Journal of Chromatography A, 652 (1993) 337–345
Elsevier Science Publishers B.V., Amsterdam

CHROM. 25 064

Computer-assisted pH optimization for the separation of geometric isomers in capillary zone electrophoresis

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ABSTRACT

With the rapid development of capillary electrophoresis, several workers have considered the theoretical pH optimization for the separation of geometric isomers. However, for samples composed of more than two isomers, these mathematical treatments lead only to an optimum pH range. In this work, the application of software based on the iterative computation of the resolution as a function of pH was studied, in order to have direct access to the optimum pH value for complex mixtures of isomers. The values deduced were compared with the experimental values for acids and bases.

INTRODUCTION

Capillary electrophoresis is becoming an increasingly used analytical tool for the resolution of complex mixtures of ionic or ionizable compounds. Nevertheless, capillary zone electrophoresis (CZE), even though more efficient, is often supplanted by micellar electrokinetic chromatography (MEKC) in studies of complex mixtures of ionizable molecules [1–4]. This is sometimes disadvantageous and is often due to a lack of evaluation of the two methods for particular applications. It is considered important to predict, as far as possible, the potential of CZE before resorting to MEKC.

In this context, we present here a computer program for the calculation of the evolution of the resolution in CZE of a complex mixture of ionizable molecules as a function of the predominant parameter of this technique, pH. Although previous workers have undertaken the optimization of the pH in CZE for one pair of compounds [5–7], for three compounds their predictions lead only to an optimum pH range. Moreover, those studies concerned the optimization of the pH with regard to the maximum charge difference between the two compounds involved (i.e., the maximum selectivity), disregarding the evolution of the electroosmotic mobility with pH depending on the ionization state of the silica. The determination of pK_a values and absolute mobilities using CZE has also been reported [8], but optimization of the analytical conditions was not achieved.

THEORETICAL

The program developed allows the prior calculation of the resolution of each compound pair (i, j) at a fixed pH according to the classical equation

$$R_{s(i,j)} = 2\left(\frac{t_j - t_i}{w_i + w_j}\right) \quad (1)$$

and then, by extraction, to retain the limiting pair of compounds for which the resolution is minimum at this pH value. By successive iteration as a function of pH, the step being fixed at 0.05 pH unit, the program calculates the pH

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0021-9673/93/$06.00

value for which the resolution of the limiting pair(s) is maximum. This pH value corresponds to the optimum conditions.

In order to perform this evaluation, a limited number of experimental applications are required, depending on two cases: if the pK_a values of the different compounds are known under the analytical temperature conditions, only one experiment is required; and if the pK_a values are not known, two electropherogams are required. This procedure is shown schematically in Fig. 1.

Taking into account the nature of the most commonly employed capillaries, *i.e.*, fused silica, the evolution of the electroosmotic flow as a function of pH cannot be neglected. Indeed, as demonstrated by Lukacs and Jorgenson [9], this evolution depends on the ionization of the silanol groups at the capillary surface and is similar to a bilogarithmic function with an approximate pK of 6 pH units. This fit is reliable if one takes account of the hysteresis effect [10] and the need for a constant ionic strength [11].

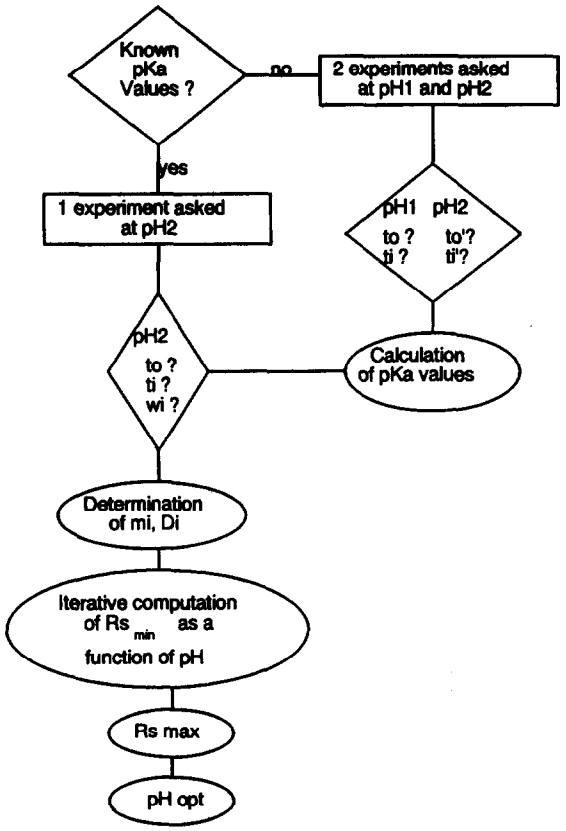

Fig. 1. Schematic diagram of the computation treatment.

Therefore, we modelled the evolution of the electroosmotic flow according to the following equations:

$$\underset{(1-x_s)}{\text{-Si-O-H}} \rightleftharpoons \underset{(x_s)}{\text{Si-O}^-} + \text{H}^+ \quad (2)$$

where x_s is the silanol fraction in its ionized form:

$$\log\left(\frac{x_s}{1-x_s}\right) = \text{pH} - \text{p}K_s \quad (3)$$

with $pK_s = 6$, where pK_s is the silanol pK_a:

$$\mu_{e0} = x\mu_0 \quad (4)$$

where μ_{e0} is the effective electroosmotic mobility and μ_0 the absolute electroosmotic mobility when the silica surface is fully ionized. As shown in Fig. 2, the fit between the experimental and the theoretical pK_s values is satisfactory, although the fit between the two curves is less satisfactory in particular for pH values between 3 and 5.

This important point have been established, it is then possible to undertake the evolution of the resolution of a mixture of ionizable compounds as a function of pH. As noted above, two cases must be considered: (i) the pK_a values of the

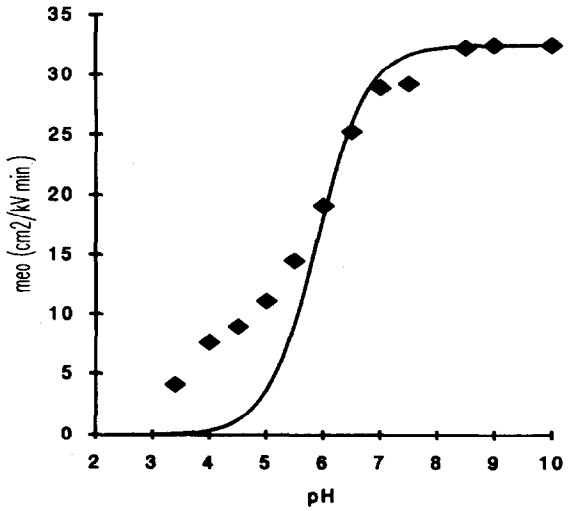

Fig. 2. Evolution of the electroosmotic mobility as a function of pH.

different compounds are known at the experimental temperature or (ii) the pK_a values of the studied compounds are not known or have not been reported in the literature under the required analytical conditions.

In the case of ionizable molecules of known pK_a values, a single experiment is necessary for the determination of the electrophoretic mobilities and the molecular diffusion coefficients of the different compounds. This unique manipulation of the sample has to be done at a pH value for which the compounds are ionized, *i.e.*, pH_1. The calculation of the migration times and the peak widths at different pH values is done through the following equations.

Acids

$$\underset{(1-x_i)}{A-H} \rightleftharpoons \underset{(x_i)}{A^-} + H^+ \quad (5)$$

$$pH = pK_a + \log\left(\frac{x_i}{1-x_i}\right) \quad (6)$$

where x_i is the fraction of the compound i in its ionized form and can be calculated at any pH value with

$$x_i = \frac{10}{1+10^{pH-pK_{ai}}} \quad (7)$$

We then have access to the apparent mobility (μapp_i) of compound i, knowing its electrophoretic mobility (μ_i) and its molecular diffusion coefficient (D_i) through the experiment at pH_1:

$$\mu_{app_i} = x_s\mu_0 - x_i\mu_i \quad (8)$$

$$t_i = \frac{lL}{V\mu_{app_i}} \quad (9)$$

$$w_i = \left(\frac{32D_it_i^3}{l^2}\right)^{1/2} \quad (10)$$

where L is the total capillary length and l the capillary length from the inlet to the detection window. Therefore, it is possible to calculate the resolution $R_{s(i,j)}$ for each pair of compounds using eqn. 1.

Bases

The determination of the electrophoretic mobilities and the molecular diffusion coefficients of basic compounds of known pK_a values is done using the same approach. The calculation is based again on one electropherogram, this time performed at a low pH value in order to visualize the compounds in their ionic form but not too low in order to visualize the electroosmotic flow also:

$$\underset{x_i}{B-H^+} \rightleftharpoons \underset{(1-x_i)}{B} + H^+ \quad (11)$$

$$pH = pK_a + \log\left(\frac{1-x_i}{x_i}\right) \quad (12)$$

$$x_i = \frac{1}{1+10^{pH-pK_{ai}}} \quad (13)$$

$$\mu_{app_i} = x_s\mu_0 + x_i\mu_i \quad (14)$$

In the case of ionizable molecules for which the pK_a values are unknown or have not been reported in the literature at the temperature of the analysis, one more electropherogram is needed at a different pH value in order to elucidate the variation of the ionized fraction of the compound as a function of pH. Hence for acidic molecules, the pK_a is deduced from the equation

$$pK_a = -\log\left(\frac{\mu_{eff_2}\cdot 10^{-pH_2} - \mu_{eff_1}\cdot 10^{-pH_1}}{\mu_{eff_1} - \mu_{eff_2}}\right) \quad (15)$$

In a similar way, the pK_a of basic molecules is deduced from the equation

$$pK_a = -\log\left[\frac{(\mu_{eff_2} - \mu_{eff_1})\cdot 10^{-pH_2-pH_1}}{\mu_{eff_1}\cdot 10^{-pH_2} - \mu_{eff_2}\cdot 10^{-pH_1}}\right] \quad (16)$$

where μ_{eff_i} is the effective mobility of the compound at pH_i and calculated with the following equation, for either acidic or basic molecules:

$$\mu_{eff_i} = \mu_{app_i} - \mu_{e0} \quad (17)$$

The choice of the pH_1 and pH_2 values in the case of basic molecules is restricted by the fact that it is necessary to visualize both the electroosmotic flow and the partially ionized compounds. Too low pH values, corresponding to high ionization of the basic molecules, induce too low electroosmotic flows which are difficult to access in reasonable times.

EXPERIMENTAL

Reagents

Buffer and sample solutions were prepared with water purified by reverse osmosis and filtered using a Milli-Ro + Milli-Q system (Millipore, Molsheim, France). The reagents used for the electrolytes, *i.e.*, borax, boric acid, phosphoric acid, dibasic sodium phosphate, sodium acetate, sodium hydroxide and sodium chloride, were of analytical-reagent grade from Aldrich (La Verpillère, France). Compounds used for the validation of the program, *i.e.*, nitrophenols, chlorophenols and chloroanilines, were of 99% purity from Aldrich. For the determination of the electroosmotic flow, mesityl oxide of analytical-reagent grade from Aldrich was used.

Apparatus

All experiments were carried out on a P/ACE 2100 system (Beckman, Fullerton, CA, USA) monitored by a PS/2 computer (IBM, Greenock, UK) using P/ACE software (Beckman), Data collection was performed with the same software. Samples were loaded by a 1-s pressure injection into a fused-silica capillary (50 cm × 50 μm I.D.). UV detection was performed at 214 nm through the capillary at 50 cm from the inlet.

The pH values of the electrolytes were measured using a Beckman Model Φ pH meter at the temperature of the analyses. The separations were performed three to five times for each pH value studied, in order to ensure good reproducibility of the measurements.

Buffer preparation

Stock solutions of borax (12.5 m*M*), boric acid (50 m*M*), dibasic sodium phosphate (50 m*M*), phosphoric acid (50 m*M*), sodium acetate (50 m*M*) and sodium hydroxide (50 m*M*) were prepared daily prior to their dilution with sodium chloride (50 m*M*) in order to prepare electrolytes with good pH buffer characteristics and a quasi-constant ionic strength.

Software

The calculation software was developed using BASIC, pre-installed on IBM DOS machines. This choice, which deliberately excluded a complex and more powerful language, appeared to be fast enough to compute the results in a satisfactory time (less than 1 min).

RESULTS AND DISCUSSION

In order to validate the treatment and establish its generality, we undertook the separation of various mixtures and compared the evolution of the minimum resolution as a function of pH with the calculated values. Three mixtures, two composed of acids and one of bases were analysed by CZE at different pH values over a wide range, *i.e.*, 2.2–10.

For weak acid compounds, we undertook the separation of the geometric isomers of the chlorophenols and the nitrophenols. The pK_a values, taken from the literature [12,13], are 8.48, 9.02 and 9.38 for *o*-, *m*- and *p*-chlorophenol, respectively, and 7.23, 8.40 and 7.15 for *o*-, *m*- and *p*-nitrophenol, respectively. The separation of the nitrophenols presents much more difficulty owing to the close pK_a values for the *ortho* and *para* isomers.

Study of the influence of pH on the resolution of the chlorophenol geometric isomers

The separation of the three monochlorophenols was performed experimentally over the pH range 7–10, the minimum resolution for this mixture being measured every 0.5 pH unit. The temperature was kept constant at 25°C, a value at which the pK_a values have been reported in the literature [12].

The values obtained were compared with the theoretical curve based on the analysis performed at pH 10. As shown in Fig. 3, a very satisfactory fit is found for the whole studied pH range. The minimum resolution of this mixture is maximum at pH 9.3, as predicted by the calculation.

Moreover, the pK_a values calculated from the electropherograms obtained at pH 9 and 10 were in excellent agreement with those reported in the literature. The fit between the experimental values and the calculation treatment using the so-deduced pK_a values is also very satisfactory (Fig. 4).

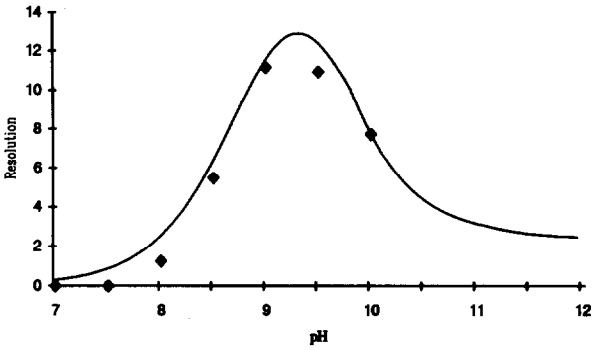

Fig. 3. Evolution of the minimum resolution as a function of pH for the geometric isomers of chlorophenol. Solid line, computed curve calculated from an electropherogram obtained at pH 10 and based on the literature pK_a values, *i.e.*, 8.48, 9.02 and 9.38 for the *ortho*, *meta* and *para* isomers, respectively.

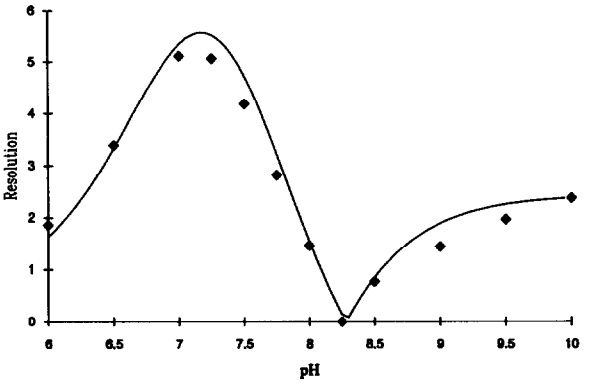

Fig. 5. Evolution of the minimum resolution as a function of pH for the geometric isomers of nitrophenol. Solid line, computed curve calculated from an electropherogram obtained at pH 10 and based on the literature pK_a values, *i.e.*, 7.23, 8.40 and 7.15 for the *ortho*, *meta* and *para* isomers, respectively.

Study of the influence of pH on the resolution of the nitrophenol geometric isomers

The validation of the program having been successfully accomplished, we undertook the separation of the three nitrophenol isomers, where the pK_a values of the *ortho* and *para* isomers are much closer and therefore present a major difficulty for the pH optimization.

We first computed the resolution of the nitrophenol geometric isomers as a function of pH using an electropherogram obtained at pH 10 and the pK_a values from the literature [12] (Fig. 5).

The computed curve shows a complete loss of resolution at pH 8.45 and two maxima of resolution at pH 7 and 10. This indicates inversion of migration for two compounds. In order to confirm this effect, and therefore to validate the treatment, we undertook, as with the chlorophenol isomers, a series of analyses at different pH values over a wide range, *i.e.*, 6–10. The corresponding electropherograms allowed us to calculate the experimental minimum resolution for each pH value considered. These values are also reported in Fig. 5.

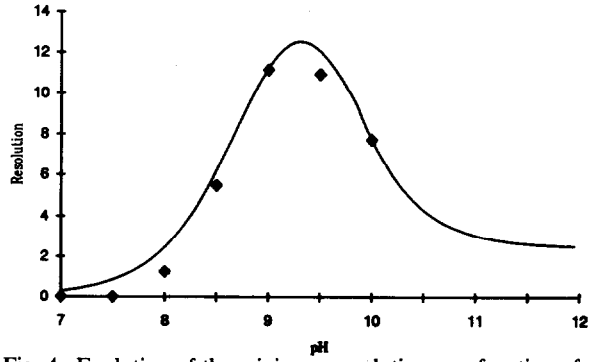

Fig. 4. Evolution of the minimum resolution as a function of pH for the geometric isomers of chlorophenol. Solid line, computed curve calculated from an electropherogram obtained at pH 10 and based on the computed pK_a values, *i.e.*, 8.472, 9.022 and 9.366 for the *ortho*, *meta* and *para* isomers, respectively.

As for the chlorophenol isomers, a good fit between the experimental and computed values is observed throughout the pH range. Furthermore, the comparison of the electropherograms obtained at the pH values for which the resolution of the mixture is a maximum, *i.e.*, pH 7 (Fig. 6) and pH 10 (Fig. 7) reveals, as predicted, an inversion in the order of migration of two peaks. Co-elution analyses confirmed the peak inversion and assigned it to the *ortho* and *para* isomers, as reported in Figs. 6 and 7.

Finally, when substituting the pK_a values from the literature for the values calculated from the electropherograms obtained at pH 10 and 9, the fit between the computed curve and the experimental resolutions appears to be less satisfactory (Figs. 5 and 8). This indicates that our method for the determination of pK_a values using CZE is less precise than that used in the literature. Nevertheless, this imprecision does

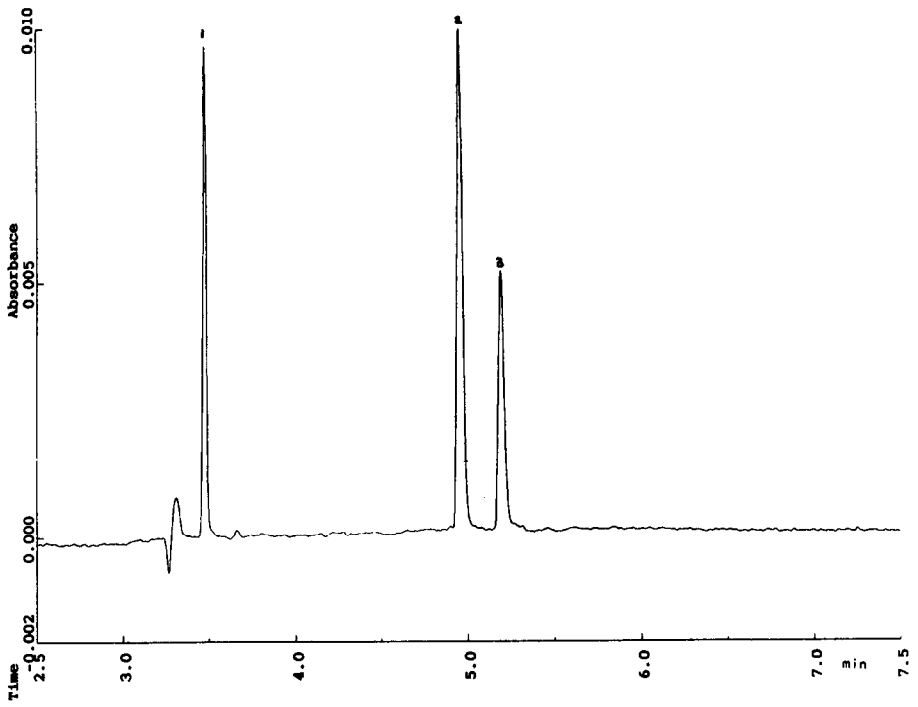

Fig. 6. Electropherogram of the geometric isomers of nitrophenol obtained by CZE at pH 7. Capillary tube, 57 cm × 50 μm I.D.; temperature, 25°C; applied voltage, 30 kV. Peaks: 1 = *m*-; 2 = *o*-; 3 = *p*-nitrophenol.

not alter the validity of the essential predictions deduced from the treatment, *i.e.*, the optimum pH values corresponding to the maximum resolution for this mixture and the inversion of migration order between the *ortho* and *para* isomers at pH 8.45.

Study of the influence of pH on the resolution of the chloroaniline geometric isomers

As the theoretical approach for the prediction of the behaviour of acidic compounds in CZE thus appeared to be very satisfactory, we completed the study by investigating the electrophoretic behaviour of basic geometric isomers. The compounds chosen were the geometric isomers of the chloroaniline, for which the pK_a values at 25°C have been reported in the literature [13] as 2.64, 3.52 and 3.98 for the *ortho*, *meta* and *para* isomers, respectively. Their electrophoretic behaviour was studied experimentally in the pH range 2.2–6.

As the computation of the resolution as a function of pH needs a complete electropherogram, the choice of the pH value at which the reference electropherogram will be measured is important. As described above, the pH value must be as low as possible in order that the fraction of the compounds in their ionic form is the maximum. Nevertheless, as the chlorophenols have very low pK_a values, this choice is limited by the ionization state of the capillary wall. Therefore, a compromise was found at pH 3.75, at which the electroosmotic flow was not too slow and the geometric isomers were partially ionized. Under such conditions, the comparison of the computed curve and the experimental results is less satisfactory than with acid solutes (Fig. 9). Moreover, the substitution of the pK_a values from the literature by those deduced from the electropherograms obtained at pH 3.75 and 4.25 does not lead to a better fit between the computed curve and the experimental results.

To explain such an observation, two considerations can be invoked: (i) a very slow equilibrium of the ionization state of the capillary wall in this pH range, as evidenced by Lambert and

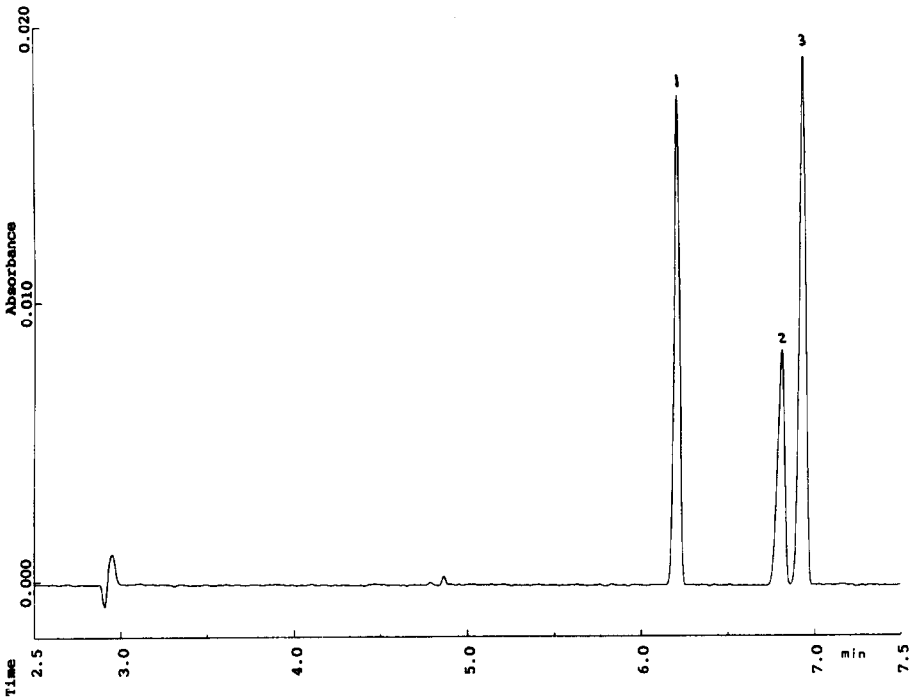

Fig. 7. Electropherogram of the geometric isomers of nitrophenol obtained by CZE at pH 10. Conditions as in Fig. 6. Peaks: 1 = *m*-; 2 = *p*-; 3 = *o*-nitrophenol.

Middleton [10]; and (ii) tailing peaks, leading to a decrease of resolution not taken into account in the computed treatment owing to the adsorption phenomena that occur between basic compounds and the capillary wall, especially as our experiments were performed on capillaries of small I.D. (50 μm).

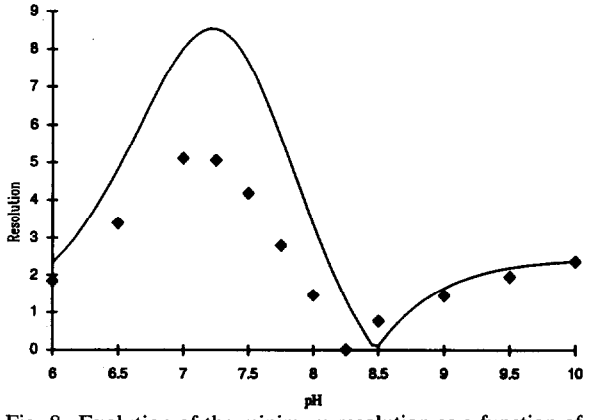

Fig. 8. Evolution of the minimum resolution as a function of pH for the geometric isomers of nitrophenol. Solid line, computed curve calculated from an electropherogram obtained at pH 10 and based on the computed pK_a values, *i.e.*, 7.272, 8.289 and 7.155 for the *ortho*, *meta* and *para* isomers, respectively.

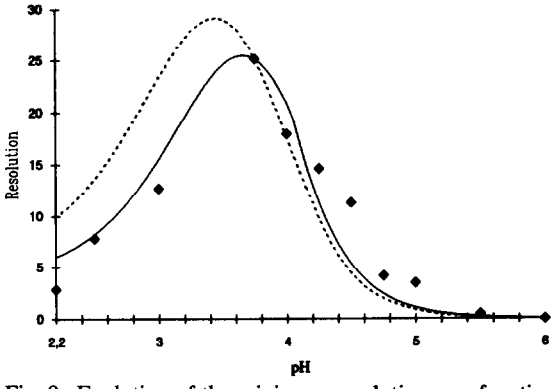

Fig. 9. Evolution of the minimum resolution as a function of pH for the geometric isomers of chloroaniline. Solid line, computed curve calculated from an electropherogram obtained at pH 3.75 and based on the literature pK_a values, *i.e.*, 2.64, 3.52 and 3.98 for the *ortho*, *meta* and *para* isomers, respectively; dashed line, computed curve calculated from an electropherogram obtained at pH 3.75 and based on the computed pK_a values, *i.e.*, 3.046, 3.244 and 3.574 for the *ortho*, *meta* and *para* isomers, respectively.

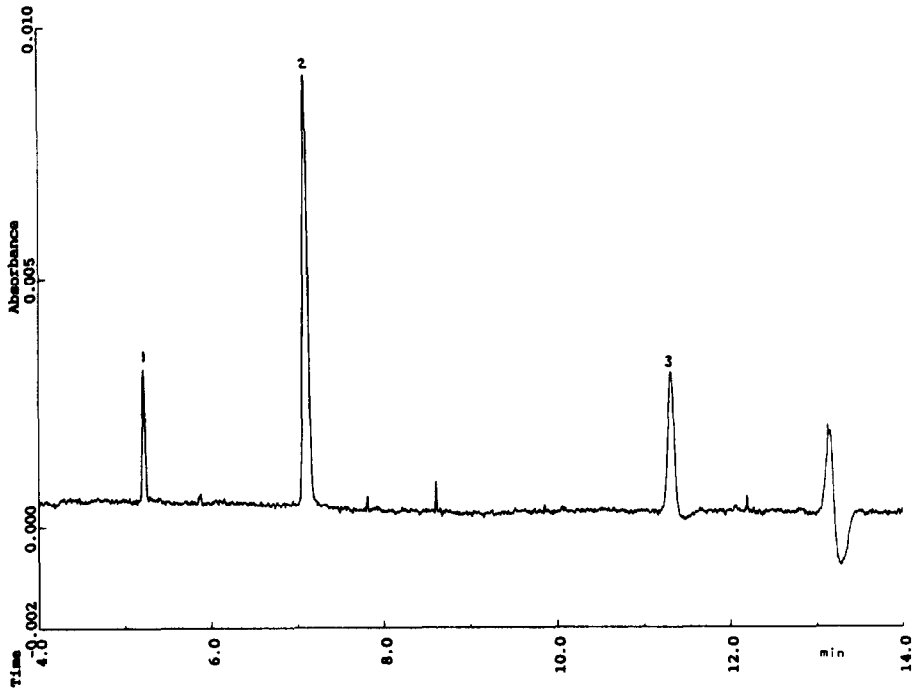

Fig. 10. Electropherogram of the geometric isomers of chloroaniline obtained by CZE at pH 3.75. Capillary tube, 57 cm × 50 μm I.D.; temperature, 25°C; applied voltage, 30 kV. Peaks: 1 = *p*-; 2 = *m*-; 3 = *o*-chloroaniline.

Nevertheless, as shown in Fig. 9, the predicted optimum pH value at which the resolution is maximum for this mixture is in good agreement with the experimental results. The corresponding electropherogram obtained at this pH value (3.75) is shown in Fig. 10.

Finally as predicted by the computation, no inversion of the migration order of the geometric isomers of chloroaniline was observed.

CONCLUSIONS

This series of experiments concerning the resolution of mixtures containing either acid or basic compounds showed a satisfactory fit between the experimental results and the computed values of the resolution as a function of pH. This good agreement leads to a precise determination of the optimum pH value *a priori*, for compounds for which the pK_a values are either tabulated or not. In addition, a good idea of the electrophoretic behaviour of the different constituents of the mixture can be predicted, allowing one to detect, *a priori*, an inversion in the migration order of the solutes. This possibility of the computation treatment appears to be very useful for the optimization of analyses of mixtures containing some compounds at low concentration levels, allowing a better detection.

Considering these very encouraging results, the extension of this treatment to amphoteric molecules is in progress.

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