Journal of Food and Drug Analysis, Vol. 12, No. 4, 2004, Pages 332-335 藥物食品分析 第十二卷 第四期

Determination of Benzoate Derivatives in Soy Sauce by Capillary Electrophoresis and In-Capillary Microextraction Procedure

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(Received: April 15, 2004; Accepted: June 14, 2004)

ABSTRACT

Benzoate derivatives in soy sauce were analyzed by capillary zone electrophoresis (CZE) coupled with a microextraction technique. The analytes were acidified and extracted with ethyl acetate. The organic extract was directly injected (30 mbar × 1 sec) into a fused silica capillary (75 μm i.d. × 34 cm) and separated in 20 mM borax buffer (pH 9.2) with 10 kV working voltage. Separation was completed in less than 10 min as monitored by A_{225}. Hippuric acid was used as internal standard to improve the reproducibility for quantification (CV = 4.5%, n = 12).

Key words: *p*-hydroxybenzoate, ethyl acetate, capillary zone electrophoresis (CZE), hippuric acid, preservative

INTRODUCTION

Benzoate and its derivatives (parabens) are important preservatives for food and pharmaceutical industries, whereas the additives are metabolized in liver by glycine or sulfate conjugation[1]. The detoxicated metabolites are more hydrophilic and with higher urinal clearance. The additives are therefore a burden to the liver and may eventually lead to irreversible damages. Several analytical methods, especially those using high performance liquid chromatography (HPLC), have been reported to determine the contents of benzoate preservatives[2-5]. However, for samples with complicated compositions, additional sample pretreatment procedures are required.

Soy sauce is a daily seasoning of traditional Asian food. The sauce is actually the fermentation broth of *Aspergillus orygae*. The fungus was cultivated for months in a salty medium containing mainly soybean and wheat; and the protein was digested into peptides and amino acids during the fermentation period. The fermentation broth is therefore rich in nutrients that will disturb the analysis of possible unhealthy additives. In addition, the high salt content (>10%) will certainly hinder a charge-based separation process such as capillary zone electrophoresis (CZE).

As the sample pretreatment method for liquid chromatography (LC), Chu *et al.*[5] used a commercialized disposable C18 solid phase extraction cartridge to extract benzoate preservatives selectively from soy sauce. The costly pretreatment method is rapid but not eco-friendly.

Our laboratory recently developed a microextraction procedure for CZE to quantify the enzymatic reaction of angiotensin converting enzyme[6]. Benzoate derivatives were acidified and extracted into the layer of ethyl acetate (EA), and the organic layer was injected into the alkaline running buffer of the CZE system. The benzoates were then extracted back into the running buffer and separated simultaneously.

In the present CZE approach, the microextraction procedure was adopted and optimized for analyzing benzoate preservatives in soy sauce.

MATERIALS AND METHODS

I. *Reagents*

Methylparaben (methyl *p*-hydroxybenzoate; MP), propylparaben (propyl *p*-hydroxybenzoate; PP), butylparaben (butyl *p*-hydroxybenzoate; BP) and *p*-hydroxybenzoate (HP) were purchased from Wako Pure Chemical Industries, Ltd. Ethyl acetate (EA) was from Mallinckrodt Baker, Inc. Benzoic acid (BA), hippuric acid (HI) and sodium tetraborate (Borax) were from Nacalai Tesque, Inc. (Kyoto, Japan). Sodium dodecyl sulfate (SDS) was from Bio-Rad Laboratories. Soy sauces were purchased from local supermarkets. Deionized water was used in sample dilutions and buffer preparations. All solutions were filtered through a 0.45 μm membrane filter (FP Vericel Membrane Filter).

II. *Instrumentation and General CZE Procedure*

Capillary electrophoresis was performed with a commercialized system (G1600A, Agilent) and an uncoated fused-silica capillary (34 cm × 75 μm i.d., Beckman). The capillary was rinsed sequentially with 1N NaOH (950 mbar × 40 min) and deionized water (950 mbar × 20 min) before use. The effective capillary length (from inlet to the

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detector) was 25.5 cm. After flushing (950 mbar) with the running buffer for 4 min, sample was injected, separated and monitored with a PDA detector. The capillary was cleaned (950 mbar) sequentially with 1 N NaOH (3 min), 10 mM SDS (1 min), deionized water (5 min) and air (2 min). The above working sequence and the temperature (25˚C) were controlled by a visualized software (Chemstation^{TM}, Agilent).

III. *Microextraction CZE Procedure*

Into a test tube was added sequentially with 1 mL of sample, 1 mL of 1N HCl and 1 mL of ethyl acetate. After mixing thoroughly with a vortex mixer, approximately 1 mL of the mixture was transferred into the vial for CZE analysis. Phase separation was finished in less than 5 min. The upper organic layer was injected (30 mbar × 1 sec) and analyzed (A_{225}) by the aforementioned procedure.

In the recovery test, soy sauce (0.5 mL) was spiked with 0.5 mL of either the standard solutions (10 mM for each analytes) or deionized water to serve as the sample solutions.

RESULTS AND DISCUSSION

I. *Determination of the Upper Limit of Working Voltage*

The ohmic behavior of the capillary filled with 20 mM borax buffer deviated from linearity in voltage higher than approximately 10 kV (Figure 1), indicating the rise in temperature and the decrease in viscosity of the running buffer[7]. Since the temperature gradient may distort the profile of a plug flow and hamper the separation, the working voltage was optimized around 10 kV.

II. *Direct CZE of Soy Sauce*

Resolution of the electropherogram of an untreated soy sauce was not sufficient for quantification purposes (Figure 2). The noise from sample matrix complicated the electrophoregram, and the signal was restricted by the maximum loading volume of a sample with high ionic strength. In spite of the separation capability of CZE, it was incompetent without pretreating the sample.

III. *Microextraction CZE and the Optimization*

After injecting into the running buffer, the preservatives (in ethyl acetate) were extracted into the alkaline running buffer and separated roughly according to the charge-to-mass ratios of the preservatives (Figure 3). As revealed by the electrophoretic current and the migration times of ethyl acetate, the electroosmotic flow was not affected by the neutral sample plug and was faster in higher electric field. By considering the theoretical plates, resolutions and separation times, 10 kV was selected for the following studies.

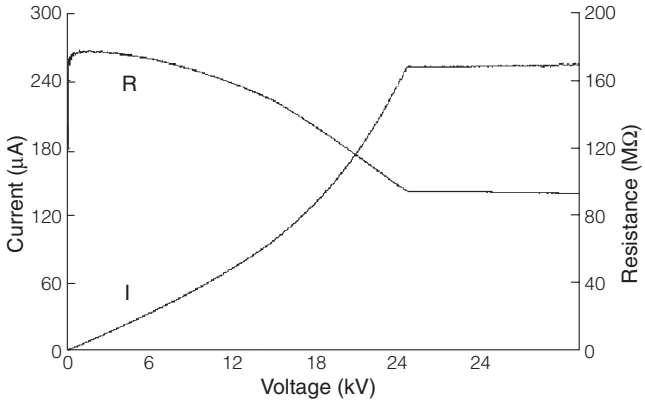

Figure 1. Effect of voltage on the current and resistance of a fused silica capillary (75 μm i.d. × 34 cm) filled with 20 mM borate buffer (pH 9.2). Other conditions were detailed in the materials and methods section.

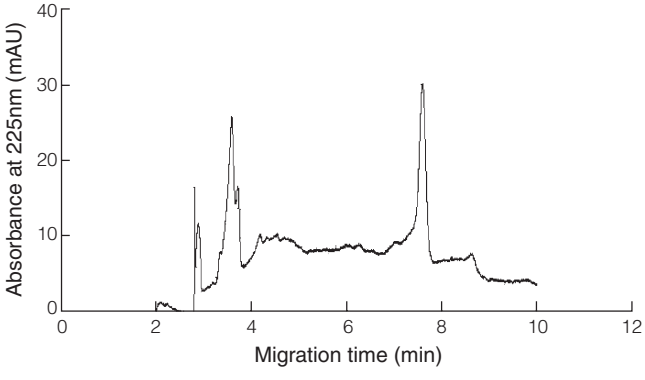

Figure 2. Electropherogram obtained by directly injecting soy sauce into the analytical capillary. Borax buffer (20 mM, pH 9.2) was used as the running buffer for the separation (10 kV). Other conditions were detailed in the materials and methods section.

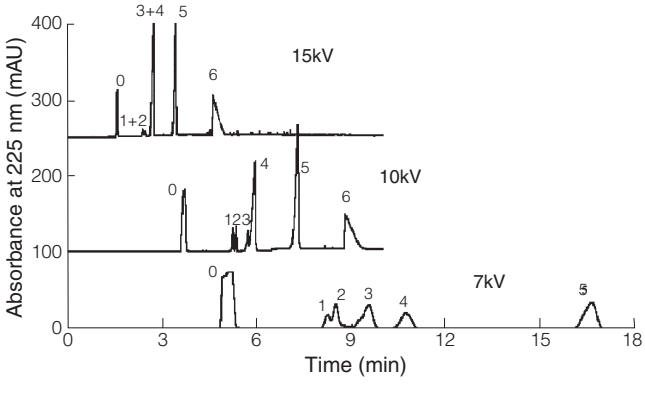

Figure 3. Effect of separation voltage on the electropherograms. The analytes (0: ethyl acetate; 1: butyl *p*-hydroxybenzoate; 2: propyl *p*-hydroxybenzoate; 3: methyl *p*-hydroxybenzoate; 4: hippuric acid; 5: benzoic acid; 6: *p*-hydroxybenzoic acid) were dissolved in ethyl acetate as 10 mM solutions, and the solutions (1 mL for each) were mixed as the CZE sample (30 mbar × 1 sec). (A) 15 kV, 118 μA; (B) 10 kV, 53 μA, (C) 7 kV, 32 μA. Other conditions were the same as Figure 2.

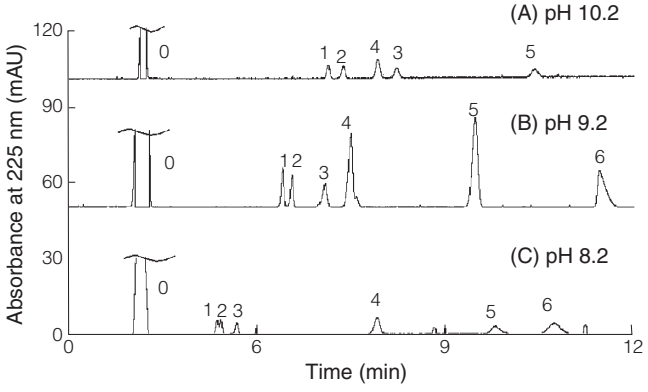

Figure 4. Effect of pH on the electropherograms. The pH was controlled by 20 mM borax as (A) pH = 10.2, (B) pH = 9.2 and (C) pH = 8.2. Other conditions and notations were the same as Figure 3.

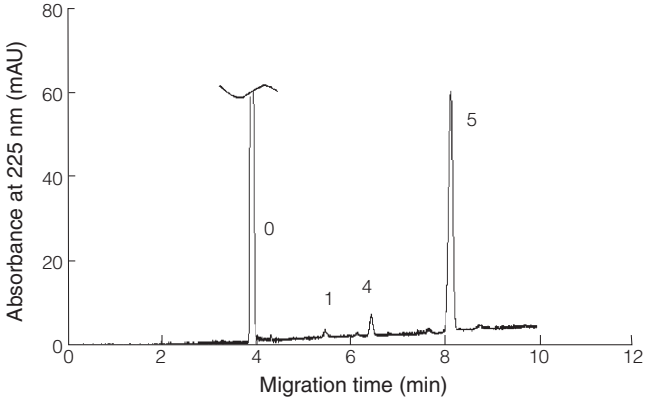

Figure 5. Electrophorogram of soy sauce obtained by the aid of a microextraction procedure. Borax buffer (20 mM, pH 9.2) was used as the running buffer for the separation (10 kV). Other conditions and notations were the same as Figure 3.

The influence of pH was also investigated (Figure 4). The phenoxy groups of the preservatives tend to dissociate at higher pH, which increases the negative charge densities and electrophoretic mobilities of the molecule. Therefore, the electropherograms shifted to the direction of longer migration time at higher pH. At pH higher than 10, the electrophoretic mobility is eventually higher than the electroosmotic mobility, and the peak of hydroxybenzoate is excluded (Figure 4-A). Also at this pH, the migration time of methyl paraben (pKa = 8.47) becomes longer than hippuric acid.

The obvious tailing profile of hydroxybenzoate (peak 6, Figure 4-B) indicates that the local electric field within the sample plug was higher than that in the background running buffer[8]. The phenomenon may be eliminated by decreasing the ionic strength of the running buffer or increasing the concentration (the conductivity) of the sample plug. Similar phenomena were observed in Figure 3.

IV. *Using Internal Standard to Improve Reproducibility*

Although the repeatability of migration time was excellent (CV < 2%, n = 12), the precision of peak area for the microextraction CZE method (CV = 34.2%, n = 12) was not acceptable for quantification purposes. This may be attributed to mechanical errors of the hydrodynamic injection process. Hippuric acid was added as the internal standard to eliminate possible injection errors; the area ratios were taken for quantification with satisfactory reproducibility (CV = 4.5%, n = 12). The linear regression coefficients between the ratios of concentration and area are higher than 0.975 (benzoate, R^2 = 0.9873; methyl paraben, R^2 = 0.9861; propyl paraben, R^2 = 0.9773; butyl paraben, R^2 = 0.9756; hydroxybenzoate, R^2 = 0.9809).

Table 1. Recoveries of benzoate preservatives in soy sauce[a]

Preservative	Recovery (%)	C.V. of recoveries (%)
Butyl *p*-hydroxybenzoate	115.7[b]	8.26
Propyl *p*-hydroxybenzoate	110.6	4.42
Methyl *p*-hydroxybenzoate	94.6	9.27
Benzoic acid	91.7	4.48
p-Hydroxybenzoic acid	117.5	13.2

[a]The same experimental conditions as Figure 5.
[b]Each datum is the average of five experiments.

V. *Analysis of Preservatives in Soy Sauce*

Figure 5 shows the electropherogram of a commercial soy sauce obtained by the aid of a microextraction technique. The existence of butyl *p*-hydroxybenzoate and benzoate in the sample was resolved by the microextraction CZE method. The microextraction procedure served as a noise filter to exclude unwanted signals in the sample matrix.

Recoveries of different preservatives from soy sauce were also investigated (Table 1).

CONCLUSIONS

The microextraction CZE approach successfully analyzed benzoate preservatives in soy sauce with minimum sample pretreatment procedures. Compared with other analytical method, this method is rapid, labor-saving and economic. Similar analytical concepts may be appropriate for other complicated biological samples and analytes. Substantial benefits to routine chemical analysis are expected.

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