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# Resolution as a Function of the pH of the Buffer Based on the Analyte Charge Number for Multivalent Ions in Capillary Zone Electrophoresis without Electroosmotic Flow: Theoretical Prediction and Experimental Evaluation

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The electrophoretic resolution,  $R$ , consists of two terms: the selectivity term (with the ratio of the effective mobilities as the selectivity coefficient) and the efficiency term. It was shown that the latter depends on the effective charge number of the separands as the only analyte-specific parameter, when solely longitudinal diffusion is causing peak broadening, and when electroosmosis does not occur. Both terms, and thus the resolution, depend on the pH of the buffering electrolyte. The resolution was calculated for all pairs of separands (polyvalent acids) as a function of the pH, with the pK values and the actual mobilities of the separands, based on the equations derived. That unique pH was calculated where a certain resolution is established within the shortest time. The theoretically predicted pH values for a given resolution were compared with the experimental data. An excellent agreement was found for di- and tribasic weak acids, where the pH could be predicted within few hundredths units.

## INTRODUCTION

If capillary zone electrophoresis (CZE) is applied not only as a method for the determination of physicochemical properties of single compounds such as  $pK_a$ , electrophoretic mobility, or characteristics like electroosmotic flow and  $\zeta$  potential, but also as a separation method to determine the kind and the concentration of sample components, it is essential to resolve these separands to a degree, which allows their identification and quantitation with sufficient precision and accuracy.

This degree of separation of two components can be described in CZE by the same dimensionless number, the resolution,  $R$ , as in elution chromatography. It is defined by the distance of the centers of the peaks (which are identical with the peak maxima for Gaussian peaks) of two compounds  $i$  and  $j$ , measured in units which are related to the peak widths. From the different definitions given in chromatography, two were applied to CZE: the one introduced by Giddings<sup>1</sup> (eq 1) and one according to Huber<sup>2</sup> (eq 2), introduced into CZE in previous papers,<sup>3,4</sup> both expressed in the time domain as follows:

$$R_{ji} = (t_j - t_i) / (2\sigma_{ti} + 2\sigma_{tj}) \quad (1)$$

$$R_{ji} = (t_j - t_i) / \sigma_{ti} \quad (2)$$

In eqs 1 and 2,  $t$  is the migration time and  $\sigma_t$  is the standard deviation based on time. It can be seen that the difference between the two definitions lies in the choice of the scaling unit, which is  $2\sigma_{ti} + 2\sigma_{tj}$  in eq 1, and  $\sigma_{ti}$  in eq 2. Baseline separation is achieved for peaks with the same areas at a resolution of 1.5 or 6, respectively, with the conversion factor of 4.

The expressions as given in eqs 1 and 2 are, however, not in a form that allows operating with appropriate working parameters to adjust the resolution. Therefore they are transformed into more operative expressions, as in chromatography.

Such a transformation was introduced by Giddings<sup>5,6</sup> into CZE and led to the expression given in eq 3 for the case without

$$R_{ji} = \frac{1}{4} \frac{\Delta v}{\bar{v}} (\bar{N})^{1/2} = \frac{1}{4} \frac{u_i - u_j}{\bar{u}} \left( \frac{\bar{u} U}{2\bar{D}} \right)^{1/2} \quad (3)$$

electroosmosis, and longitudinal diffusion being the only process which causes peak dispersion, the case this contribution will be focused on.

$\Delta v$  is the velocity difference of the separands,  $\bar{v}$  is their average velocity,  $\bar{N}$  is the mean plate number,  $u_i$  is the electrophoretic mobility of separand  $i$ ,  $\bar{u}$  is the mean electrophoretic mobility of both separands under consideration,  $U$  is the voltage, and  $\bar{D}$  is the mean diffusion coefficient of analytes  $i$  and  $j$ . The reason to avoid electroosmotic flow in the present investigation was the demand for a very high precision of measurements of migration times and ionic mobilities, respectively, which could not be reached under conditions where electroosmosis occurs. A detailed discussion of the topic, including other processes of peak broadening, is given in refs 7 and 17.

Equation 3 was modified by Terabe et al.<sup>8</sup> for the special case of two separands having equal actual mobilities,  $u^{act}$  (these are the mobilities of the fully ionized subspecies at the ionic strength of the electrolyte solution), and very similar  $pK_a$  values. It was even possible to optimize the separation conditions to resolve benzoic acids that differed only by their oxygen isotope content.

It was demonstrated in previous papers<sup>3,4,9–11</sup> that an expression for the plate number and for the resolution can be derived which does not depend on the diffusion coefficients

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of the separands or on a mean diffusion coefficient, respectively. This is valid because the absolute (and with some approximation also the actual) ionic mobility is related to the diffusion coefficient, as given by the Nernst-Einstein equation,  $D = ukT/ze_0$ . According to this equation, the ratio  $u/D$ , occurring in the equation for the plate number,  $N$ , can be substituted, leading to the following transformed expression for  $N$ , for the case without electroosmosis (see also refs 1, 5, and 6).

$$N_i = z_i e_0 U / 2kT \quad (4)$$

In these equations  $e_0$  is the electric charge,  $k$  is the Boltzmann constant,  $T$  is the absolute temperature, and  $z_i$  is the effective charge number of the analyte  $i$ . In principle, this expression is valid only under certain limitations. Because the Nernst-Einstein equation is a limiting law, it is valid strictly only for infinite dilution. For ionic strengths commonly applied in CZE the deviation from ideality is, however, in an acceptable range for our purpose. It is also assumed that the difference between the diffusion coefficients of the charged and uncharged particles of the weak electrolyte considered is not too large (see, for example, ref 12).

Further, all other effects which cause peak broadening except longitudinal diffusion, resulting either from processes within the separation capillary, such as dispersion due to Joule heating, concentration overload, and adsorption, or stemming from a flow profile, are neglected in the derivation of eqs 3 and 4 as well as extracolumn effects like contributions from the width of the detector cell or the injection plug. The former effects are suppressed by applying not too high field strengths, by using an efficient cooling system, by working at low sample concentrations, and also by excluding electroosmosis. The latter effect was minimized in the present paper by injection of the test substances from water, which leads to a stacking of the ions and generates a very narrow effective input profile.

After insertion of the appropriate values for the electric charge number and the Boltzmann constant, eq 4 can be rewritten for 25 °C as  $N \approx 20z_i U$  ( $U$  given in volts), as already shown by Giddings.<sup>5</sup> For an effective charge number of 2, the plate number calculated from eq 4 is 150 000 under the given conditions (3.74 kV, 25 °C). In agreement with previous investigations,<sup>3,10,11</sup> values about 25% lower than the theoretical ones were found experimentally in the present work.

It can be seen from eq 4 that the plate number is related to the effective charge number as the only analyte-specific property. This approach was used to interpret the extremely large plate numbers observed for oligo- and polynucleotides in gel-filled capillaries.<sup>10</sup> It was also shown that theoretical predictions based on this approach were in agreement with experimental data, when strong mono- and divalent ions and weak monovalent electrolytes were investigated.<sup>3,4,11</sup>

Based on the context given along with eq 4, it was demonstrated for monovalent weak electrolytes that the resolution,  $R$ , depends only on the charge number as analyte-specific operational parameter. As a result, from this calculation an optimum buffer pH can be predicted for a demanded resolution<sup>4</sup> when the actual mobilities and the  $pK_a$  values of the separands are known. This approach differs from the optimization scheme introduced in CZE by Li et al.,<sup>13</sup> utilizing the overlapping resolution mapping procedure, where a number of preplanned experiments are performed and the optimum separation conditions are recalculated.

In this paper, in extension to the case of monovalent electrolytes, the expression for the resolution is generalized

for multivalent, weak electrolytes in CZE without electroosmotic flow. These equations were evaluated for a number of di- and tribasic acids, for which at least  $pK_a$  values were found. For substances with higher basicity no reliable data were available, so they could not be included in this investigation.

## EXPERIMENTAL SECTION

**Reagents.** Chemicals used for the preparation of the buffer solutions were disodium hydrogen phosphate 12-hydrate, sodium dihydrogen phosphate monohydrate, acetic acid, formic acid, malonic acid, glycine, hydrochloric acid, sodium hydroxide (all analytical-reagent grade; E. Merck, Darmstadt, Germany). The following compounds were used as test substances: 1,2-benzenedicarboxylic acid (phthalic acid, 99%), 1,3-benzenedicarboxylic acid (isophthalic acid, 99%), 1,4-benzenedicarboxylic acid (terephthalic acid, 98%, all Aldrich, Steinheim, Germany), 1,2,3-benzenetricarboxylic acid, dihydrate (98%, Fluka, Buchs, Switzerland), 1,2,4-benzenetricarboxylic acid, (99%, Aldrich), and 1,3,5-benzenetricarboxylic acid (98%, Aldrich).

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). Water was distilled twice from a quartz apparatus.

**Apparatus.** The measurements were carried out with an instrument (P/ACE System 2100, using System Gold 6.01; Beckman, Palo Alto, CA) which was equipped with a UV absorbance detector. The absorbance was measured at 214 nm. The separation capillary was made from fused silica (Scientific Glass Engineering, Ringwood, Australia) with a 75- $\mu$ m i.d. and with a total length of 0.269 m. The effective length (the distance from the injector to the detector) was 0.202 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 3750 V along the migration distance from injector to detector.

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

**Procedures.** The pH of the buffers was adjusted by adding sodium hydroxide solution to the solutions of the respective acids, adding hydrochloric acid to glycine, or combining disodium hydrogen phosphate 12-hydrate and sodium dihydrogen phosphate monohydrate, respectively, using a glass-calomel electrode. The electrode must be carefully adjusted in the pH range of interest prior to each measurement. The buffers were used in a pH range of  $\pm 1$  unit around the  $pK_a$  of the respective acid (described under Reagents).

The coating procedure is described in ref 14.

The effective ionic mobilities were measured in the usual way from the migration time and distance.

The  $pK_a$  values were taken from literature.<sup>15,16</sup>

## RESULTS AND DISCUSSION

In the Introduction, it was pointed out that an expression for the resolution of any pair of monovalent separands can be derived, based on the particular  $pK_a$  values and actual mobilities of the analytes, whereby the decisive analyte parameter is the effective charge, affected by the pH of the buffer only, as shown in previous papers.<sup>3,4</sup> In analogy to the monovalent ions, a general expression for multivalent ions will be introduced below.

Whereas much more data for the  $pK_a$  values than for mobilities are published, the latter are, if known, mostly given only for the totally dissociated form of the polyvalent ions and not for the subspecies. Furthermore, values of absolute mobilities,  $u_i^0$  (limiting mobilities for infinite dilution), are published rather than actual mobilities,  $u_i^{\text{act}}$  (at the ion strength under experimental conditions). In contrast to the  $pK_a$  values, the deviation of the actual mobilities from the

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**Table I. Dependence of the Effective Mobility,  $u_i^{\text{eff}}$ , on the pH of the Buffering Electrolyte<sup>a</sup>**

compound	$u_i^{\text{eff}}$ ( $10^{-5}$ cm <sup>2</sup> /V·s) at pH														
	2.00	2.50	3.00	3.50	3.70	3.90	4.00	4.50	5.00	5.50	6.00	6.50	7.00	7.50	8.00
1,2-benzenedicarboxylic acid (2A)	–	4.6	17.09	24.15	26.71	28.43	29.17	32.90	36.59	43.02	46.56	46.56	46.80	49.49	48.82
1,3-benzenedicarboxylic acid (2B)	–	–	6.8	14.80	20.33	24.95	26.91	38.29	44.29	48.17	48.56	47.23	47.17	49.76	48.95
1,4-benzenedicarboxylic acid (2C)	–	–	8.5	17.25	22.98	27.53	29.40	40.34	45.06	48.30	48.04	47.23	47.17	49.76	48.95
1,2,3-benzenetricarboxylic acid (3A)	–	7.6	16.49	24.09	28.30	31.28	32.81	40.98	44.72	49.09	53.27	54.23	55.90	59.78	59.39
1,2,4-benzenetricarboxylic acid (3B)	6.0	13.0	21.46	28.75	33.48	36.81	38.37	46.32	50.45	55.56	58.42	56.78	57.32	60.38	59.98
1,3,5-benzenetricarboxylic acid (3C)	–	–	13.0	23.49	29.74	34.57	36.59	48.17	54.56	58.81	58.81	57.50	57.68	60.78	59.98

<sup>a</sup> The effective mobilities were measured as described in the Experimental Section. The precision of the determination given by the range of five measurements was lower than 0.5% rel. (—) values are too low to be measured in a reasonable time ( $T = 25^\circ\text{C}$ ).

**Table II.  $pK_a$  Values and Actual Mobilities,  $u_i^{\text{act}}$ , of the Separands<sup>a</sup>**

compd	$pK_{a(i1)}$	$pK_{a(i2)}$	$pK_{a(i3)}$	$u_{i1}^{\text{act}}$ ( $10^{-5}$ cm <sup>2</sup> /V·s)	$u_{i2}^{\text{act}}$ ( $10^{-5}$ cm <sup>2</sup> /V·s)	$u_{i3}^{\text{act}}$ ( $10^{-5}$ cm <sup>2</sup> /V·s)
3A	2.80	4.20	5.87	25.4	46.9	59.5
3B	2.52	3.84	5.20	24.1	48.3	60.1
3C	2.13	3.90	4.70	9.6	58.0	60.0
2A	2.94	5.43	—	31.2	50.0	—
2B	3.70	4.60	—	36.5	50.1	—
2C	3.54	4.34	—	33.4	49.3	—

<sup>a</sup> The  $pK_a$  values were taken from refs 15 and 16. In the suffix of the actual mobilities,  $u_i^{\text{act}}$ , the numbers (1, 2, 3) indicate the basicity of the subspecies (the step of dissociation). The values for the actual mobilities,  $u_i^{\text{act}}$ , are the result of an approximation of the measured effective mobilities,  $u_i^{\text{eff}}$ , given in Table I, according to eq 5 ( $T = 25^\circ\text{C}$ ).

limiting values can be substantial. For this reason the  $pK_a$  values of the separands were taken from literature, but the actual mobilities were determined by own electrophoretic measurements.

**Determination of the Ionic Mobilities of the Individual Subspecies of the Polyvalent Ions.** The mobilities of the individual ionic subspecies,  $k$ , of the particular acids,  $i$ , with maximum basicity (or valency),  $n$ , were determined by measuring the dependence of the effective mobility,  $u_i^{\text{eff}}$ , of the separand, on the pH. They are given in Table I.

The theoretical relation of  $u_i^{\text{eff}}$  to the degree of dissociation,  $\alpha$ , and thus to the pH used here, is as follows:<sup>17</sup>

$$u_i^{\text{eff}} = \sum_{k=1}^n \alpha_{ik} (u_{ik}^{\text{act}} - u_{i(k-1)}^{\text{act}}) \quad (5)$$

with  $\alpha_{ik} = 1/(1 + 10^{pK_{a(i,k)} - \text{pH}})$  for acids.

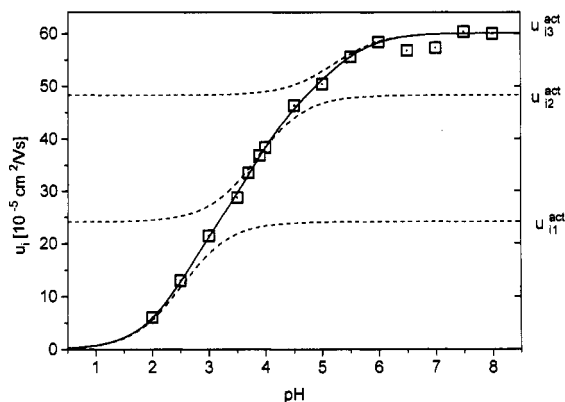
$\alpha_{ik}$  is the degree of dissociation for the subspecies of valency  $k$  for the given acid,  $i$ . Equation 5 reads, for example, for tribasic acids

$$u_i^{\text{eff}} = \alpha_{i1}u_{i1}^{\text{act}} + \alpha_{i2}(u_{i2}^{\text{act}} - u_{i1}^{\text{act}}) + \alpha_{i3}(u_{i3}^{\text{act}} - u_{i2}^{\text{act}}) \quad (6)$$

Curves for the pH dependence of the effective mobilities of the individual subspecies were approximated by an iteration procedure using eq 5 with the given  $pK_a$  values, taken from the literature (Table II). A least-squares fit of the experimental data of  $u_i^{\text{eff}}$  for different pH values of the buffers (see Table I) to the function as given in eq 5 was calculated with  $u_{ik}^{\text{act}}$  being the free parameters. A typical example of this approximation can be seen in Figure 1 for a tribasic acid.

The actual mobilities resulting from this approximation are given in Table II. They are used for further calculation of resolution and time of analysis.

**Selectivity, Efficiency, and Resolution.** It is generally accepted that the selectivity for any pair of separands,  $i$  and  $j$ , in capillary electrophoresis (for the case where no electroosmotic flow occurs) is described by the ratio of the effective



**Figure 1.** Mobility,  $u$ , of 1,2,4-benzenetricarboxylic acid (3B) as a function of the pH: (□) experimentally determined effective mobility. Solid line: calculated curve of the effective mobility, according to eq 5, with the values of  $u_k^{\text{act}}$ , obtained by iteration based on a least-squares fit (for details see text). Dotted lines: curves calculated for the individual ionic subspecies,  $u_k^{\text{eff}} = u_{(k-1)}^{\text{act}} + \alpha_k(u_k^{\text{act}} - u_{(k-1)}^{\text{act}})$ .

mobilities,  $r_{ij} = u_i^{\text{eff}}/u_j^{\text{eff}}$ , which is termed the selectivity coefficient<sup>18</sup> in analogy to chromatography. However, it was discussed in the Introduction that the efficiency of the system is also affected by the effective mobility of each separand. It was shown that, under the conditions defined, the only decisive separand property, which determines the plate height and the plate number, is the effective charge number. Thus, the resolution of two separands is determined by their actual mobility and the degree of dissociation. The latter and the charge number are influenced by the pH of the buffer only. This was demonstrated for a number of monovalent ions.<sup>4</sup> For the general case of multivalent ions, as discussed here, the appropriate derivation of the equation based on the definitions for the resolution given in eqs 1 and 2 (substituting  $\sigma_{ti}$  by  $t_i/(N_i)^{1/2}$ , and introducing eq 4 for  $N_i$ , and by substitution of  $t_i$  by  $L/u_i^{\text{eff}}E$ , where  $L$  is the effective length and  $E$  the effective field strength) leads to two cases. The one according to eq 1 gives

$$R_{ji} = \frac{(u_i^{\text{eff}} - u_j^{\text{eff}})(z_i z_j)^{1/2}}{u_i^{\text{eff}} z_i^{1/2} + u_j^{\text{eff}} z_j^{1/2}} \left( \frac{e_0 U}{8kT} \right)^{1/2} \quad (7)$$

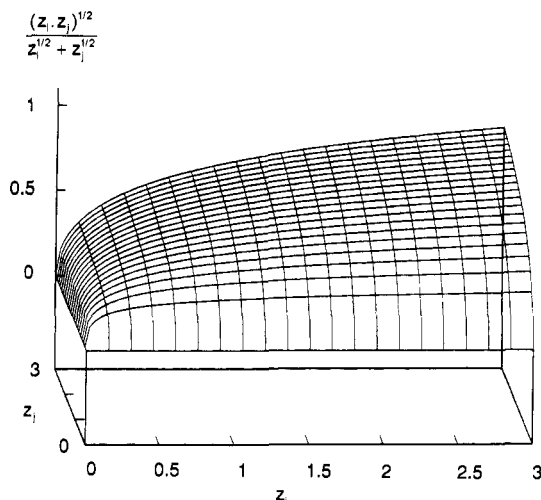
or

$$R_{ji} = (r_{ij} - 1) \frac{1}{1/z_i^{1/2} + r_{ij}/z_j^{1/2}} \left( \frac{e_0 U}{8kT} \right)^{1/2} \quad (8)$$

In these equations the effective charge number,  $z_i$ , is approximated by

$$z_i = \sum_{k=1}^n \alpha_{ik} \quad (9)$$

$\alpha_{ik}$  being the degree of dissociation of the individual ionic subspecies as defined above. It should be mentioned that some corrections must be made for highly charged species.



**Figure 2.** Charge-dependent term,  $(z_i z_j)^{1/2} / (z_i^{1/2} + z_j^{1/2})$ , of the resolution (eq 10) as a function of the charge numbers  $z_i$  and  $z_j$  of the separands  $i$  and  $j$ .

In contrast to the degree of dissociation  $\alpha$ , which can only have values between 0 and 1,  $z_i$  can range from 0 to  $n$ , the maximum valency.

Closely migrating components have very similar effective mobilities. In this case the selectivity coefficient,  $r_{ij}$ , is about unity, and the second term on the right-hand side of eq 8 becomes  $(z_i z_j)^{1/2} / (z_i^{1/2} + z_j^{1/2})$ . In this case, which is the most interesting case with respect to the optimization of the separation conditions, eq 8 can be transformed to

$$R_{ji} = (r_{ij} - 1) \left[ \frac{(z_i z_j)^{1/2}}{z_i^{1/2} + z_j^{1/2}} \left( \frac{e_0 U}{8kT} \right)^{1/2} \right] \quad (10)$$

The influence of the charge of the distinct analytes on the charge-dependent factor,  $(z_i z_j)^{1/2} / (z_i^{1/2} + z_j^{1/2})$ , of the efficiency term in eq 10 is shown in Figure 2. This factor increases with increasing charge numbers, steeper at smaller values than at larger ones. It reaches a maximum value at the highest charge numbers, where the system therefore has the highest separation efficiency, but not necessarily the highest resolving power, because the selectivity is lost drastically if the actual mobilities (of the totally dissociated acids) are close or even identical. This will indeed be the case for a number of components given in Table II, e.g., 2A–2B or 3B–3C.

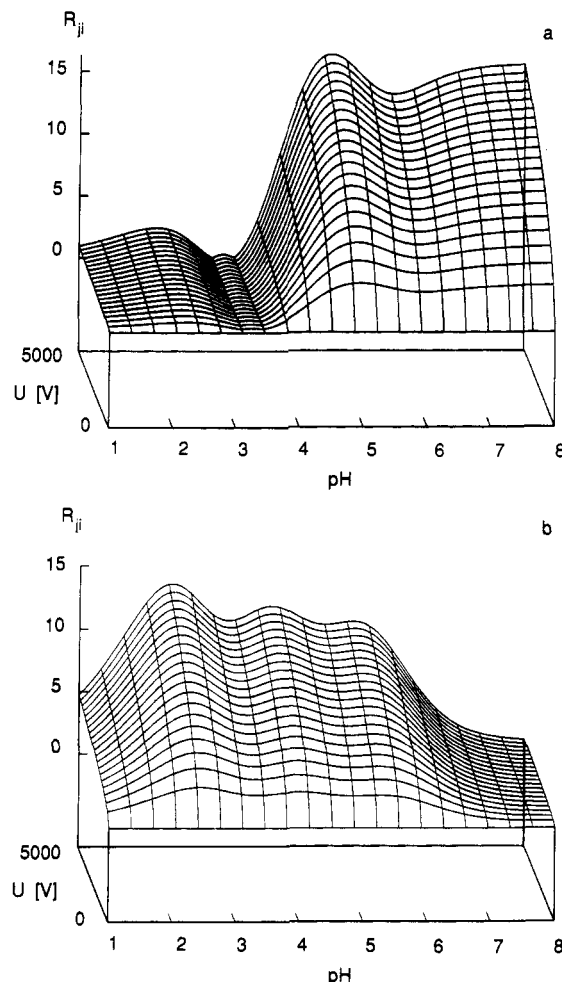
Obviously, this factor, and therefore the resolution, is not defined for the charge number of zero of both analytes, because in this case no electromigration occurs. This factor is zero for the case that  $z_j$  is zero and  $z_i$  has a finite value. The resulting resolution is, however, not defined either, because then the selectivity coefficient reads  $r_{ij} = u_i/0$  and is thus not defined. This is also obvious, because in this case one of the analytes remains at the injector end of the separation capillary.

The resolution based on the definition given in eq 2 reads as follows after an analogue substitution of the migration times,  $t_j$  and  $t_i$ , and the standard deviation  $\sigma_{ii}$ , as carried out above:

$$R_{ji} = (r_{ij} - 1) \left[ z_i^{1/2} \left( \frac{e_0 U}{2kT} \right)^{1/2} \right] \quad (11)$$

In both expressions for the resolution two main terms can be distinguished, as in the case for monovalent ions discussed in previous papers: (1) the selectivity term, given by  $r_{ij} - 1$ ; (2) the efficiency term, which consists of two parts, one affected by the voltage (a fact which is well-known) and the other determined by the effective analyte charge number(s).

It can be concluded that the resolution depends on (1) physical constants  $e_0$  and  $k$ , (2) instrumental parameters  $U$  and  $T$ , and (3) analyte parameters  $u_i^{\text{act}}$ ,  $pK_a$ ,  $\alpha$ , and  $z_i$ .

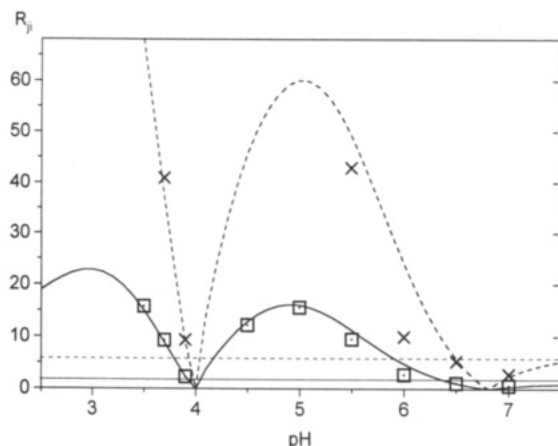


**Figure 3.** Resolution as a function of pH and voltage for two different pairs of separands: (a) 2A–3A; (b) 3A–3B. Compounds as in Table I. The resolution was calculated according to eq 8. The values of  $u_i^{\text{act}}$  and  $pK_a$  were taken from Table II.

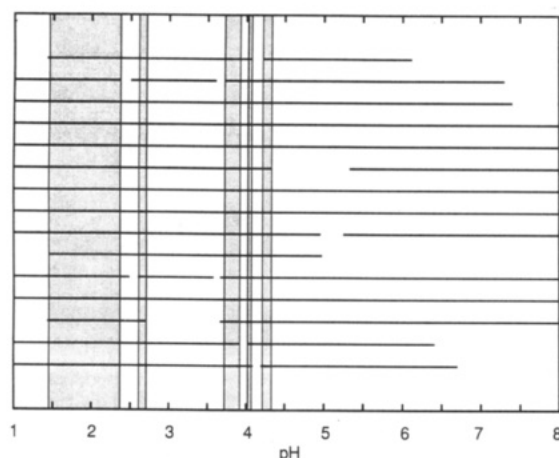
From the analyte parameters, however, only the degree of dissociation and thus the charge number is a variable in a given solvent system, namely, a function of the pH of the buffer. It follows that (besides the temperature) only two parameters exist in order to adjust the resolution in CZE: the voltage and the pH.

Whereas the voltage influences the resolution in a quite simple way (whereby the order of this dependence is still under discussion<sup>19</sup>), the dependence of the resolution on the pH is more complex. As it is not the goal of this investigation to deal with the aspects of the effect of the voltage on the plate number in detail,  $N$  is simply taken directly proportional to  $U$ .

In this way the dependence of  $R_{ji}$  on pH was computed for all 15 possible pairs of components, according to eqs 7 and 11. As expected, the resulting curves show a larger complexity than those obtained for pairs of only monobasic acids (as discussed in ref 4), because more sets of different  $pK_a/u_i^{\text{act}}$  combinations are possible. Two examples are shown in Figure 3. It can be seen that for certain cases the resolution increases with increasing pH, has a maximum, and decreases at higher pH. Depending on the mutual sequence of actual mobility and  $pK_a$  value, the curve can also fall to a resolution of zero at a certain pH. This effect is independent of the efficiency, and is the case when the migration sequence changes with varying pH, because the effective mobilities of the two separands approach each other. Such changes are analogous to those discussed for the case of monovalent acids and will



**Figure 4.** Agreement of the experimental values of the resolution,  $R_p$ , with the theoretical curve for the pair 2A-2C (Table I). Curve calculated according to eqs 7 (solid line) and 11 (dotted line) with values from Table II. (□) measured values according to eq 7; (X) measured values according to eq 11. The horizontal lines are drawn at a resolution of 1.5, and 6.0, respectively.

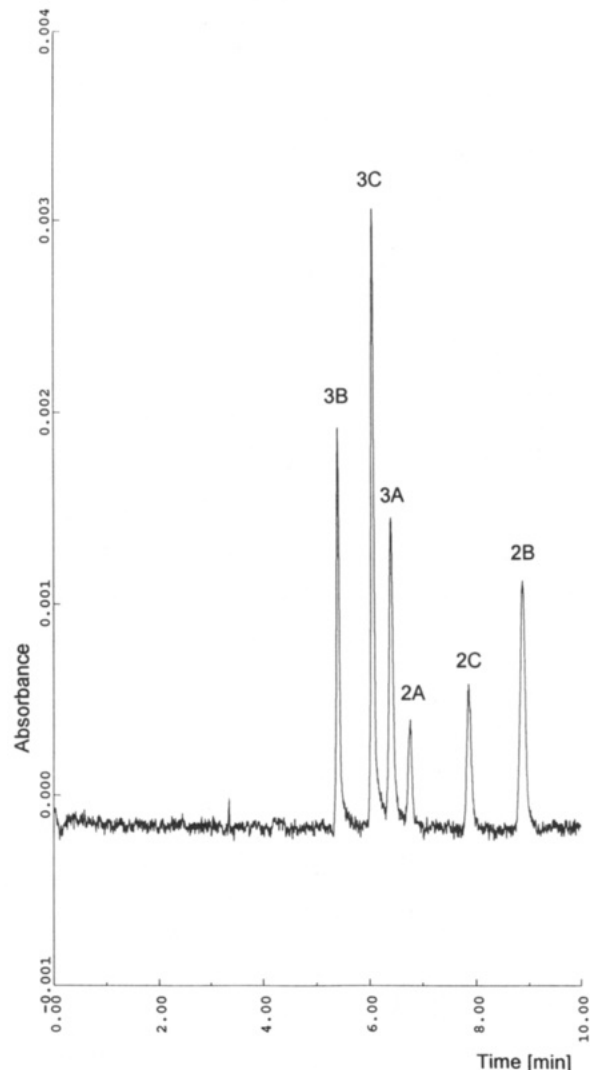


**Figure 5.** pH ranges with a resolution of  $\geq 1.5$ , according to eq 7, for all 15 possible pairs of analytes. These ranges, delineated by the horizontal lines, were obtained for all possible  $R_p$  vs pH curves, two of them shown in Figure 3. The vertical lines border those five overlapping pH ranges, where all pairs of analytes are separated with a resolution of  $\geq 1.5$ : 1.45-2.37, 2.60-2.71, 3.72-3.93, 4.02-4.07, and 4.21-4.33. Sequence of pairs (according to Table I) from bottom to top: 2A-2B, 2A-2C, 2A-3A, 2A-3B, 2A-3C, 2B-2C, 2B-3A, 2B-3B, 2B-3C, 2C-3A, 2C-3B, 2C-3C, 3A-3B, 3A-3C, 3B-3C.

not be treated here in more detail. In contrast to simple electrolytes, polyvalent ions can create more than one single pH value where the resolution falls to zero, according to the particular  $pK_a$  values. Theoretically two trivalent acids can show up to five distinct pH values with a resolution of zero.

The agreement of the experimental values of the resolution with the theoretical curve according to eqs 7 and 11, is shown for one example in Figure 4. For very high resolution values (which are normally not of interest) the experimental values follow eq 11, at least in tendency, but are in good agreement with eq 7, even in this range of  $R$ . For lower values of the  $R_{ji}$  (which is the more important case), the agreement is, however, excellent for both curves.

Based on such graphs, as shown in Figure 4, the pH ranges where a certain resolution is established can be calculated for each pair of separands. For a resolution of 1.5 (eq 7) these ranges are shown in Figure 5, represented by the vertical lines. All pairs of components should be separated with that chosen, minimum resolution within the following overlapping pH ranges: 1.45-2.37, 2.60-2.71, 3.72-3.93, 4.02-4.07, and 4.21-4.33. The results of the calculations based on eq 11 agree with that derived by eq 7 within one-hundredth pH



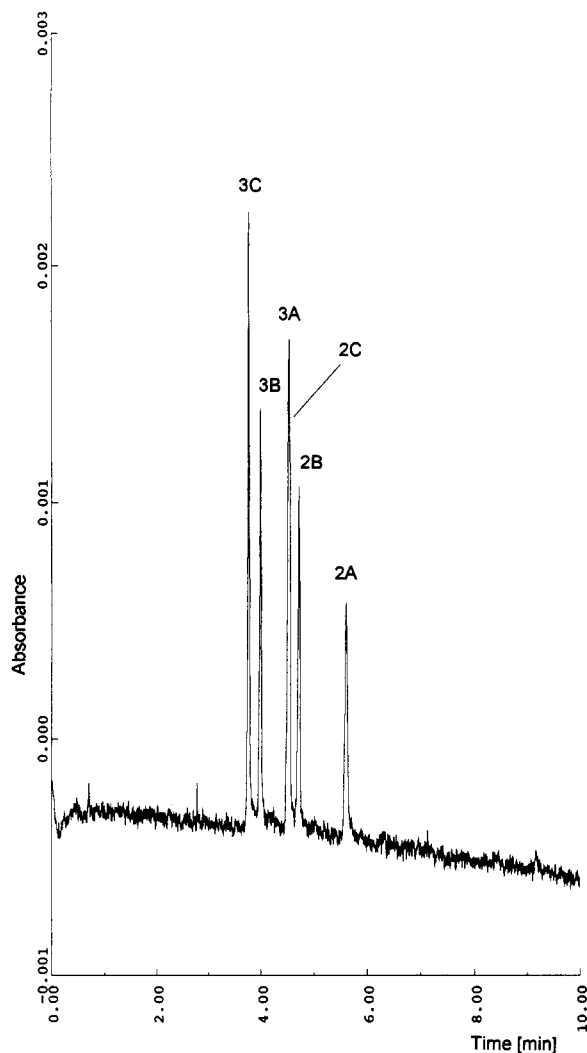
**Figure 6.** Electropherogram of the separands at pH 3.73. The test components are given in Table I. For details, see text.

unit in the range of interest. Below a pH of 2.5 the values differ slightly more.

According to the calculations, baseline separation should be obtained, for example, at pH 3.73. This can indeed be seen from the electropherogram given in Figure 6. The agreement of the experimental findings with the theoretical prediction is also demonstrated by Figure 7, where the electropherogram obtained at pH 4.37, only 0.04 pH unit outside the pH range, is shown: the resolution is indeed smaller than 1.5 (6.0) for the pair 3A-2C, as predicted from theory.

**Analysis Time.** An appropriate resolution of the components is one aim of an analysis; another important task often is the minimum time of analysis within which a given resolution can be obtained. In the case of CZE, this time of analysis is related to the retention time of the slowest migrating component. Obviously this time is determined by the effective mobility of this component, which is affected by the pH as discussed above. From all pH values, where a given resolution is reached or exceeded, the highest pH value of all ranges is that unique value, where the slowest migrating component has the highest degree of dissociation and, therefore, the highest effective mobility. It is clear that this argumentation is valid for anions; for cations it will be the lowest pH.

It follows from Figure 5 that the pH for the minimum time of analysis for the set of components under consideration (for a minimum resolution of 1.5 or 6.0, respectively) must



**Figure 7.** Electropherogram of the separands at pH 4.37. The test components are given in Table I. 2C appears as a shoulder of peak 3A. For details, see text.

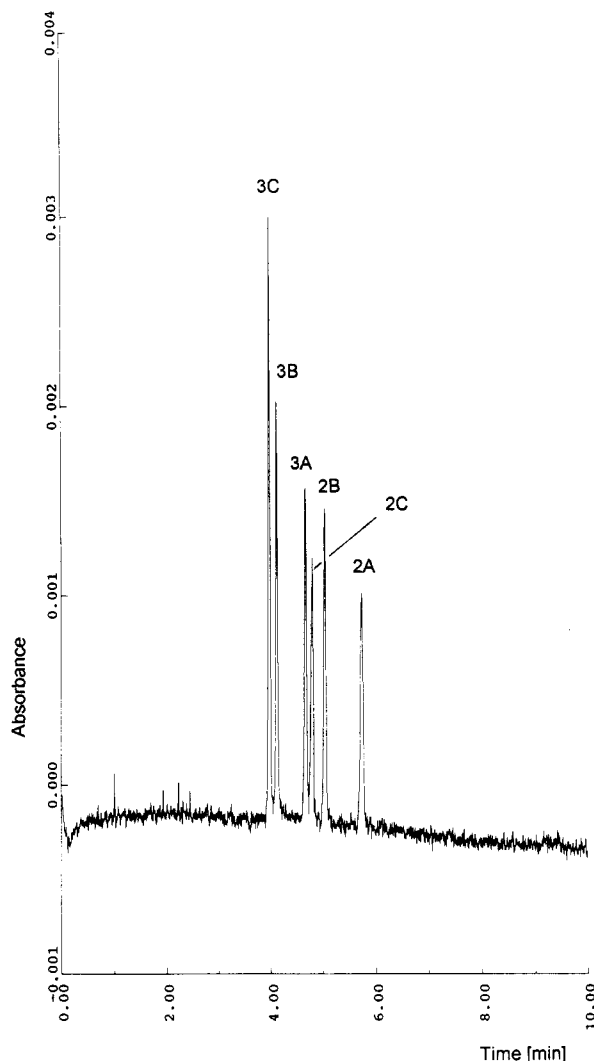
be 4.33. The electropherogram obtained at this pH is shown in Figure 8. Indeed the minimum resolution, namely, that for the pair 3A–3C, is about 1.7 (6.8), in agreement with the theoretically predicted value. It was pointed out above that even a minor shift of the pH, namely, only by 0.04 unit, leads to a drastical loss of the resolution (see Figure 7, at pH 4.37).

Compared with the result obtained at pH 3.73, where a separation with a resolution of 1.5 (6.0) or larger is also obtained in about 9 min, at pH 4.33 a reduction of the time of analysis to about 6 min can be reached. This is an improvement of the analysis time by about one-third.

### CONCLUSION

The results allow the conclusion that the model presented here is valid not only for monovalent analytes but, in general, also for multivalent constituents of the sample. The central point is that the effective charge number influences not only the selectivity, but also the efficiency in CZE and is therefore the central analyte parameter for the adjustment of the resolution under the conditions and limitations discussed in the Introduction.

However, problems still remain for the prediction of the pH for a given resolution and minimum analysis time. One is due to the very limited number of data for  $pK_a$  values and actual mobilities available for the ionic subspecies, especially for polybasic or polyacidic analytes. This was one of the restrictions which caused us to use di- and tribasic acids in this paper, but no analytes with higher valency.



**Figure 8.** Electropherogram of the separands at pH 4.33, which is predicted theoretically as that unique pH where all components are separated with a resolution of  $\geq 1.5$  (6.0) and where the minimum time of analysis is reached. The test components are given in Table I.

Another problem is given by the fact that the effective charge number, which is decisive for the resolution, may not be identical with a nominal charge number, that calculated from the  $pK$  values of the analytes and the pH of the buffer. This may be a special problem when ions are considered with high charge density, which can easily undergo association reactions with counterions (whereby such association reactions can be, on the other hand, utilized in some cases to improve the resolution for specific target ions).

Further, for highly charged oligo- or polymers the effective charge number is related to the  $\zeta$  potential of the particles rather than to a nominal charge number and is often not known exactly. For these cases the model described here may still be valid, but it can hardly be proved experimentally.

The most severe experimental limitation was found to originate from concentration overload, so that peaks in the electropherograms are nonsymmetrical, especially for samples consisting of analytes with very different mobilities. In this case the selection of the type and concentration of the buffer must be focused onto that pair of analytes which is most critically to be separated, and it must be accepted that other peaks in the electropherogram still remain asymmetric.

Within the defined limitations, however, an excellent agreement of the experimental results is found with the theoretical predictions of the resolution as a function of the pH of the buffer. Especially that unique pH value could be predicted where a certain resolution is established in the

shortest analysis time within the subtle range of a few hundredths units.

**Registry Numbers Provided by the Authors:** Disodium hydrogen phosphate 12-hydrate, 10039-32-4; sodium dihydrogen phosphate monohydrate, 10049-21-5; acetic acid, 64-19-7; formic acid, 64-18-6; malonic acid, 141-82-2; glycine, 56-40-6; hydrochloric acid, 7647-01-0; sodium hydroxide, 1310-73-2; 1,2-benzenedicarboxylic acid, 88-99-3; 1,3-benzenedi-

carboxylic acid, 121-91-5; 1,4-benzenedicarboxylic acid, 100-21-0; 1,2,3-benzenetricarboxylic acid dihydrate, 569-51-7; 1,2,4-benzenetricarboxylic acid, 528-44-9; 1,3,5-benzenetricarboxylic acid, 554-95-0; methylcellulose, 9004-67-5; formaldehyde, 50-00-0.

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