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Separation of tosylated polyamines by high-performance capillary zone electrophoresis

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Abstract

The successful separation of tosylated polyamines putrecine (PUT), cadaverine (CAD) spermidine (SPD) and spermine (SPM) by high-performance capillary zone electrophoresis in an uncoated fused-silica capillary is reported. The optimum conditions for obtaining the resolution between PUT and CAD was 5 m*M* sodium phosphate buffer (pH 2.2) containing 0.87 m*M* SPD as an electroosmotic flow modifier, with a gradient voltage that starts at 10 kV and then jumps to 30 kV in different steps. The use of different injection volumes is also discussed.

Keywords: Cadaverine; Spermidine; Spermine; Amines; Polyamines; Putrescine

1. Introduction

Polyamines (PAs) generally include spermidine [SPD, $NH_2(CH_2)_4NH(CH_2)_3NH_2$]; spermine [SPM, $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$] and their precursor, putrescine [PUT, $NH_2(CH_2)_4NH_2$], but cadaverine [CAD, $NH_2(CH_2)_5NH_2$] is also included in this paper. PUT, SPD and SPM are synthesised by decarboxylation of ornithine formed from arginine, whereas CAD is formed by decarboxylation of lysine.

PAs are ubiquitous, found both in eucaryotes and procaryotes and considered necessary for proper cell function and growth [1,2]. The mechanism by which PAs promote and regulate growth in the cell is not well understood. Since Russell [3] reported in 1971 that PA concentrations in the urine of some tumour patients were higher than normal, the relationship between PAs and tumour status has aroused the interest of many researchers and many cases of different tumours with variation in PA concentrations have been reported [4,5]. What has been established so far is that PAs, which in neutral aqueous solution are mainly present as polyammonium ion, form complexes with mononucleotides in aqueous solution [6]. They have a high affinity for DNA and RNA double helices, as would be expected for a positively charged polycation attracted to a negatively charged polyanion, although recent evidence points to interactions between ammonium groups and donor atoms on the bases [7,8].

Traditional methods for separation of PAs are largely confined to thin-layer chromatography [9,10] and HPLC. However, both methods require that PAs be derivatized or labelled before detection, since they have no chromophore and cannot be detected with an ultraviolet (UV) detector. Derivatization of PAs for posterior HPLC analysis included different methods; i.e.: tosylation with *p*-toluenesulphonyl chloride (TsCl) [11], benzoylation with benzoyl chloride and 3,5-dinitrobenzoyl chloride according to the Schotten–Baumann reaction [12–15], dansylation with dansyl chloride [16–19], postcolumn reaction with *o*-phthalaldehyde [20–22] or 2-(1-pyrenyl)ethyl

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chloroformate [23]. This makes the methods tedious and time consuming.

High-performance capillary zone electrophoresis (HPCZE) has proved to be a powerful technique in the separation of charged biomolecules with very high resolution [24,25]. There are not many works on HPCZE of PAs. Ma and co-workers in three recent papers reported measuring PAs in serum with indirect ultraviolet detection using quinine sulfate as background electrolyte [26–28]. It has been demonstrated that diaminoalkanes, such as PAs decreased electroosmotic flow (EOF) [29,30] and this can be used to enhance resolution in the absence of any permanent capillary coating.

The aim of this work was to establish the potential utility of HPCZE for the separation of tosylated PAs with direct ultraviolet detection. We also compared electrophoretic mobility of these substances under different conditions of sample injection and voltage.

2. Experimental

2.1. Chemicals

All chemicals used for the preparation of the buffers, phosphoric acid, sodium phosphate and sodium hydrogencarbonate, were of analytical-reagent grade (Merck, Darmstadt, Germany) and were used as received. Deionized water was doubly distilled and filtered through Millipore (Bedford, MA, USA) GS filters of 0.22-μm pore diameter (DIBDF-water). Ethylamine (ETAM), CAD, PUT, SPD, SPM and *p*-toluenesulphonyl chloride (TsCl) were obtained from Sigma (St. Louis, MO, USA). Acetone, sodium hydroxide (NaOH), hydrochloric acid, chloroform and *n*-hexane were also from Merck.

2.2. Extraction of polyamines from biological material

Maize grains (1 g) were homogenized with 8.0 ml of 5% (w/v) cold perchloric acid [19]. The homogenate was centrifuged at 20 000 *g* for 20 min at 2°C and the supernatant was directly used for polyamine tosylation.

2.3. Tosylation of polyamines

PAs were tosylated by the method of Sugiura et al. [11] slightly modified. PA solutions containing 75 m*M* of ETAM, CAD, PUT, SPD and SPM were prepared in DIBDF-water. To 2.0 ml of sample solutions containing ETAM, which has been used as a reference peak, and PAs, 1.0 ml of 0.5 *M* sodium hydrogencarbonate and 30 mg of TsCl dissolved in 2 ml of acetone were added. The mixture was then warmed in a water-bath at about 70°C for 1 h. After cooling, 5.0 ml of 2.0 *M* NaOH was added. The mixture was washed with three volumes of *n*-hexane and, after adding 7.5 ml of 2.0 *M* hydrochloric acid, the tosylated PAs were extracted with 10.0 ml chloroform. The organic phase was then dried and the residue redissolved in 2.0 ml DIBDF-water. The mixture was then washed again with three 5.0 ml volumes of toluene. Finally, the organic phase was dried under vacuum and the residue redissolved in 2 ml of the electrophoretic buffer, 0.5 m*M* sodium phosphate at pH 5.4 or 2.2, diluted tenfold. All the samples were filtered through a 0.45-μm disposable syringe filter (Alltech, Deerfield, IL, USA).

2.4. Instrumentation

CZE was performed using a three-dimensional capillary electrophoretic system from Hewlett-Packard (Palo Alto, CA, USA), Microbore fused-silica tubing coated with polyimide and extended light path from Hewlett-Packard (part no. G1600-62332, Germany) of 72 cm effective length×75 μm I.D., was used. This special capillary design 'bubble cell' is used to extend the optical pathway without increasing the overall capillary area. The bubble cell is made by forming an expanded region directly within the capillary. Since is located only in the detection region no increase in current occurs. The capillary was enclosed in a cassette for easy handling and thermostated at different temperatures, as indicated. On-line detection was performed with a diode array detector from Hewlett-Packard. Tosylated PAs and ETAM were monitored at 200 and 225 nm and electropherograms were recorded using a CHEMSATION three-dimensional HPCE C1030AX with a Vectra 486/66xM and monitor EVVGA D1196A from Hewlett- Packard.

2.5. Capillary electrophoresis separation conditions

A new capillary was first conditioned with 0.5 *M* NaOH for 5 min at 60°C, 0.05 *M* NaOH for 5 min at 60°C and DIBDF-water for 5 min at 60°C. Further equilibration was with the corresponding running buffer (5 m*M* sodium phosphate at pH 5.4 or 2.2) containing 0.87 m*M* SPD for 60 min, followed for another 60 min with an applied voltage of 11 kV at 30°C. These long times are required when SPD was used as EOF modifier. Regeneration of the capillary surface between runs was performed by rinsing it in the following sequence; 0.05 *M* NaOH for 3 min, DIBDF-water for 5 min and the corresponding buffer for 3 min.

PA solutions, prepared in the electrophoresis buffer, diluted tenfold, were hydrodynamically injected into the capillary with different pressures for different times, as indicated, that correspond to 5.5–19 nl of injection volume and 0.41–1.42 nmol of injected mass. Electrophoretic separations were carried out either at a constant voltage of 15 or 20 kV or at a variable voltage during run, as indicated. Temperature was maintained at 20°C or 30°C, as indicated.

2.6. Data analysis

Samples of ETAM and PAs were analysed at least four times under each set of analytical conditions and the average time for each peak was determined. Data were analysed using a BMDP-2V analysis of variance (ANOVA) program and the mean values were compared using Duncan's multiple range test.

3. Results and discussion

3.1. High-performance capillary zone electrophoresis

HPCZE allows the separation of ionized molecules, which move according to their electrophoretic mobilities [31]. Tosylation procedure was chosen because the use of this derivatization has the following advantages: (i) it proceeds in an aqueous medium; (ii) it enables PAs to be easily extracted from the reaction mixture and (iii) the derivatives of PAs can be detected with a UV monitor without the use of a fluorescence detector. Tosylated PAs have been detected at 200 and 225 nm simultaneously and the absorbance spectrum is shown in Fig. 1. For calculations, the absorbance at 200 nm was chosen since, at observed from the spectrum, it is higher than that at 225 nm.

Primarily amino group of the natural PAs behave similarly to aliphatic primary amines of comparable basicity [32]. The amino groups are ionized when the buffer pH is less than their pK_a values. In this case, as all pK_a values of PAs are higher than 8.6 [32] and the positive electrophoretic mobility of these organic cations, at the pH values (2.2. and 5.5) used, was in the same direction as EOF since all the amino groups of PAs were positively charged. Therefore, in order to minimize adsorption of PAs to charged fused-silica capillary wall and reduce retention time drift, a surface modifier such as SPD at a 0.87 m*M* concentration was added to the running buffer. This PA concentration was selected since it produced the best resolution of several peaks of ovalbumin [30].

Samples were prepared in a tenfold diluted buffer without SDP that has a very similar composition to the support buffer. These results in the formation of an enhanced electric field at the injection point and a sharp rise in the number of ions injected into the column. In the region of the capillary bubble, the electrical resistance is reduced and thus the field is

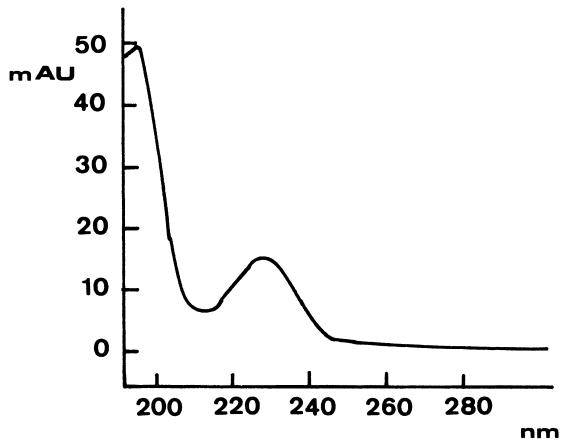

Fig. 1. Absorbance spectrum of 75 m*M* tosylated SPD in 0.5 m*M* sodium phosphate buffer. Tosylated PUT, CAD and SPM have identical spectra.

decreased. Concomitant to this is a proportional decrease in flow velocity due the expanded volume of the bubble. When the zone front enters the bubble its velocity decreases and the zone concentrates or 'stacks' in a manner similar to electrophoretic stacking during injection. As the sample zone expands radially (across the capillary) to fill the increased volume, it contracts longitudinally (along the capillary). Thus the sample concentration remains constant but the path length increases [33]. This stacking process is particularly efficient with small ions [34].

3.2. HPCZE of tosylated PAs under different hydrodynamic injections at constant voltage

To achieve satisfactory separations using HPCZE technique, the optimization of injection volume/amount, are of primary importance. In this work, the combined effects of pressure and time of hydrodynamic injection and voltage applied during runs, on the migration behaviour and selectivity of PAs, are taken into consideration for obtaining optimized separations.

HPCZE of tosylated PAs, was carried out using a 72 cm×75 μm I.D. capillary and 5 m*M* sodium phosphate buffer, pH 5.4, containing 0.87 m*M* SPD at 30°C (or 20°C were indicated) with different hydrodynamic sample injections (Figs. 2–5).

The hydrodynamic flow of a liquid in the capillary is described by Poiseuille equation

$$V = \frac{\Delta P \Pi r^4 t}{8\eta L} = \frac{\Delta P \Pi d^4 t}{128\eta L} \quad (1)$$

or

$$Q = \frac{\Delta P \Pi r^4 t}{8\eta L} C_s = \frac{\Delta P \Pi d^4 t}{128\eta L} C_s \quad (2)$$

where the volume (V) or the amount (Q) introduced is defined by the pressure difference across the capillary (ΔP), the diameter (d), or, alternatively, the duration of the injection (t), the viscosity of the fluid in the capillary (η), the total length of the capillary (L), and the concentration of analyte in the sample matrix (C_s [35]. Using the values of ΔP and t, described in Table 1, we can calculate the sample injection in the order of 5.5–19 nl which represents about 0.4–1.4 nmol of each ETAM and PAs.

Table 1
Volume (V) and amount (Q) of injected PAs as a function of different hydrodynamic injection conditions[a]

Hydrodynamic condition $\Delta P\ t$ (mbar s)	Injection	
	V (nl)	Q[b] (nmol)
17×3	5.46	0.412
5×3	8.12	0.61
17×7	12.84	0.96
25×7	18.88	1.42

[a] Calculations were performed according to Eqs. (1) and (2).
[b] Assuming $C_s = 75$ m*M* of each ETAM and PA.

Relative migration times of PUT, CAD, SPD and SPM, to ETAM, are shown in Fig. 2A (at 20 kV) and Fig. 2B (at 15 kV). The relative peak positions of PUT, SPD and SPM standards seemed to be in accordance with the expected result based on the consideration of the Z/M (charge to mass ratio) values, which are PUT 0.0227, SPD 0.0206 and SPM 0.0197. However, CAD, which has a Z/M value of 0.0196, very similar to SPM, migrates after PUT. Then, under the analytical conditions that we described here, the four standard PAs migrate in accordance with their mass, more than with their Z/M values, since SPD used as a modifier of EOF neutralized the negative charge of the capillary wall. With injection volumes of 5.46–12.84 nl, the four PAs maintained their relative migration times either when the separation voltage was 20 kV (Fig. 2A) or 15 kV (Fig. 2B). However, when injection volume was 18.88 nl, relative migration time of all PAs increased in about 0.1 units. This increase is a consequence of the reduction in the migration time of ETAM. While PAs have positive charges at pH 5.4, ETAM has only one at the N-terminus of the molecule and its migration time is lower than that of PAs. The increase in relative migration times obtained with an injection volume of 18.88 nl was nullified if temperature during running was decreased from 30°C to 20°C (represented with dashed line in Fig. 2A). When separations were carried out at 15 kV and 30°C (Fig. 2B), relative migration times of PAs were independent of injected volume at the assayed conditions. Rose and Jorgenson [36] quantitatively studied hydrodynamic injection procedures using an automated sampling system to minimize operator error. They found that hydrodynamic injection had

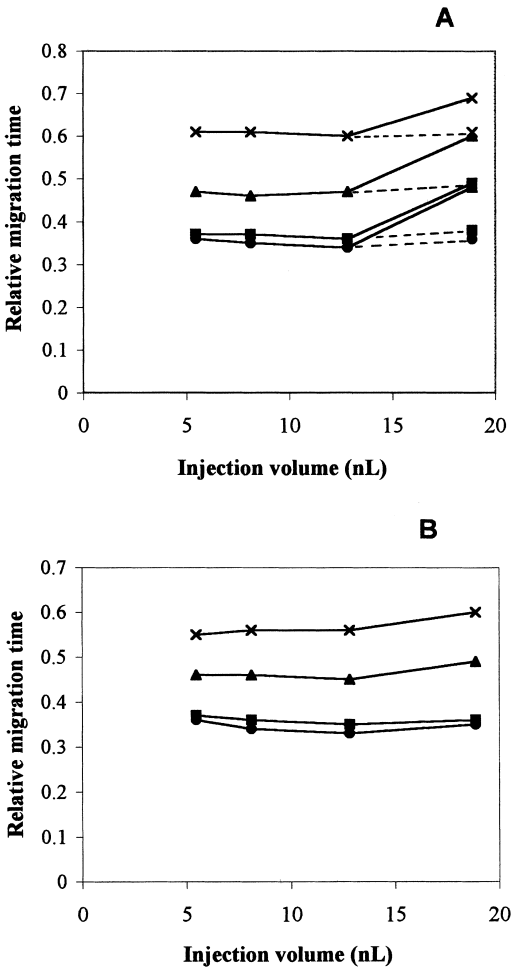

Fig. 2. Relative migration times for (●) PUT, (■) CAD, (▲) SPD and (×) SPM as a function of injected volume. Capillary: 72 cm×75 μm I.D. 'bubble cell'; running electrolyte: 5 m*M* sodium phosphate, pH 5.4, containing 0.87 m*M* SPD, at 30°C; detection at 200 nm in (a) 20 kV and in (b) 15 kV. In (a), for 18.88 nl of injected volume, a second value of relative migration time was obtained (dashed line) at 20°C.

little effect on separation efficiency and, when automated, had a reproducibility of 2.9% R.S.D..

Factors such as (a) applied voltage, (b) temperature and (c) injection volume affected the resolution in the selected experimental ranges. Thus, a relative unit of 400 (20°C×20 kV), which is similar to 450 (30°C×15 kV) was able to maintain relative migration time at different injection volumes from 5 to 19 nl. However, a relative unit of 600, which is (30°C× 20 kV) produced and increase in relative migration times when the injection volume was 18.88 nl, compared with those obtained at the same applied voltage and 20°C. There were some strong interactions among these factors and therefore, some of the effects were combined with each other.

Selectivity factors, $\alpha_{2,1}$, $\alpha_{3,2}$ and $\alpha_{4,3}$ within the same injection volume range, were also measured in order to obtain more information. Fig. 3A and B illustrate these typical plots of $\alpha_{x,y}$ versus injection volume. Resolution between CAD and PUT ($\alpha_{2,1}$ was close to 1.04 and very similar at all the assayed injection volumes (Fig. 3A and B). Both PAs have a very similar mass and a value of $Z=+2$, Whereas selectivity between SPD and CAD ($\alpha_{3,2}$) was lower than that between SPD and SPM ($\alpha_{4,3}$), at 20 kV

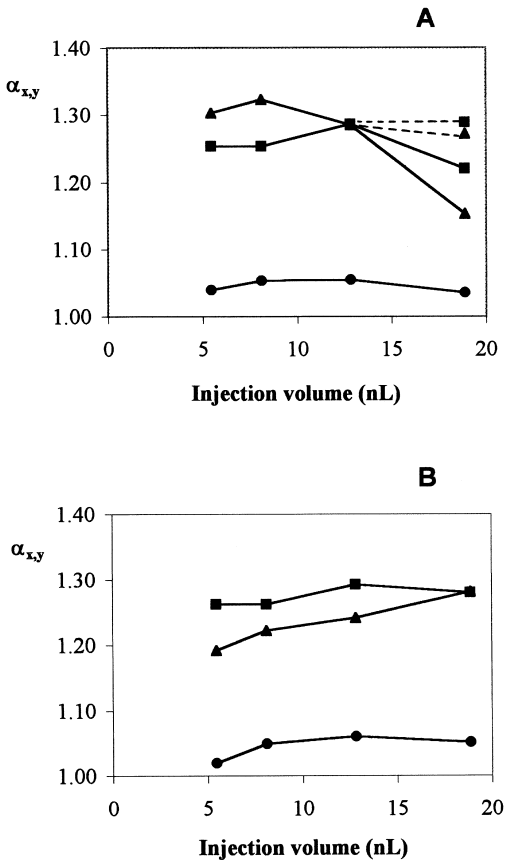

Fig. 3. Selectivity factors of (●) CAD:PUT ($\alpha_{2,1}$), (■) SPD:CAD ($\alpha_{3,2}$ and (▲) SPM:SPD ($\alpha_{4,3}$ as a function of injected volume in (a) at 20 kV and in (b) at 15 kV. In (a) for 18.88 nl of injected volume a second value of relative migration time was obtained (dashed line) at 20°C. Other operating conditions as for Fig. 2.

(Fig. 3A), the contrary occurred at 15 kV (Fig. 3B). Furthermore, both $\alpha_{3,2}$ and $\alpha_{4,3}$ decreased at the highest injection volume when the applied voltage was 20 kV (Fig. 3A). Maintaining separation temperature at 20°C, both $\alpha_{3,2}$ and $\alpha_{4,3}$ were relatively constant with values around 1.3, similar to those obtained at 15 kV and 30°C (Fig. 3B). The capillary temperature affects the resolution in two ways. First, the nature of the buffer medium will be affected by changes in temperature. As temperature increases, the viscosity decreases and both EOF and electrophoretic mobility increase. The mobility of most ions increases about 2%/°C [37]. Second, if the temperature gradients are steep enough, density gradients in the electrophoretic buffer can be induced, which in turn can cause natural convection. A convection will remix separated sample zones and reduces performance severely [38]. Our results show that the combinations of 20°C with other factors at certain levels were better than those of 30°C with the same factors. A typical electropherogram of the four standard PAs is shown in Fig. 4. SPD and SPM are baseline resolved whereas PUT and CAD coeluted. Analysis time is within a reasonable range (<14 min).

Within the sample injection volume (5.46–18.88 nl) and a separation temperature of 30°C normalized areas represented as mAU s increased by a factor of 3.8–11.4 owing to the injection of larger sample volumes either at 20 kV of separation voltage (Fig. 5A) or 15 kV (Fig. 5B). When the temperature was decreased to 20°C, the increase in mAU s is less pronounced at the highest injected volume because of the reduced viscosity of the electrolyte (Fig. 5A). An increased voltage resulted in decreased normalized areas for all the assayed PAs. Our results are in disagreement with those reported by Arentoft et al. [39] since they observed an increase in normalized areas of some oligosaccharides when the voltage increased from 10 to 20 kV.

3.3. HPCZE of tosylated PAs under different hydrodynamic injections at voltage gradients

Another power supply feature is the ability to run voltage, current or power gradients (also called field programming) during an analysis. Field programming can be used to ramp the voltage at the beginning of an analysis to avoid rapid heating, thermal expansion of buffer and expulsion of sample from capillary. Field programming is also particularly useful for decreasing the analysis time.

Five series of stepwise voltage gradients (Fig. 6), three at electrolyte pH 5.4 (gradients a, b and c) and two at pH 2.2 (gradients d and e) were performed. In these experiments, temperature during run was 30°C and injection volume was 5.46 nl. Gradients a and c are very similar, both starting at 10 kV with slow increases to 12 min and a final ramp to achieve 20 kV in gradient a or 15 kV in gradient c. Gradients d and e are similar, during the first 13 min of run, both starting at 10 kV and slowly voltage increases to 13 kV: both finishing at 30 kV, from 15 min in gradient d and from 14 min in gradient e. Gradient b starts at 7 kV, slowly increases during the first 12 min at 10 kV and rapidly now to reach a value of 30 kV at 13 min. Fig. 7A shows relative migration times of the

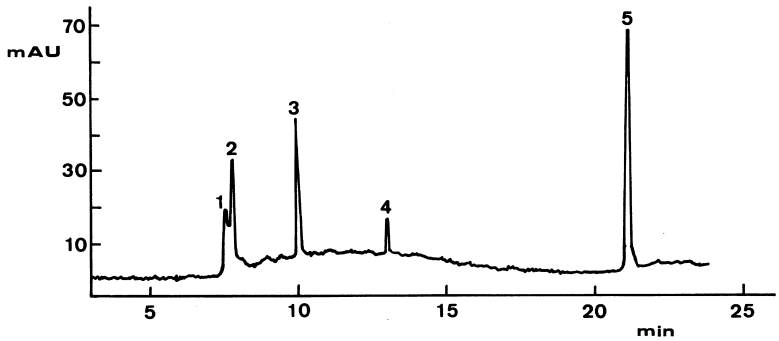

Fig. 4. Electropherogram of standard tosylated PAs (1) PUT, (2) CAD, (3) SPD, (4) SPM and (5) ETAM obtained with an injection volume of 5.46 nl, running electrolyte 5 m*M* sodium phosphate pH 5.4 at 30°C, applied voltage: 20 kV, capillary as for Fig. 2; detection at 200 nm.

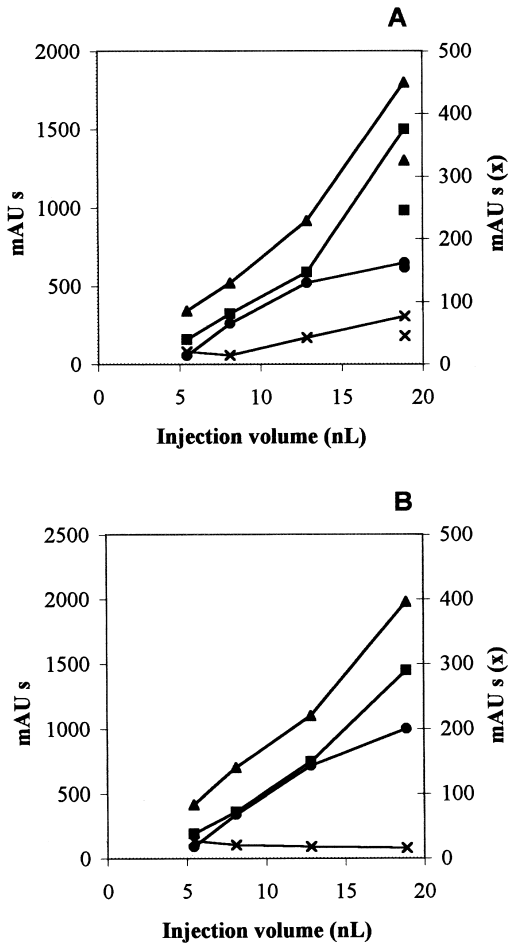

Fig. 5. Normalized areas, expressed as mAbsorbance units per second (mAU s) for (●) PUT, (■) CAD, (▲) SPD and (×) SPM as a function of injected volume in (a) at 20 kV and in (b) at 15 kV. In (a) for 18.88 nl of injected volume a second value of (mAU s) was obtained (dashed line) at 20°C. Other operating conditions as for Fig. 2.

four standard PAs at different power gradients during run. Gradients a and c provided similar relative migration of all PAs whereas these migrated nearly in gradient b.

In HPCZE, the pH of the buffer plays an important role in the separation of ionizable analytes because it determines the extent of ionization of each individual analyte [40]. As the amino groups of PAs are ionized when the buffer pH is less than their pK_a values, the positive electrophoretic mobility of these organic cations increases when the buffer pH decreases. At pH 2.2 the EOF is lower and protonation of the amino groups are higher than at pH 5.4 and so, PAs migrate slowly. Relative migration times are the lowest at pH 2.2 when power gradients d and e are employed (Fig. 7A) since migration time of ETAM was also very high. Selectivity factors between PAs is displayed in Fig. 7B. The best separation between PUT and CAD was achieved at pH 2.2 with gradient e being resolution of the other PAs also good. Gradient c provides the best selectivity of SPM/SPD. Poor resolution was achieved at pH 5.4 when applied power gradient b.

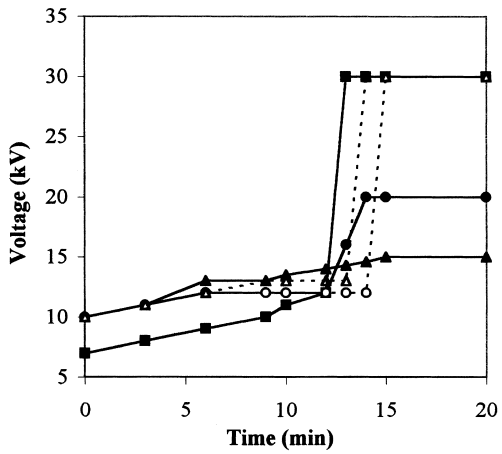

Fig. 6. Gradient voltages at two pH values of electrolyte: (●) gradient a, (■) gradient b, (▲) gradient c and (○) gradient d and (△) gradient e. Gradient a, b and c at pH 5.4; gradients d and e, at pH 2.2. Temperature, 30°C and capillary as for Fig. 2.

The charge of the capillary wall surface and the zeta potential are also influenced by buffer pH [40,41]. The negative charge is built up at the surface with increasing pH. Lin et al. [42] observed changes of electroosmotic mobility from $5.1 \cdot 10^{-4}$ at pH 5.6 to $5.8 \cdot 10^{-4}$ $cm^2\,V^{-1}\,s^{-1}$ at pH 6.8. A 50 m*M* phosphate buffer, pH 2.35, containing diaminopropane as a surface modifier was chosen as the best compromise between analysis time and resolution of 21 aromatic amines [43]. The effect of gradient voltages on normalized areas is shown in Fig. 7C. Higher values of mAU s were achieved at pH 5.4 than at pH 2.2, the highest being those observed in gradient a. Gradient d, at an electrolyte pH value of 2.2 produced the lowest normalized area values. However, the best response of SPM was achieved at

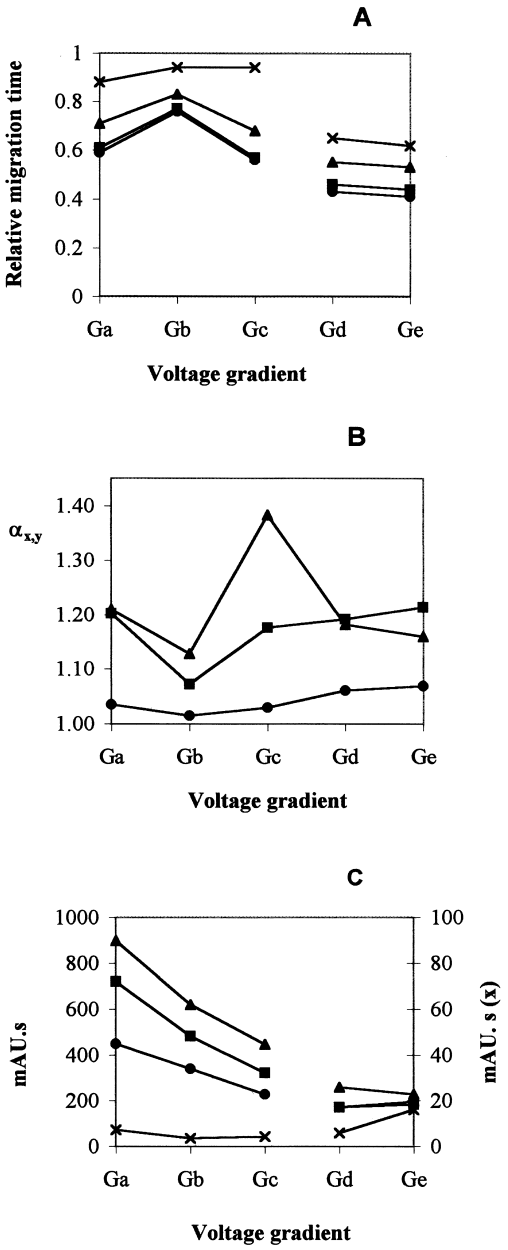

Fig. 7. Relative migration times (A), selectivity factors (B) and normalized areas (C) of (●) PUT, (■) CAD, (▲) SPD and (×) SPM obtained at gradient voltages described in Fig. 6. Symbols in Fig. 7B as described for Fig. 3. Temperature, 30°C; injection volume, 5.46 nl; capillary, as for Fig. 2.

pH 2.2 with a gradient voltage that starts and maintain the highest values.

pH is an important factor in determining selectivity in HPCZE since it affects the dissociation of analytes which in turn changes the electrophoretic mobility of charged analyte [44]. The change in the degree of dissociation (α) of an analyte is more significant when the pH is near its pK_a; α can be estimated from Eq. (3)

$$\alpha = K_a/K_a + [H^+] \quad (3)$$

where K_a is the dissociation constant of the analyte [45]. Table 2 shows α_i values depending on the pH of the buffer system used. As pH values change slightly as a function of temperature (0.25 units from 20 to 50°C in sodium phosphate buffer at pH 5.4 and 0.46 units in sodium phosphate buffer at pH 2.2; data not shown), it is possible to generate a certain pH gradient by controlling the temperature of the column using the effect of voltage-induced pH change. Whang and Yeung [46] demonstrated the effect of temperature-induced pH change on the separation of dyes. A step change in voltage from 15 to 25 kV was implemented to generate a pH gradient in the buffer solution for separating phenols which cannot be separated at fixed voltage [45].

Fig. 8 compares the separation of standard PAs at two different pH values of the electrolyte and voltage gradients. Under gradient a at pH 5.4, partial separation between PUT and CAD is achieved (Fig. 8A) whereas these two PAs are baseline resolved at pH 2.2 under gradient d (Fig. 8B). Peaks are narrower at pH 2.2 than at pH 5.4 with or without voltage gradients. From these results, we can conclude that voltage programming is advantageous for certain CE separations. As has been shown [45,47,48], groups of analytes that cannot be separated at a single pH value can be resolved by pH programming. Furthermore, the use of voltage programming is in general simpler than the use of temperature programming [45]. Different field operation techniques have been described recently to achieve better separation of different size DNA molecules, mainly with slab gel electrophoresis. Dennison et al. [49] employed ionic or wedge-shaped slab gels to linearize the logarithmic distribution of bands by a nonliner voltage gradient method. Pulsed electric fields have been used to enhance separation of DNA restriction fragments [50,51].

Separation of polyamines of a biological samples,

Table 2
Changes in the degree of dissociation (α_i) of PAs as a function of their pK_a values, at pH 5.4 and 2.2

Amine or polyamine	p$K_{a(s)}$	K_a (M)	α_i	
			pH 5.4	pH 2.2
ETAM	10.63	$2.34\cdot10^{-11}$	$5.88\cdot10^{-6}$	$3.88\cdot10^{-9}$
PUT	9.71	$1.95\cdot10^{-10}$	$4.89\cdot10^{-5}$	$3.09\cdot10^{-8}$
	11.15	$7.08\cdot10^{-12}$	$1.78\cdot10^{-6}$	$1.12\cdot10^{-9}$
CAD	10.02	$9.55\cdot10^{-11}$	$2.39\cdot10^{-5}$	$1.51\cdot10^{-8}$
	10.96	$1.09\cdot10^{-11}$	$2.75\cdot10^{-6}$	$1.74\cdot10^{-9}$
SPD	8.88	$1.32\cdot10^{-9}$	$3.31\cdot10^{-4}$	$2.09\cdot10^{-7}$
	10.15	$7.08\cdot10^{-11}$	$1.78\cdot10^{-5}$	$1.12\cdot10^{-8}$
	11.06	$8.70\cdot10^{-12}$	$2.18\cdot10^{-6}$	$1.38\cdot10^{-9}$
SPM	8.60	$2.51\cdot10^{-9}$	$6.30\cdot10^{-4}$	$3.98\cdot10^{-7}$
	9.33	$4.68\cdot10^{-10}$	$1.17\cdot10^{-4}$	$7.42\cdot10^{-8}$
	10.45	$3.55\cdot10^{-11}$	$8.92\cdot10^{-6}$	$5.63\cdot10^{-9}$
	11.23	$5.89\cdot10^{-12}$	$1.48\cdot10^{-6}$	$9.33\cdot10^{-10}$

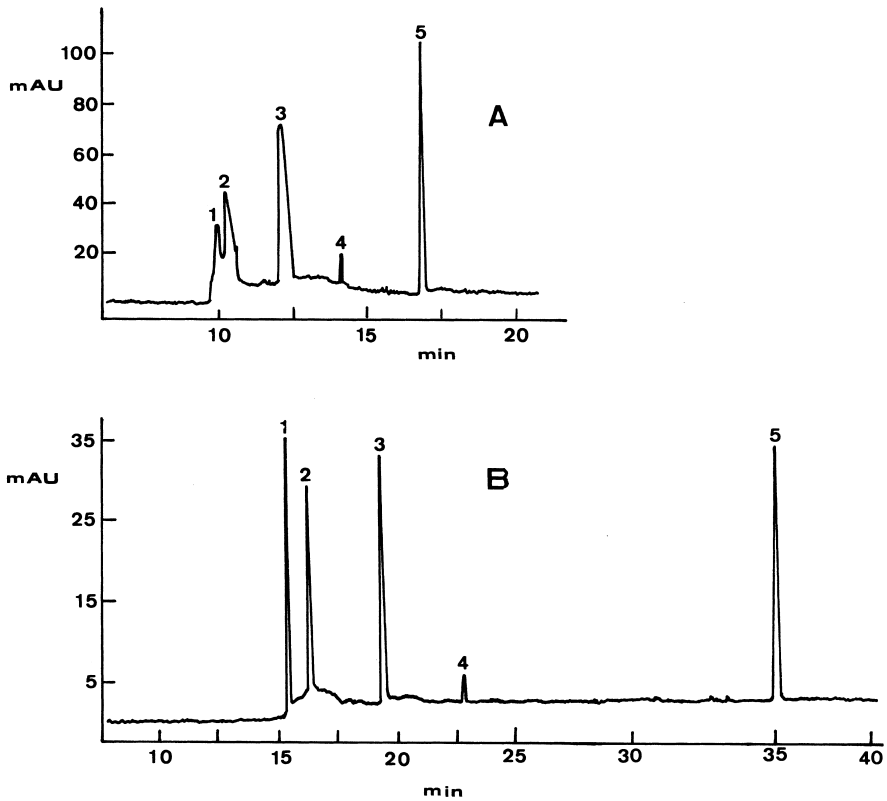

Fig. 8. Separation of tosylated (1) PUT, (2) CAD, 3 (SPD) (4) SPM in (A) gradient voltage a, pH 5.4, and in (B) gradient voltage d, pH 2.2. Temperature 30°C; injection volume, 5.46 nl; capillary, as for Fig. 2; detection at 200 nm.

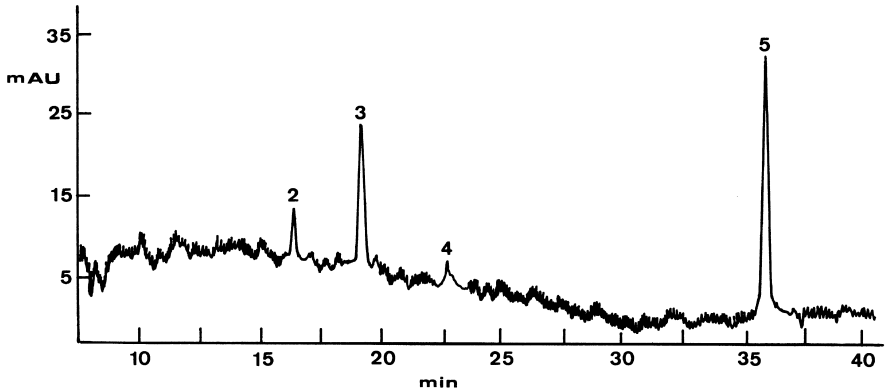

Fig. 9. Separation of tosylated (2) CAD, (3) SPD and (4) SPM from maize grains. Operating conditions as for Fig. 8B.

such as an extract from maize grains, using the method developed here is shown in Fig. 9. The sample contained CAD, SPD and SPM, but not PUT. These polyamines were separated at pH 2.2 using the voltage gradient d.

4. Conclusion

In this paper we have optimized the separation of tosylated PUT, CAD, SPD and SPM by HPCZE using two pH values of the running electrolyte (5.4 and 2.2) either at constant or variable voltage. The separation of the four standard PAs has been satisfactorily achieved using 5 m*M* sodium phosphate buffer (pH 2.2) with a voltage programming that starts at 10 kV and then progressively increases to 30 kV at 14 min for the remainder of the run. The analysis time is less than 22 min. The introduction of the internal standard ETAM prior to extraction allowed corrections for differences in injection volume among samples.

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