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Adjustment of resolution and analysis time in capillary zone electrophoresis by varying the pH of the buffer

Ernst Kenndler and Werner Friedl

Institute for Analytical Chemistry, University of Vienna, Währingerstrasse 38, A-1090 Vienna (Austria)

ABSTRACT

An expression for resolution in capillary zone electrophoresis is derived which is a function of the pH of the buffer. It is based on the conventional definitions of resolution, but takes into account the dependence of the efficiency term on the effective mobilities, or the charge numbers, of the separands (monovalent ions). The different shapes of the R *vs.* pH curves, as derived from the resolution equation, are discussed and found to agree with the experimental results. An approach for the selection of that single pH where a given resolution can be achieved within the shortest time of analysis is introduced.

INTRODUCTION

In zone electrophoresis, the degree of separation of two components, i and j, is described as in elution chromatography by a dimensionless number, the resolution, R_{ji}. It is defined by the distance of the centres of the peaks, measured not in absolute but in relative units. The definition introduced by Giddings [1] takes the sum of the twofold of the standard deviations, σ, of the peaks of components i and j as the scale unit. Because in capillary zone electrophoresis (CZE) (as in column chromatography) the peaks are represented in the time domain, the migration times, t, and the standard deviations based on time, σ_t, are applied for the definition of the resolution, which is expressed as

$$R_{ji} = \frac{t_j - t_i}{2\,\sigma_{t,i} + 2\,\sigma_{t,j}} \tag{1}$$

Using an averaged standard deviation, $\bar{\sigma}_t$, of the two peaks, eqn. 1 can be rewritten as $R_{ji} = (t_j - t_i)/4\,\bar{\sigma}_t$. Based on this definition, baseline separation is established for two peaks with equal areas at a resolution of 1.5.

Huber [2] introduced a simpler definition of the resolution for elution chromatography, taking into account the standard deviation of only one of the two peaks as the scale unit for expressing the distance between the two peaks, according to

$$R_{ji} = \frac{t_j - t_i}{\sigma_{t,i}} \tag{2}$$

This definition, which is also applicable to zone electrophoresis, is nearly equivalent to that given in eqn. 1 for peaks with very similar widths, but it can be converted into an expression that consists of operating parameters, as discussed below, without using averaged parameters. Baseline separation for peaks with equal areas is established at a resolution of 6; it can be seen that the conversion factor between these two resolution expressions is 4. Both equations are rearranged by substituting t and σ to obtain expressions with a larger optimization potential. Restricting the following discussion to CZE without electroosmotic flow, the resolution as defined by eqn. 1 then results in [1,3–5]

$$R_{ji} = \frac{1}{4} \cdot \frac{\Delta v}{\bar{v}} \sqrt{\bar{N}} = \frac{1}{4} \cdot \frac{u_i - u_j}{\bar{u}} \sqrt{\bar{N}} \tag{3}$$

Correspondence to: Prof. E. Kenndler, Institute for Analytical Chemistry, University of Vienna, Währingerstrasse 38, A-1090 Vienna, Austria.

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where Δv is the velocity difference of the separands, $\bar{v}$ is their average velocity, $u(\bar{u})$ is the (average) ionic mobility and $\bar{N}$ is the average plate number, given by

$$\bar{N} = \frac{\bar{u}\ U}{2\ \bar{D}} \tag{4}$$

In this equation U is the effective voltage applied across the effective length, L, of the capillary, resulting in a field strength E and $\bar{D}$ is the average diffusion coefficient. Eqn. 4 was derived for the case where only longitudinal diffusion causes peak broadening. All other effects are neglected, namely convective mixing (in the case of hydrodynamic or electroosmotic flow), Joule heat (occurring especially at high electrical current), electromigration dispersion (when conductivity gradients are formed between the sample zone and buffer), adsorption and extra-column effects. The influence of these effects on the plate height, H, was extensively discussed by Hjertén [6]. In the same paper it was also pointed out that under certain circumstances the latter effects are negligible compared with longitudinal diffusion, which is the aspect focused upon in this paper. For simplicity, the discussion will also be restricted to monovalent separands. The more complicated expressions for multivalent or amphoteric electrolytes will be discussed in a subsequent paper.

It was mentioned above that no averaged parameters occur in the expression for the resolution derived from the definition given in eqn. 2, leading to [7]

$$R_{ji} = \frac{u_{\mathrm{eff},i} - u_{\mathrm{eff},j}}{u_{\mathrm{eff},j}} \sqrt{N_i} = \left(\frac{u_{\mathrm{eff},i}}{u_{\mathrm{eff},j}} - 1\right) \sqrt{\frac{u_{\mathrm{eff},i}\ U}{2\ D_i}} = \left(\frac{u_{\mathrm{eff},i}}{u_{\mathrm{eff},j}} - 1\right) \sqrt{z_i} \sqrt{\frac{u_{\mathrm{act},i}\ U}{2\ D_i}} \tag{5}$$

where $u_{\mathrm{eff},i}$ is the effective mobility and D_i is the diffusion coefficient of separand i. The effective mobility, $u_{\mathrm{eff},i}$, is related by the (effective) charge number, z_i, to the actual mobility, $u_{\mathrm{act},i}$, which is equivalent to the normalized mobility for monovalent analytes, the mobility of the separand with unit charge: $u_{\mathrm{eff},i} = z_i\ u_{\mathrm{act},i}$. It should be mentioned that in contrast to the mobility, z_i always has a positive sign. It will be discussed below that the actual (or normalized) mobility can be linearly related under the conditions discussed [7–11] to the diffusion coefficient as given by the Nernst–Einstein equation, $D = u\ k\ T/z\ e_0$, where e_0 is the electric charge, k is the Boltzmann constant and T is the absolute temperature.

It can clearly be seen from eqn. 5 that the resolution consists of two terms, the selectivity term (with the ratio of the effective mobilities), and the efficiency term (the square root of the plate number). The latter also includes the effective mobility.

It was discussed in previous papers [7–11] that the last term in eqn. 5 ($u_{\mathrm{act},i}\ U/2\ D_i$) is constant for all separands at a given voltage for not too high electrolyte concentrations, where the Nernst–Einstein equation for the (normalized) mobility and the diffusion coefficient is still valid.

Applying the Nernst–Einstein equation, the plate number can be expressed by

$$N_i = \frac{z_i\ e_0\ U}{2\ k\ T} \tag{6}$$

It can be seen that the plate number depends on the charge number of the separand and on the voltage. The validity of this equation was demonstrated experimentally (at least for diluted solutions) for small ions in previous work [7,10], but this equation must also be valid for large molecules such as biopolymers, taking into account that z_i is the effective and not a nominal charge number. For such molecules this effective charge number must be related to their zeta potential rather than to their nominal charge. For polynucleotides, for example, the effective charge number may then differ from the nominal charge number as calculated from the base number [11].

Combining eqns. 5 and 6 leads to the following expression for the resolution:

$$R_{ji} = \left(\frac{u_{\mathrm{eff},i}}{u_{\mathrm{eff},j}} - 1\right) \sqrt{z_i} \sqrt{\frac{e_0\ U}{2\ k\ T}} \tag{7}$$

For weak electrolytes the charge number and thus the effective mobility are determined, according to the degree of dissociation, by the pH of the buffer in the separation unit. Hence not only the selectivity can be adjusted by the appropriate choice of the electrolyte system, but also the efficiency, and consequently the resolution. An optimization of the resolution was presented by Terabe *et al.* [12] for components with very similar pK values and equal

actual mobilities. In this paper here a concept for the adjustment of the separation of any pair of analytes is introduced and an expression for the resolution is derived and discussed for monovalent ions. It depends on the pH (and voltage and temperature) as experimental variable. From the pH range where all pairs of components can be separated with a given resolution, a single pH is selected that results in the minimum time of analysis. A more general approach for the adjustment of the resolution of multivalent separands by varying the pH and voltage is the topic of a subsequent paper.

EXPERIMENTAL

Chemicals

Chemicals used for the preparation of the buffer solutions were orthophosphoric acid, acetic acid, malonic acid and lactic acid (all of analytical-reagent grade from E. Merck, Darmstadt, Germany). The following compounds were used as test substances: 2,4-dinitrophenol (α-nitrophenol, moistened with 20% water, >97%; Fluka, Buchs, Switzerland), 2,6-dinitrophenol (moistened with 20% water, purum, >98%; Fluka), 2,3-dimethoxybenzoic acid, 3,5-dimethoxybenzoic acid, 3,4-dimethoxybenzoic acid (veratric acid) and 2,4-dimethoxybenzoic acid (all minimum 99%; EGA, Steinheim, Germany). For the coating procedure, methylcellulose (Methocel MC, 3000–5000 mPa s; Fluka) was cross-linked using formic acid and formaldehyde (both of analytical-reagent grade; Merck) as described below. Water was distilled twice from a quartz apparatus.

Apparatus

The measurements were carried out with an instrument which was equipped with a UV absorbance detector (P/ACE System 2100, using System Gold 6.01; Beckman, Palo Alto, CA, USA). The absorbance was measured at 214 nm. The separation capillary was made from fused silica (Scientific Glass Engineering, Ringwood, Australia) of 75 μm I.D. and with a total length of 0.269 m. The effective length (the distance from the injector to the detector) was 0.201 m. The capillary was thermostated at 25.0°C.

Electrophoresis was carried out without electroosmotic flow at a total voltage of 5000 V (field strength 18 600 V/m), leading to an effective potential drop of 3740 V along the migration distance.

Injection of the sample was carried out from aqueous solution (without buffer) by pressure for 1 s.

The calculations were carried out with a personal computer using MathCAD 2.08 from MathSoft.

Procedures

The pH of the buffers was adjusted by adding sodium hydroxide solution to the solutions of the respective acids (total buffer concentration 0.01 mol/l) using a glass–calomel electrode. The buffers were used in a pH range of ±1 unit around the pK_a of the respective acid (described under *Chemicals*).

The following procedure was applied to eliminate electroosmosis by coating the inner capillary surface with methylcellulose as described by Hjertén [13]: 400 mg of methylcellulose were dissolved in 100 ml of water and 7 ml of formic acid and 35 ml of formaldehyde were added with stirring. This solution can be used repeatedly for at least 6 months if stored in a refrigerator. The tube was rinsed thoroughly with distilled water for 10 min. The methylcellulose solution was drawn into the dried, clean tube by suction for *ca.* 15 min, then the capillary was sucked dry for 5 min, leaving a thin film of uniform thickness on the wall. Finally, the tube was placed in an oven at 120°C for 40 min. Throughout the coating procedure it is important that air bubbles do not adhere to the capillary wall and that the quartz tube is kept in a vertical position. A second treatment with methylcellulose is recommended. The film was found to be stable for several weeks under the given conditions. The absence of an electroosmotic flow was controlled by measuring the mobility of a reference ion from the migration distance and its migration time.

The ionic mobilities were measured in the usual way as mentioned before. The pK_a values were determined from the inflection points of the mobility *vs.* pH curves.

RESULTS AND DISCUSSION

It was pointed out in the Introduction that all definitions of the resolution of two compounds include two effects: the difference in migration and the broadening of the peaks. In CZE the difference

in migration is normally expressed by the difference in the residence times caused by the different effective mobilities, u_{eff}, of the separands, and is related to the selectivity of the separation system. It can be influenced by solvation, ionic strength, etc., but the most pronounced effect is from the degree of protolysis (or complexation). As the analytes behave as acids or bases in most instances, the degree of protolysis or dissociation, α, for a given pK_a is determined by the pH of the buffer, which is therefore the most effective tool for adjusting the selectivity.

The second effect mentioned above, decisive for the resolution, is the dispersion of the peaks, counteracting the separation, and described by the efficiency term, $\sqrt{N}$, in the expression for R. The plate count, or the plate height, H, depends on a number of effects as mentioned in the Introduction, but most of them can be reduced or even eliminated under the appropriate conditions, except longitudinal diffusion.

It follows from eqn. 5 that not only the selectivity but also the efficiency depends on the effective mobility via the charge number. For a charge number of 1 the plate number calculated from eqn. 6 is 74 800 under the given conditions (3.74 kV, 25°C). Experimentally a value of *ca.* 54 000 was found, about 25% lower than the theoretical value, which is

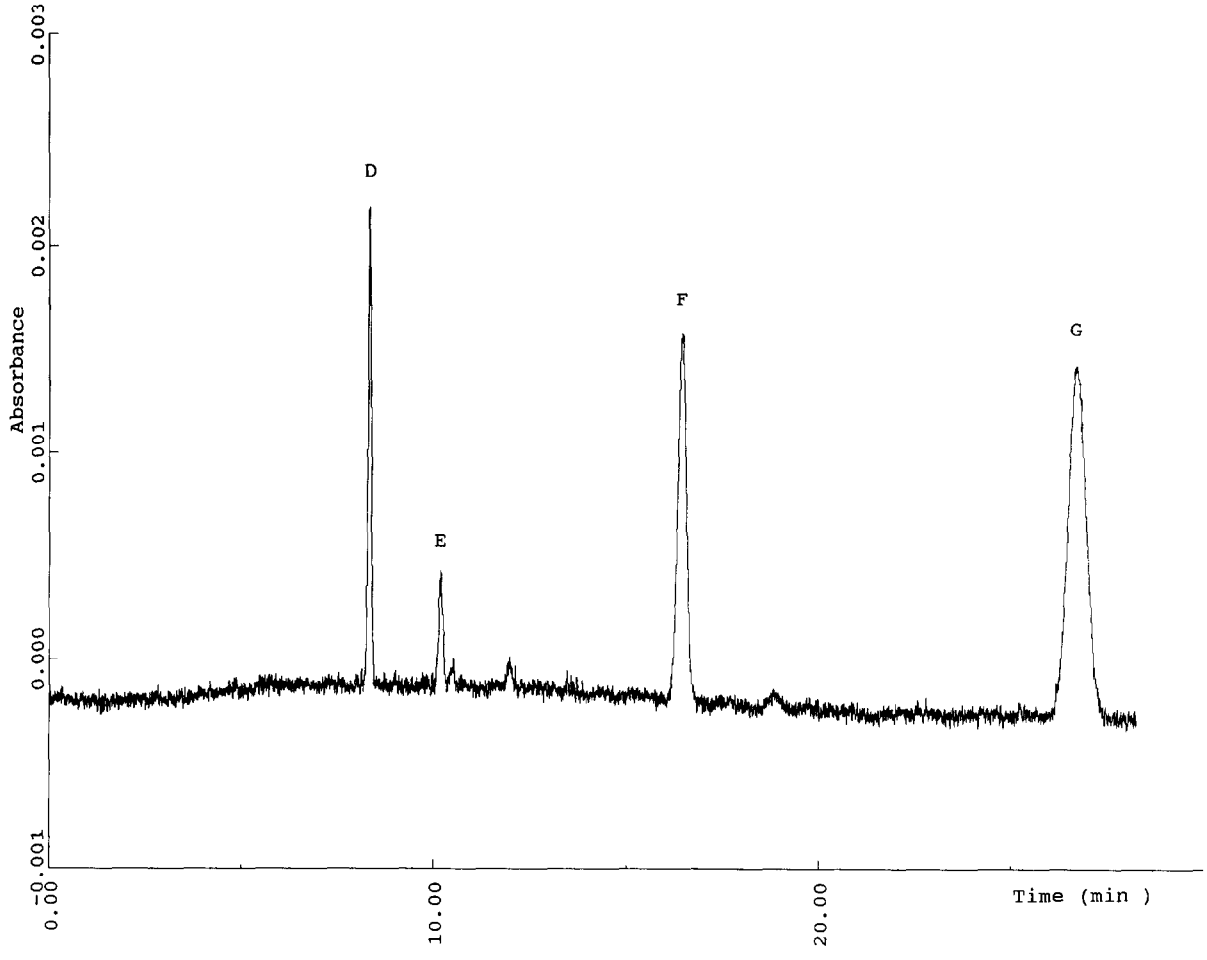

Fig. 1. Electropherogram showing the dependence of the plate number on the effective charge number, according to the degree of dissociation. The degree of dissociation of the separands decreases from D to G. Plate numbers determined from electropherogram at pH 4.17: D = 46 400, E = 35 600, F = 24 800, G = 16 200. For conditions, see Experimental. The symbols of the separands are given in Table I.

in agreement with previous investigations [7,10,11], the reason being discussed there. A voltage of 3.74 kV (5.00 kV total) was selected because it was observed that under this condition the effect of Joule heat is negligible.

The charge number of simple weak electrolytes, considered here and in previous work [7,10], is equivalent to their degree of protolysis, α. Both efficiency and selectivity are influenced by the pH of the buffer, which determines the degree of dissociation by the well known relationship $pH = pK_a \pm \log (1 - 1/\alpha)$, where the sign before the logarithm is positive for anions and negative for cations.

Effect of pH on efficiency

In Fig. 1 the electropherogram of four dimethoxybenzoic acids with very similar ionic mobilities (D, E, F and G in Table I) is shown. For this type of compound, the limiting mobilities are only slightly higher than the actual mobilities (that is, the mobility of the fully dissociated species at the given ionic strength), namely about 2–3%. The Nernst–Einstein equation should be applicable under these conditions, and the plate number must therefore depend on the charge number of the separands, which is directly related to the degree of dissociation at the given pH.

Based on the pK_a values of the analytes given in Table I, the following values for α are calculated for pH 4.17, where the electropherogram was developed: D 0.807, E 0.661, F 0.448 and G 0.304. As α decreases in this order, an analogous decrease in the plate number can be expected, according to eqn. 6 ($z_i = \alpha_i$). Indeed, it can be seen that N decreases from 46 400 for separand D to 15 000 for G, the component with the lowest degree of dissociation. If these plate counts are weighted with $1/\alpha$, the values found are 57 000, 53 900, 55 300 and 53 200. This result agrees with the predicted value of 54 000 for N/α (eqn. 6).

TABLE I

ACTUAL MOBILITIES, $u_{act,i}$ AND pK_a VALUES OF THE TEST SUBSTANCES ($T = 25°C$)

Symbol	Compound	$u_{act,i}$ (10^{-5} cm^2/V · s)	pK_a
A	Benzoic acid	32.09	4.16[a]
B	2,4-Dinitrophenol	31.09	4.04[a]
C	2,6-Dinitrophenol	32.96	3.73[a]
D	2,3-Dimethoxybenzoic acid	26.33	3.58
E	3,5-Dimethoxybenzoic acid	26.55	3.91
F	3,4-Dimethoxybenzoic acid	25.72	4.29
G	2,4-Dimethoxybenzoic acid	25.74	4.56

[a] Values taken from the literature [14].

Resolution

As both selectivity and efficiency depend on the charge number, and therefore on the pH for weak electrolytes, an appropriate expression for the resolution must reflect this dependence. Based on the definitions of the resolution, given in eqns. 1 and 2, replacement of t by $L/u_{eff}\ E$ and σ by $t/\sqrt{N}$ consequently leads to the following equations for the resolution R_{ji} for components i and j:

$$R_{ji} = \frac{(r_{ij} - 1) + (r_{ij}\,\Delta_j - \Delta_i)}{(1 + \Delta_i)^{3/2} + r_{ij}(1 + \Delta_j)^{3/2}} \sqrt{\frac{e_0\ U}{32\ k\ T}} \qquad (8)$$

according to eqn. 1 and

$$R_{ji} = \frac{(r_{ij} - 1) + (r_{ij}\,\Delta_j - \Delta_i)}{(1 + \Delta_i)^{3/2}} \sqrt{\frac{e_0\ U}{2\ k\ T}} \qquad (9)$$

according to eqn. 2, where $r_{ij} = u_{act,i}/u_{act,j}$ is the ratio of the actual mobilities (of the ions with unit charge), $\Delta_i = 10^{pK_{a,i} - pH}$ and $\Delta_j = 10^{pK_{a,j} - pH}$ for anions.

It can be seen from eqns. 8 and 9 that the resolution depends on the sample properties, namely the constants $u_{act,i}$, $u_{act,j}$, $pK_{a,i}$ and $pK_{a,j}$, and the experimental parameters pH, U and T. Whereas the former properties are determined by the analytes and cannot be influenced in a simple way (they can be affected, *e.g.*, by using organic solvents), the latter conditions can be varied much more easily (whereby a change in the temperature will lead to the least effect). It is well described in the literature that an increase in the applied voltage can cause an increase in N, but the voltage raises the resolution by the square root only, and the resulting increase in the electrical current (and therefore of Joule heat) limits the enhancement of the resolution concerning the voltage. The extent of this effect depends on instrumental conditions such as current and capillary inner diameter. This aspect is not discussed further here.

It can be concluded from eqns. 8 and 9 that the most powerful parameter in achieving an appropri-

ate resolution is the pH of the buffer which determines Δ_i and Δ_j. Its influence is demonstrated using seven test substances given in Table I.

It should be mentioned that both equations give nearly the same results for low values of the resolution (for the case of very similar peak widths for i and j). As our investigations cover a wide range of resolution values, eqn. 8 seems to have some advantage over eqn. 9, and is thus used further.

The resolution of all pairs of separands can be calculated as a function of pH from the pK_a values and the mobilities. Instead of 1.12 for the constant factor $e_0/32\,k\,T$ with the square root, a value of 0.950 is taken, considering the deviation from the theoretically reachable value of 74 800 for N as discussed above (the measured plate count was 54 000).

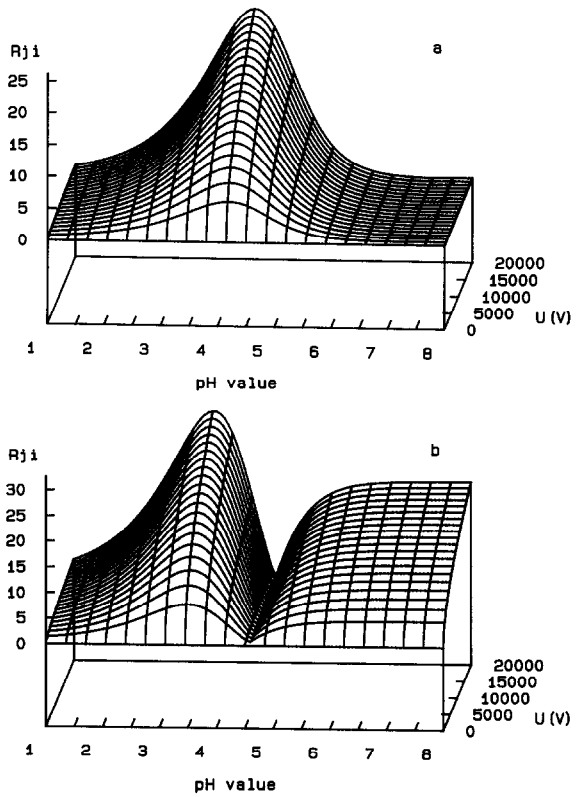

Fig. 2. Dependence of the resolution, R_{ji}, on pH and voltage U according to eqn. 8. (a) $u_{act,i} > u_{act,j}$, $pK_{a,i} < pK_{a,j}$; (b) $u_{act,i} > u_{act,j}$, $pK_{a,i} > pK_{a,j}$. $u_{act,i}$ and $u_{act,j}$ are the actual mobilities of the separands. For monovalent species, they are identical with the normalized mobilities. The curves are calculated based on the data for (a) components F and G and (b) components B and D given in Table I. For discussion, see text.

Typical examples of the resulting R *vs.* pH *vs.* U curves are shown in Fig. 2. In principle two cases can be distinguished. In Fig. 2a the case is shown where one separand has a higher actual mobility (which is reached at high pH) and a lower pK_a value than the second separand. The first separand will then always have the higher effective mobility, independent of the pH of the buffer. The R *vs.* pH curves (considered at a distinct voltage) have one maximum. These curves have a similar shape to that derived by Terabe *et al.* [12]. This derivation was limited, however, to the case where the separands have very similar pK values and identical actual mobilities.

The second case arises if the separand with the higher actual mobility is the weaker acid, having the higher pK_a of the pair considered. When varying the pH, the values of the effective mobilities will approach each other and become equal at a certain pH. Consequently, the resolution is zero at this pH. Beyond this pH the migration order of the two

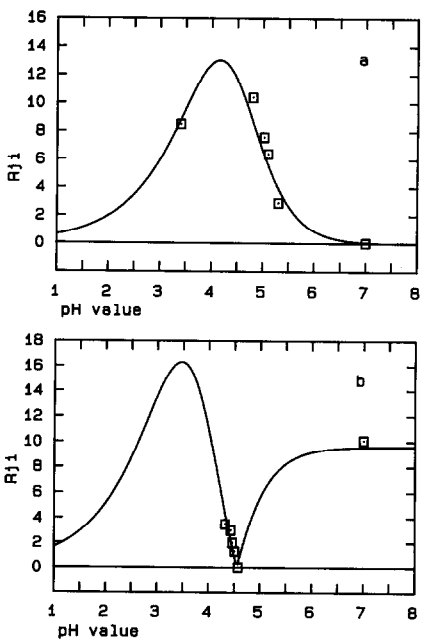

Fig. 3. Theoretical curves and experimental results of the dependence of the resolution, R_{ji}, on the pH at constant voltage. The curves were calculated for the pairs of components from Fig. 2 by eqn. 8 for an effective voltage of 3.74 kV. The correction for N was included (see text). (a) According to Fig. 2a; (b) according to Fig. 2b. □ = Experimentally determined values of R_{ji} at different pH of the buffer.

components will be reversed (and the indices in the resolution equation change).

The corresponding R *vs.* pH *vs.* U curves are shown in Fig. 2b. The resolution increases with increasing pH (at a distinct voltage), reaches a maximum, falls off to the resolution zero, increases at higher pH and reaches a plateau with a constant value of the resolution (as in Fig. 2a), because the analytes reach constant actual mobilities in this pH range, as they are fully dissociated. For both cases at constant pH the resolution increases with $\sqrt{U}$.

In Fig. 3, two typical examples are given to demonstrate the accordance of the experimental data with the curves derived from eqn. 8. The resolution is shown for the two pairs F–G and B–D (Table I) from Fig. 2a and b, as a function of the pH of the buffer for a constant voltage of 3.74 kV. The experimentally determined values for the resolution indeed follow the theoretical curves within a reasonable deviation. Similar results were obtained for all pairs of separands under investigation.

Based on graphs such as those shown in Figs. 2 and 3, the pH at which a certain resolution is established can be determined. For the case in Fig. 3b, for example, the resolution of 2.00 is obtained at pH 1.16, 4.43 and 4.68. In the pH range 1.16–4.43, and at pH >4.48, the resolution is larger than 2.00 for this pair of separands. The case shown in Fig. 3a leads to a resolution of 2.00 at pH 1.42 and 6.46. Between these pH values the resolution exceeds 2. In this way the corresponding pH values or ranges can be calculated and determined for each pair of separands. A resolution of 2.00 is chosen (rather than 1.5), because the peak areas in the examples given below are not equal.

The pH ranges where the pairs of components are separated at least with a resolution of 2.00 are shown for all possible cases in Fig. 4. Within the overlapping pH ranges all analytes are separated with that chosen, minimum resolution. This range is represented in Fig. 4 by a single vertical line, because in this example for 21 pairs (given by the combination of the seven test components in Table I) the highest and the lowest pH value of the range are equal by chance, namely 4.43. It follows that for this given set of test components no further selection of the pH can be carried out to vary the time of analysis. For that purpose another example will be given below.

It can be seen from Fig. 5 that the accurate

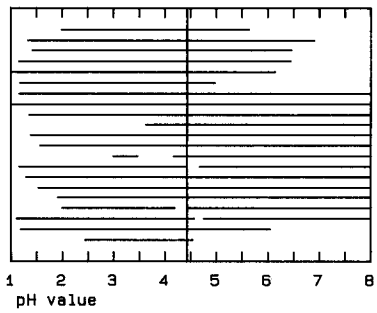

Fig. 4. Plot of the pH ranges within a resolution ≥ 2.00 is obtained for different pairs of the seven test compounds. Test components (A–G) according to Table I. The vertical line shows the pH value where the pH ranges of all 21 possible pairs overlap. Sequence of pairs from bottom to top: A–B, A–C, A–D, A–E, A–F, A–G, B–C, B–D, B–E, B–F, B–G, C–D, C–E, C–F, C–G, D–E, D–F, D–G, E–F, E–G, F–G.

selection of the appropriate pH can be very critical, because the resolution changes very rapidly with even small changes in the pH in critical regions. The slope of the R *vs.* pH curve is very steep; *e.g.*, for the pair A–E it has a value of 14 in this example, which means that the variation of the pH by only 0.01 unit causes a change in resolution of 0.14. This effect is clearly demonstrated by the electropherogram in Fig. 6, obtained at pH 4.40, only 0.03 pH unit lower than the pH 4.43 given above for a resolution of 2.00. It can be seen from Fig. 6 that this minute deviation from the optimum pH causes a total loss in the resolution of components A and E, with E appearing only as a shoulder on the peak.

The electropherogram obtained at the appropriate pH 4.43 is shown in Fig. 7. As expected from the

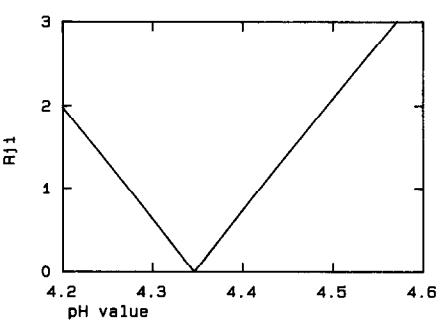

Fig. 5. Expanded graph of the dependence of the resolution, R_{ji}, on the pH around the critical pH were $R_{ji} = 0$. The curve is a section of Fig. 3b for the pair B–D at 3.74 kV.

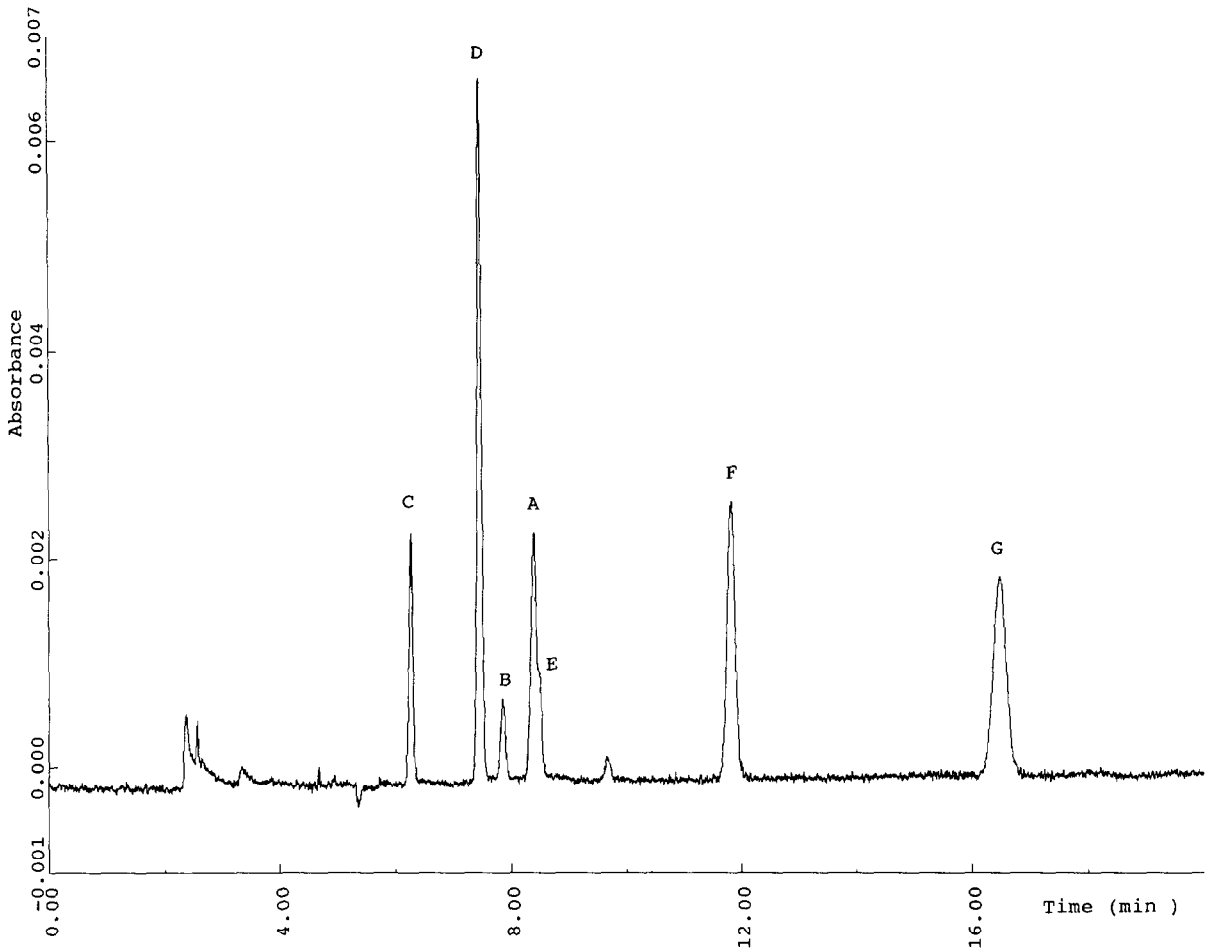

Fig. 6. Electropherogram of seven test components at pH 4.40. The test components are given in Table I. For details, see text.

calculation based on the equation for the resolution (eqn. 8), all components are separated, the most critical pair A and E with a resolution of 1.98, in agreement with the theoretically predicted value of 2.00.

Time of analysis

It must be taken into account, however, that at a given voltage not all pH values are equivalent within the calculated overlapping range, because in addition to the resolution the time of analysis is a decisive parameter. It is not favourable to select too high a resolution owing to the increase in analysis time. This time of analysis is given by the residence time of the slowest migrating component, the component with the lowest effective mobility of all separands. Generally for anions this shortest time is always established at the highest pH of the overlapping range calculated, where the selected minimum resolution is reached.

As an example, the pH ranges for a resolution of 2.00 were calculated based on eqn. 8 for four test substances (D–G in Table I). These ranges are plotted in the same way as above and are shown in Fig. 8. Here the overlapping pH ranges can be recognized within the vertical lines: between pH 1.99 and 4.99 the resolution of all components must be 2.00 or higher. For a minimum time of analysis, as a

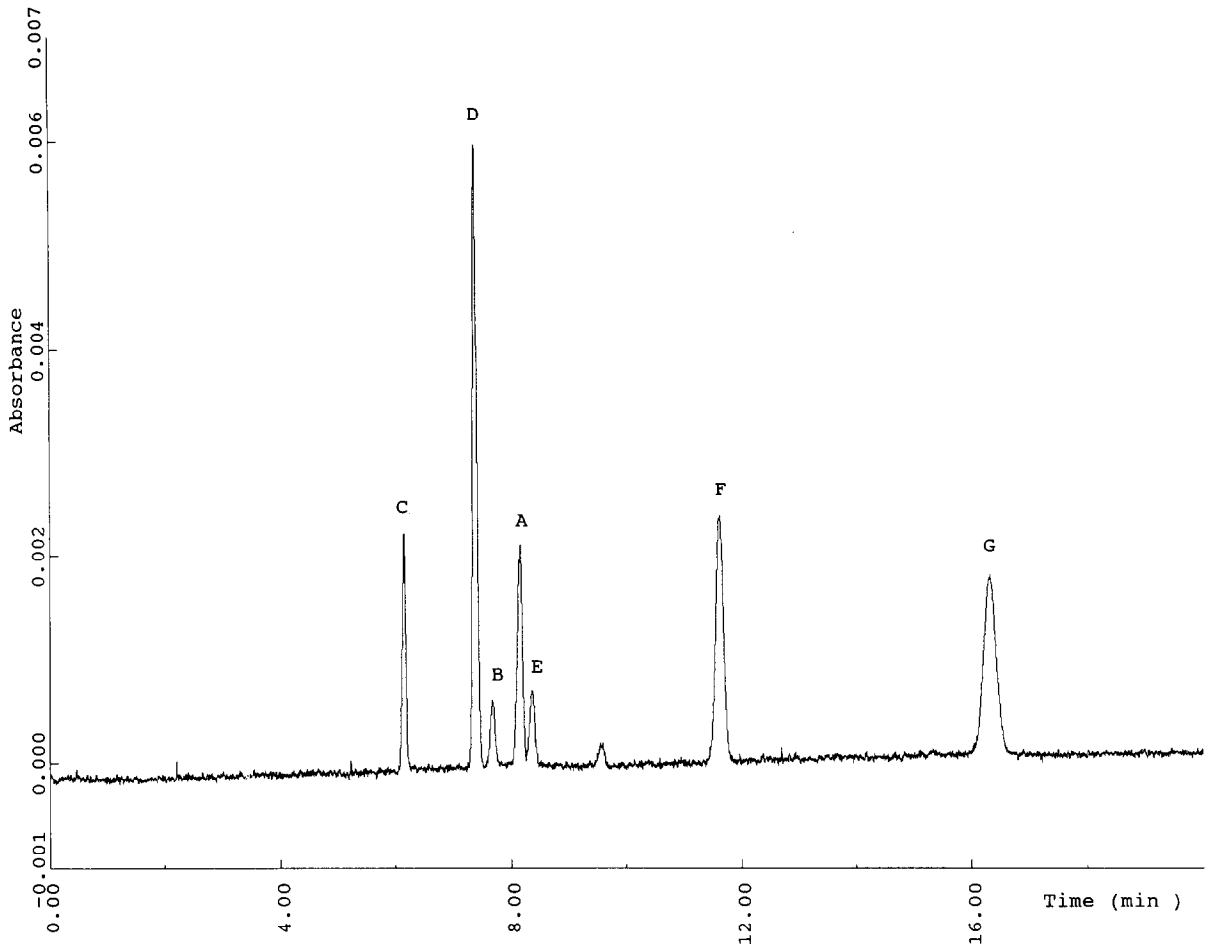

Fig. 7. Electropherogram of seven test components at pH 4.43, as calculated from eqn. 8 for a resolution of 2.00. The test components are given in Table I. The resolution determined for A and E is 1.98. For details, see text.

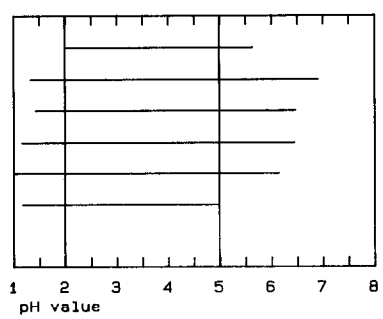

Fig. 8. Plot of the pH ranges with a resolution $R_{ji} \geqslant 2.00$ for four test components. As test components, D, E, F and G from Table I were used. The area within the vertical lines represents the pH range where this resolution is obtained or exceeded. Sequence of pairs from bottom to top: D–E, D–F, D–G, E–F, E–G, F–G.

general approach, the highest pH of this range is selected, namely pH 4.99. The electropherogram obtained under these conditions is shown in Fig. 9. The minimum resolution, namely for the pair D–E, is 1.98, again close to that theoretically predicted, and the time of analysis is about 10 min.

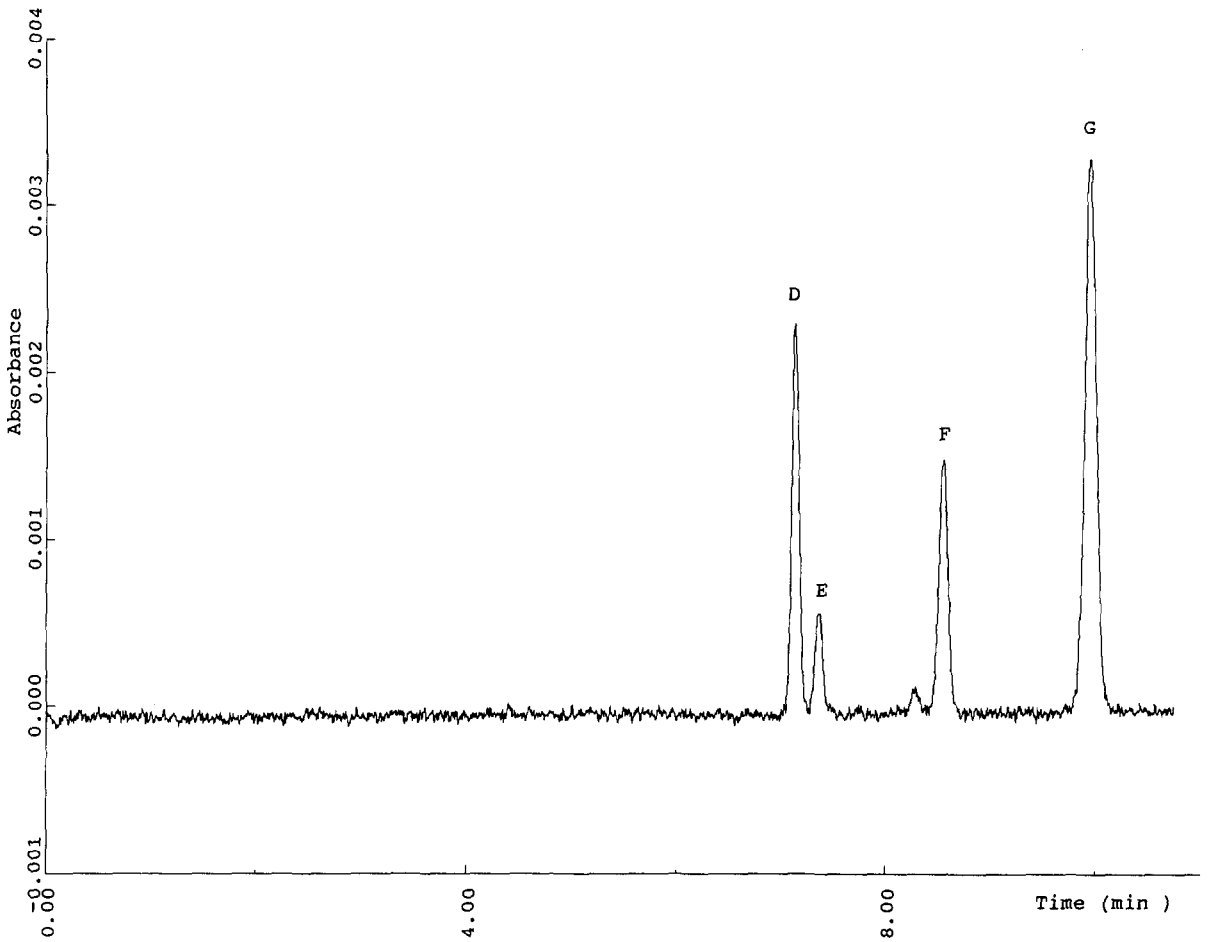

Fig. 9. Electropherogram of four test components at pH 4.99, as adjusted for a resolution of 2.00 and minimum time of analysis. The test components are given in Table I. The resolution determined for D and E is 1.98. For details, see text.

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