 30 kV using 350 mM acetic acid. Anolyte and catholyte were as described in Figure. 1.

Concentration of cIEF reagents

The anolyte, catholyte and chemical mobilization solutions must be at a high enough concentration to ensure their conductivity values are stable in repeated use. High conductivity reduces variations in detection time (Figure 4), and can reduce isotachophoresis (ITP) distortions in the pH gradient.

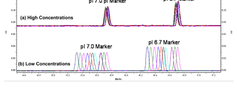

Figure 4. Twelve consecutive separations of two peptide markers (pI 7.0 and pI 6.7) by cIEF at (a) high and (b) low concentrations of anolyte, catholyte and chemical mobilization solution. Sample: 100 µL of cIEF gel, 4 µL Fluka 3-10 carrier ampholytes with 6 % TEMED, and 2.0 µL of each peptide pI marker. Focusing was 6 min at 25 kV. Chemical mobilization was 24 min at 30 kV. Concentrations: (a) High: 200 mM phosphoric acid (anolyte), 300 mM sodium hydroxide (catholyte) and 350 mM acetic acid (chemical mobilization solution); and (b) Low: 91 mM phosphoric acid (anolyte), 20 mM sodium hydroxide (catholyte) and 20 mM acetic acid (chemical mobilization solution).

Focusing Time

Because the cIEF gradient is homogeneous at the start of the separation, the focusing time must be sufficient to allow the cathodic and anodic peaks for each component to merge. Failure to achieve complete focusing will result in partial detection and/or unmerged sample peaks (Figure 5).

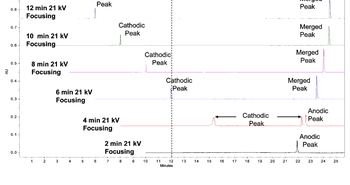

Figure 5. Separations of peptide pI 8.40a marker by cIEF using different focusing times. Experimental conditions were as described in the caption of Figure 4, except that pI 8.40a was the only peptide present in the sample and focusing was performed at 21 kV.

Some regions of the pH gradient focus faster than others. When using Pharmalyte 3-10 carrier ampholytes, the acidic region is completely focused after 5 minutes, whereas the basic region requires 10 minutes of focusing. Extension of the focusing time to 15 minutes results in the loss of the pI 3.4 marker to ITP (Figure 6).

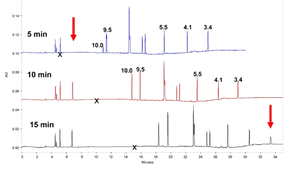

Figure 6. Separations of peptide markers by cIEF using different focusing times. Sample: 100 µL of cIEF gel, 12 µL Pharmalyte 3-10 carrier ampholytes, 18 µL of 0.5 M Arg, 4 µL of 0.2 M IDA, and 2 µL of each peptide pI marker at 1.25 mM. Focusing was at 25 kV. Chemical mobilization was at 30 kV for 30 min.

Concentration of cIEF reagents

The loss and distortion of the pH gradient due to ITP can be prevented by using stabilizers, which are chemical compounds with pI values at the extremes of the pH gradient. These compounds act as buffering zones protecting the pH gradient from ITP distortions. Stabilizers permit the use of longer focusing times thus preventing the loss of resolution. Arg (pI 10.7) has been used successfully as cathodic stabilizer, and IDA (pI 2.2) used as anodic stabilizer. The amount of cathodic stabilizer within the sample needs to be optimized to ensure the sample is focused prior to reaching the detection window (Figure 7). In addition, the amount of cathodic stabilizer has been found to affect resolution and linearity (Table 2).

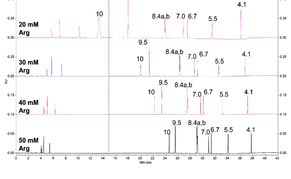

Figure 7. Separations of peptide markers by cIEF using different Arg concentrations within the sample. Experimental conditions were as described in Fig. 6, except chemical mobilization was for 30 min.

[Arginine] (mM)	Resolution	Linearity
20	1.02	0.9639
30	1.01	0.9881
40	0.96	0.9893
50	0.73	0.9892

Table 2. The effect of Arg concentration on cIEF resolution of the pI 8.4a and 8.4b peptide markers and in the linearity of the pH 4-10 gradient. The optimum concentration was found to be 40 mM Arg.

Concentration of cIEF reagents

Proteins can aggregate and precipitate as they are focused near and at their pI values. Protein precipitation and aggregation can be minimized by adding a protein solubilizer, such as urea, to the cIEF sample (Figure 8). Reproducible current profiles can be obtained by using urea in the cIEF sample and by rinsing with 4.3 M urea solution between runs (Figure 9).

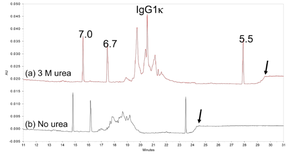

Figure 8. Separations of mouse IgG1κ and three peptide pI markers (7.0, 6.7 and 5.5) by cIEF: (a) with and (b) without 3 M urea in the sample. Sample: 100 µL of cIEF gel with and without 3 M urea, 6.0 µL of Pharmalyte 5-8 carrier ampholytes, 2.0 µL of each pI marker, 9.0 µL of 0.5 M Arg, 5.0 µL of 0.2 M IDA, and 10.0 µL of desalted IgG. Focusing was 5 min at 25 kV. Chemical mobilization was 30 min at 30 kV. Anolyte: 200 mM phosphoric acid. Catholyte: 300 mM sodium hydroxide. Chemical mobilization solution: 350 mM acetic acid. Black arrow indicates the boundary between carrier ampholytes and anodic stabilizer.

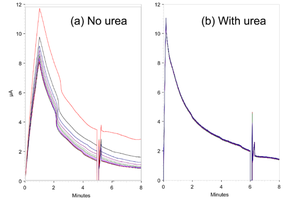

Figure 9. Electrical current profiles of cIEF separations when (a) not rinsing and (b) rinsing with urea between runs. Separations were carried out as described in the caption for Figure 8 using chemical mobilization, but with (a) 5 min and (b) 6 min of focusing.

The urea concentration needs to be optimized for each sample analyzed by cIEF. For example, the use of urea has been found to be detrimental to the cIEF analysis of metalloproteins, such as transferrin and hemoglobin (poster entitled "The Effects of Urea Concentration on cIEF Analysis of IgG1κ and other Proteins," Scott Mack et al., Beckman Coulter, presented at CE Pharm 2007). Other proteins, such as erythropoietin (EPO), require 6 M urea in order to minimize their aggregation and precipitation (poster entitled "Innovating cIEF at the Extreme," Scott Mack et al., Beckman Coulter, presented at CE Pharm 2008).

Wide- vs. Narrow-Range Carrier Ampholytes

The choice of carrier ampholytes can affect resolution in cIEF. Resolution can be increased by the use of narrow-range ampholytes, after optimization of other cIEF variables (Figure. 10).

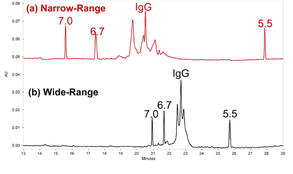

Figure 10. Separation of mouse IgG1κ and three pI markers (7.0, 6.7 and 5.5) by cIEF using (a) narrow-range ampholytes, Pharmalyte 5-8 carrier ampholytes; and (b) widerange ampholytes, Pharmalyte 3-10 carrier ampholytes. Experimental conditions are (a): as described in Fig. 8 with 3 M urea in the sample and (b): Sample: 100 µL of 3 M urea-cIEF gel, 12 µL of Pharmalyte 3-10 carrier ampholytes, 1.0 µL of each pI marker, 7.5 µL of 0.5 M Arg, 2.0 µL of 0.2 M IDA, and 10.0 µL of desalted IgG. Focusing was 10 min at 25 kV. Chemical mobilization was 25 min at 30 kV.

Separation Temperature

Separation temperature can have significant effect on the cIEF resolution of IgG1κ (Figure 11). The IgG peaks D, E, and F were not as well resolved when the temperature was > 25˚ C. However, IgG peak A was better defined at temperatures above 15˚ C. Overall detection time decreased with increasing temperature due to compression of the pH gradient resulting in the loss of resolution.

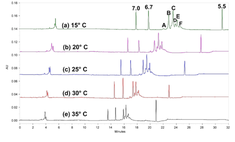

Figure 11. Separation of mouse IgG1κ by cIEF at different separation temperatures: (a) 15, (b) 20, (c) 25, (d) 30 and (e) 35 ˚ C. IgG peaks are labeled A - F. Separation conditions are as described in the caption for Figure 8 with 3 M urea in the cIEF sample. IgG1κ peaks are labeled A-F.

RESULTS OF AN INTERMEDIATE PRECISION STUDY

An intermediate precision study was carried out on the separation of mouse IgG1κ by cIEF. Experimental conditions were as described in Figure 11 at a separation temperature of 20˚ C. This study was performed by 4 operators using 5 PA 800 Plus Systems, 4 lots of neutral capillaries and 2 lots of Pharmalyte 5-8 carrier ampholytes over a period of 8 days. Figure 12 shows peak integration. The results of the study are summarized in Table 3.

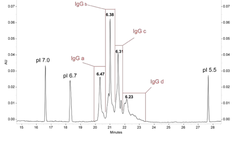

Figure 12. Peak integration of mouse IgG1κ separated by cIEF.

n = 48

Calculated pI

Peaks	Average	Std Dev	CV
IgG a	6.47	0.01	0.13%
IgG b	6.38	0.01	0.13%
IgG c	6.31	0.01	0.14%
IgG d	6.23	0.01	0.17%

Isoform Group Percent Composition

Group	Average	Std Dev	CV
IgG a	16.29%	0.57%	3.49%
IgG b	31.80%	1.03%	3.25%
IgG c	29.52%	0.82%	2.76%
IgG d	22.39%	1.21%	5.42%

Table 3. Results of an intermediate precision study of the separation of mouse IgG1κ by cIEF.

CONCLUSIONS

Separation of proteins by cIEF can be achieved with high resolution, reproducibility and robustness by optimizing key variables. These variables include: focusing time, concentration of pH gradient stabilizers, amount of protein solubilizer, separation temperature, and the type of the carrier ampholytes used.
Understanding the effect of each variable on the cIEF separation is critical when developing and troubleshooting methods.
Note: For Research Use Only; not for use in diagnostic procedures.

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