# Development of an HPLC Method For Determination of Some Preservatives in Soy Sauce

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***Abstract -- A simple, selective and precise reversed-phase high performance liquid chromatography (HPLC) method has been developed for the simultaneous quantification of benzoic acid (BA), methyl paraben (MP), and butyl paraben(BP) in soy sauce. The preservatives (BA, MP and BP) from soy sauce samples were extracted with a C18 bonded silica SPE cartridge. 10% methanol in 1% orthophosphoric acid solution followed by methanol was found to be the best solution for sample clean-up. The chromatographic separation of HPLC method was performed by using a PerkinElmer Brownlee Analytical DB C18 column (2.1 mm X 100 mm, 1.9 µm) and binary pump. The preservatives were eluted with methanol/NH4OAc buff-er and determined by a gradient elution system in one run (23 minutes) with UV detection at the maximum absorption wave-length, 230 nm for BA, and 254 nm for MP and BP. The average recoveries of benzoic acid, methyl paraben, and butyl paraben were 90, 93 and 100 %, respectively. The calibration curves showed good linearity over the concentration range of 1.0 - 60 µg/g. The regression coefficients were acceptable (R2 > 0.995). This SPE-HPLC method was proved to be a useful protocol for quantification of the preservative in soy sauce.***

***Indexed Terms: Soy sauce; preservatives; benzoic acid; parabens; solid phase extraction (SPE); HPLC-UV***

## I. INTRODUCTION

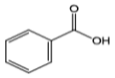
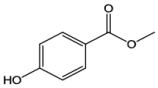
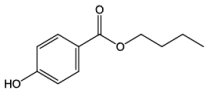
Preservative is any substance used in the food preservation process that can act as agent to prevent any alteration either of taste and appearance of the food due to microorganism and to minimize the risk of toxic food. According to directive 95/2/EC dated on 20.02.1995, "the preservatives are substances that increase the food preservation time by protecting them against the damages caused by micro-organisms"[1]. Among the available commercial food preservatives, benzoic acid (BA) and its ester derivatives are widely used. Benzoic acid is one of the weak organic acid preservative agents that inhibits the growth of both bacterial and fungal cell [2,3]. The parabens are also possible to be used as anti-microbial agents to exert the intended affects against mold and yeasts. The parabens are considered safe antimicrobial agents with relatively non-irritating and non-sensitizing effects as well as low toxicity to the human [4].

It has been previously reported that the concentration of benzoic acid and sodium benzoate used as a food preservative was ranging up to 2000 mg $kg^{-1}$ [3]. 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 (parabens) are controlled by regulation, and the quantitative analysis of these preservatives is important in routine analysis of foods. Fruit juice and beverage can be directly analyzed without clean-up procedures prior to determine by HPLC [5,6], while the other food samples, such as cheese, sauce, jam, milk, yogurt and canned seafood by HPLC technique, requires sample pretreatment, which usually involved solvent extraction or precipitation of proteins and fats by the addition of methanol or acetonitrile followed by centrifugation[6,7].

Sweet soy sauce is become one of the favorite condiments and very often used as food flavor enhancer in Asian cuisine. The commercial sweet soy sauce contains some amount of preservatives to be stored for a longer time. Sodium benzoate and methyl paraben, are the two very common preservatives added in the commercial soy sauce. The maximum allowable amount of benzoic acid and parabens added in soy sauce are 600 mg $kg^{-1}$ and 250 mg $kg^{-1}$, respectively [8]. In recent years, some analytical methods have been developed for the determination

of preservatives in food. The simple and accurate methods are very important to support consumer protection. The most common analytical method used in the analysis of benzoic acid and parabens are by using HPLC that equipped with UV or diode array detector [9-10].

Table 1. Physicochemical properties of some preservatives in soy sauce

| Name | Structure | Molecular formulae/ weight | Boiling point |
|---|---|---|---|
| Benzoic acid |  | $C_7H_6O_2$ (MW=122.13) | 250℃ |
| Methyl paraben |  | $C_8H_8O_3$ (MW=152.14) | 275℃ |
| n- Butyl paraben |  | $C_{11}H_{14}O_3$ (MW=194.22) | 309℃ |

Both benzoic acid and paraben are considered as polar compounds and also have relatively high boiling point. With regard to their properties as polar and low volatile compounds, therefore, the determination of benzoic acid and parabens can be suitably conducted by the HPLC method. Methods that have been developed to determine benzoic acid in foodstuffs include gas chromatography (GC)[7], high pressure liquid chromatography (HPLC) [8, 9]Practically, the most common sample preparation methods before analysis by the HPLC for water based condiment are direct dilution, extraction with organic solvent followed by the filtration, and extraction by using SPE (solid phase extraction) and clean-up process to minimize the matrix effect [11,12]. The SPE 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 foods followed by paired-ion liquid chromatography analysis was reported [13]. SPE method also has been used for the pretreatment of samples in the determination of the preservatives in food by gas chromatograph/mass spectrometer [14-15]. The analytical determination of these preservatives is not only important for quality assurance purposes but also for consumer interest and protection. The validation of the analysis method was attempted in terms of sensitivity, linearity range, reproducibility, repeatability, analytical recovery and robustness. The method has been developed according to the specifications of the SR EN1 2856/2001 standard [19], which, although developed for the determination of acesulfame-K, aspartame and saccharin, it also allows the determination of the benzoic acid and caffeine.

This study was aimed to develop simple and reliable method for simultaneous detection of the three preservatives (BA, MP and BP) in soy sauce by using reversed phase HPLC-UV technique. A solid phase extraction (SPE) procedure was developed in order to extract all the preservatives. Then the analytes were separated by a non-polar C18 column and identified by means of a multi-channel (UV) detector using $\lambda$ = 230 nm and $\lambda$ = 254 nm detection. Preservative amount was obtained by reference to a matrix-matched standard curve due to a relevant matrix effect. Accuracy (recovery), precision, detection, quantification, linearity range, and ruggedness of the method were evaluated.

## II. EXPERIMENTAL

### A. *Chemicals and Solvents*

Soy sauce sample and Certified Reference Materials(CRMs) were obtained from RCM-LIPI (Metrology in Chemistry, Research Center for Metrology, Indonesian Institute of Sciences). CRM as calibrants in this study were benzoic acid (99.99 ± 0.33 % purity, HRM-1002A ), methyl paraben (99.95 ± 0.32 % purity, HRM-1003A) and n-butyl paraben (99.92 ± 0.33 % purity, HRM-1004A) and they were purchased from the Health Science Authority (HSA) Singapore and used without any further purification. Orthophosphoric RPE acid (85%) for the eluent preparation of SPE was purchased from CARLO ERBA, methanol (HPLC grade) for mobile phase and used as solvent in the preparation of both standard solution and sample, and ammonium acetate ($C_2H_7NO_2$) were purchased from Merck, Germany. Solid phase extraction C18 was obtained from

PerkinElmer, USA. Deionized pure water (DI) was prepared by passing reverse osmosis system (Ultra Clear™), SIEMENS, Germany and used in all experimental runs for sample dilution and eluants preparation for SPE.

### *B. Standard Solution Preparation*

Stock solution of each standard compounds were separately prepared gravimetrically at level concentration of 1000 μg/g by dissolving BA, MP and BP in HPLC grade methanol. The solutions were stored in a brown glass bottle and kept at 4℃. The intermediate standard solution (IS) was prepared by mixing an appropriate amount of all target compounds of above standard solution and diluted gravimetrically by using methanol to produce final solution having concentration of 200 μg/g. From the IS, a series of working standard solutions containing different concentration level of the target compounds were prepared daily by gravimetrically. The working standard solutions were then utilized to establish the calibration curves. Correlation coefficients and linear equations of peak area and standard concentrations were used for the quantification methods.

### *C. Sampling by SPE Method*

This study was conducted by using a method adopted from Chu,T.Y., et. al., 2003 with a slight modification[8]. A certain amount (2 g) of soy sauce sample was accurately weighed in a centrifuge tube and about 8 mL of DI water was then immediately added into a sample contained centrifuge tube, and the final mass of the solution was recorded. After that, 1 mL of the diluted soy sauce sample, which known the mass, was passed through into the conditioned C18 SPE cartridge, and followed by washing the cartridge with 4 mL of 10% (v/v) methanol in 1% orthophosphoric acid solution. The target preservative analytes were then extracted by eluting from the cartridge with 3 mL of methanol, and the final mass was recorded. The extract solution was then filtered by using 0.22 mm syringe filters and directly injected to HPLC-UV detection.

### *D. Chromatographic Technique by HPLC-UV*

A PerkinElmer® Flexar™ FX-15 UHPLC system fitted with a Flexar FX UV/VIS detector served as a platform for this experiment. The separation was achieved by using a PerkinElmer Brownlee Analytical DB C18 column (2.1 mm X 100 mm, 1.9 μm), binary pump and data acquisition and data processing were performed by Chromera CDS 3.4.1 software. Sample data collection was optimized to 23 min per sample with UV detection at the maximum absorption wavelength $\lambda_{max}$, 230 nm for BA, and 254 nm for MP and BP. Mobile phase used was combination of methanol–ammonium acetate buffer. Chromatographic Conditions for Preservatives in soy sauce sample were conducted as shown in Table 2.

## III. RESULTS AND DISCUSSION

### *A. Development of SPE Method*

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 washing step[20]. In order to optimize the extraction, the SPE cartridge was conditioned with methanol followed by water. In order to remove unwanted substances in soy sauce samples, 10% (v/v) methanol in 1% orthophosphoric acid solution followed by MeOH was used in this study for washing.

### *B. Optimization of Chromatographic Method*

Composition of mobile phase is a key factor in resolution of chromatographic separation and the run time of analysis. Mobile phases containing acetate buffer are obviously recommended as the most suitable to assure the very good chromatographic separation of preservatives [10]. During our preliminary experiments, several different mobile phases were tested including methanol–acetate buffer (15:85); methanol–acetate buffer (35:65), methanol–acetate buffer (50:50), methanol–acetate buffer (60:40), and methanol–acetate buffer (80:20). The

results showed that in low percent of methanol (methanol–acetate buffer (35:65), the resolutions of BA was suitable; however, under this circumstance, BP was eluted so late. On the other hand, in high percent of methanol such as methanol–acetate buffer (50:50), the resolutions of MP and BP were no good. So, by evaluating these results, gradient elution program was followed to isolate three preservatives in a single run.

Finally, the optimized gradient elution program (as shown in Table 2) was applied in order to obtain good resolution of the peaks and also reasonable run time 23 min. Under optimum conditions, the retention time for BA (first peak), MP (second peak), and BP (third peak) is about 4.8, 12.9, and 20.7 min, respectively.The gradient elution was started with 85%B on 1 minute and gradually decreased to 20%B until 20minutes. The total run time was 23 minutes per sample. The preservatives were absorbed on the non-polar C18 column stationary phase. In this process, the more polar components in soy sauce were eluted preferentially as shown in Table 3. For this reason , the gradient elution was chosen as the chromatographic analysis condition . The column was equilibrated for one hour before sample injection . The temperature was set at 35℃ by using column oven and 5 µL of standard or sample solution was injected by cool-heat autosampler. Binary LC pumps with vacuum degasser were used to avoid air bubbles and the flow rate was 0.1 mL/min. After a complete series of analysis, the HPLC system was cleaned using an adequate washing method to attain the suitable conditions for the column keeping.

It is well known that the detection wavelength is one of the most important factors affecting the sensitivity of the method. The results showed that the optimized wavelength for BA was at 230 nm**,** while the optimized wavelength for MP and BP were found at 254 nm**,** respectively. The multi channel UV detector was set at 230 nm (0 – 7 min) for BA and 254 nm (7–23 min) for MP and BP absorption.The retention time and maximum absorption of the compounds for preservatives in soy sauce were found as shown in Table 3.

Table 2. Chromatographic conditions for preservatives in soy sauce

| | | |
|---|---|---|
| Pump Type | : | Flexar FX-15 (Binary pump) |
| Column | : | PerkinElmer Brownlee Analytical DB C18 column (2.1 mm X 100 mm, 1.9 µm) |
| Column (T) | : | 35℃ |
| Mobile Phase | : | A : methanol<br>B : 0.01 M Ammonium Acetate Buffer ($NH_4OAc$) |
| Flow Rate | : | 0.1 mL/min |
| Injection Vol; | : | 5 µL( Autosampler Cool-Heat) |
| Gradient Elution | : | Time(min) %A : %B<br>1 15 85<br>5 50 50<br>3 60 40<br>11 80 20<br>3 50 50 |
| UV Detector | : | Analytical Wavelength Program<br>230 nm ( 0 – 7 min)<br>254 nm ( 7 – 23 min) |
| End Time | : | 23 in |

*C. Quantitative Analysis*

*1)Peak Identification*

Peak identification of the preservatives in sample was based on the comparison between the retention times of standard compounds, and was confirmed by spiking the known standard compounds to the sample. Chromatogram of preservatives are shown in Figure 1.

*2) Validation of The Method*

To test linearity, multiple points external calibration technique was used in this study by five levels of concentration within the range 1.0 to 60µg/g. All analysis were performed in three replicates and the results proceed with Chromera software. The linearity range and the regression coefficients are listed in Table 3. To test peak area and retention time reproducibility,

Chromera CDS 3.4.1 software allows the calculation of the relative standard deviations (RSD) for the retention time of the analytes for all levels of the calibration graph and for peak area at each calibration level. It was found that the regression coefficients( $R^2$ – value) of all target compounds were greater than 0.995.The mass fraction of all three analytes were calculated based on the following equation.

$$X = \left(\frac{C \times M2 \times M4}{M1 \times M3}\right)\left(\frac{1}{Rec}\right)$$

Where,

X : Mass fraction of analyte (µg/g)
C : Measured concentration in HPLC (µg/g)
M1 : Mass of 2 mL of sample (g)
M2 : Mass of solution after addition of DI water (g)
M3 : Mass of 1 mL aliquot taken to be passed through the SPE cartridge (g)
M4 : Mass of methanol extract solution after clean up with SPE (g)
Rec : Recovery of the method

Table 3. Equations of calibration graphs (peak area vs concentration) and correlation coeofficients ($R^2$) for the three analytes

| Preserva-tives | $\lambda_{max}$ (nm) | Calibration equation | $R^2$ |
|---|---|---|---|
| BA | 230 | $y = 5.12 \times 10^4 X - 1.5 \times 10^3$ | 0.996 |
| MP | 254 | $y = 5.00 \times 10^4 X + 6.5 \times 10^3$ | 0.995 |
| BP | 254 | $y = 4.92 \times 10^4 X + 2.6 \times 10^4$ | 0.999 |

* Linearity range between 1.0 and 60µg/g (n=3)

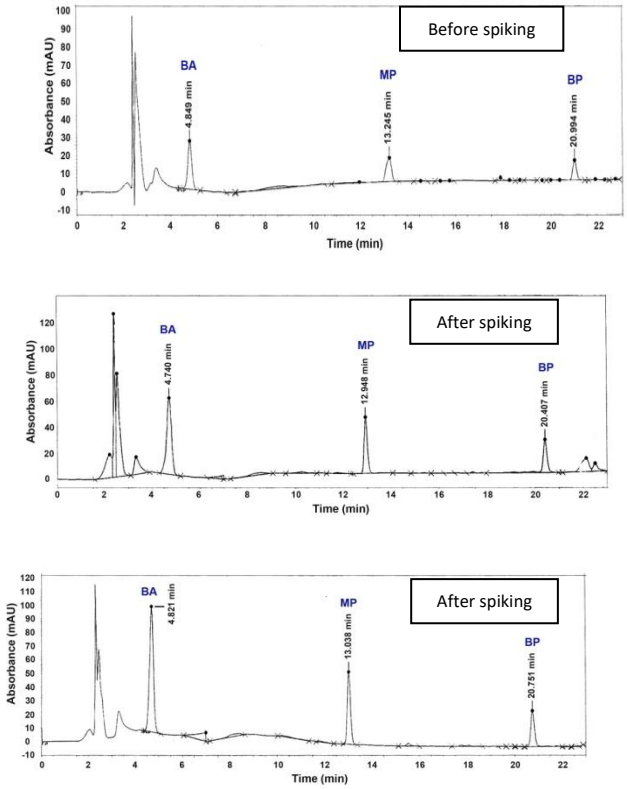



Figure 1. HPLC chromatograms of soy sauce sample before and after spiking by monitoring using multi-channel UV detector in λ = 230 nm and λ = 254 nm

*3) Recovery of the Method*

The recovery of the SPE method was studied by standard addition method. It was performed by adding a known concentration of the analytes to the sample pre-treatment method. Then it was calculated by the concentration of the analytes recovered in relation to that added as a spike sample (where value observed is divided by a reference value or an expected value). The results obtained from the average of three replications are presented in Table 4.According to Table 4, it can be concluded that the recovery study of the preservatives in the soy sauce was correct. Therefore, the proposed analytical method was sufficiently accurate for simultaneous determination of the three preservatives by the average recoveries of 90 – 100 % and relative standard deviation (RSD) of 4.28 – 5.01 %; indicating the accuracy of the method.

Table 4. The results of food preservatives' recovery study

| Preservatives | Expected value (μg/g) | Mean recovery (%) | RSD (%) |
|---|---|---|---|
| BA | 459.1 | 90.4 | 5.01 |
| MP | 108.7 | 93.1 | 4.81 |
| BP | 83.3 | 100.0 | 4.28 |

## IV. CONCLUSIONS

Nowadays, low-calorie sweeteners and preservatives are widely used in foodstuffs and soft drinks. Investigations on the toxicity of these compounds have raised questions as to whether they are safe to consume or not. As a result, their concentration in foods and beverages is regulated through legislation in order to prevent excessive intake. Therefore, the simultaneous determination of three preservatives in soy sauce were conducted by HPLC-UV detection at the maximum absorption wavelength, 230 nm for BA, and 254 nm for MP and BP. The SPE pre-treatment method showed acceptable recoveries of 90-100% and combination with the HPLC method was found to be suitable for the routine determination of these preservatives in food items. The development of proposed HPLC method for the simultaneous quantification of benzoic acid, methyl paraben and n-butyl paraben in soy sauce was found to be a selective, simple, precise, reproducible, sensitive and accurate.

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