Capillary zone electrophoresis in mixed aqueous–organic media: effect of organic solvents on actual ionic mobilities, acidity constants and separation selectivity of substituted aromatic acids. I. Methanol

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Abstract

The impact on the actual mobility, μ_{act}, of 25 substituted aromatic single charged carboxylates (with hydroxy, methyl, nitro or chloro substituents) and benzoate by addition of up to 75% (v/v) of methanol to the background electrolyte (phosphate buffer, pH 7 in water, ionic strength 20 mmol l^{-1}) was investigated systematically. Furthermore, the magnitude of the changes in the pK_a values of five of the benzoic acids in the mixed aqueous–organic environment (up to about 80%, v/v, methanol), which was found to be 1.5–2 units higher compared to pure aqueous solutions, was determined by potentiometric titration. The solutes were classified based on the change of their actual mobility by cluster analysis with a hierarchical clustering procedure. The influence of methanol on the actual mobility was discussed concerning the viscosity of the solution, showing that Walden's rule is obeyed only roughly. A different behavior of the mobilities of hydroxy-substituted acids upon addition of methanol was found compared to the methyl, nitro and chloro derivatives, as their actual mobilities were reduced to a markedly larger extent. This effect was related to the hydrogen bonding ability of the OH groups of the former class of anions. Generally, enhancement in selectivity (expressed by the selectivity coefficient, the ratio of the actual mobility of benzoate, used as reference ion, to that of the particular separand) by adding methanol as organic modifier was found only for the acids with hydroxy substituents. For the other solutes in general selectivity decreased upon addition of methanol. © 1998 Elsevier Science B.V.

Keywords: Buffer composition; Electrophoretic mobility; Selectivity; Dissociation constants; Organic acids; Methanol

1. Introduction

Resolution in capillary zone electrophoresis can be achieved adjusting two particular parameters: (1) the separation efficiency, given by the plate number, N (influenced in the ideal case by the applied voltage, the ion charge and the magnitude of the electro-osmotic flow, the EOF) and (2) the separation selectivity, expressed e.g. by the selectivity coefficient, the ratio of the mobilities of the pair of separands under investigation. It is obvious that the expression for the resolution is composed by the efficiency term, given by the square root of the plate number, and the selectivity term, given by $\Delta\mu_{tot}/\bar{\mu}_{tot}$, the relative difference of the total mobilities, μ_{tot}, of two particular separands, i and j. However,

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this relative difference can often be approximated by $(\mu_{\text{tot},i}/\mu_{\text{tot},j}) - 1$. Thus, in the present paper the selectivity coefficient is considered rather than the selectivity term throughout.

In this paper focus is directed to separation selectivity, not to efficiency (the plate number, N, is a topic addressed in a number of previous papers [1–10]); it concentrates on the mobility of the separands. Under analytical conditions the mobility is composed of three individual parameters. Two of them are solute specific, namely the actual mobility, $\mu_{\text{act},i}$ (which represents the mobility of the fully charged species, i, in respect to the chosen ionic strength and temperature), and the average charge (depending on the degree of ionization, α_i) influenced by protolysis or by complexation. These two parameters determine the effective mobility, $\mu_{\text{eff},i}$, of the species. Migration is, however, also unspecifically dependent on the extend and direction of the EOF (with mobility μ_{EOF}), occurring in untreaded fused-silica capillaries.

The effective mobility of weak electrolytes is strongly influenced by the pH of the buffering background electrolyte (BGE). The buffer pH is a common tool for adjusting selectivity in the separation of small analytes. However, there is still demand on other possibilities to affect migration selectively. One is to work with aqueous–organic solvents to influence the actual mobility as well as the pK_a value of the separands. This paper focuses (as those published previously [11–14]) on the separation selectivity, which can be expressed by the selectivity coefficient, r_{ij}, of the pair of separands, i and j, defined as:

$$r_{ij} = \frac{\mu_{\text{tot},i}}{\mu_{\text{tot},j}} \tag{1}$$

The total mobility, $\mu_{\text{tot},i}$, of a separand is

$$\mu_{\text{tot},i} = \mu_{\text{act},i}\alpha_i + \mu_{\text{EOF}} \tag{2}$$

If the dependency of the degree of ionization, α, of a weak monobasic acid on the pH of the buffering background electrolyte and the solute's pK_a value is also introduced Eq. (2) is transformed to:

$$\mu_{\text{tot},i} = \mu_{\text{act},i}\frac{1}{1 + 10^{pK_{a,i} - \text{pH}}} + \mu_{\text{EOF}} \tag{3}$$

According to Eq. (3) selectivity in CZE separations is influenced (besides the magnitude and the direction of the EOF) by the actual mobility and the pK_a value of the individual separands. The effect of organic cosolvents in the BGE of the CZE system on these two variables, of central importance in the discussion of the application of mixed media in capillary electrophoresis, is the topic of the present investigation. The variation of the mobility of the EOF in presence of organic solvents has been investigated and discussed in previous papers [15,16].

A number of solvents, including both protic and aprotic dipolar solvents are of interest [17,18]. Criteria for application of these solvents are (1) their miscability with water, (2) the ability to dissolve ionic samples and buffers, (3) the transparency to detection light beam, (4) sufficiently low evaporation properties under the experimental conditions and in regard to practical reasons their (5) low toxic properties and prices.

According to these criteria only a considerably small number of solvents remain as potential candidates in this investigation (less than for HPLC): the lower alcohols and acetonitrile. Solvents like the dipolar, aprotic N,N-dimethylformamide, N,N-dimethylacetamide or dimethyl sulfoxide, although of principle interest and already applied in CE (see e.g. Refs. [19–23] and the citations given in Ref. [18]) were not taken into consideration in the present investigation due to their high extinction coefficient at the wave length range normally used with UV detectors (below 260 nm). This fact makes the investigation of the solutes difficult at the low concentration level necessary to avoid concentration overload, resulting in peak triangulation and thus to problems in an accurate determination of the actual mobilities.

In order to investigate the effects of the organic cosolvent on the decisive electrophoretic parameters in a fundamental way actual mobilities-those of the fully deprotonized anions of the aromatic acids- were determined rather than the pH dependence of their total mobilities. This allows a more pronounced understanding of the effects caused by the organic solvent. As it is clear that the degree of dissociation, governed by the pK_a of the analyte and adjusted by the pH of the buffer is the most powerful tool to

affect selectivity in CZE, changes in pK_a of the analytes by the solvent was also a central topic. However, a discussion of the variation of the resolution by adding organic solvents is not given here, because this would hinder to clearly separate the effect of the solvent on selectivity from that on efficiency, and would thus counteract the intention of the present investigation.

It was pointed out that the selection of the solvents for this investigation is based on the criteria mentioned above, resulting in the application of binary mixtures of water with methanol (MeOH), ethanol (EtOH), 1-propanol (PrOH) and acetonitrile (ACN), respectively. Due to the large number of data obtained and the resulting comprehensive discussion only the results in aqueous-methanolic solutions are presented in this paper. The other systems are topic of separate contributions.

2. Experimental

2.1. Apparatus

Electrophoretic experiments were performed on a P/ACE 2100 CE unit (Beckman Instruments, Fullerton, CA, USA) equipped with a variable wavelength UV absorbance detector set at 214 nm. Gold Software 3.0 was used for data collection. For all measurements untreated fused-silica capillaries (Polymicro Technologies, Bloomfield, NJ, USA) were used. Runs without pressure mobilization steps, representing the majority of the measurements, were carried out in a 50 µm I.D. capillary with 27.1 cm total length and 20.4 cm distance to the detector window.

Samples were injected by pressure (3.5 kPa) for 1 s. The applied constant voltage was set at 10.0 kV during all runs with a linear voltage ramp-up of 0.17 min. This ramp was taken into account on the calculation of the mobilities. Measured currents were 11.7 µA at 0% MeOH, 8.3 µA at 20%, 6.8 µA at 40%, 5.8 µA at 60% and 3.4 µA at 75% (v/v) MeOH in the normal mode (at combined pressure-voltage mode at 75% MeOH it was 6.9 µA).

For measurements of the mobility in an electrophoretic step combined with two pressure modes as demonstrated in Ref. [24]-undertaken for some of the

determinations in 75% MeOH, where the mobility of samples and that of the EOF where too similar to allow reasonable times of measurement- the sample together with a neutral marker (dimethyl sulfoxide, DMSO) was injected (injection time 1 sec), shifted into the capillary by pressure for 2 min (pressure 3.5 kPa), separated from DMSO for 6 min by applying a voltage of 10 kV, followed by a 0.02 min injection of the neutral marker and finalized by the application of pressure (3.5 kPa) to shift all zones toward the detector. For these experiments a 75 µm I.D. capillary with a total length of 46.4 cm and 39.6 cm to the detection window was used.

The capillary cartridge was thermostatted during all runs at 25.0°C. The exact average temperature of the background electrolyte is not known accurately, because about 7 cm of the capillary (2 times 3.5 cm at each end) are situated outside the thermostatted cartridge. It should be noted, that this is a significant part of the entire capillary length. These conditions were, however, constant for all measurements.

In both modes the capillary was rinsed with high pressure (140 kPa) between each run for 1 min with bidistilled water and for 2 min with the running buffer.

The potentiometric determination of the apparent pK_a values of the acids were carried out with a titration unit (model DL 67, Mettler Toledo, Schwerzenbach, Switzerland) equipped with a glass-calomel electrode (DG 101-SC, Mettler Toledo); the same electrode was used for the preparation of the buffer electrolytes. It was filled with aqueous KCl, 3 mol l^{-1}, saturated with AgCl. Calibration was made with aqueous standard buffer solutions. Titrator was a solution of 0.1 mol l^{-1} NaOH.

2.2. Chemicals

Phosphate buffer (5 mmol l^{-1} Na$_2$HPO4 and 5 mmol l^{-1} NaH$_2$PO4, analytical grade, E. Merck, Darmstadt, Germany) was used throughout all measurements. Its solubility limited the concentration range of the mixed aqueous–methanolic solvent to 75% (v/v) methanol, as with higher methanol concentration precipitation of the phosphate occurred. The (apparent) pH of the resulting solutions measured by a glass electrode was 7.02 at 0%, 7.45 at

20%, 7.86 at 40%, 8.43 at 60% or 9.08 at 75% (v/v) MeOH (analytical grade, Merck).

Aromatic acids (concentration 0.1 mmol l^{-1} for mobility determination, 10 mmol l^{-1} for pK_a determinations) were purchased from EGA-Chemie (Steinheim, Germany) at the highest available purity.

3. Results and discussion

3.1. pK_a values

Data of the apparent pK_a values of benzoic acid and the 3-hydroxy-, 3-methyl-, 3-nitro-, 3-chloroderivatives) obtained by potentiometric titration in the mixed solvents are given in Table 1 for methanol concentrations between 0 and about 80% (v/v). In the following discussion the substances are named with symbols indicating the position of the substituent(s) by a number, and symbolizing the substituent by OH for the hydroxy group, Me for the methyl-, NO$_2$ for the nitro- and Cl for the chloro group. 2,4,6-triNO$_2$ e.g. means 2,4,6-trinitrobenzoic acid.

To better visualize the effect of methanol on the pK_a value the differences between the apparent p$K_{a,mix}$ in the mixed solvent and the p$K_{a,water}$ in pure water are depicted in Fig. 1. It can be seen that the apparent p$K_{a,mix}$ values increase nonlinearly upon addition of methanol, and reach values between 1.5 and 2 units higher in about 80% (v/v) methanolic solvent than in pure aqueous solution.

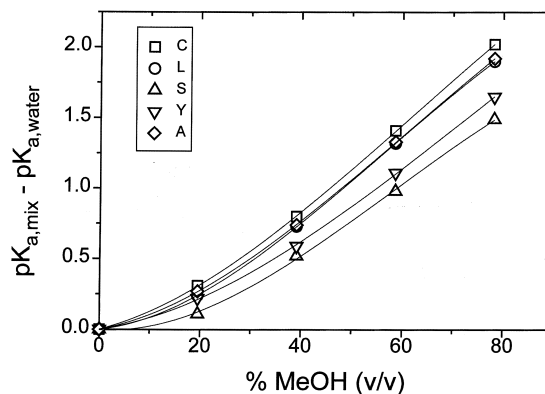The dependency of the pK_a on the content of

Fig. 1. Increase of the appearent pK_a values of five aromatic acids by addition of methanol as cosolvent, compared to the pure aqueous solution. $I = 0.005$ mol l^{-1}, $T = 25°C$. The symbols of the acids are according to Table 2.

methanol can be described by polynomial fit of the third order according to the equation

$$pK_{a,mix} = pK_{a,water} + a_1c^1 + a_2c^2 + a_3c^3 \qquad (4)$$

where a_1, a_2 and a_3 represent the coefficients of regression, and c the percentual concentration of methanol in % (v/v). The fitted curves lead to high correlation coefficients between 0.9997 and 0.99999, and match better (by one 9 in the correlation coefficients) than those obtained by the second power fit.

Besides this general tendency, it is clear from the result of the curves that methanol influences the pK_a values selectively, otherwise the curves should fall better into one single line. In fact the selective change through the addition of methanol on the pK_a values can be proved by plotting the pK_a values in water against those in the mixed aqueous-methanolic solvents. The coefficient of linear correlation in fact ranges between 0.996 and 0.984, reflecting moderate linear correlation. Thus, it can be concluded that, on the one hand, a clear tendency of the dependence of the pK_a with variable methanol contents is observable; the values can be predicted within 0.1 to 0.2 pK units in the methanol concentration range investigated. A more accurate value – in the range of few 0.01 units, essential for the prediction of the obtainable resolution [6,8] cannot be forecasted, on the other hand. It is possible, however, concerning the

Table 1
Apparent pK_a values of benzoic, 3-hydroxy (3-OH), 3-methyl- (3-Me), 3-nitro- (3-NO$_2$), and 3-chloro- (3-Cl) benzoic acids in water and mixed aqueous-methanolic solutions

MeOH (%, v/v)	pK_a value				
	Benzoic	3-OH	3-Me	3-NO$_2$	3-Cl
0	4.12	4.03	4.24	3.46	3.82
19.5	4.39	4.34	4.48	3.57	4.04
39.1	4.86	4.83	4.97	3.98	4.41
58.6	5.45	5.44	5.56	4.44	4.93
78.2	6.04	6.05	6.14	4.95	5.47

$I = 0.005$ mol l^{-1}, $T = 25°C$. Symbols in parenthesis as in Table 2.

excellent correlation coefficient of the exponential fit, to calculate very accurately the pK_a in a solution with any methanol content in the given range.

The result of the pK_a dependence on the methanol concentration can be interpreted by several concepts. One is based on electrostatic interactions, according to Born's theory, another concept consideres aqueous–methanolic mixtures as a ternary solvent, consisting of clusters of water, methanol, and combined water–methanol species [25,26]; the longest established concept is that of the medium effect, $\ln \gamma_i^m$ (or $\log \gamma_i^m$), given by

$$\ln \gamma_i^m = \frac{(\bar{\omega}_i^0)_{mix} - (\bar{\omega}_i^0)_{water}}{RT} \qquad (5)$$

Here the transfer activity coefficient or medium activity coefficient, γ_i^m, is defined as $\gamma_i^m = \gamma_{i,mix}/\gamma_{i,water}$; $\gamma_{i,mix}$ and $\gamma_{i,water}$ are the activity coefficients of species i in the mixed solvent and water respectively. $\bar{\omega}_i^0$ is the chemical potential of component i in the standard state, R is the gas constant and T the absolute temperature.

According to this model (for details cf. e.g. [27–33] and the papers cited in Refs. [11,12,14,17]), the reversible work for the transfer of 1 mol of species i from infinite dilution in water to infinite dilution in solvent S is related to the medium effect. If the mixed solvent better stabilizes the particles of species i than water, the medium effect has a negative value and vice versa. When applied to the proton, the medium effect is a measure for the basicity of the solvent S relative to water.

For the case under consideration, the neutral acid, HA, undergoes the dissociation equilibrium HA = $H^+ + A^-$ in both solvents. The reversible work in transferring 1 mol H^+ and A^- from infinite dilution in water to infinite dilution in the mixed aqueous–methanolic solvent (and transferring 1 mole of HA from the mixed solvent to water) is given by the standard free energy of transfer, ΔG_t^0. Consequently, the change of the dissociation constant, K_a, that is related to this standard free energy of transfer, is given by

$$\Delta pK_a = pK_{a,mix} - pK_{a,water} = \log \frac{\gamma_{H^+}^m \gamma_{A^-}^m}{\gamma_{HA}^m} \qquad (6)$$

where $\gamma_{H^+}^m$, $\gamma_{A^-}^m$ and γ_{HA}^m are the transfer activity coefficients of the particles involved in the ionization

equilibrium: the proton, the anion and the neutral molecule, respectively. The change of the pK_a values will be determined by the extent of stabilization or destabilization of the particles H^+, A^- and HA in the mixed methanolic solvent, compared to the pure aqueous solvent.

A problem occurs insofar as the values for the transfer activity coefficients for the individual particles can be obtained only by approximation methods. This leads to contradictious values, not only concerning the magnitude, but also the sign of the individual contributions. In the literature an average value of zero (with standard deviation of 2) for log $\gamma_{H^+}^m$ (cf. e.g. Ref. [29]) seems to be accepted. This means that methanol has a basicity comparable to water, and the medium effect on the proton should not be decisive for the shift of the pK_a value. The medium effect on HA, the neutral particle involved in the ionization equilibrium is of minor significance.

The destabilization of the anion seems to exert the major influence on the pK_a value since methanol has a worse solvating ability towards anions compared to water. Consequently log $\gamma_{A^-}^m$ has positive and large values following that the medium effect on the anion dominates according to Eq. (6), resulting in an increase of pK_a values when methanol is added to water.

It is clear that the detailed change of the pK_a values for the individual species depends on the solvation of the particles under consideration by water and methanol, respectively. As the subtle extend of solvation cannot be forecasted in detail, the accurate change in the pK_a on the methanol concentration cannot be foreseen and therefore selectivity not predicted in detail.

It should be noted that, as in the literature, the above discussion is directed to the solvation of the anion, mainly concerning the negatively charged group in the molecule. A possible lipophilic interaction of the methyl group of the alcohol with the aromatic ring or the lipophilic substituent is not taken into account in the same way as a possible hydrogen bonding of the OH groups of the solute with that of the solvent.

3.2. Mobilities

The actual mobilities of the 26 anionic solutes are given in Table 2 in pure aqueous and mixed aque-

Table 2
List of actual mobilities, $\mu_{act,i}$, of the solutes in pure aqueous and mixed aqueous–methanolic solvents

| Symbol | Name | $\mu_{act,i} \times 10^{-9}$ m^2 V^{-1} s^{-1} | | | | |
		Water	20% MeOH	40% MeOH	60% MeOH	75% MeOH
A	benzoate	33.27	24.03	20.26	19.65	22.01
B	2-OH	36.29	26.02	20.78	20.32	23.25
C	3-OH	31.17	21.96	17.21	16.42	19.01
D	4-OH	31.08	21.31	16.45	15.65	18.33
E	2,3-diOH	32.62	23.17	18.09	17.78	20.62
F	2,4-diOH	32.53	22.79	17.27	16.79	19.41
G	3,4-diOH	29.68	19.88	15.57	14.81	17.16
H	3,5-diOH	28.97	20.16	15.28	14.66	15.87
I	2,4,6-triOH	34.00	23.49	17.93	17.28	19.76
J	3,4,5-triOH	27.69	18.81	14.69	13.84	15.29
K	2-Me	31.59	23.41	19.18	18.88	21.04
L	3-Me	31.53	23.28	18.95	18.58	20.09
M	4-Me	31.51	22.66	18.50	18.24	19.98
N	2,4-diMe	29.21	21.56	17.07	17.59	20.14
O	2,5-diMe	29.36	21.91	17.68	18.11	20.68
P	3,4-diMe	29.38	21.71	17.64	17.80	20.08
Q	3,5-diMe	28.94	21.72	17.62	17.93	20.24
R	2-NO$_2$	32.78	23.52	19.37	19.14	21.10
S	3-NO$_2$	32.27	23.59	19.18	19.06	21.02
T	4-NO$_2$	32.65	23.34	19.03	18.63	20.98
U	3,4-diNO$_2$	31.03	22.24	18.23	18.30	20.17
V	3,5-diNO$_2$	30.63	22.05	18.34	18.75	20.98
W	2,4,6-triNO$_2$	28.42	20.31	16.81	17.41	20.32
X	2-Cl	32.34	23.21	19.41	19.22	20.92
Y	3-Cl	32.63	23.25	19.32	18.77	21.03
Z	4-Cl	32.25	23.01	18.85	18.42	20.49

Methanol content is given in % (v/v). The actual mobilities were determined in the mixed media at appearent pH values at least two units higher than the appearent pK_a values of the solutes. The number in the name list corresponds to the position of the substituent in the aromatic ring of the benzoic acid derivative, and the abbreviation means OH for the hydroxy-, Me- for methyl-, NO$_2$- for the nitro-, and Cl- for the chlorogroup as substituents. Temperature of the thermostatted capillary 25°C. $I = 20$ mmol l^{-1}.

ous–methanolic solvents of the electrophoresis buffer. The data presented are the mean values of duplicate measurements, with typical deviations between the two measuring values in the range below 1% relative. In contrast to the findings of the group of Vigh the values obtained by the method introduced there [24] showed worse reproducibility compared to the method based on the direct measurement of the migration time of the solute and the EOF marker, perhaps due to worse instrumental conditions in the present investigation.

In water all solutes show higher actual mobilities compared to those observed in methanolic media. Mobilities decrease with increasing proportion of methanol, and are reduced by about 30 to 45% in the case of 60% (v/v) methanol, where a minimum can

be observed. Between 60 and 75% (v/v) methanol the actual mobilities increase again slightly.

The effect of methanol on the mobility can be depicted by correlating the actual mobilities in the mixed solvents to those in pure aqueous buffer. This is shown in Fig. 2. The tendency of first decreasing and finally increasing actual mobilities with increasing methanol content can be clearly seen from these graphs. Even more pronounced shown is the scattering effect of methanol on the mobility data around the linear regression line. In fact the linear correlation coefficient decreases from 0.9137 at 20% MeOH to 0.7818 at 40%, to 0.630 at 60% and finally to 0.646 at 75% MeOH, values demonstrating the lack of linear correlation.

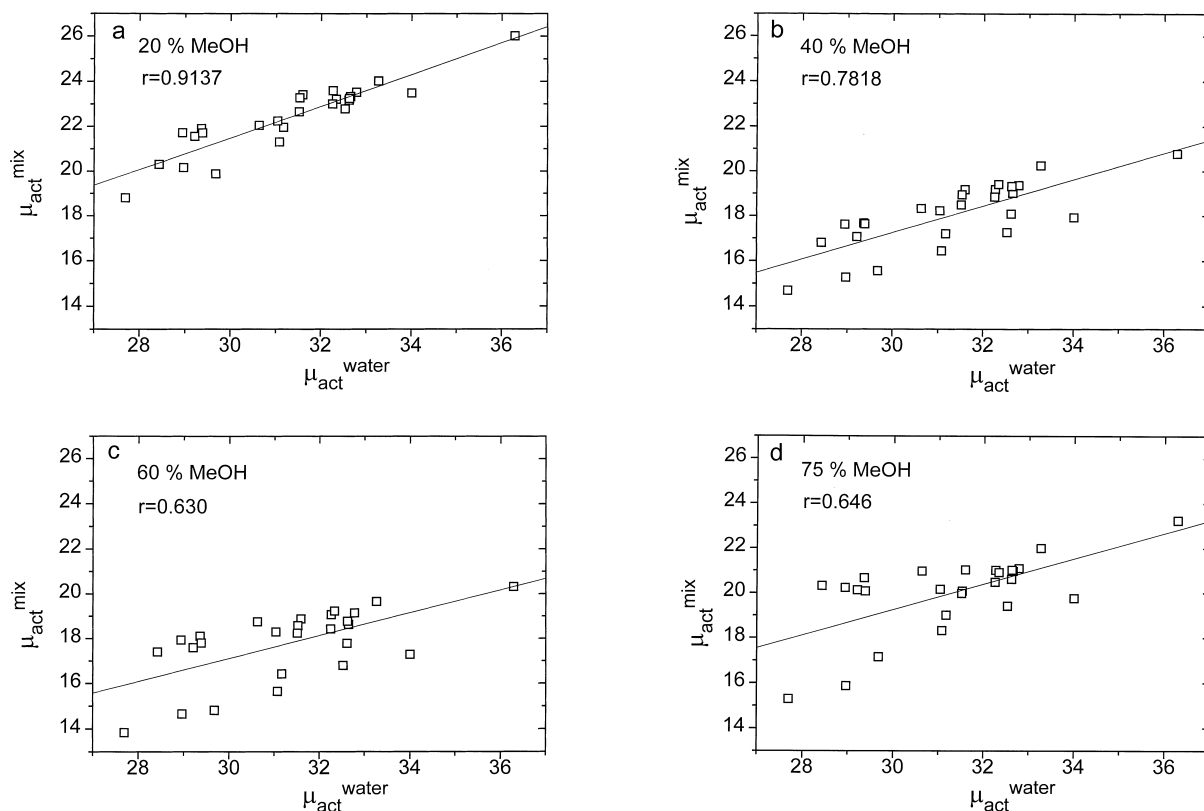The individual solutes can be classified based on

Fig. 2. Correlation of the actual mobilities of the 26 solutes in the four mixed aqueous–methanolic solvents of different methanol content to the actual mobilities in the pure aqueous system. Ionic strength of the BGE 20 mmol l^{-1}, thermostatting temperature 25°C. The straight line is the result of linear regression. r is the linear correlation coefficient. The data of the actual mobilities are those from Table 2.

the change of their actual mobilities upon variation of the solvent composition by cluster analysis. The solutes are considered as taxonomic units and the mobilities as features for classification. A hierarchical clustering method was used, with the single linkage algorithm for construction of the dendrogram from the squared Euclidian distance, depicted in Fig. 3. The mobility values were standardized to unit variance.

A well structured dendrogram results with subclusters combining at low and high similarity values as well. It can be seen that the highest dissimilarity is exhibited by 2-hydroxybenzoate (B). This is clear because the actual mobility of this solute has by far the highest distance to all other solutes (see Table 2). The next two most dissimilar subclusters (separated at the distance of 2.5) contain the three oligohydroxy
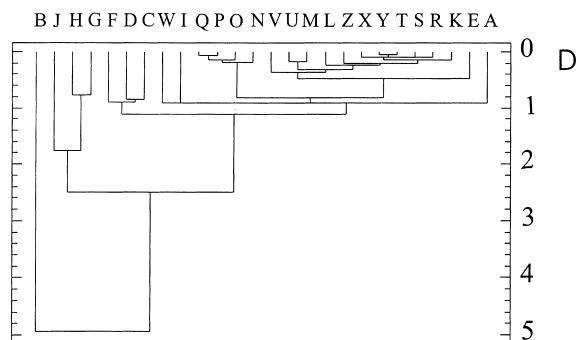

Fig. 3. Dendrogram obtained from hierarchical cluster analysis (with single linkage algorithm, using squared Euclidian distance). D = distance. Symbols as in Table 2.

acids with the common characteristic of one OH-group in position 3, on the one hand (solutes G, H and J from Table 2), and all other solutes, on the other hand. Within the latter subcluster (linked at distance 1.2) again 3 hydroxy acids form a subunit (C, D, F). The solutes with the highest similarity, in contrast, are found at the right hand side of the dendrogram, with distance values smaller than 0.5, representing mostly monosubstituted nonhydroxy derivatives (K, R, S, T, Y, X, Z, L, M, U, V). A similar subcluster is formed by all dimethyl substituted acids (N, O, P, Q).

The result of the cluster analysis can be interpreted twofold. On the one hand solutes with the same substituents show the same general tendency of the variation of the electrophoretic properties with variation of the methanol content. On the other hand methanol addition provokes a sufficiently different change on the actual mobilities for solutes with different substituents (hydroxy-groups or lipophilic groups, respectively) to introduce an interesting dimension enlarging the separation selectivity window in CE in some cases.

The tendency of decreasing mobility at lower and of increasing mobility at higher methanol concentration reflects roughly the change of viscosity of the mixed solvents, as illustrated in Fig. 4. The viscosity increases with increasing methanol content, shows a maximum between 40 and 50% (v/v) methanol, and finally decreases again. Although Walden's rule (which states that the product of the absolute mobili-
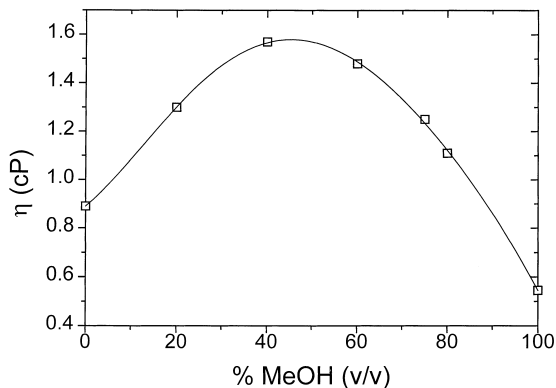
ty and the solvent viscosity is constant; it is a limiting law) is an oversimplification, as the solvent does not behave as a continuum due to the similar molecular dimensions of the solvent and the solute, at least such a tendency is observed here. The relative change of the corresponding product, $\mu_{act,i}\eta$, is shown in Fig. 5a as a function of the solvent composition for a number of methyl-, nitro- and chloro-substituted benzoates. This relative change is expressed in percent by the difference of the product in the mixed medium to that in water, related to the product in water. Due to the large number of solutes only half of those listed in Table 2 are depicted (from K every second solute from the list). The
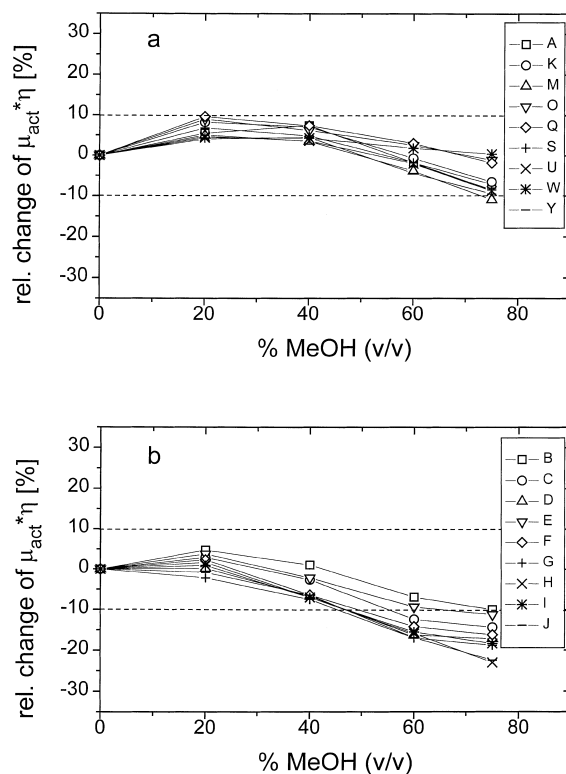

Fig. 5. Relative change of the product $\mu_{act,i}\eta$ of the actual mobility, $\mu_{act,i}$, and the solvent viscosity, η, according to Walden's rule, with the solvent composition. The symbols of the anions are given in Table 2. (a) Solutes without OH substituent; the group includes benzoate and every second separand from Table 2, after 2-methylbenzoate (K). (b) All OH-substituted solutes. The relative change in percent is expressed by the difference of the value of $\mu_{act,i}\eta$ in the mixed solvent and that in water, divided by the latter. Symbols as in Table 2.

Fig. 4. Coefficient of dynamic viscosity, η, at 25°C for the mixed aqueous-methanolic solvents used. The data are derived by interpolation from those given in Ref. [16].

others show a very similar behavior. The discussion on the behavior of the hydroxy substituted benzoates will be given below in more detail, as a larger deviation in comparison to those from Fig. 5a is observed.

It can be clearly seen that the actual mobilities corrected by the solvent viscosity vary to a significant lower extend as compared to the actual mobilities alone. Whereas the latter decrease by more than 30% upon methanol addition, the former stay in a ±10% region. This proves that Walden's rule seems to be roughly obeyed in this case, a fact that is surprising considering the complex solvent system under investigation. Application of Walden's rule so far led to consistent results only in mixtures of lower alcohols.

Further, a more detailed effect becomes visible. At lower methanol concentrations than that of maximum viscosity (at about 50% methanol) the mobility is reduced underproportionally with viscosity as compared to Walden's rule: all values of the relative changes are positive. The product of actual mobility and viscosity is higher than that in pure water. In the concentration range of maximum viscosity Walden's rule is best obeyed, showing the best distribution of the relative change of the $\mu_{act}\eta$ values around zero % relative change. Right from the maximum viscosity the mobilities are reduced overproportionally, as the relative changes of $\mu_{act}\eta$ are in the negative percentage range. However, a variation of the actual mobilities in the 10% range allows to forecast the general trend of the dependence of the actual mobility on the solvent concentration for such type of solutes.

The behavior of those solutes with hydroxy-substitution on the aromatic ring is visible from Fig. 5b. The actual mobilities of the OH acids follow moderately Walden's rule at the lower methanol concentration range, where the values of the corresponding product scatter around the zero point within a +5 and −5% range. At concentrations higher than about 50% MeOH (that of maximum viscosity) the actual mobility is reduced overproportionally as compared to water, and at 75% MeOH nearly all OH acids show negative values of the relative changes of $\mu_{act}\eta$ between −10 and −20%. The mobility here is smaller than influenced only by viscosity.

Due to the high complexity of the systems an interpretation of this different behavior of the anions which is only speculative is tried here. This interpretation obviously lacks support obtained e.g. by spectrometric methods.

It is evident (although the shape of the curves of both groups of acids is similar), that a difference is observable between the acids without OH substitution and those with OH groups in the ring. As the overall viscosity is the same for all separands, the stronger reduction of the OH-substituted anions may be caused by their ability to form hydrogen bonds with the molecules of the solvents, probably preferred with the remaining water molecules even at higher methanol content. This is not necessarily connected with a better solvation at the point of the negative charge of the anion, that clearly does not take place, as all acids show an increase in pK_a with increasing methanol content. The interpretation based on the stronger OH bonding of the hydroxy-substituted acids compared to the other solutes is supported by the fact that especially the trihydroxy and those dihydroxy with the OH group in the position 3 and 4 in the ring exhibit the strongest retardation. These are the positions where OH bonding with the solvent molecules is less sterically hindered compared to position 2, where the OH groups can interact intramolecular with the neighbored carboxylate group.

3.3. Separation selectivity

Separation selectivity in CZE is in principle given by the ratio of the total mobilities as defined by Eq. (1), and depends therefore on the magnitude and the direction of the EOF [1,2]. We can, however, focus the discussion of the effect of methanol on the selectivity to those conditions, where only the effect of the solvent on the specific solute mobility (and not on the unspecific mobility of the EOF) is of significance.

Therefore the selectivity is expressed here by the ratio of the actual mobility of benzoate (the solute without substituent except the carboxylate) in the particular solvent systems, and solute, i, in the same solvent, given by

$$r_{A,i} = \frac{\mu_{act,A}}{\mu_{act,i}} \qquad (7)$$

The result is depicted in Fig. 6, showing an interesting feature. Although selectivity is slightly enhanced by low methanol content, for the solutes without OH groups on the aromatic ring selectivity is in fact lost at higher methanol concentrations (Fig. 6a). The selectivity coefficients of this type of solutes are reduced, and therefore addition of methanol is not favorable here in general. However, for an individual pair of solutes from this group addition of methanol may still bring an increase in selectivity.

The different behavior of a number of OH-substituted benzoates can be deduced from Fig. 6b. On the one hand the selectivity range is even slightly larger in pure aqueous buffer compared to the nonhydroxy acids (the selectivity coefficients ranges up to 1.2.)

In contrast to the other group of acids, the selectivity coefficient increases with methanol content, on the other hand, and remains partially much higher than in the pure aqueous system. With two exceptions (solutes H and J, both having OH groups in position 3 and 5) the highest selectivity coefficients are at the methanol concentration with the maximum viscosity of the solvent; at higher concentrations a slight decrease is found. The result underlines the special behavior of the hydroxy-substituted anions, as discussed above for the product of mobility and viscosity.

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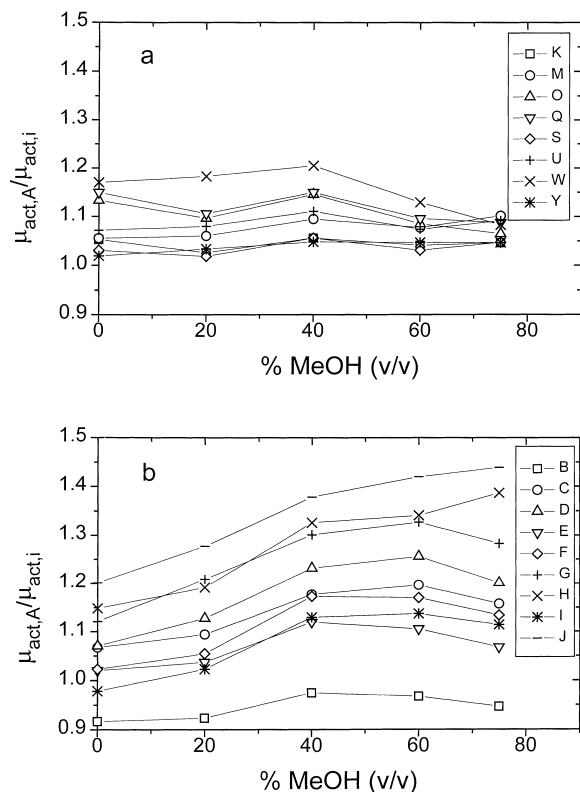
Fig. 6. Effect of methanol as cosolvent of the buffer on the selectivity coefficient, defined by the ratio of the actual mobility of benzoate (A) to that of the different anions, *i*. (a) Solutes without OH substituent; the group includes benzoate and every second separand from Table 2, after 2-methylbenzoate (K). (b) All OH-substituted solutes. Symbols as in Table 2.

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