Method Validation of Alkyl Parabens Content in Soy-sauce

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ABSTRACT

Alkyl p-hydroxybenzoates (alkyl parabens) is acidic food additive preservative prevents the microbial growth. Addition of alkyl parabens in food must meet the safety requirement from Codex. The objective of the study was to do method validation in analysis of the alkyl (methyl-, ethyl, propyl-, butyl-) parabens content in soy-sauce. The method used was applied High Performance Liquid Chromatography (HPLC) technique. Performance of the method was measured by several parameters. The R^2 value of linearity for all alkyl paraben was above 0.990. Limit of detection for methyl-, ethyl-, propyl-, and butyl paraben were 0.15, 0.13, 0.12 and 0.12 µg/ml, respectively. Limit of quantification for methyl-, ethyl-, propyl-, and butyl paraben were 5.00, 7.95, 8.17, and 8.30 ppm, respectively. Recovery of all alkyl parabens were above 87% and the coefficient of variation were below 11%. The method showed good specificity, accuracy, and precision. The method was valid based on those parameters and could be used as a routine laboratory method.

Keywords: *Preservatives, parabens, method validation, soy-sauce, HPLC*

INTRODUCTION

Parabens or *p*-hydroxybenzoic acids are group homologous series of hydroxybenzoic acid, differing in the ester group, including methyl-, ethyl-, propyl-, butyl-, heptyl-, and benzyl-group (Figure 1). It has been used singly or in combination as an antimicrobial preservative in food products, pharmaceutical, and cosmetics formulations. The parabens are stable over the

pH range and soluble in water. They are relatively safe to use (non-iritating, non-sensitizing, and low toxicity), not carcinogenic, not mutagenic, not teratogenic or embryotoxic and are negative in the uterotropic assay (Soni et al, 2005). Parabens also occur naturally in foods. Methyl paraben has been reported as a constituent of cloudberry, yellow passion fruit juice, white wine, botrytised wine, and Bourbon vanilla, and recently, propyl paraben has been detected in the aerial part of the plant Stocksia brahuica (Family: Sapindaceae) (Ali et al, 1998).

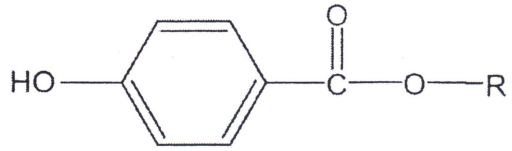

Figure 1. Chemical structure of parabens, where R = methyl (CH_3), ethyl (C_2H_5), propyl C_3H_7), butyl (C_4H_8)

Use of alkyl parabens as food additives must meet food safety requirement. The United State Food and Drug Administration (US FDA) has classified methyl and propyl paraben as Generally Recognized as Safe (GRAS) for direct addition to food. Heptyl paraben is allowed by the FDA for direct addition to fermented malt beverages in amounts maximum 12 ppm and in non-carbonated soft drinks and fruit in various foods. FDA has also approved methyl-, propyl- and butyl paraben as synthetic flavoring substances and adjuvants for addition to foods at minimum quantity to beverages. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) suggested the group Acceptable Daily Intake (ADI) for the methyl-, ethyl-, and propyl- esters of p-hydroxybenzoic acid as 0–10 mg/kg body weight/day (JECFA, 1974). Ishiwata et al. (1997) reported the highest detection rate of parabens in Japan was soy sauce with the average concentration was 34.2 mg/kg as p-hydroxybenzoic acid.

A laboratory analysis was needed in order to determine parabens content in food products. There were some methods for determination of parabens in cosmetics, food products and pharmaceuticals (Wang and Chang, 1998; Ali et al, 1999; Kreuz et al, 1999; Driouich et al, 2000; Lin Yu et al, 2000; Labat et al, 2000). The method analysis should be validated to assure validity of the result. Validation of the method detection is one of the most important

factors for assuring precise and accurate analyses (Žel et al, 2008). The objective of this research was to do the method validation of alkyl parabens in soy-sauce using High Performance Liquid Chromatography (HPLC) instrument. Method validation was done by evaluating a series of method performance characteristic in addition to precision and accuracy: analytical selectivity, analytical specificity, linear range, limit of detection, limit of quantification, and recovery.

MATERIALS AND METHODS

Raw Materials

One brand of commercial soy-sauce was used as food matrix and was purchased from market in Bogor, Indonesia. Chromatographic grade of acetonitrile and methanol were obtained from Merck (Germany). Analytical grade of potassium dihydrogen phosphate, acetonitrile, ethanol, and silica gel 60 pellet were obtained from Merck (Merck, Germany). The standards of methyl paraben, ethyl paraben, propyl paraben, and butyl paraben were obtained from Agency of Drug and Food Control-Republic of Indonesia, Jakarta.

Preparation of Sample and Standards

Soy-sauce sample was digested, homogenized, and weight as much as 2.5 g. Sample was extracted three times with 25 ml of ethanol (80%). Extract was diluted to 100 ml using ethanol (80%) and filtered. A 20 ml of filtrate was eluted through the solid phase extraction (SPE) column contain silica 60. Eluted sample then filtered using membrane filters 0.45 µm. Final filtrate was placed in a small vial for further analysis. The paraben standards were diluted in ethanol in several concentrations before injecting into the separation instrument.

HPLC Configuration

The method used in this research was modification from The United States Department of Agriculture (USDA) method using High Performance Liquid Chromatography (HPLC) technique. An Agilent model 1200 series HPLC with MWD detector set on UV wave-length of 254 nm (Agilent Technologies,

USA) was used for the analysis of paraben compounds. An Eclips Xdb C18 column (15 cm×4.6 mm×5 µm; Supelco, USA) was used in separation process. A degassed and filtered mixture of 0.05 M potassium phosphate, pH 6.80, and acetonitrile (65:35 v/v) was used as mobile phase. The flow rate was maintained at 1.0 ml/min with sample loop at 20 µl. All separations were carried out at ambient temperature.

RESULTS AND DISCUSSION

Specificity of Alkyl Parabens

The analytic specificity refers to the ability of a method to detect only the analyte of interest (Walton, 2001). Specificity of methyl-, ethyl-, propyl-, and butyl paraben standards were examined by the HPLC method and the responses observed. Injection was done firstly for each single standard, followed by four standards injection simultaneously. Figure 1 part A-D shows sample chromatograms obtained from the analysis of individual paraben standards. Every standard was gave single peak in each chromatogram. A chromatogram of simultaneous injection of four paraben standards was given in the part E of Figure 1.

It shows that each single standard was come up in different retention time with similar order with the individual standard injection. The methyl paraben was come up first, followed by ethyl-, propyl-, and butyl paraben.

As comparison data, the alkyl parabens standards were also added to the food samples (soy-sauce) and unspiked soy-sauce used as a control. Specificity of four parabens standards in unspiked and spiked soy-sauce was given in Figure 2. In Figure 2 part A clearly shows that there was no peak of the alkyl parabens standards detected in chromatogram for soy-sauce without spiking. Using the same method, the chromatogram of spiked parabens pointed the presence of the alkyl paraben standards with the same pattern to the simultaneous standards injection in Figure 1 (E). Based on those results, HPLC-MWD method used in this study (from extraction to clean-up steps) was specific for analysis of methyl-, ethyl-, propyl-, and butyl parabens.

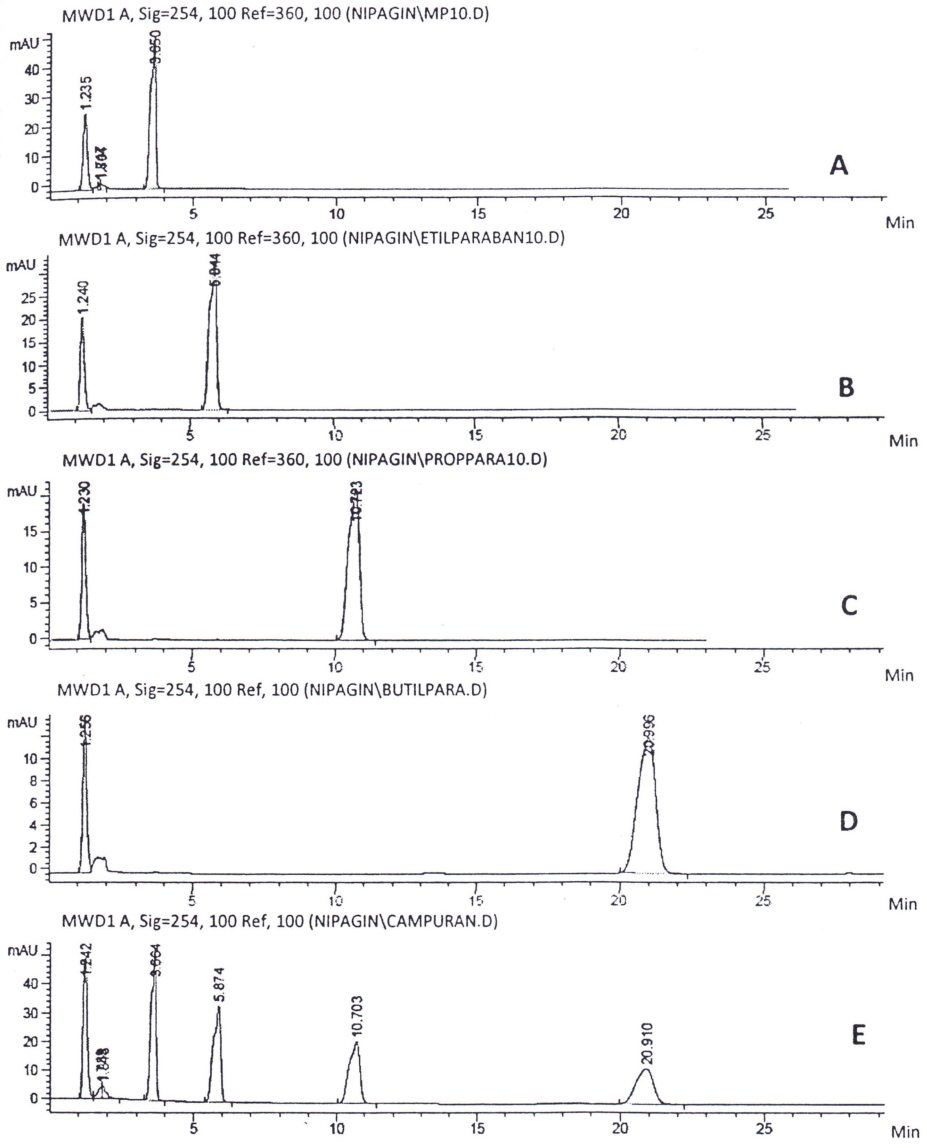

Figure 1. The chromatogram of HPLC-MWD for alkyl paraben standards (A-methyl paraben, B-ethyl paraben, C-propyl paraben, D-butyl paraben) detected in different retention time, so that could be analyze simultaneously as shown in part E

Linearity

The linearity or linear range of the method defines the effective working range of the test (Walton, 2001). This range was validated by evaluating a set of reference (standard) solutions or by creating a series of dilutions (a minimum 5 samples) that range from an analyte concentration approaching the lowest detection limit to one with a concentration nearing the expected upper limit of the working range (Lumsden, 2000).

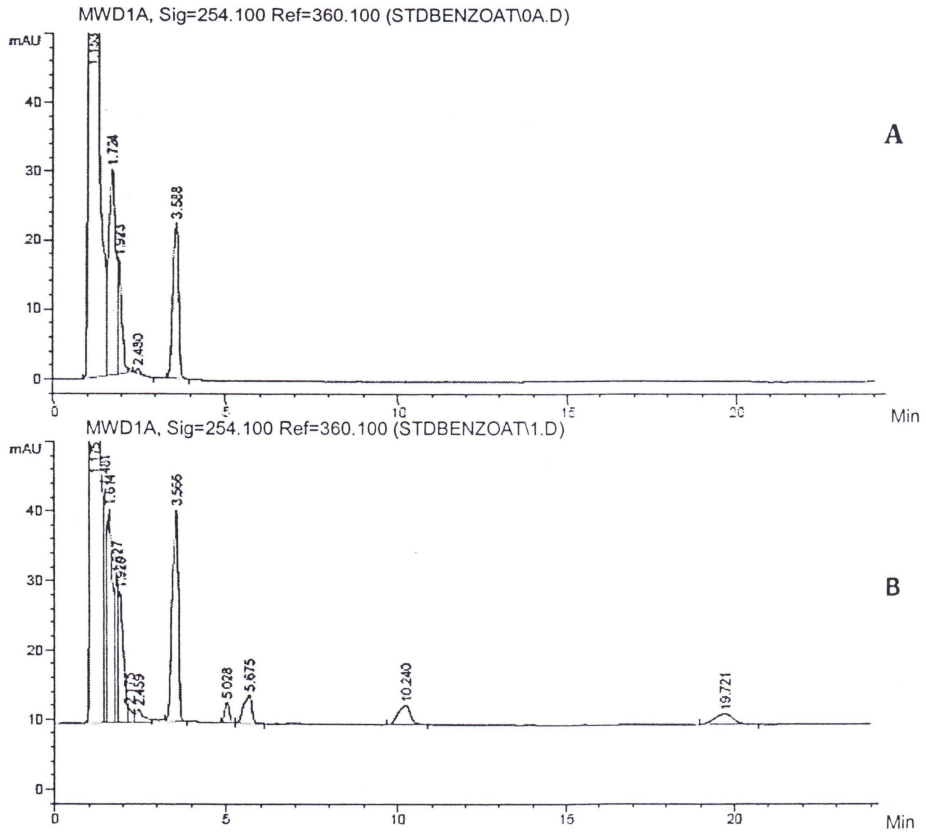

Figure 2. The chromatogram of HPLC-MWD of sample (sweet soy-sauce) without spike (A); and the chromatogram HPLC-MWD alkyl parabens standards (methyl-, ethyl-, propyl-, and butyl paraben) spiked to the samples (B). Alkyl parabens were detected as separate individual peaks

Individual test standards containing 50, 100, 250, 500, and 750 µg of each of the paraben standards was added to the 2.5 g of sample and were

examined by the HPLC method and the responses measured. Figure 3 shows the chromatograms obtained from the response of HPLC-MWD instrument.

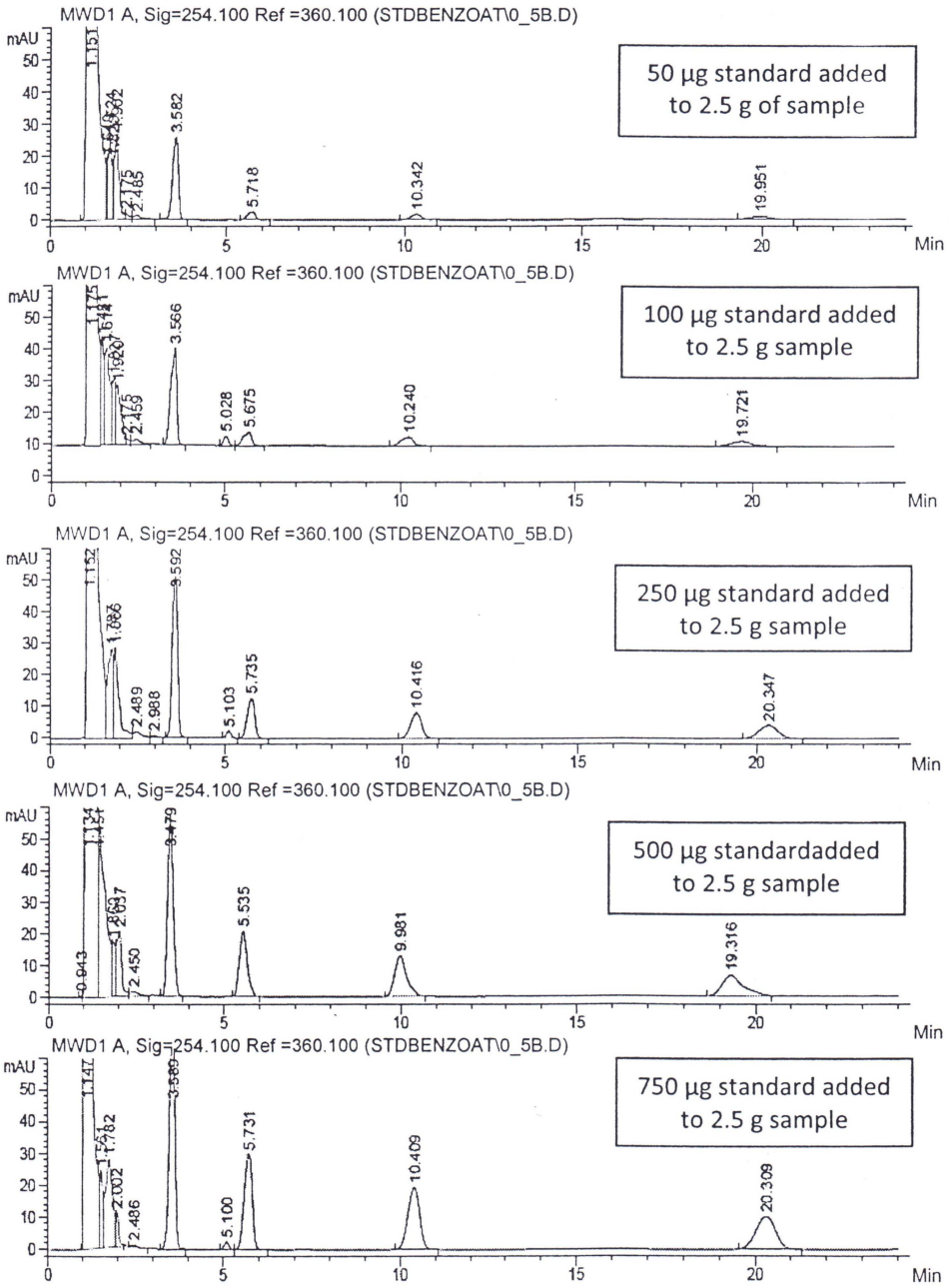

Figure 3. HPLC-MWD response for analysis of parabens standards added to the samples in various concentrations

The four parabens standards added to the sample were in proportion to concentration added. In each case, a linear relationship between peak height and concentration was observed. Higher the spiked parabens standards concentration will make the peak area in chromatogram higher.

The analytical range (linear range) of methyl-, ethyl-, propyl-, and ethyl paraben were evaluated during this study. Regression analysis of data for each component gave the value for slope, intercept and correlation coefficient for each calibration curve. The linear range of the alkyl parabens were obtained in range of 50-755 µg/2.5 g or 20-300 µg/g sample. The regression curves of standard parabens linearity were shown in Figure 4. Each figure showed the R^2 value more than 0.990. It means that the method has good linearity. These curves were also used as standard curve or calibration curve for MDL (method detection limit) and recovery.

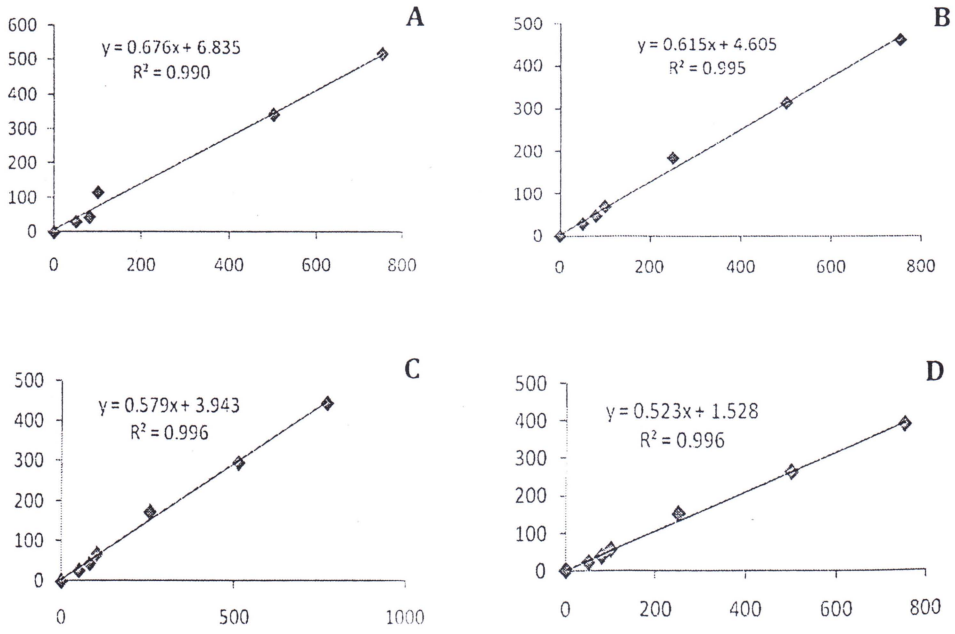

Figure 4. Linearity of paraben standards: A-methyl paraben, B-ethyl paraben, C-propyl paraben, D-butyl paraben. X-axis: µg standard/ 2.5 g sample; Y-axis: absorbance unit

Instrument Limit of Detection ($LoD_{instrument}$)

The limit of detection (LoD) is defined by The International Union of Pure and Applied Chemistry (IUPAC) as the smallest quantity or the lowest concentration of analyte in a sample that can be detected with reasonable certainty. This test would be necessary for trace analysis. The limit of detection and analytical selectivity are related, but are not synonymous. Sensitivity is related to precision and refers both to a test's ability to detect small quantities of the analyte and to detect small differences between samples (Walton, 2001). A 'sensitive' methodology has a high level of analytical sensitivity and a low limit of detection.

$LoD_{instrument}$ of alkyl parabens were determined by performing seven injection of 0.8 µg/ml methyl-, ethyl-, propyl-, and butyl paraben standards solution, respectively, to the HPLC-MWD with same condition. The standard deviation was calculated and LoD was defined as 3 times of the standard deviation. LOD illustrates the limit detection of HPLC-MWD in detection of each alkyl paraben. $LoD_{instrument}$ of methyl-, ethyl-, propyl-, and butyl paraben was 0.15, 0.13, 0.12, and 0.12 µg/mL, respectively.

Method Detection Limit (MDL) or Limit of Quantification (LoQ)

Method detection limit (MDL) is based on a sample, which have gone through the entire sample preparation scheme prior to analysis. This test is performed based on different types of sample matrices. It takes into account the LoD, the performance of the operator, the performance of the method itself and the effect of the matrix on the analyte of interest. The 'limit of quantitation' (LoQ) is strictly the lowest concentration of analyte that can be determined with an acceptable level of repeatability precision and trueness (Eurachem, 2000).

Determination of LoQ was similar with the procedure for LoD determination. The analysis used paraben standards solution added to the sample in concentration of 20 µg/g or 20 ppm. The standard deviation was calculated and LoQ was defined as 5 times of the standard deviation. There were the lowest concentrations of paraben that can be reported. The LoQ for methyl-, ethyl-, propyl-, and butyl paraben was 5.00, 7.95, 8.17, and 8.30 ppm, respectively. The value below the LoQ is reported as undetected. From the

result indicates that the method could be used to detect the presence of parabens in very low concentration.

Accuracy and Precision

Accuracy is defined as the closeness of the agreement between the measured value of an analyte and its true value (Walton, 2001). Method accuracy can be determined in three ways: by comparing the performance of the candidate method with that of a definitive or reference method (gold standard), by performing a recovery experiment, or by comparing the candidate method with the established method that is being replaced (Koch and Peters, 1999). The determination of accuracy in this study was done by recovery experiment. This experiment estimates the ability of an analytical method to correctly measure an analyte when a known amount of the analyte is added to sample.

Precision is defined as the reproducibility of result obtained by testing identical sampel (Walton, 2001). Imprecision reflects the amount of variation inherent in the method and may be both positive and negative. Imprecision is quantitated by calculating the mean, standard deviation (SD), and coefficient of variation (CV) of data collected from an analytical run. The CV calculated as the SD multiplied by 100 and divided by mean value.

Recovery analysis was done in two different concentrations; represent low and high concentration of parabens in sample. Concentration used to represent low concentration was 200 µg/g and to represent high concentration was 1000 µg/g. Each concentration was injected seven times to the HPLC method. Recovery and coefficient of variation (CV) of alkyl parabens analysis were listed in Table 1 as follow.

The recovery of simultaneous alkyl parabens was 87-99.8% and the CV was 4.5-10.7%. The result showed good accuracy and precision as revealed by the percentage recovery and CV. Acceptable recovery based on AOAC standard is 80-110%, and acceptable CV is less than 15%. The results indicate that the method was accurate and covered all range of acceptable values.

Table 1. Simultaneous determination of alkyl parabens by the HPLC method

Standard	Spike 200 µg/g		Spike 1000 µg/g	
	Recovery (%)	Coefficient of variation (%)	Recovery (%)	Coefficient of variation (%)
Methyl paraben	99.8	10.7	88.2	5.9
Ethyl paraben	94.5	5.8	87.3	5.0
Propyl paraben	97.3	5.9	89.8	4.5
Butyl paraben	95.7	6.3	87.1	4.5
AOAC	80-110	< 15	80-110	< 15

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