Practical Capillary Electrophoresis

SECOND EDITION

Robert Weinberger

Second Edition

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It is a pleasure to dedicate the second edition of this book to my wife Lisa and children Julie and Jeremy.

PREFACE TO THE SECOND EDITION

It is hard to believe that seven years have passed since I wrote the first edition of this book. The time is ripe for a second edition. Not only has capillary electrophoresis matured, but my ability to articulate the field has improved as well.

I have reorganized this book to better reflect usage in the field. There are now ten chapters instead of twelve. The material on isotachophoresis has been combined with the section on stacking, and the special topics chapter has been eliminated. With the exception of the introduction and the chapter on basic concepts, all of the other material has been extensively reorganized and rewritten. Emphasis has been placed on commercially available apparatus and reagents, although gaps in the commercial offerings are discussed as well. Note that micellar electrokinetic capillary chromatography (MECC) is considered as a variant of capillary zone electrophoresis (CZE) and is included in the chapter on secondary equilibrium. Cyclodextrins and chiral recognition are covered here as well.

Many thanks to Dr. Bruce McCord, Mr. Ira Lurie, and Professor Ira Krull for reviewing some of the chapters in this second edition. The author gratefully acknowledges the support of Hewlett-Packard and in particular Dr. David Heiger.

Much has been said about the ability of capillary electrophoresis (HPCE) to replace liquid chromatography (HPLC). Clearly it has not. As the first high-performance condensed phase technique, HPLC quickly replaced gas chromatography as the method of choice for separating polar molocules. As food for thought, imagine if capillary electrophoresis had a 25-year head start over HPLC. Then perhaps the chromatographers would be fighting the uphill battle of displacing HPCE. As noted in this text, HPCE is clearly superseding the slab gel, at least in the fields of DNA separations.

Robert Weinberger
Chappaqua, NY
June 1, 1999

PREFACE TO THE FIRST EDITION

Capillary electrophoresis (CE) or high-performance CE (HPCE) is making the transition from a laboratory curiosity to a maturing microseparations technique. Now used in almost 1000 laboratories worldwide, CE is employed in an ever-widening scope of applications covering both large and small molecules.

The inspiration for this book arose from my popular American Chemical Society short course entitled, as is this text, "Practical Capillary Electrophoresis." During the first 18 months since its inception, nearly 500 students have enrolled in public and private sessions in the United States and Europe.

I have been amazed at the diversity of the scientific backgrounds of my students. Represented in these courses were molecular biologists, protein chemists, analytical chemists, organic chemists, and analytical biochemists from industrial, academic, and government laboratories. Interestingly enough, CE provides the mechanism for members of this multidisciplinary group to actually talk with each other, a rare event in most organizations.

But the diverse nature of the group provides teaching challenges as well. Most of the students are well versed in the art and science of liquid chromatography. However, CE is not chromatography (usually). It is electrophoresis, and it is governed by the art and science of electrophoresis. For those skilled in electrophoresis, CE offers additional separation opportunities that are not available in the slab-gel format. Furthermore, the intellectual process of methods development differs from that in either slab-gel electrophoresis or liquid chromatography.

The key to grasping the fundamentals of CE is to develop an understanding of how ions move about in fluid solution under the influence of an applied electric field. With this background, it becomes painless to wander through the electrophoretic domain and explain the subtleties and permutations frequently illustrated on the electropherograms. Accordingly, a logical approach to methods

development evolves from this treatment. This is the goal of my course, and hopefully, I have translated this message into this text.

Since I work independently, without academic or industrial affiliations, the writing of this text would have been impossible without the help of my friends and colleagues. In particular, I am grateful to Professor Ira Krull and his graduate student, Jeff Mazzeo, from Northeastern University for reviewing the entire manuscript; Dr. Michael Albin from Applied Biosystems, Inc., for providing his company's computerized bibliography on HPCE; and the Perkin-Elmer Corporation including Ralph Conlon, Franco Spoldi, and librarian Debra Kaufman and her staff for invaluable assistance. I am also thankful to my associates throughout the scientific instrumentation industry for providing information, intellectual challenges, hints, electropherograms, comments, etc., many of which are included in this text. Last, I thank my students for helping me continuously reshape this material to provide clear and concise explanations of electrophoretic phenomena.

Finally, many of the figures in this text were produced by scanning the illustration in a journal article with subsequent graphic editing. While all efforts were made to preserve the integrity of the original data, subtle differences may appear in the figures produced in this book.

Robert Weinberger
Chappaqua, NY
August 1992

MASTER SYMBOL LIST

A_{corr}	Corrected peak area
A_{raw}	Raw peak area
a	Fraction ionized
a	Molar absorptivity
α	Separation factor
b	Detector optical pathlength
C, c	Concentration
C_m	Coefficient for resistance to mass transfer in the mobile phase
C_s	Coefficient for resistance to mass transfer in the stationary phase
CLOD	Concentration limit of detection
CMC	Critical micelle concentration
$\%C$	Percentage of crosslinker in a gel
D	Capillary diameter
D, D_m	Diffusion coefficient
D_{sm}	Solute diffusion in stagnant mobile phase
DR	Dynamic reserve
d_p	Particle diameter, chromatography
ΔH	Height differential between capillary inlet and outlet
$\Delta\mu_{ep}$	Difference in mobility between two solutes
ΔP	Pressure drop
δ	Debye radius
ξ	Zeta potential
e	Charge per unit area
E	Field strength
E	Acceptable increase in H
E	Detector efficiency
ε	Dielectric constant
ε	Molar absorptivity
ε_o	Permittivity of vacuum

f	Frictional force (Stoke's law)
g	Gravitational constant
γ	Field enhancement factor
γ	Obstructive factor for diffusion, Van Deemter equation
H	Height equivalent of a theoretical plate
dH/dt	Rate of heat production
I	Current
I_f	Fluorescence intensity
I_o	Excitation source intensity
k	Conductivity
k'	Capacity factor
$\tilde{k}'$	Capacity factor in MECC
K, λ	Thermal conductivity
K	Equilibrium constant
L	Length of capillary
L_d	Length of capillary to detector
l_d	Length of the detector window
L_f	Length of capillary from detector to fraction collector
L_{open}	Length of the unpacked portion of a CEC capillary
L_{packed}	Length of the packed portion of a CEC capillary
L_t	Total length of capillary
l_{inj}	Length of an injection plug
λ	Tortuosity factor, Van Deemter equation
m	Mass
M	Actual mass
MLOD	Mass limit of detection
N	Number of segments in a polymer chain
N	Number of theoretical plates
n	Number of charges
η	Viscosity
P_{wm}	Partition coefficient between water and micelle
ΔP	Pressure drop
Φ	Polymer concentration, size separations
Φ	Quantum yield
Φ	Overlap threshold
Φ_f	Fluorescence quantum yield
Φ^*	Entanglement threshold, size separations
ρ	Density
ρ	Resistivity
Q	Quantity of injected material
q	Ionic net charge
R	Resistance
R	Peak ratio
R	Displacement ratio

R_s	Resolution
r	Ionic radius (Stokes' law)
r	Capillary radius
S/N	Signal to noise ratio
σ	Peak variance
σ_{cap}	Peak variance due to capillary wall effects
σ_{det}	Peak variance due to the detector
σ_{diff}	Peak variance due to diffusion
σ_{ed}	Peak variance due to electrodispersion
σ_{heat}	Peak variance due to Joule heating
σ_{inj}	Peak variance due to injection
σ_L	Peak variance in units of length
σ_{tot}	Peak variance from all sources
t	Time
t_a	Absorption time to a stationary phase or wall
t_d	Desorption time from a stationary phase or wall
t_L	Lag time
t_m	Migration time
t_{mc}	Migration time for a micellar aggregate
t_o	Migration time for a neutral "unretained" solute
t_r	Retention time
T	Temperature
TR	Transfer ratio
$\%T$	Percentage of monomer and crosslinker in a gel
μ	Ionic mobility
μ_{app}	Apparent (measured) mobility
μ_{eo}	Electroosmotic mobility
μ_{ep}	Electrophoretic mobility
V	Partial molar volume of micelle
V	Voltage
υ	Ionic velocity
υ	Mean linear velocity
υ_{ep}	Electrophoretic velocity
υ_{eo}	Electroosmotic velocity
υ_{open}	Solute velocity in the unpacked portion of a CEC capillary
υ_{packed}	Solute velocity in the packed portion of a CEC capillary
W	Power
W_{inj}	Width of an injection plug
W_s	Spatial width of a sample zone
W_t	Temporal width of a sample zone
X_i	Intital length of an injection plug
X_s	Zone length after stacking
Z	Number of valence electrons
z	Charge

CONTENTS

9. Detection

10. Putting It All Together

CHAPTER 1

Introduction

1.1 ELECTROPHORESIS

Electrophoresis is a process for separating charged molecules based on their movement through a fluid under the influence of an applied electric field. If two solutes have differing electrophoretic **mobilities**, then separation will usually occur. The separation is performed in a medium such as a semisolid slab-gel. Gels provide physical support and mechanical stability for the fluidic buffer system. In some modes of electrophoresis, the gel participates in the mechanism of separation by serving as a molecular sieve. Nongel media such as paper or cellulose acetate are alternative supports. These media are less inert than gels, as they contain charged surface groups that may interact with the sample or the run buffer.

A carrier electrolyte is also required for electrophoresis. Otherwise known as the **background electrolyte** (BGE), the carrier electrolyte, or simply the run buffer, this solution maintains the requisite pH and provides sufficient conductivity to allow the passage of current (ions), necessary for the separation. Frequently, additional materials are added to the BGE to adjust the resolution of the separation through the generation of secondary equilibria. Additives can also serve to maintain solubility and prevent the interaction of solutes or excipients with the gel matrix or, in the case of capillary electrophoresis, with the

capillary wall. The theory and practice of electrophoresis have been the subject of many textbooks and conference proceedings (1–9).

Apparatus for conducting electrophoresis, such as that illustrated in Figure 1.1, is remarkably simple and low cost. The gel medium, which is supported on glass plates, is inserted into a Plexiglass chamber. Two buffer reservoirs make contact at each end of the gel. Electrodes immersed in the buffers complete the electrical circuit between the gel and power supply. Many samples can be separated simultaneously, since it is possible to use a multilane gel. One or two lanes are frequently reserved for standard mixtures to calibrate the electropherogram. Calibration is usually based on molecular size or, in isoelectric focusing, pI.

Gels such as polyacrylamide or agarose serve several important functions:

1. they may contribute to the mechanism of separation;
2. they reduce the dispersive effects of diffusion and convection; and
3. they serve to physically stabilize the separation matrix.

The gel composition is adjusted to define specific pore sizes, each for a nominal range of molecular sizes. This forms the basis for separations of macromolecules based on size. By proper calibration, extrapolation to molecular weight is straightforward.

Reduction of convection and diffusion is an important function of the gel matrix. The production of heat by the applied field induces convective movement of the electrolyte. This movement results in band broadening that reduces the efficiency of the separation. The viscous gel media inhibits fluid movement in the electric field. Such a material is termed anticonvective. Since the gel is of high viscosity, molecular diffusion is reduced as well, further enhancing the efficiency of the separation.

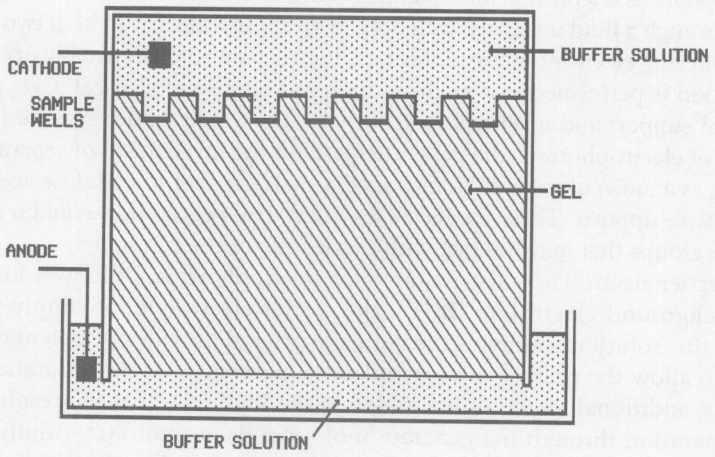

FIGURE 1.1 Drawing of an apparatus for slab-gel electrophoresis.

Finally, the gel must be sufficiently viscous to provide physical support. Low viscosity solutions or gels would flow if the plate is not held level. Immersion in detection reagents would be impossible, since handling or contact with fluid solutions would destroy the matrix and separation. In the capillary format, the gel is unnecessary since the walls of the capillary provide the mechanical stability for the separation.

The basic procedure for performing gel electrophoresis is as follows:

1. prepare, pour and polymerize the gel;
2. apply the sample;
3. run the separation;
4. immerse the gel in a detection reagent;[1]
5. destain the gel;
6. preserve the gel; and
7. photograph or scan the gel for a permanent record.[2]

These steps are extremely labor intensive. High performance capillary electrophoresis (HPCE) is the automated and instrumental version of slab-gel electrophoresis. In the DNA applications arena, the most important of which include DNA sequencing, human identification, and genetic analysis, HPCE is rapidly replacing the slab-gel as the separation method of choice.

The separation of some polymerase chain reaction (PCR) products is shown in Figure 1.2. A restriction digest, used as a sizing standard, appears in the outer lanes. The middle three lanes of the gel show a triplicate run of a 500-mer double-stranded DNA PCR reaction. Quantitation for such a separation is difficult and often imprecise, but such information can be obtained with the aid of a gel scanner. Recoveries of material from the gel are performed using procedures such as the Southern blot (10). Sufficient material is recoverable for sequencing or other bioassays.

Separations of the sizing standard and 500-mer PCR product by HPCE using a size selective polymer network are shown in Figure 1.3. Quantitation is readily performed using peak area comparison with the standard. However, fraction collection is difficult relative to the slab-gel, particularly for trace impurities, since only minuscule amounts of material are injected into the capillary.

1.2 MICROCHROMATOGRAPHIC SEPARATION METHODS

The evolution of chromatographic methods over the last 40 years has produced a systematic and rational trend toward miniaturization. This is particularly true

[1]On-line detection is performed on an instrument such as an automated DNA sequencer.
[2]Automated gel scanners can be used in place of gel archiving or photography.