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A Rapid Method for the Simultaneous Determination of Preservatives in Soy Sauce

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ABSTRACT

A rapid method for the simultaneous determination of five preservatives is presented. The preservatives from soy sauce samples were extracted with a C18 bonded silica SPE cartridge from soy sauce samples. 10% Methanol in 1% phosphoric acid solution was found to be the best solution for clean-up. The preservatives were eluted with methanol and determined by high-performance liquid chromatography using a gradient elution system in one run. The average recoveries of *p*-hydroxybenzoic acid, benzoic acid, ethyl *p*-hydroxybenzoate, propyl *p*-hydroxybenzoate and butyl *p*-hydroxybenzoate are 97, 96, 95, 93 and 92 %, respectively. The calibration curves arewere linear between 1.8 and 54 mg/kg. The regression coefficients arewere acceptable ($R^2 > 0.992$). This method is a useful protocol for routine examination of the preservative constituents in soy sauces.

Key words: solid phase extraction, benzoic acid, *p*-hydroxybenzoate, soy sauce, HPLC

INTRODUCTION

The most commonly used preservatives in soy sauce are benzoic acid and *p*-hydroxybenzoates. Excess amounts of these additives can be harmful to human health. Therefore, the minimum permissible concentrations of benzoic acid and the esters of *p*-hydroxybenzoic acid are controlled by regulation, and the quantitative analysis of these preservatives is important in routine analysis of foods. The analytical methods for determining these preservatives in food samples have been previously described in the literature. Fruit juice and beverage can be directly analyzed without clean-up procedures prior to determination by HPLC[1,2], while the estimation of the preservatives in other food samples, such as cheese, sauce, jam, milk, yogurt and canned seafood by using HPLC, require sample pretreatment was necessary, which usually involveds solvent extraction[2,3] or precipitation of proteins and fats by the addition of methanol or acetonitrile followed by centrifugation and filtration[4]. Gas chromatography was also used in the determination of preservatives[5,6] which included sample preparation by steam distillation, derivatization or several extractions. Unfortunately, all of these methods are laborious, as well as time and solvent consuming. In addition, the applications of capillary electrophoresis[7,8] and micellar electrokinetic capillary chromatography[9,10] in preservatives analysis have been reported recently.

For the examination of the preservatives in soy sauce, direct dilution prior to HPLC analysis was announced as a CNS method[11]. However, there are many types of enzymes and proteins in fermented foods and those having lower molecular weights are difficult to remove. To prolong the useful life of liquid chromatographic columns, reducing these components in the sample matrix is crucial. In recent years, solid phase extraction (SPE) methods have been widely used for cleaning up food samples[12-14]. These methods are experimentally simpler, time-saving and require less volume of organic solvents for sample preparation. The utilization of SPE to extract the additives from in foods followed by paired-ion liquid chromatography analysis was reported[15]. SPE method also has been used for the pretreatment of samples in the determination of the preservatives in food by gas chromatograph/mass spectrometer[16-18]. The main objective of this study was to develop a fast, simple and reliable method for the routine analysis of the preservatives in soy sauce that can be accomplished by readily available instruments in most laboratories.

MATERIAL AND METHODS

I. *Chemicals and Solvents*

p-Hydroxybenzoic acid (PHB) and butyl *p*-hydroxybenzoate (butyl paraben, BP) were purchased from Chem Service (West Chester, PA, USA). Benzoic acid (BA) was obtained from Sigma (St. Louis, MO, USA). Ethyl *p*-hydroxybenzoate (ethyl paraben, EP), propyl *p*-hydroxybenzoate (propyl paraben, PP) and sodium dihydrogen phosphate were purchased from Wako (Osaka, Japan). HPLC grade acetonitrile was purchased from Mallinckrodt (Paris, Kentucky, USA) and reagent grade phosphoric acid was obtained from Union Chemical (Hsinchu, Taiwan, ROC). Deionized pure water was prepared by passing reverse osmosis water through a Barnstead Nanopure D4741 deionization pure water system (Dubuque, IA,

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USA). All samples of soy sauce were purchased from the local markets.

II. *Instrument*

A Shimadzu high performance liquid chromatograph was used (Tokyo, Japan). It consists of two Model LC-9A pumps with a mixing chamber for high-pressure binary gradient elution; Model 7125 manual sample injector with 5 μL sample loop; and Model SPD-6AV UV-VIS spectrophotometric detector operating at 215 nm, at which wavelength the absorbances of all the preservatives are closest to each other (Figure 1). The column was a Merck Lichrospher RP-18 analytical column (4.0 mm i.d. × 25 cm) (Darmstadt, Germany), and the SISC ChemStation (Taipei, Taiwan) was used for data acquisition and data processing.

III. *Chromatographic Conditions*

Known volume (e.g., 5 μL) of standard or sample solution was injected. Two LC pumps were used. The flow rate was 1.2 mL/min and the UV detector was set at 215 nm.

(I) *Isocratic elution*

The mobile phase was acetonitrile:0.03 M NaH_2PO_4 (42:58, v/v).

(II) *Gradient elution*

The mobile phase gradient started from acetonitrile:0.03 M NaH_2PO_4 (26:74), held for 5 min, then changed to 50:50 within 2.5 min. This mobile phase was used until the completion of the determination.

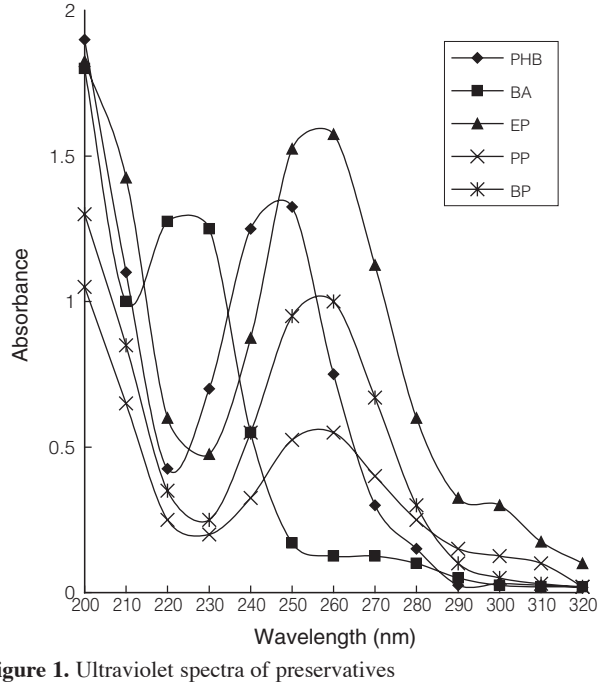

Figure 1. Ultraviolet spectra of preservatives

IV. *Sample Preparation*

The soy sauce samples were diluted 5-fold with water. An Accubond ODS cartridge was conditioned with 4 mL of methanol followed by 3 mL of water. one mL of diluted sample was passed through the conditioned cartridge, and the cartridge was washed with 4 mL of 10% (v/v) methanol with 1% phosphoric acid solution. The preservatives were eluted from the cartridge with 3 mL methanol, then diluted with methanol to 5.0 mL and filtered through a 0.45 mm syringe filter.

RESULTS AND DISCUSSION

I. *Selection of an Appropriate Condition and Washing Solution*

The critical factor in the solid phase extraction (SPE) of benzoic acid on a C18 sorbent is the pH of the solvent systems in the adsorption and wash step[12]. Two condition of procedures were tested. In the first one, the cartridge was conditioned with methanol followed by water. In the second one, the cartridge was conditioned with methanol, water, followed by 1% H_3PO_4. For evaluating the results, a standard solution (20 mg/kg) of five preservatives was analyzed without passing through the SPE cartridge as a comparison. Table 1 compared the results of two different procedures, lower yield of ethyl *p*-hydroxybenzoate resulted by using 1% H_3PO_4. In order to optimize the extraction, three 1 mL fractions of eluent were collected separately. The amounts of analytes in each fraction were quantitized, and the profile of analytes on the sorbent were evaluated. The preservatives were retained much longer in the cartridge when it was conditioned with 1% H_3PO_4. Significant differences were found at the second and third mL of eluent between the two procedures ($P < 0.05$). Thus,

Table 1. Comparison of the effects of different conditioning and washing solutions on recoveries.

Conditioning Solution[a]	Washing Solution[b]		Recovery(%)				
			PHB	BA	EP	PP	BP
A	C	1st fr.	0.7	0	0	0.4	0.2
		2nd fr.	94	107	96	97	98
		3rd fr.	11	2	4	6	8
		total	106	109	100	103	106
B	C	1st fr.	0.1	0	1	0.6	1
		2nd fr.	101	104	79	82	72
		3rd fr.	9	10	14	24	31
		total	110	114	94	107	104
A	D	1st fr.	20	20	9	7	5
		2nd fr.	96	91	84	80	73
		3rd fr.	9	12	25	29	36
		total	125	124	118	116	113

a: The cartridge was conditioned with: A (MeOH and H_2O); B (MeOH, H_2O and 1% H_3PO_4)

b: The samples were washed with: C: MeOH/ 1% H3PO4 (1/9); D: 1% H_3PO_4

methanol and water were selected as the condition solvents.

In order to remove unwanted substances in soy sauce samples, two solvent systems were assessed respectively. Table 1 demonstrated that by washing with methanol:1% phosphoric acid (1:9), the second fraction of eluent gave good recoveries (94~106%) in which all the analytes were almost flushed out. In contrast, when the samples were washed with 1% phosphoric acid, the aqueous solution without an organic modifier caused the ester analytes to be more spread out in the cartridge. As a result, a diffusion of different analytes from polar to less polar occured on the sorbent, and thus decreasing the extraction efficiency. In addition, the total recoveries of the interested preservatives were much better (100~109%) than that obtained by washing with 1% phosphoric acid only. Therefore, 10% methanol in 1% phosphoric acid solution was preferred in this study.

II. *Optimization of the Chromatographic Analysis*

Figure 2 displayed the chromatograms of isocratic and gradient elution, respectively. Each soy sauce sample was spiked with preservatives standard solutions. It is obvious in Figure 2(A) that PHB was coeluted with components from soy sauce. Hence, an isocratic liquid chromatographic method was only capable of analyzing BA, EP, PP and BP but not PHB in soy sauce samples. Figure 2(B) showed that the isolation of five preservatives could be accomplished by gradient elution in one run. When the starting polarity of the mobile phase was increased by reducing the organic portion in the mobile solution in the gradient elution, the preservatives were absorbed on the nonpolar C18 stationary phase. In this process, the more polar components in soy sauce were eluted preferentially. Therefore, the preservatives were separated from the more polar components in soy sauce. For this reason, the gradient elution was chosen as the chromatographic analysis condition.

III. *Validation of the Method*

The linearity of each preservative was examined. The results are summarized in Table 2. The calibration curves are linear in the concentration range between 1.8 and 54 mg/kg. The regression coefficients are acceptable (R^2 > 0.992). Five replicates were checked by applying the t-test, and were in agreement at the 95% confidence level. For the accuracy study, three different concentration levels of standards were spiked to a selected sample of soy sauce. The average recoveries of *p*-hydroxybenzoic acid, benzoic acid, ethyl *p*-hydroxybenzoate, propyl *p*-hydroxybenzoate and butyl *p*-hydroxybenzoate were 97, 96, 95, 93 and 92 %, respectively (Table 3).

IV. *Comparisons Between the CNS Method11 and this Reported Method*

The soy sauce sample was diluted with methanol/water (50/50) solution in the CNS method and was analyzed by HPLC directly. On the other hand, the preservatives in soy sauce samples were extracted with methanol from the SPE cartridge and then diluted with methanol in our method. Comparisons of the CNS method and this reported method are shown in Table 4. The results clearly demonstrated that the recoveries of EP, PP and BP using this reported method were significantly higher than those obtained by the CNS

Table 2. The calibration curves of preservatives, peak area vs concentration*

preservatives	Calibration equation	R^2
p-Hydroxybenzoic acid	$Y = 1.72 \times 10^4X - 6.67 \times 10^3$	0.9960
Benzoic acid	$Y = 1.11 \times 10^4X - 4.67 \times 10^3$	0.9973
Ethyl paraben	$Y = 1.89 \times 10^4X - 4.09 \times 10^4$	0.9961
Propyl paraben	$Y = 1.80 \times 10^4X - 4.57 \times 10^4$	0.9934
Butyl paraben	$Y = 1.57 \times 10^4X - 5.29 \times 10^4$	0.9929

*: Linear range between 1.8 and 54 mg/kg (n = 5)

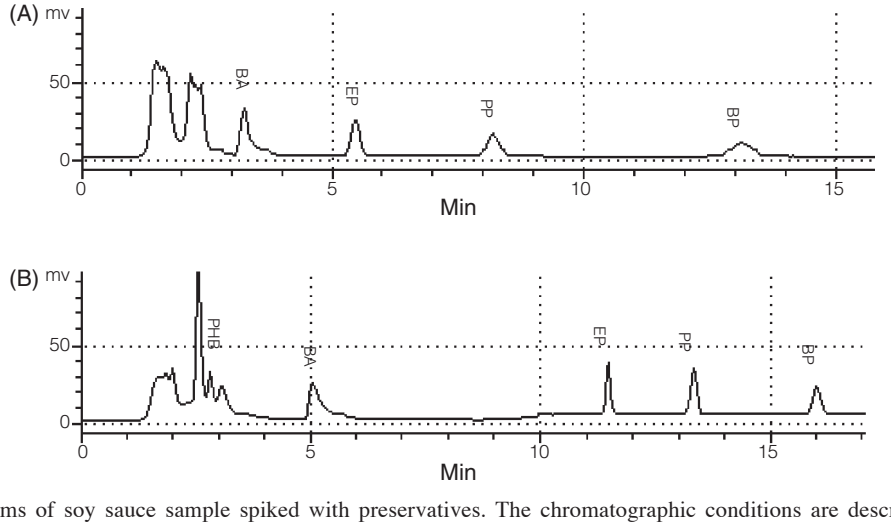

Figure 2. Chromatograms of soy sauce sample spiked with preservatives. The chromatographic conditions are described in "Material and Methods". (A) isocratic elution, (B) gradient elution.

method, suggesting that methanol extraction recovers these less polar preservatives more effectively than simple dissolution of the samples in the methanol/water solution. Furthermore, the CNS method requires two different solvent systems for two categories of preservatives respectively, i.e. methanol/acetonitrile/5 m*M* citric acid buffer (1:2:7) for PHB and BA; methanol/5 m*M* citric acid buffer (6:4) for EP, PP and BP while the current method accomplishes the analysis of all the preservatives in one run.

V. *Determination of the Preservatives in Different Soy Sauce Samples*

The amounts of PHB, BA, EP, PP and BP were determined using an external calibration curve (Table 5). The data in Table 5 clearly indicated that none of the concentrations of BA in the samples analyzed is higher than the maximum permitted level of 0.6 g/kg. *p*-Hydroxybenzoic acid and parabens were not detected in brand A. The contents of *p*-hydroxybenzoic acid and parabens in brand D are far below the allowed level of 0.25 g/kg. On other hand, the PHB in brands B and C were found to be higher than the maximum permitted level.

Table 3. Recoveries of preservatives in spiked samples (%)[a]

Preservatives	Spiked level (mg/kg)		
	63	72	108
p-Hydroxybenzoic acid	93.7 (± 2.8)[b]	101.5 (± 7.1)	95.2 (± 7.7)
Benzoic acid	98.6 (± 2.4)	95.7 (± 3.3)	94.1 (± 10.1)
Ethyl paraben	94.6 (± 1.5)	95.1 (± 1.8)	96.2 (± 9.5)
Propyl paraben	94.6 (± 0.6)	92.2 (± 4.5)	92.4 (± 9.6)
Butyl paraben	90.6 (± 6.6)	94.4 (± 7.7)	92.3 (± 6.7)

a: The average recovery (%) of triplicates
b: The coefficient of variation

Table 4. Comparison of the CNS method and this reported method for the recoveries of preservatives from commercial soy sauce[a]

Methods	Added amount	Recovery (CV %)				
	(μg/g)	PHB	BA	EP	PP	BP
Reported method	300	93.7 (± 2.8)	98.6 (± 2.4)	94.6 (± 1.5)	94.6 (± 0.6)	90.6 (± 6.6)
CNS method	250	95.0 (± 3.3)	92.0 (± 4.4)	81.5 (± 0.1)	73.8 (± 6.0)	61.0 (± 3.7)

a: The average recovery (%) of triplicates

In conclusion, we have successfully developed a selective, rapid and reliable method for the determination of soy sauce preservatives such as benzoic acid, *p*-hydroxybenzoic acid, ethyl-, propyl- and butyl-parabens. The method is simple and requires less time and solvent than traditional methods.

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Table 5. Determination of the amount of preservatives in commercial soy sauce samples (mg/kg)

Preservatives	Soy sauce			
	Brand A[a]	Brand B[a]	Brand C[b]	Brand D[a]
p-Hydroxybenzoic acid	ND[c]	596.5 ± 1.7	392.1 ± 33.1	ND
Benzoic acid	529.3 ± 5.1	3.8 ± 0.4	ND	179.5 ± 7.5
Ethyl paraben	ND	ND	ND	ND
Propyl paraben	ND	ND	ND	ND
Butyl paraben	ND	ND	7.3 ± 0.4	7.6 ± 0.4

a: Data are average values of five replicates (n = 5)
b: Data are average values of four replicates (n = 4)
c: ND = not detectable

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