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# Poly(vinylamine)-coated capillaries with reversed electroosmotic flow for the separation of organic anions

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**Abstract**

A simple method for the preparation of poly(vinylamine) (PVA) coating on the inner surface of a fused-silica capillary for CE is reported. A high quality PVA was synthesized from acidic hydrolysis of N-vinyl-*tert*.-butylcarbamate. Based on the reactivity of PVA primary amino groups a coating procedure consisting of two steps: (1) dynamic adsorption of PVA from an aqueous solution, (2) cross-linking of the polymer amino functions with N,N′-methylenebisacrylamide and end-capping of the primary amino groups with an acrylamido derivative bearing quaternary amino functions was devised. A permanent coating that generates a strong anodic electroosmotic flow was produced which is fully compatible with the separation of polyanionic acids. A complete evaluation of reproducibility, efficiency and long-term stability of the coating was carried out using a mixture of eight organic mono-, di- and tricarboxylic acids. The usefulness of PVA-coated capillaries in the analysis of real samples containing UV-transparent organic acids was demonstrated. 

*Keywords:* Coated capillaries; Organic acids

## 1. Introduction

Capillary electrophoresis (CE) has gained acceptance over the past few years for the analysis of small ions. The success of the technique derives from its ability to provide rapid and efficient separations covering a wide application range. CE ion analysis is advantageous over ion chromatography as it requires simple instrumentation and inexpensive fused-silica capillary columns. The technique offers the possibility of analyzing anions or cations simply by switching buffers and allows simultaneous analysis of inorganic anions, organic acids and transition metals in a single run. The presence of ionizable groups on the surface of a fused-silica capillary generates upon application of an electric field an electroosmotic flow (EOF) whose control is of fundamental importance to achieve optimal separation in ion analysis. The direction and the magnitude of EOF has been controlled through: (i) manipulation of pH [1,2], ionic strength [3], buffer composition [1,4], (ii) dynamic or permanent modification of the capillary wall [5–10], (iii) application of a radial potential to the capillary [11–13].

The analysis of fast moving ions by CE in an uncoated capillary represents a serious problem as the analytes migrate against EOF according to their electrophoretic mobility with a velocity of the same magnitude of the flow. This implies that these analytes would never emerge from the column. The

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problem cannot be solved by changing the pH as EOF and anions mobilities vary with pH in the same manner, as the pH is decreased both EOF and analyte mobility are simultaneously reduced. The use of capillaries with a reversed EOF represents a convenient solution to this problem. In capillaries with a reverse EOF, both electroosmotic and electrophoretic forces drive the analytes towards the anode shortening transit times and improving the reproducibility of transit times. Reversal of EOF in the capillary can be achieved by addition of cation surfactants to the electrophoretic buffer [14–16], by dynamic adsorption of cation polymers from the running buffer [17–20] or by permanent modification of the wall with poly(ethyleneimine) (PEI) [21]. This method first proposed by Regnier to prevent adsorption of cationic macromolecules to the wall, allows reversal of EOF in a permanent way without any need for additives in the background electrolyte (BGE). The use of a PEI coating for the separation of divalent acids is hindered by the occurrence of strong electrostatic interactions between the acids and the amine groups of PEI causing a dramatic decrease in the separation efficiency for this type of analytes [17].

In this article we report on the use of a different polyamine, poly(vinylamine) (PVA), to produce a permanent coating on the wall of a CE capillary that generates a strong anodic EOF and is fully compatible with the separation of polyanionic acids. PVA presents several advantages over the well-known PEI in the production of coatings as it is strongly adsorbed on silica like other polyamines and it has reactive amino groups that allow relative easy derivatization. Based on the reactivity of PVA primary amino groups a coating procedure was devised consisting of two steps: (1) dynamic adsorption of PVA from an aqueous solution, (2) cross-linking of the polymer amino functions with N,N′-methylenebisacrylamide and end-capping of the primary amino groups with an acrylamido derivative bearing quaternary amino functions. A complete evaluation of reproducibility, efficiency and long-term stability of the coating was carried out using a mixture of eight organic acids. The usefulness of PVA-coated capillaries in the analysis of real samples containing UV-transparent organic acids was demonstrated.

## 2. Experimental

### 2.1. Materials

Glacial acetic, phosphoric and hydrochloric acids were from Carlo Erba (Milan, Italy). Tris(hydroxymethyl)aminomethane was purchased from Sigma (St. Louis, MO, USA). Citraconic, benzoic, 2-nitrobenzoic, 3-nitrobenzoic, *o*-toluic, *p*-toluic, benzoic, 3-hydroxybenzoic, phthalic acids were from Aldrich (Steinheim, Germany). N,N,N,-Trimethylaminoethylacrylamide (Immobiline p$K_a$>12) was purchased from Fluka (Switzerland).

N,N-methylenebisacrylamide was from Bio-Rad.

### 2.2. Apparatus and electrophoresis

CE separations were carried out in a Spectra-Phoresis 1000 capillary system (Thermo Separation, Freemont, CA, USA). Data were collected on a personal computer using SW-Phoresis 1000 software. In all the experiments, 50 μm fused-silica capillaries (Polymicro, Phoenix, AZ, USA) were used. The samples were detected at 254 nm. Before each run the capillary, 40 cm long (32 cm to the window) was washed with the running buffer by applying a pressure of 15 p.s.i. for 4 min (1 p.s.i.=6894.76 Pa). The samples were loaded electrophoretically by applying 75 V/cm for 5 s. EOF measurements were carried out in 50 m*M* acetate–Tris buffer adjusting the pH to 4.0, 5.0, 7.5 and 8.1 with Tris. Benzyl alcohol was used as the EOF marker.

CZE separation of UV-transparent organic acids was carried out in 5 m*M* benzoic acid adjusted to pH 4.5 by adding NaOH. The samples (1 m*M* water solutions) were loaded electrophoretically by applying 6 kV for 6 s. Detection wavelength was 214 nm.

### 2.3. Synthesis of PVA

PVA was synthesized from polymerization and acidic hydrolysis of *N*-vinyl-*tert*.-butylcarbamate according to the method described by Hart [22,23]. Chlorohydrated amino groups of PVA were freed in 1 *M* KOH solution followed by dialysis in 6-8000 cellulosic dialysis tubing. The number-average molecular mass of PVA ($M_n$ = 36 600) was obtained

from viscosimetric measurements in 0.1 *M* NaCl–0.1 *M* NaOH using the relation $[\eta] = 6.2 \cdot 10^{-3} \overline{M}_n^{0.88}$ [24].

### 2.4. Capillary coating

#### 2.4.1. Capillary pretreatment

The capillaries were first pretreated with 1.0 *M* NaOH for 1 h followed by 45 min of washing with distilled water, by 1 h of washing with 0.1 *M* HCl and again 45 min with water. Residual water was evaporated by connecting the capillary overnight to a gas chromatography oven at 100°C under a nitrogen pressure of 3 atm (1 atm = 101 325 Pa).

#### 2.4.2. Dynamic adsorption of PVA (first step)

The capillaries were flushed at room temperature with a 0.5% w/v solution of PVA in water for 6 h and subsequently washed with water for 1 h.

#### 2.4.3. PVA cross-linking and derivatization (second step)

A solution of 4% Bis, 3% Immobiline $pK_a > 12$ and triethylamine 0.38 *M* in water was forced overnight through the capillaries treated as described in the first step under a nitrogen pressure of 3 atm, at room temperature, by using a laboratory-made device, followed by 90 min of washing with water.

## 3. Results and discussion

The use of capillary coatings to manipulate EOF and wall interactions is an expanding field which is generating increasing interest. A general method to reverse EOF consists of coating and cross-linking a polymer onto the surface of a fused-silica capillary. Stationary phases of this type, modified by grafting appropriate ligands, have found large application in chromatography in the production of stationary phases for ion-exchange [25], affinity [26], size exclusion [27] or liquid chromatography [28,29]. Poly(alkylamines) are water-soluble polymers of considerable potential application. Over the last 15 years, these polymers have been the subject of much interest both industrial and academic. The interest towards these polymers is mostly focused on the high chemical reactivity of their primary amino groups. These amino functions allow formation, by an appropriate chemical reaction, of a wide range of novel polymers, which may be used in liquid chromatography [28,29], catalysis [30], metal chelation [31] or cleaning up pollution [32]. Among these polyamines the most popular are poly(allylamine) (PAA) and linear and branched PEI, all of which are commercially available. In comparison to the two others, PVA has been the object of relatively few studies in the literature. This is undoubtedly because of the fact that it is not commercially available.

In this work we used PVA, a member of the poly(alkylamine) family, as the base for the preparation of a novel coating for CE. PVA is a linear and hydrosoluble polyelectrolyte of simple structure which has two main properties: (i) like other poly(alkylamine)s it is strongly adsorbed on silica; (ii) it possesses reactive primary amino groups which allow relatively facile functionalization. In previous works [31–33], using this polyamine as the supporting polymer several stationary phases for high-performance liquid chromatography were prepared using the coating technology.

There are several precursor polymers for the synthesis of PVA: poly(N-vinylphthalimide), poly(N-vinylsuccinimide), poly(N-vinylacetamide), poly(acrylamide) or poly(acrylic) acid. In order to obtain PVA with a degree of purity of 100% we used for the synthesis a method reported by Hart [22,23] which comprises polymerization of N-vinyl-*tert*.-butylcarbamate followed by acidic hydrolysis of the polymer. This method leads to the formation of a high quality PVA with a linear backbone bearing almost 99% of primary amino functions. The polymer composition plays an important role on the conformation of the polymer which in turn affects adsorption and coating stability.

### 3.1. Coating procedure

The coating procedure is depicted in Fig. 1. A simple wash of the capillary with a low-viscous 0.5% (w/v) solution of PVA with the primary amino functions in the deprotonated form, is sufficient to generate a coating that can be used at pH 4.0 for the

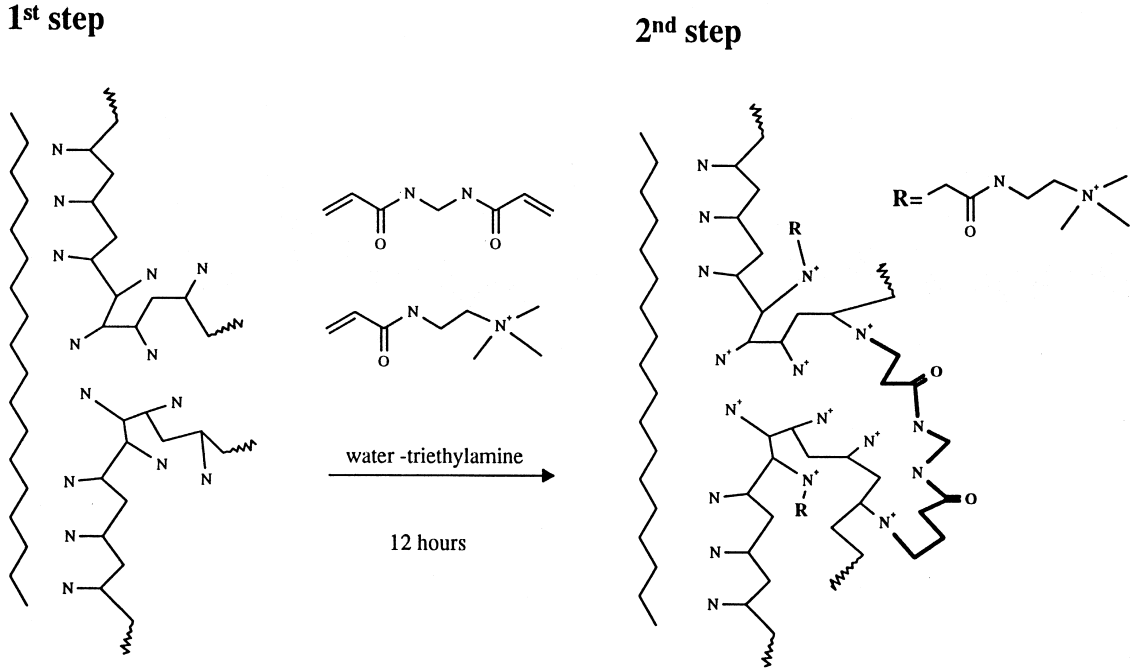


Fig. 1. Schematic representation of the coating procedure.

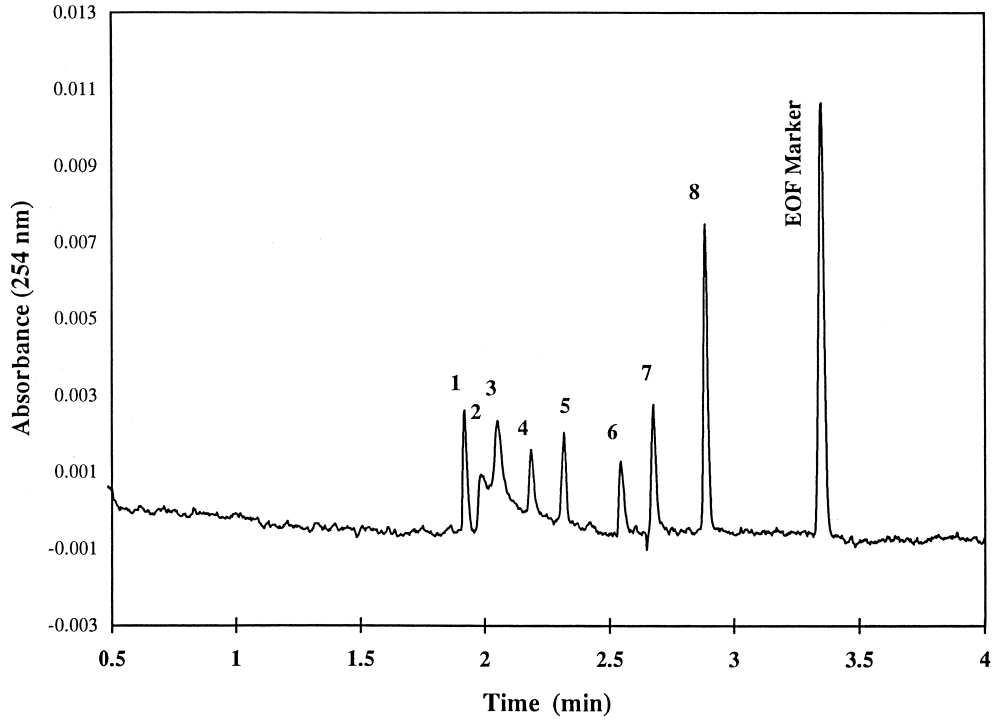


Fig. 2. CZE separation of eight organic acid standards. Capillary, PVA dynamic coating (coating solution 0.5% w/v PVA in water) (first step), 40 cm (32 cm to the detector)×375 μm O.D.×50 μm I.D.; buffer, 50 m*M* sodium acetate buffer, pH 4.0; applied voltage 500 V/cm, detector anodic; electrokinetic injection, 75 V/cm for 5 s; detection, 254 nm. Peaks: 1=*o*-nitrobenzoic acid, 2=citraconic acid, 3=*m*-nitrobenzoic acid, 4=*o*-toluic acid, 5=benzoic acid, 6=*p*-toluic acid, 7=*p*-OH-benzoic acid and 8=*p*-$NH_2$-benzoic acid.

separation of organic acids. In practice the main problems of this coating are (i) limited lifetime and (ii) tendency to adsorb bi- or tricarboxylic acids which probably results in the formation of stable, five or six membered ring structures between carboxyl groups of the analyte and the easily accessible amino groups on the coating. Fig. 2 shows the separation of a set of eight organic acids contained in the test mixture used to assess the quality of the coating. The recovery of citraconic acid (peak 1) is very limited and this compound gave a tailed peak that could hardly be detected. In order to avoid adsorption of organic acids, primary amino groups were transformed into quaternary ammonium functions by means of a nucleophilic addition of primary amines to the conjugated double bond of a commercially available acrylamido derivative, N,N,N-trimethylaminoethylacrylamide (Immobiline p$K_a$>12). The reaction was carried out inside the capillary on the accessible amino functions dynamically adsorbed on the wall. Fig. 3 shows the CE separation profile of eight organic acids in the capillary dynamically coated with PVA, as described in Section 2.4 and derivatized overnight with a 3% solution of the Immobiline p$K_a$>12 in water. Although not completely resolved from *o*-nitrobenzoic acid, citraconic acid was eluted as a sharp and symmetric peak. Fig. 4(a and b) show the electropherogram of citraconic and phthalic acids run (a) in the capillary with underivatized primary amino groups, and (b) in the same capillary after derivatization of amino groups with quaternary ammonium functions. Both acids are strongly adsorbed onto the wall in the first capillary, however their profile improved dramatically after derivatization.

The derivatization of PVA amino functions with Immobiline p$K_a$>12 improved the capillary performance in the separation of organic acids but unfortunately the capillary lifetime was limited to only 24 h. Although not sufficient for routine use in

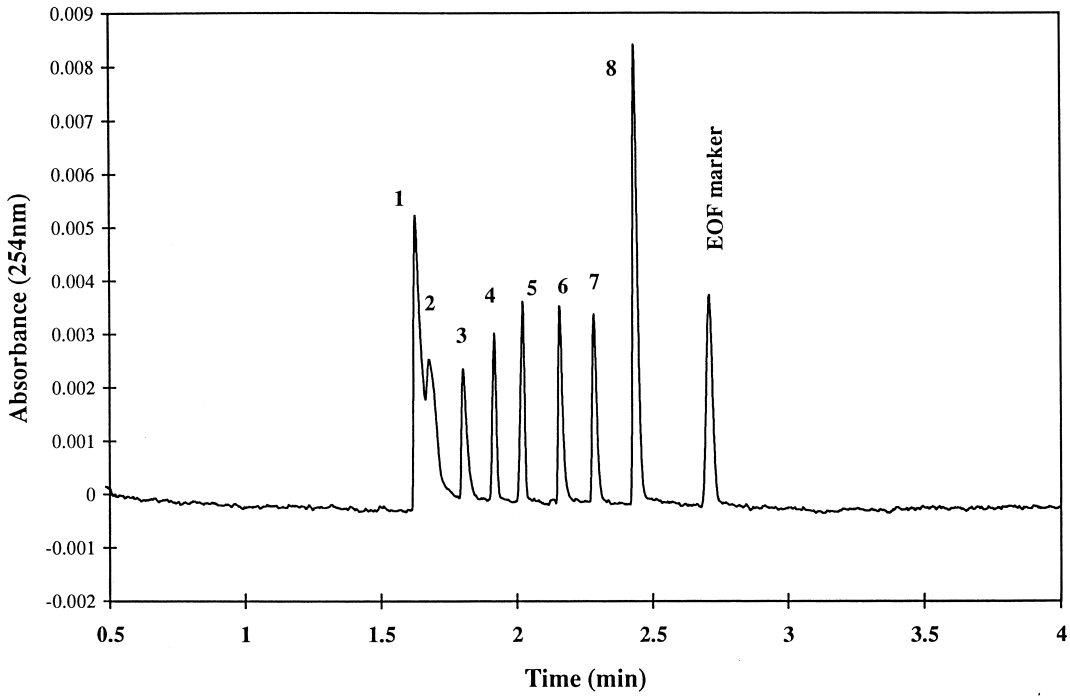


Fig. 3. CZE separation of eight organic acid standards. Capillary, PVA dynamic coating derivatized with N,N,N-trimethylaminoethylacrylamide, 40 cm (32 cm to the detector)×375 μm O.D.×50 μm I.D.; buffer, 50 m*M* sodium acetate buffer, pH 4.0; applied voltage 500 V/cm, detector anodic; electrokinetic injection, 75 V/cm for 5 s; detection, 254 nm. Peaks 1=citraconic acid, 2=*o*-nitrobenzoic acid, 3=*m*-nitrobenzoic acid, 4=*o*-toluic acid, 5=benzoic acid, 6=*p*-toluic acid, 7=*p*-OH-benzoic acid and 8=*p*-$NH_2$-benzoic acid.

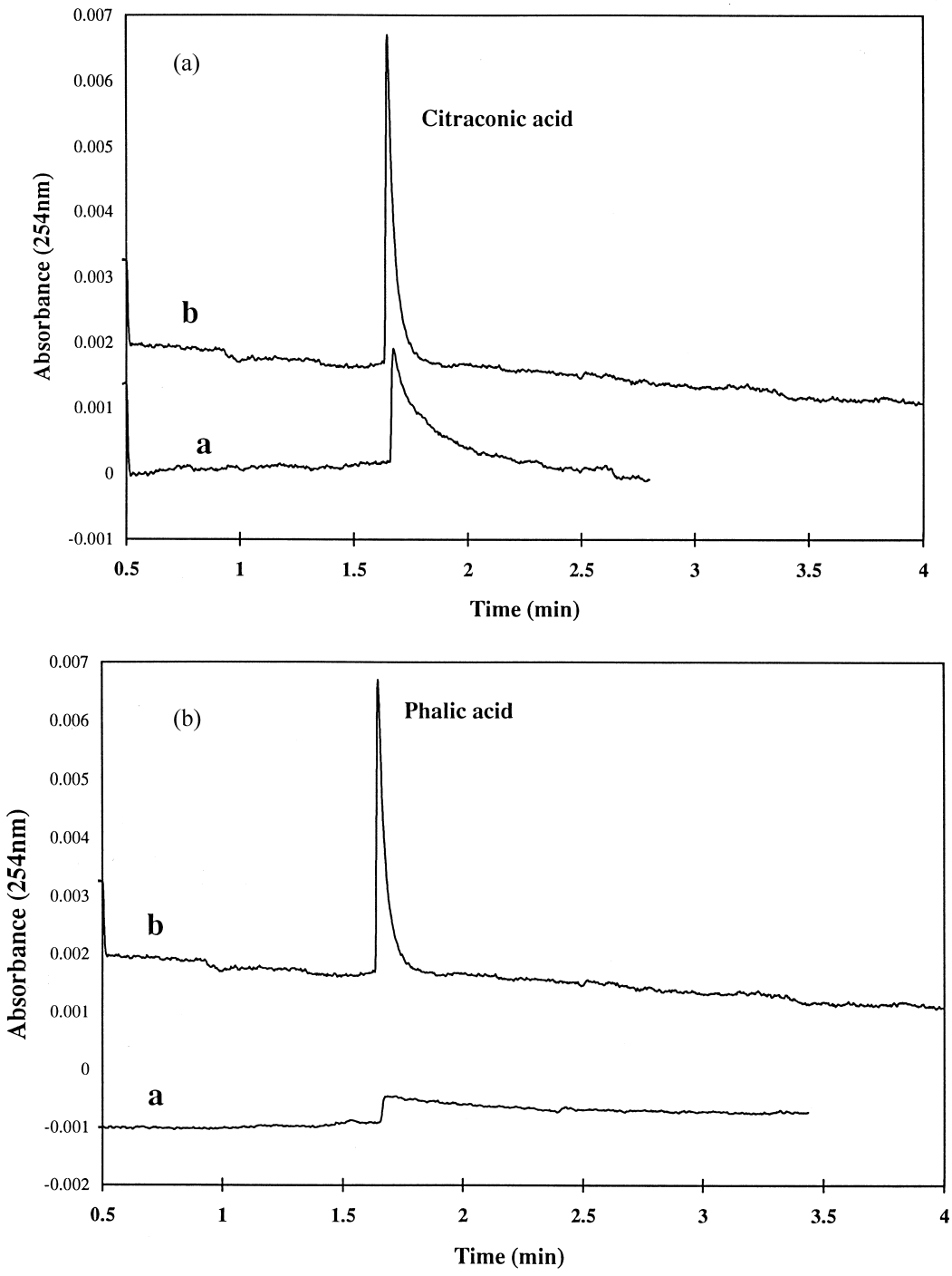


Fig. 4. CZE separation of citraconic (a) and phthalic acid (b) in (a) uncross-linked (first step) and (b) cross-linked PVA coating (second step), 40 cm (32 cm to the detector)×375 μm O.D.×50 μm I.D.; buffer, 50 m*M* sodium acetate buffer, pH 4.0; applied voltage 500 V/cm, detector anodic; electrokinetic injection, 75 V/cm for 5 s; detection, 254 nm.

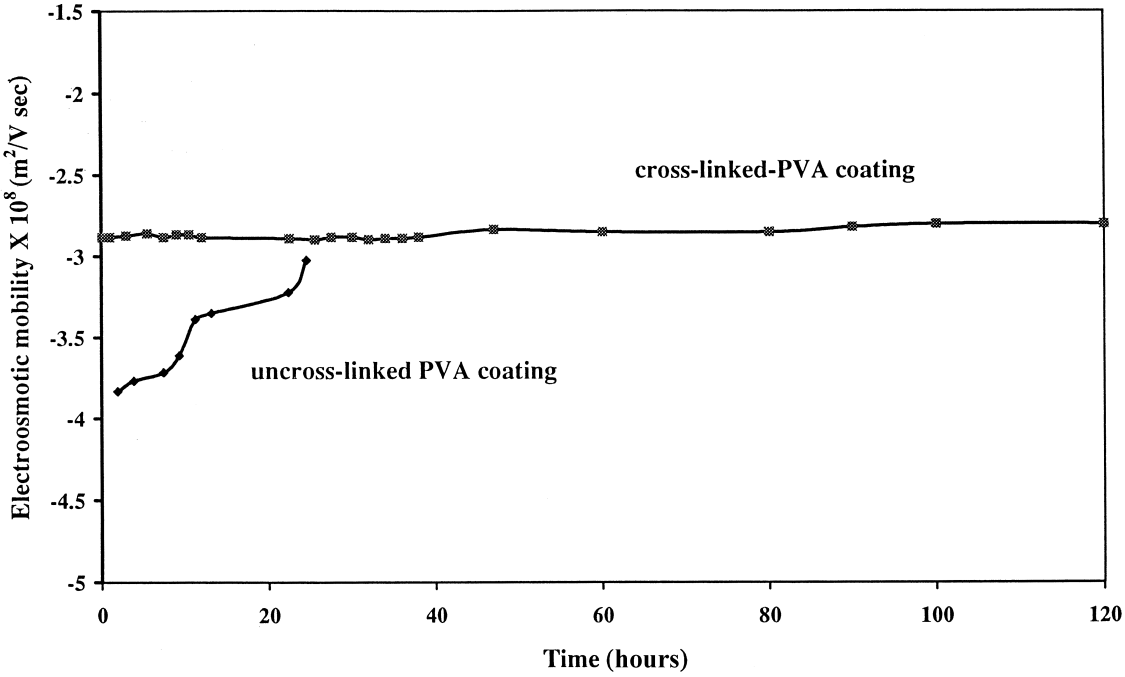


Fig. 5. Electroosmotic mobility versus capillary lifetime in cross-linked and uncross-linked coated capillary. EOF was measured in 50 m*M* acetate–Tris buffer, pH 4.0. Benzyl alcohol was used as the neutral marker.

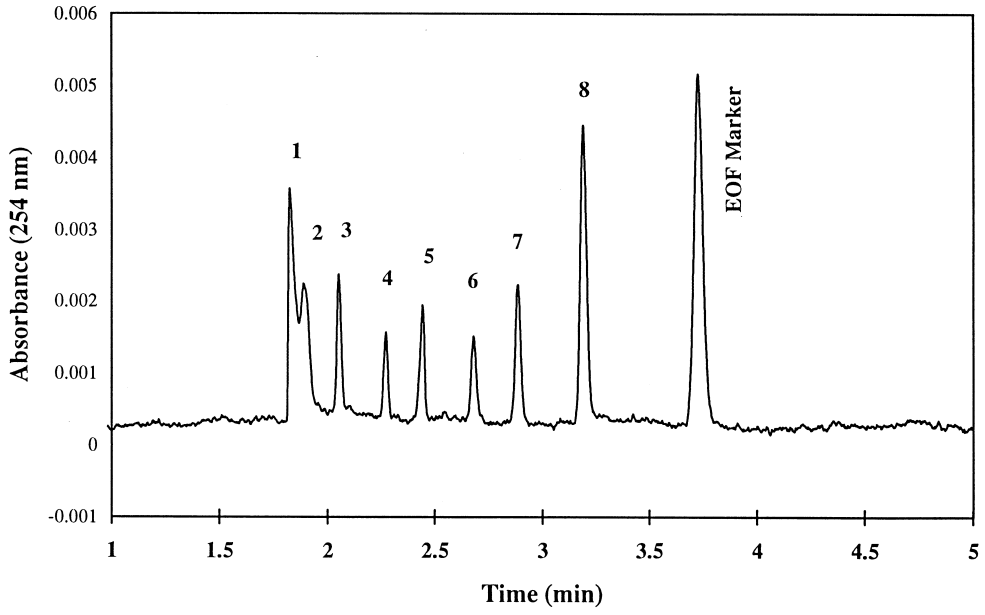


Fig. 6. CZE separation of organic acid standards. Capillary, cross-linked PVA coating (second step), 40 cm (32 cm to the detector)×375 μm O.D.×50 μm I.D.; buffer, 50 m*M* sodium acetate buffer, pH 4.0; applied voltage 500 V/cm, detector anodic; electrokinetic injection, 75 V/cm for 5 s; detection, 254 nm. Peaks: 1=citraconic acid, 2=*o*-nitrobenzoic acid, 3=*m*-nitrobenzoic acid, 4=*o*-toluic acid, 5=benzoic acid, 6=*p*-toluic acid, 7-*p*-OH-benzoic acid and 8=*p*-$NH_2$-benzoic acid.

automated systems, the lifetime indicates that a strong binding occurred between the polymer and the wall. The strength of the binding between PVA and the silanols depends on the pH at which the polymer is adsorbed onto the surface. When the binding takes place between PVA with free amino functions and the wall, a more strong bonding occurs by a hydrogen bonding mechanism. On the contrary adsorption of a polymer with amino functions partially protonated leads to the formation of a less stable coating which is mainly adsorbed by electrostatic interaction. Due to its lower binding constant, this coating degrades more rapidly. These capillaries would required the presence of a small amount of PVA (0.001%, w/v) in the running buffer to displace the equilibrium toward adsorption (data not shown).

The technique we adopted in the present work to stabilize the coating was the cross-linking of PVA chains by reacting primary amino functions of the polymer with N,N-methylenebisacrylamide, a cross-linker commonly used to produce poly(acrylamide) gels. Amino groups belonging to different chains reacted with Bis functional groups resulting in the formation of an insoluble network on the capillary surface. The two conjugated double bonds of Bis easily undergo nucleophilic addition of primary amines in a alkaline water solution. This reaction stabilized the coating but did not solve the problem of adsorption of organic acids. In order to stabilize the coating while overcoming this problem, two simultaneous reactions were carried out on PVA amino groups: (i) cross-linking of PVA polymer chains adsorbed on the capillary surface and (ii) derivatization of easily accessible primary amino functions with Immobiline $pK_a > 12$ to transform these groups in less reactive quaternary amino functions. In this way, a new highly stable phase was obtained in a single step.

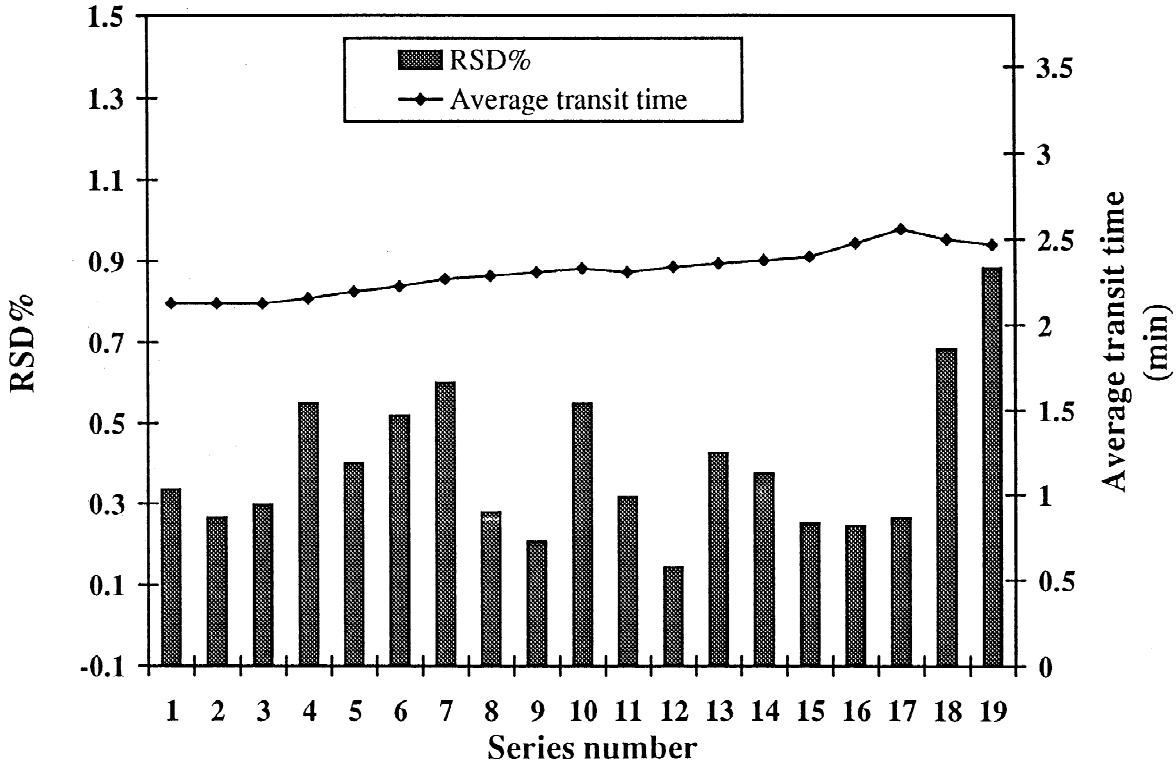


Fig. 7. Migration time reproducibility for benzoic acid in a cross-linked PVA coated capillary. R.S.D.% of 10 injections were taken every 6 h over a test period 120 h.

### 3.2. Stability test

The gain in stability achieved with the cross-linked PVA in comparison with the non cross-linked one is depicted in Fig. 5. In the new phase, there is practically no change in the EOF after 120 h of continuous use at pH 4.0. Fig. 6 shows the separation profile of the test organic acids in a cross-linked PVA capillary. All the components gave symmetric peaks and migrated in less then 3.5 min due to the fast EOF. The reproducibility of transit times for the peak of benzoic acid is given in Fig. 7. R.S.D.% and average transit times of a series of 10 injections were taken every 6 h during the 120 h of the whole test period. The values oscillate from 0.2 to 1% indicating an excellent reproducibility.

### 3.3. EOF

The immobilization on the capillary surface of ionizable groups that bear a positive charge at a pH lower than 8 generated an EOF directed towards the anode. Typically the rate of such EOF at pH 4 was $2.9 \cdot 10^{-8}$ $m^2$ V s. As shown in Fig. 5 a decrease in the flow velocity is observed for cross-linked capillaries compared to uncross-linked ones. This reduction might result from the fact that the cross-linking step required flushing the capillary overnight with an alkaline solution of Bis and Immobiline p$K_a$ > 12. In these conditions adsorbed polymer chains might be eroded from the surface. In fact this coating does not completely mask all surface silanols. An increase in pH results in a significant decrease in EOF, this decrease continues up to pH 8 where the flow is suppressed to negligible values. Fig. 8 shows the variation of EOF as a function of the pH of the running buffer in cross-linked PVA-coated capillaries in the absence and in the presence of variable amounts of PVA in the BGE. This graph clearly indicates that this coating can be successfully used

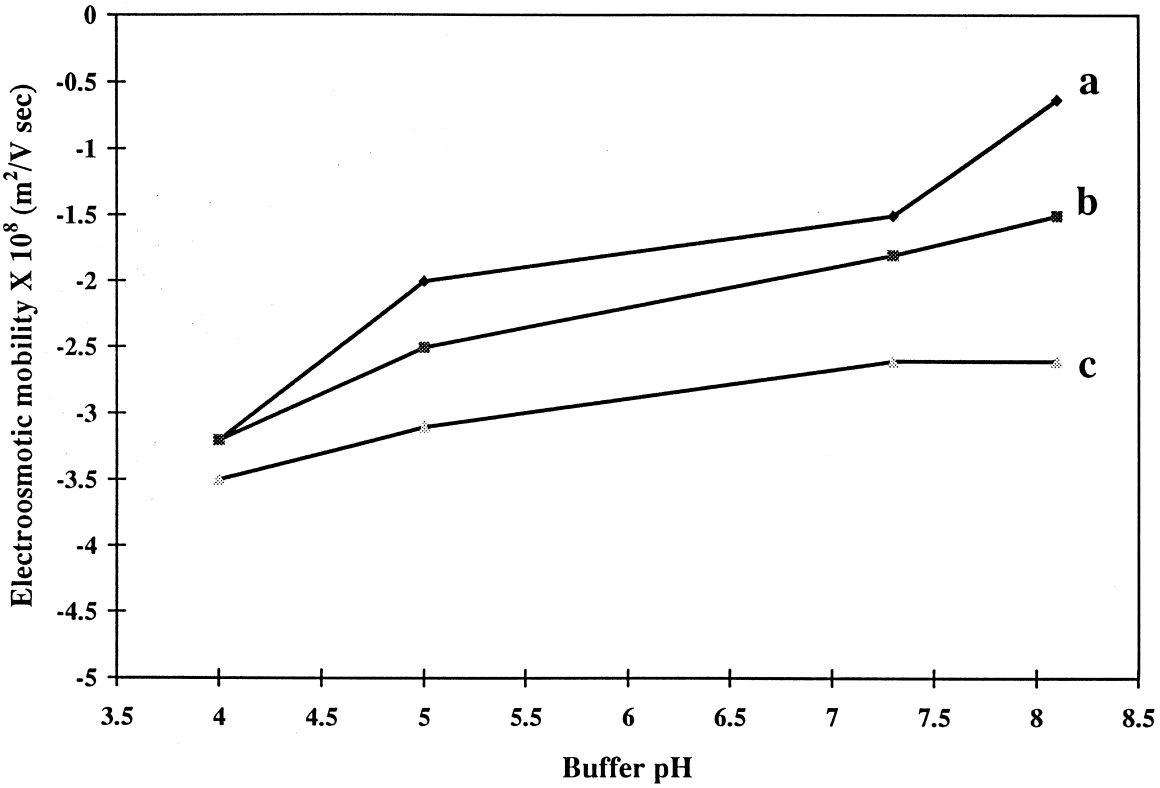


Fig. 8. Electroosmotic mobility versus pH in a cross-linked PVA coated capillary, (a) without PVA added to the running buffer, (b) with 0.001% (w/v) of PVA and (c) with 0.01% (w/v) PVA in the running buffer. Running buffer 50 m*M* acetate titrated to pH 4.6 and 7.5 with Tris. Anodic migration; applied voltage, −20kV; neutral marker, benzyl alcohol.

up to pH 5.0, above this value a significant EOF can only be generated in the presence of a small amount of polymer in the BGE that produces a more efficient masking of surface silanols. Given the insufficient coverage of silanols this coating does not seem to be useful to separate basic proteins, actually all the attempts to separate basic proteins in the pH interval from 4 to 7 were unsuccessful with the exception of myoglobin that was analyzed in a PVA-cross-linked-coated capillary in 0.01% (w/v) PVA-containing acetate–Tris buffer at pH 7.0 (data not shown).

## 4. Conclusions

A novel coating procedure was devised consisting of two steps: (i) dynamic adsorption of synthetic high quality PVA, (ii) cross-linking and derivatization of PVA through nucleophilic addition of primary amino groups to the conjugated double bonds of N,N-methylenebisacrylamide and N,N,N-trimethylaminoethylacrylamide. This second step generated an insoluble coating that made it possible to use these capillaries for the separation of a test mixture of organic acids in the 4–5 pH interval for several weeks with excellent reproducibility of transit times. The tendency of polyamine functions of the coating to adsorb organic acids was efficiently counteracted by transforming primary functions in quaternary ammonium groups. Although very stable this coating is not completely effective in shielding wall silanols at high pH and therefore it was not suitable for separating proteins. Given the simplicity of the coating procedure good reproducibility was obtained in different batches with a R.S.D. for the EOF of five different capillaries lower than 0.2%. Finally the usefulness of the coating in the separation of UV-transparent anions by indirect UV detection have been demonstrated. The new phase was successfully used in the separation of malic, citric and succinic acids (Fig. 9) by applying indirect UV detection with a nonadsorbing UV-counter ion and a UV-adsorbing co-ion.

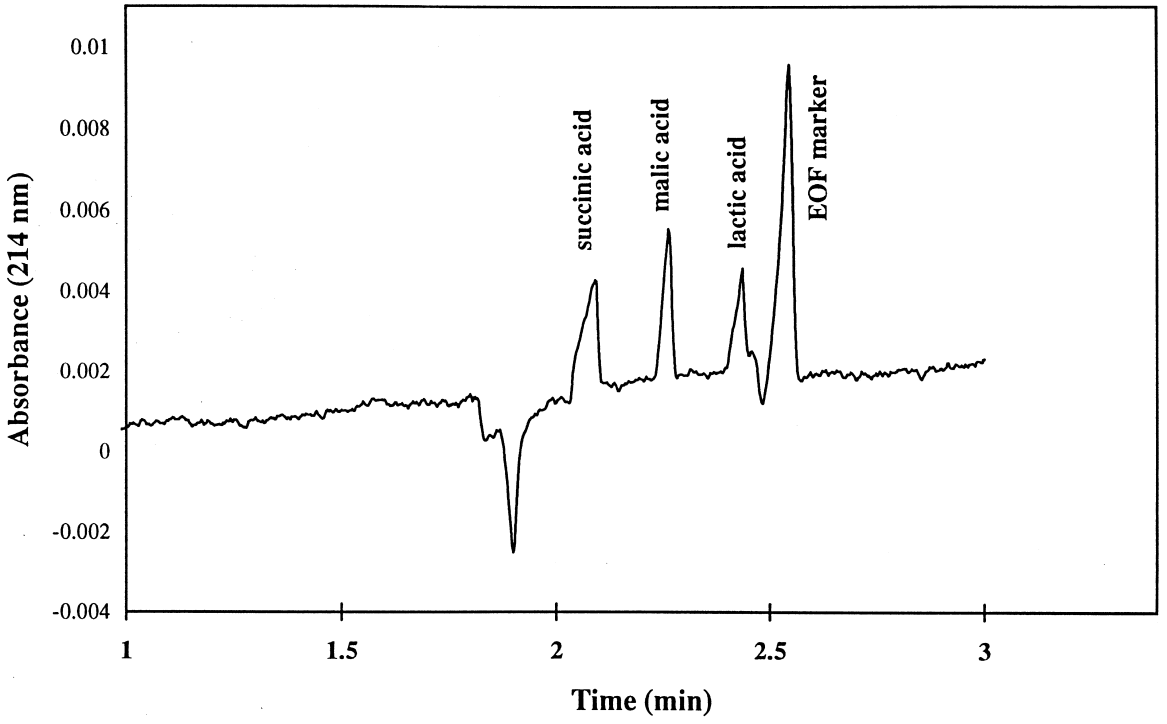


Fig. 9. CZE separation of UV-transparent organic acids in a cross-linked PVA-coated capillary. Separation buffer, 5 m*M* sodium benzoate, pH 4.5; applied voltage, 20 kV, 3 μA; UV indirect detection, 214 nm; electrokinetic injection, 75 V/cm 5 s.

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