ELSEVIER

Journal of Chromatography A, 823 (1998) 561–571

JOURNAL OF CHROMATOGRAPHY A

Treatments of fused-silica capillaries and their influence on the electrophoretic characteristics of these columns before and after coating

Alejandro Cifuentes, Patricia Canalejas, Alicia Ortega, José Carlos Díez-Masa*

Institute of Organic Chemistry (CSIC), Juan de la Cierva 3, E-28006 Madrid, Spain

Abstract

Making coated columns in capillary electrophoresis (CE) is a laborious task involving many preparation steps such as etching, leaching, dehydration, silylation and coating of the inner wall of fused-silica capillaries. In this work we demonstrate, by testing more than 250 columns, that it is possible to follow up the influence of the different steps on both the electrophoretic behavior of CE columns and the reproducibility of their preparation. This study was done by carrying out triplicate measurements of electroosmotic flow values from columns at four different pH values after each step. The effectiveness of the coatings was also investigated by injecting a test-group of basic proteins. It is demonstrated that etching the columns with sodium hydroxide followed by a leaching treatment with hydrochloric acid provides higher reproducibility than by either leaching or etching alone. Dehydration of the capillary tubing affects the yield of the subsequent silylation reaction while the best results seem to be obtained by dehydrating the columns overnight at 160°C. The silylation degree achieved is demonstrated to also depend on the time of reaction and the concentration of silyl reagent. Moreover, a conclusive demonstration about the effect of both silylation and polymerization reactions on the final coating performance is given.

Keywords: Capillary columns; Electroosmotic flow; Capillary electrophoresis; Coated capillaries; Proteins; Protein adsorption

1. Introduction

Fused-silica capillaries covalently coated with different polymeric substances are increasingly employed in capillary electrophoresis (CE). These coated columns provide, apart from the well known features of fused-silica material (good thermal conductivity and transparency to UV–visible light), a reduction in the noxious effects originated by the negative charges on the inner capillary wall and a substantial decrease in the electroosmotic mobility for most of the polymeric coatings used. Thus, coated capillaries diminish adsorption of analytes onto the wall and overcome the limited applicability of bare columns to isoelectrofocusing and CE separations based on size.

However, the preparation of this type of polymeric layer is, in general, a laborious task. It requires many treatment-steps of the inner wall, e.g., leaching, silylation, etc., prior to covalently bonding a chemically and mechanically stable polymeric layer to the capillary surface [1–9]. This multistep process frequently brings about lack of reproducibility of the coating preparation as has been observed by several

*Corresponding author.

0021-9673/98/$ – see front matter
PII: S0021-9673(98)00295-7

authors [10–12]. Moreover, the effectiveness of the different treatments has mainly been studied by just carrying out a single test of these columns after the final coating step.

Although many procedures to coat capillaries for CE have been developed, e.g., see [1–9,13], little attention has been paid to two factors that should be of importance in the coating preparation: (a) the reproducibility of the different treatments prior to deposition of the polymer, and (b) the effect of such treatments on the final quality of the coating. Following this idea, Towns et al. [14] carried out a systematic study at which the improvement of efficiency and recovery of a test protein along the progress of the coating procedure was shown. Some works have also dealt with the influence in CE of the nature of the fused-silica surface on the electrophoretic behavior of capillary tubing after different treatments. Thus, Hjertén and Kubo [12] demonstrated that the quality of the coating is strongly affected by the surface structure of the commercial fused-silica tubing even when capillaries of the same manufacturer but from different batches were employed. This different behavior among capillaries from different batches has been further corroborated by electroosmotic flow (EOF) measurements [15] and by scanning electron microscopy [16]. Furthermore, other authors have mentioned that even the storage time of the capillary can modify the silica properties [17]. However, Coufal et al. [18] have demonstrated that the magnitude and repeatability of the EOF (related to the silica surface charge) depends mainly on the type of buffer employed whereas the manufacturer of the tubing is less important.

Controversies on silica surfaces are old [19,20] and although a great deal of work has been put into their characterization, see e.g. references [19–23], there is a lack of systematic studies in CE about the effect of the different treatments of silica tubing on both the electrophoretic behavior of capillaries before being coated and the effectiveness of the final coating. The aim of this work was to carry out a more systematic study on the effect of such treatments during the preparation of coated columns. As a second goal, we tried to establish a procedure to modify in a regular and reproducible manner the capillary surface prior to be coated.

2. Experimental

2.1. Chemical and reagents

Lysozyme chicken egg white, ribonuclease A bovine pancreas, and α-chymotrypsinogen bovine pancreas were purchased from Sigma (St. Louis, MO, USA) and used as received. The proteins were solved at concentrations of 1 mg/ml in Milli-Q water (Millipore, Bedford, MA, USA), stored at −5°C, and warmed to room temperature before use. 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-morpholinoethanesulfonic acid (MES, pH 6) and 2-N-cyclohexylaminoethanesulfonic acid (CHES, pH 10) (both from Sigma) 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 at the values indicated in parenthesis. Hydrochloric acid, sodium hydroxide (both from Merck), 7-oct-1-enyltrimethoxysilane (ABCR, Karlsruhe, Germany), acrylamide, ammonium persulfate (APS), and N,N,N;N-tetramethylethylenediamine (TEMED) (all from Schwarz, Cleveland, OH, USA) were used for the preparation of the polyacrylamide coated capillaries.

2.2. Instrumentation

Analyses were carried out in a P/ACE 2100 (Beckman, Fullerton, CA, USA) capillary electrophoresis apparatus. Fused-silica capillaries used were from Polymicro Technologies (Composite Metal Services, Worcester, UK) and Siemens (Munich, Germany) and they were 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 thermostated at 25°C. Detection took place at 254 nm when acetone was injected and 214 nm when proteins were used. Data were collected and analyzed using 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.

2.3. Capillary treatment

A set of different conditions were tested in order to obtain a reproducible procedure for treating the capillaries before coating. The tentative procedure employed as starting point was as follows: capillaries were first etched and/or leached, washed, dehydrated, silylated, washed again and finally coated with linear polyacrylamide as described in Section 2.4. The specific conditions for each treatment are discussed in Section 3.

2.4. Capillary coating

Capillaries were coated with polyacrylamide as previously described by Hjertén [1]. The coatings were prepared as follows: 0.04 g of acrylamide was dissolved in 1 ml of Milli-Q water and degassed using vacuum or bubbling with helium. The monomer solutions were polymerized by addition of 2 μl of 10% APS and 3 μl of 10% TEMED.

2.5. Electroosmotic flow measurements

The electroosmotic mobility (μ_{eo} or EOF) of treated capillaries was measured at the different stages of the treatment using acetone solved in Milli-Q water (5% v/v) at five different values of pH, i.e., 4, 5, 6, 8 and 10. 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.

2.6. Data treatment

A one-way analysis of variance was carried out to test at each pH the differences between the EOF means for the different capillaries shown in Table 1. The study was done by using the Biomedical computer program (BMDP) through a test of Student–Newman–Keuls of multiple comparison of the mean values [24] for a 95% confidence interval.

Error bars are given for all the EOF vs. pH plots in this work. They were calculated as $t\sigma_{n-1}/n^{1/2}$ to represent a 95% confidence interval, where t is the t of Student, σ_{n-1} is the standard deviation for each mean value and n is the number of measurements, i.e., 3 or 6 as will be indicated below.

Table 1
Average EOF values vs. pH for capillaries of the same manufacturer-same batch (PMB1C1, PMB1C2, PMB1C3, PMB1C4, PMB1C5), different batch (PMB2C1) and different manufacturer (SIB1C1, S1B1C2) after being etched with sodium hydroxide 1 *M* for 30 min

pH	PMB1C1[a]	PMB1C2	PMB1C3	PMBIC4	PMB1C5	PMB2C1	S1B1C1[b]	SIB1C2
4	1.96[c 1,2]	2.00[1,2]	1.99[1,2]	1.93[2,3]	1.90[3]	1.98[1,2]	2.04[1]	2.02[1]
	(1.8)[d]	(1.1)	(3.3)	(0.1)	(0.8)	(0.4)	(1.01)	(0.4)
5	2.26[1]	2.32[1]	2.34[1]	2.33[1]	2.27[1]	2.46[1]	2.25[1]	2.45[1]
	(14.7)	(12.7)	(6.3)	(8.9)	(10.6)	(6.3)	(11.2)	(8.0)
6	4.14[1]	4.93[2]	5.58[3]	5.02[2]	4.79[2]	5.38[4]	4.50[5]	5.00[2]
	(5.6)	(1.9)	(2.3)	(2.4)	(2.7)	(2.2)	(1.0)	(2.9)
8	7.55[1]	7.75[2]	7.88[3]	7.72[2]	7.65[2]	7.76[2]	7.57[1]	7.52[1]
	(0.7)	(0.7)	(0.2)	(0.6)	(0.7)	(0.3)	(0.4)	(0.8)
10	5.72[1]	5.50[1]	5.64[1]	5.60[1]	5.54[1]	5.70[1]	5.54[1]	5.38[1]
	(0.1)	(2.4)	(1.1)	(0.7)	(1.3)	(1.3)	(0.6)	(0.4)

[a] PMB1C1 to C5 are capillaries from Polymicro Technologies; PMB1C1 to C3 are consecutive capillaries from a 50-m reel; PMB1C4 and PMB1C5 are capillaries taken at the middle and at the end of such a reel; PMB2C1 is a capillary from a different batch.
[b] SIB1C1 and S1B1C2 are capillaries from Siemens from the same batch.
[c] EOF values are given in 10^8 m^2/V s.
[d] Values in parenthesis correspond to R.S.D.s, $n=3$.
[1,2,3] Common superscripts within rows indicate nonsignificant differences ($P<0.05$) among EOF mean values at a given pH.

3. Results and discussion

Although some microscopic techniques have been used to obtain information about the inner surface of fused-silica capillaries, e.g. scanning electron microscopy [16] or atomic force microscopy [25,26], these techniques remain too expensive to characterize a material with a small added value. In CE the technique more often employed for obtaining information about the inner surface of capillaries consists of measuring the dependence of the EOF on the pH of the buffer, see e.g. refs. [14,15,17,18], correlating such a value with the number of negative charges onto the capillary wall. In this work we have used this approach to characterize the capillary surface after each treatment step. Moreover, the coating effectiveness has also been checked through the CE separation of a test-set of basic proteins.

3.1. Etching

As we have stated in Section 1 the behavior of fused-silica capillaries in CE seems to differ depending on the author. Differences in silica tubing have been assigned to factors such as the manufacturer [14], batch [12], and even the piece of capillary used within the same reel [15]. In order to diminish the effect of irreproducibility among tubing a usual practice in CE and GC is to clean the silica tubing prior to any treatment. This cleaning is done by performing an acidic or basic treatment of silica surfaces. Obtaining a fresh and clean inner tubing surface will logically improve the reproducibility of the results achieved after subsequent modifications of such surfaces.

In order to study this point we first etched several capillaries with 1 *M* sodium hydroxide for 30 min, including columns from the same manufacturer and batch, from the same manufacturer and different batch, and from different manufacturers. The average of the triplicate measurements of EOFs at each pH value after this treatment, together with their respective R.S.D.s ($n=3$) are shown in Table 1. Also, the results of the statistical study about significant differences ($P<0.05$) among mean EOF values of capillaries at a given pH are indicated in this table by superscripts, e.g., 1, 2, 3, etc. Common superscripts within each row mean no significant differences.

As shown in Table 1, the results obtained at pH 5 and 10 seem to indicate that the eight capillaries studied behave in a similar way after this etching-treatment independently of manufacturer and batch. However, at pH 5 the R.S.D. ($n=3$) values are too large, ranging from 6.3 to 14.7%, what can explain the statistically nonsignificant differences obtained at this pH. Due to the poor reproducibility obtained with this buffer at pH 5 its use in subsequent tests was abandoned. Results obtained at pH 4 and 8 indicate that under these testing conditions capillaries seem to arrange into three different groups. These three different groups do not correspond either to different manufacturers or batches as can be observed in Table 1, which seems to indicate that the origin of fused-silica tubing does not affect the EOF values obtained after our etching-treatment. However, at pH 6 four out of the eight capillaries seem to be significantly different from each other and also different from the group formed by the four remaining columns. Moreover, at this pH at which a 50 m*M* MES buffer was used, a general trend toward larger differences among the average EOF values was obtained (ranging from 4.14 to $5.58 \cdot 10^{-8}$ $m^2\,s/V$) for columns from the same batch and manufacturer (Table 1). When this buffer was replaced for a malic buffer at the same pH and concentration, the precision of the EOF measurements clearly improved. Using malic buffer, three columns from the same batch and manufacturer rendered average EOF values of 2.8, 2.8 and $2.9 \cdot 10^{-8}$ $m^2\,s/V$ with R.S.D. ($n=3$) values ranging from 1.7 to 2.7%, without statistically significant differences ($P<0.05$). Therefore, this malic buffer was used for all the subsequent measurements at pH 6. A similar study to that shown previously was carried out using a 2.5-h treatment of the capillaries with 1 *M* sodium hydroxide. Very similar results to those obtained by treating the capillaries for 30 min were achieved after this longer treatment. Therefore, the shorter treatment was selected.

Results obtained at pH 5 (with acetic–acetate buffer) and pH 6 (with MES and malic–malate buffers) seem to corroborate what had been observed by Coufal et al. [18], who demonstrated that the main influence on the irreproducibility of the EOF comes from the nature of the separation buffer employed.

Table 1 shows that the higher the pH, the higher the EOF value, except at pH 10 where lower EOF values than at pH 8 were obtained. This behavior can be explained as follows. EOF is proportional to the zeta potential, which is known to depend, among other factors, on the ionized silanol groups on the capillary wall and to be inversely proportional to the square root of the ionic strength of the running buffer. In our case at pH 8 and 10 the number of ionized silanols must be nearly the same. However, the ionic strength is quite different for both buffers, because to reach pH 10 (CHES buffer) a quantity of sodium hydroxide nearly eight times higher than that for reaching the value of pH 8 (boric–borate buffer) is needed. Therefore, at pH 10 the ionic strength is much higher than at pH 8 which can explain the lower EOF observed at the higher pH.

The treatment proposed seems to provide a relatively reproducible silica surface with no appreciable influence of the manufacturer or batch from which tubing were obtained. Moreover, the type of buffers chosen in this study do not seem to affect the reproducibility of the EOF measurements [18]. This allowed us to establish a further study in order to improve the aforementioned irreproducible behavior of capillary silica surface. This approach is shown next by describing the effect of leaching treatments of the fused-silica capillary wall.

3.2. Leaching

In order to improve the reproducibility of the EOF values obtained using an etching treatment with sodium hydroxide we tested the use of an acidic treatment. Acidic treatments have also been routinely employed among researchers working on silica tubing for chemically homogenizing and preparing columns prior to any subsequent manipulation. To analyze the effect of the acidic treatment we tested different conditions for leaching capillaries, i.e., different concentrations of hydrochloric acid, different temperatures and different times of exposure. In all the cases, after leaching with an acidic solution, a rinsing step with water for 1 h was carried out before measuring the EOF. First, we tested whether by using the acidic cleaning procedure, i.e. leaching, instead of etching with sodium hydroxide, we could improve the reproducibility of the EOF values obtained in Table 1. For this experiment capillaries from the same batch and manufacturer were treated with solutions of 0.1 *M* or 1 *M* hydrochloric acid for 1 or 2 h at room temperature or 70°C. The best results in terms of EOF reproducibility were obtained using a solution 0.1 *M* hydrochloric acid at 70°C for 2 h. As an example, Fig. 1A shows the results of EOF vs. pH obtained for four capillaries after this treatment. Each line corresponds to a single capillary and each point represent the average EOF value of its triplicate measurement at that pH. Error bars were calculated as in Section 2.6 and they represent a 95% confidence interval. Reproducibility among columns is poorer than that obtained by using sodium hydroxide, since at practically every pH tested larger differences in EOF values are obtained in Fig. 1A compared with those given in Table 1 for capillaries from the same batch. Also, Fig. 1A indicates that the

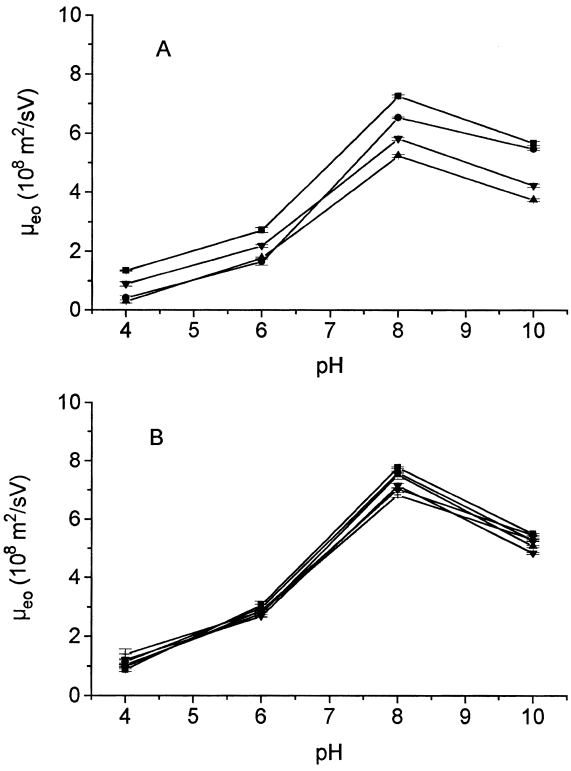

Fig. 1. Plots of electroosmotic mobility vs. pH for (A) four capillaries from the same batch leached with 0.1 *M* hydrochloric acid at 70°C for 2 h and (B) six capillaries from the same batch as above treated with 1 *M* sodium hydroxide for 30 min and leached with 0.1 *M* hydrochloric acid at 70°C for 2 h.

four capillaries behave in a significantly different ($P<0.05$) way because error bars from different columns practically do not overlap at any of the pH values.

Consecutive use of etching and leaching was also tested. As in the experiment of Fig. 1A, several pieces of tubing from the same batch and manufacturer were used. The results for six capillaries are given in Fig. 1B. As in the previous experiment, each line corresponds to a single capillary and each point represents the average EOF value of the triplicate measurements at that pH. Similar EOF values together with numerous overlaps among the error bars of the different capillaries at the four pH values tested were obtained (i.e., only a few statistically significant differences ($P<0.05$) within the six capillaries shown in Fig. 1B were observed at pH 8). Therefore, etching plus leaching gave much more reproducible column-to-column results compared with those shown in Fig. 1A. This improvement can probably be explained by the cleaning and removing of impurities through etching with sodium hydroxide which brings about a more reproducible silica surface as demonstrated above together with the removal of trace metals through leaching by an acidic attack. It has been demonstrated that trace metals can modify the chromatographic properties of silica and the reproducibility of the different treatments of such a material [19,22,27,28]. Although the level of metals in fused-silica capillaries is expected to be lower than that of the silica for chromatographic supports, such impurities can still play an important role in the chemical homogeneity of the inner capillary surface [29,30] which can affect both the EOF values and the coating properties [30]. The acidic washing of silica seems to remove from one third to two thirds of metals from silica [22,31], although the careful choosing of the acidic washing conditions can lead to a reduction of the impurities of the surface higher than 80% [32].

Therefore, from the results presented above it seems that an etching attack with sodium hydroxide followed by a leaching treatment with HCl (0.1 *M* at 70°C for 2 h) leads to good results.

3.3. Dehydration

The importance of removing physically adsorbed water from the silica surface prior to the silanization step has been discussed by many authors [21,30,33,34]. The impact of the adsorbed water content on the silanization reaction has to be taken into account, since under noncontrolled conditions it can bring about polymerization of the silane molecules [14,21,34] resulting in a thick, irreproducible, and irregular surface layer [21]. Moreover, it has been shown that thermal treatment can reduce the number of active silanols on the silica surface by giving rise to siloxane bridges [30,33,35].

Since in CE the stability and the effectiveness of the coating increases with increasing number of its bonds to the capillary surface [15], it is helpful to have a silanol concentration high enough when chemical surface modification has to be achieved. However, it is also of importance in the development of functionalized silica for both chromatographic and electrophoretic techniques to take into account the number of unreacted silanols on the silica surface due to their detrimental effect on the separation mechanism. In CE the unreacted silanols can bring about, among other effects, undesired adsorption of positively charged compounds what will dramatically reduce the efficiency of their separation.

To assess the effect of dehydration on the silanization yield of the capillary surface we carried out a study as follows. In each experiment, four capillaries were etched, leached and rinsed. Next, each group of four capillaries was heated overnight at a different temperature ranging from room temperature to 250°C (see Table 2) under a gentle stream of nitrogen. After dehydration, each group treated at the same temperature were divided in two subgroups: two capillaries were silylated using a solution containing 7-oct-1-

Table 2
ϕ[a] values vs. pH from capillaries of the same manufacturer and batch dehydrated at different temperatures

Temperature (°C)	pH			
	4	6	8	10
25	0.47	0.03	0.06	0.17
80	0.32	−0.01	0.07	0.13
120	0.22	0.05	0.08	0.05
160	0.51	0.13	0.17	0.13
200	0.39	0.06	0.12	0.16
250	0.19	0.04	−0.02	−0.15

[a] $\phi=(\mu_{oc}-\mu_{osi})/(\mu_{oc}+\mu_{osi})/2$.

enyltrimethoxysilane, methanol and acetic acid and the other two capillaries were used as controls, that is, they were treated with a blank solution containing only methanol and acetic acid. The four capillaries were then rinsed with methanol for 1 h and installed in the CE equipment to measure their respective EOFs. To estimate the silylation degree of these capillaries we defined the parameter $\phi=(\mu_{oc}-\mu_{osi})/(\mu_{oc}+\mu_{osi})/2$, where μ_{oc} and μ_{osi} are the average EOFs of the control and silylated capillaries, respectively. According to the definition of ϕ, the higher ϕ, the larger the difference between the EOF values of both columns and, consequently, the larger the number of reacted silanols. The results obtained for ϕ at the different dehydration temperatures are given in Table 2. The R.S.D. values of the different EOFs measured ranged from 0.2 to 3.1% at the four pH values employed, in good agreement with that mentioned above for the same buffers in Table 1. Under these conditions the highest number of reacted silanols with 7-oct-1-enyltrimethoxysilane was obtained at 160°C. As a general trend, at dehydration temperatures higher than 120°C lower EOF values were obtained, which seems to corroborate the theory about the decreasing number of free silanols on the silica surface promoted by thermal treatment [22,30,33,35].

From our results, it could be speculated that at dehydration temperatures lower than 160°C the water physically adsorbed on silica is not sufficiently removed. At temperatures higher than 160°C the number of free silanols seems to become smaller probably due to the formation of siloxane bridges, as has been observed by other authors working on amorphous silica [33,36], which brings about a decrease in the reactivity of such a surface with the silyl derivative.

3.4. Silylation

In CE several authors have studied the effect of different silyl derivatives [11,15,17,37,38] as well as the solvent used to dissolve the silyl reagent [12,17] on the properties of the coating. However, it seems that there is a lack of systematic studies on the effect of different parameters, e.g., temperature of silylation, concentration of silane, etc, affecting the silylation reaction during the preparation of polymeric coatings for CE. This lack of systematic studies may account for the low reproducibility of the results when comparing the same CE coatings e.g., compare results from refs. [11,17,37]. In order to optimize the silylation yield a systematic study was carried out, investigating the effect of the temperature of reaction, catalyst used, time of reaction, and silyl derivative concentration.

To study the effect of temperature of silylation on the amount of silyl reagent 7-oct-1-enyltrimethoxysilane bonded to the capillary wall, several groups of four capillaries were etched, leached, rinsed and dehydrated under optimal conditions for each step. After dehydration, two capillaries were taken from each group as test-set, and their EOFs measured. The other two columns were filled with the silylating solution (200 μl silyl reagent, 4 ml methanol and 20 μl acetic acid) and after sealing both ends, they were heated for 3 h at the temperature studied. EOF values of these silylated columns were measured after rinsing with methanol for 1 h. Results obtained showed that within the range of temperature tested, i.e., 25, 40, 60 and 80°C, there were no significant differences ($P<0.05$) among the silylated columns. Significant differences were not observed either between capillaries silylated at 25°C and those treated at 60°C even when undiluted silyl reagent and longer reaction times (24 h) were used. However, using undiluted silane higher silanization yields were obtained as will be discussed below.

Although the importance of the catalyst used during the silanization reaction has been studied [15,22,39,40], according to Kinkel and Unger [39] it is very difficult to predict the optimum solvent–catalyst combination in this type of reaction. In the present work we tested the use of acetic acid and triethylamine as catalysts of the silanization reaction under identical conditions, i.e., 200 μl of silyl reagent, 4 ml of methanol plus 20 μl of catalyst. The results did not show significant differences between capillaries for which an acidic or basic catalyst had been employed, what seems to be in good agreement with the observation of Kinkel and Unger [39]. Furthermore, we carried out the silylation reaction without catalyst and with acetic acid as a catalyst. Under these conditions, better silylation yields were achieved by using a catalyst as in reference [15].

The effect of both the reaction time and the

concentration of silyl reagent onto the degree of reaction were also analyzed in this work. The results obtained are given in Fig. 2A and B, where each line represents the average of two capillaries treated under identical conditions and the error bars indicate again a 95% confidence interval. At pH values of 6, 8 and 10 there are significant differences ($P<0.05$) between a reaction time of 2 h and an overnight reaction using a solution consisting on 200 μl of silyl reagent, 4 ml of methanol and 20 μl of acetic acid. However, at pH 4 there are not significant ($P<0.05$) differences among the capillaries as can be deduced from the overlap of their error bars. This result can be explained through the lower ionization of silanol groups having place at pH 4 compared to that occurring at pH 6, 8 and 10. Thus, at pH 4 the differences between tubing in terms of negatively charged groups on the capillary wall are expected to be smaller and consequently the differences between EOF values too. Besides, these differences for silylated capillaries must be even smaller, since for this tubing a noticeable number of silanol groups of the surface have already reacted with the silyl reagent reducing in this way the number of ionizable silanol groups.

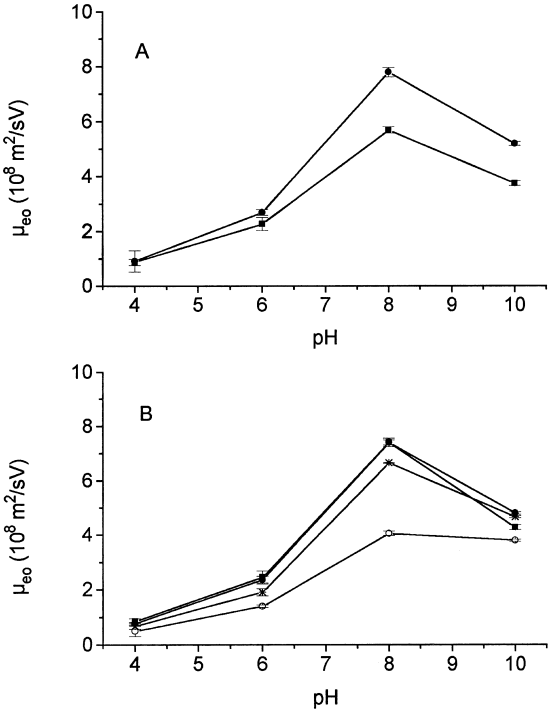

Fig. 2. Plots of electroosmotic mobility vs. pH for (A) capillaries treated with 1 *M* sodium hydroxide for 30 min and leached with 0.1 *M* hydrochloric acid at 70°C for 2 h, washed with water for 1 h, dehydrated at 160°C overnight and silylated with 200 μl of 7-oct-1-enyltrimethoxysilane in 4 ml methanol and 20 μl of acetic acid for 2 h (●) and overnight (■); and (B) capillaries silylated overnight with different concentration of silane: 1 μl (■), 10 μl (●), 200 μl (*) all of the three amounts solved in 4 ml of methanol and 20 μl of acetic acid, and undiluted silane plus 0.5% acetic acid (○); the rest of conditions as in (A).

The effect of the silyl derivative concentration is shown in Fig. 2B. At pH 6 and higher, significant differences ($P<0.05$) among the EOF values are observed among the capillaries treated with undiluted silyl reagent compared with those treated with silyl reagent diluted in methanol at different concentrations. Better yields of the silanization reaction obtained with undiluted reagents has also been described for other different organosylanes [34]. This effect can be explained through both the higher silyl concentration and the adsorption of solvent on the silica surface [34]. Thus, the stronger the adsorption of the solvent on the surface, the denser is the shielding towards attacking by the silyl reagent. Also, in Fig. 2B, no significant ($P<0.05$) differences were observed at pH 4, corroborating the results in Fig. 2A at this pH. Moreover, in Fig. 2B it is observed that the difference in a given capillary between the EOF values at pH 8 and pH 10 is smaller when the silylation yield increases. This is due to the reduction of free silanols having taken place during silylation reaction. In this way, the number of ionizable silanol groups of the wall is diminished and, therefore, lower zeta potential are obtained bringing about a lower difference due to the effect of ionic strength on the EOF values.

Therefore, from the results presented above it can be concluded that for 7-oct-1-enyltrimethoxysilane used in this work the best silylation conditions consisted of using undiluted silyl reagent, acetic acid as catalyst and performing the reaction overnight at room temperature.

3.5. Coating

The coating properties and their dependence on the previous treatments of the silica support have been much studied in chromatographic techniques

[19,20,22] but has received scarce attention in the preparation of coatings in CE [11,14,17,37,38]. In many cases these works compared different types of silyl derivatives [11,17,38] and their effect on the final coating properties, e.g., EOF, efficiency, stability, etc.

To prove the correlation between the effectiveness of the silanization step (which has been demonstrated above to depend on the other previous treatments) and the properties of the covalently bound polymeric coating in CE, we carried out the following set of experiments.

For each experiment, sets of three capillaries were silylated and, after washing with methanol for 1 h, two of the silylated capillaries were installed in the CE instrument and their EOFs measured, therefore, each line at the EOF vs. pH plots (Figs. 3 and 4) represents the average of two columns. The third capillary was coated with linear polyacrylamide following the Hjertén's procedure [1]. At this stage, it was necessary to develop a reproducible procedure for removing oxygen from acrylamide solution, since it is known that oxygen can modify the polymerization degree achieved in this reaction. This modification is expected to affect the CE behavior of coated capillaries. To demonstrate this point, two series of three capillaries were silylated with very similar reaction yields, as it was deduced from the similar EOF values at the different pH values tested (see insert at Fig. 3). Two capillaries, one from each set, were coated with two individual acrylamide solutions treated with a different degassing procedure. Fig. 3A and B demonstrate that capillaries gave very different results in the separation of basic proteins depending on the degree of polymerization of the acrylamide. Such a polymerization degree could be evaluated through its direct relation with the viscosity [41] estimated from the remaining polymerized acrylamide solution. Fig. 3A was obtained using a coated capillary corresponding to a polyacrylamide solution of viscosity much lower than that obtained for the coating employed in Fig. 3B. This behavior was repeatedly observed in our experiments until an optimized procedure for degassing the acrylamide solution was developed.

Once the degassing procedure was optimized (flowing helium through the acrylamide solution at a given pressure and for a given time of exposure) and

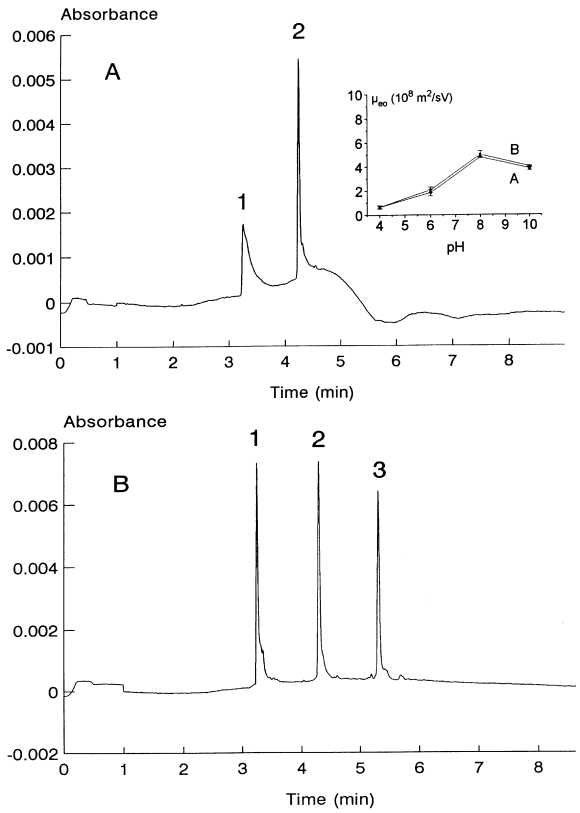

Fig. 3. Electropherograms showing the separation of three basic proteins using two capillaries with similar silylation degree and coated using a solution of acrylamide of (A) low and (B) high viscosity after polymerization. Separation conditions: coated capillaries 27 cm (20 cm effective length)× 50 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* formic acid at pH 4. Detection at 214 nm. The insert shows the average plot of EOF vs. pH of two couples of silylated capillaries treated identically to those used in (A) and (B), viz., 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 and silylated with undiluted silane overnight.

proved to work in a reproducible way in terms of viscosity of the polyacrylamide solution, the effect of different silylation conditions on the coating properties was studied. An example of the results obtained is shown in Fig. 4. The insert of Fig. 4 gives the EOF values obtained for two pairs of capillaries silylated at different conditions, showing that the yield of silanization obtained with both procedures is significantly different ($P<0.05$). Fig. 4A and B show separations of a group of basic proteins after coating

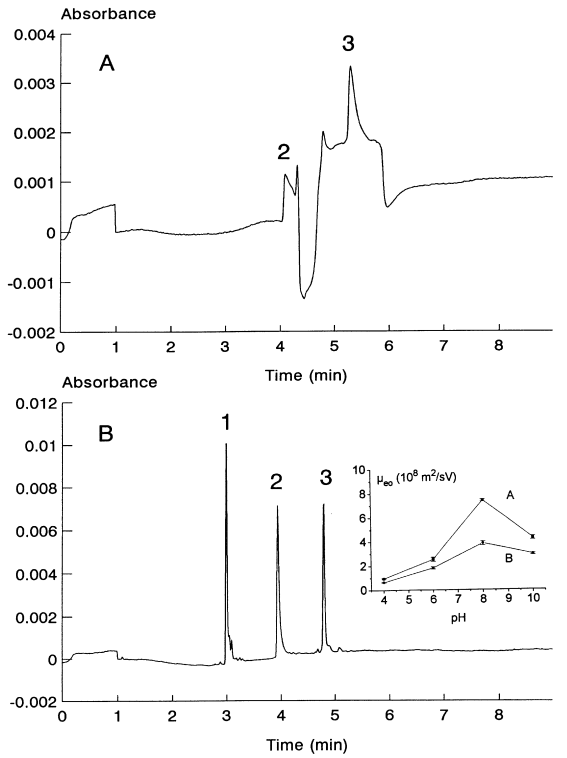

Fig. 4. Electropherograms showing the separation of three basic proteins using two capillaries silylated using different conditions (A) 10 μl of 7-oct-1-enyltrimethoxysilane in 4 ml methanol and 20 μl of acetic acid and (B) undiluted 7-oct-1-enyltrimethoxysilane plus 0.25% acetic acid. Identical solution of acrylamide was employed for both columns. All separation conditions as in Fig. 4. The insert shows the average plot of EOF vs. pH of two couples of capillaries treated identically to those used in (A) and (B), viz., 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 and silylated as indicated.

the silylated columns. The lower the quantity of silane anchored to the silica surface (estimated through the EOF measuring), the worse the coating obtained in terms of efficiency in the separation of proteins. A similar result was obtained in four identical experiments (data not shown) done to study the reproducibility of this effect.

When the EOF values from these two coated capillaries were measured very similar results were obtained, namely, after 60 min running at 12 kV and using a pH 6 buffer no EOF peak was observed (data not shown). However, Fig. 4 shows that the separation of basic proteins in terms of efficiency were very different from column to column. Therefore, it is suitable to compare the properties of different polyacrylamide coatings in terms of separation efficiency instead of comparing them in terms of the EOF of a neutral marker, since the latter can lead to misleading conclusions due to the extremely low EOF value provided by the polyacrylamide coated capillaries.

Acknowledgements

Thanks are given to Dr. P. Martin-Alvarez (Instituto de Fermentaciones Industriales, CSIC, Spain) and Dr. M. de Frutos (Instituto de Quimica Organica General, CSIC) for fruitful discussions. This work was supported by a CYCIT project (MAT96-0564).

References

[1] S. Hjertén, J. Chromatogr. 347 (1985) 191.
[2] G.J.M. Bruin, J.P. Chang, R.H. Kulman, K. Zegers, J.C. Kraak, H. Poppe, J. Chromatogr. 471 (1989) 429.
[3] J.K. Towns, F.E. Regnier, J. Chromatogr. 516 (1990) 69.
[4] A. Cifuentes, J.M. Santos, M. de Frutos, J.C. Diez-Masa, J. Chromatogr. A 652 (1993) 161.
[5] J.T. Smith, Z. El Rassi, Electrophoresis 14 (1993) 396.
[6] M. Gilges, M.H. Kleemiβ, G. Schomburg, Anal. Chem. 66 (1994) 2038.
[7] M. Huang, W.P. Vorkink, M.L. Lee, J. Microcol. Sep. 4 (1992) 233.
[8] W. Nasabeh, Z. El Rassi, J. Chromatogr. 559 (1991) 367.
[9] Y.F. Maa, K.J. Hyver, S.A. Swedberg, J. High Resolut. Chromatogr. 14 (1991) 65.
[10] G. Schomburg, Trends Anal. Chem. 10 (1991) 163.
[11] M. Gilges, H. Husmann, M.H. Kleemiβ, S.R. Motsch, G. Schomburg, J. High Resolut. Chromatogr. 15 (1992) 452.
[12] S. Hjerten, K. Kubo, Electrophoresis 14 (1993) 390.
[13] M. Chiari, M. Nesi, P.G. Righetti in: P.G. Righetti (Editor), Capillary Electrophoresis in Analytical Biotechnology, CRC Press, Boca Raton, FL, 1996, p. 1.
[14] J.K. Towns, J. Bao, F.E. Regnier, J. Chromatogr. 599 (1992) 227.
[15] J. Kohr, H. Engelhardt, J. Chromatogr. A 652 (1993) 309.
[16] S. Kaupp, R. Steffen, H. Watzig, J. Chromatogr. A 744 (1996) 93.
[17] H. Engelhardt, M.A. Cuñat-Walter, J. Chromatogr. A 716 (1995) 27.
[18] P. Coufal, K. Stulik, H.A. Claessens, C.A. Cramers, J. High Resolut. Chromatogr. 17 (1994) 325.
[19] J. Nawrocki, Chromatographia 31 (1991) 177.
[20] J. Nawrocki, Chromatographia 31 (1991) 193.

[21] P. Van der Voort, F. Vansant, J. Liq. Chromatogr. 19 (1996) 2723.
[22] J. Nawrocki, B. Buszewski, J. Chromatogr. 449 (1988) 1.
[23] R.K. Iler, The Chemistry of Silica, Wiley, New York, 1982.
[24] J.W. Dixon, Biomedical Computer Program, Universitiy of California Press, Los Angeles, CA, 1983.
[25] A. Cifuentes, J.C. Díez-Masa, J. Fritz, D. Anselmetti, A.E. Bruno, Anal. Chem., in press.
[26] R. Barberi, M. Giocondo, R. Bartolino, P.G. Righetti, Electrophoresis 16 (1995) 1445.
[27] J. Nawrocki, J. Chromatogr. 362 (1986) 117.
[28] C. Giddings, Anal. Chem. 35 (1963) 1999.
[29] Q. Wu, H.A. Claessens, C.A. Cramers, Chromatographia 33 (1992) 303.
[30] K. Grob, Making and Manipulatig Columns for Gas Chromatography, Hüthig, Heidelberg, 1986.
[31] M. Verzele, C. Dewaele, J. Chromatogr. 217 (1981) 399.
[32] J. Sonnefeld, Colloid Polym. Sci. 274 (1996) 1137.
[33] A.B. Scholten, J.W. Haan, H.G. Janssen, L.J.M. van de Ven, C.A. Cramers, J. High Resolut. Chrom. 20 (1997) 17.
[34] K.D. Behringer, J. Blumel, J. Liq. Chromatogr. 19 (1996) 2753.
[35] R.P.W. Scott, P. Kucera, J. Chromatogr. Sci. 13 (1975) 337.
[36] L.T. Zhuravlev, Langmuir 3 (1987) 316.
[37] M. Nakatami, A. Skibukawa, T. Nakagawa, J. Chromatogr. A 661 (1994) 315.
[38] C.L. Ng, H.K. Lee, S.F.Y. Li, J. Chromatogr. A 659 (1994) 427.
[39] J.N. Kinkel, K.K. Unger, J. Chromatogr. 316 (1984) 193.
[40] L. Boksanyi, O. Liardon, E. sz. Kovats, Adv. Colloid Interface Sci. 6 (1976) 95.
[41] A.V. Tobolsky, Properties and Structure of Polymers, Wiley, New York, 1960.