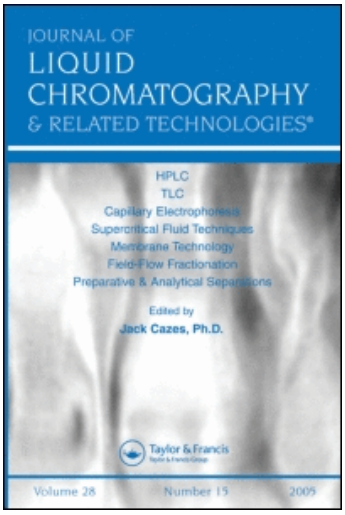

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ABSTRACT

The possibilities of capillary electrophoresis for the analysis of several basic and acid proteins by using different capillary modification procedures are shown. In order to reduce the interaction of proteins onto the capillary inner wall, potassium chloride (KCl), polyethyene glycol (PEG), poly(vinyl alcohol) (PVA), and hydroxyethylcellulose (HEC) were tested as additives in the running buffer. Furthermore, a PVA coated capillary was examined to compare the suitability of coating to separate different nature proteins (lysozyme, carbonic anhydrase, trypsin inhibitor, and bovine serum albumin) by capillary electrophoresis.

The experiments were carried out at different concentrations of the additive, voltages and, in some cases, pH. This study showed that the PVA provides the better results, since both kinds of proteins could be analysed simultaneously without important adsorption drawbacks. When a poly(vinyl alcohol)

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coated capillary was used, the improved performance over bare fused-silica capillaries were emphasized. Efficiencies higher than 500,000 theoretical plates per meter were obtained.

INTRODUCTION

In recent years, the emergence of several new areas of analysis, like the biotechnology industry, is creating a demand for new and more efficient methodologies for the separation and identification of biological compounds. The analytical requirements for these new methodologies are on the basis of their speed, efficiency, quantitation, and automation. As a consequence of this, capillary electrophoresis (CE) seems to be well suited for the separation of biopolymers such as proteins. However, for CE to become stabilised as a routine separation methodology for proteins, the challenges associated with the capillary surface chemistry must be addressed. The adsorptivity of the proteins onto the inner wall of the silica-based capillaries has been investigated so far, but it is still the major drawback for these analyses.

In the protein analysis by CE, significant tailing or irreversible adsorption of the proteins on the inner wall of a fused-silica capillary was observed, because a coulombic and/or hydrophobic interaction exists between the proteins and the inner wall of the capillary.[1] The walls of the fused-silica capillaries are negatively charged at physiological pH as a result of ionization of SiOH to SiO^- (pK_a of $SiOH \approx 3$).[2] A protein having a positive effective charge, or one having a high molecular weight, tends to adsorb on the surface of these capillaries. Minimising this adsorption is important in extending the utility of CE to a broader range of proteins.

The adsorption results in irreproducible separations, reduced efficiency, and, in some cases, the inability to detect the analytes due to adsorption of the entire analyte sample to the capillary wall. To overcome these challenges, several approaches have been proposed so far:

a) To control the pH or ionic concentration of background electrolytes[3,4] to reduce coulombic interactions by eliminating wall and solute charge differences;
b) To modify the capillary inner wall by dynamic deactivation[5–11] through the addition of modifiers to the running buffer;
c) To cover the inner wall permanently by means of the physical adsorption of polymers (polyethyleneimine, cellulose PEG, PVA)[12–15] or by means of the covalent bonding via silylation followed by deactivation or direct Si-C coupling.[16–22]

The objective of this work is to carry out a comparative study about the efficiency of the different procedures developed to prevent protein capillary wall interactions. Several additives, namely potassium chloride, PEG, HEC, and PVA have been tested. So, a poly(vinyl alcohol)-coated capillary was used and the results obtained have been compared with the preceding.

EXPERIMENTAL

Instrumentation

All experiments were performed using a HP^{3D} CE capillary electrophoresis system (Hewlett-Packard GmbH, Waldbronn, Germany) equipped with UV diode array detection and a computer controlled via HP Chemstation software. The fused-silica capillaries used were 50.0 μm I.D., with 64.5 cm of total length and 56.0 cm effective length, and they were from Teknokroma (Barcelona, Spain). The PVA permanent coated capillary was purchased from Hewlett-Packard and it has the same dimensions as the fused-silica one.

The capillaries were thermostatized (25°C) with a high-speed air stream. Injection was carried out by pressure at 50 mbar for 3.7 s. Detection took place at 215 nm.

Capillary Conditioning

In order to increase area and migration time reproducibility, the capillaries have to be conditioned from run to run. For the different capillaries, several conditioning procedures were employed. The fused-silica capillaries were successively rinsed between injections with 0.1 M NaOH for 3 min and buffer for 5 min. Before the first run, the capillary was rinsed with 1 M NaOH for 10 min. The PVA-coated capillary does not have to be rinsed with NaOH before its first run, and between injections the capillary was only flushed with buffer for 5 min.

Chemicals

Standard proteins, i.e., anhydrase carbonic, lysozyme, bovine serum albumin, and trypsin inhibitor were obtained from Sigma (St. Louis, MO, USA) and used as received. PVA, PEG (both from Sigma), HEC (from Hewlett-Packard), and KCl (from Merck, Darmstadt, Germany) were used as additives.

Sodium dihydrogenphosphate, sodium hydroxide, and phosphoric acid (all of analytical-reagent grade) were purchased from Sigma and used in the running buffers. Milli-Q water (Millipore, Mildford, USA) was used throughout.

Buffers and Sample Preparation

A 50 mM phosphate solution was used in all the running buffers. The required amount of the sodium dihydrogenphosphate and, in this case the additive, was dissolved in the Milli-Q water and, subsequently, 1 M NaOH or H_3PO_4 was added to adjust the desired pH. Standard proteins were dissolved at a final concentration of 0.5 mg l^{-1} in Milli-Q water. These samples were stored at $-5^{\circ}C$ and used at room temperature.

RESULTS AND DISCUSSION

Table 1 shows the different additives and conditions of separation employed. As can be seen in these tables, four buffer additives were used to minimize protein-wall interactions: potassium chloride since the salt and protein compete for adsorption sites on the capillary wall; PEG to modify fused-silica capillaries in order to provide a hydrophilic surface that would be essentially inert to proteins; HEC as cellulose derivative. These additives decrease the zeta potential on the surface of the capillary wall; and PVA as neutral polymer. Table 1 also shows different concentrations, voltages, and pH tested in the range or values indicated in each case.

Ohm's Law Plots and Electrosmotic Flow Control

When voltage is applied through a capillary containing buffer solution a current is generated according to Ohm's law. This current leads to heating Joule effect, which causes a temperature rise inside the capillary. If the generated heat is adequately vanished, a good higher voltage application would be possible. That would provide a better effectiveness, smaller migration times, and greater resolution. The point in which linearity gets lost in the graphic representation of the Ohm's law determines the maximum voltage that could be employed.

Table 1. Additives Added to a 50 mM Phosphate Buffer and Conditions of Separation

Additive	Concentration	Voltage (KV)	pH
KCl	10–75 mM	9–15	7.0
PEG	0.1%–3.0% (w/w)	15	7.0
HEC	0.05% (w/w)	30	2.5
PVA	0.05%–1.0% (w/w)	10–30	3.0

We have prepared phosphate 50 mM (pH 7.0) buffer solutions with increasing concentrations of KCl (0, 10, 25, 50, and 75 mM). In order to plot the evolution of the intensity versus potential (Ohm's plot), a potential gradient ($2.5\,kV\,min^{-1}$) is applied to every prepared buffer.

As can be observed in Figure 1, the intensity evolution with the potential follows an increasing lineal tendency in all the buffer solutions until approximately 15 kV. Higher voltages cause an increase in current, which is indicated by a deviation from linearity. The point at which nonlinearity occurs, marked in the graph with a dotted vertical line, indicates the maximum voltage that should be employed. As is observed, the current intensity increases until 300 μA. Keeping in mind the maker's recommendations (not to exceed 200 μA under the normal system operation) and considering the previous discussion, a potential range from 9 to 15 kV will be used.

Similar experiments were carried out with phosphate 50 mM buffer solutions with 0.1%, 1.0%, and 3.0% of PEG. Only at low PEG percentages (0.1%–1.0%) is a detriment of linearity detected when voltages of more than 15 kV were applied.

Using hydroxyethylcellulose as an additive, the heat dissipation is effective for every applied voltage. So, it generates moderate currents that allow their use in our equipment. Consequently, the voltage will be set to 30 kV.

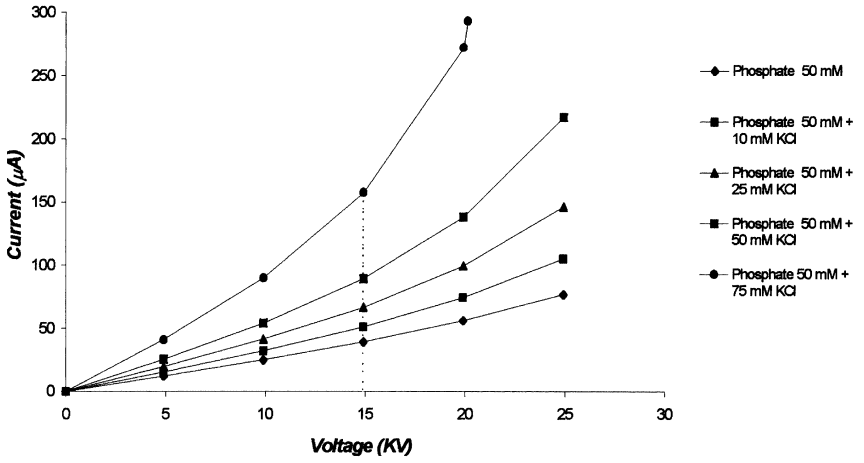

Figure 1. Ohm's law plots. The dashed line indicates the maximum voltage that should be used. Buffers: pH 7.0 50 mM phosphate plus the additive. Capillary: fused silica 50 μm, i.d., 56.0 cm effective length; 64.5 cm total length. Temperature: 25°C. Detection: UV, 215 nm. Injection by pressure (50 mbar, 3.7 s).

Although it seems adequate to choose pH values close to the physiological pH when PVA was used as additive, the working pH must be lower than 5, because the higher pH values lead to a loss of PVA adsorption to the capillary wall. That effect seriously modifies the protein separation.[23] So, pH 3 was the choice for these tests. A practically constant electrical current was observed when 0.05–1.0% PVA concentration was tested. This additive has no charge and it contributes negligibly to the electrical conductivity, so PVA is an adequate buffer additive since there is a smaller temperature rise within the capillary.

On the other hand, when CE separations are performed in the presence of additives, it is particularly essential to carry out the control of EOF for reproducible analyses. If it varies, the migration times will change and several problems, such as wrong peaks and identification and quantification mistakes will be generated. In our experiment, EOF measurement was made producing a hydrodynamic injection of methanol as a neutral marker (50 mbar, 3.7 sec) in the buffers solution. The baseline distortion on an electropherogram corresponds to migration time of the EOF marker. This experiment was repeated for three consecutive days in order to establish the migration time reproducibility for every one of the studied buffer solutions. When the KCl additive was used, the experiment was made employing the three different working potentials, namely 9, 12, and 15 kV, and the obtained results show that the EOF reproducibility was, in all cases, below 5%. As can be seen in Figure 2, when

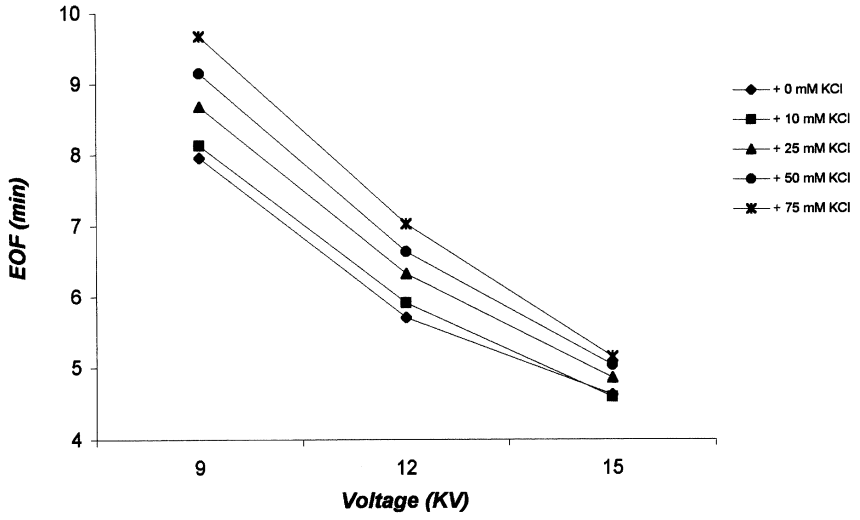

Figure 2. Effect of applied voltage on electroosmotic flow. Other conditions are as discribed in Figure 1.

the applied potential increased, the migration time of the marker decreased and when the capillary temperature is controlled, as it happens in this case, an increase of the running buffer ionic strength originates a zeta potential decrease which leads to a EOF decreasing. This is in agreement with the theoretical reasoning.

The presence of the PEG as additive in the running buffer contributes negligibly to the EOF migration time, although as can be seen in Figure 3, EOF decreased when an increase in pH was observed. The use of PVA as an additive increases the viscosity of the buffer and, as a result of that, an EOF decrease was noticed when the PVA percentage increased.

Optimization of Protein Separation

KCl

We tested the four concentrations cited for KCl for the protein separation and compared the results obtained by using the buffer without additive. It is observed that when a buffer without additive was employed, no carbonic anhydrase peak appears. Even when the KCl percentage was increased this protein peak was poorly observed because its adsorption on the capillary wall was still notable. Soybean trypsin inhibitor and BSA were, in all cases, separated, and more symmetric and bigger peaks were obtained with KCl. Figure 4 shows the electropherogram obtained at 12 kV using 50 mM KCl as additive. Under all conditions, the tested basic protein, lysozyme, was retained on the capillary wall by the silanol group.

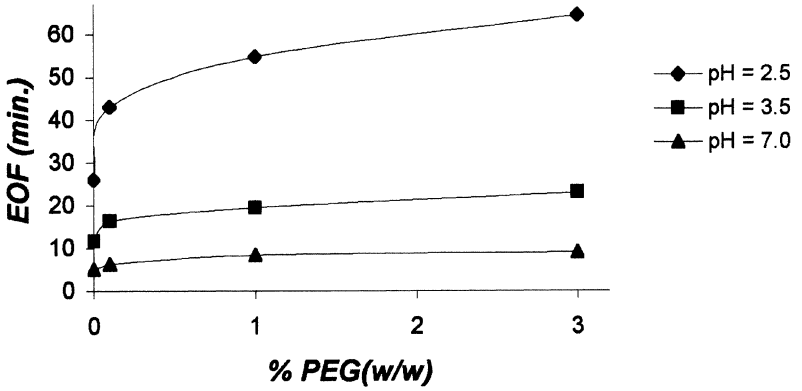

Figure 3. Effect of % PEG on the electroosmotic flow at different pH. Run voltage: 15 kV. All other conditions are as described in Figure 1.

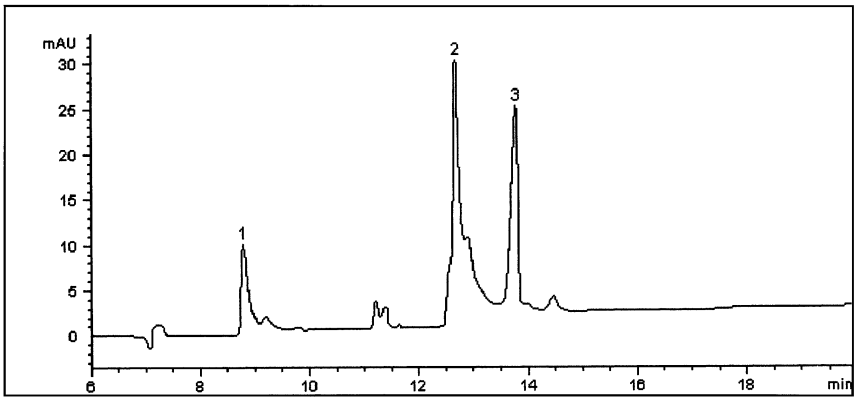

Figure 4. Electropherogram of proteins mixture. Peaks: 1) Carbonic anhydrase; 2) Soybean trypsin inhibitor; 3) Serum albumin bovine. Additive: 75 mM KCl. Voltage: 12 kV. The rest of conditions are as described in Figure 1.

In order to estimate the efficiency of separation, a number of plates per meter for each concentration was obtained. In the maximum effectiveness point, a number of plates/m between 5.0×10^4 and 1.2×10^5 was reached. Relative standard deviations (RSD) of migration time and corrected area calculated for six repetitive runs were in the range of 0.4 and 3.5% and 1.2 and 3.9%, respectively. Reproducibility between days, made along three days and six runs every day, exhibited values of RSD until 10%. Sample injection by pressure, followed by post-injection of a buffer plug, did not improve the results. This lack of reproducibility between injections indicates that the capillary reconditioning process is not effective.

PEG

Initially, we tested several concentrations of PEG. Three different phosphate 50 mM buffer solutions with 0.1%, 1.0%, and 3.0% of PEG, respectively, were prepared. Under 15 kV potential, changes in the protein migration times with the pH of the running buffer were tested. Figure 5 reflects that the use of this additive at pH 2.5 does not allow the protein separation. Nevertheless, when the pH of the electrolytic solution was increased the separation was considerably improved. At pH 7.0, the proteins migration times are different enough to assure their separation. When this pH value was employed, a suitable separation was found when 1.0% or 3.0% of PEG was employed. The analysis time reaches to 25 minutes. In these conditions, lysozyme remains adsorbed.

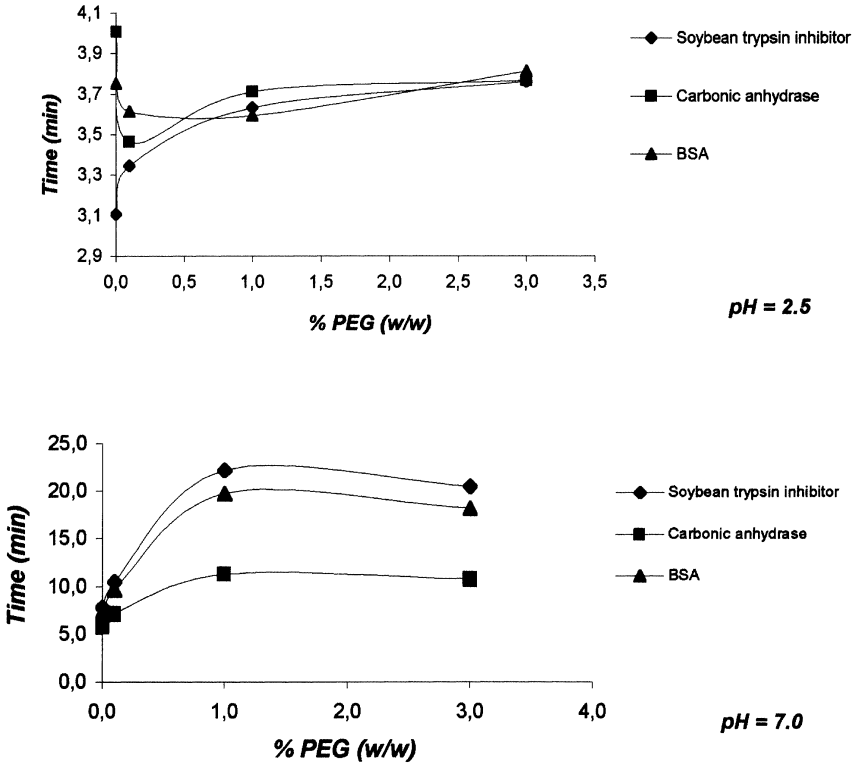

Figure 5. Evolution of proteins migration time with the % PEG at different pH. Run voltage: 15 kV. Other conditions are as described in Figure 1.

A smaller theoretical plates number was observed when there was an increase in the additive concentration in the running buffer (Figure 6). This is probably because of the polymer interaction with the proteins generating thermal effects and modifications on the electrophoretic mobility.[24]

HEC

Figure 7 shows the electropherogram obtained when a commercial running buffer containing a phosphate 50 mM buffer solution with 0.05% (w/w) of hydroxyethylcellulose was used. As can be verified, the lysozyme peak was present in the electropherogram. The HEC originates peaks more symmetric than the preceding additives, although their width and tails indicate that the

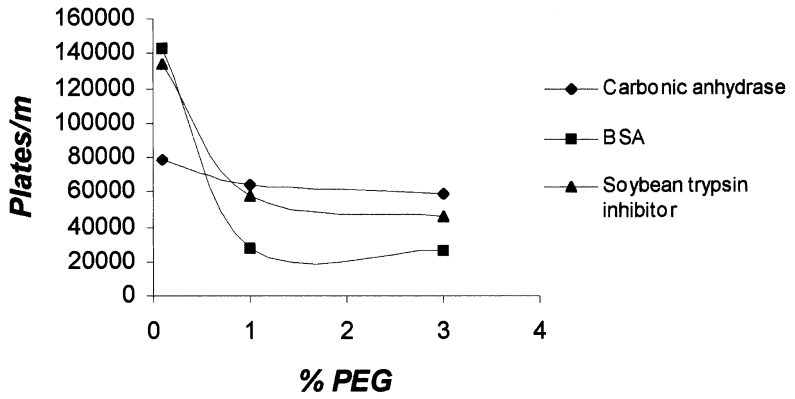

Figure 6. Effect of % PEG on efficiency. Buffer pH 7.0. Voltage: 15 kV. Other conditions are as described in Figure 1.

adsorptions were still significant. On the other hand, carbonic anhydrase and soybean trypsin inhibitor appear overlapped. Greater HEC percentages are inadequate since it origins a sieving effect.[25,26] The separation efficiency with this additive oscillates between 1.6×10^4 plates/m for the carbonic anhydrase and 3.0×10^4 for lysozyme.

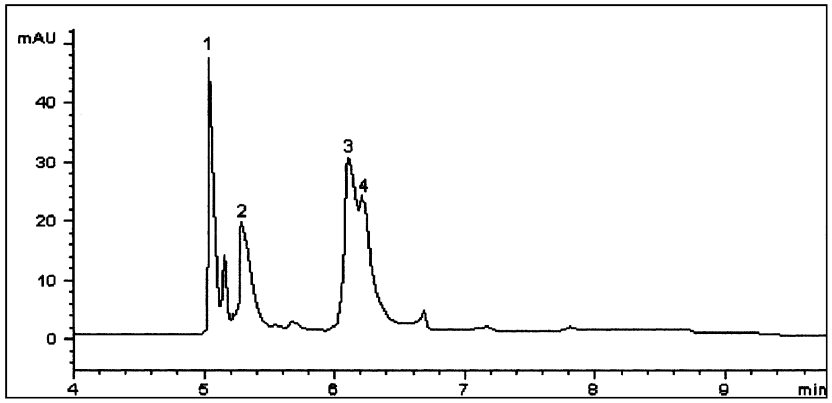

Figure 7. Electropherogram of proteins mixture. Peaks: 1) Lysozyme; 2) BSA; 3) Carbonic anhydrase; 4) Soybean trypsin inhibitor. Additive: 0.05% (w/w) HEC. Run voltage: 30 kV. Buffer pH 2.5. The rest of conditions are as described in Figure 1.

PVA

Three different phosphate 50 mM buffer solutions with 0.05%, 0.3%, and 1.0% of PVA were prepared and used to separate the proteins mixture. Although an increase of the PVA percentage in the running buffer causes the number of theoretical plates to increase for proteins, its use is not prudent because it could cause important distortions on the baseline in that, in some cases, rise to make protein identification and quantification impossible. This effect is not observed when the experiment is designed at a lower polymer percentage, as is seen in Figure 8. There is a noticeable effect probably due to a running buffer viscosity increase and the incipient creation of a polymeric separation network. At low concentration levels, although a differentiated graphical registry for proteins is obtained, adsorptions of some of these substances (displayed in the electropherogram as tailed peaks) are still substantial and effectiveness in separation decreases. The number of theoretical plates in separation was unusually low for this technique.

PVA Permanent Coated Capillary

The PVA employed as a dynamic covering agent (as has been described in a previous section) can experiment a particular aggregation process. It takes place by molecular interactions of hydrogen bridges between the hydroxyl groups of polymer chains.[27] This process, that is maintained when PVA solutions are used with fused-silica capillaries, is probably initiated and accelerated by the presence

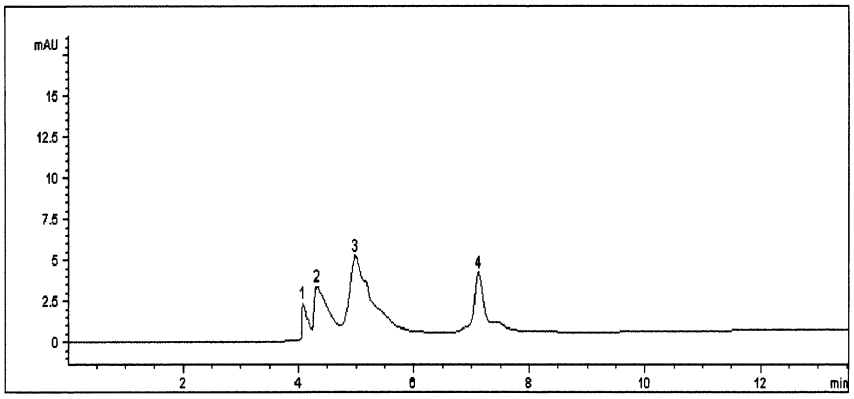

Figure 8. Electropherogram of proteins mixture. Peaks: 1) Lysozyme, 2) BSA; 3) Carbonic anhydrase; 4) Soybean trypsin inhibitor. Additive: 0.3% PVA. Run voltage: 30 kV. Buffer pH 3.0. Other conditions are as described in Figure 1.

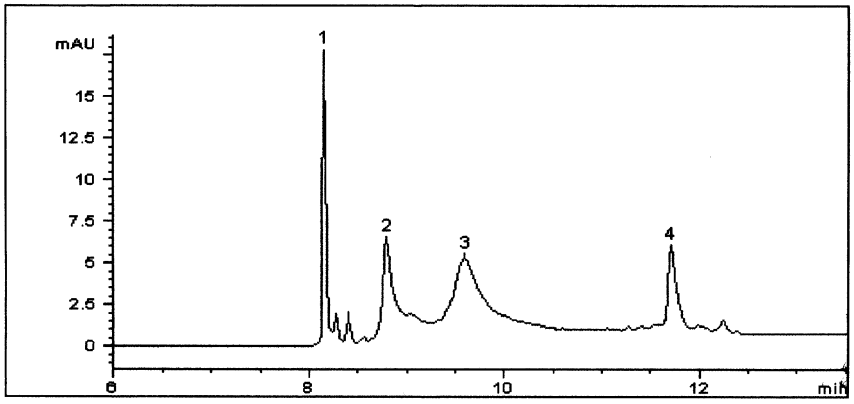

Figure 9. Electropherogram of four proteins standards. Peaks: 1) Lysozyme, 2) BSA; 3) Carbonic anhydrase; 4) Soybean trypsin inhibitor. Buffer: pH 3.0 50 mM phosphate. Run voltage: 30 kV. PVA coated capillary 50 μm, i.d., 56.0 cm effective length; 64.5 cm total length. Temperature: 25°C. Detection: UV, 215 nm. Injection by pressure (50 mbar, 3.7 s).

of the capillary wall silanol groups, which cause the reorganisation of PVA molecules adsorbed to the silica wall. This could facilitate fixation on this surface of additional PVA layers, originating EOF variations that cause irreproducibility. Thus, variations between 15% and 20% within lots are usual.

On the other hand, large molecules such as proteins can establish hydrophobic or electrostatic interactions with the internal capillary wall,[28] intensifying the previous described problems. All the dynamic coating designed to diminish these problems present instability in high pH range. These disadvantages can be solved by means of the use of PVA permanently covered capillaries.

The PVA permanent coated capillary is based on a highly hydrophilic polymer (polyvinyl alcohol) that is permanently linked to the surface of the

Table 2. Resolution Obtained Using Dynamically and Permanently PVA-Coated Capillaries

Resolution	Dynamically Coated	Permanently Coated
$R_{(1,2)}$	1.3	1.6
$R_{(2,3)}$	1.0	1.1
$R_{(3,4)}$	1.4	3.7

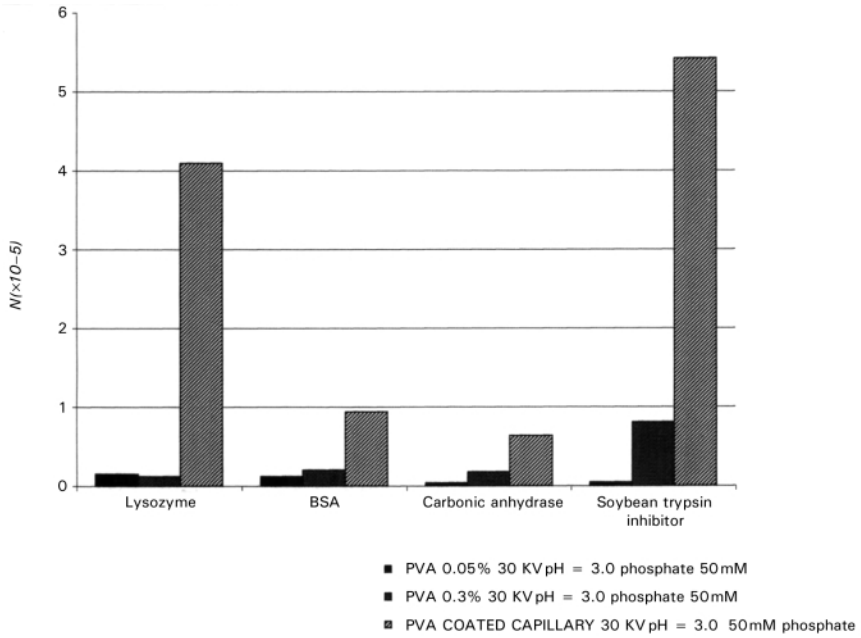

Figure 10. Comparison of protein separation efficiency using dynamically or permanently PVA coated capillaries. Conditions: Buffers: pH 3.0 50 mM phosphate. Voltage: 30 kV. All other conditions are as described in Figure 1.

capillary, forming a covering that makes the proteins separation possible. The PVA permanent coated capillaries are characterised by a non-covalent union between the polymer and the capillary surface. This is, because in their construction, heating around 160°C generates a pseudocrystalline layer, non-water soluble that is probably linked to silica surface by hydrogen bridges. This coating is designed to suppress interactions with the capillary inner surface and to minimise electroosmotic flow in the fused-silica capillaries. Their application is centred in acid and basic protein separation, allowing its use in the pH range between 3.0 and 9.5.

In our study with this variety of capillaries, we have used a 50 mM phosphate buffer fitted to pH 3.0 and 30 kV as operating potential. Under these conditions, the four protein mixtures were investigated. As observed in Figure 9, the acid and basic protein mixture separation was carried out in the same conditions of pH and ionic strength that was described for the dynamic coating. Nevertheless, it is verified that results obtained with this sort of coating are substantially better than in the case of PVA as additive. So, a better resolution (Table 2) was obtained employing PVA permanent coated capillaries; and in all

cases the number of theoretical plates working with a permanently coated capillary is greater than in the dynamic coating capillary (Figure 10). On the other hand, the proteins show remarkably improved peak symmetry with this capillary. The effect is particularly observed with lysozyme and carbonic anhydrase.

CONCLUSIONS

Of the four separation additives (ionic and polymeric) studied, the polyvinyl alcohol (PVA), so dynamic as a permanent covering, revealed better results. This compound allows the studied protein separation (basic and acid nature) against other additives such as KCl or PEG.

A fused-silica capillary with a PVA permanent coating exhibits improved performance over bare fused-silica capillaries using PVA as a running buffer additive in the protein separation.

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