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Novel, trifunctional diamine for silica coating in capillary zone electrophoresis

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Abstract

A novel compound {quaternarized piperazine [(*N*-methyl,*N*-4-iodobutyl)-*N*′-methylpiperazine] (QPzI)} for the coating of a silica capillary able to reduce or invert the electroosmotic flow (EOF) in capillary zone electrophoresis is reported. Unlike standard oligoamines (like spermine and tetraethylene pentamine) which are very efficient in quenching macromolecule interaction with the silica wall, but only in acidic pH ranges, QPzI acts all along the pH scale, including alkaline pH ranges. It is believed that QPzI behaves like a trifunctional derivative: it forms ionic bonds with dissociated silanols via its quaternary nitrogen, hydrogen bonds via its tertiary nitrogen and, most importantly, a covalent bond via alkylation of ionized silanols through the terminal iodine atom in the butyl chain. Excellent separations are obtained with a variety of organic compounds, such as aromatic carboxylic acids, tryptophan metabolites and arylalkanoic acids. Such separations could not be obtained in naked capillaries in the presence of oligoamines and on some occasions not even with capillaries coated with a covalent layer of neutral polymers. In separations taking place in alkaline media, QPzI is not added to the background electrolyte, but is used simply in the capillary pre-conditioning step, a unique feature strongly supporting the hypothesis of its covalent binding to the silica surface. In difficult separations, such as in the case of *o*-/*p*-OMe-phenylacetic acids or nicotinic/picolinic acid, which would not normally occur under standard conditions, it is believed that QPzI acts as a discriminator, thus playing an active role in the separation process, rather than simply modulating the EOF. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Silica; Coated columns; Electroosmotic flow; (*N*-Methyliodobutyl)-*N*′-methylpiperazine; Piperazines; Diamines; Organic acids

1. Introduction

Fused-silica columns, as developed by Dandenau and Zerenner [1], are at the heart of capillary zone electrophoretic (CZE) technology. The most important characteristic of the CZE capillary columns is associated with the chemical structure of the fused silica. The presence of different types of silanol groups (SiOH), which are weakly acidic in character, on the silica surface can confer some unique properties that greatly influence CZE separations, by inducing an electroosmotic transport of ions during the analysis. The density of isolated, geminal and vicinal silanols, the ionogenic species most represented on a silica surface, is given as 8.31 $\mu M/m^2$, corresponding to about 5 silanols per nm^2, with average p*K* values assessed as 5.3 [2] or 6.3 [3]. This

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means that at any pH value above 2 there will be a progressively higher ionization of silanols, which will induce, upon application of a voltage gradient, a net flow of solvent, carried by the cations in the diffuse double layer, towards the cathode, called electroosmotic flow (EOF). Per se, the EOF is not detrimental in the CZE separation process and could even be beneficial, such as in the case of micellar electrokinetic chromatography, where the interplay between the net liquid transport towards the cathode and the retro-migration of sodium dodecyl sulfate (SDS) micelles towards the anode provides an ample time window for separation of neutral and hydrophobic analytes partitioned to different extents in the charged micelles [4]. However, in the case of separation of macromolecules, such as proteins [5], peptides [6] and DNA [7], and even in the case of small metabolites, it could have rather negative effects, ranging from skewed peaks to completely adsorbed species onto the Debye–Hückel layer of the silica wall. Thus, for many applications, control and/or suppression of EOF is necessary for exploiting the full potential of the CZE technique.

Several approaches have been employed for controlling and possibly eliminating EOF. In general, the various methods can be divided into three main groups [8]: (i) changes of the buffer pH, concentration and composition through the addition of organic solvents or additives; (ii) dynamic modification of the wall surface, via adsorption of neutral or charged polymers, including surfactants; (iii) chemical derivatization of surface silanols with concomitant covalent binding of a variety of polymers (e.g., polyacrylamides, celluloses).

Each method offers advantages and disadvantages that should be carefully considered in the selection of the appropriate system.

Among all the possible additives, perhaps the class which has received the greatest attention is that of the amine modifiers, ever since Nahum and Horváth [9] recommended them as additives to the mobile phases employed in reversed-phase chromatography of ionogenic substances, in order to suppress the untoward effect of the residual unmasked silanolic groups in the stationary phase. This class comprises a vast number of compounds, starting from monoamines (e.g., triethylamine and propylamine, morpholine, glucosamine and galactosamine, *N*,*N*-diethylethanolamine, *N*-ethyldiethanolamine, triethanolamine, ethanolamine, hydroxylamine, ethylamine, tetramethylammonium chloride), to diamines [e.g., 1,3-diaminopropane, 1,4-diaminobutane (putrescine), 1,5-diaminopentane (cadaverine), ethylenediamine, *N*,*N*,*N*′,*N*′-tetramethyl-1,3-butanediamine and the α,ω-bis-quaternary ammonium alkanes, such as hexamethonium and decamethonium bromides] and to oligoamines [e.g., diethylentriamine and triethylentetramine, *N*,*N*′-bis(3-aminopropyl)1,4-butanediamine (spermine) and 1,4,7,10-tetraazocyclodecane (cyclen)] (for reviews, see Refs. [5] and [10]).

We have recently evaluated the merits of these additives, which have the great advantage of not requiring any chemistry or any manipulation of the silica surface, and are thus appealing to a vast number of CZE users. As expected, the best additives are those that act by a multipoint attachment to the silanols, such as spermine and TEPA (tetraethylenepentamine) [11]. However, even these oligoamine additives (which are already very effective in the millimolar range) suffer from serious drawbacks: they are useless in the alkaline pH range, due to deprotonation of the oligoamino backbone. Since a vast number of CZE applications exploit analysis in an alkaline medium, this problem considerably diminishes the usefulness of such compounds. We report here a unique chemical (and a possible family of derivatives) able to quench and control most efficiently EOF all along the pH scale. Although such a compound is a simple diamine (which in principle should have a very modest efficacy in modulating EOF) it will be shown here that, due to its peculiar structure, it is in fact the most powerful chemical ever reported and that it could represent a true step forward for resolving the serious problem of EOF, while offering unrivalled separation capabilities.

2. Experimental

2.1. Reagents

The following acids: nicotinic, quinolinic (1), xanturenic (2), antranylic (3), picolinic (4), 3-hydroxyantranylic (5), kynurenic (6), phenylacetic (8), *m*-NO_2-phenylacetic (9), *o*-OMe-phenylacetic (10),

p-OMe-phenylacetic (11), naphthylacetic (12) acids, ketoprofen (13), phenoprofen (14) and the EOF marker acrylamide (7) were obtained from Aldrich. Fused-silica capillaries (50 μm I.D.×375 μm O.D.) were from Polymicro Technologies (Phoenix, AZ, USA) and were used as such, without inner coating.

2.2. Synthesis of the quaternarized piperazine [(N-methyl,N-4-iodobutyl)-N′-methylpiperazine] (QPzI)

An 11.4-g amount of *N*,*N′*-dimethylpiperazine (0.1 mol) is dissolved in acetone (100 ml) and the resulting solution is added to a stirred mixture of 1,4-diiodobutane (31 g, 0.1 mol) in diethylether (100 ml). The reaction is run for 24 h. The precipitate formed is filtered, washed with acetone and dried under vacuum at 0.5 mmHg (1 mmHg=133.322 Pa) for 3 h to yield QPzI (38.2 g, 90%, m.p. 278–280°C).

^{1}H-nuclear magnetic resonance (NMR) (dimethyl sulfoxide, DMSO) δ (ppm): 1.71–1.88 (m, 4H), 2.28 (s, 3H), 2.58–2.68 (m, 2H), 2.68–2.78 (m, 2H), 3.05 (s, 3H), 3.3 (t, 2H), 3.35–3.45 (m, 6H).

Mass spectrometry (MS) (matrix-assisted laser desorption ionization, MALDI): 296 ($M^{+}-I$, 100), 169 (28).

2.3. Capillary electrophoresis

When operating with a new capillary, it is necessary to perform a washing with 0.1 *M* NaOH for 2 h and water for 3 h. Then, a brief pre-conditioning, consisting in a few washing cycles, as described below, is applied until reaching constant (inverted) EOF flux values. Pre-conditioning: washing (5 bar for 1.5 min) with a modifier solution (2 m*M* in tetraborate buffer, 25 m*M*, at pH 9.0), followed by a brief washing (5 bar for 3 min) with running buffer.

Sample analysis is performed according to the following procedure: washing (5 bar for 2 min) with the modifier solution (2 m*M* in tetraborate buffer, 25 m*M*, at pH 9.0), followed by a washing (5 bar per 3 min) with running buffer, sample injection (10–20 mbar for 10–20 s), injection of a running buffer plug (25 mbar for 5 s). Analysis have been performed with a Hewlett-Packard ^{3D}CE instrument, in capillaries of 50 μm I.D., typical length of 60 (or 100) cm, applied voltage: −20 or −25 kV; UV–Vis detection at 210 nm, temperature of 25°C.

3. Results

3.1. Separation of aromatic carboxylic acids

The analysis of carboxylic acids containing heterocyclic rings with nitrogen groups, when performed in an uncoated capillary, is besieged by problems due to adsorption of such molecules to the capillary wall. The analysis of dilute solutions of such compounds is thus impossible, unless properly coated capillaries are adopted. In Fig. 1a the electropherogram pertaining to the analysis of a solution of picolinic acid, at 1 m*M* concentration, in an uncoated capillary, is reported. It is clear that the wall adsorption originates a broad peak, with very poor plate count. Fig. 1b gives the analysis of a mixture of picolinic and nicotinic acids at 50 micromolar concentration, performed with the procedure of wall modification described in Section 2.3, in the presence of the quaternarized piperazine. It is evident here how the brief capillary pre-treatment with the modifier effectively abolishes the analyte adsorption to the capillary wall, allowing thus proper quantitation of species present even at low levels, as often required in the analysis of biological fluids, in environmental and in food analysis.

3.2. Analysis of tryptophan metabolites (kynurenine pathway)

Tryptophan can be degraded via the kynurenin pathway, originating a series of metabolites: the separation of a mixture of these six compounds is illustrated in Fig. 2, when pre-conditioning the capillary with QpzI. The analysis of the mixture is performed according to the procedure of Section 2.3, thus in the absence of the modifier in the running buffer. One should note the excellent separation of all six compounds in the mixture, coupled to very short analysis times (<6 min). It has not been possible to obtain such results in an uncoated capillary both due to unfavourable EOF flow and to adsorption of analytes. Also the use of conventional oligoamines (spermine and TEPA) has not given any

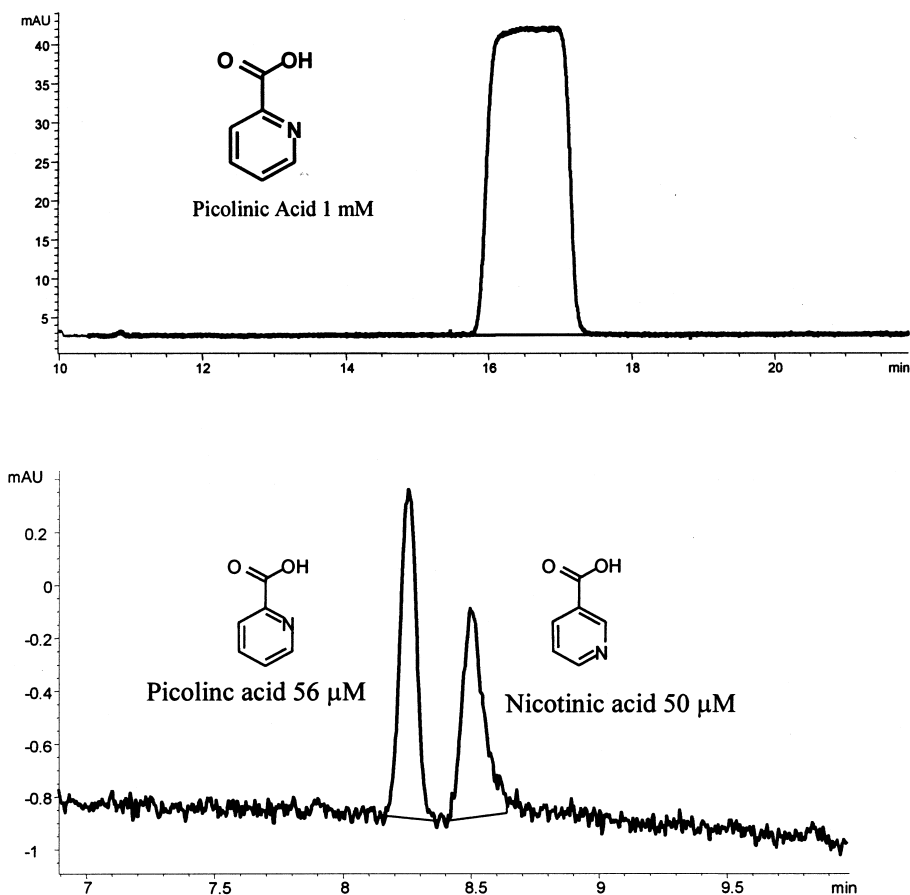

Fig. 1. Electropherogram of (a) 1 m*M* picolinic acid (uncoated capillary) and (b) 50 μ*M* picolinic and nicotinic acids following the wall modification procedure. Analysis conditions: (a) fused-silica capillary, 60 cm total length×50 μm I.D., 50 m*M* borate buffer, pH 9.0, +15 kV, 20°C, λ=210 nm, (b) fused-silica capillary, 60 cm total length×50 μm I.D., 25 m*M* borate buffer, pH 9.0, −20 kV, 20°C, λ=210 nm. Conditioning: washing (5 bar for 2 min) with the modifier solution (2 m*M* in tetraborate buffer, 25 m*M*, at pH 9.0), followed by a washing (5 bar per 3 min) with running buffer, sample injection (20 mbar for 20 s), injection of a running buffer plug (10 mbar for 5 s).

appreciable result. The flux inversion, due to an excess of positive charges present in QPzI bound to the silica wall, has been verified via the elution of a neutral marker (compound 7: acrylamide), eluted in ca. 10 min.

3.3. Separation of arylalkanoic acids

A mixture of seven alkanoic acids, some of them with closely related structures, such as compounds (10) and (11), which would render their separation by CZE extremely difficult under normal experimental conditions, were analysed by capillary pre-conditioning with QpzI. Fig. 3 shows the corresponding electropherogram, as obtained. Also in this case no separation has been possible with either coated capillaries or with background electrolytes containing conventional oligoamines (e.g., spermine, TEPA), alone or in a mixture. It is believed, in fact, that the separation of Fig. 3 has been made possible by the interaction of the analytes with the QPzI compound present on the wall surface. In this case, thus, QPzI would act by both inverting the EOF flux and by becoming an active player in the separation process, due to its interaction with some analytes. It is of interest to see how different pH values during the pre-conditioning step, as well as during the CZE run, would affect the same separation. Fig. 4 illustrates

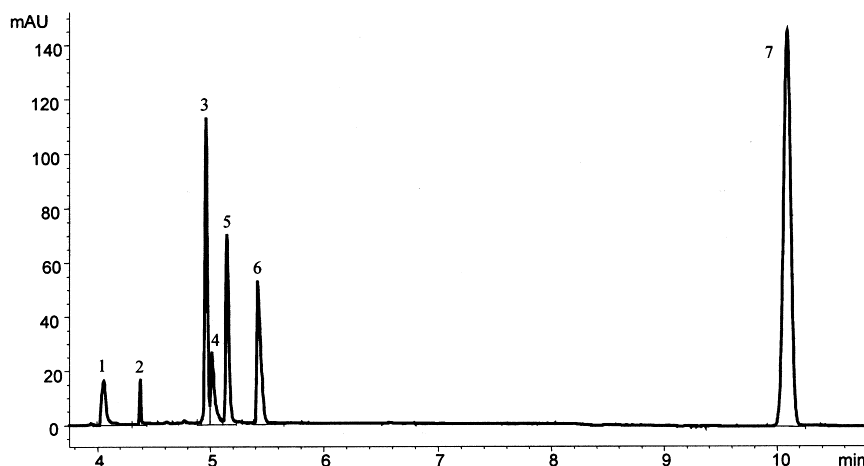

Fig. 2. Electropherogram of tryptophan metabolites following the wall modification procedure. Analysis conditions: fused-silica capillary, 60 cm total length×50 μm I.D., 25 m*M* borate buffer, pH 8.5, −20 kV, 20°C, λ=210 nm. Conditioning: washing (5 bar for 2 min) with the modifier solution (2 m*M* in tetraborate buffer, 25 m*M*, at pH 9.0), followed by a washing (5 bar per 3 min) with running buffer, sample injection (10 mbar for 10 s), injection of a running buffer plug (10 mbar for 5 s). Analyte concentration: 0.2 mg/ml. [Compound **7** is an EOF marker (acrylamide). The migration time of the L-tryptophan, under the same analytical conditions, is 8.5 min].

the CZE results obtained by pre-treatment and analysis using a background buffer at pH 7.0. Two effects are immediately apparent: the elution time is considerably lengthened and the peaks (in any case still well separated) are not only broadened but also skewed. We believe that this is due to two concomitant phenomena: diminished binding of QPzI to the wall, with possible partial interaction of some of the derivatives with the silica surface, coupled to diminished, inverted EOF (see Discussion).

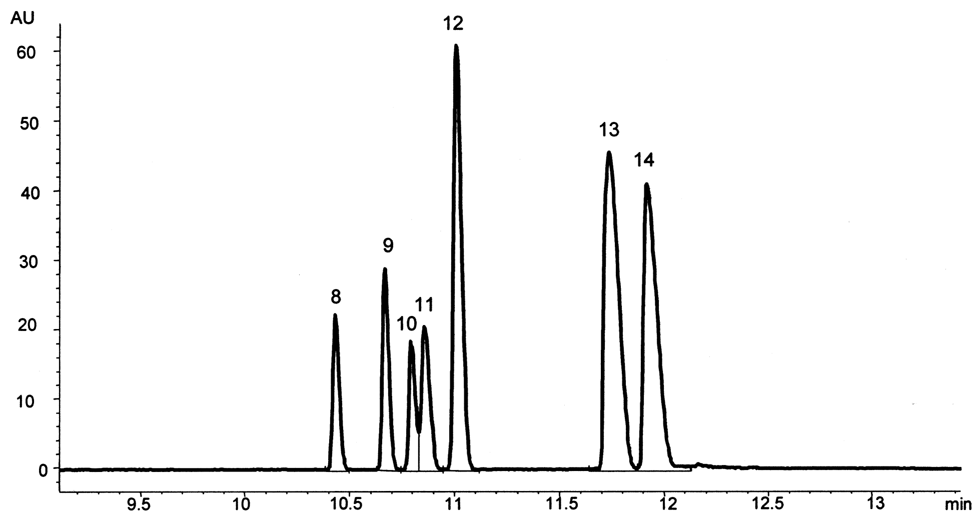

Fig. 3. Electropherogram of arylalcanoic acids derivatives 8–14 following the wall modification procedure at pH 8.5. Analysis conditions: fused-silica capillary, 100 cm total length×50 μm I.D., 25 m*M* borate buffer, pH 8.5, −25 kV, 25°C, λ=210 nm. Analyte concentration: 0.14 mg/ml. Conditioning: washing (5 bar for 2 min) with the modifier solution (2 m*M* in tetraborate buffer, 25 m*M*, at pH 9.0), followed by a washing (5 bar per 3 min) with running buffer, sample injection (15 mbar for 15 s), injection of a running buffer plug (20 mbar for 5 s).

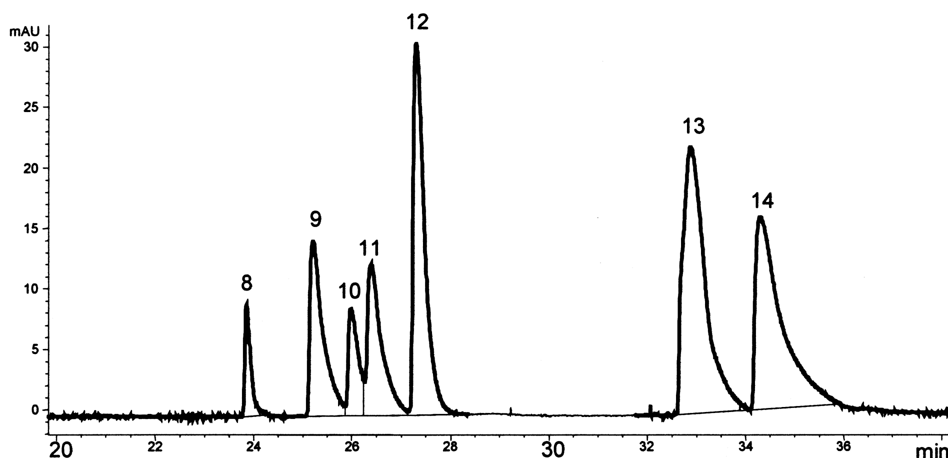

Fig. 4. Electropherogram of arylalcanoic acids derivatives 8–14 following the wall modification procedure at pH 7. Analysis conditions: fused-silica capillary, 100 cm total length×50 μm I.D., 25 m*M* dihydrogenphosphate+NaOH buffer at pH 7.0, −25 kV, 25°C, λ=210 nm. Analyte concentration: 0.14 mg/ml. Conditioning: washing (5 bar for 2 min) with the modifier solution (2 m*M* in tetraborate buffer, 25 m*M*, at pH 9.0), followed by a washing (5 bar per 5 min) with running buffer, sample injection (15 mbar for 15 s), injection of a running buffer plug (20 mbar for 5 s).

3.4. On the mechanism of action of QPzI

It is of interest to note that all the above-mentioned separations have been performed only by pre-conditioning the capillary, in the absence of QPzI in the background electrolyte during the CZE analysis. It must thus be asked how stable is this wall-coating and how reproducible the EOF from run to run. Fig. 5 explores these phenomena and offers some clues to the action of QPzI on the wall. The electropherogram represents four consecutive CZE separations of 8, 9, 11 and 12. Prior to run No. 1, the capillary was conditioned just once as described in Section 2.3. At each subsequent run after this, the pre-conditioning procedure is applied and then the following run performed. It is thus seen that, already after run No. 2, the capillary wall is stabilised and every run after that gives highly reproducible peak profiles and elution times. The effect of temperature on the coating has also been explored, as shown in Fig. 6, which represents an overlay of three different runs, at 16, 20 and 30°C, respectively, of the same mixture of the arylalkanoic acids 8, 9, 11 and 12. It is seen that, in this temperature range, the peak profile and separation efficiency is well maintained, the apparent acceleration of transit times originating solely from the diminished solvent viscosity at higher temperatures. Both experiments suggest that QPzI does not simply interact with the siliceous surface via ionic bonding, but must be covalently bound to the silanolic moiety (see also the following Discussion section).

4. Discussion

We believe that the unique properties of QPzI originate from its structural characteristic, which can be summarised as:

(a) The presence of one quaternary nitrogen able to form ionic bonds with silanols at any operative pH value.

(b) The presence of one tertiary nitrogen able to form hydrogen bonds and/or electrostatic interactions when protonated.

(c) The presence of one alkyl chain (typically but not exclusively C_4), possessing terminal carbon atoms substituted with one electronegative atom able to react with silanolic groups to such an extent as to form covalent bonds with the capillary wall.

It is thus seen that QPzI is a trifunctional derivative and as such is able to bind in a completely different fashion from that of conventional amino additives, including oligoamines, which typically

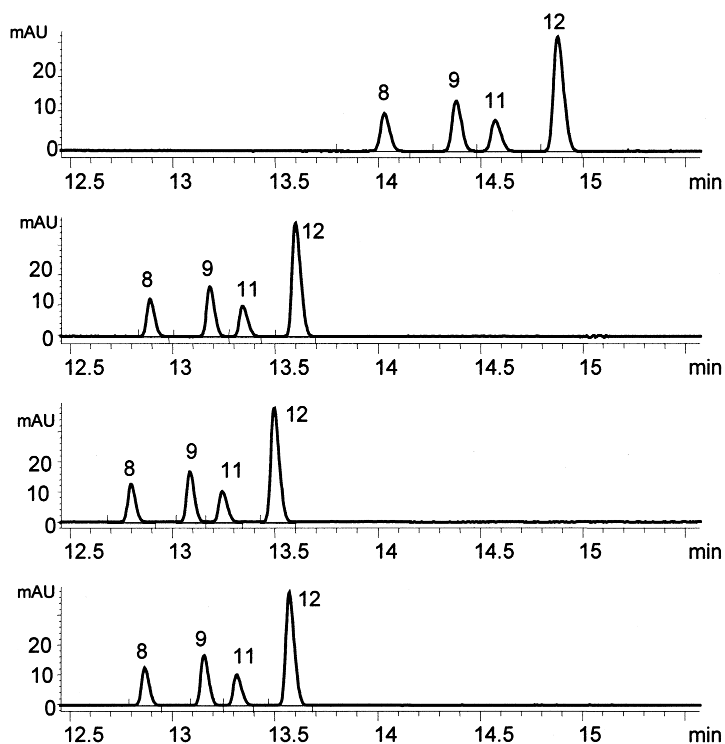

Fig. 5. Stabilization of the silica wall modification (see text). Analysis conditions: fused-silica capillary, 100 cm total length×50 μm I.D., 25 m*M* borate buffer, pH 8.5, −20 kV, T=25°C, λ=210 nm. Conditioning: washing (5 bar for 2 min) with the modifier solution (2 m*M* in tetraborate buffer, 25 m*M*, at pH 9.0), followed by a washing (5 bar per 3 min) with running buffer, sample injection (15 mbar for 15 s), injection of a running buffer plug (20 mbar for 5 s).

bind only by ionic interactions. That is why even the most powerful oligoamines (spermine, TEPA), as soon as they are brought into an alkaline medium, become completely ineffective. On the contrary, QPzI not only binds via ionic and H-bonds, but must be covalently bound to the silica wall, otherwise one could not possibly explain the overall features described in the present paper obtained in the absence of the additive in the background electrolyte, but solely by pre-conditioning the capillary prior to the electrophoretic step. Figs. 5 and 6 are a strong, albeit indirect, evidence of such a covalent bonding. To support such an assumption, two other analogues of QPzI (compounds 16 and 17 of Fig. 7) have been synthesized. In the first the tertiary amino group (with attached methyl) was lacking, whereas in 17 the terminal iodine in the butyl chain was lacking. When each of them was tested separately, the following results were obtained: compound 16 was able to partially suppress EOF (by a factor of 6, diminishing it from $6\cdot10^{-4}$ down to $1\cdot10^{-4}$ cm^2 V^{-1} s^{-1}), gave acceptable separations but was unable to invert the EOF; runs could still be performed by a simple pre-conditioning step, in the absence of 16 during the electrophoretic step; compound 17 does not induce any appreciable effect: it could not reduce properly the EOF, it did not give reproducible transit times and good separations and could not be used in a pre-conditioning step.

On the basis of the above findings, we have nicknamed QPzI ‘‘the scorpion’’, since its behaviour resembles that of an (asymmetric) scorpion, possessing two ‘‘chelae’’ and a ‘‘stinging’’ tail, as depicted in Fig. 8. It is seen that this molecule first docks onto the silica wall via the net positive charge of the quaternary nitrogen. Once hooked onto the wall, QPzI is further pasted to it via hydrogen bonding occurring on the deprotonated nitrogen. Finally,

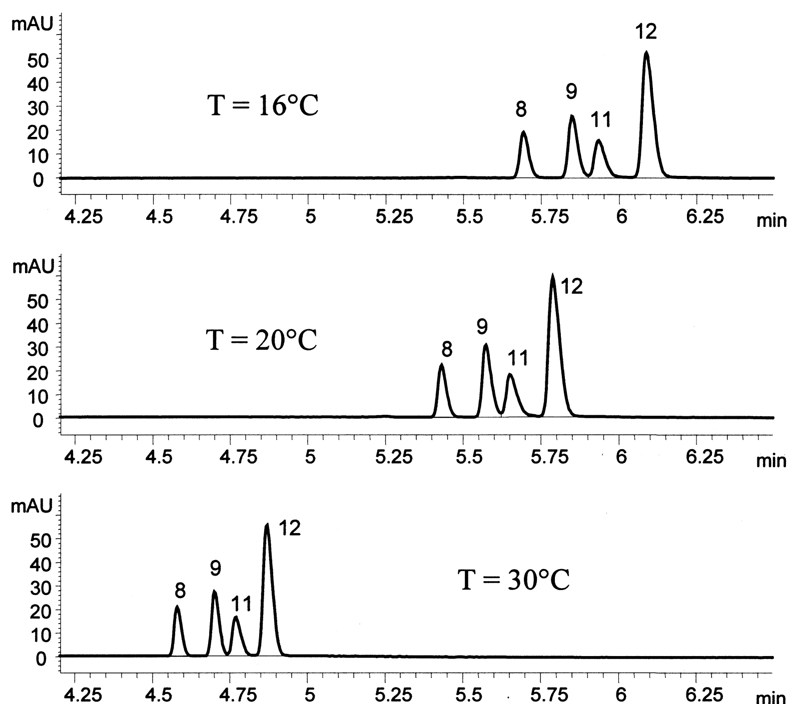

Fig. 6. Effect of the temperature on the silica wall stabilization. Analysis conditions: fused-silica capillary, 60 cm total length×50 μm I.D., 25 m*M* borate buffer, pH 8.5, −20 kV, 20°C, λ=210 nm.. Conditioning: washing (5 bar for 2 min) with the modifier solution (2 m*M* in tetraborate buffer, 25 m*M*, at pH 9.0), followed by a washing (5 bar per 3 min) with running buffer, sample injection (15 mbar for 15 s), injection of a running buffer plug (10 mbar for 5 s).

given the close surface contact, the tail is able to "sting" and form a covalent bond with a neighbouring silanol anion, thus inducing the alkylation of the surface through a siloxane bridge. The close contact arising from the relatively week H-bond and ionic interaction enormously accelerates the nucleophilic substitution of iodine atom for entropic reasons. A unique property of the QPzI would be that, by properly dosing its amount during the preconditioning step, one can achieve full control of the silica surface charge: partial quenching of its negative charge, achievement of a null-charge point and charge reversal, with progressively higher positive charge. This property renders this molecule highly attractive for properly modulating the surface properties of silica, all of this without resorting to the

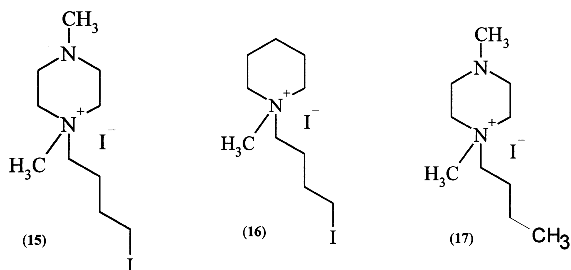

Fig. 7. Quaternary ammonium salts tested in the coating of capillary silica wall.

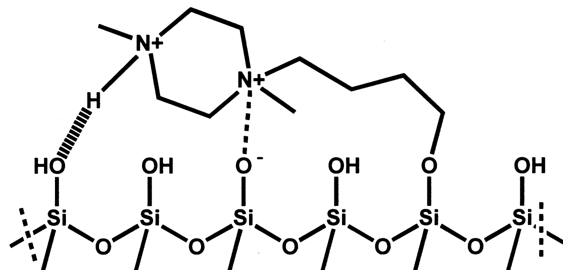

Fig. 8. Proposed silica surface interactions with quaternarized piperazine 15.

very complex and lengthy procedures typically needed when, e.g., trying to anchor covalently neutral polymers to the silica surface. It might sound strange that we can achieve even charge reversal, considering that QPzI is a simple diamine and that, at alkaline pH values, only the quaternary nitrogen is protonated. By assuming that this last, positively-charged nitrogen, always finds a negative counterpart on the silanolic moiety, one should at most reach a point of null charge, but not charge reversal. We hypothesize that, upon docking onto the siliceous surface, the tertiary nitrogen (which normally has a pK of 6.0) might change its pK value, thus being able to acquire a partial positive charge, which might not always be able to find a negatively charged silanol in the immediate environment, due to the ring rigidity. It is hoped that this novel compound will represent an important step forward in solving the serious problem of EOF and its control.

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