# Focusing Strategy and Influence of Conductivity on Isoelectric Focusing in Immobilized pH Gradients

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## Summary

Sample preparation is a key component of successful two-dimensional (2-D) gel electrophoresis of proteins. No buffer is universally suitable for the extraction and solubilization of protein samples. On one hand, it is necessary to extract the maximum number of proteins and maintain their solubility during 2-D electrophoresis. This is commonly done by adding agents like detergents and chaotropic agents to solubilization cocktails. On the other hand, isoelectric focusing (IEF) is most successful with samples containing few ionic compounds, although the contribution of ions to IEF is not often recognized. We therefore studied the effects of water quality, DTT, and salt-containing buffers on IEF. We also investigated different factors that affect the applied current during IEF, such as the method of rehydration with sample and different modes of voltage ramping. Our initial observations indicate that the total number of spots per gel is almost the same under different run conditions. However, variations in spot count were observed outside the 25–100 kD and pI 5–8 ranges under different running conditions.

## Introduction

2-D gel electrophoresis is a critical technique for proteomic research, since it is the only method currently available that is capable of simultaneously separating thousands of polypeptides for quantitative studies (Hochstrasser 1998). One of the most important improvements in 2-D electrophoresis was the introduction of immobilized pH gradients, or IPGs (Bjellqvist et al. 1982, 1993). Various types of apparatus that allow direct incorporation of proteins into the IPG during rehydration have contributed to higher resolution and higher protein loading capacity. Nevertheless, methods for proper control of electrical parameters during IEF are not often fully appreciated. Recording electrical parameters during an IEF run allows evaluation of the entire run and analysis of the resulting electrical profile. This analysis is useful to determine the optimal migration parameters and for troubleshooting. Although numerous protocols are available in the literature (Gorg et al. 2000, Laboratoire de Biochimie des Protéines et Protéomique server at http://www-smbh.univ-paris13.fr/lbtp/index.htm) as well as in electrophoresis equipment manufacturers' instructions, it is difficult to identify the best protocol for a new sample.

IEF protocols include three major steps: a step at low voltage to initiate desalting of the sample; a progressive gradient to high voltage to mobilize the ions, polypeptides, and proteins; and a final high-voltage step to complete the electrofocusing.

Establishment of a voltage gradient between two electrodes results in mass transfer between them. Three processes contribute to mass transfer: 1) electrical migration, where charged species move in response to the electrical field; 2) diffusion, where species move under a chemical potential gradient (for example a concentration gradient); and 3) convection, which occurs as a result of diffusion or hydrodynamic transport (Garfin 2000). The movement of charged matter is observable as an electrical current (I) and follows Ohm's Law, $V = I \times R$, where V is the voltage and R is the resistance. The three mass transfer processes, namely the moving current, the equilibrium current, and the convection current, contribute to the observable (net) current.

During electrophoresis in a pH gradient, as in IEF, the moving current is due to amphoteric molecules that move in response to the field until they reach their steady-state positions. In addition, ionic species move through the gradient to the oppositely charged electrode. The transport of ionic species through, and out of, the focusing matrix can take considerable time — a fact that is not often recognized. As each species reaches an electrode or the point in the IPG at which it is uncharged, its contribution to the net current decreases.

The equilibrium current is the current generated by all the species as they diffuse around their equilibrium position, including ions close to the electrode and proteins in the IPG as they are converted between their neutral and ionic forms. The equilibrium current is proportional to the concentration of all ionized species in the sample. In contrast to the moving current that decreases with time at constant voltage, the equilibrium current is linearly proportional to the voltage.

The convection current may be driven by electroendosmotic movement, a component of hydrodynamic transport.

For safety from overheating, a limit of 50 µA per 3 mm wide strip is advised for commercial apparatus. If the current reaches 50 µA when the voltage is set to increase linearly, the voltage will then be regulated by the current limit, and the resulting voltage increase will no longer be linear. Once the current decreases below 50 µA, the voltage will again drive the linear progression of the gradient. One of the major problems encountered by novices to 2-D electrophoresis is the inability to reach the voltage they want.

We evaluated the contribution of various compounds to the electrical field. We show how recording the electrical parameters helps to identify artifacts, allowing adjustment of the profile and the duration of each IEF step, and adaptation of the protocol to the sample and the buffers used.

## Methods

### First-Dimension Electrophoresis

All reagents and materials were obtained from Bio-Rad unless otherwise indicated. The loading volumes were 125 µl and 300 µl for ReadyStrip™ 7 cm and 17 cm IPG strips, respectively. Passive rehydration was carried out in the PROTEAN® IEF cell at 20°C. The rehydration step was 2 hr long for test samples that contained buffers in the absence of proteins, and 6–9 hr for in-gel protein incorporation.

Three different premade reagents were tested: ReadyPrep™ reagent 1, reagent 2, and reagent 3.* For protein in-gel incorporation, the salt-free rehydration solution was 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 0.24% (v/v) Triton X-100, 0.2% (w/v) Bio-Lyte 3/10. Rehydration solution (280 µl) was mixed with 20 µl (100 µg) of soluble proteins extracted from the lymphoblastoid cell line PRI as previously described in Joubert-Caron et al. (2000). To evaluate the effect of DTT on the current, lyophilized reagent 2 was reconstituted with deionized water and with either 10 mM or 20 mM DTT.

IEF was carried out in the PROTEAN IEF cell. The 7 cm pH 3–10 strips were subjected to the following IEF program: 50 V for 15 min, 250 V for 15 min, linear gradient to 4,000 V over 2 hr, and 4,000 V for 2 hr. The longer 17 cm pH 3–10 strips were subjected to three different IEF program strategies: 1) a “direct progressive voltage” (50 V for 15 min, 250 V for 15 min, linear gradient to 10,000 V over 5 hr, and 10,000 V for 5 hr, or, for the protein-containing mixture, for a total of 40 kV-hr ); 2) a “desalting step and progressive voltage” (50 V for 9 hr, 200 V for 1 hr, linear gradient to 1,000 V over 1 hr, linear gradient to 10,000 V over 6 hr, and 10,000 V for a total of 40 kV-hr for the entire run) as described in Joubert-Caron et al. (2000); and 3) a “desalting step and rapid voltage” (50 V for 9 hr, 10,000 V to 40 kV-hr). The current was limited to 50 µA per strip, and the running temperature was 20°C. The strips were run in duplicate for samples that contained buffers in the absence of proteins, and in triplicate for in-gel protein incorporation. For protein-containing samples, the strips were stored at -20°C until used for the second dimension.

### Connection of a PROTEAN IEF Cell to a Computer

To monitor electrical properties during IEF, a modem cable was connected from the RS-232 port of the PROTEAN IEF cell to the serial port of a PC. The HyperTerminal software was opened from the Start menu of the Windows 95 operating system. The setup configuration was 9,600 bits/sec, 8 data bits, no parity, stop bit at 1, material control flow. The PROTEAN IEF cell exported run data every 5 min to HyperTerminal software. At the end of the run, the data were entered into an Excel spreadsheet for plotting.

### Second-Dimension Electrophoresis

Prior to SDS-PAGE, IPG strips were equilibrated as previously described in Joubert-Caron et al. (2000). They were loaded on PROTEAN II Ready Gel® 8–16%T precast gels and run for 1 hr at 40 V followed by 15 hr at 150 V in a PROTEAN II XL cell. The gels were silver stained and one gel per condition was scanned with a GS-700 scanner and quantitated as described by Joubert-Caron et al. (1999) and Poirier et al. (2001) with Melanie 3 software (Genebio, Geneva, Switzerland).

* Reagent 1 contains 40 mM Tris. Reagent 2 contains 40 mM Tris, 8 M urea, 4% (w/v) CHAPS, and 0.2% (w/v) Bio-Lyte® 3/10 ampholyte. Reagent 3 contains 40 mM Tris, 5 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (w/v) sulfobetaine (SB 3-10), and 0.2% (w/v) Bio-Lyte 3/10.

## Results and Discussion

We evaluated the contribution to the current of different compounds in the sample as well as in the rehydration buffers. In all tests, the default current limit of 50 µA per strip of the PROTEAN IEF cell was used.

### Contribution of Water Quality to Electrofocusing

The effect of the quality of the distilled water on IEF was tested by reconstituting lyophilized ReadyPrep reagent 2 with either partially purified, lower quality demineralized water or deionized (<0.6 µS/cm) water. Water (125 µl) was loaded on 7 cm strips as described in Methods. Figure 1A illustrates the impact of the water quality on the conductivity of the buffers. The equilibrium current when using lower quality water was almost twice (41 µA versus 24 µA) that of deionized water.

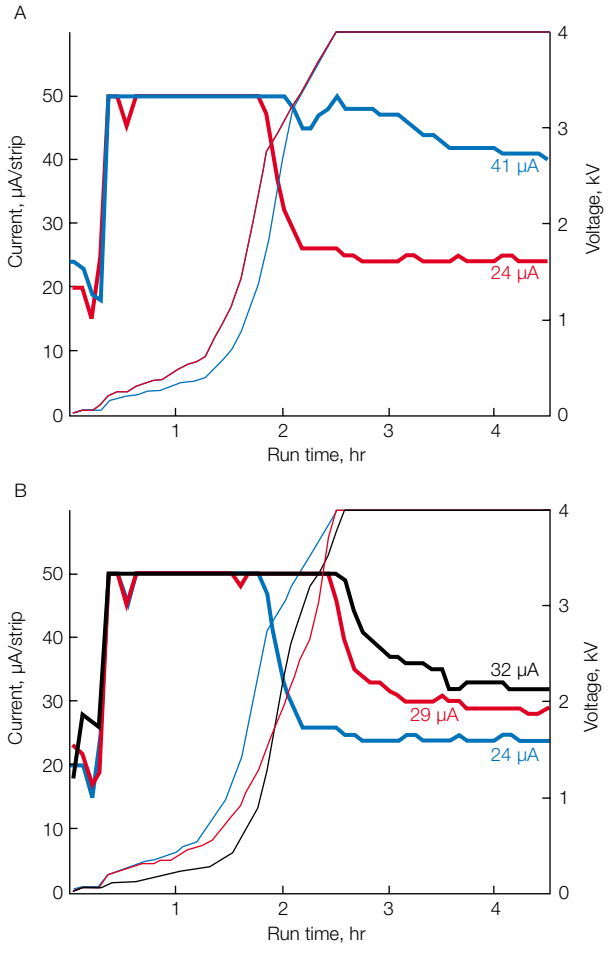


Fig. 1. Current and voltage profiles of different solutions loaded on IPG strips (current, thick lines; voltage, thin lines). Solutions (125 µl) were loaded onto 7 cm pH 3–10 strips. A, lyophilized reagent 2 reconstituted with either demineralized water (blue) or deionized water (red). B, reagent 2 supplemented with no DTT (blue), 10 mM DTT (red), or 20 mM DTT (black). The IEF program was 50 V for 15 min, 250 V for 15 min, linear gradient to 4,000 V over 2 hr, and 4,000 V for 2 hr.

### Effect of DTT on Electrical Properties

The effect of dithiothreitol (DTT) was determined as above. DTT is an ionic compound ($pK_a$ 9.5) used to reduce disulfide bonds in proteins. Figure 1B illustrates the effect of the concentration of DTT. The electrical field was maintained over 25 hr for reagent 2 containing 20 mM DTT. After 8 hr, the current, which had been stable at 32 µA, decreased progressively to reach a new stable level at 24 µA, identical to the equilibrium current value observed with the same buffer without DTT (data not shown). Thus, DTT migrated in the field and became neutralized over long runs, which would allow the proteins to reoxidize. This effect can be counterbalanced by laying an extra paper strip soaked with 20 mM DTT onto the IPG gel surface near the cathode when running basic IPG strips (Gorg et al. 1995). However, this has the disadvantage of providing a source of DTT ions that will continuously enter the electrical field. Several protocols recommend tributyl-phosphine (TBP) instead of DTT (Herbert et al. 1998). TBP is neutral and does not migrate in the electrical field. Moreover, it is used in smaller quantities (3–5 mM). Current profiles of reagent 2 with and without 2 mM TPB are similar (data not shown). Thus, TBP offers a clear advantage over DTT.

### Effect of Salts on IEF

At 50 µA per strip, the 40 mM Tris (reagent 1) required in excess of 20 kV-hr over 5.5 hr to completely exit the 17 cm IPG strips (Figure 2A, black lines). When 40 mM Tris was added to a rehydration solution, resulting in a solution similar to reagent 3, the current stayed at the limit of 50 µA for 8 hr (Figure 2A, green lines). In contrast, for the rehydration solution alone (without Tris and protein), the current stayed at 50 µA for only 1 hr 50 min, and the moving current reached zero in 2.5 hr (Figure 2A, blue lines), whereas adding 100 µg of proteins required only 30 min more for the moving current to reach zero (Figure 2A, red lines). After that time, strips with and without protein both showed the same equilibrium current profile and values. The difference in migration time can be attributed to the proteins, which are amphoteric molecules, and to the presence of ions in the protein extract. Assuming an average protein molecular weight of 40 kD, loading 100 µg in 300 µl corresponds to an 8.3 µM concentration. The associated conductivity is insignificant compared to the contribution of any salts or ionic contaminants. The high current contributed by a salt (such as Tris at only 40 mM) versus 100 µg of protein, emphasizes the importance of desalting samples as extensively as possible.

### Electrical Strategies

Three different voltage regimens were tested. In the experiment illustrated in Figure 2A, the molecules were first mobilized at low voltage for a short time. Then the voltage was increased linearly to 10,000 V over 5 hr. Complete focusing was achieved at 10,000 V for 5 hr or a total of 40 kV-hr for the protein-containing solution. For both the rehydration solution alone, and the protein-containing sample, it took only 3 hr for the moving current to reach zero. The remaining current, due to the equilibrium current, was then determined only by the voltage according to Ohm's Law. The graphical representation

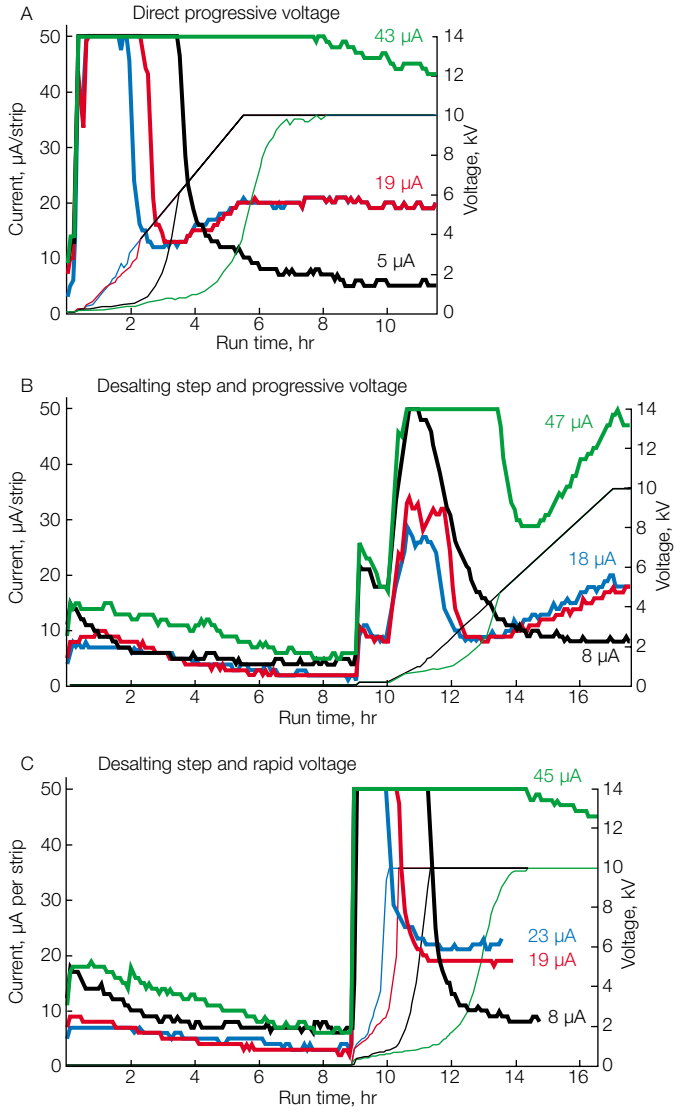


Fig. 2. Current and voltage profiles of solutions subjected to three electrical strategies (current, thick lines; voltage, thin lines). Blue, rehydration solution; red, rehydration solution with 100 µg protein; black, reagent 1; green, reagent 3. Solutions (300 µl) were loaded on 17 cm pH 3–10 IPG strips and then subjected to different IEF electrical strategies.

allowed us to deduce that the gradient duration might be reduced in this case for optimization of the IEF. The current profiles of the two samples (with and without proteins) were the same for almost 2 hr. After 2 hr, the voltage increased rapidly for the sample without protein, whereas the protein-containing solution remained under the 50 µA limit for a longer time. This data illustrates the order in which the molecules are mobilized, first the high-velocity ions, then the proteins. Reagent 3 without the addition of potentially ionic constituents (that is, without protein) was current limited at the end of the voltage-ramp step. An 8 hr run was required for the current to drop below the 50 µA limit, and 40 kV-hr were reached in 9 hr 20 min at a current of 46 µA. To reduce the current significantly below the 50 µA limit for this particular case, it may be necessary either to decrease the mixture's salt composition, or to increase the duration of the gradient step to allow the moving current to drop near zero.

To evaluate the impact of so-called active rehydration, which is proposed to facilitate the entry of some proteins into the gel (Gorg et al. 1999), an initial extended step at 50 V was inserted. This step has also been described as a preliminary desalting step (Righetti and Bossi 1997). This second strategy was used in the "desalting step and progressive voltage" program described in the Methods section.

As shown in Figure 2B, the global current profile looked very different from the previous profile shown in Figure 2A. A desalting process was apparent in the first step at 50 V for almost 7 hr, after which the current stabilized. This step could hence be reduced from 9 hr to 7 hr. After this initial step, all tested mixtures except reagent 3 followed the voltage ramp without current limitation. The rehydration solutions, with or without proteins, showed similar profiles, with a return to equilibrium after 2 hr at the last gradient step. These data suggest that the duration of the gradient could be reduced from 6 hr to 2–3 hr. Reagent 1 (40 mM Tris) reacted to the gradient step with a high but short peak of current, due to the previous extended desalting step at 50 V. Even reagent 3 (the mixture with Tris) showed a zero moving current before the end of the gradient step, which could therefore be reduced from 6 hr to 4 hr. It is noteworthy that in both electrical strategies, the values of the equilibrium current were the same.

In the first "direct progressive voltage" strategy (Figure 2A), part of the run was under the control of the 50 µA limit. Adding an initial long desalting step at low voltage in the second "desalting step and progressive voltage" strategy (Figure 2B) allowed the voltage to follow subsequent programmed steps. However, 50 µA is only a compromise value, which maximizes the possible current delivered without disturbing the final reproducibility of the pattern by overheating (the Joule effect) and while preventing strip burning. The third electrical strategy, "desalting step and rapid voltage" (Figure 2C), was designed to examine the effect of a 50 µA limit regulation after an active rehydration and desalting step: 50 V for 9 hr, 10,000 V to 40 kV-hr. After the desalting step, the current reached the 50 µA limit immediately after the high voltage was applied (Figure 2C). The current then decreased progressively, finally reaching the same values as in the two previous electrical strategies.

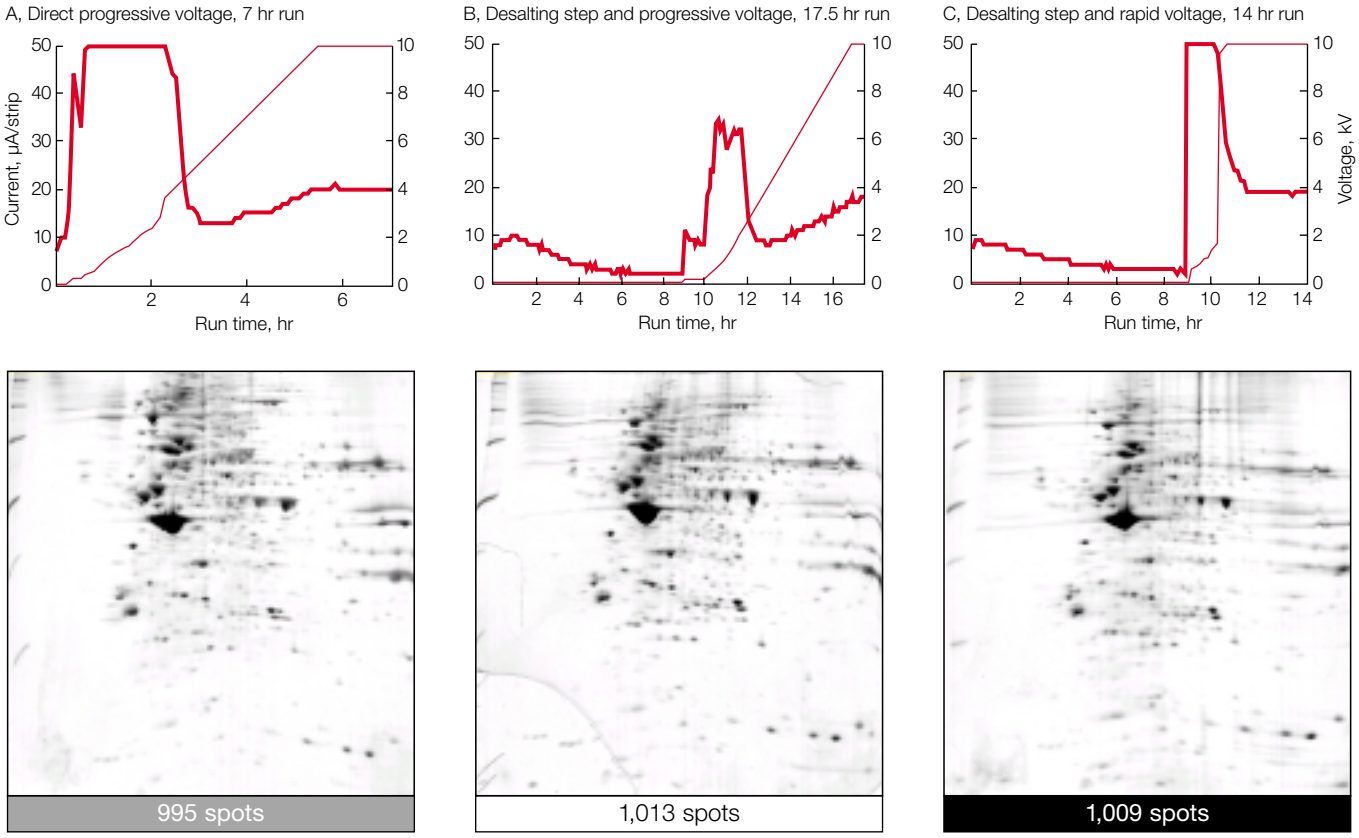


Fig. 3. 2-D analytical gels of proteins focused using different electrical strategies. Proteins were extracted from the lymphoblastoid cell line PRI in extraction buffer containing 2 mM tributylphosphine. Aliquots (100 µg) of the extracts were loaded onto 17 cm pH 3–10 IPG strips. Top row, current (thick lines) and voltage (thin lines) profiles obtained during IEF of the samples (data from Figure 2). Proteins focused in these runs were separated in the second dimension on 8–16%T Ready Gel precast gels, with Precision Protein™ molecular weight markers on the left side of each gel. Proteins were detected by silver staining.

Analysis of 2-D gels allowed further comparison of the three protocols. Each of the 2-D gels of samples subjected to the "direct progressive voltage", the "desalting and progressive voltage", and the "desalting and rapid voltage" IEF runs (Figure 3A, B, and C, respectively) appeared similar and showed almost the same number of spots (1,005, SEM <1%). The IEF run durations were 7 hr, 17.5 hr, and 14 hr, respectively. Image analysis of the gels showed that the distribution of protein spots, however, was not identical. The number of spots was determined in three molecular weight intervals: small proteins <25 kD; proteins between 25 kD and 100 kD; and large proteins >100 kD (see Table). The sum of the spots counted in the three intervals slightly differed from the total number of spots per gel; some spots could have been counted twice because they lie near the boundary between intervals. The three electrical strategies gave similar numbers of spots for the proteins between 25 kD and 100 kD (739, SEM <1%). This corresponds to the mass range that is well separated by 2-D electrophoresis. For these common proteins, intense evaluation of different electrical strategies is not necessary once the moving current reaches zero. The fastest strategy (7 hr in this case) can be chosen. For both large and small proteins, the first two strategies, which have a progressive voltage gradient in common, showed similar numbers of spots. The initial low-voltage step at 50 V did not give a significant increase in the number of large protein spots.

The third strategy, however, with its sudden voltage increase, showed fewer large protein spots and many more small protein spots compared to the two other strategies. One can hypothesize that the small, more mobile molecules in a complex mixture respond first to the suddenly applied field. As the applied voltage increases, the molecules could be progressively subjected to the field and mobilized, and the movement of the larger ones would be better regulated by the applied current.

**Table. Number and distribution of protein spots focused using different electrical strategies by mass intervals. Data from gels in Figure 3.**

| | Focusing Strategy | | |
|---|---|---|---|
| **Mass Range, kD** | **Direct Progressive Voltage** | **Desalting Step and Progressive Voltage** | **Desalting Step and Rapid Voltage** |
| >100 | 119 | 132 | 76 |
| 25–100 | 740 | 745 | 732 |
| 0–25 | 143 | 132 | 191 |

Looking at the distribution of spots in various pH intervals (Figure 4), proteins with pIs in the ranges of 5–6 and 6–7, which are the most abundant in the sample (Joubert-Caron et al. 2000), were present in the same proportion at all of the voltage regimens applied (SEM around 1%). The extremely acidic (pH 3.5–4) and alkaline (pH 9–9.5) regions also showed similar numbers of spots but the small number of spots

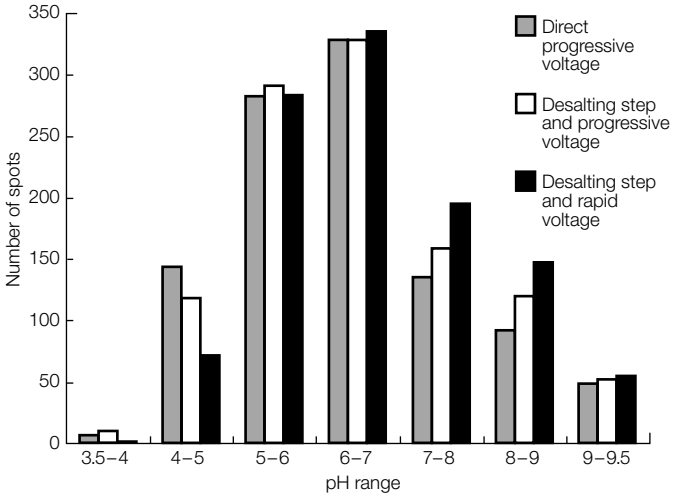


Fig. 4. Number and distribution of the spots by pI intervals focused using different electrical strategies. Data from gels in Figure 3.

counted may not allow detection of any significant discrepancy. In contrast, the pH 4–5 range, and the pH 7–8 and 8–9 ranges showed a different number of spots depending on the strategy (SEM around 20–30%). Interestingly, compared to the two other strategies, the "desalting and rapid voltage" strategy yielded more proteins in these two alkaline ranges and fewer proteins in the acidic range. Further investigations with other samples are needed to confirm this observation before proposing any explanation.

## Conclusions

Recording the electrical parameters during the IEF process allows tracking of the electrical behavior of the samples. Tracking emphasizes the role of the small ions in the sample, which move in response to the applied voltage more readily than the proteins. Protocols can also be adjusted based on these records (for instance to optimize step duration). Sample conductivity influences IEF runs in IPG strips more than in capillary IEF because larger amounts of sample solution can be loaded into IPGs. Contrary to common perception, mobile ions in an IPG sample do not migrate rapidly to the electrodes when voltage is applied, but may require a long time to clear from the strips. The present data confirm the usefulness of a progressive voltage gradient with a duration sufficient to lower the moving current to near zero.

The current through IPG strips is often limited to 50 µA per strip to minimize heating. This is the case with the most popular commercial IPG cells, the PROTEAN IEF cell and the IPGPhor. The 50 µA per strip limit helps to keep sample temperatures below 30°C. Above 30°C, urea in the sample solutions can begin to break down to cyanates, thereby increasing the risk of protein carbamylation. The current limit can be increased in cases where carbamylation is not an issue.

The best advice that we can give when running IEF on conductive (high-salt) samples under current-limiting IPG conditions is to be patient and allow sufficient time for ions to clear from the IPG strips before expecting the voltage to reach high levels.

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