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# Separation of basic proteins in free solution capillary electrophoresis: effect of additive, temperature and voltage

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**Abstract**

A comparative study on the separation of basic proteins using different buffer additives in free solution capillary electrophoresis is presented. Five additives, potassium chloride, morpholine, cetyltrimethylammonium bromide (CTAB), poly(vinyl alcohol) and polyethyleneimine, used to reduce the adsorption of the proteins onto the capillary wall were tested. The study was done at different concentrations of additive, voltages and temperatures of separation. The advantages and drawbacks of each additive are discussed. From our study, it is deduced that the use of CTAB renders the best protein separations provided that adequate additive concentration, run voltage and, more interestingly, temperature of separation are chosen. Using this simple procedure, efficiencies higher than 300 000 theoretical plates per meter were obtained for the separation of basic proteins under optimized conditions.

*Keywords:* Proteins; Potassium chloride; Morpholine; Cetyltrimethylammonium bromide; Poly(vinyl alcohol); Polyethyleneimine

## 1. Introduction

Protein–capillary wall interactions in capillary electrophoresis (CE) analysis of proteins seems to be the main reason for the loss in efficiency [1], poor reproducibility of migration time [2] and low protein recovery [3] obtained during the separation of these biopolymers. The adsorption is believed to be due to the electrostatic interactions between positively charged residues of the protein and the negatively charged silanol groups of the fused-silica surface.

Several methods have been developed to avoid interactions between proteins and the silica surface, for instance, either adjusting the pH of the buffer to such a high value that the charge of proteins and surface have the same sign or selecting a pH<2 whereby the silanol groups are not charged. The application of very basic or acidic pHs has been successful used for the separation of some proteins [1,4], but generally most of the proteins are not stable or soluble at these extreme pH values.

Another approach is to shield the silanol groups by covering the surface with a polymeric layer. The use

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of such polymeric coatings has led to high efficiencies and reproducible protein separations. A large variety of polymers such as linear polyacrylamide [5], poly(ethylene glycol) [6], polyethyleneimine [7,8], poly(vinyl alcohol) (PVA) [9], cross-linked polyacrylamide [10,11], hydroxylated polyether [12] and proteins [13] have been applied. Also, $C_{18}$ moieties, in combination with surfactants or polymers adsorbed on them [3], have been used in the preparation of these capillaries. However, many of the coatings show a limited life-time while the preparation is laborious and time consuming. Moreover, the reproducibility of the coating-making procedure is often poor [14].

It has been also shown that it is possible to reduce the protein–capillary interaction by using different additives in the separation buffer. Following this idea, the use of inorganic salts [15], amines [11,16], cationic surfactants [17,18] or polymers [9] have been reported in the literature. However, relatively high ionic strength is frequently necessary to suppress the adsorption of proteins and this increases the heating effect [15] while some additives are only useful in a fixed range of pH [9]. The main advantage arising from this procedure is its simplicity while allowing separations of proteins with relatively high efficiencies. Besides, prior to each separation it is possible to wash the capillary wall under harsh conditions, e.g., by using sodium hydroxide, which can improve the separation reproducibility.

In spite of these advantages, little attention has been paid to this procedure for diminishing the protein–capillary interactions. We believe that a comparative study about the drawbacks and advantages arising from the use of additives of different natures into the buffer, as previously done for different capillary coatings [14], could provide some valuable insight on this methodology.

The goal of this work is to carry out such a comparison. Five additives, namely potassium chloride (KCl), morpholine, cetyltrimethylammonium bromide (CTAB), PVA and polyethyleneimine (PEI), have been tested at different concentrations, voltages and temperatures of separation. The choice of these additives was based on their different successful employments reported in the literature [7,9,15,18,19]. The comparison between additives was done in terms of heating generation, reproducibility and separation efficiency using basic proteins as test compounds.

## 2. Experimental

### 2.1. Instrumentation

Experiments were carried out in a P/ACE 5510 HPCE (Beckman, Fullerton, CA, USA) electrophoresis apparatus with a diode array detector. The fused-silica capillaries used were 75 $\mu$m I.D. with 57 cm of total length and 50 cm of effective length (Composite Metal Services, Worcester, UK). Injections were carried out using $N_2$ pressure (0.5 psi) for 2 s. The external temperature of the capillaries was from 10 to 40°C depending on each experiment. Detection took place at 210 nm. Data were collected and analyzed using a System Gold software (Beckman) running on a 486DX2-66 MHz computer. In order to increase migration time reproducibility, the capillaries were successively rinsed between injections with NaOH 0.1 mol/l and buffer for a period of 3 min with each solution.

### 2.2. Samples and chemicals

Basic proteins, i.e., lysozyme, cytochrome C, ribonuclease A, $\alpha$-chymotrypsinogen and trypsinogen, were purchased from Sigma (St. Louis, MO, USA) and used as received. The proteins were dissolved at the concentrations indicated in each case in Milli-Q water. These samples were stored at −4°C and warmed at room temperature before use.

PEI (molecular weight range $6.10^5-1.10^6$, Fluka, Buchs, Switzerland), KCl and morpholine (both from Aldrich, Milwaukee, WI, USA), PVA (molecular weight 72 000) and CTAB (both from Sigma) were tested as buffer additives.

2-[N-Morpholino]ethanesulfonic acid (MES) (Aldrich) was used in all the running buffers. These buffers were 50 m*M* MES pH 7, varying the concentration of additive as indicated in each case. Buffers were prepared by dissolving the weighted amounts of MES and the additive in Milli-Q water and adding 0.1 mol/l sodium hydroxide (NaOH) or 0.1 mol/l hydrochloric acid (HCl) until pH 7.

Table 1
Different additives and conditions of separation employed

| Additive | Concentration | Voltage (kV) | Temperature (°C) |
|---|---|---|---|
| $K^+$ | 10–100 m*M* | +8, +15 | 20 |
| Morpholine | 10–200 m*M* | +8, +15 | 20 |
| PVA | 0.05–1% | +8, +15, +20 | 20 |
| PEI | 0.05–0.5% | −8, −15, −22 | 20 |
| CTAB | 1–50 m*M* | −5 to −25 | 10 to 40 |

All the additives were added to a 50 m*M* MES buffer and adjusted to pH 7.

## 3. Results and discussion

Table 1 shows the different additives and conditions of separation tested. As stated above, we selected these additives based on their different successful employment reported in literature [7,9,15,18,19]. In our case a restriction was included with reference to the separation pH, i.e., pH 7 was used in all cases. The choice of a pH value close to the physiological seems to us quite adequate, when mainly the separation of large biopolymers, i.e., proteins is the main goal.

As can be seen in Table 1, five types of additives were used; an inorganic cation, $K^+$; an organic cation, morpholine; a neutral polymer, PVA; a cationic polymer, PEI and a cationic surfactant, CTAB. At least five different concentrations were tested in the range indicated in each case. Table 1 also shows the different voltages employed with each concentration of additive. When PEI or CTAB were used, negative voltages had to be employed due to the anodal electroosmotic flow induced by these two substances as will be discussed below. The study on the temperature influence was carried out with CTAB as additive.

### *3.1. Heating generation*

In Fig. 1 the electrical current obtained depending on the type and concentration of each additive is shown. As can be seen, for KCl [Fig. 1(3)] and CTAB [Fig. 1(4)] the higher the concentration of additive the higher the electrical current. This effect arises from the increase in electrical conductivity of the buffer produced by the addition of these two substances. Furthermore, in Fig. 1 it can also be seen that the electrical current, and with that, the induced

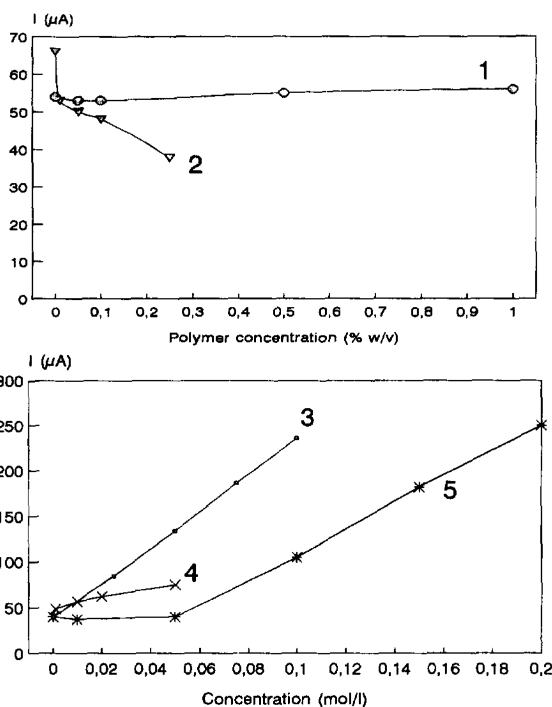


Fig. 1. Electrical current versus additive concentration for the five additives: 1, PVA at 20 kV; 2, PEI at 22 kV; 3, KCl at 15 kV; 4, CTAB at 15 kV; 5, morpholine at 15 kV. Buffer: 50 m*M* MES plus the additive and adjusted at pH 7 as indicated in Section 2. Capillary: 75 μm I.D.; 50 cm effective length; 57 cm total length.

temperature rise within the capillary, obtained by using 50 m*M* KCl, is twice as much as that obtained with the same concentration of CTAB. The concentration range of CTAB was not further increased due to precipitation of the surfactant at values higher than 50 m*M*.

When PVA [Fig. 1(1)] was employed as additive a practically constant electrical current was observed. This polymer has no charge so, at the concentrations employed, its contribution to the electrical conductivity is expected to be almost negligible.

A different behaviour is observed when PEI [Fig. 1(2)] or morpholine [Fig. 1(5)] were used as buffer additives. Since the additives were added to the buffer and then the pH was adjusted to pH 7, the basic character of these two substances reduced the amount of NaOH needed to adjust the buffer pH. Their lower electrical conductivity compared to that of NaOH explains the decrease in electrical current observed with PEI along the range of concentrations

studied as well as with morpholine at concentrations lower than 50 m*M*. At higher concentrations of morpholine, HCl had to be added to adjust the pH to 7, which explains the increase in electrical current observed in that range of concentrations.

From these results it can be deduced that, in terms of heating generation, PVA, PEI and CTAB are better suited as additives to the buffer since they produce lower electrical current and, therefore, smaller temperature rise within the capillary.

### *3.2. Efficiency and reproducibility*

Initially we tested at least five concentrations of each additive, in the range indicated in Table 1, for the separation of basic proteins. Analyses were done at different voltages, keeping constant the temperature of separation, 20°C, for all the additives employed. In Fig. 2, the best separations obtained with each additive are shown and compared to that obtained by using the buffer without additive (Fig. 2A). As can be seen, by using the separation buffer with no additive, the proteins adsorb on the capillary wall which makes impossible their separation and even their detection.

The use of PVA as additive to the buffer had been shown to work as a good dynamic coating allowing the separation of basic proteins with high efficiency values [9]. However, in that work the limitations of this procedure in terms of the separation pH, since it

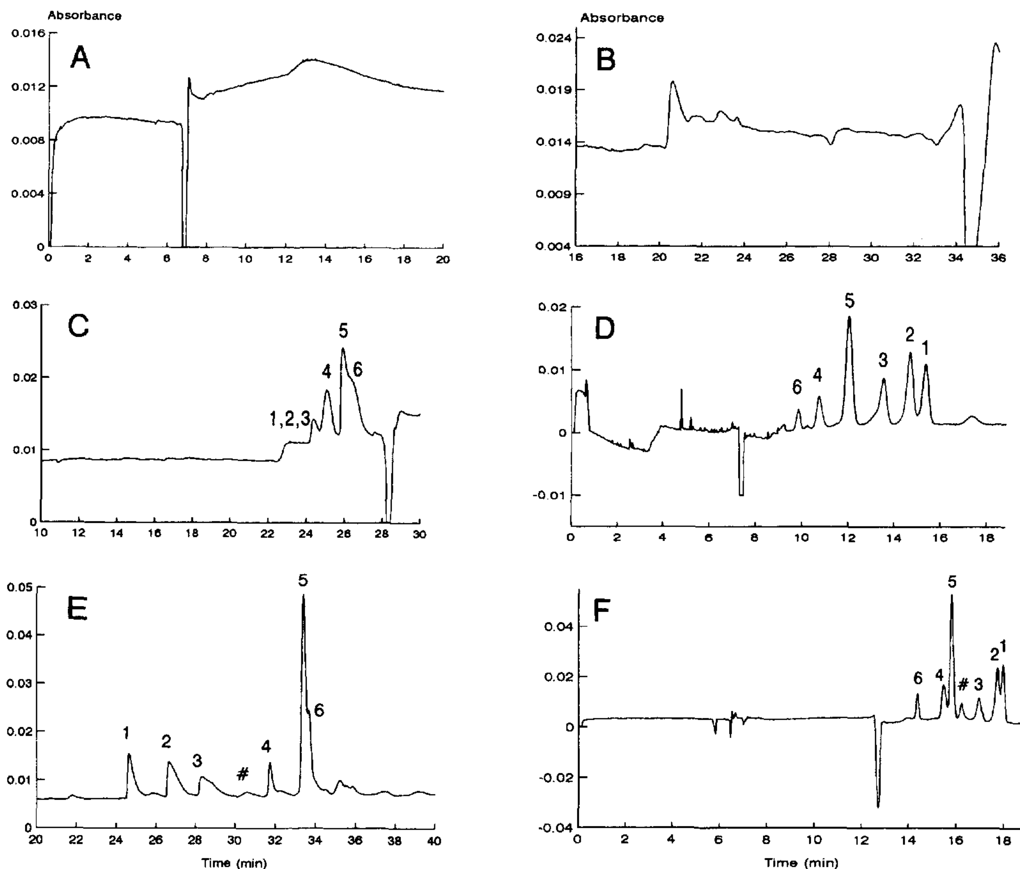


Fig. 2. Comparison of the separation of basic proteins using different additives: (A) 50 m*M* MES pH 7 with no additive, run voltage 15 kV; (B) 0.5 % (w/v) PVA added, run voltage 20 kV; (C) 75 m*M* KCl, run voltage 8 kV; (D) 0.1 % (w/v) PEI, run voltage −22 kV; (E) 150 m*M* morpholine, run voltage 8 kV; (F) 10 m*M* CTAB, run voltage −8 kV. Peaks: 1=cytochrome *c*, 0.55 mg/ml; 2=lysozyme, 0.3 mg/ml; 3 and # = impurities from cytochrome *c*; 4=trypsinogen, 0.28 mg/ml; 5=$\alpha$-chymotrypsinogen, 0.6 mg/ml; 6=ribonuclease A, 0.45 mg/ml. All the separations were carried out at 20°C. Detection took place at 210 nm.

needs to be lower than 5, were mentioned. Fig. 2B shows that the use of this polymer at pH 7 does not allow the separation of the basic proteins, probably due to the lack of adsorption of PVA on the capillary wall at this pH [9]. Under these conditions, a decrease of the electroosmotic flow was noticed, likely, as a result of the higher viscosity of the buffer induced by the addition of PVA.

In Fig. 2C the separation obtained at 8 kV using 75 m*M* KCl as additive is shown. Under these conditions, poor separation of proteins was achieved while their adsorption on the capillary wall was still important. When higher concentrations of KCl was used i.e. 100 m*M*, worse separations were obtained probably due to the higher heating generation which could bring about degradation of the proteins. A similar effect was observed with morpholine (Fig. 2E) since better efficiencies were obtained at a concentration of 150 m*M* than those obtained with 200 m*M*. Under these conditions, i.e., 150 m*M* morpholine and 8 kV (Fig. 2E), an acceptable separation of proteins was obtained although some adsorption of the proteins on the capillary wall still ruins the efficiency of the separation. This can be deduced from the tailing peaks, mainly number 1 and 2, which correspond to the most basic proteins, cytochrome *c* and lysozyme respectively, as well as from peaks indicated as 3 and # corresponding to impurities from cytochrome *c*. Besides, under these conditions the analysis time needed for the separation was quite long since more than 30 min were required.

The use of PEI as buffer additive gave much better separation as can be observed in Fig. 2D, in which 0.10% w/v of this additive was used at −22 kV. The adsorption of the polymer, positively charged, onto the ionized silanol of the capillary wall seems to change the sign of the charge on the capillary wall from negative to positive originating an anodal electroosmotic flow. Therefore, a negative voltage had to be applied in order to displace the proteins to the detection point. This results in a reverse order of migration of the proteins compared to that obtained under normal conditions, i.e., as obtained in Fig. 2E. The PEI molecule can interact with the negative groups of proteins [20], which can explain the change in migration order observed for peak 5 compared to that observed in Fig. 2E. Moreover, these interactions can bring about more complex protein–polymer–capillary wall associations [20], which could explain the poor efficiency observed under these conditions (i.e. lower than 50 000 plates/m). Using this polymer, an additional problem was observed related to the lack of reproducibility of the analysis time. Under these conditions and even when 0.1 *M* NaOH was systematically used for reconditioning the capillary between runs, the R.S.D. ($n=5$) was higher than 4%, probably due to the irreversible adsorption of this polymer onto the capillary [8], which makes it difficult to obtain reproducible conditions between injections.

The use of CTAB in CE, as reported by Altria and Simpson [21] and Tsuda [22], also originates a reverse charge on the capillary wall inducing an electroosmotic flow towards the anode. This effect is explained as a result of the adsorption of the positively charged ammonium moieties onto the negatively charged silanol groups. At CTAB concentrations higher than 0.35 m*M*, a bilayer is formed through hydrophobic interactions between the nonpolar chains. Under these conditions the cationic heads are facing the buffer, reversing the charge of the capillary wall from negative to positive.

The use of CTAB for the separation of proteins has generated little attention among the CE community [18,23]. This can be mainly due to the denaturing characteristics of this substance when interacting with proteins [24]. However, this cationic surfactant presents some interesting features that need to be discussed. First, by using CTAB as additive to the buffer, both surface and proteins have the same positive charge. Under these conditions, the proteins are repelled from the surface in analogy to what has been described by Lauer and McManigill [1]. Another interesting aspect is related to the differential adsorption of CTAB on proteins [24]. Unlike sodium dodecyl sulfate (SDS) which binds to proteins to the same extent providing to all of them the same charge-to-mass ratio, CTAB interacts with each protein to a different degree which can enlarge the differences between proteins [18,24] making their separation easier.

In Fig. 2F, the separation of the basic proteins obtained by using 10 m*M* CTAB as additive at 20°C and a run voltage of 8 kV is shown. It can be observed that, under these conditions, no adsorption

is taking place since rather symmetrical peaks are obtained.

Comparing Fig. 2D with Fig. 2F, it is observed that the same migration order of proteins is obtained with PEI and CTAB. Moreover, with CTAB both the efficiency (e.g. up to 200 000 plates/m for peaks 6 and 5) and reproducibility (e.g. analysis time %R.S.D.$_{n=5}$ lower than 1.3% using NaOH between runs) were higher than those obtained with PEI. In spite of these acceptable results, the resolution between the different peaks was not as good as that obtained with PEI. Therefore, we performed a further optimization of this separation through the study of some other parameters, i.e., temperature and run voltage and to test their effect on the separation of proteins at different CTAB concentrations.

### *3.3. Optimization of protein separation using CTAB as additive*

The effect of concentration of cationic surfactants on the separation of proteins by CE still remains unclear. This is mainly due to the great difficulty in understanding the different effects taking place during the CE separation of proteins in a micellar system where too many equilibria are involved, e.g., protein–micelle, protein–surfactant monomer and protein–surfactant monomer–micelle. Moreover, the difficulty increases when one takes into account the different protein–surfactant interactions taking place, i.e., electrostatic and hydrophobic, and their dependence upon protein conformation. Then, this dependence is a function of i.a., pH, ionic strength and temperature of the separation.

The influence of CTAB concentration on the separation of proteins is shown in Fig. 3. As can be seen, there is a variation in the selectivity of the separation depending on the concentration of additive. This variation is mainly observed for trypsinogen and $\alpha$-chymotrypsinogen, peak number 4 and 5 respectively, which according to their isoelectric point, 8.7 for trypsinogen and 9.2 for $\alpha$-chymotrypsinogen, are two of the most negative among the five proteins studied. Therefore, at the separation pH employed, a higher tendency to interact with the positive micelles may be expected for these two proteins.

A similar effect to that observed for trypsinogen and $\alpha$-chymotrypsinogen could be expected for ribonuclease A according to their similar isoelectric points (9.3 for ribonuclease A). However, this variation does not take place for this protein. This different behaviour can be explained through some other interactions, e.g., hydrophobic, taking place between the surfactant and the proteins which would result in a different quantity of surfactant adsorbed onto each protein and, therefore, different charge and electrophoretic mobility values for each biopolymer [24–26]. Moreover, the small number of binding sites for CTAB that proteins normally present and their relatively small binding constants [24] have to be mentioned which can also explain why ribonuclease A does not behave like trypsinogen and $\alpha$-chymotrypsinogen.

Besides, as shown in Fig. 3, the higher the concentration of CTAB the longer the analysis time for trypsinogen and $\alpha$-chymotrypsinogen, what suggests that the positive charge of the proteins increases as a result of a higher number of protein–CTAB interactions. This can be explained through the absence of saturation of the CTAB–$\alpha$-chymotrypsinogen, and CTAB–trypsinogen complexes as usually observed for proteins with this type of cationic surfactant [24]. These results have been explained by considering that some biopolymers, even in the presence of an excess of cationic surfactant, would be poised at a steep portion of the binding isotherm [24]. Therefore, unlike SDS which binds to the same extent to each protein, when using CTAB there is not equivalent saturation of the surfactant–protein interaction which precludes the use of CTAB for the calculation of molecular weight of proteins.

To provide more insight on this point, a deeper study about some structural features that can influence the CTAB–protein binding for these three proteins is in order. As stated above, the binding of detergents to proteins involves hydrophobic and electrostatic interactions. Therefore, the number of glutamyl and aspartyl side chains as well as the hydrophobicity of proteins play an important role in the complexes formation with CTAB. The number of negatively charged residues is 10 for ribonuclease A and 14 for $\alpha$-chymotrypsinogen and trypsinogen. Moreover, the average hydrophobicity is 870 for ribonuclease A and 940 for $\alpha$-chymotrypsinogen and

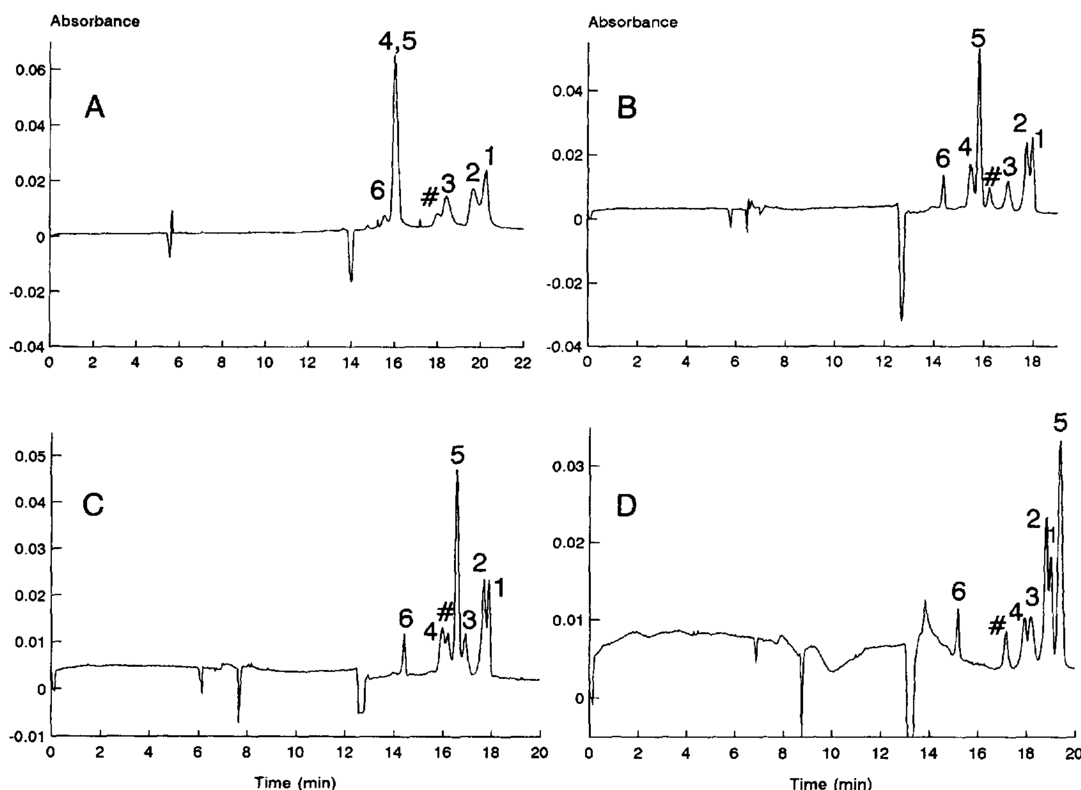


Fig. 3. Effect of CTAB concentration on the separation of basic proteins. Buffer 50 m*M* MES at pH 7 with: (A) 1 m*M*; (B) 10 m*M*; (C) 20 m*M*; (D) 50 m*M* CTAB as buffer additive. All the conditions as Fig. 2F.

trypsinogen [27]. Thus, both parameters indicate that a lower CTAB–ribonuclease A interaction is expected compared to that of CTAB with $\alpha$-chymotrypsinogen and trypsinogen.

Also in Fig. 3, efficiency and resolution were better for concentrations of CTAB higher than 1 m*M*. Since the critical micelle concentration (cmc) of CTAB in water is 0.94 m*M*, it seems that for achieving reproducible proteins separations, concentrations of CTAB higher than the cmc are required. A similar effect has been observed by other authors [18]. Besides, it can also be observed in Fig. 3 that the electroosmotic flow value kept relatively constant for CTAB concentrations higher than 1 m*M*, while the best separations in terms of efficiency and resolution were obtained for 10 m*M* CTAB.

Next, we proceeded to study the temperature effect on the separation by employing different temperatures ranging from 10°C to 40°C. The results obtained are shown in Fig. 4. As can be seen, when 40°C were used (Fig. 4A) the separation of proteins was ruined, probably as a result of the thermal degradation of proteins and/or the variation of the protein–surfactant interaction arising from the temperature change as observed for smaller molecules [26,28]; a broader explanation of this effect is given below. Moreover, the lower the temperature employed, the better the separation as shown in Fig. 4D for which a temperature of 10°C was utilized. To our knowledge, this is the first time that the influence of the thermal effect on this type of protein–CTAB system has been addressed in CE.

A possible explanation for this effect can be found in the difficulty in achieving saturation of the cationic surfactant–protein complexes [24]. Under these conditions, small variations in the protein structure and its environment can produce large modifications on the CTAB–protein binding process,

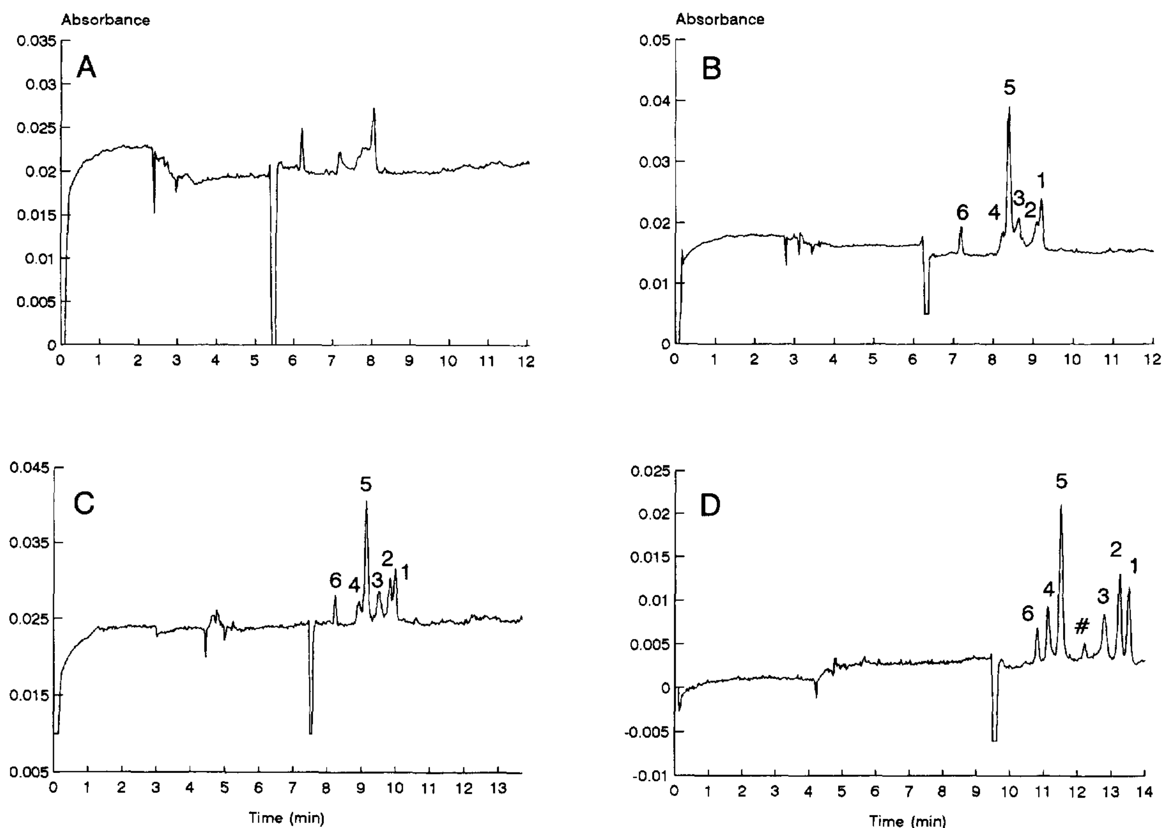


Fig. 4. Effect of temperature on the separation of basic proteins using 10 m*M* CTAB as buffer additive. Temperature: (A) 40°C; (B) 30°C; (C) 20°C; (D) 10°C. Run voltage: −15 kV. The rest of the conditions as in Fig. 2F.

which eventually leads to a change of electrophoretic mobility of the CTAB–protein complex. Thus, the variation of temperature can induce, apart from other effects on the protein conformation such as that of denaturation stated above, changes on the buffer pH which can modify the charge of proteins. This hypothesis is also supported by the marked pH dependence of binding of cationic detergents to proteins [24]. Moreover, the dependence of hydrophobic interactions on temperature can not be neglected. As already known [25,29], the higher the temperature the larger the hydrophobic interaction, which seems to be in good agreement with the higher variation of electrophoretic mobility of CTAB–protein complexes with temperature compared to that for electroosmotic flow. For instance, the effective electrophoretic mobility (i.e. apparent electrophoretic mobility minus electroosmotic flow) calculated from Fig. 4 for $\alpha$-chymotrypsinogen is $1.2 \cdot 10^{-8}$ at 30°C and $0.57 \cdot 10^{-8}$ $m^2/V$ s at 10°C, while the electroosmotic flow values obtained were $4.9 \cdot 10^{-8}$ at 30°C and $3.3 \cdot 10^{-8}$ $m^2/V$ s at 10°C.

From these results it seems clear that temperature plays an important role in the separation of biopolymers using CTAB. Following this idea and taking into account the dependence of the heating generation on the run voltage applied, we studied the effect of this parameter on the efficiency of the separation. In Fig. 5 the efficiency values obtained for two proteins, i.e., cytochrome *c* and ribonuclease A, using 10 m*M* CTAB versus the run voltage employed are shown. As can be seen, the higher the voltage the lower the efficiency, probably as result of the thermal effect as mentioned above.

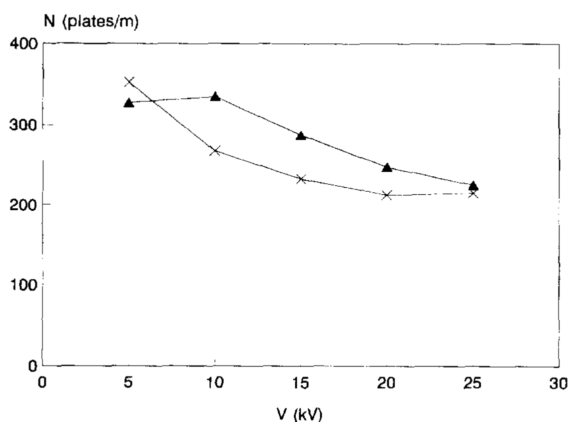


Fig. 5. Efficiency versus run voltage for cytochrome *c* (▲) and ribonuclease A (×) for a buffer 50 m*M* MES at pH 7 containing 10 m*M* CTAB as additive. The rest of the conditions as in Fig. 4D.

Fig. 6 shows the separation of the basic proteins used in this work under optimized conditions, i.e., 10 m*M* CTAB, −10 kV run voltage and 10°C separation temperature. Under these conditions, efficiencies higher than 300 000 plates/m were normally obtained, while slightly better analysis time reproducibilities, i.e., R.S.D. ($n=5$) lower than 1.1%, were obtained for the proteins compared to those obtained under the conditions given in Fig. 2F.

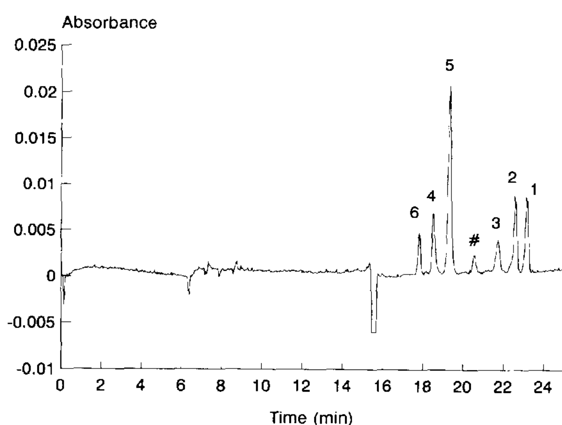


Fig. 6. Separation of basic proteins under optimized conditions using CTAB as buffer additive. Buffer: 50 m*M* MES, 10 m*M* CTAB, pH 7. Run voltage: −10 kV. Temperature: 10°C. Other conditions as in Fig. 4D.

## 4. Conclusions

The use of CTAB as buffer additive brings about the best efficiencies in the separation of basic protein compared to those obtained with KCl, PVA, morpholine or PEI.

The great influence of temperature, surfactant concentration and run voltage on the CE analysis of basic proteins in a cationic micellar system was demonstrated. By using this simple procedure, efficiencies higher than 300 000 plates/m were obtained, under optimized conditions, for the separation of these biopolymers.

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