Separation of Positional Isomers of Organic Acids and Dyestuff Food Additives by Nonaqueous Capillary Electrophoresis

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The merits of capillary electrophoresis in pure organic solvents were investigated for the separation of closely related positional isomers of dinitrobenzoic acid and isomeric pairs of dyestuff food additives, amaranth-new coccine and light green SF yellowish-brilliant blue. Instead of ordinary buffer salts, cetyltrimethylammonium bromide (CTAB), in methanol or methanol–acetonitrile, and sodium dodecyl sulfate (SDS), in methanol–ethanol, were the media used for the separation, in which a complete resolution of the tested compounds was achieved in a relatively short time. Both nonaqueous systems showed low levels of generated electric current, especially SDS in methanol–ethanol, which showed submicro amperic levels. As a result of low-joule heating and diminished adsorption of solutes on the capillary wall, a relative standard deviation of <0.4% was obtained for the migration times without use of temperature control and a washing step between runs. Addition of urea to the CTAB/methanol system was used for manipulation of electroosmotic flow, analysis time, and resolution. Although elution of the tested compounds in CTAB/acetonitrile was very fast, methanol–acetonitrile was found to be better for optimizing separation and analysis time. For the CTAB/methanol system, the separation pattern was independent of the sample matrix because of the opposite directions of the electroosmotic flow and the electrophoretic mobility of the anionic organic acid isomers. This caused removal of samples constituents including neutral and positively charged species from the capillary after the start of the electrophoretic run.

Separation of closely related isomeric compounds is the state-of-the-art in any separation method. Liquid chromatography separated such compounds by using special techniques including recycle chromatography with conventional columns or very long (e.g., 6 m) microbore columns. However, successful separation required long separation times such as 5–20 h (1–4). Soon after development of capillary electrophoresis (CE) in the field of separation science, its power of high resolution was demonstrated by several successful separations in a wide area of analysis (5–7).

Nonaqueous capillary electrophoresis (NACE) is a relatively new CE technique that has recently gained much attention because of its features complementary to, as well as its superiority over, conventional CE methods; NACE provides a wider application range, different selectivity, and resolution ability. Because the nature of the solvent has considerable effect on the behavior of solutes, shifting from an aqueous to a nonaqueous medium in CE may provide some advantages, including better resolution, different method selectivity, faster analysis time, and wider application range. Using organic solvents with the aqueous electrolyte buffer in CE and micellar electrokinetic capillary chromatography has been reported and established as a successful approach to improve separation efficiency (8–10). Our literature search showed some reports based on CE in pure nonaqueous media; they included separation of quinolines in acetonitrile (11), investigation of formamide as a medium for CE with the study of current, voltage, efficiency, and detectability (12), study of the separation of a series of inorganic anions in methanol and dimethylformamide with electrochemical and indirect UV detection (13), application of nonaqueous media for the separation of long-chain surfactants (14), and a comparative study between the resolution ability of aqueous and nonaqueous CE (15).

In this study, we aimed to show the merits of performing CE in nonaqueous media consisting of cetyltrimethylammonium bromide (CTAB) in methanol as well as sodium dodecyl sulfate (SDS) in methanol–ethanol for the fast and efficient separation of closely related isomeric organic acids and dyestuff food additives. Such separations have not been achieved with conventional (aqueous) CE. The study was carried out on the following model compounds with pKₐ...
values given in parentheses: picric acid, internal standard (0.29), 2,4-dinitrobenzoic acid (0.95), 3,4-dinitrobenzoic acid (1.78), and 3,5-dinitrobenzoic acid (1.80). Two pairs of isomeric dyestuff food additives, amaranth-new coccine and brilliant blue-light green SF yellowish, which do not possess any acidic or basic functional groups, were also tested to see the impact of solvation in nonaqueous media on resolution. Complete separation between peaks of tested compounds was achieved in a short time. In aqueous CE with high resolution ability, separation of isomeric organic acids, which have close pKa values, is a challenge and cannot be achieved without time-consuming optimization steps and the addition of several modifiers including organic solvents to the carrier electrolyte (16–18). However, we successfully separated 2,4-, 3,4-, and 3,5-isomers in a very short analysis time (<4 min) by taking advantage of differences in the ionization and/or solvation of carboxylic acid groups in methanol. The separation of dye-stuff isomers shows that although they have the same charge and molecular mass, solvation in methanol produces moieties with different molecular masses that can move with different velocities and thus be separated from each other. Because in nonaqueous media the pKa of silanol groups is shifted to higher values (19), the number of exposed SiO⁻ groups is lower in nonaqueous CE than in aqueous CE. This lower exposure can reduce the unwanted adsorption of solutes onto the capillary wall during the electrophoretic run. The direct effect of this behavior was improved reproducibility of the migration times of the solutes, which is quite important in both qualitative and quantitative studies. The advantages and effects of using the monomeric surfactants CTAB and SDS in non aqueous solvents for the separation of positional isomers of organic acids and food additive dyestuffs are discussed in this report.

Experimental

Instrumentation

A high-voltage power supply (Model HCZE-30 PNO.25-LDS, Matsusada Precision Devices, Tokyo, Japan)

![Image](image_url)

Figure 1. Change in migration times of tested compounds as a result of different concentrations of SDS in methanol–ethanol. Electropherograms were obtained in (A) 10mM SDS, (B) 20mM SDS, and (C) 30mM SDS. Peaks: 1 = picric acid; 2 = 3,5-dinitrobenzoic acid; 3 = 3,4-dinitrobenzoic acid; and 4 = 2,4-dinitrobenzoic acid. Conditions: 30kV applied voltage, reversed polarity mode, 39 cm long separation capillary, and hydrodynamic injection of sample for 5 s in the cathodic vial.
was used to generate an electric field of \( \pm 30 \) kV. Separations were performed at 30 kV. On-column detection of separated peaks was performed at 254 nm with a 875-CE UV/Vis detector (Jasco, Tokyo, Japan). Electropherograms were processed and recorded on a Chromatopac Model CR3A (Shimadzu, Tokyo, Japan). For the CTAB-in-methanol study, separations were performed by using a fused-silica capillary tube, 57 cm long, 39 cm from anode to detector, 50 µm id, and 375 µm od. Sample injection was performed in a cathodic vial, from where the anionic species started to separate in an 18 cm distance of capillary from cathode to detector. For SDS in the methanol–ethanol solvent system, the polarity of the power supply was reversed and the same lengths of capillary as mentioned above were used, except that because sample injection was performed in a cathodic vial, the length of the separation capillary was 39 cm. Capillaries were obtained from G.L. Sciences (Tokyo, Japan).

**Procedure**

Hydrodynamic injection was performed by gravity and by raising the cathodic vial 5 cm higher than the level of the an-
odic vial for 5 s. Before use, each new capillary was washed by flushing with 1M NaOH for 15 min and with 1M HCl for 15 min, before washing with methanol for 15 min for complete removal of the aqueous phase. Then the capillary was conditioned with carrier electrolyte for 30 min before use.

Chemicals and Reagents

All reagents were analytical grade if not otherwise stated. Dyestuff food additives were received as a gift from the National Institute of Health Science (Tokyo, Japan). Methanol, ethanol, acetonitrile, 3,5-dinitrobenzoic acid, and 3,4-dinitrobenzoic acid were obtained from Kanto Chemical Co. (Tokyo, Japan). 2,4-Dinitrobenzoic acid was obtained from Wako Chemical Industries (Osaka, Japan).

CTAB was obtained from Nakarai Chemicals (Kyoto, Japan). Electrophoresis grade SDS was obtained from Katayama Chemical (Osaka, Japan).

Results and Discussion

The dielectric constant (ε) of most organic solvents is lower than that of water (e.g., methanol, 32.7, and water, 78.5, at 25°C); in the case of methanol, its polarity is also lower than that of water (5.1 and 10.2, respectively). These factors could affect the acid-base ionization and dissociation of a solute and might produce different charge-to-molecular mass values for 2 closely related compounds. Because the charge-to-molecular mass value of a given solute has a direct effect on its electrophoretic mobility, the impact of ε and the polarity of a solvent on the migration behavior of that solute is obvious. With acids and bases, the autoprotolysis constant (Ks) of the solvent should also be taken into consideration. The smaller the Ks, the greater the range of acid or base strength that can exist in a solvent, and the greater the likelihood that it will be a differentiating solvent [Ks(H2O) = 14, Ks(MeOH) = 16.7]. As a result of the above considerations, the separation of 2,4-, 3,4-, and 3,5-dinitrobenzoic acid isomers was tested in nonaqueous systems consisting of SDS monomers in methanol-ethanol (4 + 1) and CTAB in pure methanol.

Table 1. Reproducibility of migration times of compounds tested in the methanol–ethanol/SDS system

<table>
<thead>
<tr>
<th>Run</th>
<th>Migration time, mina</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Peak 3</th>
<th>Peak 4</th>
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<tr>
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<td>10.51</td>
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<td>Standard deviation</td>
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<td>0.09</td>
<td>0.06</td>
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<tr>
<td>RSD, %b</td>
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<td>0.85</td>
<td>0.42</td>
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</table>

a For peak identification, see Figure 1 legend.

b RSD = relative standard deviation.

Table 2. Reproducibility of migration times of compounds tested in the methanol/CTAB system and improvement of relative standard deviation (RSD) with picric acid as internal standard

<table>
<thead>
<tr>
<th>Run</th>
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<th>Peak 2</th>
<th>Peak 3</th>
<th>Peak 4</th>
<th>Peak 2/Peak 1</th>
<th>Peak 3/Peak 1</th>
<th>Peak 4/Peak 1</th>
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<tr>
<td>RSD, %b</td>
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<td>1.30</td>
<td>1.35</td>
<td>1.52</td>
<td></td>
<td></td>
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</tbody>
</table>

a For peak identification, see Figure 1 legend.

b With picric acid as internal standard.
or methanol–acetonitrile. Ethanol was added to the methanol to reduce the background absorption.

Characterization of the electroosmotic flow (EOF) is important as a fundamental phenomenon in CE and for the evaluation of the individual electrophoretic mobility of solutes. The behavior of EOF was examined for 2 systems: solutions containing 0–30 mM SDS in methanol–ethanol and solutions containing 0–50 mM CTAB in methanol. No bulk flow of solvent was observed in the absence of SDS and/or CTAB, because no ionic species was dissolved in the solvent; the presence of ionic species in the solvent is necessary for initiation of zeta potential and consequently EOF.

In the case of the methanol-ethanol/SDS system, the counter ion of SDS (i.e., Na⁺), builds up near the surface of the capillary (which exposes some SiO⁻ groups) to maintain charge balance, forms a double layer, and creates a potential difference, known as zeta potential (ζ), very close to the wall. Then the anionic SDS monomers are oriented in the area near this region. When the voltage is applied across the capillary, the Na⁺ ions are less likely to move because of their strong adsorption on the surface, but the SDS monomers are attracted and move toward the anode. Because SDS anions are solvated by the mixture of methanol and ethanol, their movement drags the bulk solution in the capillary from the cathode to the anode. The direction and magnitude of EOF was determined by using 1,2-dinitrobenzene as a neutral marker. In contrast, in the case of methanol/CTAB, the positively charged CTAB monomers build up near the negatively charged surface of the capillary and are attracted toward the cathode when a potential is applied across the capillary, and they drag the bulk of the capillary solvents from the anode to the cathode. The magnitude of EOF in these 2 systems, which is lower than that of the corresponding aqueous CE system, can be explained as follows. The physical parameters than influence EOF mobility (μ_eof) are dielectric constant (ε), viscosity (η), and zeta potential (ζ). These parameters are related to (μ_eof) by the equation 1:

\[
μ_{\text{eof}} = \varepsilon \times \frac{ζ}{η}
\]

The ratio of ε/η for methanol is ca 67% of that for water (the ratio of ε/η for water is 80/0.89 = 89.88); the same ratio

Figure 3. Comparison of (A) methanol/CTAB and (B) aqueous solution of sodium citrate (pH = 3) for the separation of closely related isomeric dinitrobenzoic acids. See Figure 1 legend for peak identification and Figure 2 legend for separation conditions.

Figure 4. Chemical structures of isomeric pairs of dyestuffs separated by the MeOH/CTAB system.
Figure 5. Comparison of separation efficiency in (A) methanol/CTAB and (B) sodium tetraborate in water. Peaks: 1 = amaranth; 2 = new coccine; 3 = brilliant blue; 4 = light green SF yellowish. See Figure 2 legend for separation conditions.

Figure 6. Effect of adding urea to the carrier electrolyte on migration time and peak resolution of the compounds tested. Electropherograms: (A) without urea, (B) with 50mM urea, (C) with 100mM urea, (D) with 150mM urea, and (E) with 200mM urea. See Figure 1 legend for peak identification and Figure 2 legend for separation conditions.
for methanol is 32.7/0.54 = 60.55. In addition, methanol can decrease the zeta potential at the silica surface via an increase in the pK_a value of the silanol group, which causes ionization of fewer silanol groups and thus diminishes the number of negative charges on the surface. Both of these parameters cause a decrease in EOF in the methanol–ethanol/SDS and methanol/CTAB systems in comparison to the EOF in an aqueous system.

The use of SDS or CTAB in methanol as the carrier electrolyte system is a novel approach for nonaqueous CE with advantages that are discussed here. Both systems showed lower generated electric current in comparison with that in aqueous CE, because of the lower conductivity and higher viscosity of the media. The lower electric current (up to submicro ampere levels) observed in tests of nonaqueous systems was advantageous in removing the heat generated during electrophoretic runs. This behavior can produce more reproducible results even without the use of a capillary thermostat. In addition, generation of low electric current makes it possible to run electrophoretic separations under higher applied voltages, which in turn lead to improvements in resolution efficiency.

**Effect of SDS and CTAB Concentrations on EOF**

As in conventional CE (20), EOF in both systems decreased with increasing concentration of SDS or CTAB. Because the direction of EOF in the methanol–ethanol/SDS system was the same as that of the solute’s electrophoretic mobility, it was found that a reverse polarity mode and injection of sample in the cathodic vial caused anionic organic acids to migrate from the cathode toward the anode, passing through the detector. Consequently, a decrease in EOF caused the migration time of the solute to increase (Figure 1). In MeOH/CTAB the direction of EOF and the direction of the solute’s electrophoretic mobility were opposite, and the magnitude of the EOF was not sufficient for the anionic species to migrate toward the cathode; therefore, the power supply was applied in the normal polarity mode with injection of sample in the cathodic vial. This procedure caused the anionic organic acids to migrate toward the anode and pass through the detector. Thus, a decrease in EOF resulted in faster elution of the organic acids tested (Figure 2).

The proposed nonaqueous CE systems were compared with a conventional aqueous CE system for the separation of isomeric dinitrobenzoic acids and food additive dyestuffs. Typical electropherograms obtained in aqueous and nonaqueous (methanol/CTAB) systems are shown in Figure 3. As can be seen, although complete separation between peaks was achieved in the nonaqueous system, the peaks of the 3,4- and 3,5-isomers could not be separated in the aqueous system because of their very close pK_a values (1.78 and 1.80, respectively), which resulted in almost similar mobility, and thus co-elution of these 2 isomers. The reason for the successful separation of the 3,4- and 3,5-isomers in methanol/CTAB could be the different degrees of ionization and/or solvation of the carboxylic groups of the 2 isomers in methanol, which produced different charge-to-mass ratios for these 2 moieties.

![Figure 7](image_url)

**Figure 7.** Effect of different percentages of acetonitrile in the carrier electrolyte on migration time and peak solution of the tested compounds in the methanol/CTAB system. Volumes of acetonitrile–methanol were (A) 90 + 10, (B) 50 + 50, (C) 30 + 70, and (D) 20 + 80. See Figure 1 legend for peak identification and Figure 2 legend for separation conditions.
Reproducibility of Migration Time

To check the reproducibility of migration time, consecutive analyses were performed, without a washing step between runs. The results are shown in Tables 1 and 2 for the methanol–ethanol/SDS and methanol/CTAB systems, respectively. The relative standard deviation (RSD) was <2% (whereas the RSD for aqueous CE was almost double), which clearly shows that the method is highly reproducible with respect to migration time. Using picric acid as an internal standard enhanced reproducibility, and the RSD was <0.5% (Table 2). The improvement may be due to the diminished adsorption of the solutes onto the capillary wall because of the decreased number of silanol groups. This decrease comes from the increased pKₐ of the silanol groups in methanol (19).

Separation of Isomeric Dyestuffs with the MeOH/CTAB System

In previous sections, an improvement was described for the separation efficiency of isomeric dinitrobenzoic acids in the nonaqueous systems tested. This improvement could be due to a different dissociation of carboxylic acid groups and/or a different solvation of solute in methanol or methanol–ethanol, compared with that taking place in an aqueous system. The successful separation of the dinitrobenzoic acid isomers led us to investigate the possibility of resolving 4 isomeric dyestuff food additives which possess ionizable sulfonyl groups. The structures of the 2 isomeric pairs (i.e., light green SF-yellowish-brilliant blue, and amaranth-new coccine) are shown in Figure 4. As can be seen, the ionic charge and molecular mass of the compounds in each pair are identical, suggesting similar electrophoretic mobility; however, complete separation between these dyestuffs was achieved with the methanol/CTAB system, whereas no resolution was obtained between isomeric pairs with an aqueous CE system (Figure 5). The different electrophoretic mobilities among the isomeric pairs which resulted in complete resolution of the corresponding peaks is based on the differences in solvation of the dyestuffs in methanol. These differences in solvation are based on differences in geometry, which lead to the differences in molecular mass that result in resolution. The resolution between the peaks of brilliant blue and light green SF-yellowish is very important from...
a quality control point of view; the latter is prohibited as a food additive because of its potential toxicity. Thus, the proposed nonaqueous system may have uses in areas of food inspection and safety, to detect illegal use of light green SF yellowish in the presence of brilliant blue.

**Effect of Urea Addition**

Urea was added to the carrier electrolyte in the methanol/CTAB system to affect the EOF and electrophoretic mobility of the tested compounds. The magnitude of these parameters changed as a function of urea concentration. The decrease in EOF upon addition of urea to the carrier electrolyte may be due to the increased viscosity of the medium or the interaction of urea with CTAB via lone pairs of amide or carbonyl groups with the positive charge of the quaternary ammonium ion. These interactions can increase the molecular mass of CTAB and thus decrease its mobility (i.e., decrease EOF). The electropherograms obtained with different amounts of urea are shown in Figure 6. As can be seen, adding urea up to the optimal amount can be used to manipulate migration time and reduce analysis time while still achieving acceptable separations.

**Effect of Acetonitrile**

Acetonitrile was used as a solvent instead of methanol to investigate its potential limitations and advantages for the separation of the tested compounds. With 20 mM CTAB in acetonitrile, fast elution of the tested organic acids was obtained in <2 min without proper separation. Further studies showed that both the EOF and the electrophoretic mobility of solutes were increased in acetonitrile. The faster EOF in acetonitrile is due to the higher ratio of dielectric constant to viscosity ($\varepsilon/\eta = 10\, \text{cm}^2/\text{J.m}$) for this solvent, compared with that of water ($\varepsilon/\eta = 7$) and that of methanol ($\varepsilon/\eta = 6$), which can affect EOF according to equation 1. The higher values of electrophoretic mobility for the solute in acetonitrile can be explained by the aprotic dipolar nature of acetonitrile, which leads to a lower degree of solvation. As a consequence, a lower molecular mass was obtained for the solutes, which resulted in a higher charge-to-mass ratio and, thus, higher electrophoretic mobilities for the solutes. Accordingly, changing the percentage of acetonitrile was used as an optimizing tool to manipulate resolution and analysis time of the tested dinitrobenzoic acid isomers (Figure 7).

**Effect of Sample Matrix in the Methanol/CTAB System**

The potential impact of different sample matrices that may come from different methods of sample preparation and/or sources was studied by dissolving the tested compounds in different solvents including water, methanol, water-methanol, and carrier electrolyte (methanol/CTAB). No considerable differences were found for resolution, peak shape, migration time, and separation pattern in the electropherograms obtained (Figure 8). These parameter are independent from the sample matrix because after the start of the electrophoretic run, while the main components of the sample migrate toward the anode, the constituents of the matrix, including the solvent and neutral or positively charged species, migrate through the cathode and are removed from the capillary. This means that any potential interference effect from neutral or positively charged compounds in the sample matrix can be simply removed.

**Conclusions**

In this study, the merits of performing CE in pure nonaqueous media (methanol, methanol–ethanol, and methanol–acetonitrile) were investigated for the separation of closely related positional isomers of dinitrobenzoic acids and isomeric pairs of dyestuff food additives (amaranth–new coccine and light green SF yellowish–brilliant blue). Instead of ordinary buffer electrolytes, CTAB and/or SDS monomers were used to prepare the carrier electrolyte; under the low level of electric current generated, a complete resolution of the tested compounds was achieved in a relatively short time. An RSD of <0.4% was obtained for the migration times, without temperature control and a washing step between runs. Addition of urea to the carrier electrolyte was shown to be a tool for the manipulation of EOF, analysis time, and resolution. Although elution of the tested compounds in methanol/CTAB was very fast, methanol–acetonitrile appeared to be better for optimizing separation. Our results showed that for the separation of positional organic acid isomers with close pKa values and isomeric organic moieties with ionizable functional groups, the methanol/CTAB system can achieve complete resolution in short capillaries (<20 cm). In addition to urea, the effect of mixed anionic, neutral-anionic, and neutral-cationic surfactants could be studied. The results from this study show the superiority of nonaqueous CE for the separation of closely related organic acids and other ionic compounds, it produces different degrees of ionization and/or solvation that provide different charge-to-mass ratios for the moieties, which have similar charges and masses in aqueous media.

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**References**