ELSEVIER

Journal of Chromatography B, 699 (1997) 77–89

JOURNAL OF CHROMATOGRAPHY B

Review

Isoelectric focusing in immobilized pH gradients: an update

Pier Giorgio Righetti*, Alessandra Bossi

Department of Agricultural and Industrial Biotechnologies, University of Verona, Strada le Grazie, Ca' Vignal 37134 Verona, Italy

Abstract

The latest trends on isoelectric focusing (IEF) in immobilized pH gradients (IPG) are here reviewed. The major advances on IPG technologies have been made when interfacing this technique with sodium dodecyl sulfate-polyacrylamide gel electrophoresis to produce two-dimensional (2-D) maps. Previous 2-D maps were routinely performed using conventional IEF as a first dimension, which typically resulted in poor reproducibility of spot position. With IPGs, correlation between experimental and calculated protein p*I* values is as good as ±0.01 to 0.02 pH units. A new software has also been released, permitting easy calculation and optimization of linear, concave and convex exponential gradients, even in very complex recipes utilizing all ten Immobiline chemicals. It has also been proven that IPGs can be interfaced with mass spectrometry, thus obtaining a novel 2-D map with the best of p*I* measurements in the first dimension coupled with the best of mass determination in the second dimension. Recently, it has been shown that IPGs can be exploited to charter forbidden grounds, with the creation of non-linear pH gradients covering the extreme alkaline pH 10–12 gradient. In such basic regions, excellent steady-state patterns of histones and subtilisin mutants have been reported. Different families of histones could be mapped not only in this pH 10–12 interval, but also in 2-D maps exploiting this very alkaline gradient in the first dimension. Although the IPG technique is now a trouble-free, user-friendly technique, some annoying artefacts, producing severe protein smears and precipitation, were very recently reported, but found to be linked to some commercial Immobiline preparations containing up to 5% oligomers. Better quality control on the part of the company producing such chemicals should eliminate even this last source of troubles.

Keywords: Immobilized pH gradients; Reviews; Proteins; Immobilines

Contents

1. Introduction 78
2. Two-dimensional maps 78
3. Correlating protein p*I*s with amino acid composition 79
4. Computer modelling 81
5. Interfacing IPGs with mass spectra 82
6. Very alkaline pH gradients and 2-D maps 83
7. The latest artefacts 86
8. Conclusions 87

*Corresponding author. Address for correspondence: L.I.T.A., University of Milano, via Fratelli Cervi 93, Segrate 20090 (Milano), Italy.

0378-4347/97/$17.00
PII S0378-4347(96)00151-X

9. Abbreviations 87
Acknowledgements 87
References 99

1. Introduction

Isoelectric focusing (IEF) in immobilized pH gradients (IPG) represent the latest development in focusing methods, i.e., electrophoretic techniques able to create and maintain a pH gradient in an electric field throughout the duration of the separation process until the attainment of steady-state conditions. Conventional IEF is based on the use of a multitude (perhaps a few thousand) of soluble, amphoteric buffers (carrier ampholytes), able to migrate electrophoretically to their isoelectric point (p*I*) values and, once there, to maintain a constant pH value by exerting substantial buffering capacity and providing good conductivity [1]. Conversely, in IPGs, the pH gradient is generated prior to the electrophoretic step by casting a gradient gel with the aid of a two-vessel gradient mixer. The buffers (8 weak acrylamido derivatives containing either carboxyl groups or tertiary amino groups, supplemented by one strongly acidic and one strongly basic derivative) are covalently affixed to the polyacrylamide matrix, and this provides an indefinitely stable pH gradient [2].

The first report on the IPG technique appeared in 1982 [3], but the method had a remarkably slow growth, owing to some problems connected with the chemicals used to create and maintain the pH gradient (available commercially under the tradename Immobiline from Pharmacia Biotechnologies, Uppsala, Sweden). These problems, particularly vexing with the alkaline species, could be grouped into three categories: (a) auto-polymerization, to form oligomers and *n*-mers; (b) hydrolysis to free acrylic acid and a diamine; (c) formation of N-oxides due to persulphate oxidation during polymerization [4]. The second generation of these compounds (Immobiline II), launched in the summer of 1988 [5], now offers a trouble free technique of unrivalled versatility and resolving power. In fact, with a resolution capability (ΔpI, expressed in difference in p*I* values between a protein and the nearest resolved contaminant) of 0.001 pH units [6], this technique has no matching counterpart in the field of separation science.

The specific advantages of focusing techniques over other conventional electrophoretic procedures are as follows: (a) to a large extent, the results do not depend on the mode of sample application, the total protein load or the time of operation, as both IEF and IPG are steady-state techniques [an intrinsic physicochemical parameter of the amphoteric analyte (its p*I*) can be measured]; (b) excellent resolution is possible between variants whose p*I* values differ by only 0.02 (in conventional IEF) or by ca. 0.001 pH unit (with IPGs) [the protein bands are extremely sharp (focusing effect)].

Of course, with the growth of the new star, capillary zone electrophoresis (CZE), the situation has changed dramatically. Unlike IEF and IPG, which are labor-intensive and require largely manual operation, CZE offers complete automatic procedures, with on-line detection and quantitation, and short analysis times. Additionally, CZE requires truly minute sample amounts and volumes (a few μl at the injection port, but only a few nl in the analyte zone!) [7]. Nevertheless, while CZE is offering spectacular results in analysis of DNA fragments, its usage in IEF is still in its infancy, as documented in some recent reviews [8–11]; moreover, it has been impossible up to the present to adapt the IPG technique to the capillary format.

Since the IPG technique, which had already plateaued in 1990, has been extensively described in the manual which appeared in the same year [2], we will refer the reader to this book for an in depth view of the field. Additionally, two reviews have appeared in 1991, describing its applications in clinical chemistry and forensic analysis [12], as well as in two-dimensional (2-D) protein mapping [13]. Thus, we will be plucking (and hopefully not plundering, so as to avoid leaving a trail of smouldering ruins on our wake, like Attila the Hun) the last three years of literature, highlighting only the last few important developments in the field.

2. Two-dimensional maps

Already in 1988 the unique advantages of 2-D maps using the IPG technique in the first dimension were well documented [14]. One of the major difficulties in establishing 2-D maps had been the variability of spot position in the first (focusing) dimension, due to batch to batch variation of carrier ampholytes and to pH gradient decay in conventional IEF. This rendered quite problematic spot identification, pattern matching and inter-laboratory comparison. On the contrary, with the use of IPGs, constant zone position, even over several days, and pattern constancy had been amply demonstrated and verified. As an extra bonus, IPGs allowed the creation of reproducible, non-linear pH gradients tailored to spot distribution and frequency along the pH scale [15]. A flurry of articles appeared at that time describing important applications of IPGs in 2-D analysis of human skin fibroblast and myeloblast proteins [16], of tubulin proteins in neuronal and non-neuronal tissues [17], of platelet polypeptides [18], to name just a few. Yet, the vast majority of laboratories routinely using and developing 2-D techniques remained adamantly attached to the conventional IEF technique, refusing to convert to the new system.

One of the groups to finally yield to the new wind was Hochstrasser's laboratory in Geneva. This group had been among the first to apply 2-D plasma/serum protein patterns to the diagnosis of diseases (and of their remittal) in medicine. Yet, conventional 2-D maps had a high coefficient of variation, were relatively expensive and time consuming; the progress was thus quite slow. The switch to IPGs [19] and the use of polyvinylidene difluoride (PVDF) membranes for protein transfer and microsequencing [20] proved to be winning strategies. The potential clinical usefulness of 2-D maps was studied by comparative analysis of plasma/serum obtained from apparently healthy individuals and from patients with a few, selected, known diseases. Despite their apparent complexity, patient plasma/serum protein maps revealed readily detectable modifications of the "reference" protein profile for some selected diseases. Abnormal profiles could be characterized by the presence or absence of particular spots, by the reduction or enlargement of spot size, or by alterations of spot microheterogeneity. Combination of several modifications enabled different "disease-associated spot pattern" to be distinguished on the protein maps of patients with monoclonal gammopathies, hypogammaglobulinemia, hepatic failure, chronic renal failure, hemolytic anemia etc. Blotting on PVDF membranes, followed by microsequencing, allowed the discovery of more than 40 new polypeptide spots previously unreported in human sera. As an extra bonus, the use of IPGs permitted N-terminal sequencing of a much larger number of spots than in conventional IEF gels. It was found that fewer proteins were N-terminal blocked than those eluted from conventional gels. This could possibly be due to the fact that IPG gels are devoid of any residual ungrafted monomers, since they are extensively washed prior to use. Addition of the free acrylamide monomer, present in un-washed gels, has been clearly demonstrated also by NMR spectra of the adduct with amino acids [21,22]. Fig. 1 gives an idea of the wealth of information which can be obtained in 2-D maps of human plasma proteins. It is an enlargement of the more acidic (pH 3.5 to 6.5) and medium molecular mass area of a full 2-D map (the latter revealing >600 spots). The July 1995 issue of Electrophoresis reports the proceedings of a meeting on 2-D maps organized in September 1994 by Drs. V. Pallini, L. Bini and D.F. Hochstrasser at the University of Siena (a second meeting on the same topic will be held in Siena in September 1996 and the proceedings will be available in Electrophoresis sometime in 1997). Several articles, in this issue, highlight the dramatic progress made in 2-D maps, especially in regard to the use of IPGs in the first dimension. We will recall just a few: sequence analysis of wheat grain allergens in IPG-Dalton (DALT) [23]; inside Swiss 2D-PAGE database [24]; phenotyping of apolipoprotein E [25]; protein patterns of spermatocytes and round spermatids of rat testis [26]; genetic variability of carrot seed proteins [27].

3. Correlating protein p/s with amino acid composition

In recent literature [28,29], excellent agreement was found among theoretical p/s predicted from a

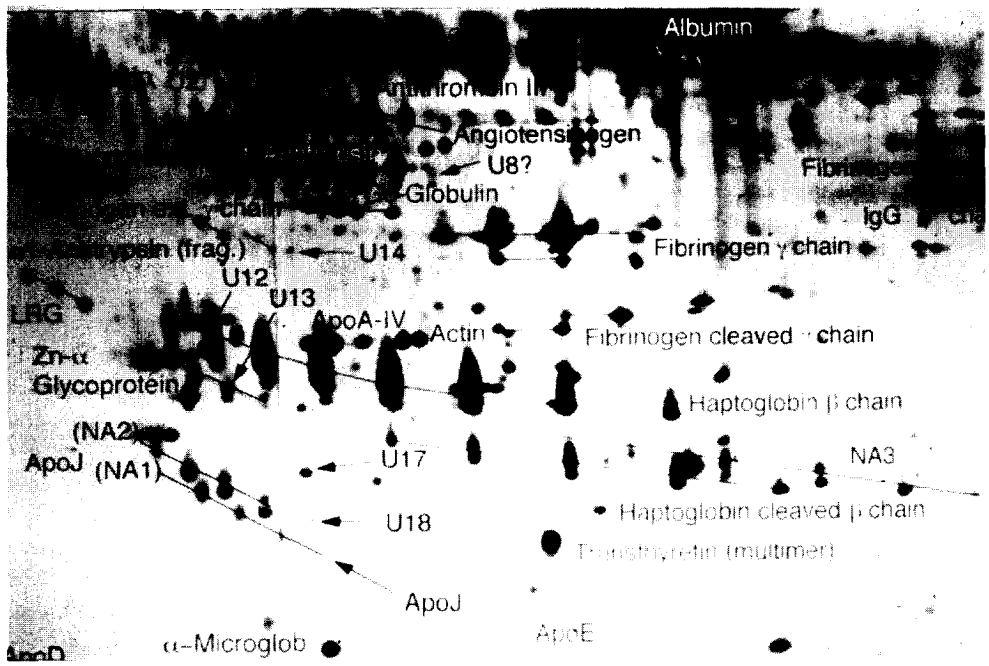

Fig. 1. Enlargement of the more acidic (pH 3.5 to 6.5) and medium M_r area of a full 2-D map of human plasma protein. The latter map was obtained in a non-linear pH 3.5-10 IPG interval. Plasmatic proteins (0.75 μl or 45 μg) were loaded at the cathodic gel side. After the second dimensional separation, the gel was silver stained. The newly identified spots are indicated by red arrows or lines. "U" stands for an unknown sequence in the Swiss-Prot database. Spots U12 and U13 are in the position of the Zn-α-glycoprotein, leucin rich glycoprotein (LRG) (from Hughes et al. [19], with permission).

protein amino acid sequence and experimental p*I*s as derived from an IPG gel. The agreement between the two sets of data was, in the case of a set of 29 proteins, as good as ±0.01 to 0.02 pH units, quite remarkable indeed. Such a straight correlation permitted a re-evaluation of the few outliers: when their composition was re-assessed, it was found that either the original amino acid sequence first reported was wrong, or that some post-synthetic modifications had occurred altering the predicted p*I* value. Fig. 2 gives an example of such a unique correlation. Here, 36 polypeptides from normal human keratinocytes are plotted, with a fairly large scatter of points in the pH 6 to 7 range (Fig. 2A). However, when the same data are re-calculated assuming N-terminal blockage for a number of polypeptide chains, a much superior data fit is observed (Fig. 2B). However, two major points should be highlighted: (i) these data refer to randomized structures denatured in 9.8 *M* urea; (ii) the agreement was only proven for acidic proteins, the correlation ending at pH 7.0. Thus, it remains to be seen if such a good correlation actually exists, even at moderately to very alkaline p*I* values, in view of the many existing difficulties (e.g. uncertainties on the true p*K* values of alkaline Immobiline species, especially when incorporated into the gel matrix; lack of precise data on the true incorporation of such alkaline compounds as the quaternary base, which moreover is not even a pure acrylamide, but a methacrylamide derivative). Other factors might contribute to p*I* uncertainties in alkaline milieus. One is the matrix effect, since Bossi et al. [30] have demonstrated that, when substituting conventional acrylamide with the very hydrophilic N-acryloylaminoethoxyethanol matrix, an appreciable p*K* shift ensues. Another one is the effect of additives to the gel matrix. In alkaline intervals, it is customary to add, e.g., 30% sorbitol or other polyols to the gel, so as to minimize electrosmotic flux due to the net charge of the gel matrix. While Gelsema et

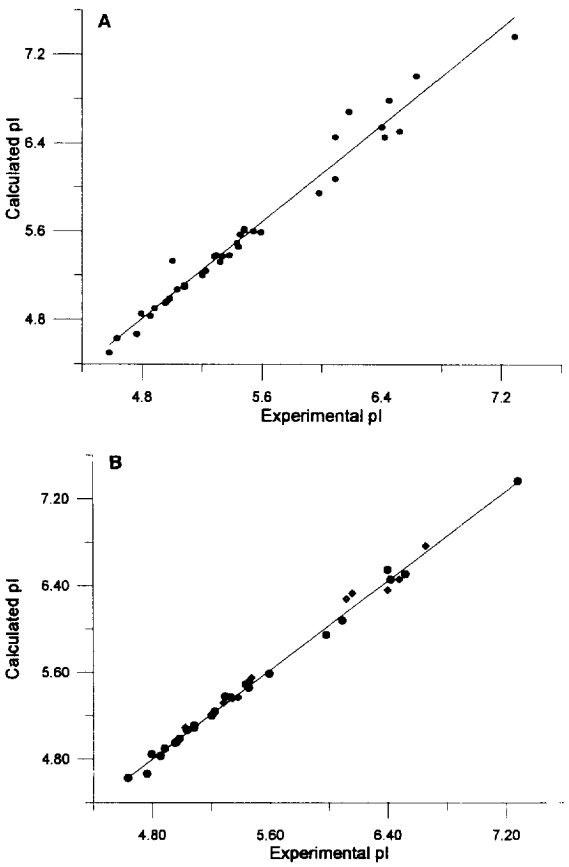

Fig. 2. Calculated vs. experimental p*I* values (the latter obtained in IPG gels in 9.8 *M* urea, 2% 3-[(cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS) and 10 m*M* DTT). Lines are fitted using the least squares' criterion. (A) 36 polypeptides from normal human keratinocytes; (B) same as A, in which the p*I* values were re-calculated for 12 polypeptides assuming N-terminal blockage, the open diamonds indicate re-calculated p*I* values (from Bjellqvist et al. [29], with permission).

al. [31–33] demonstrated that the addition of up to 60% sucrose or glycerol to the focusing media produced only small errors in p*I* assessments, of the order of ±0.1 pH units, this effect was only explored in the interval $3<pI<9$ and it might not apply to much higher pH values, up to pH 12, as obtainable in modern IPG recipes. That the problem with alkaline proteins could be particularly severe is also suggested by Watts and Singh [34]: e.g. with bovine carbonic anhydrase, a theoretical p*I* of 9.6 (corrected for temperature and 8 *M* urea) was given vs. an experimental p*I* of 7.0; for horse myoglobin (skeletal) a theoretical p*I* of 9.7 vs. an experimental p*I* of 8.0 (however their data refer to conventional IEF in amphoteric buffers, not to IPGs).

4. Computer modelling

Since the late seventies, computers have been used as tools in the development of high-performance liquid chromatography (HPLC) separations. During the past decade, a number of international groups have worked intensively in the area of computer-assisted chromatographic method development, as summarized in some recent books [35–37]. All of these activities have produced a number of software packages for optimization of HPLC separations, available from several companies [38]. If we do not consider the software for image acquisition and analysis in 2-D maps, not much had been developed for helping electrophoresis users. Perhaps one of the first computer programs has been the one developed by our group to help in calculating extended IPG recipes, a formidable computing program which took almost 10 years to arrive at the final product. The first approach to the formation of extended pH gradients was through the sequential elution of buffering species of increasing p*K* from a five-chamber mixer [39]. This procedure was soon abandoned in favour of standard two-vessel gradient mixing, for which we studied the conditions for gradient linearity, as a function of the p*K* distribution of the buffers and of the titrants [40,41]. The above programs had as a target, for producing linear pH gradient, the minimisation of the coefficient of variation of the buffering power [C.V.(β)], which had several shortcomings. We thus abandoned C.V.(β) in favour of a novel target function: minimisation of the sum of squares of residuals. The new simulator performed considerably better than the previous programs, and allowed calculation of recipes having deviations from linearity well below 1% of the given pH interval (a limit set for the previous programs) [42,43]. The final evolution came in 1993, when we developed a novel software package, on a windows platform, including several new features as compared with previous simulators [44]. First, the estimation of the pH gradient (a non-linear problem) was transformed into a linear programming problem, thus

allowing the use of the simplex as an optimization algorithm. Second, several types of pH gradients could be simulated and optimized, including linear, exponential, logarithmic and sigmoidal. Finally, an equation was implemented in the program to account for the variation of the activity coefficient of ions as a function of the prevailing ionic strength in solution. The simulator was checked experimentally by eluting solutions from a two-vessel gradient mixer and verifying the shape of the various pH gradients: an excellent correlation between simulated and experimental data could be obtained. Fig. 3 gives some examples of different types of linear and non-linear gradients which can be calculated: it should be noted that the deviation from the expected shape (error) is truly minute.

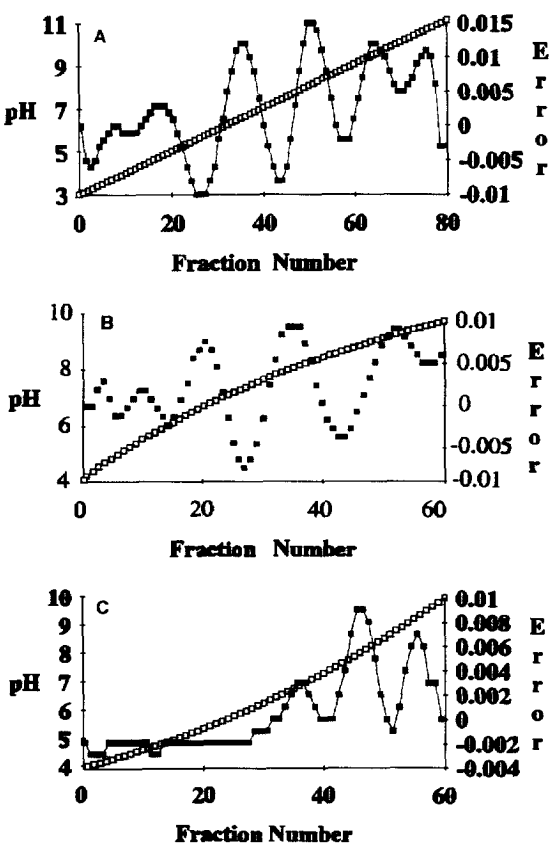

Fig. 3. Examples of different shapes of IPG gradients which can be simulated with the program of [44]. (A) Linear IPG in the pH 3–11 interval; (B) logarithmic pH 4–10 gradient; (C) exponential pH 4–10 gradient. Symbols: open squares, pH gradient profile; solid squares, deviation from the target shape. All gradients have been calculated with the constraint of an average β of 6 mequiv. L^{-1} pH^{-1} (from Giaffreda et al. [44], with permission).

5. Interfacing IPGs with mass spectra

While polyacrylamide gel electrophoresis (PAGE), conducted either as 1- or 2-D separations, is an almost universally used technique in protein biochemistry, up to recent times it has been used essentially as a descriptive method, since it could not provide direct chemical characterisation of analytes. Two major events have occurred in the last decade, which have transformed PAGE into a most refined physico-chemical tool for unequivocal protein identification. After 1985, a few groups developed general methods for direct N-terminal [45] and internal sequencing [46] of gel separated proteins, not only from 1-D, but also from 2-D matrices [47]. The second event has been the coupling of mass spectrometric (MS) to electrophoretic techniques. The breakthrough in MS of proteins and peptides came in 1988 with the development of two new methods for introducing large, intact molecules containing excess charge into the gas phase. One is electrospray ionization mass spectrometry (ESI-MS) [48], in which ions are formed from a solution at atmospheric pressure; the other is matrix-assisted laser-desorption time of flight (MALDI-TOF) [49], in which ions are formed from a solid state containing an intimate mixture of analyte and a weak aromatic acid. Both methods allow precise mass determinations with an error not exceeding ±0.01%, i.e., typically ±1 over 10 000 mass units. ESI is characterized by the formation of multiple ionized species. Ions from a pure polypeptide sample differing in their charge state are therefore detected as an envelope of peaks of different m/z (mass/charge) values. From the series of measured m/z values, the molecular mass of the polypeptide is easily calculated by a process commonly referred to as deconvolution. This fact of developing a number of peaks of different m/z values has some important practical implications: molecular masses well in excess of the nominal mass range of the mass analyzer, and extending up to 200 000, can be determined. Addi-

tionally, due to the ease of operation in eliciting ions, ESI-MS could be connected on line with, e.g., CZE units [50,51]. However, for gel phases, the macroion has to be eluted, typically by electroblotting onto a membrane, thus the coupling can only be off-line (at present, at least). Two groups have independently developed procedures for analyzing proteins blotted from 1-D [typically sodium dodecyl sulfate (SDS)-gels] and 2-D matrices: Patterson [52–54] and Lottspeich [55,56]. In one approach, developed for SDS-PAGE, after blotting onto Immobilon-CD membranes, Patterson [53] has illustrated 3 methods for obtaining MALDI-TOF-MS data from a single protein band: (i) direct MALDI-MS of ca. 10% of the band; (ii) cyanogen bromide cleavage of another 10% of the band and (iii) enzymatic digestion of the remaining 70–80% of the band, followed by MALDI-MS. With the peptide fragments, sufficient data on the mass values could be obtained from as little as 5 pmol of protein loaded onto the gel. Also, Sutton et al. [57] have attempted identification of myocardial proteins from 2-D gels by peptide mass fingerprinting, again using MALDI-TOF-MS. Interestingly, alkylation of Cys and oxidation of Met residues were found to be two significant modifications that influenced successful identification of a protein spot. Taken together, these few examples show the impressive progress made in protein identification by interfacing PAGE techniques with MS. However, when one looks carefully at the data, one can notice that, in reality, most of the mass values are obtained after an SDS-PAGE step, which already is an intrinsic measure of mass. Thus, the data are repetitive in that a raw mass measurement (as derived from SDS-PAGE) is further refined by a precise M_r evaluation (as assessed by MS). We have adopted another strategy: directly interfacing an IPG gel (which already gives the best in p*I* evaluation) with mass spectra (which, of course, offer the best in mass determination) [58]. It should be noted that, in this last case, a true 2-D technique is generated. An example of this method is given in Fig. 4. Some

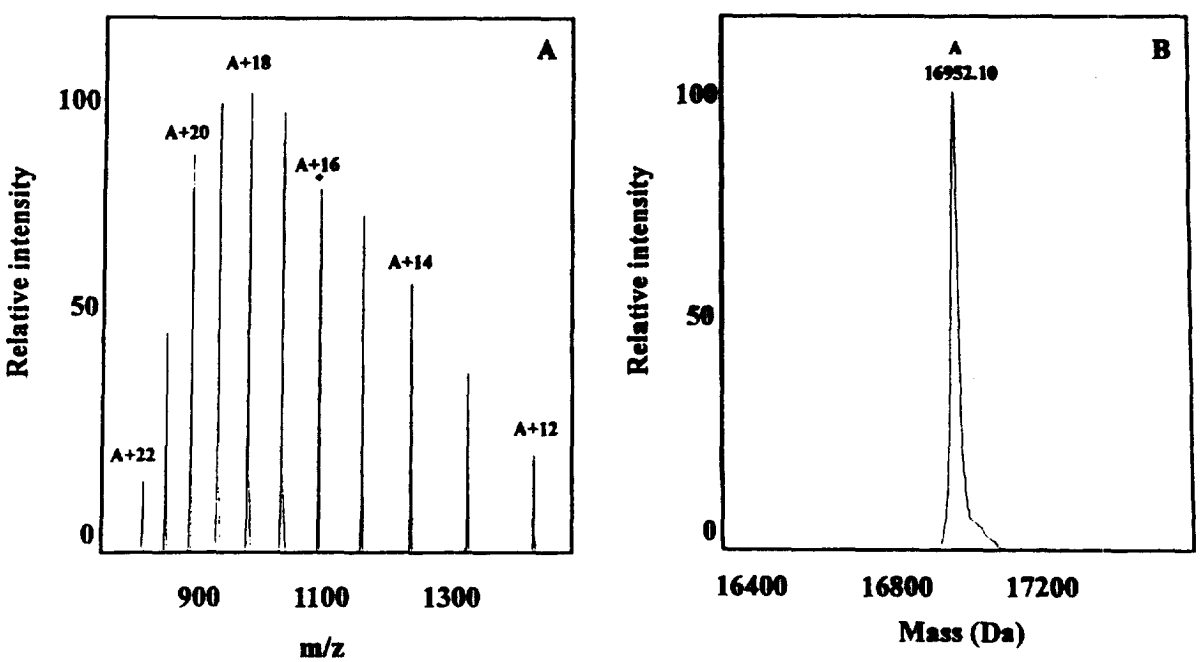

Fig. 4. ESI-MS of the main myoglobin band (p*I* 7.3) eluted from an IPG gel, showing the same multiply charged ions distribution (A) of a myoglobin sample analyzed directly (not shown). The deconvoluted mass spectrum (B) gave an M_r of 16 952, which closely matched the calculated value of 16 951 (from Breme et al. [58], with permission).

more information on interfacing MS with planar electrophoresis can be found in [59].

6. Very alkaline pH gradients and 2-D maps

With the introduction of the p*K* 10.3 Immobiline (N,N-diethylaminopropylacrylamide) we were able to report some rather alkaline IPG intervals, spanning the pH 10–11 gradient [60,61]. In such gradients, we could focus proteins, such as cytochrome C (p*I* 10.45), typically lost in the cathodic compartment in conventional IEF. Other macromolecules, such as elastase, could not only be analyzed in such basic gradients, but were found, by zymogramming, to retain their enzyme activity. Recently, however, we were able to optimize an extremely alkaline gradient, covering the pH 10–12 interval and to successfully apply it to the analysis of very basic proteins, such as subtilisins [62] and histones [63]. In the former case, we could focus and assess the purity of two subtilisins, Savinase and Durazym, produced in large scale by Novo Nordisk, and used as additives for household laundry detergents. These enzymes are serine endopeptidase with an extended binding cleft comprising at least eight binding subsites. They are both relatively small proteases, composed of 269 amino acids. They could not be analyzed by conventional IEF in soluble, amphoteric buffers, as they were regularly lost in the cathodic compartment. In the pH 10–12 IPG interval, Savinase was found to have a p*I* of 11.15±0.15 and Durazym a p*I* of 10.95±0.17. Both enzymes were proven to be active by an in situ zymogramming consisting of a casein/agar overlay. Fig. 5 gives an example of such a separation, demonstrating the unique resolving power of IPGs even under such extreme conditions. Even more spectacular is the histone separation shown in Fig. 6: here all histone families were found to focus in the upper gel region, covering the pH 11 to 12 range. When over-developing the pH 10–11 region, which seemed to be devoid of proteins, a fine spectrum of sharp bands were found to cover this interval. This separation is truly unique, since never before had histones been seen focused at their p*I* position: typically, they had been analyzed in acetic-acid–urea gels, i.e., under conditions in which they bear a high net positive charge [64]. It should be noted, in addition, that the focusing pattern in Fig. 6 is consistent with the basicity of the various classes of histones; additionally, their p*I* values (in the range of 11 to 12 and perhaps even higher) are in agreement with their relative amino acid composition, as calculated with our computer program (which can also calculate p*I* values by entering the p*K* values of amino acids instead of Immobilines) [44].

Interestingly, we have ben able to report, for the first time, true steady-state, 2-D maps of histones. An example of such 2-D separation is shown in Fig. 7: a total of 35 individual spots, exhibiting p*I* values between pH 11 and 12 and M_r values from 13 000 to 32 000 (with the heaviest distribution around 18 000) could be counted [65].

7. The latest artefacts

With this brief excursus, we could stop here and the readers would only remember the glorious notes of this IPG paean. As luck goes, we recently found some disturbing phenomena, which for a moment threw us back to an ante-1986 condition, when the first artefacts of the IPG technique were denounced. As stated in the introduction, the most noxious problems of IPGs (connected with the alkaline Immobilines), namely auto-polymerization and hydrolysis, had been fully eliminated in 1988. Yet, we recently found [66] that, in recipes as wide as pH 4–9, encompassing neutrality and containing the p*K* 7.0 Immobiline as one of the buffering ions, smears were directly proportional to the total amount of this p*K* 7.0 species present in the gradient formulation. At a total level of 10 m*M* p*K* 7.0 in these recipes, severe smears occurred not only for mildly hydrophobic proteins (e.g., recombinant alcalase and termamylase) but also for the relatively hydrophilic p*I* marker proteins. Streaks and smears were essentially abolished in recipes devoid of the p*K* 7.0 Immobiline or in formulations containing a maximum of 3 m*M* of this compound. Although, by partitioning in water/*n*-octanol, it has been found that the p*K* 7.0 Immobiline is quite hydrophobic (partition coefficient $P=0.5$), the problem was traced back to the presence of oligomers in some commercial prepara-

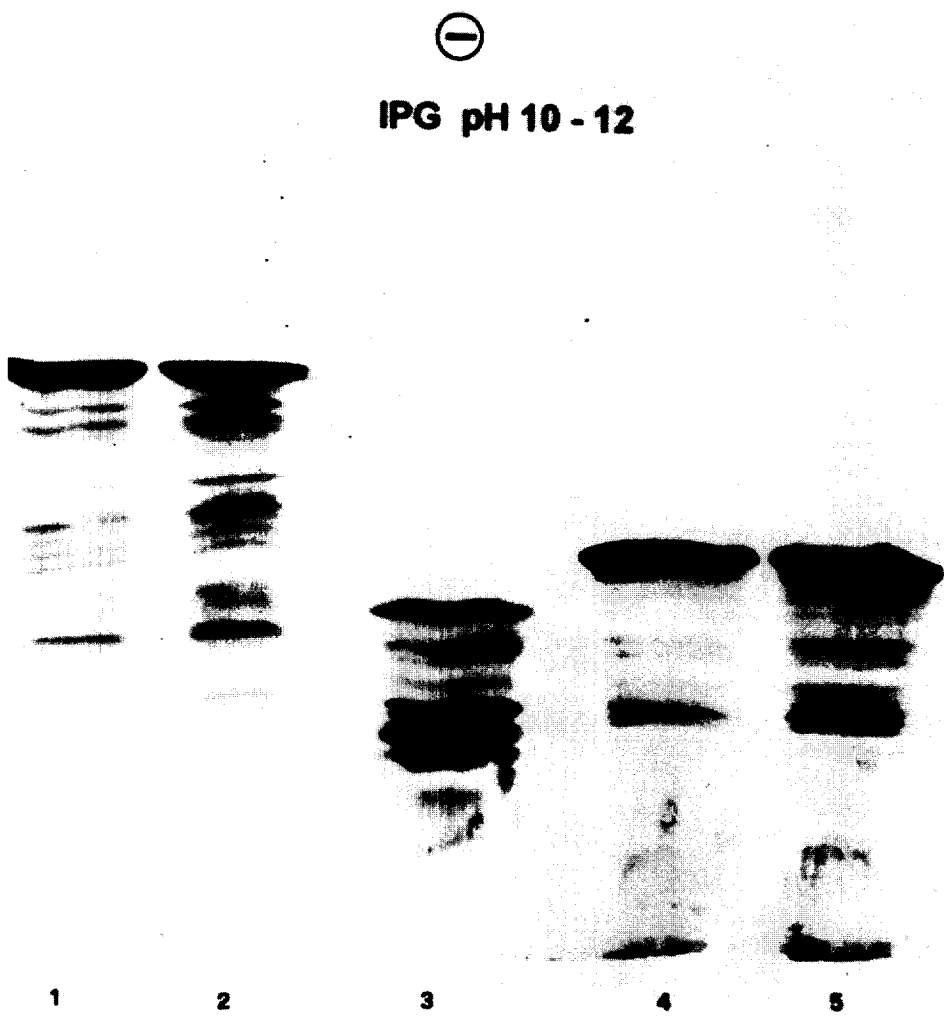

Fig. 5. Focusing of: (1, 2) Savinase; (3) cytochrome *c*; (4, 5) Durazym in a non-linear, IPG 10–12 interval. Average β: 8 mequiv. L^{-1} pH^{-1}. Conditions: gel run under light paraffin oil; sample loaded in surface basins at the anodic side; focusing at 1500 V for up to 4 h. Staining with Coomassie Brilliant Blue R-250 (from Bossi et al. [62], with permission).

tions (see Fig. 8). Thus, the users should be aware of the fact that, even when dissolved in *n*-propanol, some batches of Immobilines might still contain oligomers, probably formed during the synthetic step. This problem did not occur with the Pharmacia Immobilines (since this company routinely checks their products for the presence of oligomers) but was particularly evident with the Fluka products. It is hoped that this last inconvenience will also be rapidly eliminated with appropriate steps in quality control.

8. Conclusions

We hope that this brief review on the most recent aspects of IPGs will convince the readers that this technique has much to offer, is now a trouble-free

Fig. 6. Focusing of histones in an IPG pH 10–12 interval. Gel: 6% T, 4% C polyacrylamide matrix, containing an IPG 10–12 gradient, reswollen in 7 *M* urea, 1.5% Nonidet P-40 and 0.5% Ampholine pH 9–11. The gel was run at 10°C under a layer of light paraffin oil at 500 V for the first hour, followed by increasing voltage gradients, after sample penetration, up to 1300 V for a total of 4 h. The samples (2 mg/ml; 50 μl seeded) were loaded in plastic wells at the anodic gel surface. Staining with Coomassie Brilliant Blue R-250 in Cu^{2+}. Tracks (from left to right): (1–3) VIII-S histones; (4–6) II-AS histones; (7) cytochrome *c* (the main upper band has a p*I* of 10.5). Note the fine spectrum of sharp bands (more than a dozen) focusing in the pH 10–11 region (from Bossi et al. [63]. with permission).

operation and is also user friendly, due to the availability of appropriate software and of ready made, commercially available gel strips covering a range of IPG intervals. The latest developments, such as interfacing IPGs with MS, now provide a unique environment for unambiguous identification of proteins and peptides. It is anticipated that the technique will grow substantially, especially in its application as first dimension of 2-D maps, and in its preparative aspects, owing to the remarkable concepts of multicompartment electrolyzers with isoelectric membranes (see the appropriate review in this monograph).

9. Abbreviations

2-D Two-dimensional maps

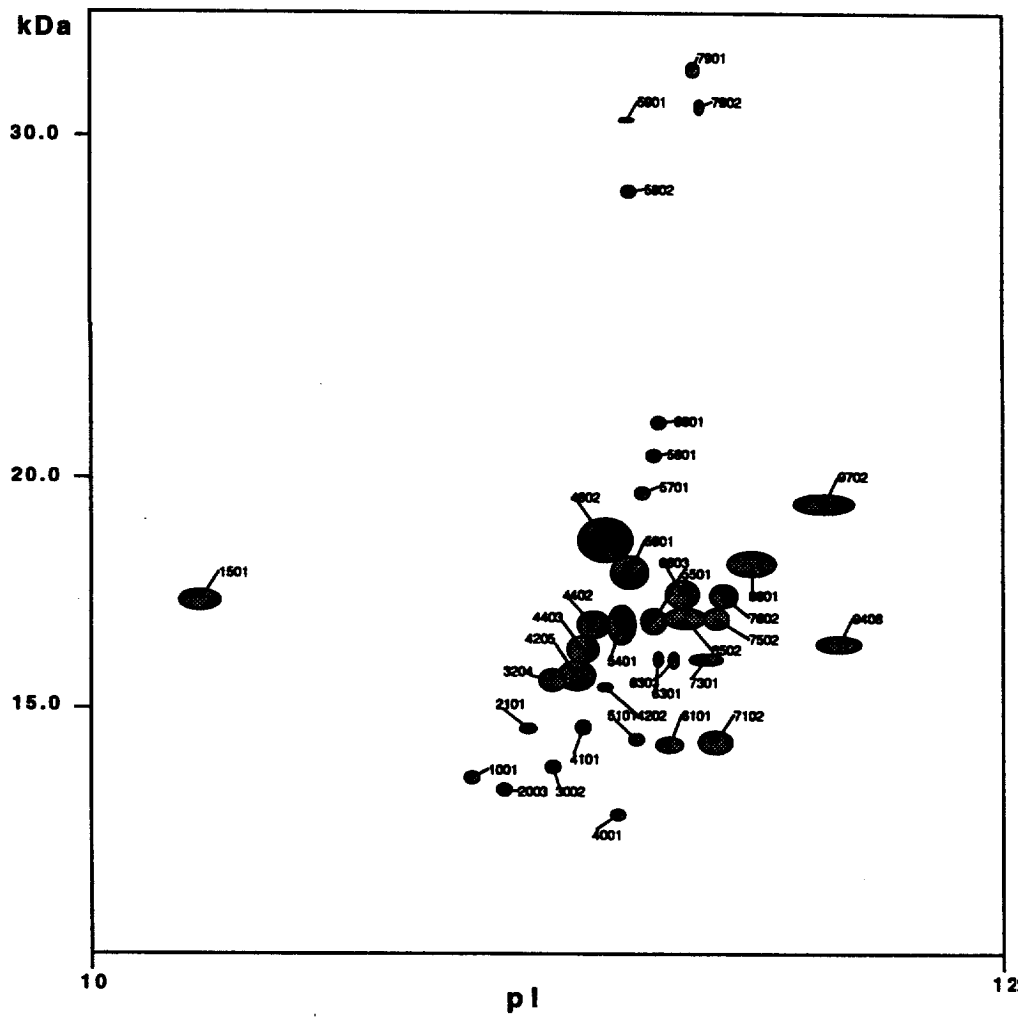

Fig. 7. Computer image analysis of a 2-D map of a mixture of all histone fractions of Fig. 6. A total of 35 spots is detected and quantified. Spot No. 1501 is cytochrome *c* added as a p*I* marker (from Righetti et al. [65], with permission).

CZE	Capillary zone electrophoresis
DALT	For Dalton: second dimension of a 2-D map
ESI	Electrospray ionization
HPLC	High-performance liquid chromatography
IEF	Isoelectric focusing
IPG	Immobilized pH gradients
MALDI	Matrix-assisted laser desorption ionization
MS	Mass spectra
NMR	Nuclear magnetic resonance
P	Partition coefficient
PAGE	Polyacrylamide gel electrophoresis
p*I*	Isoelectric point
PVDF	Polyvinylidene difluoride
TOF	Time of flight

Acknowledgements

Support from ASI (Agenzia Spaziale Italiana, Roma) from ESA (European Space Agency) and from Radius of Biotechnology (ESA, Paris, France)

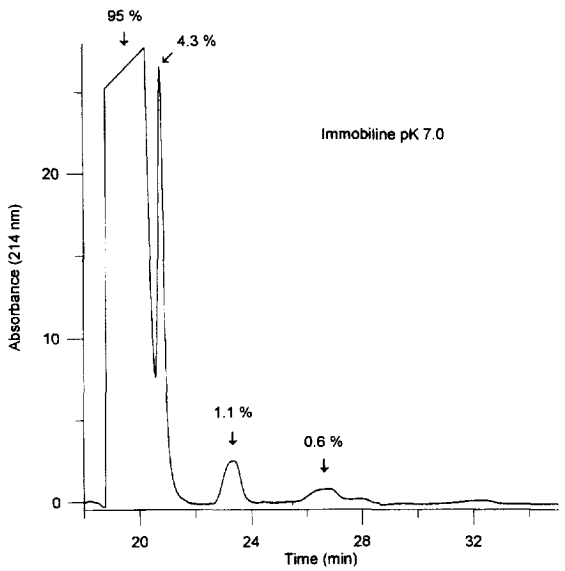

Fig. 8. Determination of the extent of oligomer formation in the Fluka's p*K* 7.0 Immobiline by micellar electrokinetic chromatography. Buffer: 100 m*M* borate, pH 9.0, supplemented with 20 m*M* SDS. The run was in the catodic direction, at 3 kV and 22 μA. Samples (20 m*M*) were loaded for 10 s by the hydrostatic injection method. Sample zones were revealed at 214 nm. The run was performed with a Waters Quanta 4000-E instrument (from Esteve-Romero et al. [66], with permission).

during the development of the various research projects reviewed here is gratefully acknowledged.

References

[1] P.G. Righetti, Isoelectric Focusing: Theory, Methodology and Applications, Elsevier, Amsterdam, 1983.
[2] P.G. Righetti, Immobilized pH Gradients: Theory and Methodology, Elsevier, Amsterdam, 1990.
[3] B. Bjellqvist, K. Ek, P.G. Righetti, E. Gianazza, A. Görg, R. Westermeier and W. Postel, J. Biochem. Biophys. Methods, 6 (1982) 317–339.
[4] P.G. Righetti, M. Chiari, E. Casale and C. Chiesa, Theor. Applied Electrophor., 1 (1989) 115–121.
[5] B.M. Gåveby, P. Pettersson, J. Andrasko, L. Ineva-Flygare, U. Johannesson, A. Görg, W. Postel, A. Domscheit, P.L. Mauri, P. Pietta, E. Gianazza and P.G. Righetti, J. Biochem. Biophys. Methods, 16 (1988) 141–164.
[6] G. Cossu and P.G. Righetti, J. Chromatogr., 398 (1987) 211–216.
[7] P.G. Righetti (Editor), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, Boca Raton, FL, 1996.
[8] P.G. Righetti and C. Tonani, in F. Dondi and G. Guiochon (Editors), Theoretical Advancement in Chromatography and Related Separation Techniques, NATO ASI Series C: Mathematical and Physical Sciences, Vol. 383, Kluwer, Dordrecht, 1992, pp. 581–605.
[9] P.G. Righetti and M. Chiari, in N. Guzman (Editor), Capillary Electrophoresis: Theory, Methodology and Applications, Marcel Dekker, New York, 1993, pp. 89–116.
[10] P.G. Righetti and C. Gelfi, J. Cap. Electrophor., 1 (1994) 27–35.
[11] P.G. Righetti, C. Gelfi and M. Chiari, in P.G. Righetti (Editor), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, Boca Raton, FL, 1996, pp. 509–538.
[12] P.G. Righetti, E. Gianazza, A. Bianchi-Bosisio, P.K. Sinha and E. Kottgen, J. Chromatogr., 569 (1991) 197–228.
[13] E. Gianazza and P.G. Righetti, J. Chromatogr., 569 (1991) 43–62.
[14] A. Görg, W. Postel and S. Günther, Electrophoresis, 9 (1988) 531–546.
[15] E. Gianazza, P. Giacon, B. Sahlin and P.G. Righetti, Electrophoresis, 6 (1985) 53–56.
[16] K. Patel, M.J. Dunn, S. Günther, W. Postel and A. Görg, Electrophoresis, 9 (1988) 547–554.
[17] D.J. Field and J.C. Lee, Electrophoresis, 9 (1988) 555–562.
[18] J.I. Asakawa, Electrophoresis, 9 (1988) 562–568.
[19] G.J. Hughes, S. Frutiger, N. Paquet, F. Ravier, C. Pasquali, J.C. Sanchez, R. James, J.D. Tissot, B. Bjellqvist and D.F. Hochstrasser, Electrophoresis, 13 (1992) 707–714.
[20] J.C. Sanchez, F. Ravier, C. Pasquali, S. Frutiger, N. Paquet, B. Bjellqvist, D.F. Hochstrasser and G.J. Hughes, Electrophoresis, 13 (1992) 715–717.
[21] M. Chiari, A. Manzocchi and P.G. Righetti, J. Chromatogr., 500 (1990) 697–704.
[22] M. Chiari, P.G. Righetti, A. Negri, F. Ceciliani and S. Ronchi, Electrophoresis, 13 (1992) 882–884.
[23] A. Posch, W. Weiss, C. Wheeler, M.J. Dunn and A. Görg, Electrophoresis, 16 (1995) 1115–1119.
[24] J.C. Sanchez, R.D. Appel, O. Golaz, C. Pasquali, F. Ravier, A. Bairoch and D.F. Hochstrasser, Electrophoresis, 16 (1995) 1131–1151.
[25] O. Golaz, J.C. Sanchez, R.W. James and D.F. Hochstrasser, Electrophoresis, 16 (1995) 1184–1186.
[26] G. Cossio, J.C. Sanchez, O. Goldz, R. Wettstein and D.F. Hochstrasser, Electrophoresis, 16 (1995) 1225–1230.
[27] A. Posch, B.M. van den Berg, H.C.J. Burg and A. Görg, Electrophoresis, 16 (1995) 1321–1316.
[28] B. Bjellqvist, G.J. Hughes, C. Pasquali, N. Paquet, F. Ravier, J.C. Sanchez, S. Frutiger and D. Hochstrasser, Electrophoresis, 14 (1993) 1023–1031.
[29] B. Bjellqvist, B. Basse, E. Olsen and J.E. Celis, Electrophoresis, 15 (1994) 529–539.
[30] A. Bossi, P.G. Righetti and M. Chiari, Electrophoresis, 15 (1994) 1112–1117.
[31] J.W. Gelsema and C.L. De Ligny, J. Chromatogr., 130 (1977) 41–50.
[32] J.W. Gelsema, C.L. De Ligny and N.G. Van Der Veen, J. Chromatogr., 140 (1977) 149–155.

[33] J.W. Gelsema, C.L. De Ligny and N.G. Van Der Veen, J. Chromatogr., 154 (1978) 161–174.
[34] N.R.M. Watts and R.P. Singh, Electrophoresis, 16 (1995) 22–27.
[35] J.C. Berridge, The Techniques for the Automated Optimization of HPLC Separation, Wiley, New York, 1985.
[36] P.J. Schoenmakers, Optimization of Chromatographic Selectivity, a Guide to Method Development, Elsevier, Amsterdam, 1986.
[37] J.L. Glajch and L.R. Snyder (Editors), Computer-Assisted Method Development for High-Performance Liquid Chromatography, Elsevier, Amsterdam, 1990.
[38] A. Drouen, J.W. Dolan, L.R. Snyder, A. Poile and P.J. Schoenmakers, LC·GC Int., 5 (1992) 28–37.
[39] G. Dossi, F. Celentano, E. Gianazza and P.G. Righetti, J. Biochem. Biophys. Methods, 7 (1983) 123–142.
[40] E. Gianazza, G. Dossi, F. Celentano and P.G. Righetti, J. Biochem. Biophys. Methods, 8 (1983) 109–133.
[41] F. Celentano, E. Gianazza, G. Dossi and P.G. Righetti, Chemometr. Intell. Lab. Syst., 1 (1987) 349–358.
[42] C. Tonani and P.G. Righetti, Electrophoresis, 12 (1991) 1011–1021.
[43] P.G. Righetti and C. Tonani, Electrophoresis, 12 (1991) 1021–1027.
[44] E. Giaffreda, C. Tonani and P.G. Righetti, J. Chromatogr., 630 (1993) 313–327.
[45] R.H. Aebersold, J. Leavitt, R.A. Saavedra, L.E. Hood and S.B.H. Kent, J. Biol. Chem., 261 (1986) 4229–4238.
[46] R.H. Aebersold, J. Leavitt, R.A. Saavedra, L.E. Hood and S.B.H. Kent, Proc. Natl. Acad. Sci. USA, 84 (1987) 6970–6974.
[47] R.H. Aebersold, J. Leavitt, L.E. Hood and S.B.H. Kent in K. Walsh (Editor), Methods in Protein Sequencing Analysis, Humana Press, Clifton, NJ, 1986, pp. 277–294.
[48] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong and C.M. Whitehouse, Science, 246 (1989) 64–71.
[49] M. Karas and F. Hillenkamp, Anal. Chem., 60 (1988) 2299–2301.
[50] J. Cai and J. Henion, J. Chromatogr. A, 703 (1995) 667–692.
[51] M.W.F. Nielen, J. Chromatogr. A, 712 (1995) 269–284.
[52] S.D. Patterson, Anal. Biochem., 221 (1992) 1–15.
[53] S.D. Patterson, Electrophoresis, 16 (1995) 1104–1114.
[54] S.D. Patterson and R. Aebersold, Electrophoresis, 16 (1995) 1791–1914.
[55] C. Eckerskorn, K. Strupat, M. Karas, F. Hillenkamp and F. Lottspeich, Electrophoresis, 13 (1992) 664–665.
[56] K. Strupat, M. Karas, F. Hillenkamp, C. Eckerskorn and F. Lottspeich, Anal. Chem., 66 (1994) 464–470.
[57] C.W. Sutton, K.S. Pemberton, J.S. Cottrell, J.M. Corbett, C.H. Wheeler, M.J. Dunn and D.J. Pappin, Electrophoresis, 16 (1995) 308–316.
[58] U. Breme, J. Breton, C. Visco, G. Orsini and P.G. Righetti, Electrophoresis, 16 (1995) 1381–1384.
[59] K.L. Busch, J. Chromatogr. A, 692 (1995) 275–290.
[60] C. Gelfi, M.L. Bossi, B. Bjellqvist and P.G. Righetti, J. Biochem. Biophys. Methods, 15 (1987) 41–48.
[61] P.K. Sinha and P.G. Righetti, J. Biochem. Biophys. Methods, 15 (1987) 199–206.
[62] A. Bossi, P.G. Righetti, G. Vecchio and S. Severinsen, Electrophoresis, 15 (1994) 1535–1540.
[63] A. Bossi, C. Gelfi, A. Orsi and P.G. Righetti, J. Chromatogr. A, 686 (1994) 121–128.
[64] S. Panyim and R. Chalkley, Arch. Biochem. Biophys., 130 (1969) 337–343.
[65] P.G. Righetti, A. Bossi, A. Görg, C. Obermaier and G. Boguth, J. Biochem. Biophys. Methods, 31 (1996) 81–91.
[66] J. Esteve-Romero, E. Simò-Alfonso, A. Bossi, F. Bresciani and P.G. Righetti, Electrophoresis, 17 (1996) 704–708.