Reiner Westermeier

Electrophoresis in Practice

A Guide to Methods and Applications
of DNA and Protein Separations
Fourth, revised and enlarged Edition

in collaboration with

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Contents

Foreword

The number of electrophoretic separation methods has increased dramatically since Tiselius' pioneer work for which he received the Nobel Prize. Development of these methods has progressed from paper, cellulose acetate membranes and starch gel electrophoresis to molecular sieve, disc, SDS, and immunoelectrophoresis and finally to isoelectric focusing but also to high resolution two-dimensional electrophoresis. Together with silver and gold staining, autoradiography, fluorography and blotting, these techniques afford better resolution, sensitivity and specificity for the analysis of proteins. In addition, gel electrophoresis has proved to be a unique method for DNA sequencing while high resolution two-dimensional electrophoresis has smoothed the fascinating path from isolation of the protein to the gene through amino acid sequencing and after gene cloning, to protein synthesis.

The spectrum of analytical possibilities has become so varied that an overview of electrophoretic separation methods seems desirable not only for beginners but also for experienced users. This book has been written for this purpose.

The author belongs to the circle of the bluefingers and experienced this in Milan in 1979 when he was accused of being a money forger when buying cigarettes in a kiosk after work because his hands were stained by Coomassie. Prof. Righetti and I had to extricate him from this tricky situation. According to Maurer's definition (Proceedings of the first small conference of the bluefingers, Tübingen 1972) an expert was at work on this book and he can teach the whitefingers, who only know of the methods by hearsay, fore example, how not to get blue fingers.

As it is, I am sure that this complete survey of the methods will not only help the whitefingers but also the community of the bluefingers, silverfingers, goldfingers etc. and will teach them many technical details.

Weihenstephan, February 1990

Prof. Dr. Angelika Görg
FG Proteomik,
Technische Universität München,
Freising-Weihenstephan

Preface

German version

This book was written for the practician in the electrophoresis laboratory. For this reason we have avoided physico-chemical derivations and formulas concerning electrophoretic phenomena.

The type of explanation and presentation stems from several years of experience in giving user seminars and courses, writing handbooks and solving user problems. They should be clear for technical assistants as well as for researchers in the laboratory. The commentary column offers room for personal notes.

In part I, an introduction – as short as possible – to the actual state of the art will be given. The references are not meant to be exhaustive.

Part II contains exact instructions for 11 chosen electrophoretic methods, which can be carried out with one single piece of equipment. The sequence of the methods was planned so that an electrophoresis course for beginners and advanced users can be established afterwards. The major methods used in biology, biochemistry, medicine and food science methods have been covered.

If – despite following the method precisely – unexplained effects should arise, their cause and the remedies can be found in the trouble-shooting guide in the appendix.

The author would be thankful for any additional comments and solutions for the trouble-shooting guide which the reader could supply.

Freiburg, March 1990 *R. Westermeier*

English version, First Edition

The author is grateful to Dr. Michael J. Dunn, Senior Lecturer at the National Heart and Lung Institute, Harefield, Middlesex, UK, for his kind engagement of reading the manuscript, correcting the english and for his excellent and informed advices.

In this version, some updates have been made to methodological aspects, new experiences, applications, and the references. A new drawing program is used, which allows higher resolution in the explanatory figures.

Leonberg, February 1993 *R. Westermeier*

English version, Second Edition

The author thanks Professor Görg for her tips for the state of the art of high resolution two-dimensional electrophoresis, and Dr. Gabriel Peltre, Institute Pasteur, Paris, for valuable hints on the practice of immunoelectrophoresis, agarose isoelectric focusing, blotting, and titration curves.

This version has been updated in the wording, the way of quoting the references, and in the methodology. A few figures, hints for problem solving, and a few very important references have been added. The main differences to the previous issue, however, are constituted by the addition of the lately developed methods for DNA typing and the methodology for vertical gels. Thus, section II contains now 15 chosen electrophoretic methods.

Freiburg, November 1996 *R. Westermeier*

English version, Third Edition

Three years ago, just when the second issue reached the book stores, "Proteome analysis" became a buzz word. Proteomics seems to become the continuation of the genome sequencing approach with even greater efforts. Many molecular biology laboratories start to work with two-dimensional electrophoresis of proteins now. It was thus necessary to update the book again, this time with the further developed state of the art of 2-D electrophoresis, and a short overview on the proteome analysis methodology. Some space could be gained for this, because image analysis of electrophoresis gels has become much easier in the last few years. Thus its description became much shorter. Also the front picture has been renewed to express the importance of 2-D electrophoresis nowadays. Of course, all the other technical areas have been checked for new developments and updated as well.

Freiburg, July 2000 *R. Westermeier*

English version, Fourth Edition

During the last few years the Proteomics technologies have been further developed and became more robust. A very powerful new method for quantitative 2-D electrophoresis, difference gel electrophoresis (DIGE), has meanwhile been established. It is the only method, which allows sample multiplexing in 2-D gels and enables the use of an internal standard. Therefore its description was added to the Proteomics chapter. Also in some other areas of electrophoretic methodology progresses have been accomplished; they are now included. As with the other editions, the occasion of preparing a new edition was used to revise the entire manuscript again, and to fix some errors. Finally, the author highly appreciates the complete layout refurbishment of the book, which has been carried out to give it a more professional look and improve its readability.

Freiburg, July 2004 *R. Westermeier*

Abbreviations, symbols, units

2-D electrophoresis	Two-dimensional electrophoresis
A	Ampere
acc.	according
A,C,G,T	Adenine, cytosine, guanine, thymine
A/D-transformer	Analog-digital transformer
ACES	N-2-acetamido-2-aminoethanesulfonic acid
AEBSF	Aminoethyl benzylsulfonyl fluoride
AFLP	Amplified restriction fragment length polymorphism
API	Atmospheric pressure ionization
APS	Ammonium persulphate
ARDRA	Amplified ribosomal DNA restriction analysis
AU	Absorbance units
16-BAC	Benzyldimethyl-n-hexadecylammonium chloride
BAC	Bisacryloylcystamine
Bis	N, N′-methylenebisacrylamide
bp	Base pair
BSA	Bovine serum albumin
C	Crosslinking factor [%]
CA	Carbonic anhydrase
CAF	Chemically assisted fragmentation
CAM	Co-analytical modification
CAPS	3-(cyclohexylamino)-propanesulfonic acid
CCD	Charge-coupled device
CDGE	Constant denaturing gel electrophoresis
CHAPS	3-(3-cholamidopropyl)dimethylammonio-1-propane sulfonate
CE	Capillary electrophoresis
CID	Collision induced dissociation
conc	Concentrated

CM	Carboxylmethyl
CMW	Collagen molecular weight marker
const.	Constant
CTAB	Cetyltrimethylammonium bromide
Da	Dalton
DAF	DNA amplification fingerprinting
DBM	Diazobenzyloxymethyl
DDRT	Differential display reverse transcription
DEA	Diethanolamine
DEAE	Diethylaminoethyl
DGGE	Denaturing gradient gel electrophoresis
DHB	2,5-dihydroxybenzoic acid
DIGE	Difference gel electrophoresis
Disc	Discontinuous
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
DPT	Diazophenylthioether
dsDNA	Double stranded DNA
DSCP	Double strand conformation polymorphism
DTE	Dithioerythreitol
DTT	Dithiothreitol
E	Field strength in V/cm
EDTA	Ethylenediaminetetraacetic acid
ESI	Electro spray ionization
EST	Expressed sequence tag
FT-ICR	Fourier transform - Ion cyclotron resonance
GC	Group specific component
h	Hour
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanane-sulfonic acid
HMW	High Molecular Weight
HPCE	High Performance Capillary Electrophoresis
HPLC	High Performance Liquid Chromatography
I	Current in A, mA
IEF	Isoelectric focusing
IgG	Immunoglobulin G
IPG	Immobilized pH gradients
ITP	Isotachophoresis
kB	Kilobases
kDa	Kilodaltons
KR	Retardation coefficient
LDAO	Lauryldimethylamine-N-oxide
LMW	Low Molecular Weight
M	mass
mA	Milliampere

MALDI	Matrix assisted laser desorption ionization
MEKC	Micellar electrokinetic chromatography
MES	2-(N-morpholino)ethanesulfonic acid
min	Minute
mol/L	Molecular mass
MOPS	3-(N-morpholino)propanesulfonic acid
m_r	Relative electrophoretic mobility
mRNA	messenger RNA
MS	Mass spectrometry
Ms^n	Mass spectrometry with n mass analysis experiments
MS/MS	Tandem mass spectrometry
MW	Molecular weight
NAP	Nucleic Acid Purifier
Nonidet	Non-ionic detergent
NEPHGE	Non equilibrium pH gradient electrophoresis
O.D.	Optical density
P	Power in W
PAG	Polyacrylamide gel
PAGE	Polyacrylamide gel electrophoresis
PAGIEF	Polyacrylamide gel isoelectric focusing
PBS	Phosphate buffered saline
PCR®	Polymerase Chain Reaction
PEG	Polyethylene glycol
PFG	Pulsed Field Gel (electrophoresis)
PGM	Phosphoglucose mutase
pI	Isoelectric point
PI	Protease inhibitor
pK value	Dissociation constant
PMSF	Phenylmethyl-sulfonyl fluoride
PPA	Piperidino propionamide
PSD	Post source dissociation (decay)
PTM	Post-translational modification
PVC	Polyvinylchloride
PVDF	Polyvinylidene difluoride
r	Molecular radius
RAPD	Random amplified polymorphic DNA
REN	Rapid efficient nonradioactive
R_f value	Relative distance of migration
RFLP	Restriction fragment length polymorphism
R_m	Relative electrophoretic mobility
RNA	Ribonucleic acid
RPA	Ribonuclease protection assay
s	Second
SDS	Sodium dodecyl sulfate

SNP	Single Nucleotide Polymorphism
ssDNA	Single stranded DNA
T	Total acrylamide concentration [%]
t	Time, in h, min, s
TBE	Tris borate EDTA
TBP	Tributyl phosphine
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TCEP	Tris carboxyethyl phosphine
TEMED	N,N,N′,N′-tetramethylethylenediamine
TF	Transferrin
TGGE	Temperature gradient gel electrophoresis
TMPTMA	Trimethylolpropane-trimethacrylate[2-ethyl-2(hydrohymethyl) 1,3-propandiol-trimeth-acrylate]
ToF	Time of Flight
Tricine	N,tris(hydroxymethyl)-methyl glycine
Tris	Tris(hydroxymethyl)-aminoethane
U	Volt
V	Volume in L
v	Speed of migation in m/s
v/v	Volume per volume
VLDL	Very low density lipoproteins
W	Watt
w/v	Weight per volume (mass concentration)
ZE	Zone electrophoresis

Part I:
Fundamentals

Introduction

Electrophoretic separation techniques are at least as widely distributed as chromatographic methods. With electrophoresis a high separation efficiency can be achieved using a relatively limited amount of equipment. It is mainly applied for analytical rather than for preparative purposes. However, with the advent of new technology like amplification of DNA fragments with Polymerase Chain Reaction (PCR®), and highly sensitive and powerful mass spectrometry analysis of proteins and peptides, so called "analytical amounts" of electrophoretically separated fractions can now be further analysed.

The main fields of application are biological and biochemical research, protein chemistry, pharmacology, forensic medicine, clinical investigations, veterinary science, food control as well as molecular biology. It will become increasingly important to be able to choose and carry out the appropriate electrophoresis technique for specific separation problems.

The monograph by Andrews (Andrews 1986) is one of the most complete and practice-oriented books about electrophoretic methods. In the present book, electrophoretic methods and their applications will be presented in a much more condensed form.

Andrews AT. Electrophoresis, theory techniques and biochemical and clinical applications. Clarendon Press, Oxford (1986).

Principle: Under the influence of an electrical field charged molecules and particles migrate in the direction of the electrode bearing the opposite charge. During this process, the substances are usually in aqueous solution. Because of their varying charges and masses, different molecules and particles of a mixture will migrate at different velocities and will thus be separated into single fractions.

The electrophoretic mobility, which is a measure of the migration velocity, is a significant and characteristic parameter of a charged molecule or particle. It is dependent on the pK values of the charged groups and the size of the molecule or particle. It is influenced by the type, concentration and pH of the buffer, by the temperature and the field strength as well as by the nature of the support material. Electrophoretic separations are carried out in free solutions as in capillary

Chrambach A. The practice of quantitative gel electrophoresis. VCH Weinheim (1985).

Mosher RA, Saville DA, Thormann W. The dynamics of electrophoresis. VCH Weinheim (1992).

and free flow systems, or in stabilizing media such as thin-layer plates, films or gels. Detailed theoretical explanations can be found in the books by Chrambach (1985) and Mosher *et al.* (1992).

The relative mobility is abbreviated as m_r or R_m.

Sometimes the *relative* electrophoretic mobility of substances is specified. It is calculated relative to the migration distance of a standard substance, mostly a dye like bromophenol blue, which has been applied as an internal standard.

There is a fourth method: "Moving Boundary Electrophoresis", which is described on page 9. However this technique has no practical importance anymore. "Electrophoresis" is a general term for all these methods. Blotting is not seen as a separation, but as a detection method.

Three basically different electrophoretic separation methods are employed in practice nowadays:

a) Electrophoresis, sometimes called zone electrophoresis (ZE).
b) Isotachophoresis or ITP
c) Isoelectric focusing or IEF

The three separation principles are illustrated in Fig. 1.

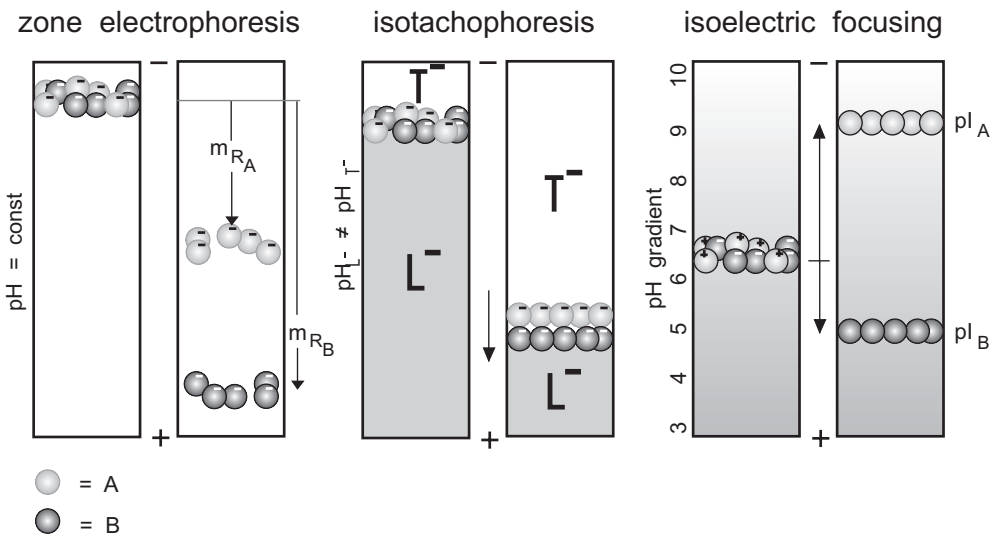

Fig. 1: The three electrophoretic separation principles. Explanations in the text. A and B are the components of the sample.

a) In *zone electrophoresis* a homogeneous buffer system is used over the whole separation time and range so as to ensure a constant pH value. The migration distances during a defined time limit are a measure of the electrophoretic mobilities of the various substances. It can be applied to nonamphoteric as well as amphoteric molecules. During the separation diffusion can lead to blurred zones, which reduces the sensitivity of detection and the resolution.

This is also valid for disc electrophoresis, because a discontinuous system exists only at the beginning of the separation and changes into a homogeneous one.

b) In *isotachophoresis* (ITP), the separation is carried out in a discontinuous buffer system. The ionized sample migrates between a leading electrolyte with a high mobility and a terminating – sometimes called trailing – ion with a low mobility, all of them migrating with the same speed. The different components are separated according to their electrophoretic mobilities and form stacks: the substance with the highest mobility directly follows the leading ion, the one with the lowest mobility migrates directly in front of the terminating electrolyte. In ITP there is a concentration regulating effect which works against diffusion.

In comparison to other electrophoretic and chromatographic separation methods, ITP is considered exotic because there are no spaces between the zones: the bands are not "peaks" (Gaussian curves) but "spikes" (concentration dependent bands). ITP is mostly applied for stacking of the samples during the first phase of disc electrophoresis.

c) *Isoelectric focusing* (IEF) takes place in a pH gradient and can only be used for amphoteric substances such as peptides and proteins. The molecules move towards the anode or the cathode until they reach a position in the pH gradient where their net charges are zero. This pH value is the *"isoelectric point"* (pI) of the substance. Since it is no longer charged, the electric field does not have any influence on it. Should the substance diffuse away, it will gain a net charge again, and the applied electric field will cause it to migrate back to its pI. This concentrating effect leads to the name *focusing*. Thus also with IEF there is no problem with diffusion.

In IEF it is important to find the correct place in the pH gradient to apply the sample, since some substances are unstable at certain pH values (see below).

Areas of applications: Mainly proteins, peptides, sugars, and nucleic acids are separated. Electrophoretic methods are used for the qualitative characterization of a substance or mixture of substances, for control of purity, quantitative determinations, and preparative purposes. The most prominent fields are the Genome and the Proteome analysis. The word "Proteome" was introduced by Mark Wilkins during a congress in Sienna 1994, in written form in the publication by Wasinger *et al.* one year later.

Wasinger VC, Cordwell SJ, Cerpa-Poljak A, Yan JX, Gooley AA, Wilkins MR, Duncan MW, Harris R, Williams KL, Humphery-Smith I. Electrophoresis 16 (1995) 1090–1094.

The scope of the applications ranges from whole cells and particles to nucleic acids, proteins, peptides, amino acids, organic acids and bases, drugs, pesticides and inorganic anions and cations – in short – everything that can carry a charge.

Sample application on gels which are immersed in buffer (e.g. vertical and submarine gels) is done with syringes into sample wells polymerized into the gel or into glass tubes, the sample density is raised with glycerol or sucrose.

The sample: An important criterion for the choice of the appropriate electrophoretic method is the nature of the sample to be analyzed. There must be no solid particles or fatty components suspended in the solution. Those interfere with the separation by blocking the pores of the matrix. Sample solutions are mostly centrifuged, sometimes also desalted, before electrophoresis.

Substances which are exclusively negatively or positively charged are easy to run: Examples of such anions or cations are: nucleic acids, dyes, phenols and organic acids or bases. Amphoteric molecules such as amino acids, peptides, proteins and enzymes have positive or negative net charges depending on the pH of the buffer, because they possess acidic as well as basic groups.

In "Proteome analysis", where complex mixtures of several thousand proteins have to be separated in one gel; the sample preparation procedure greatly influences the result.

Proteins and enzymes are often sensitive to certain pH values or buffer substances; conformational changes, denaturation, complex formation, and intermolecular interactions are possible. The concentration of the substances in the solution also plays a role. In particular, when the sample enters the gel, overloading effects can occur when the protein concentration reaches a critical value during the transition from the solution into the more restrictive gel matrix.

For open surfaces as in horizontal systems (e.g. cellulose acetate, agarose gels and automated electrophoresis) either sample applicators are used, or the sample is pipetted into sample wells with a micropipette. Capillary systems usually have an automated sample applicator.

For sodium dodecyl sulphate electrophoresis, the sample must first be denatured; which means it must be converted into molecule-detergent micelles. The method of selective sample extraction, particularly the extraction of not easily soluble substances often determines the nature of the buffer to be used.The nature of the stabilizing medium, e.g. a gel, is dependent on the size of the molecule to be analyzed.

The buffer: The electrophoretic separation of samples is done in a buffer with a precise pH value and a constant ionic strength. The ionic strength should be as low as possible so that both the contribution of the sample ions to the total current and their speed will be high enough.

Yet a minimum buffering capacity is required so that the pH value of the samples analyzed does not have any influence on the system.

During electrophoresis, the buffer ions are carried through the gel just like the sample ions: negatively charged ions towards the anode, positively charged ones towards the cathode. This should be achieved with as little energy as possible so that not much Joule heat is developed.

Elemental charge: 1.602×10^{-19} *As;*
Avogadro constant: 1.602×10^{23} *elemental units per mol.*

With the help of the Second Law of Electrolysis by Michael Faraday it is possible to calculate the amount of ions migrating in an electrophoresis experiment: The amounts of electricity are equal to the amount of substances, which are eliminated from different electrolytes. Taking the Avogadro constant and the elemental charge, this means: 1 mAh equals 36.4 μmol.

To guarantee constant pH and buffer conditions the supplies of electrode buffers must be large enough. The use of buffer gel strips or wicks instead of tanks is very practical, though only feasible in horizontal flatbed systems.

For anionic electrophoresis very basic, and for cationic electrophoresis very acidic buffers are used.

In vertical or capillary systems, the pH is very often set to a very high (or low) value, so that as many as possible sample molecules are negatively (or positively) charged, and thus migrate in the same direction.

In these systems the sample is loaded at one end of the separation medium.

When a gel matrix does not contain any ions from polymerization, amphoteric buffers can be applied, which do not migrate during electrophoresis. Such a buffer substance, however, must possess a high buffering capacity at its isoelectric point. For some applications, no buffer reservoirs are necessary with this method.

A polyacrylamide gel, which is covalently bound to a plastic film can be washed after polymerization. See method 4 in this book.

Electroendosmosis: The static support, the stabilizing medium (e.g. the gel) and/or the surface of the separation equipment such as glass plates, tubes or capillaries can carry charged groups: e.g. carboxylic groups in starch and agarose, sulfonic groups in agarose, silicium oxide on glass surfaces. These groups become ionized in basic and neutral buffers: in the electric field they will be attracted by the anode. As they are fixed in the matrix, they cannot migrate. This results in a compensation by the counterflow of H_3O^+ ions towards the cathode: electroendosmosis.

Electroendosmosis is normally seen as a negative effect, yet a few methods take advantage of this effect to achieve separation or detection results (see page 12: MEKC and page 19: counter immunoelectrophoresis).

In gels, this effect is observed as a water flow towards the cathode, which carries the solubilized substances along. The electrophoretic and electroosmotic migrations are then additive (see Fig. 2). The results are: blurred zones, and drying of the gel in the anodal area of flatbed gels.

In capillary electrophoresis mostly the term "electroosmotic flow" is applied, the term "electroendosmosis" is only used in gel electrophoresis.

When fixed groups are positively charged, the electro-osmotic flow is directed towards the anode.

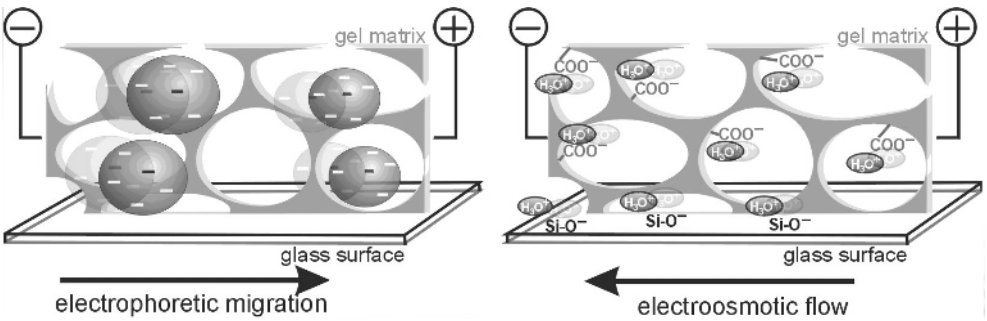

Fig. 2: Electroendosmosis: Negatively charged groups fixed to the gel matrix or to a surface cause the flow of water ions. This results in a water transport into the opposite direction of the electrophoretic migration of the sample ions, leading to blurred band pattern.