**High Speed and Reproducible Analysis of Nitrosamines by Capillary Electrophoresis with a Sulfonated Capillary**

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Abstract: Recently environmental control is regarded as important for good human health conditions, and toxic substances, including carcinogens and endocrine disruptors should be eliminated from our living environment. Hence easy quantitative methods are expected for a high level of environmental control. Our previous paper describes an easy quantitative analysis of nitrosamines (NAs) by capillary electrophoresis with an untreated fused silica capillary installed in an ordinary apparatus. In this paper, utilizing a novel type capillary column having sulfonated inner wall was investigated for improvements of separation performance and reproducibility. A sulfonated capillary causes fast and stabile electroosmotic flow because its inner wall is strongly negative charged. On a performance comparison of a sulfonated capillary with an untreated fused silica, analysis time reduction of c.a. forty percent was achieved, and relative standard deviations of migration times and peak responses were less than one third. In addition sample concentrations giving detection and quantitation limits were also reduced to a half.

**Key words:** capillary electrophoresis, micellar electrokinetic chromatography, sulfonated capillary, nitrosamine, carcinogen, food

1 INTRODUCTION

To ensure a healthy diet, monitoring of the constituents of food is indispensable. Therefore a quantitative analytical methodology for determining food contents is becoming more important and has been studied by many scientists¹⁴. Also many hazardous chemicals, such as mutagenic substances, carcinogens, and endocrine disruptors have been studied from multiple angles for a long time. In these studies a high performance system for quantitative analysis of targets has been necessary. Novel methodologies of capillary electrophoretic separations have been developed for analyses of foods, hazardous chemicals and pharmaceuticals⁵⁻¹⁰. We have reported on the conveniences of this separation technique for quantitative analysis of nitrosamines (NAs) in our previous paper⁹. Though capillary electrophoresis was quite reasonable for simultaneous quantitation of NAs, capillary electrophoretic separation of NAs with an untreated fused silica as a separation column did not show so high reproducibility of migration times and peak responses. In many cases of capillary electrophoretic analyses with untreated fused silica, relatively low reproducibility of migration time caused by variation of electroosmotic flow rate is a serious problem for reliabilities of estimated values. To solve this problem many techniques have been tried and innovated. The simplest contrivance is capillary inner wall conditioning, for example rinsing with an aqueous solution of hydroxide followed by a running buffer for enough time to equilibrate electric charge of the capillary inner wall. With this treatment, the equilibrium of the electric charge of a silanol group on the inner surface of the capillary is kept in a similar condition for each run, which creates stabile electroosmotic flow rate. However controlling electric charge completely is difficult with this treatment, because the silanol group on a capillary wall is a weak acid. As another contrivance to improve reproducibility a thermostated capillary oven can be given.

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Recent commercial apparatuses are equipped with air or coolant liquid circulated capillary ovens. Controlling electroosmotic flow rate with an untreated fused silica as a separation column is still difficult, though this system is reasonably able to keep the electric charge of not only the silanol group but also ionization of ionic samples. We presented using a capillary having sulfonyl group on its inner wall, in our previous paper including principle of preparation and comparison of reproducibility with an untreated fused silica using standard specimens as model samples. In this paper we proposed the utilization of this capillary having a strong acidic group on its inner wall to improve separation performance for simultaneous quantitative analysis of NAs.

2 EXPERIMENT

2.1 Chemicals

Reagent grade samples of chemicals were obtained from the following sources: N-nitrosodimethylamine (NDMA) from Wako Pure Chemical (Osaka, Japan), N-nitrosodiethylamine (NDEA) and N-nitrosopiperidine (NPIP) from Sigma (St. Louis, MO, USA), N-nitrosopyrrolidine (NPYR) from Aldrich (Milwaukee, WI, USA). All other chemicals were also of the highest commercially available grade. Glassware-distilled deionized water was used for preparation of running buffers and sample solutions. Each stock sample solution of NDMA, NDEA, NPYR and NPIP was prepared individually by dissolving in water at a concentration of 40 mM. These solutions were mixed in a common volume to prepare a sample solution containing each NA at the same concentration of 10 mM. This solution was diluted to 2 mM for optimization, and to appropriate concentrations for calibration curves.

2.2 Capillary electrophoresis

Waters Quanta 4000 equipped with an auto sampler, an air circulating system for capillary cooling and an UV detector was used as a capillary electrophoresis apparatus. FunCap CE/Type S (total length, 58 cm; effective length, 50 cm; internal diameter, 50 μm) from GL Sciences Inc. (Fukushima, Japan) as a sulfonated capillary was installed in the apparatus. An untreated fused silica capillary was also used for a reference.

A stock solution of phosphate buffer was prepared by adding 50 mM disodium hydrogen phosphate aqueous solution to a solution of 50 mM sodium dihydrogen phosphate until a solution with pH 6.8 was achieved with a pH meter. Sodium dodecyl sulfate (SDS) was dissolved in the stock solution of phosphate buffer at a concentration of 150 mM. Phosphate buffers containing SDS at various concentrations were used as electrophoretic solutions for capillary electrophoretic analysis. Those electrophoretic solutions were prepared by mixing phosphate buffer and SDS containing phosphate buffer in various ratios for optimization of separation conditions. Sample solutions were introduced from the anodic end of the capillary for 20 s by the hydrostatic introduction system. Potential of 20 kV was applied between both ends of the capillary. The capillary tube was conditioned by rinsing with an electrophoretic solution for 5 min using flush mode of the introduction system before each sample introduction. Detection was carried out by monitoring UV absorption at 214 nm. The capillary electrophoretic analyses were carried out at room temperature (22 ~ 23°C).

2.3 Calibration curve

Calibration graphs for the four kinds of NAs were obtained simultaneously. A sample solution of NA mixture was diluted with water to each concentration of 0.05, 0.07, 0.1, 0.2, 0.5, 0.7, 1, 2, 5, 7, 10, 15 and 20 mM. These standard solutions were analyzed by capillary electrophoresis under the optimized conditions. The peak responses of each NA were plotted against the concentration.

3 RESULTS

3.1 Optimization of separation conditions

Samples of nitrosamines (NAs) are hydrophobic compounds predicated by their structures as shown in Fig. 1. For capillary electrophoretic separation of hydrophobic compounds, micellar electrokinetic chromatography (MEKC) is generally the most suitable separation mode. This is especially true when separating hydrophobic compounds having weakly or no electric charge.

We have already reported usefulness of MEKC for NAs separation in a previous paper. Also in this work of improving separation performance, MEKC mode was adopted for NAs analysis by capillary electrophoresis (CE). It was expected that utilization of this new type capillary which has a sulfonyl group on its inner wall would allow

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\text{Fig. 1 Chemical Structures of Nitrosamines.}
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High speed and reproducible analysis of nitrosamines by capillary electrophoresis with a sulfonated capillary.

Fast electroosmotic flow however may reduce separation efficiency due to shorter analysis time. Concentration of SDS in the running buffer was reinvestigated to optimize the analytical conditions, though electrophoretic conditions were optimized in the previous paper. Electropherograms of NAs at various concentrations of SDS in the running buffers are shown in Figure 2.

Values of theoretical plate numbers, resolutions and migration times calculated from electropherograms in Figure 2 were plotted against SDS concentration (Figure 3). The values of theoretical plate number of fast moving NAs increased with SDS concentration. The plot of theoretical plate number of NPYP reached a plateau at the concentrations of 75 mM. The values of theoretical plate number of slow moving NAs did not change significantly. Resolution and migration time gave larger values with higher concentration of SDS. From these results, a solution of 50 mM phosphate buffer (pH 6.8) containing SDS at a concentration of 75 mM is the most suitable running buffer among used buffers, because it gives a high enough resolution and a shorter analysis time.

Reliability of quantitation under the optimum analytical conditions was validated from calibration curves of each NA. Plots of peak responses toward concentrations of each NA at the range of 50 μM to 20 mM gave straight lines with good linearity (correlation coefficient were 0.997~0.999). The equations of each calibration graph as peak response vs. concentration (μM) are given below; NDMA, Y = 9.68·10^3X + 2.16·10^3; NDEA, 8.21·10^X + 1.71·10^2; NPYR, 8.33·10^X + 1.70·10^2; NPIP, 7.96·10^X + 1.18·10^2. The given concentration range of 50 μM to 20 mM is sufficient for quantitation of NA contents in foods or drinking water at harmful concentration for human health.

The NAs mixtures at common concentrations are made by successive dilution of standard solution. These were analyzed to estimate the detection and quantitation limits as shown in Figure 4. The limits of detection and quantitation were 20 μM and 50 μM of the concentration giving the values of 3 and 5 as the signal to noise ratios (S/N). In the case of using an untreated capillary, determination and quantitation limits were 50 μM (S/N = 3) and 100 μM (S/N = 5), respectively (data not shown). Therefore, both limits for a sulfonated capillary were lower when compared with an untreated fused silica.

Reproducibility

Reproducibility when using an untreated fused silica and a sulfonated capillary were compared. Electropherograms of NAs by repetition with an untreated fused silica (a) and
sulfonated capillary (b), each under the optimum analytical conditions are shown in Fig. 5.

All electropherograms gave sharp peaks separated completely from each other, and each set of repetitions gave high repeatability. The data of these electropherograms were summarized in Table 1.

Repeatability of migration times with an untreated capillary and sulfonated capillary were 0.53 to 0.92% and 0.11 to 0.16% respectively as relative standard deviations (RSDs). Repeatability of peak responses were 1.6 to 2.9% and 0.68 to 0.95% respectively. The day to day variations of migration time and peak response over three days with an untreated fused silica were 3.0 to 4.6% and 4.2 to 8.8% respectively. These with sulfonated capillary were 1.8 to 1.9% and 1.9 to 2.6% respectively. In comparison of these values, both repeatability and the day to day variation given by using a sulfonated capillary displayed higher performance than an untreated capillary.

4 DISCUSSION

By using a sulfonated capillary, faster and reproducible analysis of NAs was achieved with baseline separation and less than 1% of relative standard deviations for both migration times and peak responses. These high performances are based on fast and stable electroosmotic flow caused by strong electric charge of the sulfonyl group dissociating completely on a capillary inner wall. The comparison of the performances between an untreated capillary and a sulfonated capillary is summarized in Table 2.

Table 2 includes data of the analysis with an untreated fused silica under the conditions giving similar analysis time as a sulfonated capillary under the optimum conditions. Though both capillaries gave similar theoretical plate numbers under their respective optimum conditions, analysis time with a sulfonated capillary was reduced considerably and shorter analysis time caused slightly lower resolution. In addition, fast electroosmotic flow made sharper peaks causing reduction of a sample concentration for detection and quantitation limits. By comparison of the separation performance factors between each capillary under the conditions giving similar analysis time, using an untreated fused silica with a running buffer containing SDS at a low concentration of 25 mM gave much lower values of theoretical plate numbers and resolutions.

5 CONCLUSION

The separation performance of a sulfonated capillary was compared with an untreated fused silica using a mixture of NAs as a sample. The most remarkable feature of
**Table 1** Comparison of Repeatability.

<table>
<thead>
<tr>
<th>Anal. No.</th>
<th>Migration time</th>
<th>Peak response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NDMA</td>
<td>NPYR</td>
</tr>
<tr>
<td>1</td>
<td>6.98</td>
<td>7.82</td>
</tr>
<tr>
<td>2</td>
<td>6.98</td>
<td>7.84</td>
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<tr>
<td>3</td>
<td>7.01</td>
<td>7.85</td>
</tr>
<tr>
<td>4</td>
<td>7.03</td>
<td>7.88</td>
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<tr>
<td>5</td>
<td>7.02</td>
<td>7.87</td>
</tr>
<tr>
<td>6</td>
<td>7.08</td>
<td>7.97</td>
</tr>
<tr>
<td>SD</td>
<td>0.037</td>
<td>0.0527</td>
</tr>
<tr>
<td>AVE</td>
<td>7.02</td>
<td>7.87</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>0.53</td>
<td>0.67</td>
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</tbody>
</table>

**Table 2** Comparison of Separation Efficiency.

<table>
<thead>
<tr>
<th></th>
<th>Untreated fused silica (75 mM SDS)</th>
<th>Sulfonated capillary (75 mM SDS)</th>
<th>Untreated fused silica (25mM SDS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NDMA</td>
<td>NPYR</td>
<td>NDEA</td>
</tr>
<tr>
<td>Migration time (min)</td>
<td>7.02</td>
<td>7.87</td>
<td>8.78</td>
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<tr>
<td>Theoretical plate number</td>
<td>96216</td>
<td>92227</td>
<td>64570</td>
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<tr>
<td>Resolution</td>
<td>7.64</td>
<td>6.76</td>
<td>649</td>
</tr>
<tr>
<td>Repeatability (time, n=6)</td>
<td>0.53</td>
<td>0.67</td>
<td>0.82</td>
</tr>
<tr>
<td>Repeatability (area, n=6)</td>
<td>2.9</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Detection limit (S/N=3)</td>
<td>50 μM</td>
<td>20 μM</td>
<td></td>
</tr>
<tr>
<td>Quantitation limit (S/N=5)</td>
<td>100 μM</td>
<td>50 μM</td>
<td></td>
</tr>
</tbody>
</table>

sulfonated capillary is fast and stable electroosmotic flow caused by the strong electric charge of the sufonyl group on the capillary inner wall. It makes not only short analysis time but also improvements of reproducibility and detection limits possible. As a comprehensive result, a sulfonated capillary has much higher performance than an untreated fused silica, and it is shown that by using this capillary, reliable analysis could be carried out with short analysis time. For a future work this capillary should display its power as a separation and transfer tube in mass spectrometry joined with capillary electrophoresis.

References