Advances in Electrophoresis

Edited by A. Chrambach, M. J. Dunn, B. J. Radola

Volume 2

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Preface

It seems appropriate to reflect on the reasons behind the production of an annual review series on electrophoresis. The monthly journal *Electrophoresis* provides space for reviews and so do many other methodological journals. Moreover, the meetings of the international and some national electrophoresis societies produce proceedings also containing review articles. The purpose of *Advances in Electrophoresis* is to assemble these multiple sources into a central "review bank" that is readily available to everyone using electrophoretic methods.

A central review bank should provide a forum for the authoritative voices in each specialized field of electrophoresis, thereby helping to resolve problems created by discordant advice at different levels of expertise. It should serve to unify research areas whose results are published in a wide range of journals, for example, those of the two most challenging classes of substances – proteins and nucleic acids. Rather than summarizing all available information, the reviews in *Advances in Electrophoresis* present the essence of each topic and demonstrate its potential. The reviews are directed to the great many readers who already use electrophoretic techniques but do not follow their development in the original literature. Also, the reviews should be indispensible to those interested in the application of a new technique or entering a field requiring the use of electrophoresis. Ideally, the reviews will be the key references for the following years in a particular area.

Advances in Electrophoresis contains reviews dealing either with selected techniques or important areas of application of electrophoresis. We have already alluded to the need for reviews on methodological progress. However, we consider it equally essential to provide reviews on important areas of application. Electrophoresis is not an esoteric method employed by only a small group of experts. On the contrary, its range of applications is increasing at an astonishing pace and, in many areas, it is already established as an indispensible tool. By publishing in *Advances in Electrophoresis* a balanced blend of reviews covering applications and techniques we expect a crossfertilizing effect which should stimulate further developments in the field of electrophoresis.

In order to accomplish these aims, we should like to encourage our readers to send us their comments, criticisms and suggestions for important topics to be included in forthcoming volumes. Finally, we wish to thank the authors for the sacrifice they have made in filling these pages and thereby in providing the field of electrophoresis with its first centralized retrieval bank.

November 1988

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THE CURRENT STATE OF ELECTROFOCUSING IN IMMOBILIZED pH GRADIENTS

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Abbreviations: Bis, *N,N'*-methylenebisacrylamide; **%C**, crosslinking agent (g)×100/%T; **CA**, carrier ampholytes; **CHAPS**, 3-[3-(cholamidopropyl)-dimethylammonio]-1-propane sulfonate; **2-D**, two-dimensional; **DATD**, *N,N'*-diallyltartardiamide; **DHEBA**, dihydroxyethylbisacrylamide; **DTT**, dithiothreitol; **ICAPG**, IPG in presence of CA; **IEF**, isoelectric focusing; **IPG**, immobilized pH gradient; **IPGEF**, IEF in IPGs; **ICAPG-EF**, IEF in ICAPGs; **SDS-PAGE**, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **%T**, acrylamide(g)+Bis(g)/100 mL; **TEMED**, *N,N,N',N'*-tetramethylethylenediamine; **Vh**, volt×hours

1 Principles of isoelectric focusing in immobilized pH gradients

Immobilized pH gradients (IPG) were introduced in 1982 [1], utilizing substituted acrylamides with acidic or basic functional groups designated by the tradename Immobiline (LKB, Bromma, Sweden); the concept of their application to pH gradient gels had been described in 1975 [2]. By incorporating Immobilines into the polymerization mixture, the substituted groups are covalently bonded to the polyacrylamide matrix (Fig. 1). The substitutions are either carboxylic acid or tertiary amine functional groups R, varying in pK, with the general structure of $CH_2 = CH - CO - NH - R$ [3]. When these are mixed in various proportions, the mixture can in principle be of any desired pH across the pH scale. Using the presently commercially available six Immobilines, a pH gradient of 4 to 9.5 can be generated by forming a gradient from the most acidic to the most basic monomer mixtures. The appropriate mixtures of the six Immobilines to produce the desired

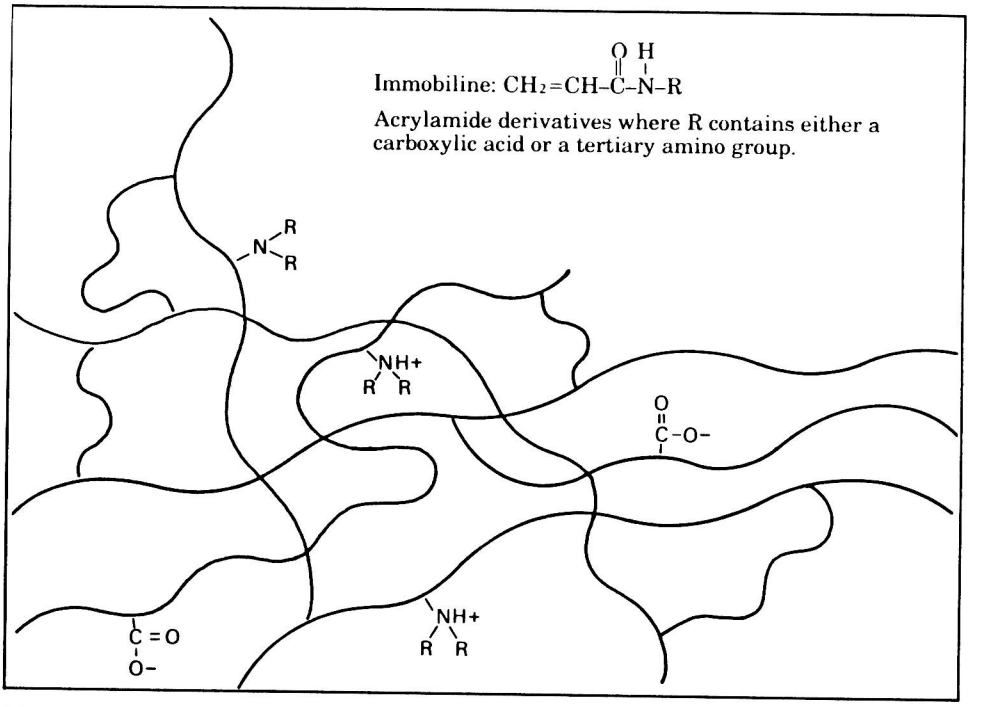


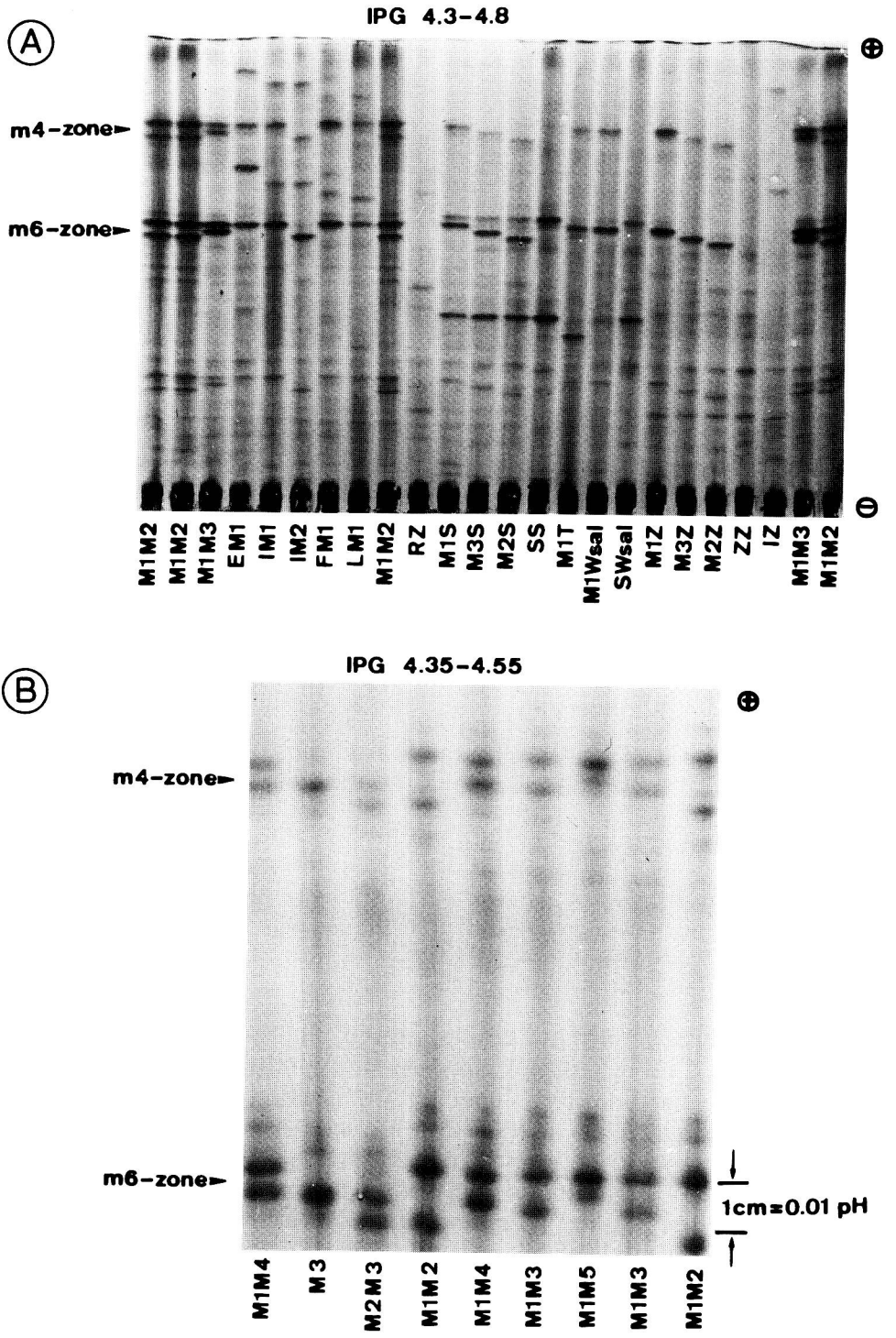
Figure 1. Schematic representation of an Immobiline [1].

pH gradient were initially restricted to two Immobilines and a range of 1 pH unit and were calculated using the Henderson-Hasselbalch equation [1]. Subsequently, Righetti and coworkers [4, 5] devised a computer program capable of producing recipes [6] for monomer mixtures for both narrow and broad pH gradients (up to 6 pH units) from pH 4–10. The computed pH gradients may be linear or in any desired way nonlinear, and they may contain flat regions if so desired [7]. Formulations for IPGs encompassing 7 pH units (pH 3–10) are given in [5]. They make use of additional substituted vinyl compounds with p*K*s 0.8 and 12 which are at present commercially unavailable as Immobiline preparations. Mosher *et al.* [8] computed pH gradients covering the alkaline extreme of pH 10–11 by use of a postulated substituted vinyl compound with p*K* 11. The acidic extreme pH 3–4 was experimentally demonstrated with the commercially available Immobilines [8, 9].

Application of Immobiline pH gradients to electrofocusing required a suitable gradient forming technique [10, 11] and polymerization procedure and the first successful electrofocusing in IPG (IPGEF) was not reported until 1982 [1], a full 7 years after the concept was patented. An immediate advance made by the method consisted of the practical feasibility of setting up very narrow pH ranges (0.1 pH/cm or less) in most regions of the pH scale, with a resulting dramatic improvement in resolution for some applications (Fig. 2) [1, 12–14]. This had not been readily possible with pH gradients formed by carrier ampholytes (CA) [15]. However, the central promise inherent in IPGEF was a pH gradient stable with time, generated by the covalent anchoring of the pH gradient to a polymer matrix (Fig. 3). It is now a demonstrated fact that such stability of the pH gradient can be attained even when the time needed to reach the steady state of IPGEF is considerable in shallow pH gradients [16] or in application to complex protein mixtures [17].

The publication, six years ago, of the promise and basic techniques of IPGEF [1] did not immediately supplant the wide application of isoelectric focusing (IEF) in biochemistry. This is presumably due to the relatively larger experimental difficulty of the technique, need for attention to detailed procedure and skillfulness of the operator in IPGEF compared to IEF. It is also due to the unavailability, when the method was first introduced, to explicit

Figure 2. Increase in protein-band separation as a function of the pH range in IPGEF. (A) pH Range 4.3–4.8, gel length of 20 cm, pH/cm: 0.025. (B) pH Range of 4.35–4.55, gel length of 20 cm, pH/cm: 0.01. IPG conditions as in Fig. 3, 16 h. Protein: α_1-antitrypsin phenotypes [12].


A
IPG 4.3–4.8
m4-zone
m6-zone
⊕
⊖
M1M2
M1M2
M1M3
EM1
IM1
IM2
FM1
LM1
M1M2
RZ
M1S
M3S
M2S
SS
M1T
M1Wsal
SWsal
M1Z
M3Z
M2Z
ZZ
IZ
M1M3
M1M2
B
IPG 4.35–4.55
m4-zone
m6-zone
⊕
1cm≡0.01 pH
M1M4
M3
M2M3
M1M2
M1M4
M1M3
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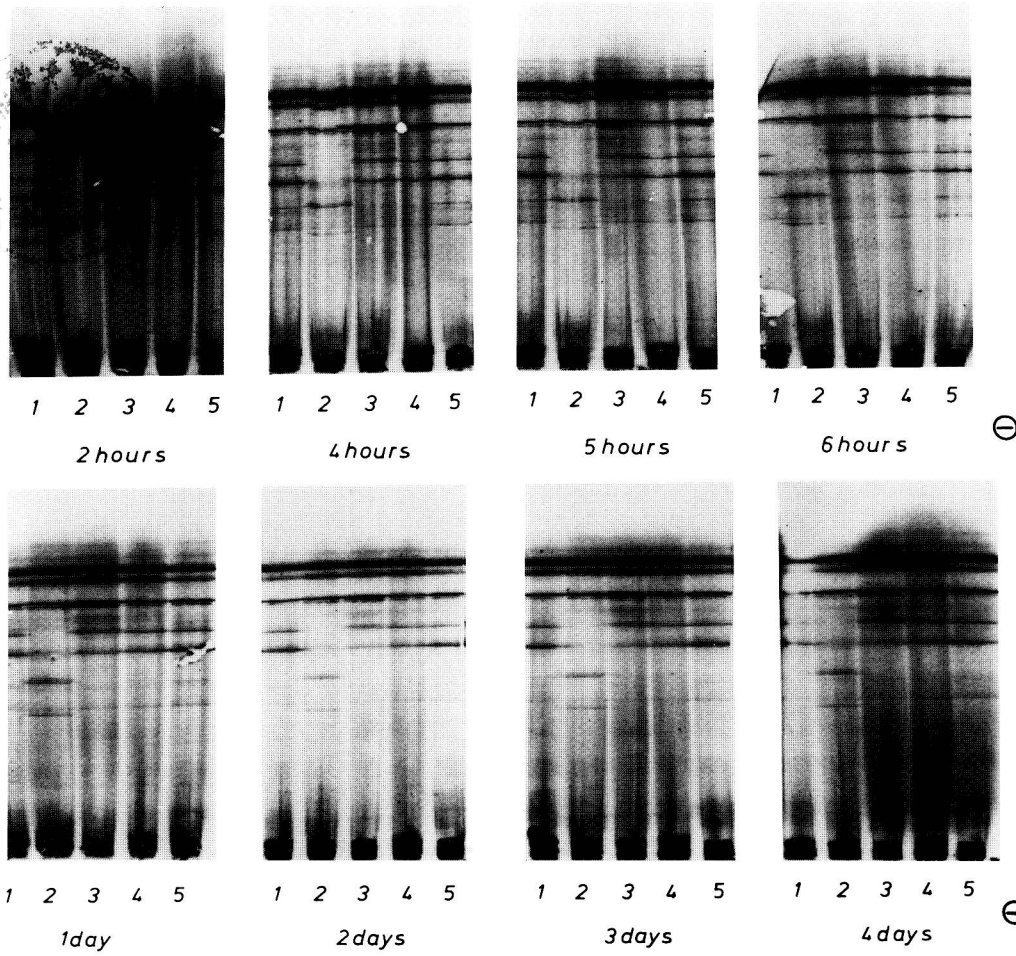

Figure 3. Stability of protein-zone positions with time in IPGEF (pH range 4–6, 4 %T, 4 %C, 15% glycerol, 10 °C, 250 V/cm). Samples (1) to (5): Proteins from 5 soybean varieties. Protein stain: Coomassie R-250 [16].

and sufficiently elaborated and detailed procedures. The inherent trickiness of the method, and the increasingly numerous and conflicting procedures, led to a situation where the novice, by contrast with classical IEF, nearly inevitably failed in his first attempts at IPGEF. To reduce this problem, it will be one of the main purposes of this review to provide both a sufficient degree of procedural detail and a selection of recipes from among the jungle of published directions, suggestions and procedures. In consequence, no attempt will be made to cite exhaustively all of the various experimental or computational approaches in the literature since these are apt to confuse and mislead the user.

2 Techniques of immobilized pH gradient formation and isoelectric focusing

2.1 Storage and dilution of Immobiline

Immobilines are vinyl derivatives and are therefore prone to spontaneous vinyl polymerization catalyzed by light, elevated temperature and amines in presence of water. It is therefore advisable to store Immobiline dry, dark, at −20 °C, or if available, −70 °C, and not admixed with other Immobiline species – to avoid contact between those species containing catalytically active amine groups. Of the two most alkaline Immobilines (p*K* 8.5 and 9.2), which are anhydrous liquids, the former is distributed in presence of a polymerization inhibitor. In spite of these precautions, one must expect a slow spontaneous polymerization of the Immobiline monomers to occur with prolonged storage. Upon use, the required Immobiline monomer should be diluted to the concentration of 0.2 M (by the addition of 25 mL of distilled water). Sealed aliquots of the dilute solution are stored at −20 °C or below, preferably under inert gas (argon or, if not available, nitrogen).

2.2 Formulation of the light and heavy solutions containing Immobilines

Almost all of the presently used formulations for forming IPG gels derive from the computations of Righetti *et al.* (*e.g.* [4, 5]). These formulations, however, do not provide the weights or volumes of the desired stock solutions directly. This has been done by the manufacturer in the relevant Application Note [6] and is reprinted here (Tables 1 and 2). Formulations for gradients narrower than 1 pH unit must be arrived at by interpolation of plots of volume of appropriate Immobiline solutions *vs.* pH (Fig. 4). The appropriate Immobiline mixtures which define the range of the pH gradient are selected from Tables 1 and 2. From these mixtures heavy and light solutions containing acrylamide monomer and crosslinking agent are prepared; the heavy solution contains glycerol to establish the desired density difference between

Table 1. Narrow pH gradients: volumes of Immobiline for 15 mL of each starting solution (2 gels)

Acidic dense solution Volume (μL) 0.2 M Immobiline						pH range	Basic light solution Volume (μL) 0.2 M Immobiline					
p*K* 3.6	4.6	6.2	7.0	8.5	9.3		3.6	4.6	6.2	7.0	8.5	9.3
–	904	–	–	–	129	3.8 – 4.8	–	686	–	–	–	477
–	817	–	–	–	141	3.9 – 4.9	–	707	–	–	–	525
–	755	–	–	–	157	4.0 – 5.0	–	745	–	–	–	584
–	713	–	–	–	177	4.1 – 5.1	–	803	–	–	–	659
–	689	–	–	–	203	4.2 – 5.2	–	884	–	–	–	753
–	682	–	–	–	235	4.3 – 5.3	–	992	–	–	–	871
–	691	–	–	–	275	4.4 – 5.4	–	1133	–	–	–	1021
–	716	–	–	–	325	4.5 – 5.5	–	1314	–	–	–	1208
562	600	863	–	–	–	4.6 – 5.6	–	863	863	–	–	105
458	675	863	–	–	–	4.7 – 5.7	–	863	863	–	–	150
352	750	863	–	–	–	4.8 – 5.8	–	863	863	–	–	202
218	863	863	–	–	–	4.9 – 5.9	–	863	863	–	–	248
158	863	863	–	–	–	5.0 – 6.0	–	863	803	–	–	338
113	863	863	–	–	–	5.1 – 6.1	–	863	713	–	–	443
1251	–	1355	–	–	–	5.2 – 6.2	337	–	724	–	–	–
1055	–	1165	–	–	–	5.3 – 6.3	284	–	694	–	–	–
899	–	1017	–	–	–	5.4 – 6.4	242	–	682	–	–	–
775	–	903	–	–	–	5.5 – 6.5	209	–	686	–	–	–
676	–	817	–	–	–	5.6 – 6.6	182	–	707	–	–	–
598	–	775	–	–	–	5.7 – 6.7	161	–	745	–	–	–
536	–	713	–	–	–	5.8 – 6.8	144	–	803	–	–	–
486	–	689	–	–	–	5.9 – 6.9	131	–	884	–	–	–
447	–	682	–	–	–	6.0 – 7.0	120	–	992	–	–	–
416	–	691	–	–	–	6.1 – 7.1	112	–	1133	–	–	–
972	–	–	1086	–	–	6.2 – 7.2	262	–	–	686	–	–
833	–	–	956	–	–	6.3 – 7.3	224	–	–	682	–	–
722	–	–	857	–	–	6.4 – 7.4	195	–	–	694	–	–
635	–	–	783	–	–	6.5 – 7.5	171	–	–	724	–	–
565	–	–	732	–	–	6.6 – 7.6	152	–	–	771	–	–
509	–	–	699	–	–	6.7 – 7.7	137	–	–	840	–	–
465	–	–	683	–	–	6.8 – 7.8	125	–	–	934	–	–
430	–	–	684	–	–	6.9 – 7.9	116	–	–	1058	–	–
403	–	–	701	–	–	7.0 – 8.0	108	–	–	1217	–	–
381	–	–	736	–	–	7.1 – 8.1	103	–	–	1422	–	–
1028	–	–	750	750	–	7.2 – 8.2	548	–	–	750	750	–
983	–	–	750	750	–	7.3 – 8.3	503	–	–	750	750	–
938	–	–	750	750	–	7.4 – 8.4	458	–	–	750	750	–
1230	–	–	–	1334	–	7.5 – 8.5	331	–	–	–	720	–
1037	–	–	–	1149	–	7.6 – 8.6	279	–	–	–	692	–
885	–	–	–	1004	–	7.7 – 8.7	238	–	–	–	682	–
764	–	–	–	893	–	7.8 – 8.8	206	–	–	–	687	–
667	–	–	–	810	–	7.9 – 8.9	180	–	–	–	710	