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Preparation of linear polyacrylamide-coated capillaries Study of the polymerization process and its effect on capillary electrophoresis performance[1]

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Abstract

The effect of different parameters controlling the characteristics of linear polyacrylamide coatings deposited on the inner wall of fused-silica capillaries and their influence on capillary electrophoresis (CE) performance of these coated columns is investigated. To carry out this study, a reproducible procedure to obtain capillaries with similar extent of modification of the surface silanols with 7-oct-1-enyltrimethoxisilane was first approached. Next the polymer attachment to the silica wall, via covalent linkage to the silyl reagent grafted onto the silica, was investigated. In this way, by using columns with a similar silylation extent, differences in CE performance observed among capillaries coated under diverse conditions could be assigned to the characteristics of the polyacrylamide layer. It is demonstrated that the characteristics and reproducibility of these polymeric coatings depend on the adequate control of both the temperature of polymerization and the degassing of the polymerizing dissolutions used. More interestingly, it is also demonstrated that the quantities of monomer (acrylamide), initiator (ammonium persulfate) and activator (*N,N,N′,N′*-tetramethylethylenediamine), and the ratio among them used in the preparation of the coating polymer have a large influence on the performance of CE columns. The optimum conditions for preparing the polyacrylamide coatings are discussed. The applicability of these linear polyacrylamide-coated capillaries to the separation of basic and acidic proteins in free zone CE is demonstrated. Besides, the use of these coated columns in capillary gel electrophoresis for the separation of DNA fragments is shown. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Coated capillaries; Capillary columns; DNA

1. Introduction

Since Hjerten demonstrated for the first time in 1985 the possibility of coating fused-silica capillaries with linear polyacrylamide [1], capillaries coated with this polymer have been widely employed in several applications of capillary electrophoresis (CE) such as separation of basic proteins [1–14], analyses of proteins and DNA fragments based on molecular mass [15–20], separation of peptides and proteins according to their isoelectric point [11,21–23], etc. The procedure employed to prepare polyacrylamide-coated capillaries is laborious, involving many steps [1,24–26]. These steps can be arbitrarily divided into

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two main groups. The first one includes the different pretreatments (etching, leaching, dehydration and silylation) required to obtain high surface coverage of the inner capillary wall by the silylating agent employed. The second part of the process consists of coating of the capillary wall with an uniform layer of linear polyacrylamide covalently anchored to the fused-silica tubing through the silyl derivative introduced in the previous steps.

Despite the huge number of references showing the utility of Hjerten's procedures (see for instance Refs. [1–33]) to our knowledge, no works have been reported demonstrating the effect of the polymerization process itself, which concomitantly controls the polyacrylamide characteristics, on the CE performance of coated capillaries. To carry out such a study, the use of a reproducible silylation procedure is mandatory; otherwise it would be impossible to differentiate the effects on the CE performance of the polymeric coating itself from those resulting of the pretreatment of the capillaries [13]. For instance, it has been demonstrated that inadequate conditions for etching, leaching or dehydrating the columns can decrease the silylation yield or its reproducibility [26]; similarly, it has been shown that capillaries poorly silylated and subsequently coated with polyacrylamide present limited CE performance [26]. Conversely, it could be assumed that behavior of columns with the same surface silylation extent and presenting differences in CE performance after coating, could be mainly attributed to the formation of the polyacrylamide layer. For the sake of the methodology, the first goal of this work is to test a silylation procedure, using 7-oct-1-enyltrimethoxysilane, able to provide reproducible columns in terms of silylation coverage irrespective of the fused-silica tubing employed.

Once silyl-bonded capillaries with similar extent are obtained, the effect of the different polymerization conditions and their influence on the CE behavior of these coated capillaries can be explored. Thus, taking into account that polymerization of acrylamide is carried out via free radicals [34–36], the size of the polymer and its probability to interact with the vinyl groups on the capillary wall is controlled, among other factors, by the concentration of monomer, initiator and activator and the ratios among them [34–36]. Moreover, temperature of reaction and oxygen content of the different dissolutions employed also play an important role in polymerization reaction when a redox system, such as the mixture *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) used in this work, is employed to generate free radicals [34–36]. These polymerization conditions can be expected to modify the polyacrylamide properties and, therefore, the CE behavior of polyacrylamide-coated columns. Consequently, the second goal of this work is to study the effect of the concentrations of monomer, initiator and activator and the ratios among them, as well as reaction conditions on the polymerization itself and how they can modify the CE performance of capillaries coated with this polymer.

2. Experimental

2.1. Chemical and reagents

Formic acid (pH 4), acetic acid (pH 5) and malic acid (pH 6) (all from Merck, Darmstadt, Germany), boric acid (pH 8) (from Aldrich, Steinheim, Germany), and 2-*N*-cyclohexylamino-ethanesulfonic acid (CHES, pH 10) (from Sigma, St. Louis, MO, USA) were used as received for preparing the different running buffers at concentrations of 50 m*M* and their pH adjusted with 1 *M* sodium hydroxide to the values indicated in parentheses. Hydrochloric acid, sodium hydroxide (both from Merck), 7-oct-1-enyltrimethoxysilane (ABCR, Karlsruhe, Germany), acrylamide, APS and TEMED all from Schwarz (Cleveland, OH, USA) were used for the preparation of the polyacrylamide-coated capillaries. Lysozyme chicken egg white, ribonuclease A bovine pancreas, α-chymotrypsinogen bovine pancreas, bovine serum albumin (BSA), β-lactoglobulin A, β-lactoglobulin B and α-lactalbumin were purchased from Sigma and used as received to test the capillaries coated with polyacrylamide. The proteins were solved in Milli-Q water (Millipore, Bedford, MA, USA), stored at −5°C, and warmed at room temperature before use.

The eCAP dsDNA 20 000 kit from Beckman was employed (except the enclosed coated capillary) for the separation of a standard mixture of double stranded DNA (ds-DNA) fragments. A linear poly-

acrylamide-coated column prepared as described in this work was used to carry out such separations.

2.2. Toxicity

Acrylamide monomer is toxic and should be handled with care; avoid skin contact.

2.3. Instrumentation

Analyses were carried out using a P/ACE 2100 (Beckman Instruments, Fullerton, CA, USA) CE apparatus. Fused-silica capillaries used were from Polymicro Technologies (Composite Metal Services, Worcester, UK), SGE (Ringwood, Victoria, Australia) and Siemens (Munich, Germany) and they were of 27 cm (20 cm effective length)×50 μm I.D. Injections were made using nitrogen pressure (0.5 p.s.i.; 1 p.s.i.=6894.76 Pa) for a given time. Capillaries were thermostatted at 25°C. Detection took place at 254 nm when acetone was injected and at 214 nm when proteins were used. Data were collected and analyzed using a System Gold software from Beckman running on a 486DX2-66 MHz computer. For the thermal treatment of capillaries a gas chromatograph Model 8310B from Perkin-Elmer (Norwalk, CT, USA) was employed.

2.4. Capillary pretreatment

Capillaries were etched with 1 *M* sodium hydroxide for 30 min, leached with 0.1 *M* hydrochloric acid at 70°C for 2 h, washed with water for 1 h, dehydrated at 160°C overnight under a flow of dry nitrogen, silylated overnight by using a solution of 7-oct-1-enyltrimethoxysilane plus (0.5%, v/v) acetic acid at 120°C, and finally washed with methanol for 1 h. After each treatment a few millimeters at both ends of the capillary were cut off to discard the damaged polyimide external coating.

2.5. Capillary coating

A set of different conditions were tested to coat the capillaries based on the method previously described by Hjerten [1]. The coatings were prepared as follows. A given quantity of acrylamide was first weighted in an airtight vial provided with a silicone septum and dissolved in 1 ml of Milli-Q water. One end of the capillary, previously silylated as indicated in Section 2.4, was dipped into the acrylamide solution by insertion into the vial trough the septum. The other end was sealed by puncturing it in a piece of silicone rubber. After degassing the acrylamide dissolution, by bubbling nitrogen for a given time, the airtight vial was closed and a gas pressure of 1.5 bar was applied into the vial. A given volume (microliters) of TEMED previously degassed with nitrogen was then added to the acrylamide solution with a 10-μl syringe and the mixture was gently shaken. The piece of silicon rubber from the capillary end was then removed and the solution allowed to flush into the tubing under the gas pressure (1.5 bar). While pressurizing the dissolution into the column, a given volume of activator APS previously degassed as above was added with a 10-μl syringe, the solution was gently shaken and after letting the polymerizing solution to flow for 1 min, the tube was sealed again with the silicon rubber and the reaction allowed to complete overnight into the capillary. Polymer not bonded to the capillary wall was washed away with water.

The whole procedure consumed only a few microliters of the polymerizing solution. Therefore, in order to prepare the solution of the polymer for viscosity measurements (see below), the final quantity of polymerizing dissolution remaining into the vial was considered to be equal to 1 ml.

2.6. Electroosmotic flow (EOF), analysis time and efficiency measurements

The electroosmotic mobility (μ_{eo} or EOF) of silylated capillaries was measured at pH values of 4, 6, 8 and 10 using acetone solved in Milli-Q water (5%, v/v). Experiments were always made at increasing pH starting at pH 4 and finishing at pH 10. The acetone sample was injected by pressure (0.5 p.s.i., 3 s). For all the capillaries each EOF measurement at the different pH values was performed in triplicate. Efficiency values (N) and analysis times (t_a) in polyacrylamide-coated capillaries were measured using the buffers described in Section 2.1. at pH 4 and pH 5 (both performed in quadruplicate) using a test-set of basic proteins. These separations were carried out at a running voltage of 12 kV.

2.7. Viscosity measurements

The polyacrylamide prepared was characterized using viscosity measurements. It was hypothesized that after having reacted overnight the polyacrylamide remaining in the vial and that contained into the capillary had similar characteristics. Thus, the polymerized solution into the vial was employed to calculate the viscosity of the polymer bound to the column. To do so, the polyacrylamide gels prepared in each reaction conditions were diluted to a final volume of 5 ml. Therefore, polymer viscosity refers henceforth to the viscosity of these dissolutions. Viscosity values were calculated using Poiseuille's equation and the CE instrument. The data on total length, effective length, and internal diameter of the capillary, as well as the pressure applied are readily known from the experimental conditions. Thus, by determining the time required for each solution to go through the effective length of capillary under a pressure of 20 p.s.i. at a constant temperature of 30°C, the determination of the viscosity using the Poiseuille's equation is straightforward. However, it has to be mentioned that under our conditions, long capillaries, e.g., 57 cm, are more convenient for these experiments, because both the not thermostatted parts of the capillary (ca. 8 cm) and the time needed for the system to reach a steady state flow when the gas pressure is applied will have a much lower influence on the measurements.

3. Results and discussion

Although it seems that coating performance depends on both the extent of the silanization reaction and the characteristics (e.g., thickness and homogeneity) of the attached polymeric layer [2,13,26,29], little attention has been paid to the influence of the polymerization step itself on the CE performance of these coated columns. This can be attributed to the difficulty to individualize the effects of silylation and polymerization steps separately on the CE performance of the coated capillary obtained. For doing so, it was first tested the reproducibility of the silylation procedure employed throughout the present work for different fused-silica capillaries.

3.1. Reproducible silylation of capillaries

In a previous publication [26] we demonstrated that by optimizing the different conditions employed for etching, leaching, dehydrating and silylating fused-silica capillaries, it was possible to obtain columns with reproducible silylated extent. In this regard, the similarity among these derivatized capillaries was concluded by comparing their EOF values measured at four different pH values. However, in that work it was not shown that these treatments could be useful to obtain reproducible silylation coverage using capillaries from different manufacturers, what would extend the applicability of this silylation procedure. This point has been investigated in the present work. To do so, a group of capillaries from three different manufacturers were etched, leached, dehydrated and silylated as indicated in Section 2.4. Note that a slight modification, consisting of using higher silylation temperature (120°C) than the previously reported (room temperature) [26], has been now introduced, what provides more reproducible silylation degrees [37].

In Fig. 1A, EOF values obtained at different pH values using fused-silica columns from Polymicro, Siemens and SGE silylated using the pretreatment previously described are given. For comparison, EOF values obtained for a capillary from Polymicro which has only been etched with 1 mol/l NaOH are also given (etched capillaries from Siemens or SGE rendered similar EOF values [26]). As can be deduced from Fig. 1A, this procedure is useful to obtain reproducible silylation degrees even with fused-silica tubings from different manufacturers, which are supposed to contain impurities of different nature and concentration [4]. Moreover, comparison of the EOF values before and after silylation indicates that the silanization of the inner wall took place, since a large decrease in the EOF values is observed after silylation. This decrease in the EOF can be explained considering that the EOF value depends, among other factors, on the number of ionized silanol groups on the capillary wall. As silylation reaction takes place via covalent bonding of the silane reagent to silanol groups, the number of ionizable groups after silanization decreases, and so do the EOF values.

Since the whole treatment developed allows the

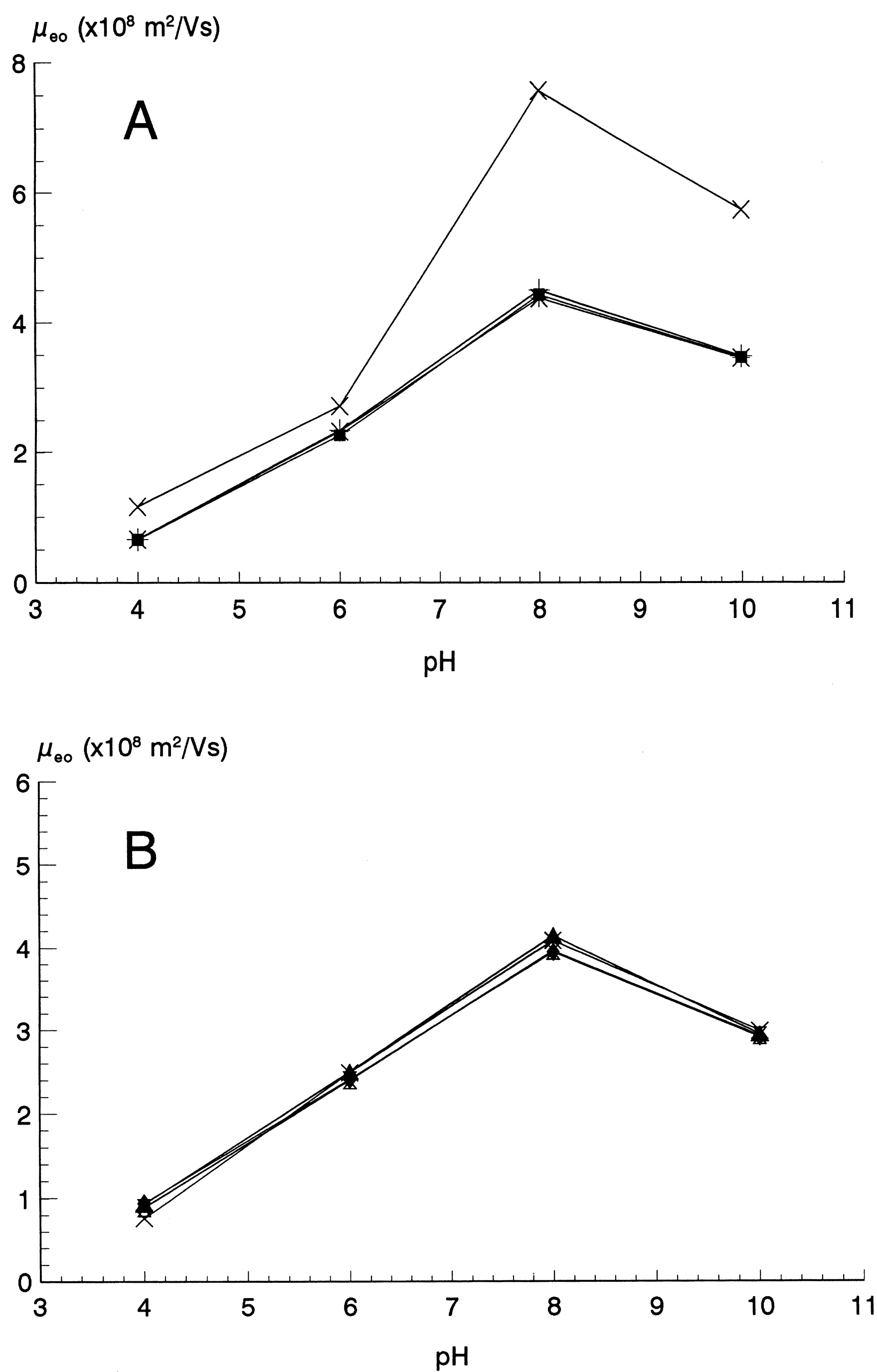

Fig. 1. (A) Variation of electroosmotic mobility with pH for a capillary from Polymicro etched with 1 *M* sodium hydroxide for 30 min (×) and three capillaries from Polymicro (+), Siemens (■) and SGE (*) after silylation. (B) Variation of electroosmotic mobility with pH for four capillaries from Polymicro after silylation. For silylation conditions see Section 2.4.

preparation of very similar silylation coverages of the inner wall of fused-silica capillaries, it could be assumed that the pretreatment of the capillaries, as it has been defined in this work, has an even influence on the CE performance of the columns. Under this assumption, the study about the effect of the polymerization step on the CE behavior of polyacrylamide coated columns was undertaken. Moreover, along all the experiments described below at least two silylated capillaries were added and checked as silylation controls, corroborating the good reproducibility shown in Fig. 1A.

3.2. Reproducible polymerization of acrylamide

Acrylamide, in the presence of free radicals, rapidly polymerizes to high-molecular-mass polymers [34–36]. Redox catalysts, such as the pair TEMED/APS, are generally used for aqueous polymerization as initiator systems at room temperature. The mechanism behind this free-radical polymerization has been extensively studied indicating that the number of molecules of monomer polymerized per initiating event, or free radical, is very high for acrylamide, what means that polymerization will take place rapidly and the molecular masses of the resulting polymer will be high. The rate of acrylamide polymerization is proportional to the square root of the concentration of initiator [I] and it also depends on the concentration of acrylamide monomer [A] to the power of ca. 1–1.5 [38–41]. A bimolecular reaction between radicals is believed to be the main mechanism of chain termination at moderated temperatures [34,41,42], while kinetics obtained at low conversion rates have been observed to apply also at high conversions [43]. According to the information presented above, the molecular mass of the polyacrylamide obtained in aqueous solution depends in a first approach on the ratio $[A]/[I]^{1/2}$. Consequently, in absence of side reactions, high monomer concentrations should bring about large polymers, while increasing the initiator concentration should cause shorter polymeric chains. It has also been shown that oxygen dissolved in the polymerizing solution strongly quenches persulfate initiation [44], what limits the reproducibility of the properties for the different polyacrylamide polymers prepared under the same conditions [17]. Furthermore, the reproducibility among preparations has been shown to depend to a large extent on the temperature of reaction [35].

In this work, the reproducibility of polyacrylamide preparations was first tested by determining the viscosity (η) of the polymer solutions prepared. The usefulness of this method for calculating the viscosity (see Section 2.7) was assessed by determining both its repeatability and accuracy. The accuracy was tested by determining the viscosity of water under our experimental conditions and the repeatability by measuring the η values of a dissolution of polyacrylamide. The results gave a η value of 0.87 cP for water at 30°C (0.80 cP is the expected value at that temperature [45]) and a relative standard deviation (R.S.D.) equal to 0.4% for five η measurements using the polyacrylamide solution. From these results it can be concluded that the procedure employed seems to be suitable to determine η values for our polymeric solutions.

Using viscosity measurements, the reproducibility of polyacrylamide preparations was first investigated by testing more than 80 polyacrylamide dissolutions prepared in groups of four polymers synthesized under identical conditions. It could be observed that using a 2-min degassing time and carrying out the polymerization reaction in an ice-bath, R.S.D. values higher than 30% ($n=4$) for viscosity values were usually obtained. By increasing the degassing time up to 30 min and using previously degassed solutions of TEMED and APS for 10 min, the reproducibility improved to a value of R.S.D.=19% ($n=4$). When these longer times were used and the polymerization reaction was carried out at room temperature, R.S.D. values ranging from 4.7% to 8% ($n=4$) were obtained. Further efforts to improve these values were not successful. Therefore, polymerization at room temperature of acrylamide solutions (1 ml solution volume) degassed for 30 min in an airtight vial and added with previously degassed (10 min) solutions of TEMED and APS was considered the optimum to obtain reproducible polyacrylamide preparations. Besides, using these conditions, a value of R.S.D.= 10% ($n=12$) for polyacrylamide polymers prepared in three different days was obtained.

Once the method for preparing reproducible polyacrylamide polymers in a vial was optimized, it was tested if the same reproducibility could be obtained

for polyacrylamide prepared inside the capillaries using the same polymerization procedure.

3.3. Coating capillaries with polyacrylamide

To test the reproducibility of the preparation method for polyacrylamide coatings, the two following experiments were carried out. A first group of six capillaries were etched, leached, dehydrated and silylated (see Section 2.4). The EOF values of two capillaries of this group were measured, and the four remaining capillaries were coated in parallel as indicated in Section 2.5, that is, they were all inserted into the same polymerizing solution. In another experiment a second group of six capillaries was pretreated as indicated in Section 2.4, and the EOF values of two of them were measured. The remaining four capillaries of this second group were coated by inserting each of them in one different polymerizing solution prepared under identical conditions. The two pairs of capillaries whose EOFs were measured rendered very similar values as can be seen in Fig. 1B, corroborating the good reproducibility of the silylation coverage achieved [26]. After overnight polymerization, the viscosity of the polymers was measured (see Section 2.7) and the performance of the coated capillaries was characterized measuring the reproducibility of the migration time and efficiency of model proteins (see Section 2.6). The R.S.D. values for analysis time and efficiency obtained with the parallel prepared capillaries of the first group, were 1% ($n=4$) and 3.8% ($n=4$), respectively, for lysozyme, the most basic proteins of the three tested. When the same values were calculated for the capillaries of the second group, the R.S.D. values obtained were 1% ($n=4$) and 4.3% ($n=4$), for the analysis time and efficiency, respectively, of lysozyme. Moreover, the R.S.D. value obtained for the η values measured for the four polyacrylamide preparations was 8%. Therefore, it can be concluded that the method developed in this work for preparing polyacrylamide coatings seems to render high reproducibility.

3.4. Polymerization parameters controlling the CE performance of polyacrylamide-coated capillaries

As already stated by Barberi et al. [46], in the preparation of polyacrylamide coatings for CE, polymer size and the number of polymer chains covalently bonded to the capillary wall must have a clear influence on the CE performance of these coatings. However, Srinivasan et al. [13] have indicated that, although both the silane and polymeric layer can determine the coating behavior, polymer size and concentration have a minimal influence on the coating performance. Furthermore, since the reaction to bind the polymer to the capillary wall is governed, among other factors, by the radicals present inside the capillary when the reaction takes place [13], the use of different concentrations of initiators should bring about both different quantities of active radicals and coatings of different polymer sizes. The effect of the concentration of TEMED, APS and monomer in the polymerizing solution on the capillaries performance was explored in this section.

3.4.1. Effect of the total numbers of mols of TEMED and APS for a given concentration of monomer

A given concentration of acrylamide (0.56 mol/l) and a determined molar ratio between TEMED/APS (ca. 2.25) were arbitrarily chosen for this experiment. By varying the total mass added of APS and TEMED, seven polymers of different viscosity were obtained. The results are shown in Fig. 2A where the viscosity of these polymers is plotted against the number of mmol of both TEMED and APS added per mol of monomer. As can be seen, the higher the amount used of TEMED and APS the lower the viscosity of the polymer obtained. Assuming that nearly all the monomer reacts, roughly the same polymer concentration applies for the seven cases. Results shown in Fig. 2A can be explained by the well known relationship between viscosity η and the chain length or polymer size M, $\eta=aM^b$, where a and b are constants which depend mainly on the experimental conditions [35], together with the relationship mentioned above according to which M is proportional to the ratio $[A]/[I]^{1/2}$. Thus, it is possible to establish, in a first approach, the relationship among viscosity, monomer concentration, and initiator concentration as: $\eta \sim a\{[A]/[I]^{1/2}\}^b$. According to this equation, the higher the monomer concentration and/or the lower the initiator concen-

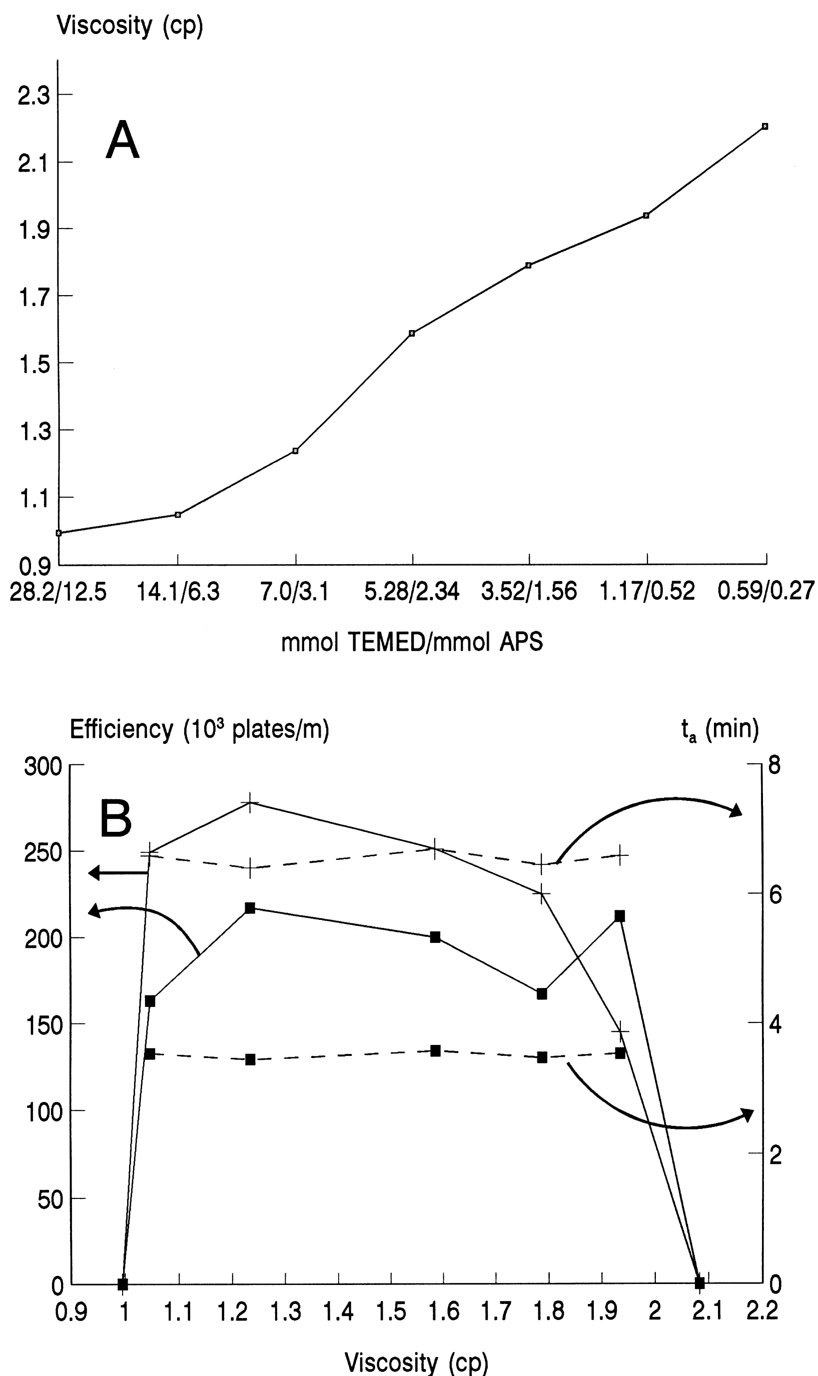

Fig. 2. (A) Variation of the viscosity of different polyacrylamide solutions (for their preparation see Section 2.7) with the total number of mmol of TEMED and APS (for a given TEMED/APS ratio ca. 2.25) added per mol of monomer. (B) Variation of the efficiency values (solid lines) and the analysis time values (t_a) (dashed lines) obtained for lysozyme (■) and α-chymotrypsinogen (+) with the viscosity given in A of the polyacrylamide solutions used as coatings. Separation conditions: coated capillaries of 27 cm (20 cm effective length)×50 μm I.D. Hydrodynamic injection (2 s, 0.5 p.s.i.). Separation buffer: 50 m*M* acetic acid at pH 5. Separation voltage: 12 kV. Detection at 214 nm.

tration the higher the polymer size and, therefore, the higher the viscosity, as is shown in Fig. 2A.

Capillaries silylated as indicated above were coated with the same seven polyacrylamide polymers prepared for the previous experiment. Fig. 2B shows the values of N and t_a for lysozyme and α-chymotrypsinogen obtained from these seven columns depending on the type of polyacrylamide used as coating and represented by the viscosity of their respective solutions measured as indicated in Section 2.7. Using capillaries coated with polyacrylamide of the highest (2.1 cP) or lowest (1.0 cP) viscosity neither the peak of lysozyme nor the peak of α-chymotrypsinogen were observed after 30 min of analysis time. This fact could be related to the poor quality of the polyacrylamide coatings obtained with both polymers. These results can be explained considering that when too high concentrations of initiator are employed the average size of the polymers forming the coating is too short, giving rise to residual adsorption phenomena between proteins and the derivatized capillary wall. At very low concentrations of initiator, although the average size of the polymer might be very high, the number of radicals inside the capillary able to activate the polymerization of the vinyl groups attached to the wall with acrylamide molecules might be small, and, as a result, the number of polymer chains anchored to the capillary might be low, which brings about residual adsorption between proteins and capillary wall [46]. Moreover, from Fig. 2B it can be concluded that efficiency measurements are more sensitive to the properties of the coating than analysis time determinations. This can be deduced from the very similar values of t_a obtained irrespective of the polymer viscosity, while different N values were observed for columns prepared with the same polymer (e.g., the N values for α-chymotrypsinogen were 145 000 and 278 000 using coated capillaries with polymers of viscosity 1.94 cP and 1.24 cP, respectively, while their respective analysis times were very similar, 6.4 and 6.5 min). These results agree with the fact that in order to adequately characterize a CE polymeric coating, data on efficiency provide more information than data on analysis time, as we already mentioned elsewhere [26].

In conclusion, from Fig. 2 it can be deduced that in order to prepare polyacrylamide coatings suitable for CE, there is a useful range of TEMED/APS per mol of acrylamide that can be employed. This range, under the preparation conditions of this work, seems to be between ca. 14/6 and 1/0.5 mmol of TEMED/mmol APS per mol of acrylamide. Lower or higher values give rise to CE coatings of poor quality as shown in Fig. 2B.

3.4.2. Effect of the TEMED/APS ratio for a given concentration of monomer

In the synthesis of polyacrylamide, the ratio between the concentrations of TEMED and APS is known to control the quantity of free radicals generated in the initiation step [34,47,48], what in turn modifies the characteristics of the polymer obtained [36]. To study the influence of the TEMED/APS ratio on the properties of polyacrylamide coatings, five capillaries were silylated as mentioned above, and five polyacrylamide polymers prepared as indicated in Section 2.5 using a given quantity of monomer (0.56 mol/l) and different TEMED/APS ratios ranging from 0.4 to 6, (maintaining the total number of mol of TEMED plus APS approximately equal to 5 mmol). The viscosity values obtained for the different polymers are shown in Fig. 3A. As can be seen, a maximum in viscosity is obtained for a TEMED/APS ratio of 1, what seems to be in good agreement with the 1:1 initiation mechanism of the system TEMED/APS [47,48]. Moreover, from Fig. 3A it can be also deduced that the product of the concentrations of TEMED and APS ([TEMED][APS]) has a large influence on the polymerization reaction of acrylamide. Thus, for a given amount of TEMED plus APS, the higher the mentioned product, the higher the viscosity obtained, as it is the case for TEMED/APS ratios of 1, 1.5 and 2.25 corresponding to millimolar concentrations of 2.36/2.36, 2.95/1.96 and 3.54/1.57, respectively. This behavior seems to confirm the fact that radicals are produced from APS and TEMED by a bimolecular mechanism [47–50]. Fig. 3B shows the efficiency values for lysozyme and α-chymotrypsinogen obtained from the CE separations carried out using the capillaries coated with the five mentioned acrylamide polymers. As can be seen, by comparing Fig. 3A with Fig. 3B, the tendency is that polymers of higher viscosity give rise to coated capillaries that provide higher N values in the separation of basic proteins.

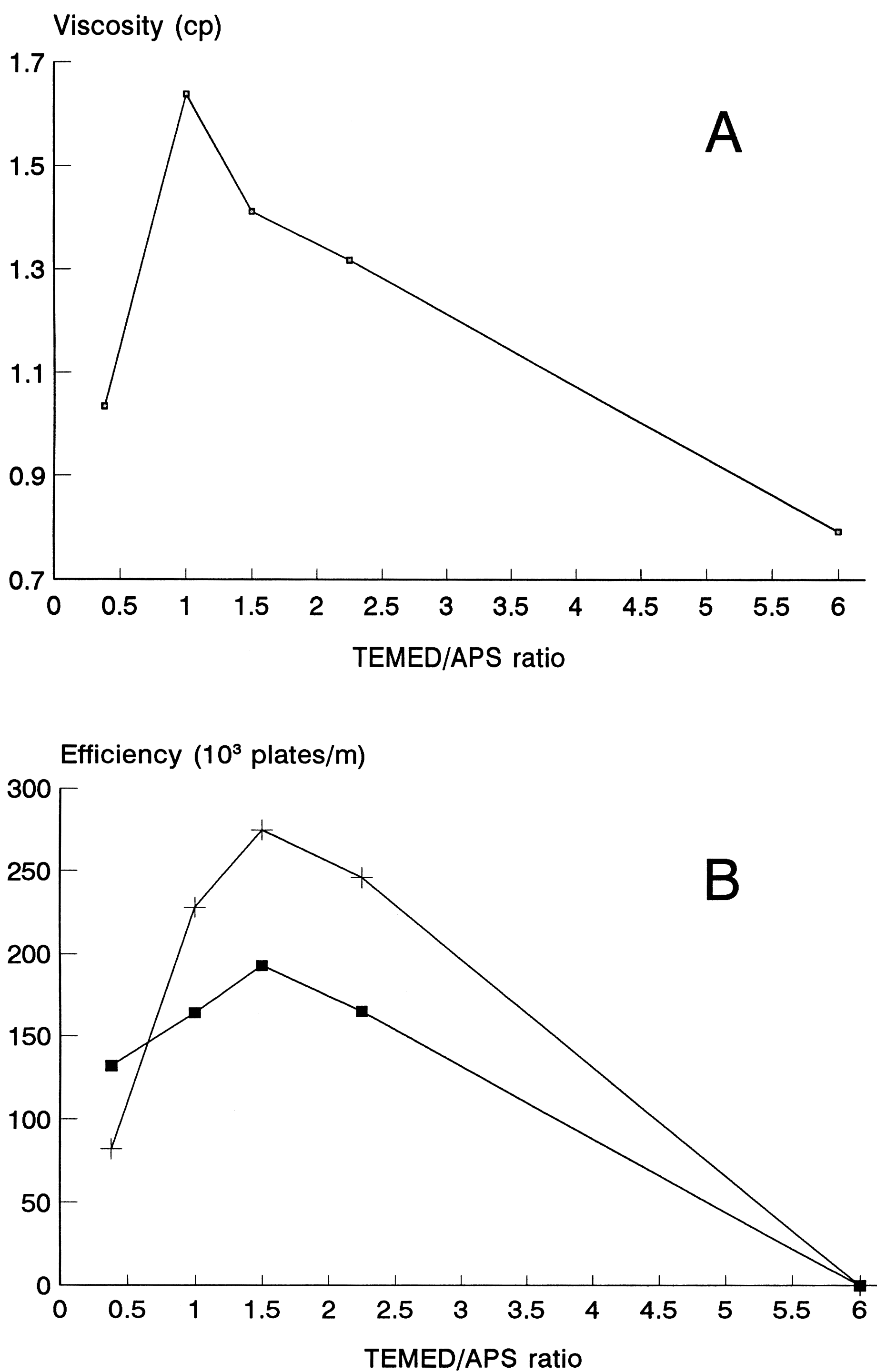

Fig. 3. (A) Variation of the viscosity of five different polyacrylamide solutions with the molar ratio of TEMED/APS (namely, 1.18/3.14, 2.36/2.36, 2.95/1.96, 3.54/1.57 and 4.74/0.79 mmol of TEMED/mmol of APS per mol of acrylamide) employed in the polymerization of 0.56 mol/l of acrylamide. (B) Variation of the efficiency values for lysozyme (■) and α-chymotrypsinogen (+) with the molar ratio of TEMED/APS employed in the preparation of the polyacrylamide polymers used as coatings. Separation conditions as in Fig. 2B.

These results are also in good agreement with those shown in Fig. 2, where it was demonstrated that the use of capillaries coated with polymers of low viscosity gives rise to low *N* values in the separation of basic proteins.

It can be concluded from the results presented in this section, that within the useful range of concentrations of TEMED/APS per mol of acrylamide (see Section 3.4.1), TEMED/APS ratios between 1 and 2.5 seem to be adequate for obtaining polyacrylamide-coated capillaries suitable for the CE separation of basic proteins. Molar ratios between TEMED and APS far from this range seem to originate polyacrylamide coatings ineffective for CE separations of such proteins.

3.4.3. *Effect of the monomer concentration for given concentrations of TEMED and APS*

In order to confirm that both the number of chains of polyacrylamide attached to the silica wall and their molecular size influence the CE behavior of polyacrylamide-coated capillaries, the following experiment was carried out. Six columns previously silylated were coated with linear polyacrylamide prepared using given quantities of TEMED and APS (1.65 μmol of TEMED and 1.1 μmol of APS were added per ml of monomer dissolution) and increasing concentrations of acrylamide monomer to a maximum of 0.56 mol/l. Although higher concentrations of acrylamide (i.e., 0.84, 0.98 and 1.12 mol/l) were also tested, they gave rise to highly viscous polymeric solutions which could not be removed from the capillary even using a HPLC pump. The *N* values obtained for the CE separation of lysozyme and α-chymotrypsinogen using these coated capillaries are shown in Fig. 4. As can be seen, there is a minimum concentration of acrylamide also called monomer effective concentration (MEC) (about 0.14

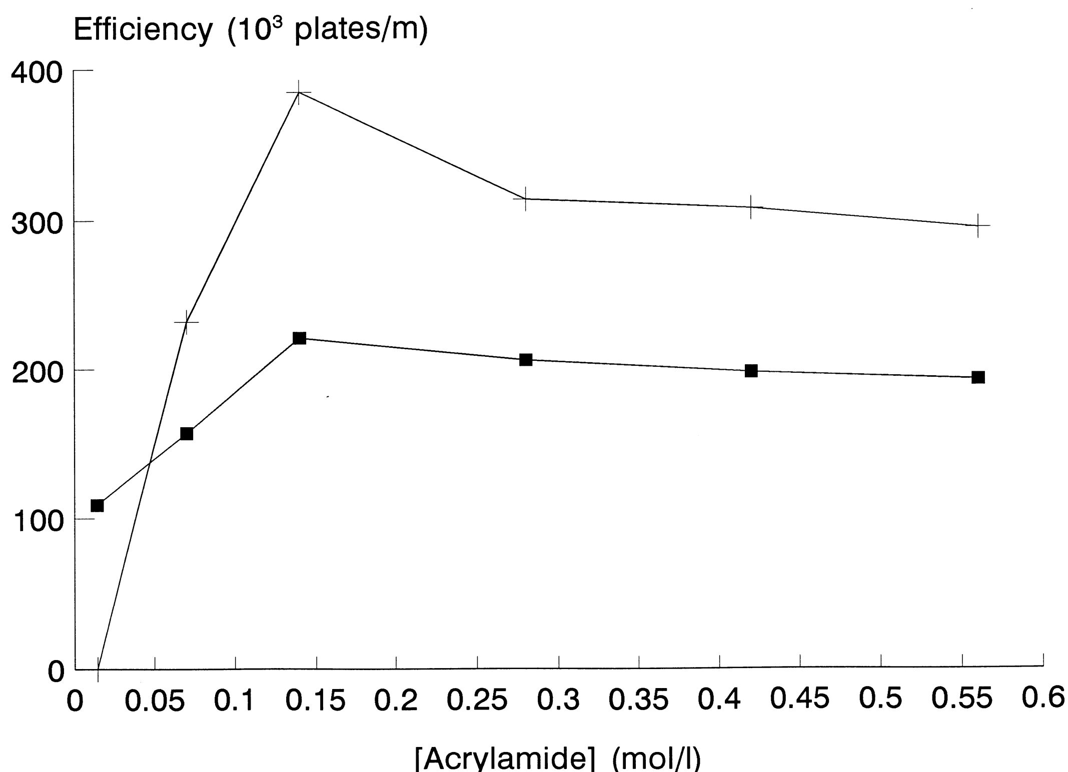

Fig. 4. Variation of the efficiency values obtained for lysozyme (■) and α-chymotrypsinogen (+) with the concentration of acrylamide monomer employed for the coating preparation. A constant quantity of 1.65 μmol of TEMED and 1.10 μmol of APS (TEMED/APS ratio 1.5) per ml of dissolution was used. Separation conditions as in Fig. 2B.

mol/l under these conditions) above which the behavior of the capillaries seems to be similar, irrespective of the acrylamide concentration used in the preparation of the coating. The use of acrylamide concentrations lower than the MEC value brings about CE coatings of poor quality, as can be deduced from the lower N values obtained at these low concentrations as shown in Fig. 4.

The results from this section seems to support the idea that a minimum chain size of polymer is necessary in order to avoid the noxious effect caused by the adsorption of the proteins on the capillary wall. Thus, too low monomer concentrations can give rise to coatings formed by a small number of too short polyacrylamide chains that, although attached to the capillary wall, can be unable to eliminate the electrostatic interaction between the protein and the remaining silanol groups of the silica wall [46]. Moreover, it is observed that once this MEC value is achieved, coated columns show similar CE behavior.

3.5. Optimum preparation conditions of linear polyacrylamide-coated capillaries and applications

The capillary preparation conditions suitable for the separation of basic proteins can be summarized as follows. After silylating the capillary tubing as indicated in Section 2.4, weigh in a airtight vial 10 mg of acrylamide and dissolve it in 1 ml of Milli-Q water at room temperature. Degas the dissolution by bubbling nitrogen for 30 min in the airtight vial and add 2.5 μl of TEMED (10%, v/v) and 2.5 μl of APS (10%, w/v) both previously degassed by bubbling nitrogen for 10 min. Fill the silylated capillary with the polymerizing mixture, seal both ends, and allow the reaction to complete overnight.

Using the above conditions for preparing polyacrylamide-coated capillaries and after optimizing the CE conditions of separation (mainly diminishing the injected protein volume) separations of basic proteins like the one shown in Fig. 5 can be routinely obtained. Under these conditions efficiencies up to 800 000 plates/m are obtained at pH 5. At this running pH, the existence of adsorption phenomena between these basic proteins and the bare fused-silica capillary has been shown to prevent the separation of these biopolymers [27,28], what demonstrates the usefulness of the present approach.

The stability of the coating has been checked by using this linear polyacrylamide coating in capillary isoelectric focusing of recombinant erythropoietin [51], where ampholytes in the range pH 3–10 with focalization time of 6 min, 90 m*M* phosphoric acid as anolyte, and 20 m*M* NaOH as catholyte were used. Under these harsh test conditions for the coating more than 30 separations were carried out without noticing any increase in band broadening.

The applicability of these coated columns was further tested by employing them in the separation by free zone capillary electrophoresis (FZCE) of a standard mixture of the major whey proteins. As can be seen in Fig. 6A, it is possible to separate the four acidic proteins, BSA, β-lactoglobulin A, β-lactoglobulin B and α-lactalbumin in less than 6 min of analysis time. Band broadening of the BSA could be mainly related to the fact that the sample used had several aggregates with broad range of molecular masses [52], although an interaction of this hydrophobic protein with the coating cannot be ruled out [53]. Despite the fact that separations of whey proteins have been already described in the literature using FZCE in uncoated capillaries [54] and capillary gel electrophoresis (CGE) [55,56], the procedure employed in the present work required much shorter analysis times.

The applicability of these polyacrylamide-coated capillaries was also tested by using them for the separation of fragments of DNA by CGE. As already known, the size separation of these molecules is usually achieved using polymeric networks dissolved in the separation buffer and coated capillaries. The use of coated capillaries is required to diminish the influence of EOF allowing the adequate sieving of the fragments of DNA in the separation buffer [15–20]. As can be seen in Fig. 6B, the use of these coated capillaries allows the separation of eight DNA fragments ranging from 126 to 23 130 base pairs (bp). A tailing peak for the 23 130 bp fragment has also been reported by other authors using the same type of DNA digest [57] which could be related to the interaction of this fragment with the hydrophilic polymer added to the separation buffer.

4. Conclusions

In the preparation of linear polyacrylamide-coated

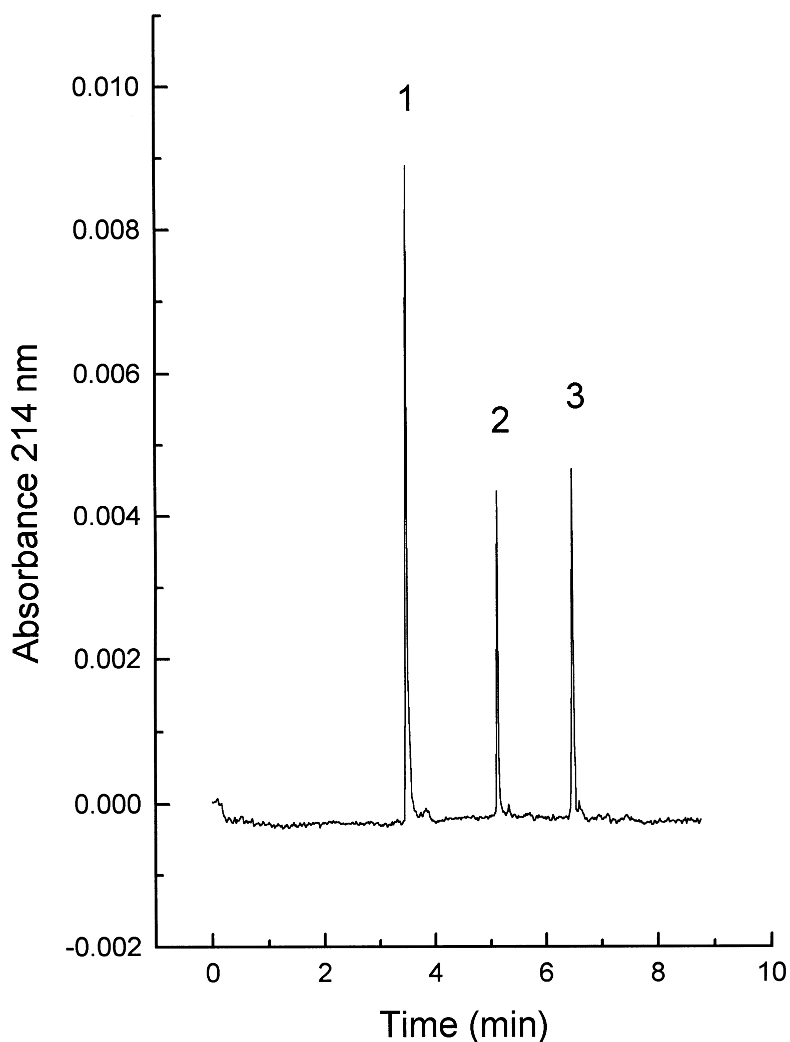

Fig. 5. Electropherogram showing the separation of three basic proteins using a polyacrylamide-coated capillary prepared under optimized conditions. Separation conditions: capillaries of 27 cm (20 cm effective length)×50 μm I.D. Hydrodynamic injection (1 s, 0.5 p.s.i.) of (1) lysozyme, (2) ribonuclease A and (3) α-chymotrypsinogen. Separation buffer: 50 m*M* acetic acid at pH 5. Run voltage 12 kV. Detection at 214 nm.

columns for CE it is demonstrated that for a given concentration of acrylamide and a determined ratio of initiator to activator, there is an optimum range of concentrations of TEMED and APS per mol of monomer at which polyacrylamide coatings suitable for CE are obtained. Furthermore, for coatings obtained using a fixed concentration of acrylamide, it is demonstrated that there is an optimum TEMED/APS ratio at which these coatings give rise to the best CE performances. For a given concentration and ratio of TEMED and APS, it is shown that there is a minimum effective concentration of monomer above which all the coatings obtained behave similarly and below which polyacrylamide coatings of very poor CE performance are obtained. The highest concentration of acrylamide that can be used in the preparation of these coatings is limited by the viscosity of the polymer obtained, because highly viscous polymers cannot be removed from the capillaries.

The optimum conditions for preparing linear polyacrylamide-coated capillaries described in this work allow the separation of basic proteins with high

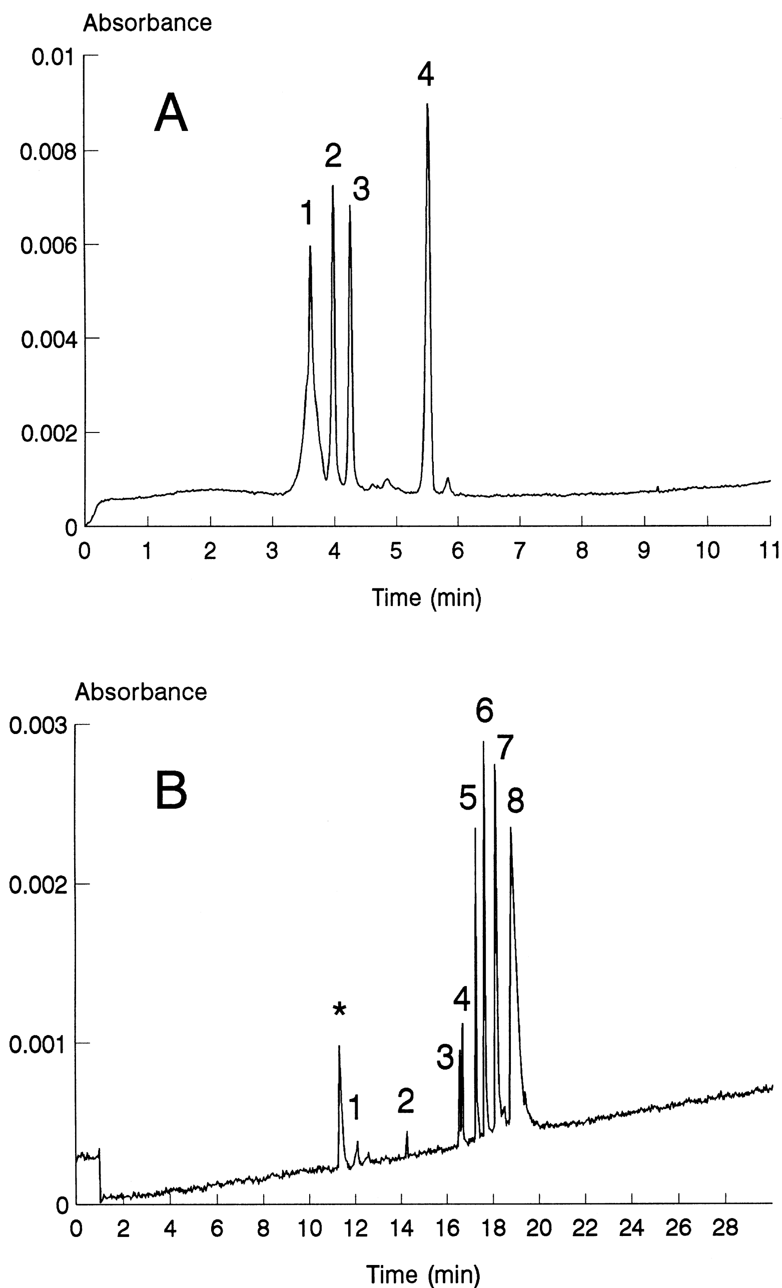

Fig. 6. (A) Electropherogram showing the separation of a standard mixture of the major whey proteins at pH 8.2 using a polyacrylamide-coated capillary. Separation conditions: capillary of 27 cm (20 cm effective length)×50 μm I.D. Hydrodynamic injection (1 s, 0.5 p.s.i.) of (1) BSA, (2) β-lactoglobulin A, (3) β-lactoglobulin B and (4) α-lactalbumin. Separation buffer: 100 m*M* borate buffer at pH 9.3. Run voltage −12 kV. Detection at 214 nm. (B) Electropherogram showing the separation of a test mixture of ds-DNA fragments using a polyacrylamide coated capillary. Separation conditions: capillary of 27 cm (20 cm effective length)×50 μm I.D. Hydrodynamic injection of (*) orange G, (1) 126 bp, (2) 564 bp, (3) 2027 bp, (4) 2322 bp, (5) 4361 bp, (6) 6557 bp, (7) 9416 bp and (8) 23 130 bp. Separation buffer: dsDNA 20 000 Gel Buffer (Beckman). Run voltage −9 kV. Detection at 254 nm.

efficiencies, up to 800 000 plates/m. Moreover, these coated capillaries have been shown to be useful for the separation of other biopolymers such as acidic proteins by FZCE, and DNA fragments in other modes of CE such as CGE.

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