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Impact of experimental parameters on the resolution of positional isomers of aminobenzoic acid in capillary zone electrophoresis

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ABSTRACT

The separation process in capillary zone electrophoresis is governed by many experimental parameters, such as the choice of the electrolyte, field strength, ionic strength, pH, temperature and additives (including organic modifiers). Positional isomers of substituted benzoic acids were used as model compounds in order to study the contributions of pH, electrolyte, ionic strength, addition of alcohols, the choice of the counter ion and the temperature to the electrophoretic separation and the magnitude of the electroosmotic flow. The results thus obtained were compared critically with those of previous studies, in which only the modifier content had been varied. Obviously, pH was the most effective parameter in optimizing the resolution. However, thermostating of the capillary at elevated temperatures turned out to be a very effective additional feature; the resolution was increased and the analysis time was shortened considerably. Other options, such as the addition of alcohols or the choice of the counter ion, will also increase the resolution but also increase the analysis time. The improvement in the concentration sensitivity was studied using the technique of sample stacking. Volumes of up to 15 nl could be introduced in this way without serious separation problems, yielding a detection limit of 10^{-6} *M*.

INTRODUCTION

Capillary zone electrophoresis (CZE) offers fast and efficient separations of ionic and ionizable compounds [1,2]. The separation is primarily based on the different mobilities of analytes in an electric field. For a given set of experimental parameters, the effective mobility depends on the net charge, size and conformation of a molecule. For each separation, the experimental parameters have to be optimized in order to exploit fully the potential of CZE. Some of these parameters are pH, ionic strength, buffer additives (including organic solvents), counter ion, capillary material and temperature.

We studied the effects of these parameters on resolution, plate number, electroosmosis and electrophoresis, using positional isomers of aminobenzoic acid as model compounds. The dissociation constants of these model compounds are similar: *p*-aminobenzoic acid, $pK_1 = 2.41$ and $pK_2 = 4.85$; *m*-aminobenzoic acid, $pK_1 = 3.12$ and $pK_2 = 4.74$ [3]. Fujiwara and Honda [4] were able to improve the resolution of

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these compounds by the addition of methanol (resolution from 0.8 to 1.5) or acetonitrile (from 0.8 to 2.2). In addition to their work, we have also studied other parameters as mentioned above. Particularly the impact of temperature and the counter ion on the resolution in CZE have been neglected in the past. We also studied the potential of sample stacking [5] in order to improve the concentration sensitivity of the model compounds.

EXPERIMENTAL

Apparatus

An Applied Biosystems (San Jose, CA, U.S.A.) Model 270A capillary electrophoresis system [5] was used, equipped with a variable wavelength UV absorbance detector, operated at either 200 or 254 nm. CZE was performed in a 72 cm × 50 μm I.D. fused-silica capillary (Applied Biosystems) at 25 kV (constant-voltage mode). The capillary was thermostated at 30.0 ± 0.1°C unless indicated otherwise. Data were recorded using a Nelson Analytical Model 4400 integration system.

Chemicals

Distilled water was purified in a Milli-Q apparatus (Millipore, Bedford, MA, U.S.A.). Morpholinoethanesulphonic acid (MES) was obtained from Sigma (St. Louis, MO, U.S.A.), *p*- and *m*-aminobenzoic acid from laboratory stock and mesityl oxide from Aldrich (Steinheim, Germany). All the other chemicals were of analytical-reagent grade from J. T. Baker (Deventer, The Netherlands).

Methods

Buffers were prepared in Milli-Q water and adjusted to a specific pH using a Philips (Cambridge, U.K.) Model PW 9409 pH meter. Stock solutions of *p* and *m*-aminobenzoic acid (10^{-2} *M*) were prepared in methanol. Each day, samples were freshly diluted with buffer solution or mixtures of the buffer solution with Milli-Q water, yielding 10^{-4} *M* sample solutions. Buffers and samples were filtered through 0.45-μm Spartan 30/B filters (Schleicher & Schüll, Dassel, Germany) prior to use.

Samples were introduced to the capillary via a controlled vacuum system. Unless stated otherwise, the injection time was 1.0 s, which corresponds to a volume of *ca.* 3.0 nl. The coefficient of electroosmotic flow was calculated using mesityl oxide as a neutral marker.

RESULTS AND DISCUSSION

The coefficient of electroosmotic flow, μ(eo) in $cm^2\ V^{-1}\ s^{-1}$, and the effective electrophoretic mobility, μ(ep) in $cm^2\ V^{-1}\ s^{-1}$, were calculated as follows:

$$\mu_{(eo)} = \frac{L_{(d)}L_{(t)}}{t_{(eo)}V} \tag{1}$$

$$\mu(ep) = \frac{L_{(d)}L_{(t)}}{V}\left[\frac{1}{t_{(m)}} - \frac{1}{t_{(eo)}}\right] \tag{2}$$

where $L_{(t)}$ (cm) is the total length of the capillary, $L_{(d)}$ the distance between the inlet of the capillary and the detector (50 cm), V (V) the applied voltage $t_{(eo)}$ (s) the migration time of the neutral marker and $t_{(m)}$ (s) the migration time of the analyte. The resolution, R_s, was calculated using

$$R_s = 0.25\, N^{0.5} \left[\frac{\mu_{(ep1)} - \mu_{(ep2)}}{\mu_{(epm)} + \mu_{(eo)}} \right] \tag{3}$$

where $\mu_{(epm)}$ (s) is the mean value of $\mu_{(ep1)}$ and $\mu_{(ep2)}$. The plate number, N, was determined using the equation

$$N = \left[\frac{t_{(m)}}{\sigma_{(t)}} \right]^2 \tag{4}$$

in which $\sigma_{(t)}$ (s) represents the peak half-width at 0.6 of the peak height.

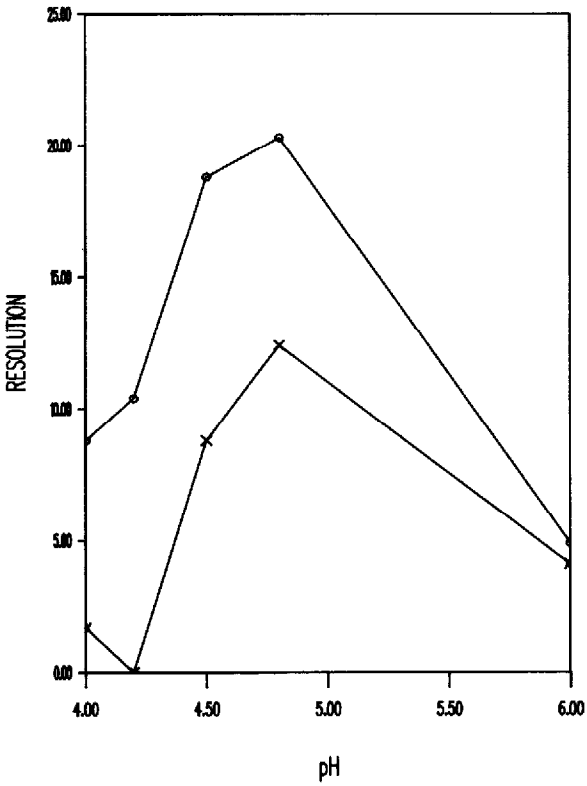

Fig. 1. Influence of pH on the resolution of *p*- and *m*-aminobenzoic acid. Conditions: CZE at 25 kV in 40 m*M* ammonium acetate buffer. Capillary thermostated at (×) 30.0°C and (○) 60.0°C.

Influence of pH

The pH of a 40 m*M* ammonium acetate buffer was varied between 4.0 and 6.0. As expected [6], the resolution was found to be optimum at a pH value close to the p*K* values of the analytes (Fig. 1). A minimum was found at pH 4.2 (co-migration). Below pH 4.2, a reversal of the migration order of *p*- and *m*-aminobenzoic acid was observed, following their p*K* values (*p*-aminobenzoic acid, pK_1 = 2.41 and pK_2 = 4.85; *m*-aminobenzoic acid, pK_1 = 3.12 and pK_2 = 4.74). Typical plate numbers ranged between 170 000 and 200 000 throughout the pH range investigated. Both $\mu_{(eo)}$ and $\mu_{(ep)}$ were reduced on decreasing the pH from 6 to 4, owing to the increased neutralization of the capillary wall and the decreased ionization of the analytes, respectively.

Similar experiments were carried out using 40 m*M* phosphate buffers between pH 5.8 and 6.8. In contrast with ref. 4, baseline separation could easily be obtained at pH 6.8 (Fig. 2). Probably band broadening due to Joule heating was significantly higher in that study (current 100 μA *versus* 38 μA in our phosphate system).

Influence of ionic strength

The ionic strength was varied using 10–100 m*M* MES buffers at pH 6.0. At lower buffer concentrations, both $\mu_{(eo)}$ and $\mu_{(ep)}$ increased owing to an increase in the zeta potential. This increase was found to be more pronounced for $\mu_{(eo)}$, yielding up to twice as fast separations at 10 m*M*. The plate number, however, decreased dramatically (from 210 000 to 40 000) and the resolution was lost completely at 10 m*M* (Fig.

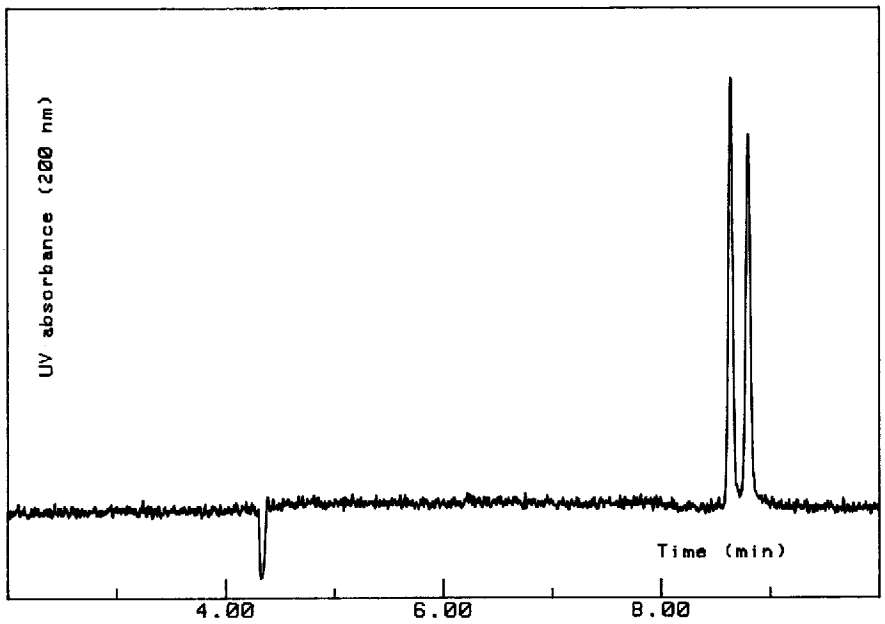

Fig. 2. CZE separation of *p*- and *m*-aminobenzoic acid at 25 kV in 40 m*M* sodium phosphate buffer (pH 6.8). Capillary thermostated at 30.0°C.

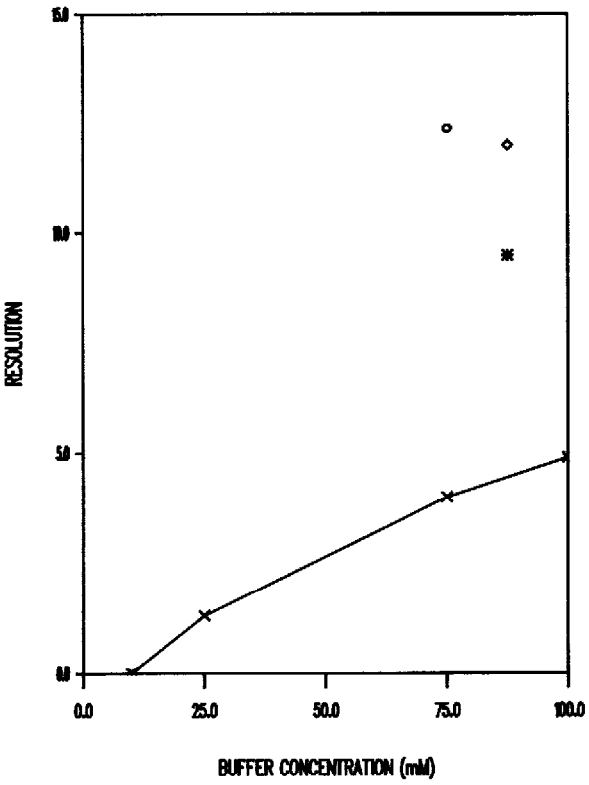

Fig. 3. Influence of buffer concentration on the resolution of *p*- and *m*-aminobenzoic acid. (×) MES buffer (pH 6.0); (○) MES buffer–methanol (75:25); (✳) MES buffer–isopropanol (87.5:12.5). Capillary thermostated at 30.0°C, except for (◇) MES buffer–isopropanol (87.5:12.5) at 60.0°C.

3). A similar trend was observed in a series of experiments using phosphate buffers at pH 6.8.

The decrease in plate number might be attributed to band broadening caused by the sample volume, migrational dispersion [7], inhomogeneous electroosmosis or time constants, etc.

Influence of alcohols

Only a few organic solvents are compatible with the materials used in our CZE apparatus. We investigated the addition of both methanol and isopropanol (IPA) to phosphate (Table I) and MES (Table II) buffer systems.

The resolution obtained with a 40 m*M* phosphate buffer (pH 6.8)–methanol (75:25) system did not differ from that obtained with a purely aqueous system. The analysis time, however, was doubled owing to a strong decrease in $\mu_{(eo)}$ and a minor decrease in $\mu_{(ep)}$. The decrease in plate number from 200 000 to 74 000 can be explained at least partially by the impact of the decreased apparent mobility [$\mu_{(ep)} + \mu_{(eo)}$] on the diffusion. Similar results were obtained with phosphate–IPA (87.5:12.5). In addition to the different wetting characteristics, the stronger effect of IPA can be attributed to its higher viscosity.

TABLE I

EFFECT OF THE ADDITION OF ALCOHOLS ON THE CZE SEPARATION OF AMINOBENZOIC ACID ISOMERS

Conditions: 40 m*M* phosphate buffer (pH 6.8); 25 kV constant voltage; samples, 3 nl, 10^{-4} *M*; capillary thermostated at 30°C; detection by UV absorbance at 200 nm. Analytes: P = *p*-aminobenzoic acid; M = *m*-aminobenzoic acid. Other conditions are given under Experimental.

Parameter	Aqueous phosphate	Phosphate–methanol (75:25)	Phosphate–IPA (87.5:12.5)
$\mu_{(ep,P)}$ (10^{-3} cm^2 V^{-1} s^{-1})	−0.269	−0.189	−0.185
$\mu_{(ep,M)}$ (10^{-3} cm^2 V^{-1} s^{-1})	−0.274	−0.193	−0.189
$\mu_{(eo)}$ (10^{-3} cm^2 V^{-1} s^{-1})	+0.546	+0.333	+0.320
N	200 000	74 000	64 000
i (μA)	38	23	24
R_s	2.0	2.0	1.9
Analysis time (min)	9	20	20

Generally, the influence of alcohols was found to be much more pronounced than reported in ref. 4. This discrepancy can be explained by the smaller I.D. of our capillary and hence the larger impact on electroosmosis.

With regard to the analysis time, *and* with regard to $\mu_{(ep)}$, *and* with regard to $\mu_{(eo)}$, Table II shows similar trends for the MES–methanol system as for the phosphate systems in Table I. The plate number decreased from 210 000 to 120 000. The resolution between *p*- and *m*-aminobenzoic acid, however, was improved considerably (see also Fig. 3). Substituting 12.5% IPA for methanol yielded similar results and again an improved resolution (asterisk in Fig. 3).

Although a direct comparison of the phosphate and the MES buffer systems is not allowed (slightly different ionic strengths and pH conditions), it is clearly suggested that zwitterionic buffers, such as MES, not only are beneficial because of their lower conductivity at relatively high concentrations, but also might have a significant

TABLE II

EFFECT OF THE ADDITION OF ALCOHOLS ON THE CZE SEPARATION OF AMINOBENZOIC ACID ISOMERS

Conditions: 100 m*M* MES buffer (pH 6.0); detection by UV absorbance at 254 nm. Other conditions as in Table I.

Parameter	Aqueous MES	MES–methanol (75:25)	MES–IPA (87.5:12.5)
$\mu_{(ep,P)}$ (10^{-3} cm^2 V^{-1} s^{-1})	−0.256	−0.161	−0.160
$\mu_{(ep,M)}$ (10^{-3} cm^2 V^{-1} s^{-1})	−0.266	−0.179	−0.174
$\mu_{(eo)}$ (10^{-3} cm^2 V^{-1} s^{-1})	+0.480	+0.294	+0.304
N	210 000	120 000	140 000
i (μA)	21	12	13
R_s	4.9	12.4	9.5
Analysis time (min)	11	19	18

TABLE III

EFFECT OF THE COUNTER ION ON THE CZE SEPARATION OF AMINOBENZOIC ACID ISOMERS

Conditions: 40 m*M* acetate buffer (pH 5.4); 25 kV constant voltage; samples 1.5 nl, 10^{-4} *M*; the mobility data are mean values of duplicate experiments; other conditions as in Table I.

Parameter	Lithium (μ^a = 0.401)	Sodium (μ^a = 0.519)	Potassium (μ^a = 0.762)
$\mu_{(ep,P)}$ (10^{-3} cm^2 V^{-1} s^{-1})	−0.228 ± 0.001	−0.242 ± 0.001	−0.250 ± 0.001
$\mu_{(ep,M)}$ (10^{-3} cm^2 V^{-1} s^{-1})	−0.245 ± 0.001	−0.260 ± 0.001	−0.268 ± 0.001
$\mu_{(eo)}$ (10^{-3} cm^2 V^{-1} s^{-1})	+0.486 ± 0.001	+0.464 ± 0.002	+0.442 ± 0.001
N	200 000	170 000	165 000
i (μA)	22	26	27
R_s	7.6	8.7	10.0
Analysis time (min)	10	12	14

[a]Cationic mobility at infinite dilution (10^{-3} cm^2 V^{-1} s^{-1}).

impact on the selectivity of the CZE system. However, more data will be required to support this statement statistically.

Influence of the counter ion

Lithium, sodium and potassium acetate buffers at pH 5.4 were applied to the separation of *p*- and *m*-aminobenzoic acid. The results are given in Table III.

As shown by the data in Table III, the choice of the counter ion does have an important effect on the electrophoretic mobility of the analytes: the $\mu_{(ep)}$ values of *p*- and *m*-aminobenzoic acid increase in the order lithium < sodium < potassium. Actually, one would expect a reversed order as the absolute ionic mobilities (at infinite dilution) of these cations increase from lithium to potassium. The cationic environment around the anionic analytes would yield enhanced relaxation and retardation forces [8] and, consequently, a diminished electrophoretic mobility of the anionic analytes, instead of the observed increase.

In order to exclude possible effects caused by the dissociation equilibria of the weakly acidic analytes, the experiment was repeated using the strongly aromatic acid, *p*-toluenesulphonic acid. However, the results given in Table IV clearly show the same trends as in Table III.

Another possible explanation, namely an increase in electrophoretic mobility due to the increase in current and increased Joule heating, is not supported by the

TABLE IV

EFFECT OF THE COUNTER ION ON THE CZE DATA FOR *p*-TOLUENESULPHONIC ACID

Conditions as in Table III.

Parameter	Lithium	Sodium	Potassium
$\mu_{(ep)}$ (10^{-3} cm^2 V^{-1} s^{-1})	−0.305 ± 0.001	−0.317 ± 0.001	−0.327 ± 0.001
$\mu_{(eo)}$ (10^{-3} cm^2 V^{-1} s^{-1})	+0.485 ± 0.001	+0.448 ± 0.001	+0.431 ± 0.001
Analysis time (min)	14	19	24

observed trend in the $\mu_{(eo)}$ values. Obviously, this experimental set-up is far from the theoretical requirement for infinite dilution, but apart from that we did not find a sound explanation for the discrepancies observed.

From a practical point of view, the choice of the counter ion might be used to optimize the resolution of the anionic analytes. Despite the slightly lower plate number obtained with the potassium acetate system, an increased resolution between *p*- and *m*-aminobenzoic acid was observed, owing to the increased matching of $\mu_{(epm)}$ and $\mu_{(eo)}$ (*cf.*, eqn. 3). However, the analysis time will increase from lithium to potassium, because of the reduced electroosmotic flow and the increased electrophoretic mobility (in the opposite direction).

Influence of temperature

In CZE, temperature is usually considered in a negative context, *e.g.*, in terms of Joule heating. However, temperature can also be exploited as a selectivity parameter. It has been observed that an increased temperature will shorten the analysis time, owing to a lower viscosity. It has also been suggested that chemical equilibria can be influenced by temperature [9] or pH gradients can be thermally generated [10]. In this study, we used the temperature dependence of chemical equilibria as a selectivity parameter. Ammonium acetate buffer (40 m*M*) was used at 30.0 and 60.0°C in the pH range 4.0–6.0. The results at 60.0°C are included in Fig. 1. It can be seen that the resolution improved over the entire pH range investigated. A resolution of more than 20 could be obtained for the positional isomers of aminobenzoic acid. In contrast to the experiments at 30.0°C (see *Influence of pH*), co-migration and a reversal of the migration order did not occur within this pH range, but they probably will below pH

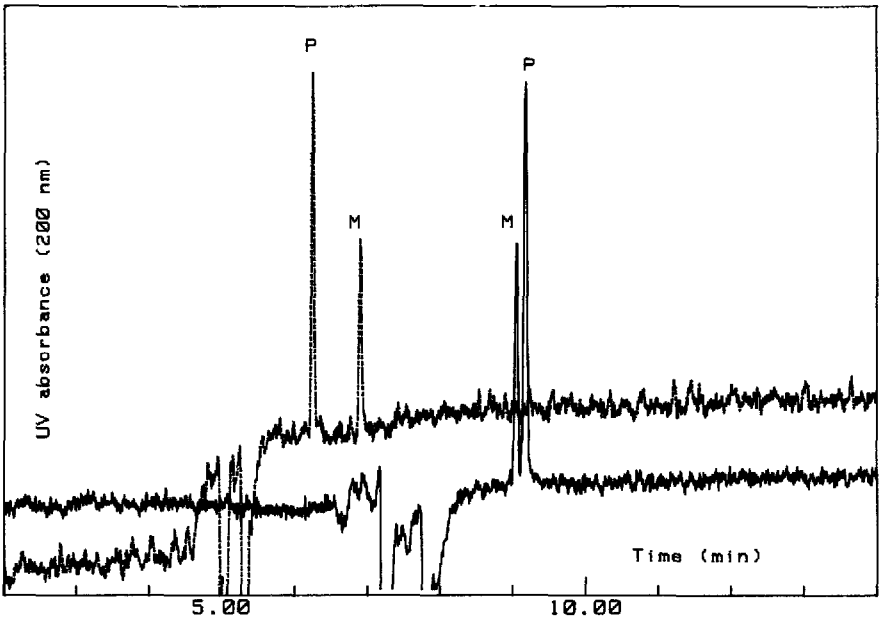

Fig. 4. CZE separation of *p*- (P) and *m*-aminobenzoic acid (M) at 25 kV in 40 m*M* ammonium acetate buffer (pH 4.0). Solid line, 30.0°C; dashed line, 60.0°C.

4.0. The migration order at pH 4.0 can be completely reversed, *i.e.*, the original migration order can be restored, just by increasing the temperature (Fig. 4).

The increase in resolution over the pH range investigated and the restoring of the original migration order at elevated temperatures can be explained by the temperature dependence of the chemical equilibria involved. Both positional isomers show increased effective mobilities, $\mu_{(ep)}$, owing to the lowered viscosity and the increase in their dissociation constants (lower pK values). However, only the *meta* isomer will be subjected to an additional chemical equilibrium. The analytes will exist either in the zwitterionic form or in the deprotonated zwitterionic form. Two molecules of the *meta* isomer will be able to form a dimer because of the electrostatic interaction and/or hydrogen-bonding ability between each other's amino and carboxyl groups. This additional equilibrium will result in a decrease in the effective mobility of *m*-aminobenzoic acid at 30.0°C. At 60.0°C, however, this chemical equilibrium will be much less pronounced, resulting in a much larger increase (relative to the *para* isomer) of $\mu_{(ep)}$ than would be expected from the viscosity and acid–base equilibrium alone. Thus an increase in selectivity and resolution will be obtained and the point of co-migration and peak reversal will be shifted towards a pH value below 4.0. The validity of this explanation was supported by the separation of the corresponding N-acetamides of *p*- and *m*-aminobenzoic acid. Obviously, these molecules will not be able to form a zwitterion, and the *meta* isomer would not be subjected to the above-mentioned additional chemical equilibrium. Indeed, an increase in temperature did not result in any increase in selectivity or resolution. The $\mu_{(ep)}$ values for the respective isomers increased exactly to the same extent.

At 60.0°C, the analysis time was reduced by only 30%. A larger reduction would have been expected, based on the dependence of viscosity on temperature ($-2\%°C^{-1}$). However, the reduced viscosity will influence both $\mu_{(eo)}$ and $\mu_{(ep)}$ in opposite directions. Additionally, it should be noted that our apparatus allows only $L_{(d)}$ to be thermostated, *i.e.*, about 70% of $L_{(t)}$. Improved resolution at higher temperature was also observed with the MES–IPA buffer system (Fig. 3).

Influence of sample volume

The influence of the sample volume on peak broadening has been emphasized recently [11,12]. On the other hand, when the sample is dissolved in a buffer of much lower ionic strength than that of the run buffer, peak compression will occur (sample stacking) [5] at the interface between the sample plug and the run buffer.

p-Aminobenzoic acid was dissolved in 40 mM ammonium acetate buffer and separated using 40 mM ammonium acetate at pH 4.8. The sample volume was varied between 0 and 90 nl and the calibration graphs thus obtained are shown in Fig. 5. The peak area was found to increase linearly within the range tested. A plot of the peak height, on the other hand, showed that serious band broadening occurred at larger injection volumes.

From the point of view of improvement of the concentration sensitivity, a volume of up to 15 nl can be introduced, thereby still obtaining a reasonable increase in peak height, provided that the ionic strength of the sample is only one-tenth of that of the run buffer. Using the sample stacking technique, the detection limit of *p*- and *m*-aminobenzoic acid was found to be 10^{-6} M.

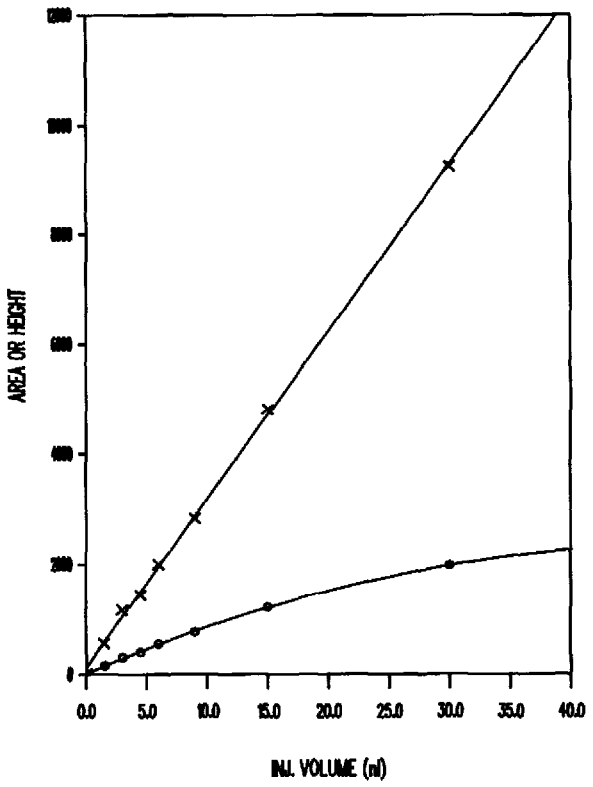

Fig. 5. Calibration graphs for *p*-aminobenzoic acid (sample stacking). (×) Peak area; (○) peak height in arbitrary units.

CONCLUSION

The influence of several experimental parameters on the resolution of *p*- and *m*-aminobenzoic acid has been investigated. Not surprisingly, pH had the strongest impact on resolution and selectivity. However, an increase in the temperature of the thermostated capillary was also very beneficial. The resolution improved and the analysis time was shortened considerably.

Unexpected results were obtained on varying the counter ion in acetate buffers. The electrophoretic mobilities of both strongly and weakly acidic aromatic model compounds increased significantly in the (cationic) order lithium < sodium < potassium. An improved resolution was obtained using potassium acetate; however, the analysis time increased by up to 40%.

The addition of alcohols resulted in an improved resolution at the expense of an increase in analysis time. Addition of 12.5% of IPA yielded similar results to those obtained with 25% of methanol. For the alcohol and cation experiments, the improved resolution is mainly due to an increased matching of $\mu_{(eo)}$ and $\mu_{(ep)}$.

The detection limit of *p*- and *m*-aminobenzoic acid in aqueous samples could successfully be lowered to 10^{-6} *M* using the sample stacking technique while maintaining the separation of the two positional isomers.

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