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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 $\mu$L 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 $\mu$L) 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.

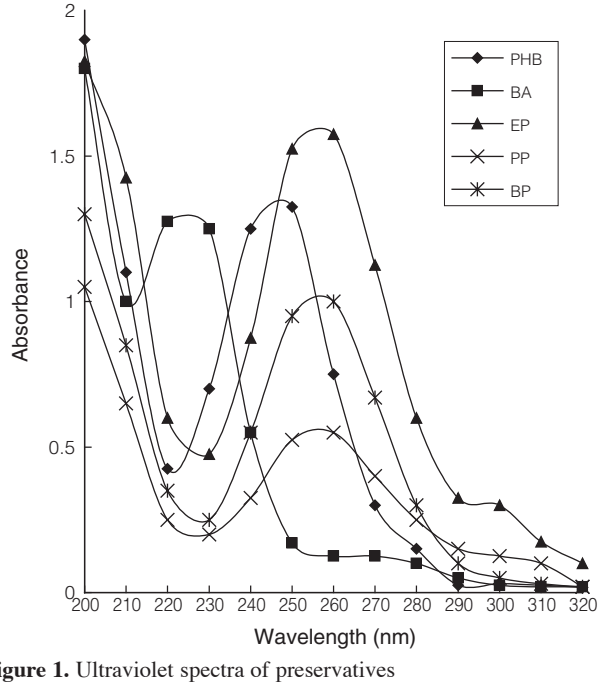


**Figure 1.** Ultraviolet spectra of preservatives

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)

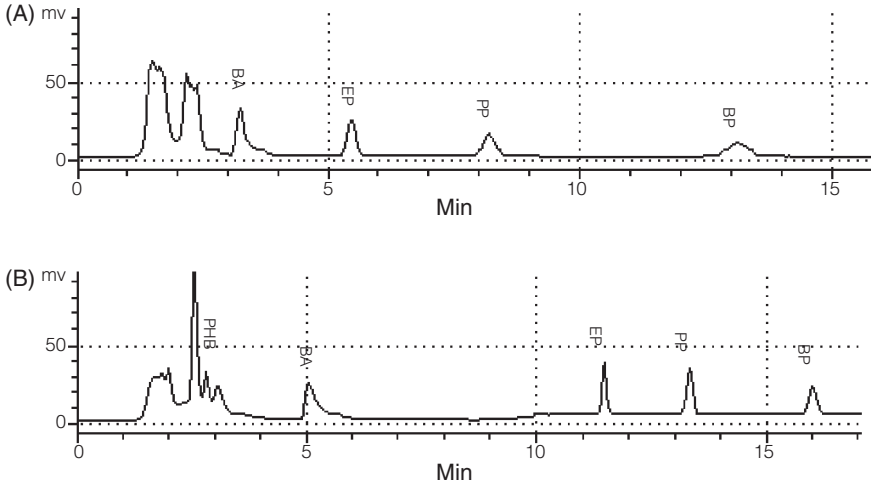


**Figure 2.** Chromatograms of soy sauce sample spiked with preservatives. The chromatographic conditions are described in "Material and Methods". (A) isocratic elution, (B) gradient elution.

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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 %) | | | | |
|---|---|---|---|---|---|---|
| | (*μ*g/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.

## ACKNOWLEDGEMENTS

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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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## 腸炎弧菌O3:K6和環境菌株的耐酸性與和假單胞菌和原生細菌的存活競爭

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### 摘　要

腸炎弧菌是常見與海產相關的食品中毒細菌，自1996年從印度起至亞洲流行的新O3:K6型菌株是第一個大流行的菌株。本研究分析這些新型菌株的耐酸性，同時探討它和假單胞菌與原生菌叢的存活競爭性。結果顯示這些新O3:K6的對數期菌體對於pH 3.0的耐受性顯著地高於環境菌株。混合培養的實驗顯示這些菌株的生長會受到所使用的菌株、培養基和溫度的影響。和假單胞菌或原生菌叢的競爭實驗中顯示，所有腸炎弧菌在1/10 TSB培養基中具有相似的競爭性，但在牡蠣培養基中，腸炎弧菌環境菌株在4 或25°C下都較新O3:K6菌株有更好的存活率。兩株新的O3:K6菌株（1121，1137）則具有類似的存活表現。這些數據提供新O3:K6腸炎弧菌在食品與環境存活與散播的參考。

**關鍵詞**：腸炎弧菌，O3:K6，假單胞菌，牡蠣，耐酸性

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## 蔬菜中硝酸鹽及亞硝酸鹽含量之高效液相層析法定量分析

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### 摘　要

本研究係探討蔬菜中硝酸鹽及亞硝酸鹽之定量分析，發展出一種簡單、快速、精確及敏感度佳的高效液相層析方法（high performance liquid chromatography, HPLC）。分析較適條件為以30%（v/v）的甲醇水溶液添加0.01 M辛胺（octylammonium），並以正磷酸調整pH值至7.0，作為移動相溶液，流速為0.8 mL/min；每一樣品在十分鐘內即可分析完成。硝酸鹽及亞硝酸鹽之回收率介於96.6%至105.7%間；硝酸鹽及亞硝酸鹽於0.1~100.0 mg/L濃度範圍內之標準曲線線性迴歸係數均大於0.9990 ，線性關係非常佳。因此，高效液相層析法相當適用於定量分析蔬菜中硝酸鹽及亞硝酸鹽含量。將HPLC方法應用於檢測12種市售蔬菜，結果硝酸鹽及亞硝酸鹽含量範圍分別為225~4,410 mg/kg 及＜5~200 mg/kg 。

**關鍵詞**：蔬菜，硝酸鹽，亞硝酸鹽，高效液相層析

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## 台灣地區食品中毒之金黃色葡萄球菌腸毒素C型菌株次分型分析

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### 摘　要

金黃色葡萄球菌在世界各地均是重要的食品中毒原因菌，產生的腸毒素分為許多型別，腸毒素C型可細分為C1、C2、C3等次分型。除腸毒素外，金黃色葡萄球菌會產生多種胞外蛋白質及毒素，包含凝固酶（coagulase）、溶血素（hemolysins）、溶菌素（lysostaphin）、核酸酶（nuclease）、蛋白質A（protein A）等三十餘種，其中凝固酶及蛋白質A之產生為金黃色葡萄球菌重要的生化特性之一。本研究收集台灣地區36個食品中毒案分離之229株金黃色葡萄球菌，利用聚合酶鏈反應（PCR）方法進行腸毒素C型產毒菌基因之次分型，並以PCR-RFLP方法檢測腸毒素C型菌其凝固酶及蛋白質A基因，進而加以分型。結果顯示由中毒案所分離的10株產腸毒素C型之金黃色葡萄球菌中有3株為腸毒素C2型，7株為C3型。凝固酶基因以PCR方法偵測可區分為兩型，以限制酶*Aul* I將PCR產物進行反應亦可分為兩型。蛋白質A基因分型不論以PCR或以限制酶*Hin*d III將PCR產物進行反應之結果均屬同一類型。本研究發展的快速分型檢測方法應用於食品中毒案可輔助污染源之分析。

**關鍵詞**：金黃色葡萄球菌，聚合酶鏈反應，腸毒素C，PCR-RFLP，凝固酶基因、蛋白質A基因

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## 醬油中防腐劑之快速多重定量分析

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### 摘　要

本研究建立了一個快捷並可同時檢測醬油樣品中五種防腐劑的高效液相層析法。本實驗以C18固相萃取匣淨化樣品，分別收集第一、第二及第三毫升沖提液以高效液相層析儀檢測結果，最適沖洗液為含10%甲醇之1%磷酸溶液，沖提液則以甲醇較佳。高效液相層析梯度流析法之平均添加回收率分別為對-羥基苯甲酸97%，苯甲酸96%，對-羥基苯甲酸乙酯95%，對-羥基苯甲酸丙酯95%及對-羥基苯甲酸丁酯95%。五種防腐劑於1.8-54 mg/kg均呈現良好線性關係，其相關係數（$R^2$）大於0.992。本方法可應用於醬油產品中防腐劑含量之例行檢測。

**關鍵詞**：固相萃取，苯甲酸，對-羥基苯甲酸酯，醬油