What Every Analytical Chemist Should Know about CE-MS

Summer Teaching Series 2019
Part 1

Scott Mellors
You should leave here understanding these things:

- CE is really simple
- CE separates molecules based on *mobility*
  - A function of molecular charge/size
- CE column diameters have to be small because of Joule Heating
- CE has no fundamental speed limit or size limit!
- CE + MS is a great match, but coupling is a little tricky
- Surface chemistry is very important
Capillary Zone Electrophoresis

James W. Jorgenson and Krynn DeArman Lukacs

Electrophoresis has developed into a powerful technique for the separation and analysis of charged substances, especially biopolymers. In large part, the success of modern electrophoresis rests on the effective utilization of media such as polymer gels. These gels stabilize the separation medium against convection and flow, which would otherwise disrupt separations. A large part of the science of modern electrophoresis is devoted to understanding and controlling the formation of these gels (1, 2). Still, electrophoresis as commonly practiced would not be considered an instrumental method of analysis. True instrumentally

versions of electrophoresis analogous to modern column chromatography are rather rare. In part, this can be traced to the essential role of stabilizing gels in electrophoresis. Because of the presence of gels, the method has not been easily adapted to on-line sample application, detection, quantification, or automated operation. Instead, modern electrophoresis is a powerful and yet manual-intensive methodology.

Instrumental versions of electrophoresis have been developed. Among these is the "rotating tube" system of Hjertén (3) and the "transient-state electrofocusing" technique of Catsimpoolas (4) are particularly notable. These techniques, although novel and quite powerful, have failed to come into routine use due to their complexity. Capillary isoelectrofocusing is probably the only instrumental version of electrophoresis to see extensive application, although here, too, acceptance has been slow. The unconventional format of data output in isoelectrofocusing, coupled with the fact that it appears better suited for separations of relatively small molecules, is the probable reason for its slow acceptance (5).

In the course of considerations of causes for zone broadening in zone electrophoresis, it occurred to us that an "open" capillary tube—that is, one containing buffer without stabilizer—offered a unique and simple situation in which to study electrophoresis in such a system electrophoresis could be studied with minimal interferences, and at the same time causes of zone broadening could be

\[ r = \frac{L^2}{\nu} \]  

where \( r \) is the solute's migration time, \( L \) is the tube length, \( \nu \) is the solute's electrophoretic mobility (electrophoretic velocity in an unit electric field), and \( V \) is the applied voltage. The separation efficiency, in terms of the total number of theoretical plates, \( N \), is

\[ N = \frac{\mu V}{2D} \]  

where \( D \) is the solute's diffusion coefficient. These two equations are the basis of some interesting predictions. First, high separation efficiencies are best achieved through the use of high voltages. Electrophoretic mobilities and diffusion are in open tubular capillaries. A buffer-filled capillary is suspended between two reservoirs filled with buffer. Samples are introduced at one end and, under the influence of an applied electric field, migrate toward the other end of the capillary. Just before leaving the capillary, sample zones migrate through a detector, which senses their passage, yielding a recording of detector response versus time which is analogous to a chromatogram but is called an electrophogram. Because of the parallelism between this system and a chromatographic system, it is natural to borrow such concepts as migration time (retention time), theoretical plates, and resolution from chromatography.

In capillary zone electrophoresis, the migration time for a solute is given by

\[ \mu = \frac{V}{r} \]  

where \( \mu \) is the mobility, \( V \) is the applied voltage, and \( r \) is the migration time. The separation efficiency is given by

\[ N = \frac{\mu V}{2D} \]  

where \( D \) is the diffusion coefficient and \( \mu \) is the electrophoretic mobility.

The flow velocity is constant over most of the tube cross section and drops to zero only near the tube walls (1). This flow velocity is found to be a factor in the flat flow profile of electrophoresis which will add the same velocity component to all solutes, regardless of their radial position, and will thus not cause any significant dispersion of the zone. The more familiar parabolic laminar flow profile, such as occurs in capillaries in ordinary hydraulic flow, would lead to serious zone spreading. Electrophoretic flow does, however, modify the equations for migration time and separation efficiency.

The migration time becomes

\[ r = \left( \frac{\mu + \mu_{\text{wall}}}{2} \right) V \]  

and the separation efficiency is now

\[ N = \frac{(\mu + \mu_{\text{wall}}) V}{2D} \]  

where \( \mu_{\text{wall}} \) is the electrophoretic mobility of the wall, \( \mu \) is the electrophoretic mobility of the solute, and \( V \) is the applied voltage. The only effect of rapid electroosmotic flow is to sweep all solutes quickly through the capillary, leaving little time for zones to separate. The resulting zones will be sharper (increased theoretical plates) but more poorly resolved. It is resolution of zones that we ultimately wish to accomplish. Following the approach of Giddings (12), we have derived an equation to predict the resolution of two zones in capillary zone electrophoresis.

\[ R_s = \frac{0.177 (\mu + \mu_{\text{wall}})^2}{D (\mu + \mu_{\text{wall}})} \]  

where \( R_s \) is the resolution, \( \mu \) and \( \mu_{\text{wall}} \) are the electrophoretic mobilities of the two solutes, and \( \mu_{\text{wall}} \) is their average mobility (10). From this equation it is clear that the resolution of two zones will be poorer if there is a large component of electroosmotic flow in the same direction as electrophoretic migration. In fact, good resolution of substances having very similar mobilities can be achieved by balancing electroosmotic flow against electrophoretic migration (13, 14). The cost of this approach is long analysis time. This is readily apparent by referring to Eq. 3 and assuming that the electrophoretic flow coefficients and the electrophoretic mobility are equal in magnitude but opposite in sign (direction).

Description of System

A schematic diagram of the system we use to perform electrophoresis in capillaries is shown in Fig. 1. In almost all cases the strong electroosmotic flow carries solutes, regardless of charge, toward the negative (grounded) electrode. For this reason samples are usually introduced at the positive (high-voltage) end, and a detector capable of sensing zones within the capillary (on-column detector)
CE Basics

- Capillary Zone Electrophoresis (CZE)
  - About the simplest method you could imagine!

\[ \mu_{EP} = \frac{q}{6\pi\eta a} \]

- More charge = faster migration
- Bigger hydrodynamic radius = slower migration

CE Basics - Definitions

- Electropherogram
- Migration time
- Background Electrolyte (BGE)
- Field strength
- Mobility

The CE equivalent of a chromatogram
CE Basics - Definitions

- Electropherogram
- Migration time ($t_m$)
- Background Electrolyte (BGE)
- Field strength
- Mobility

The CE equivalent of retention time

$t_m(l) = 1.32$ minutes
CE Basics - Definitions

- Electropherogram
- Migration time
- Background Electrolyte (BGE)
- Field strength
- Mobility

BGE is just the liquid that fills the CE column. For CE-MS this liquid is often not technically buffered, so calling it a buffer is not accurate. It’s the CE equivalent of a mobile phase.
CE Basics - Definitions

- Electropherogram
- Migration time
- Background Electrolyte (BGE)
- Field strength \((E)\)
- Mobility

\[ E = \frac{(V1-V2)}{L} \text{ (Volts/cm)} \]
CE Basics - Definitions

- Electropherogram
- Migration time
- Background Electrolyte (BGE)
- Field strength ($E$)
- Mobility ($\mu$)

Mobility defines the velocity of a molecule as a function of field strength:

$$E = \frac{(V_1 - V_2)}{L} \text{ (Volts/cm)}$$

Velocity ($v$) = $\frac{L}{t_m}$ (cm/s)

$$v = \mu E$$
A Quick Example

L = 22 cm
E = 500 V/cm

• $t_m = 6.05 \text{ m} = 363 \text{ s}$
• $v = L/t_m = 22 \text{ cm} \div 363 \text{ s} = 0.061 \text{ cm/s}$
• $v = \mu E$, so
• $\mu_1 = v/E = 0.061 \text{ cm/s} \div 500 \text{ V/cm} = 1.21 \text{ E}-4 \text{ cm}^2/\text{Vs}$

+K • $t_m = 351.6 \text{ s}$
• $v = 0.0626 \text{ cm/s}$
• $\mu_2 = 1.25 \text{ E}-4 \text{ cm}^2/\text{Vs}$

This is a CE-MS separation of a monoclonal antibody. The +K peak is identical to the main peak except it has an additional lysine. That gives it an extra positive charge, which increases its mobility.
Why does it matter if CE is done in a narrow capillary?

- The CE column is a resistor in an electrical circuit
- All the electronics you need to know:
  - \( V = IR \) \( \text{voltage} = \text{current} \times \text{resistance} \)
  - \( P = IV \) \( \text{power} = \text{current} \times \text{voltage} \)
- From that you can combine to see that:
  - \( P = \frac{V^2}{R} \)
- In a CE separation:
  - Too much heat in the column is bad
  - Resistance scales inversely with the square of the column diameter, so
  - Power (and therefore heat generation) scales with \( d_c^2 \)
  - Heat dissipation scales with \( \frac{1}{d_c} \)
- The take home message: **Smaller diameter capillaries generate way less heat and are more efficient at dissipating that heat**
- Most CE is done with column diameters in the range of 30 µm to 75 µm
**CE Basics – Separation Efficiency**

- Better Separation
- Faster
Van Deemter Curve for Liquid Chromatography

\[ H = H_A + H_B + H_C \]

- A term = Multi flow path
- B term = Longitudinal diffusion
- C term = Resistance to mass transfer
CE Basics – Separation Efficiency

Van Deemter Curve for CE versus LC

\[ H_{LC} = H_A + H_B + H_C \]
\[ H_{CE} = H_B \]
Protein vs small molecule for LC

Proteins have very slow diffusion coefficients (D), so they need to be run very slowly in LC columns to achieve good efficiency. That means very long run times.

D = 1e-6 cm²/s

D = 1e-5 cm²/s
Because CE has no C-term, slow diffusion improves the separation efficiency and does not restrict speed. Faster is still better.

**Protein vs small molecule for CE**
CE Basics – Separation Efficiency – Key Points

- Band-broadening is simpler for CE than for LC
- Good CE separations are “diffusion limited”
- Faster is better for CE!
- Bigger is better for CE!
Can we predict the efficiency of CE separations?

Theoretical Plates (N) = \( \frac{L^2}{\sigma^2} \)

Diffusion limited separation means that \( \sigma^2 = 2Dt_m \)

\( v = \frac{L}{t_m} \)

\( v = \frac{\mu E}{\mu \Delta V/L} \)

\( N = \frac{L^2}{2Dt_m} \)

\( N = \frac{vL}{2D} \)

\( N = \frac{\mu E L}{2D} \)

\( N = \frac{\mu \Delta V}{2D} \)

- Column length doesn’t matter!
- To separate better, just turn up the voltage!

Efficiency is only a function of mobility, diffusion coefficient and voltage applied
Why is it hard to couple CE with MS?

Why can’t we just do this?

- CE current = microAmps
- ESI current = nanoAmps

The electrical current of the CE separation is much greater than the current of the electrospray. So we need an electrode connected to the end of the separation column to complete the circuit.
Why is it hard to couple CE with MS?

Why can’t we just do this?

DEAD VOLUME!

- 10 cm x 30 μm i.d.
- Column volume = 70 nL
- \( u = \frac{10 \text{ cm}}{80 \text{ s}} = 0.125 \text{ cm/s} \)
- Peak width = 1 s → 1.25 mm
- Volume of bands = 0.88 nL
**What's a typical peak volume for LC?**

- **Nano-LC**
  - 75 $\mu$m i.d. capillary column run at 300 nL/min (5 nL/s)
  - Peak width $\sim$10 s
  - Band volume = 50 nL

- **UPLC**
  - 1 mm i.d. column run at 100 $\mu$L/min (1.67 $\mu$L/s)
  - Peak width $\sim$5 s
  - Band volume = 8.3 $\mu$L = 8300 nL

Even the smallest LC columns generate peak volumes that are much bigger than good CE separations. So fittings that could be used to attach a spray tip to an LC column would ruin a CE separation.
The fundamental challenge for coupling CE with MS

• We need a junction to terminate the CE circuit, but...
• The column has to be very narrow to prevent joule heating, and...
• The separation is super efficient, so the peaks are very narrow, so...
• We can’t afford to have any dead volume in the flow path, or else we’ll ruin the great separation and defeat the whole purpose!!!

• So how do we create the junction without introducing too much dead volume?
Early attempts at CE-MS

Capillary Zone Electrophoresis–Mass Spectrometry Using an Electrospray Ionization Interface

Richard D. Smith,* José A. Olivares,† Nhung T. Nguyen, and Harold R. Udseth
Chemical Methods and Separations Group, Chemical Sciences Department, Pacific Northwest Laboratory, Richland, Washington 99352

Instrumentation developed for capillary zone electrophoresis–mass spectrometry (CZE–MS) is described. The interface is based upon direct electrospray ionization from the end of the CZE capillary. The electrospray ionization source functions at atmospheric pressure and provides excellent sensitivity for wide ranges of compounds, with detection limits generally in the femtomole range (although significant improvements appear feasible). The instrumentation allows the high separation efficiencies feasible with CZE to be exploited and offers potential advantages compared with LC–MS methods, particularly when only small samples are available or high-resolution separations are necessary. The performance of the electrospray interface and the techniques and operating conditions for CZE–MS separations are described. CZE–MS separations and mass spectra are shown for mixtures that include polypeptides and quaternary ammonium salts. Separation efficiencies and detection limits vary widely from compound to compound and are shown to be sensitive to buffer selection. Separation efficiencies exceeding half a million theoretical plates are demonstrated for some compounds. Wider application and improved performance are anticipated with minimization of CZE band spread (due to adsorption and possibly other processes) and optimization of CZE buffers (for both the separation and their compatibility with electrospray ionization).

detection methods (UV and fluorescence). Clearly, however, if detection limitations could be addressed CZE would provide a powerful analytical tool for a wide range of problems, particularly where only extremely small samples are available or where high separation efficiencies are required.

In a recent communication we described the first on-line combination of CZE with mass spectrometry (5), which also represented the first reported direct combination of any electrophoretic separation technique with mass spectrometry. This development was based upon the recognition that both ends of the CZE capillary did not have to be immersed in buffer reservoirs and provided a basis for new detection methods in which the electrosprayedly induced flow could be analyzed at the column exit. The strong electrosprayed flow in CZE, which results from the substantial $f$ potential of most suitable capillary surfaces, is sufficiently large under many conditions to result in elution of ions with both positive and negative electrophoretic mobilities. In our initial work it was shown that an electrospray could be produced at the capillary terminus, providing the basis for a viable CZE–MS interfacing method (5). Here we report details of the CZE–MS interface and describe new instrumentation and methods. The application of electrospray ionization (ESI) in CZE–MS is described, typical spectra and CZE–MS separations are presented, and current limitations related to both CZE and ESI are discussed. Future approaches to realizing the impressive potential of CZE–MS are brieﬂy described.

EXPERIMENTAL SECTION

Metal coatings applied to the surface of the capillary terminus solve the CE-MS junction problem, but they are not stable enough to be a practical solution.
Optimization of Capillary Zone Electrophoresis/Electrospray Ionization Parameters for the Mass Spectrometry and Tandem Mass Spectrometry Analysis of Peptides

M. A. Moseley* and J. W. Jorgenson
Department of Chemistry, University of North Carolina, Chapel Hill, North Carolina, USA

J. Shabanowitz and D. F. Hunt
Department of Chemistry, University of Virginia, Charlottesville, VA, USA

K. B. Tomer
Laboratory of Molecular Reproduction, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA

The solution chemistry conditions necessary for optimum analysis of peptides by capillary zone electrophoresis (CZE)/electrospray ionization mass spectrometry have been studied to maximize the signal-to-noise ratio. In the electrospray ionization tandem mass spectrometry experiments, the solution chemistry conditions have been studied to maximize the signal-to-noise ratio.

The use of acidic buffers promotes the peptides, which is advantageous for mass spectrometry and tandem mass spectrometry analysis, but is problematic with CZE whose ions are lost from CZE because of the low buffer pH. This not only removes the weak ion current, but also adds to the sensitivity of the peptides, minimizing the detection of the current across the ensemble of charge states.

The performance of CZE is affected by the peptides' buffer pH. Buffers that provide higher peptide sensitivity result in lower peptide detection limits. Unfortunately, both CZE and CapLC require a low buffer pH in order to obtain the best detection limits.

The authors conclude that the buffer pH significantly affects the sensitivity and selectivity of protein detection in CZE and CapLC. They recommend using buffers with a pH of 2-3 for optimal peptide detection.

Richard D. Smith, Jon H. Wahl, David R. Goodsell, and Steven A. Holtmeyer
Current address: National Institutes of Health, National Institute on Aging. Institute for Drug Development, Bethesda, MD 20892, USA

*Current address: Department of Biochemistry, University of North Carolina, Chapel Hill, North Carolina, USA

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Some newer examples of coupling strategies

Figure 1. Schematics of (A) Sheathless interface with nanochannel, adapted from [8]. Copyright © 2016, with permission from Elsevier; (B) Sheathless interface with 24 holes using laser ablation with cellulose acetate, adapted by permission from Springer Nature [9], Copyright © 2017. (C) Tapered tip sheath liquid interface, adapted by permission from Springer Nature [21]. Copyright © 2016; (D) Sheath flow interface with extendable separation capillary, adapted from [23]. Copyright © 2018, with permission from Elsevier.
**Electroosmotic Flow (EOF)**

- Charge on the capillary surface causes a layer of complimentary ions to form near the wall (aka the electrical double layer).
- This layer moves in the electric field, dragging all of the BGE with it.

- EOF has a uniform (flat) flow profile.
- This means that it doesn’t broaden the analyte bands as it moves them.
Electroosmotic Flow (EOF)

- The magnitude of EOF depends on:
  - **Surface chemistry**
  - **pH of BGE**
  - **Viscosity of BGE**
    - Organic content
    - Temperature
  - **Ionic strength of BGE**
- The velocity of EOF scales with field strength, just like electrophoresis, so it can be directly added to electrophoretic mobility
  - \( \mu_{total} = \mu_{EOF} + \mu_{electrophoresis} \)
  - Or more simply: \( \mu = \mu_{EO} + \mu_{EP} \)
Electroosmotic Flow (EOF)

- Unmodified fused silica or glass has a negative surface charge, due to the presence of silanol groups
  - The mobile layer of complimentary ions is positively charged, so EOF moves from high positive voltage to lower voltage
  - We give this direction of EOF a positive charge
- $\mu_{EO}$ for fused silica is relatively high compared to $\mu_{EP}$ for most analytes, therefore:
  - The EOF of an uncoated capillary will push all analytes in the same direction, regardless of their charge
- EOF can be controlled by altering the surface chemistry of the column
EOF and Surface Chemistry

- Connecting CE to ESI-MS limits the tricks available for controlling surface chemistry
  - **Need clean BGE for ESI**
    - Background/interfering ions
    - Ionization suppression etc..
  - **Need static surface coatings**
    - Could be covalent or ionic, but the coating needs be attached so that it doesn’t interfere with ESI-MS
- Must prevent analyte sticking
- Must be uniform to prevent pressure gradients
EOF and Surface Chemistry – Some examples

- Uncoated silica/glass
  - Strong negative surface charge
- Aminopropyl-silane coating
  - Strong positive surface charge
- Neutral polymers
  - Polyacrylamide
  - Polyethylene glycol
- Charged polymers
  - Polyethylenimine (++)
  - Dextran sulfate (--)
Electroosmotic Flow (EOF) - Example

- We know that fluorescein is neutral at this pH, so its total mobility is equal to the electroosmotic mobility
- Neutral Marker (fluorescein)
  - $t_m = 1.24 \text{ m} = 74.4 \text{ s}$
  - $v = L/t_m = 23 \text{ cm} ÷ 74.4 \text{ s} = 0.309 \text{ cm/s}$
  - $v = \mu E$, so
  - $\mu = v/E = 0.309 \text{ cm/s} ÷ -410 \text{ V/cm} = -7.54 \text{ E-4 cm}^2/\text{Vs} = \mu_{EO}$

- Peak #5 (thymopentin)
  - $t_m = 84 \text{ s}$
  - $v = 0.274 \text{ cm/s}$
  - $\mu_5 = -6.68 \text{ E-4 cm}^2/\text{Vs}$
  - $\mu = \mu_{EO} + \mu_{EP}$
  - $\mu_{EP} = \mu - \mu_{EO} = +0.86 \text{ E-4 cm}^2/\text{Vs}$

Figure 4. Base peak electropherogram showing: fluorescein (1), methionine enkephalin (2), angiotensin II (3), bradykinin (4), and thymopentin (5) separated using a CE-ESI microfluidic device coated with APDIPES using a field strength of 410 V/cm and 50% acetonitrile and 0.1% formic acid (pH 2.8) BGE. Mass spectra were acquired with a Synapt G2 mass spectrometer at a rate of 8 summed scans per second.