*Journal of Chromatography*, 559 (1991) 479–488
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 486

# Optimizing separation parameters in capillary isoelectric focusing

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## ABSTRACT

Several modifications have been developed for capillary isoelectric focusing (IEF) to improve separation and detection of proteins. A basic compound was incorporated into the ampholyte mixture to block the segment between the monitor point and capillary end during focusing, enabling detection of basic proteins during mobilization. Use of a low-p*I* zwitterionic mobilization agent increased mobilization efficiency of the acidic region of the pH gradient, improving detection of acidic proteins. Mobilization with a neutral-p*I* zwitterion selectively mobilized neutral and basic proteins with improved resolution. Observation of colored proteins in glass capillaries mounted on thermosensitive liquid crystal was used to determine the heat generation patterns along the capillary and the effect of salt on the IEF process. The presence of salt in the sample resulted in long focusing and mobilization times. Incorporation of a non-ionic detergent in the sample plus ampholyte mixture reduced precipitation and improved reproducibility in capillary IEF of $\gamma$-globulins.

## INTRODUCTION

Isoelectric focusing (IEF) is widely used for separation of complex protein mixtures based on differences in isoelectric points. Conventional IEF performed in gel media can provide extremely high resolution, separating proteins with isoelectric point differences as small as 0.02 p*I* units [1]. The technique is limited by the time required for preparing, running and staining the gel, and non-linearity of the staining reaction.

Isoelectric focusing in capillaries was first described by Hjertén and Zhu [2]. In this variation of the technique, a mixture of proteins in the ampholyte solution is introduced into a gel-free capillary by pressure and focused at high field strength. Detection of proteins by on-tube UV monitoring requires a means of mobilizing focused zones past a monitoring point. This can be accomplished by replacement of a basic catholyte with acid or replacement of an acidic anolyte with base [2]. More commonly, mobilization is accomplished by addition of salt to the catholyte or anolyte [3]. Compared to conventional gel isoelectric focusing, capillary IEF is rapid (separations are typically complete within 15–20 min) and mobilized zones can be detected directly by on-tube monitoring. Capillary IEF requires the use of internally-coated capillaries since the magnitude of electroendosmosis in uncoated capillaries

0021-9673/91/$03.50 

prevents attainment of stable focused zones [2,4]. Capillary IEF has been applied to the separation of hemoglobins [2], transferrins [5,6], and immunoglobulins [7,8].

Capillary IEF has several limitations. First, positioning the monitor point at some distance from the end of the capillary prevents detection of proteins which focus between the monitor point and the capillary end during mobilization. Second, when mobilizing focused zones by addition of salt to either of the electrolytes, the mobilization efficiency of proteins which focus at the distal end of the tube is low. These proteins may appear as broad peaks or be undetected. Third, the presence of salts in the sample changes the distribution of the pH gradient during focusing, and increases the time required for focusing and mobilization. We have developed improvements to the capillary IEF process which address each of these limitations.

## EXPERIMENTAL

### *Materials*

Cytochrome *c* (horse heart) and γ-globulins (bovine) were obtained from Sigma (St. Louis, MO, USA). Hemoglobins A, F, S and C were obtained from Isolab (Akron, OH, USA). Phycocyanin, the IEF protein standard mixture, Bio-Lyte pH 3/10 ampholytes, and N,N,N′,N′-tetramethylethylenediamine (TEMED) were all obtained from Bio-Rad Labs. (Richmond, CA, USA). The monoclonal antibody preparation was donated by a local biotechnology company. Reduced Trition X-100 was obtained from Aldrich (Milwaukee, WI, USA). Temperature sensitive liquid crystal sheets (25–30°C) were obtained from Edmund Scientific (Barrington, NJ, USA).

### *Capillary isoelectric focusing*

Instrumental capillary IEF was performed with the Microsampler 100 high performance capillary electrophoresis system (Bio-Rad Labs.). Fused-silica capillaries (14 cm or 20 cm length × 25 μm I.D.) were coated internally with linear polyacrylamide covalently attached to the wall using a modification of the method of Hjertén [4]. Capillaries were purged with water and 10 m*M* phosphoric acid between separations. Samples were mixed with ampholytes at a final ampholyte concentration of 2%. Prior to focusing, the capillary was filled with the sample + ampholyte mixture by pressure loading. Focusing was performed at 6 kV constant voltage (unless indicated otherwise) using 10 m*M* phosphoric acid as anolyte and 20 m*M* sodium hydroxide as catholyte. When focusing was complete (as determined by the rate of current drop), cathodic mobilization was initiated by replacing the 20 m*M* sodium hydroxide catholyte with the appropriate mobilization catholyte. Mobilization was performed at 8 kV constant voltage (unless indicated otherwise). Mobilized zones were detected by on-tube monitoring at 280 nm.

To monitor the IEF process visually under various experimental conditions with colored proteins, 14 cm × 200 μm I.D. glass capillaries were used. These were coated by the same procedure used for 25 μm I.D. fused-silica capillaries. The IEF protein standard mixture (containing the colored proteins cytochrome *c*, myoglobin, hemoglobins A and C, and phycocyanin) was diluted 1:10 in 2% Bio-Lyte 3/10 ampholytes. Heat generation along the length of the glass capillary during the IEF process was monitored by mounting the capillary on a 0.7 × 12 cm piece of temperature-sensitive liquid crystal sheet. Focusing and mobilization were carried out under constant voltage 3.5 kV with a current limit of 25 μA.

## RESULTS AND DISCUSSION

### *The IEF process*

The mechanism of isoelectric focusing centers on the formation of a stable pH gradient in the capillary by the ampholytes upon application of high voltage. A protein will migrate along the gradient to the point where the pH is equivalent to its isoelectric point and migration ceases. Zone broadening is minimized, since diffusion of the protein away from the zone results in acquisition of charge; because of this focusing effect, zones are very sharp. Ideally, the pH gradient spans the entire length of the capillary and the gradient range is defined by the chemical composition of the ampholytes. A significant level of electroendosmosis would interfere with formation of a stable pH gradient and the attainment of focusing protein zones. Therefore, the use of coated capillaries is an absolute requirement for high-resolution capillary IEF. Because the entire capillary is filled with sample during loading, and because the focused zones are very narrow, the protein concentration in the focused zones can be 200- to 300-fold higher than in the unfocused sample. Therefore, of all modes of capillary electrophoresis, capillary IEF shows greatest potential for micropreparative applications.

At the completion of the focusing stage, only hydroxyl ions enter capillary at the cathodic end and only protons enter the capillary at the anodic end. When cathodic mobilization is initiated by addition of sodium chloride to the catholyte, the movement of chloride ions into the capillary causes a reduction in the hydroxyl ion concentration at the cathodic end of the capillary. As the pH drops, focused proteins and carrier ampholytes acquire charge and thus are mobilized towards the cathode [3]. By a similar mechanism, addition salt to the anolyte in anodic mobilization causes a pH increase in capillary and migration of proteins and ampholytes towards the anode. Because the pH gradient is maintained during mobilization, zone broadening and loss of resolution are minimal. In the mobilization electropherogram of hemoglobin variants presented in Fig. 1, the separation of hemoglobin A (p*I* 7.10) and hemoglobin F (p*I* 7.15) indicates a resolving power of about 0.02 p*I* units.

### *Use of TEMED in the focusing step*

In a high-performance capillary electrophoresis system, good detector stability requires placement of the monitor point some distance from the electrode. This distance is 2.8 cm in the Microsampler 100 system. Since mobilization is in one direction only, proteins which focus in this "blind" segment distal to the monitor point will be undetected during mobilization. Using a 14 cm capillary, this blind segment accounts for 20% of the pH gradient. For example, when using pH 3–10 ampholytes, proteins with p*I* values above 8.6 (*e.g.* cytochrome *c*, Fig. 2A) will not be detected. Yao-Jun and Bishop [9] have described the use of TEMED in the ampholyte mixture to extend the pH range in gel isoelectric focusing. We have used this approach to block the blind segment of the capillary and shift the pH gradient so that it occupies only the segment of the capillary proximal to the monitor point. In Fig. 2B, TEMED was added to the ampholyte + sample mixture at a final concentration of 0.5%. This basic compound filled the cathodic segment of the capillary during focusing, shifting the pH gradient away from the blind segment. In this case cytochrome *c* (p*I* 9.6) was detected during mobilization. Addition of TEMED will reduce resolution somewhat due to increased steepness of the pH gradient after focusing.

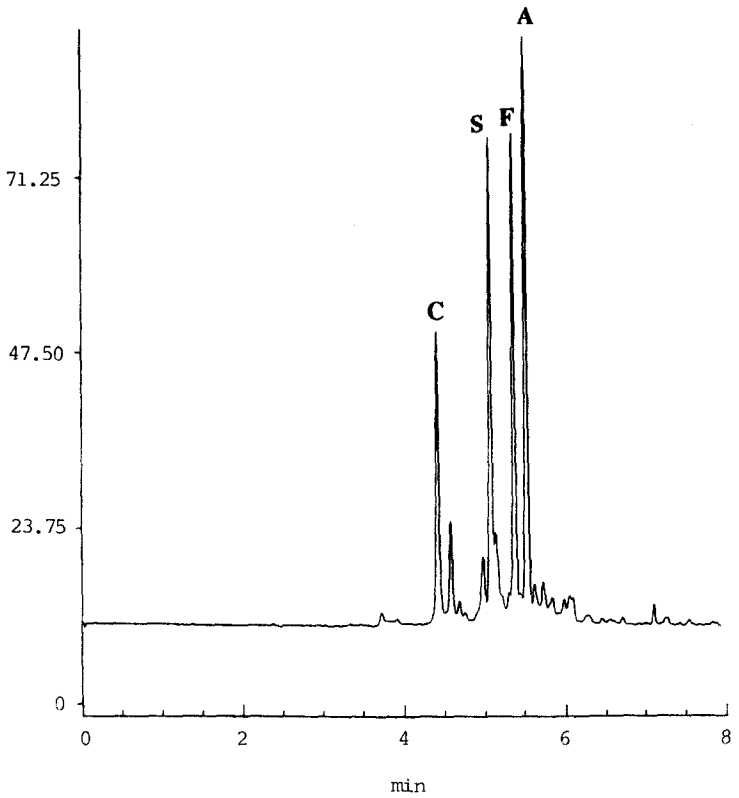


Fig. 1. Separation of hemoglobin variants by capillary IEF in a 12 cm × 25 μm coated capillary using pH 3–10 ampholytes. Focusing and mobilization were carried out at 8 kV constant voltage. Protein concentration was approximately 250 μg/ml for each protein. Isoelectric points are: hemoglobin A, p*I* 7.10; hemoglobin F, p*I* 7.15; hemoglobin S, p*I* 7.25; hemoglobin C, p*I* 7.50.

*Alternative mobilization agents*

The ideal mobilization mechanism should cause focused zones to maintain their relative order and distance during migration, *i.e.* zones should be mobilized as a train past the monitor point (Fig. 3A). In practice, salt mobilization causes movement of sodium or chloride ions into the capillary at one end. The pH change occurs at this end initially, then gradually progresses deeper into the capillary. The rate of change depends upon the amount of co-ion moving into the capillary, the mobility of the co-ion, and the buffering capacity of the carrier ampholytes. The actual slope of the pH gradient changes across the capillary, becoming shallower in the direction opposite to mobilization. Therefore protein zones at the far end of the capillary are mobilized with lower efficiency and are more likely to exhibit diffusion and defocusing (Fig. 3B). For example, the acidic protein phycocyanin (p*I* 4.65) was not detected during cathodic mobilization with sodium chloride (Fig. 4A). The shallower pH gradient at the far end of the capillary may provide better resolution for proteins which are mobilized, however.

We have developed an alternative mobilization technique employing zwitter-

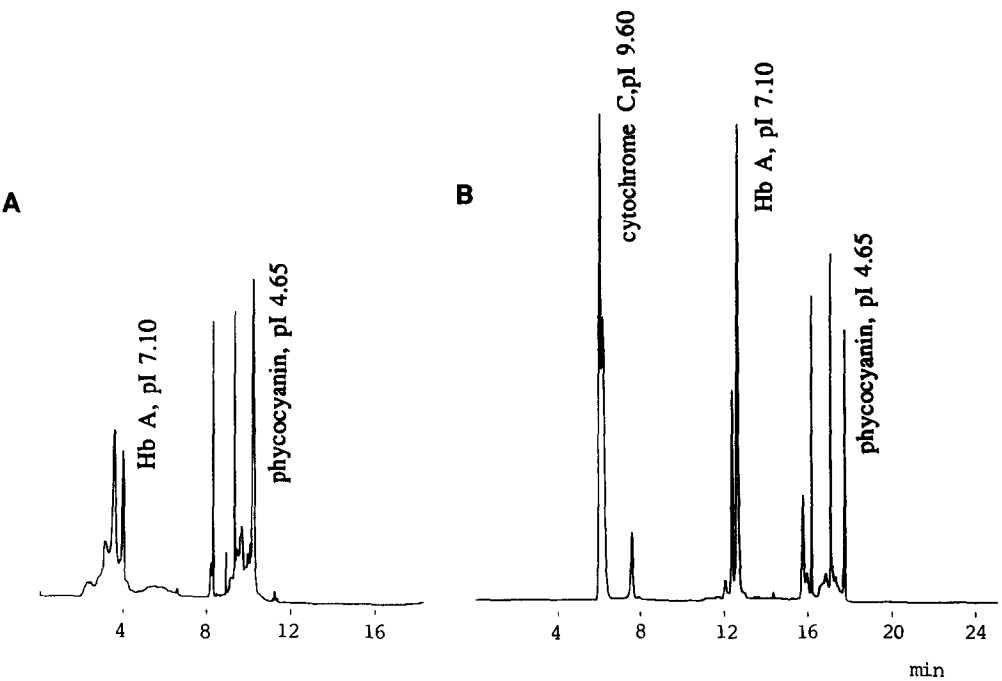


Fig. 2. Use of TEMED to block the blind segment. (A) Separation of cytochrome *c*, hemoglobin A, and phycocyanin without TEMED (cytochrome *c* undetected). (B) Separation with 0.5% TEMED added to sample + ampholyte mixture. Protein concentrations were approximately 300 μg/ml for each component.

ionic agents which allows protein zones across the entire pH gradient to be mobilized with good efficiency. The procedure is identical to sodium chloride mobilization except that the basic catholyte or acidic anolyte is replaced with a solution of a zwitterion of a given isoelectric point. We believe that zwitterion mobilization occurs via two mechanisms. First, movement of the zwitterion into the capillary causes a pH shift in the same fashion as sodium chloride mobilization. Second, the zwitterion migrates to a point in the gradient equivalent to its isoelectric point, where migration ceases and an ever-widening zwitterion zone displaces contiguous zones towards the monitor point.

In the example shown in Fig. 4B, mobilization was initiated by replacing the catholyte (20 m*M* NaOH) with a p*I* 3.22 zwitterion in basic solution. In this case, proteins across the entire pH gradient were mobilized and acidic proteins such as phycocyanin were detected. This approach can also be used to selectively mobilize portions of a broad pH gradient. For example, a zwitterion with a p*I* value near neutrality mobilizes only neutral and basic proteins. In Fig. 5A, proteins across the pH gradient were detected using a p*I* 3.22 zwitterion in basic solution while in Fig. 5B, only proteins with p*I* values greater than that of the p*I* 6.90 mobilization zwitterion were detected.

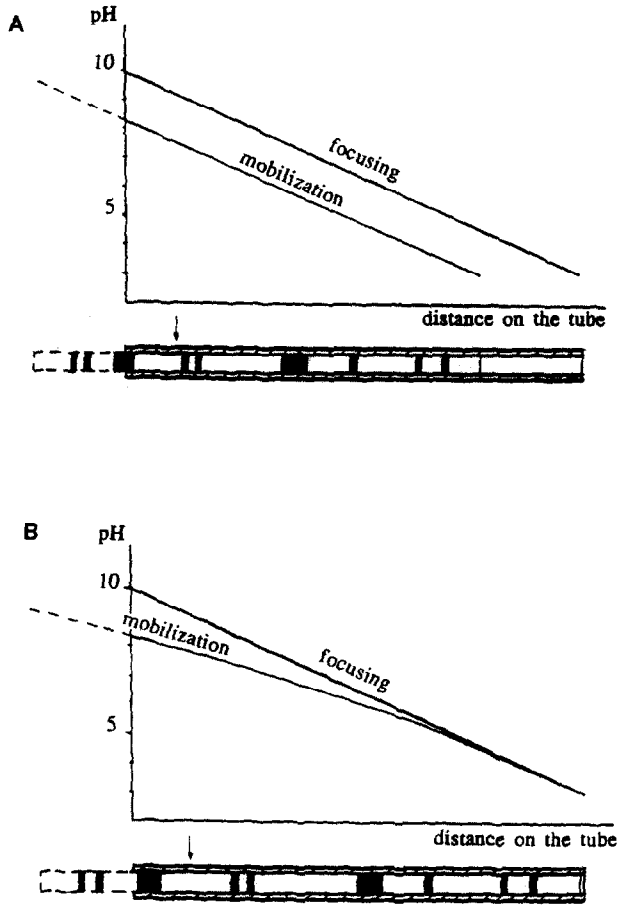


Fig. 3. Variation of pH with distance along the capillary after focusing and during mobilization. (A) Ideal profile (zones mobilize as a train); (B) actual profile (mobilization of zones at the far end of the tube is minimal).

*Effect of salt in capillary IEF samples*

Ionic species are usually present in biological samples; these can be neutral salts, buffers, or ionic detergents. The effect of salt on the IEF process was observed in a 200 $\mu$m I.D. glass capillary using colored proteins (cytochrome *c*, myoglobin, hemoglobin variants, phycocyanin). Under standard conditions without added salt, these proteins formed focused zones distributed across the entire length of a 14 cm capillary using a pH 3–10 ampholyte gradient. When the experiment was repeated with the addition of 100 m*M* NaCl to the sample + ampholyte mixture, focused zones were confined to the central 3–4 cm of the capillary. The time required for focusing and mobilization increased 4- to 5-fold and mobilized zones were more diffuse. Generation of heat during the IEF process was monitored in 200 $\mu$m capillaries mounted to temperature sensitive liquid crystal sheets. It was observed that heat

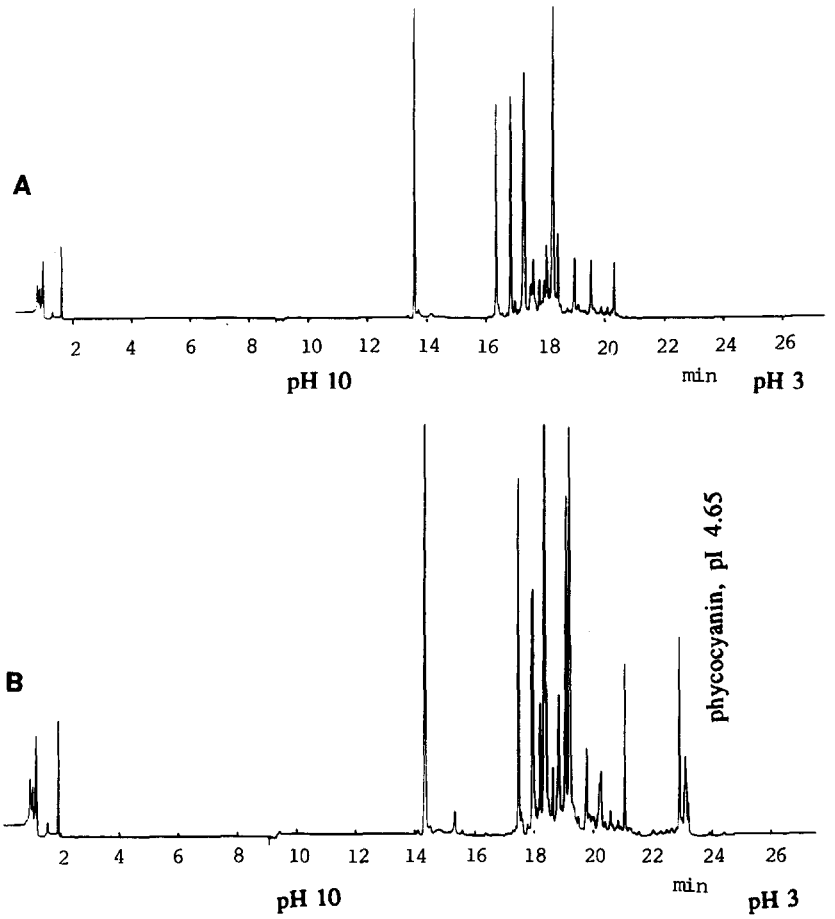


Fig. 4. Comparison of different mobilization agents. (A) Mobilization with sodium chloride (phycocyanin undetected); (B) mobilization with p*I* 3.22 zwitterion in basic solution. IEF protein standards mixture was diluted 1:50 in 2% pH 3–10 ampholytes.

generation closely follows the focusing process. Primary focused zones appeared initially at the margins of the capillary and heat generation was evident due to the increased electrical resistance in these segments. As focusing proceeded, heat generation extended to the central part of the capillary and, a steady state, heat was generated across much of the length of the tube. In contrast, when the sample + ampholyte contained 100 m*M* salt, initial current was very high, which increased the risk of overheating. Later in focusing, heat generation was localized in the central segment of the capillary containing the ampholyte gradient. Confinement of protein zones in a small segment of the capillary where heat generation was localized increased the risk of protein precipitation. For this reason, desalting of biological samples prior to capillary IEF is recommended.

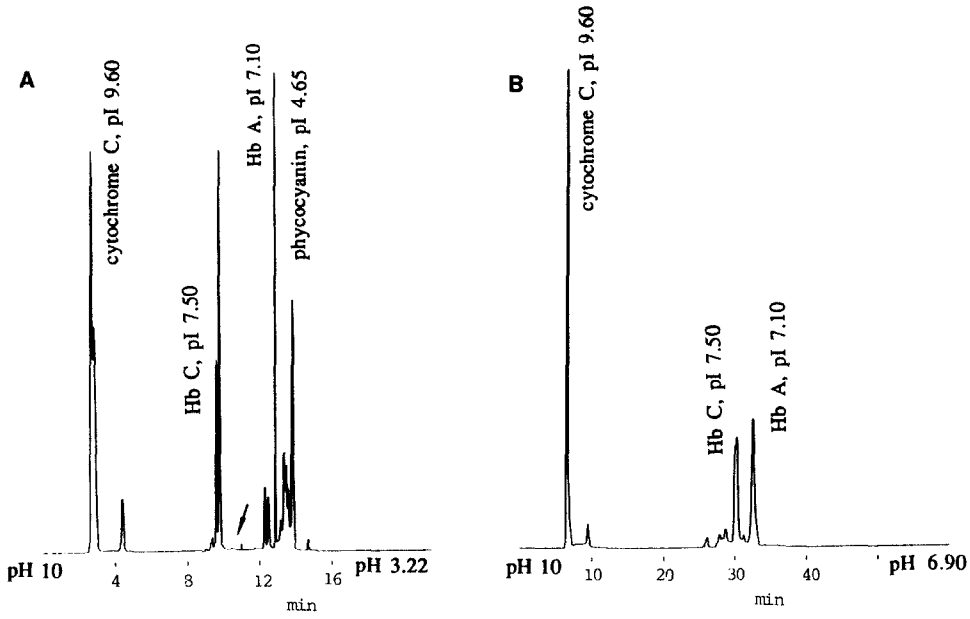


Fig. 5. Comparison of mobilization agents. (A) Mobilization with p*I* 3.22 zwitterion in basic solution; (B) mobilization with p*I* 6.90 zwitterion. Protein concentrations were approximately 300 μg/ml for cytochrome *c* and phycocyanin, and approximately 150 μg/ml for hemoglobins A and C.

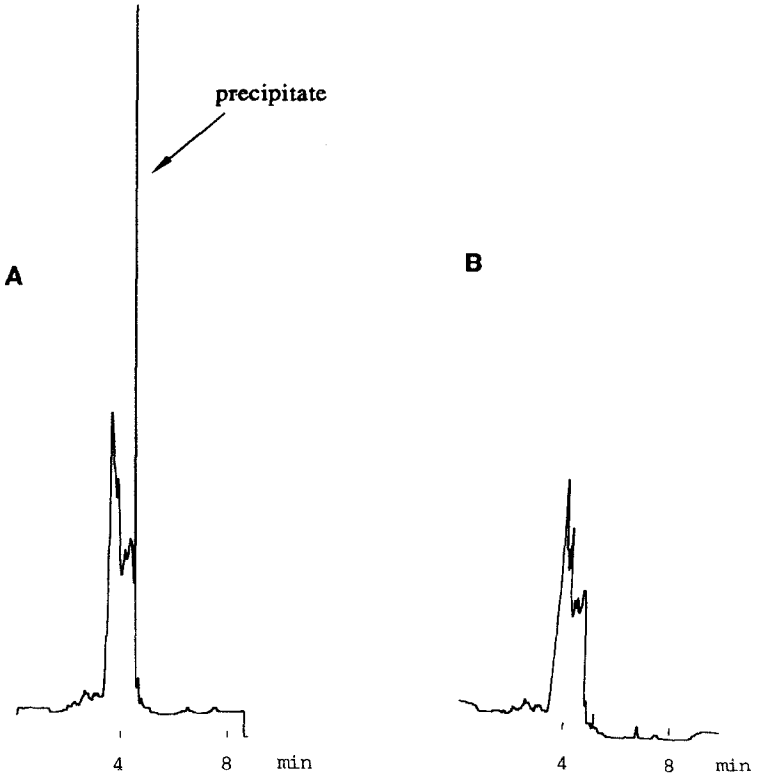


Fig. 6. Separation of monoclonal antibody preparation (A) with and (B) without precipitation.

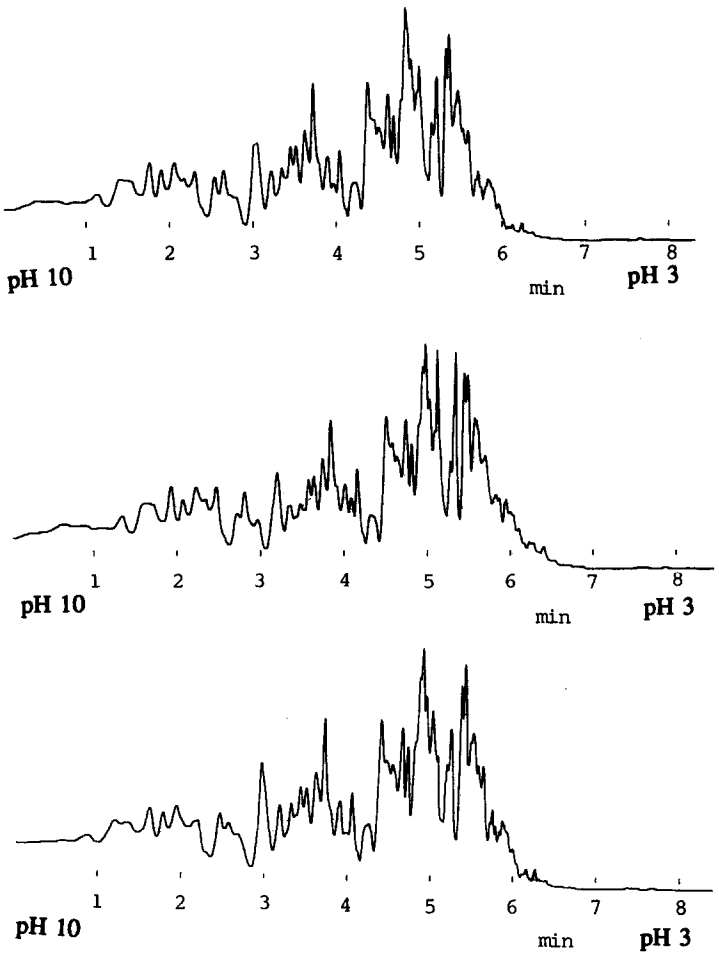


Fig. 7. Replicate separations of γ-globulins. Sample + ampholyte mixture was supplemented with 1% reduced Triton X-100.

*Protein precipitation in capillary IEF*

Protein precipitation has been a common problem in conventional gel isoelectric focusing, and capillary IEF is no exception. In both versions of the technique, proteins are confined in zones at extraordinarily high concentrations in a state of zero net charge, conditions which favor reduced solubility and aggregation. Moreover, stabilizing counterions are stripped from the protein as it is focused, and heat generation is localized in the focused zone. In capillary IEF, precipitation can cause poor reproducibility of peak size and migration time. Very sharp peaks or spikes are evidence of particulate precipitates passing the monitor point (Fig. 6). Protein precipitation can be suppressed by adding nonionic detergents or ethylene glycol to the sample + ampholyte solution [3,10]. We found that protein solubility was enhanced and reproducibility was improved in IEF separations of γ-globulins by addition of Triton X-100 to the sample + ampholyte mixture (Fig. 7).

## CONCLUSIONS

Capillary IEF is a high-resolution separation technique which can resolve protiens based on small differences in isoelectric point. In the most common approach, focused proteins are detected by cathodic mobilization past a monitor point. Basic proteins which normally focus beyond the monitor point were detected by including TEMED in the ampholyte + sample mixture to displace the gradient towards the anode. Using sodium chloride as the mobilizing agent, acidic proteins at the far end of the gradient were mobilized with poor efficiency. An alternative mobilizing technique using zwitterionic agents enabled acidic proteins to be detected as sharp peaks. The same technique could be used for selective mobilization of neutral and basic proteins with enhanced resolution. The presence of salt in the sample resulted in increased focusing and mobilization time, and increased risk of precipitation. Desalting of biological samples to 10 m*M* salt concentration prior to analysis is recommended. Precipitation could be reduced by incorporating a non-ionic detergent in the sample + ampholyte mixture. With these improvements, the capillary IEF technique provided reproducible high-resolution separations of difficult samples such as immunoglobulins.

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