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Young J. Lee
*University of Wollongong*

William E. Price
*University of Wollongong, wprice@uow.edu.au*

Margaret Sheil
*University of Wollongong, msheil@uow.edu.au*

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Abstract
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Keywords
capillary, acids, benzoic, separation, solvents, electrophoresis, organic, effect, CMMB

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Effect of Organic Solvents on the Separation of Benzoic Acids by Capillary Electrophoresis

Young Joon Lee, William E. Price* and Margaret M. Sheil
Department of Chemistry, University of Wollongong, Northfields Avenue, NSW 2522, Australia

The effect of organic modifiers on the separation of a number of closely related isomeric benzoic acids by capillary electrophoresis is described. It is shown that while a single modifier concentration cannot help resolve the entire electropherogram, organic modifiers do significantly enhance the resolution of parts of the separation system by comparison with 40 mmol l⁻¹ phosphate buffer. The effects on separation and retention times are discussed in terms of the effects on electroosmotic flow and the electrophoretic mobilities of the charged solutes. The effects were found to be modifier specific, although the trends were in the same direction (i.e., decreasing electroosmotic flow with increased percentage of organic modifier). The major influence is the manipulation of the electroosmotic mobility.

Keywords: Capillary zone electrophoresis; benzoic acid mixture; organic modifier; electrophoretic mobility; electroosmotic mobility

Introduction

Capillary electrophoresis (CE), first introduced by Mikkers et al., and by Jorgenson and Lukacs, has been demonstrated to have wide applicability to the separation of charged species in solution, particularly aqueous media. This technique is characterized by excellent mass sensitivity, low sample consumption, and high resolution. It is based on the principle of electroosmotic flow. When an electrical field is applied along an electrolyte-containing capillary a bulk flow of the liquid is induced. For the case of quartz glass the induced flow is towards the cathodic electrode. This flow is caused by a double layer on the sides of the capillary. A fused-silica capillary has a negatively charged surface when brought into contact with a buffer solution. The source of this charge is two-fold: surface silanol groups, which undergo protolysis, and OH⁻ ions adsorbed onto the surface. Electrosomotic mobility in capillaries originates from an electric double layer between the capillary wall and the liquid present in the capillary. All species in the electrolyte are therefore carried down the capillary in a manner analogous to a liquid chromatography pumped flow. However, all charged components will themselves be directly influenced by the electrical field and have an electrophoretic migration contribution to their over-all velocity. It is the combination of the two, an electroosmotic mobility (μₑₒ) and an electrophoretic mobility (μₑₚ), that leads to the separation of components.

Organic solvents may be used as modifiers in the electrolyte buffers to influence the mobilities of the analytes in a particular direction, which can improve the selectivity in a single system. Furthermore, organic reagents have been used for the separation of organic acids; the combination of aqueous and organic solvents can be used in a multi-dimensional approach to increase the separability of analytes and the probability of their identification. For example, Kenndler and Schwer studied the effect of a series of organic solvents on the electrokinetic properties of fused silica² and Kenndler and Jenner investigated the effect of two different mixed aqueous-organic solvent buffer systems. It has been demonstrated by a number of other workers. The relationship between electroosmotic mobility, electrophoretic mobility and buffer constituents has been investigated. However, there have only been a few studies that demonstrate the effects of addition of organic modifiers. Organic solvents in a buffer will directly influence both the electroosmotic and electrophoretic mobility. Both these mobilities contribute to the observed migration behaviour of each analyte in the capillary. The electrophoretic mobility of an analyte can be influenced by a variety of factors. Since the electrophoretic mobility of an ion is directly proportional to its charge, which in turn is strongly affected by pH, manipulation of the buffer pH becomes one of the key strategies in optimizing a separation. Solute-solute and solute-solvent interactions can lead to pronounced specific changes in the electrophoretic and electroosmotic mobility. Organic modifiers can have effects on the surface nature of the capillary as well as having an influence on the pKₐ of the ionization of the silanol groups and solute molecules. In addition, modifiers may change the electric field gradient by altering the resistivity of the solution and modify the diffusion coefficient of solute species by causing changes in the viscosity and hydrophobicity of the solution matrix.

The current work looks at the influence of a range of widely used organic modifiers on the separation of benzoic acids by capillary electrophoresis (CZE). In particular, the emphasis is on elucidating the effect of the modifier of the individual contributions of electroosmotic and electrophoretic flow to the over-all migration times and hence separation and resolution. A better understanding of the relative effects of modifiers on these controlling factors is needed. An enhanced knowledge will allow simpler optimization of separations using organic additives and establish clearer guidelines as to when their use is the best approach to improve resolution for a particular application.

Experimental

Apparatus

The capillary electrophoresis instrument used was an ISCO-3850 (ISCO, Lincoln, NE, USA). The separations were carried out using a conventional fused-silica capillary (approximately 84 cm × 50 μm id) also obtained (generally) from ISCO. Pressure injection was used as the sample introduction mode for all this work. Electrolytes were prepared daily, using de-ionized
water (18 MΩ cm) and filtered with a Millipore filter system (Millipore, Bedford, MA, USA) before use. Detection was carried out by measuring the absorbance at 254 nm on the column at a position 27 cm from the inlet end of the capillary tube. Conditioning of a new capillary followed the method reported by Lauer and McMannigill.14 The capillary was filled with 0.1 mol L⁻¹ NaOH overnight, then flushed with 0.1 mol L⁻¹ NaOH and Milli-Q water and finally operated with buffer only before an experiment was performed. After completing the experiments the capillary was flushed and filled with the operating buffer. Data was recorded using a Shimadzu (New York, USA) Model C-R6A integration system.

**Reagents**

Distilled water using a Mill-Q water purification system (Millipore) was used for all solutions. Benzoic acids, KH₂PO₄ and Na₂HP₀₄ were obtained from Aldrich (Milwaukee, WI, USA). Organic solvents used were analytical-reagent grade (Millipore, Bedford, MA, USA). Organic solvents were added to improve the peak selectivity.16,17 Fig. 1 shows the separation of a mixture of seven benzoic acids in 40 mmol L⁻¹ phosphate buffer optimized using only an aqueous system. It can be seen that under these electrophoresis conditions the isomeric dihydroxybenzoic acids are only resolved into two groups. The first peak in the electropherogram in Fig. 1 is the neutral marker (acetone) which may be used to relate migration times to the electroosmotic flow. This may then be used to determine the contribution of the electrophoretic mobility of the charged solute benzoic acids. In order to investigate how organic modifiers can improve this separation, a number of different modifiers were tried. To improve our understanding of the effects of organic reagents in the carrier buffer upon the separation several aspects of the separation were considered.

**Effect of Organic Solvents on Voltage**

In Fig. 2, summarized data from measurements of the voltage for varying organic modifier concentrations are shown using a constant applied electric current of 25 μA. It is seen that an increasing amount of modifier results in a larger resistance to the passage of charge and hence an increased voltage. This might be expected; however, it is interesting to note the shape of the curve is very dependent on the type of modifier used. In particular, acetonitrile shows little increase in the voltage with increasing concentration, indicating that these mixtures have high conductance which allows easy transport of charged species under the influence of the applied field. This is in accord with fundamental studies on the diffusion properties in acetonitrile–water mixtures.18 Although, conventionally, in CZE a constant potential is used (thereby ensuring a constant electroosmotic flow), these results highlight another general point about the use of organic modifiers. When adding modifiers such as methanol or ethyl acetate the current required to maintain the voltage increases sharply. This may have a deleterious effect on the separation owing to excessive amounts of Joule heating. As a consequence this may result in having to use a reduced voltage for a particular percentage of modifier, which of course in turn will affect the electric field, the

**Measurement of the Electroosmotic Velocity**

Electroosmotic velocities were calculated from the migration times of a neutral marker substance. Acetone was adopted as a neutral marker to obtain the values. Acetone was diluted in the phosphate buffer electrolyte to a concentration of 0.5% (v/v). The background electrolyte is transported by the liquid flow and should not be influenced by the electric field gradient of any adsorption effect. Measurements of the electroosmotic velocity were carried out at a field strength of 353 V cm⁻¹ at an ambient temperature of 25 °C.

Owing to the generation of Joule heat, the temperature inside the capillary may be higher, but this effect is less than 2°C because the working current was kept below 60 μA in all cases.

The magnitude of electroosmotic flow, \( \mu_{eo} \), can be calculated from an electropherogram as the retention time of a neutral marker is given by:

\[
\mu_{eo} = \frac{IL}{t_{eo}V}
\]  

where \( L \) is the distance between the inlet and the detector, \( t \) is the total length of column, \( t_{eo} \) is the migration time of the marker, and \( V \) is the applied voltage.

Electrophoretic mobility, \( \mu_{ep} \), is equal to \( V_{eo} - V_{app} \), where \( V_{app} \) is the apparent migration velocity of a sample ion, and \( V_{eo} \) is the velocity of the electroosmotic flow. The electrophoretic mobility was calculated as follows:

\[
\mu_{ep} = \frac{IL(t_{eo} - t)V}{t}
\]

where \( t \) is the migration time of sample. In the analysis of cations, electrophoretic migration is toward the cathode and is also affected by electroosmotic flow. Consequently cations elute before neutral species under these conditions.

**Results and Discussion**

In a previous study15 we were interested in the separation, by CZE, of isomeric and closely related mixtures of phenolic acids. It was found that it was not possible to resolve completely these mixtures using aqueous buffer, and to attempt to overcome this we studied the influence of the addition of organic solvents on the electrophoretic behaviour for the compounds. Organic solvents were added to improve the peak selectivity.16,17 Fig. 1 shows the separation of a mixture of seven benzoic acids in 40 mmol L⁻¹ phosphate buffer optimized using only an aqueous system. It can be seen that under these electrophoresis conditions the isomeric dihydroxybenzoic acids are only resolved into two groups. The first peak in the electropherogram in Fig. 1 is the neutral marker (acetone) which may be used to relate migration times to the electroosmotic flow. This may then be used to determine the contribution of the electrophoretic mobility of the charged solute benzoic acids. In order to investigate how organic modifiers can improve this separation, a number of different modifiers were tried. To improve our understanding of the effects of organic reagents in the carrier buffer upon the separation several aspects of the separation were considered.

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**Fig. 1** Electropherogram of a model mixture of the benzoic acids: 3,4,5-trihydroxybenzoic acid (a); 3-hydroxy-4-methoxy benzoic acid (b); 3,4-dihydroxybenzoic acid (c); 3,5-dihydroxybenzoic acid (d); 2,4-dihydroxybenzoic acid (e); 2,3-dihydroxybenzoic acid (f); and 2,5-dihydroxybenzoic acid (g). S is the neutral marker (acetone). Buffer, 40 mmol L⁻¹ phosphate (Na₂HP₀₄–KH₂PO₄, pH 7.0); column, fused silica (55 cm × 50 μm id); injection, pressure mode; 1 s (2.5 ml); injected amounts (ng), 0.2 ng (a,d); 0.07 ng (b); 0.15 ng (c); 0.41 ng (e,g); and 0.36 ng (f); voltage, 30 kV; current 50–60 μA; detection, UV at 254 nm; temperature, 25 °C.
Electroosmotic and electrophoretic mobilities of the species and hence the resolution.

**Effect of Organic Additives on Retention Time**

Fig. 3 shows the changes in retention times of the seven substituted benzoic acids, observed when organic solvents were mixed with 0.04 mol l⁻¹ phosphate buffer, pH 7.0, to make from 5 to 30% (v/v) organic solutions using a constant applied voltage of 30 kV. When acetonitrile or ethyl acetate was added, retention times were only slightly increased with modifier concentration. By contrast, with the others, e.g., ethanol or methanol, retention times were quite dramatically altered. This would appear to be in keeping with the previous conclusions based on the voltage–current curves. However, as mentioned earlier, there are two influencing factors controlling the retention: electroosmosis and electrophoresis of charged species. In addition, those which exhibit a steeper retention versus percentage modifier added, also showed the greatest increase in the resolution. Before looking in detail at the optimum modifier conditions producing the best resolution, it is perhaps a useful expedient to investigate the effect of modifier concentration on the electroosmotic and electrophoretic flows separately to ascertain their importance. This will enable a clearer picture of whether modifier addition is a valid strategy for improving the quality of a separation.

**Effect of Organic Solvent on Electroosmotic Mobility**

In Fig. 4, the electroosmotic mobility of the neutral marker is plotted against the concentration of organic solvents added to the phosphate buffer. Acetone was adopted as a neutral marker to obtain the \( \mu_{eo} \) values. The addition of organic solvents to a buffer is known to lead to a reduction in the electroosmotic mobility. The change in \( \mu_{eo} \) with modifier concentration followed a very similar pattern to that shown in the previous figure with retention times. The data for the addition of acetonitrile (and ethyl acetate) were different from those in the other organic solvents where the \( \mu_{eo} \) value only slightly decreased as the organic content of the buffer increased. When organic solvents are constituents of the buffer electrolyte, two effects can be observed. Firstly, there is a decrease of the electroosmotic velocity with increasing concentration of the organic solvent. This occurs even at high pH, where all of the...
surface silanol groups should be dissociated. This is thought to be caused by a change in the viscosity of the electrolyte. The second effect is that the pK_a of the silanol groups is shifted toward higher values when an increasing amount of organic solvent is added. The magnitude of the changes in pK_a is such that it is likely to be a major cause of differences in the electropherogram on addition of an organic modifier.

**Effect of Organic Solvents on Electrophoretic Migration**

The values of electrophoretic mobility, \( \mu_{ep} \), of individual compounds may be calculated from eqn. (2). Fig. 5 shows the effect of modifier concentration upon the electrophoretic mobility of 3,4-dihydroxybenzoic acid, one of the isomers that is not resolved in the absence of organic solvents. It can be seen that the modifier does have an effect on \( \mu_{ep} \), which is modifier specific. Acetonitrile causes a much slower variation in the mobility compared to, for example, ethanol where the changes are large (100% decrease at 25% ethanol content). The change in the value of the mobility may be attributed to two factors. Firstly, as the electrophoretic flow and the electroosmotic flow are both caused by the same phenomenon (i.e., the application of potential along a capillary), it is obvious that the presence of the organic modifier affecting the viscosity of the buffer will alter the mobility of the electrophoretically active species. Secondly, and more importantly, the organic solvent will have an effect on the ionization equilibrium of the charged, electrophoretically active species, and alter the effective charge on the ion and hence its mobility in an electric field. It is thus to be expected that the most dramatic effects of modifier addition on species mobility will occur near the species' pK_a. However, as far as improving resolution and separation are concerned, what is desirable are the differential changes in \( \mu_{ep} \) with addition of modifier resulting in the movement of peaks in the electropherogram. Consequently, this is only likely if the pH of the buffer is chosen to be close to the pK_a values of unresolved peaks.

**Electropherograms of Benzoic Acid Mixtures**

The electropherograms resulting from various concentrations of methanol in the electrophoresis buffer are shown in Fig. 6. The phosphate concentration in the buffer was maintained at 40 mmol l\(^{-1}\) in this experiment. No change in migration order for the benzoic acids was observed. When the content of methanol in the buffer increased, the electroosmotic mobility decreased (Fig. 3). As shown in Fig. 6, the addition of methanol did improve the separation of benzoic acids. The optimum conditions for the separation of benzoic acids was the buffer with 10% (v/v) methanol, but even then the isomeric pair 2,4- and 2,3-dihydroxybenzoic acids could not be separated. Addition of
higher concentration improves the resolution of the 2,3 and 2,5 pair and also the 3,4,5-trihydroxy, 3,4-dihydroxy and 3,5-dihydroxy peaks. However, a longer separation time is observed, compared with the lower content of methanol.

With the simple addition of an organic modifier it was found impossible to resolve completely the benzoic acid isomers using one set of conditions. This is perhaps not surprising with chemically similar compounds and changes which are likely to be non-specific. However, enhanced resolution of all parts of the electropherogram was achieved as shown in Fig. 7, using ethanol as the organic additive. The best resolution of the four benzoic acids (3,4,5-trihydroxy, 3-hydroxy-4-methoxy and 3,4- and 3,5-dihydroxy) was achieved by adding 10% (v/v) of ethanol. Under these conditions the complete resolution of five of the benzoic acids was achieved, leaving unresolved the two 2,3- and 2,4-dihydroxybenzoic acids. For these, the best separation was obtained from a mixture of 25% acetonitrile with 0.04 mol l⁻¹ phosphate as shown in Fig. 8.

Conclusions

The effect of organic modifiers upon electrophoresis in a capillary column has been studied. For the particular separation considered, seven benzoic acids with similar chemical formulae, addition of organic solvents did not enable complete resolution/separation to take place. However, enhancement of separation in parts of the electropherogram was achieved. Analysis of the results also allowed the effect of organic modifiers on both the electroosmotic and electrophoretic flows to be studied. It was apparent that changes in both electrophoretic and electroosmotic mobility contribute to variations in the separation of the benzoic acid mixture upon addition of organic solvent to the buffer. The effect on electroosmotic flow was to decrease it, thereby increasing the elution range within the electropherogram. The effect of modifier on electrophoretic flow was also to decrease it.

For the particular solutes considered here there were no dramatic specific shifts in electrophoretic flow that might result in change in elution order. Consequently the main effect on the resolution was through increasing the elution time range. Fujiwara and Honde³⁷ have also reported that the addition of methanol and acetonitrile to the background electrolyte was

![Fig. 7 Electropherograms of a mixture of benzoic acids as a function of ethanol concentration in the buffer; carrier was a mixture of ethanol with 0.04 mol l⁻¹ phosphate buffer, pH 7.0. Other conditions as in Fig. 1.](image)

![Fig. 8 Electropherogram of a mixture of benzoic acids using a buffer of 25% v/v acetonitrile in 0.04 mol l⁻¹ phosphate solution, pH 7.0. Other conditions as in Fig. 1.](image)
effective in expanding the elution range and changing the selectivity in CE. This work also concludes that organic modifiers can be effective in enhancing CE separations mainly through manipulation and control of the electroosmotic flow. The use of organic modifiers to change substantially and preferentially the electrophoretic mobility of solutes is dependent very much on the $pK_a$ of the solute concerned. Its use specifically to alter solute mobilities is, therefore, not likely to be widely applicable and at best confined to an ancillary role used in conjunction with manipulation of buffer pH which produces large and more predictable changes in mobilities.

References


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