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## Nonaqueous capillary electrophoresis. Application to the separation of complex mixtures of organic acids by ion-pairing mechanism

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### Abstract

Separation of a ten-membered model mixture of aromatic compounds possessing a carboxylic moiety along with other functional groups was investigated in the pH range 4.5–8.5. Methanol–acetonitrile (1:1) containing 10 mmol/l sodium acetate and an equal concentration of Tris was used as the background electrolyte. It was demonstrated that within the model set three categories of acids were present, namely those which moved nearby the endosmotic flow (on the anodic side) and increased slowly their anodic mobility with the increasing apparent pH (1), those which revealed a strong increase with increasing pH and could be best discerned at apparent pH 7–7.5 in the negative operation mode (2) and those (a single member only) which possessed a strong anodic mobility even at low pH values and could be revealed within reasonable time in the negative operational mode only (3). The mobility of the latter was only slightly affected by the pH change of the background electrolyte. Further addition of hydrophobic moiety possessing ion pairing reagents (typically trimethyloctadecyl ammonium bromide) helped to refine the resolution at neutral pH. The results demonstrate clearly the wide differences in selectivity that it is possible to obtain by running electrophoretic separations in nonaqueous buffers. © 1998 Elsevier Science Ireland Ltd.

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## 1. Introduction

The majority of electrodriven separations (capillary electrophoresis) are currently done in aqueous buffers which offers the possibility of distinguishing between individual solutes present in the sample on the basis of the differences in the  $pK_a$  values. As the  $pK_a$  values are known to change in the presence of organic modifier, solutes that are difficult to separate in purely aqueous buffers frequently offer good results if an organic modifier, typically acetonitrile or methanol, is added to the run buffer ([1,2] and references therein). Another advantage of using organic modifiers is that in this way solutes that are poorly soluble in aqueous media can be separated [3]. In this sense separations done in nonaqueous electrolytes offer an alternative to micellar electrokinetic chromatography; in nonaqueous media the separations are based on differences in the apparent  $pK_a$  values while in micellar separations the partition results from the equilibrium established between the micellar and aqueous phases.

So far, a limited number of papers have been published on the nonaqueous systems for capillary electrophoresis [4–6]. Walbroehl and Jorgenson [7] who were the first to exploit separations by purely nonaqueous media, examined acetonitrile for the separation of quinolines. They also mentioned the possibility of not only using diverse solvents but also of using a number of additives (buffers, cyclodextrins, surfactants) acting as specific selectors. Some of the electrophoretic studies in nonaqueous solvents included separation of dipeptides in formamide [4], of alkali and alkaline earth metals [8,9], lubricating oil additives [10], organic bases [11], and carboxylic acids and drugs [3].

The applications involved a wide range of solvents including methanol, ethanol, acetonitrile, nitromethane, pyridine and glacial acetic acid.

The main advantage of nonaqueous systems is, of course, the possibility of separating compounds which are insoluble (barely soluble) in aqueous media. On the other hand application of nonaqueous phases for electrokinetically driven separations brings about a number of problems to be solved. The first is the choice of the organic solvent. Typically formamide which can yield solutions with sufficient conductivity and good endosmotic flow has the disadvantage of absorbing at short wavelengths which precludes the use of UV detector set at about 200 nm; indeed this solvent was shown to be applicable for the separation of aromatic amino acids possessing peptides which can be detected at considerably longer wavelengths. As reported by Altria and Bryant [3], the methanol acetonitrile mixture (1:1) appears applicable to a wide range of solutes separated at considerably different pH values.

Another point is that owing to the slow endosmotic flow, the capillaries used should be generally shorter than those used in standard aqueous systems. This, on the other hand, allows for relatively fast separations. Other advantages are high selectivity and symmetric peak shapes; typically with solutes poorly soluble in water no distortions due to differences of the electrolyte concentration between the background electrolyte and the sample zone have been revealed.

To our best knowledge the potentials of this method involving the action of ion-pairing additives have been not sufficiently investigated. In this paper we would like to present our results obtained with a set of ten model carboxylic acids (poorly soluble in aqueous buffers) separated in acetonitrile–methanol with trimethanolamine as ion-pairing additive.

## 2. Materials and methods

### 2.1. Capillary electrophoresis

All separations were performed on a Hewlett–Packard HP 3D instrument model G 1600 A (Hewlett Packard, Cernuseo sul Naviglio, Italy) equipped with a HP 3D Chemstation software program delivered by the producer. The capillary used was uncoated and had the dimensions 27 cm (20 cm to the detector) × 50 μm I.D. Separations were run with 10 mM sodium acetate in acetonitrile–methanol (1:1) containing 10 mmol/l Tris acetate. The separation voltage was 15 kV; because some of the analytes exhibited very fast anodic mobility (particularly at pH 7–8.5) the separations were run both in the positive and negative mode (to cut the run time). Runs were performed at 25°C at 20 kV with 17–21 μA per capillary. Hydrodynamic sample application at 10.3 kPa overpressure for 1–2 s was used. Detection was by UV absorbance at 200 nm. Changes within ±2% of the migration time was considered sufficient for both within-day and day-to-day variability. Sample concentration of 200 μg/ml was routinely used.

However, for obtaining good results, careful stabilization of the capillary was needed. The final washing procedure ran as follows:

1. 1 M Aqueous NaOH 3 min
2. Water 3 min
3. 20 mM Aqueous sodium dodecyl sulphate 5 min
4. 1 M Aqueous NaOH 10 min
5. Water 2 min
6. Acetonitrile–methanol 10 min

### 2.2. Chemicals

The following chemicals were used throughout the study

#### 2.2.1. Solvents

Methanol (p.a) and acetonitrile (p.a) were products of Ashland Italia (San Giuliano Milanese, Italy).

#### 2.2.2. Salts and ion-pairing agents

Sodium acetate (p.a), trimethylheptyl and trimethyloctadecyl ammonium bromide were purchased from Sigma–Aldrich (Steinheim, Germany).

#### 2.2.3. Test mixture

The following aromatic acids constituted the test mixture (Fig. 1): 2-biphenylcarboxylic acid (7), 5-formylsalicylic acid (8), 2-methoxyphenylacetic acid (9), 4-chlorophthalic acid (10), 4-chloro-3-nitrobenzoic acid (11) and S-benzylthioacetic acid (12). All of these were the products of Sigma–Aldrich; routinely 200 μg/ml of each analyte dissolved in methanol was used for preparing the sample mixtures and spiking if

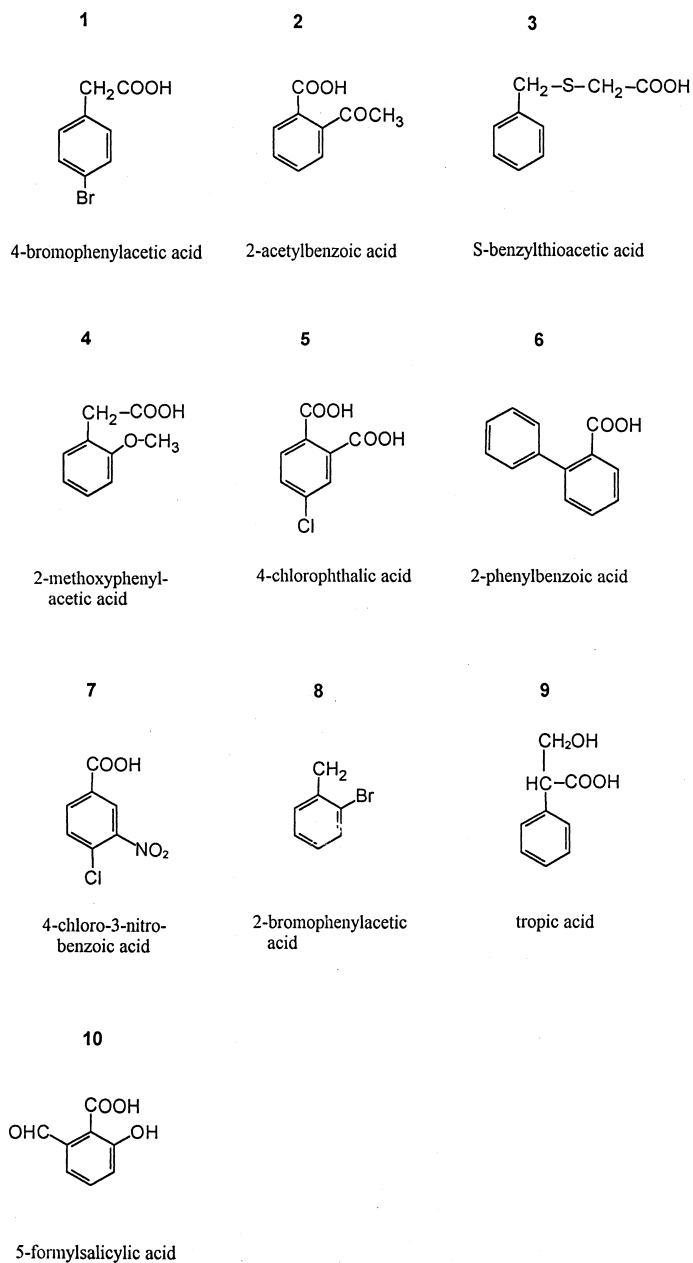


Fig. 1. Formulae of the carboxylic acids used in the test mixture. Numbers attached to individual acids were used for peak identification in electropherograms.

necessary for peak identification. Acetonitrile and methanol were used as delivered without special drying.

### 3. Results and discussion

A number of advantages can be seen in nonaqueous capillary electrophoresis. The main one is the possibility of improving solubility of water-insoluble/barely soluble analytes, without introducing micellar pseudophase into the background electrolyte. Another fact to be taken into consideration is the use of solvents in which the mobility of added electrolyte ions is much lower than in water. The main result is lower current and reduction of Joule heating produced inside the capillary. This allows the use of high field strengths to achieve rapid separations. The speed of separation is favourably influenced also by the use of low viscosity solvents such as acetonitrile and methanol; this means, though in our runs it was in the range of 5 min in a 27 cm long capillary, that some decrease in the endosmotic flow velocity could be expected. There are several nonaqueous media that may be used for nonaqueous electrophoresis; each of them exhibits specific advantages and disadvantages. At the moment it appears that, at least for carboxyl moiety containing solutes, mixtures of methanol (a protic solvent) and acetonitrile (an aprotic solvent) offer the right span of versatility in terms of additives solubilization.

In the present communication we investigated the behaviour of a set of aromatic carboxylic acids bearing different other substituents in the molecule (as specified in Fig. 1) using a methanol–acetonitrile (1:1) mixture. Separation of this rather complex mixture of aromatic acids was investigated in a broad pH range (4.5–8.5). As expected at low pH values the whole set of analytes moved closely to the endosmotic flow as shown in Fig. 2. Four peaks were discerned, namely a fused peak of two (No. 1+2) carboxylic acids followed by the peak of 2-biphenylcarboxylic acid; next came a second complex peak of three other solutes (acids No. 3,4 and 5) and the last complex peak comprising acids (No. 7,9 and 10). The peak of 2-bromophenylacetic acid could not be assigned to either of the two complex peaks on the electropherogram. If, however, the polarity of the system was reversed, the peak of 4-chlorophthalic acid appeared at 5.93 min; this means that even at pH 4.5 the migration of this peak towards anode is quite fast and escapes detection in a 30 min run if the electrophoretic system is run in the positive polarity mode.

With increasing pH of the background electrolyte, better resolution was step-by-step observed as a result of the increased dissociation of the individual members of the test mixture (Figs. 3–6) at pH 5.5 (Fig. 3). As expected the peak of 2-bromophenylacetic acid was discerned only in the negative mode of operation. However, the complex peak (from Fig. 2) of 4-chloro-3-nitrobenzoic acid, tropic acid and 2-formylsalicylic acid starts to move faster to the anodic side; at pH 5.5 this peak contains the first two of the formerly mentioned acids (No. 7 and 9). The mobility of acid No. 10 is so fast that it does not enter the positive mode recording; however its speed is not sufficient to reveal its presence in the negative mode operation. At pH 6.5 (Fig. 4) a partial resolution of the complex peak moving in the vicinity of the endosmotic flow was recorded (see the inset

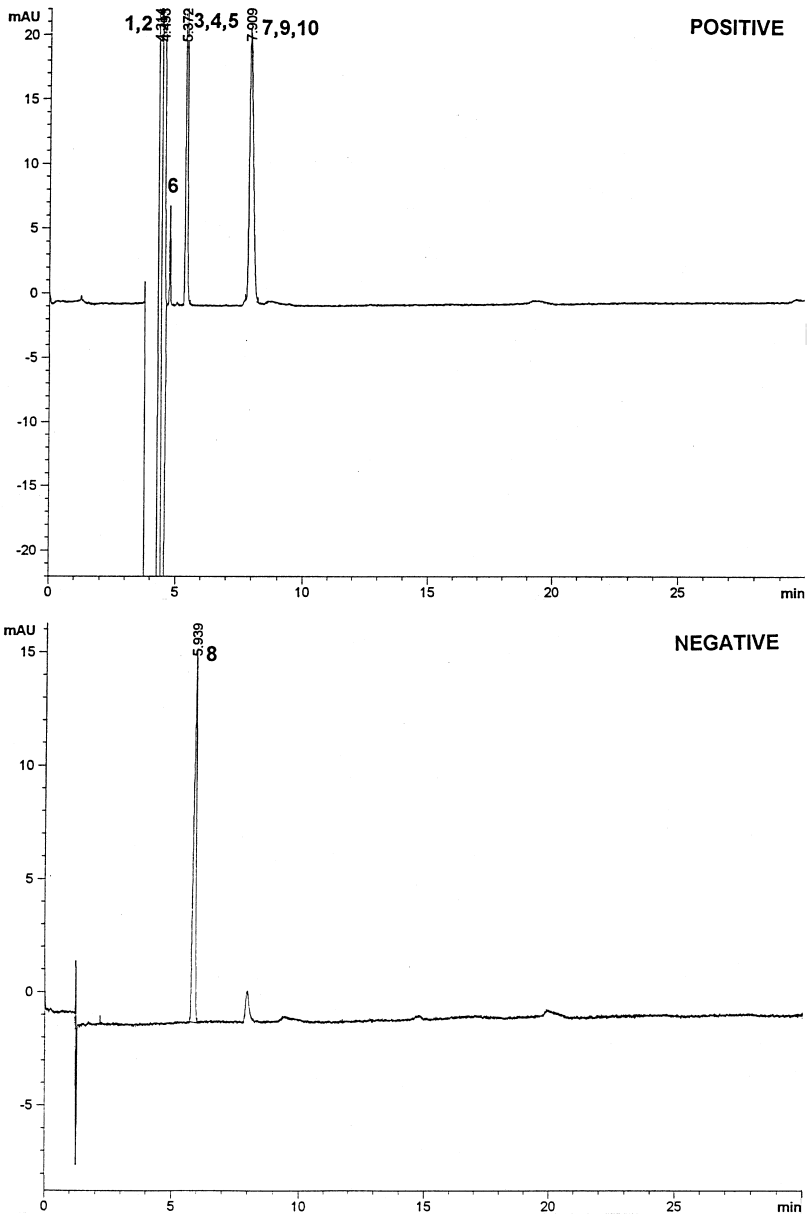


Fig. 2. Separation of the test mixture at pH 4.5 (both in the positive and negative mode). Numbers at individual peaks correspond to those used in Fig. 1.

in Fig. 4). Peaks of 2-methoxyphenylacetic acid and 2-biphenylcarboxylic acid remain distinctly resolved (see Fig. 4, inset).

However, the complex peak of 4-chloro-3-nitrobenzoic acid and tropic acid did not

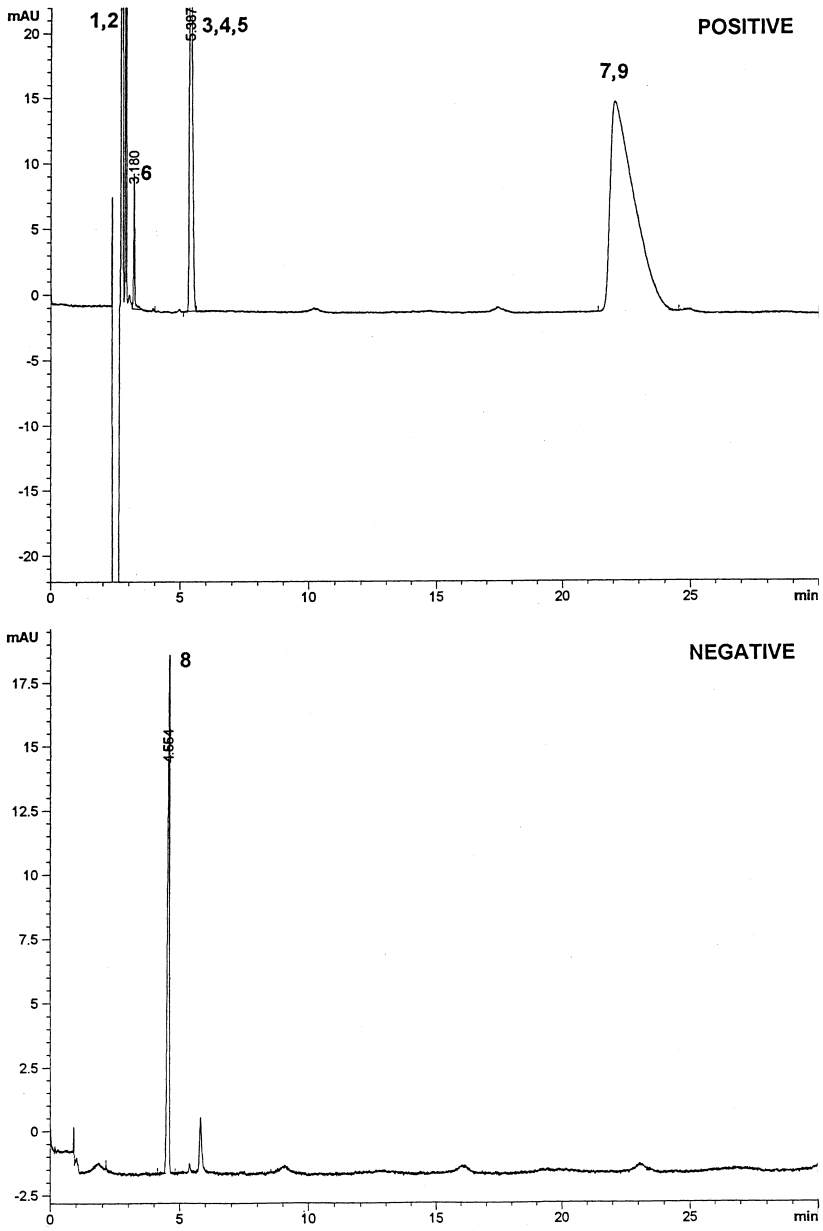


Fig. 3. Separation of the test mixture at pH 5.5 (both in the positive and negative mode). Numbers at individual peaks correspond to those used in Fig. 1.

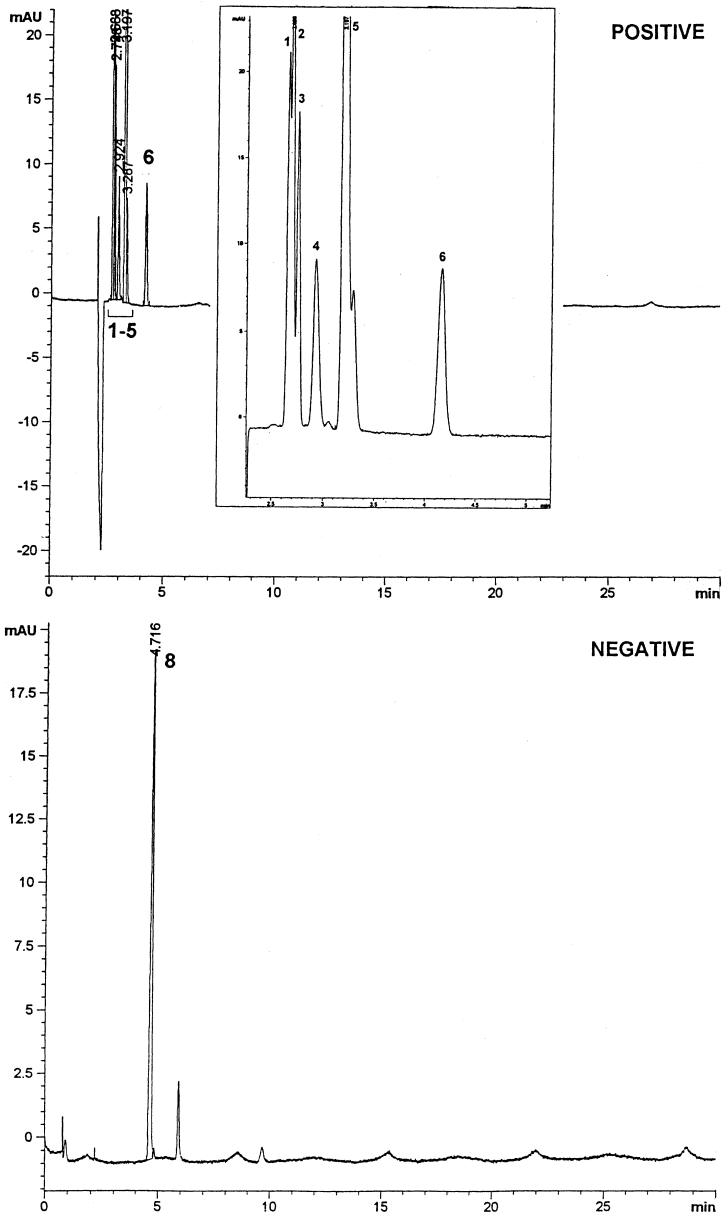


Fig. 4. Separation of the test mixture at pH 6.5 (both in the positive and negative mode). Numbers at individual peaks correspond to those used in Fig. 1.



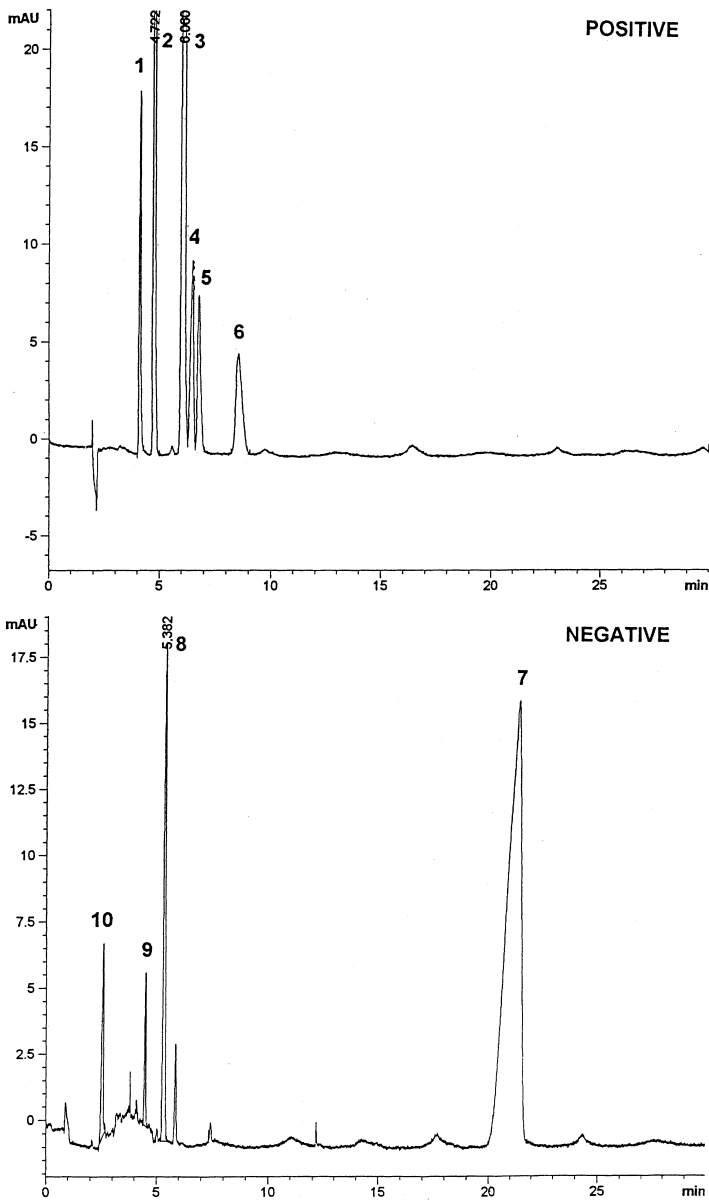


Fig. 5. Separation of the test mixture at pH 7.5 (both in the positive and negative mode). Numbers at individual peaks correspond to those used in Fig. 1.

reach the detector's window within the 30 min run time, which means that at this pH its anodic mobility was considerably increased. However, this increase was not large enough to yield the peaks of these acids in the reversed polarity mode where only the

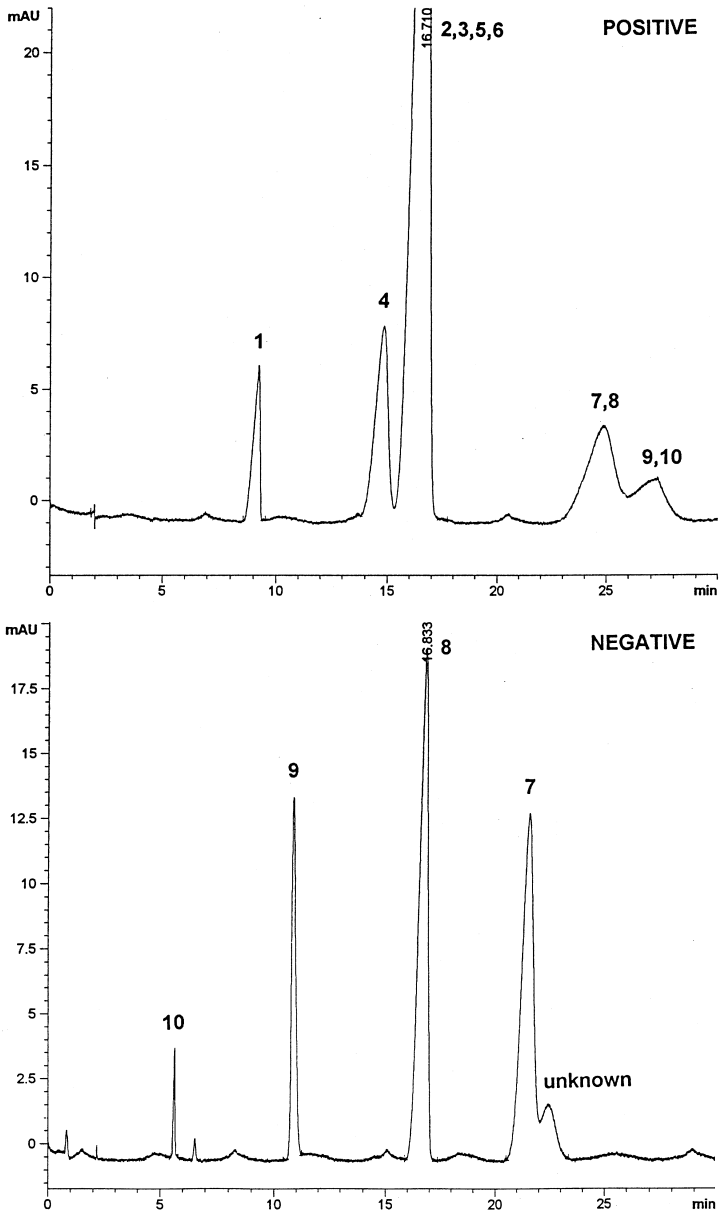


Fig. 6. Separation of the test mixture at pH 8.5 (both in the positive and negative mode). Numbers at individual peaks correspond to those used in Fig. 1.

peak of 2-bromophenylacetic acid was seen. At pH 7.5 (Fig. 5) complete resolution of the solutes moving close to the endosmotic flow was achieved (acids No. 1–6). In the negative polarity mode four peaks were well resolved corresponding to 4-chloro-3-nitrobenzoic, 2-bromophenylacetic, tropic and 5-formylsalicylic acid (acids No. 7–10).

A further increase in pH (Fig. 6) offered a good resolution of all the solutes observed in the negative operation mode. However in the positive mode a considerable decrease in selectivity was observed and four acids, namely 2-acetylbenzoic, S-benzylthioacetic, 4-chlorophthalic and 2-biphenylcarboxylic acid formed a single massive peak in the middle of the electropherogram. At this pH two fused peaks corresponding to acids No. 7+8 and No. 9+10 could be seen even in the positive mode. Also noticeable is the reversed order of peaks 1–3 where the peak of 2-methoxyphenylacetic acid (No. 4) appeared before the joint peak of acids No. 2,3,5 and 6.

The observed electrophoretic behavior of individual solutes reflects apparently the changes in their  $pK_a$  values, their solubility and their affinity to the ion-pair formation. In particular the apparent  $pK_a$  values are different from aqueous  $pK_a$  values. The  $pK_a$  value involved is influenced not only by the presence of the organic solvent based background electrolyte but also by the presence of the electrolyte (sodium acetate in our case). It has been reported that using such an electrolyte moves the apparent dissociation constant to a higher value (both for acidic and basic analytes). Of course, high apparent pH of the organic solvent based background electrolyte leads to acceptable and consistent separation of acidic species.

While no adequate separation of the model mixture was observed with methanol–acetonitrile (1:1) containing 20 mM sodium acetate, inclusion of Tris acetate (10 mM) improved selectivity significantly, apparently by an ion-pairing mechanism involving the acidic functionality of the compounds separated and added to the selectivity of the system investigated.

If an aqueous electrolyte is used for the separation of the same test mixture (20 mM borate, 10 mM with respect to Tris acetate, pH adjusted to 8.5), the result presented in Fig. 7 was observed. Note the changed position of acids No. 7 and 8 in the electropherogram as well as the presence of peaks corresponding to acids No. 9 and 10. This clearly reflects the gross changes in apparent  $pK_a$  in aqueous and nonaqueous background electrolytes. Though a nearly complete resolution of the test mixture was achieved, the run time was at least of 10 min longer as compared to nonaqueous background electrolyte.

In the next stage of our experiments we attempted to replace the Tris acetate by quaternary bases containing a large hydrophobic domain in their structure (heptyl- and octadecyl-ammonium bromide). While no good results were obtained in these cases (data not shown), using an electrolyte containing both Tris acetate and one of the hydrophobic moieties containing quaternary bases (also at 10 mmol/l concentration) offered the profiles shown in Figs. 8 and 9. The addition of the trimethylheptyl ammonium bromide at pH 7.5 resulted in a separation similar to that seen in the presence of Tris acetate only (Fig. 5) though it influenced considerably the migration times of individual solutes; in the positive separation mode the run times were only slightly longer, however no resolution of acids No. 4 and 5 was obtained. In the negative mode the run times of individual solutes were slightly shorter.

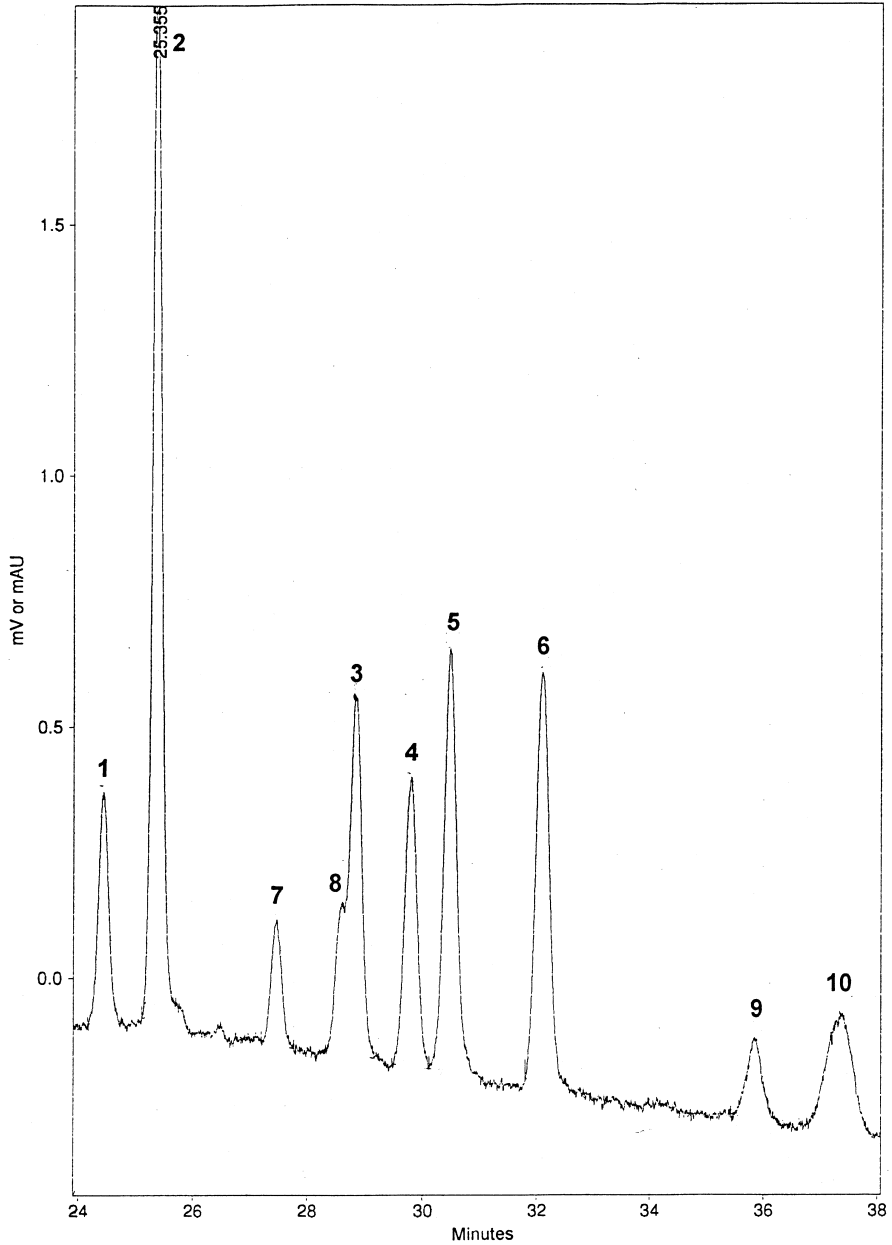


Fig. 7. Comparative electropherogram of the test mixture in 20 mM borate, 10 mM with respect to Tris (other conditions identical to those specified in Section 2). Note the changed position of 4-chloro-3-nitrobenzoic and 2-bromophenylacetic acid. The mixture contained only 100 mg/ml of these acids.

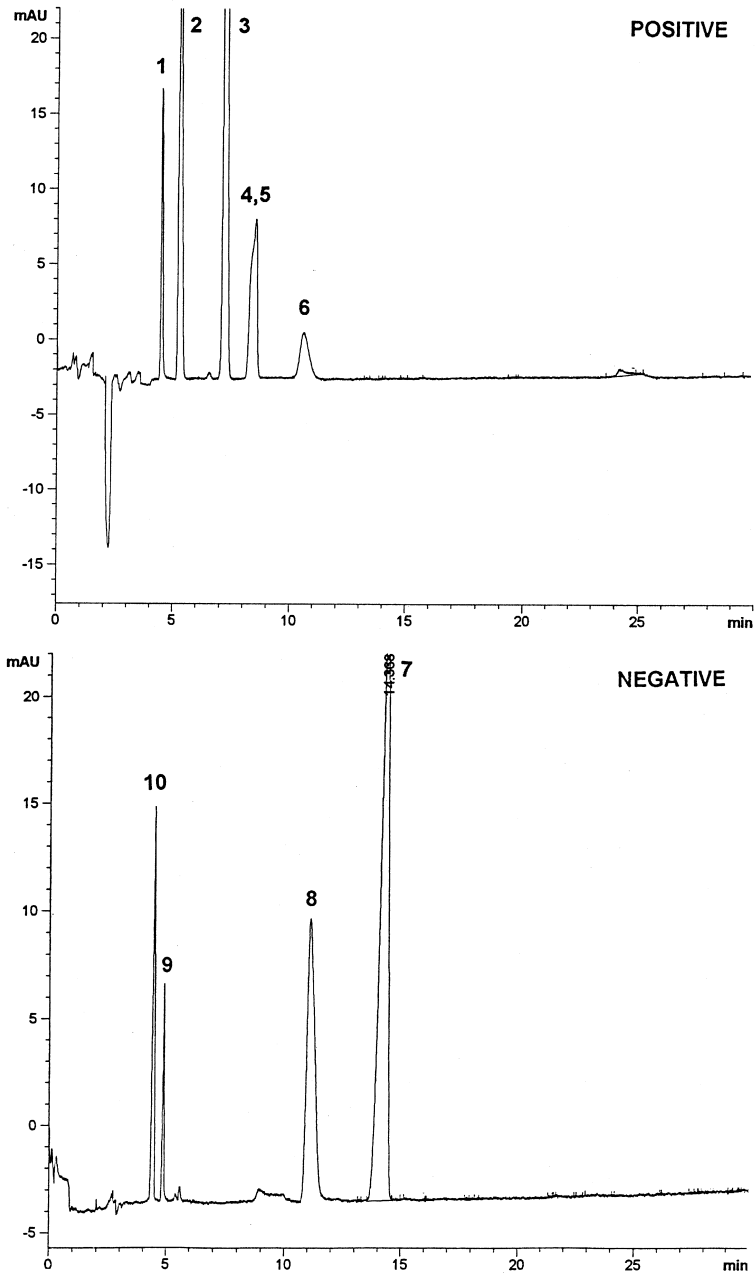


Fig. 8. Separation of the test mixture in the presence of trimethylheptyl ammonium bromide (10 mmol/l); other conditions as specified in Section 2. Apparent pH of the background electrolyte was 7.5.

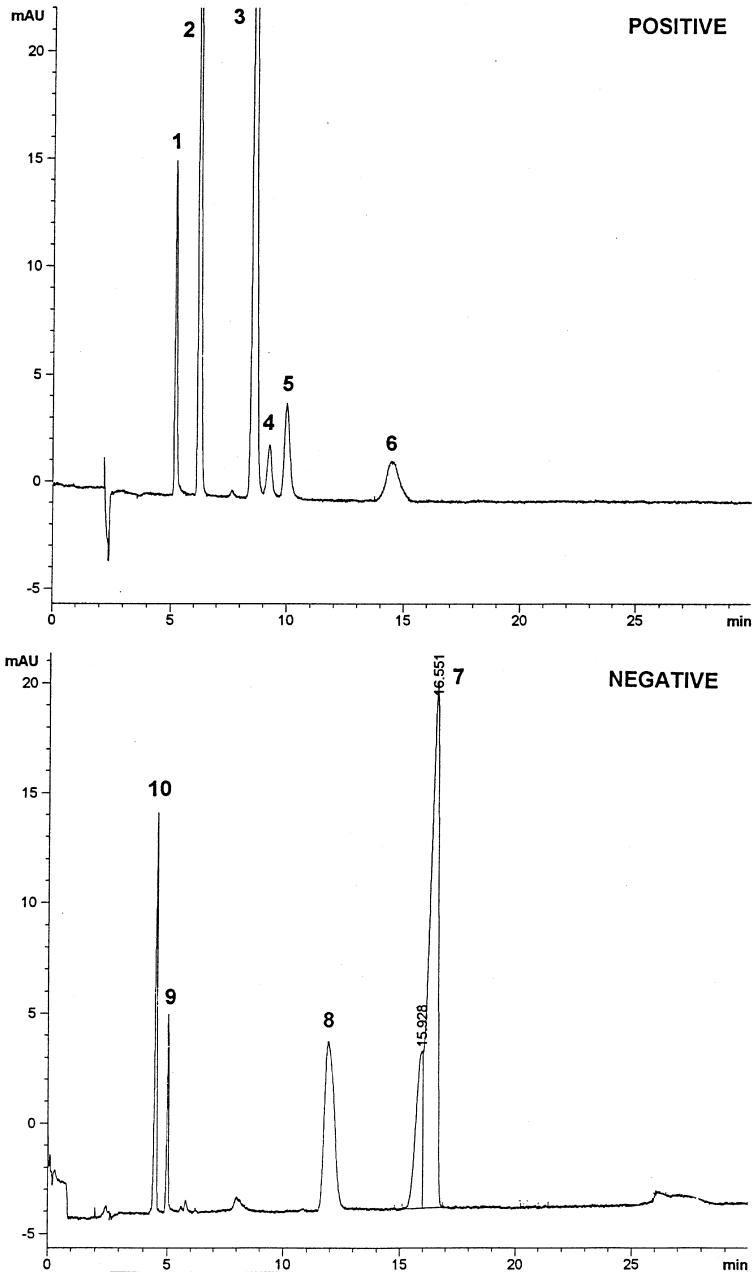


Fig. 9. Separation of the test mixture in the presence of trimethyloctadecyl ammonium bromide (10 mmol/l); other conditions as specified in Section 2.

#### 4. Conclusions

The application of nonaqueous background buffers in capillary electrophoresis offers apparently additional possibilities during method development as compared to the application of aqueous buffers only. As demonstrated with our rather randomly taken test mixture of aromatic carboxylic acids this approach allows exploitation of several additional physico–chemical factors for the separation.

1. Using an organic phase and conductivity ensuring salt results in apparent  $pK_a$  changes which allows manipulation with the peak mobility of individual solutes.
2. Ion-pairing mechanisms apparently help resolution. Based on the application of three different ion-pairing reagents two of which possessed a large hydrophobic functionality it is feasible to assume that both the ion-pairing mechanism and hydrophobic interaction contribute to the final result.

As there are more factors contributing to the final separation of solutes by capillary electrophoresis it is difficult at this stage to formulate rules that would allow prediction of electrophoretic behavior of individual analytes though, clearly, the changes in the apparent  $pK_a$  play the dominant role.

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