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Validation and Uncertainty Evaluation of an LC-DAD Method for Simultaneous Quantification of Benzoic Acid, Methylparaben, and N-Butylparaben in Soy Sauce

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Abstract

Chemical food preservation is the common strategy used by human to preserve the natural properties and to increase the shelf life of food. Although preservatives are useful to keep the food fresh and to stop the bacterial growth, there are certain preservatives that are harmful if taken in more than the prescribed limits. Some of the typical used—benzoic acid, methylparaben, and n-butylparaben—were employed in this work with the aim of establishing a simultaneous liquid chromatography (LC) method for detecting each in soy sauce matrices. Liquid and C18 solid phase extraction were performed in this procedure prior to LC using Diode Array Detector analysis. In gradient elution of a format buffer (pH 4.4) and acidified acetonitrile, the target components were successfully separated. Calibration curve ranged from 0.61–140 mg/kg linearly while the limit of quantification for benzoic acid, methylparaben, and n-butylparaben were 0.41, 0.10, and 0.11 mg/kg, respectively. The intermediate precision and recovery were in the range between 0.15-1.89% and 100.5-103.3%, respectively. The expanded uncertainty (k=2) in sample measurement was estimated at 3.4-6.5%. The offered method was conformed to the validation acceptance criteria and can be applied as a routine method in the laboratory at ppm level.

Keywords: *liquid chromatography; preservatives; food analysis; analytical method validation*

Introduction

The existence of food additives in food supply for humankind cannot be disputed in life. The U.S. FDA defines a food additive as "any substance the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of any food". This definition includes any substance used in the production, processing, treatment, packaging, transportation, or storage of food[1]. Preservatives, as one of food additives, offer features to maintain food from damaged or spoiled, and thus, prolong the food's shelf life. In 2011, FAO has reported that only two-thirds

of food manufactured worldwide is consumed by humans, which makes one third is wasted globally, about 1.3 billion tons per year[2]. This fact supports why food additives and preservative industries still exist until now.

Organic acids and their derivatives are often used as preservatives in food sector. Their properties in reducing the pH and retaining the water content of food products cause unfavorable conditions for microbial to grow[3],[4]. In addition, they can act as antioxidants and sequestrants to avoid unwanted chemical reactions from lipids[3]. Benzoic acid and parabens are two acidic compounds commonly used in food and beverages industries.

Benzoic acid is widely used for many foods with production capacity is estimated reaching 600,000 tonnes per year[5]. Benzoic acid has pK 4.20 and belongs to antimicrobial preservative[6]. It can maintain food appearance by disturbing microbial cells. The undissociated organic acids can easily penetrate the microbial cells and immediately dissociates. This intracellular dissociation will acidify the cytoplasm and are extruded to the bacteria's cell structure[7]–[9]. Foods with a pH lower than 4.5 or naturally acidic food are the most suitable for the addition of benzoic acid[5],[6]. Naturally, benzoic acid can be found at concentrations up to about 40 mg/kg in many plants and fermented/cultured dairy products such as cranberries, grapes, strawberries, apples, cinnamon, honey, yogurts, cheese, and teas[5],[10].

The use of parabens (p-hydroxybenzoic esters) as antibacterial and antifungal agents in pharmaceuticals, cosmetics, and foods is started after the success finding in 1924[11]. Parabens are most active to stop the growth of molds and yeasts and slightly less active against bacteria[12]. The antimicrobial activity of the parabens tends to increase with increasing of the alkyl chain length. But for effectiveness in the application, shorter esters are preferred because of their high solubility in water[13],[14]. Methylparaben, propylparaben, and n-butylparaben are the most used parabens. In comparison to other parabens, methylparaben is the least active of the parabens and n-butylparaben is the best antifungal agent[12],[15]. Parabens are frequently applied in combination to improve the antimicrobial activity since they have synergistic effects[15].

The exposure of benzoic acid at a high concentration could affect the central nervous system, kidney, liver, and weight gain (for certain cases without controlling food intake)[5]. European Union (EU) lists methylparaben, ethylparaben, propylparaben, and n-butylparaben as potential endocrine disruptors. Considering the toxic potential of benzoic acid and parabens, the use of them as preservatives is limited and regulated. Commission Regulation (EU) No 1129/2011 of 11 November 2011 on food additives, amending the (EC) No 1333/2008, permits benzoic acid, methylparaben (E 218) and ethylparaben (E 214) in various food such as snacks, food enzyme, jelly coating, sweeteners, condiments, and confectionary, but not propylparaben and n-butylparaben[16],[17]. FDA through Generally Recognized as Safe (GRAS) states methylparaben and propylparaben is allowed to be used in food ingredients up to 0.1%[18].

The usage of preservatives is still a challenge in sauce, dressings, and condiments sector to meet the today's consumer expectation, that the products feature long lifetime, delicious taste, but keep healthy and free of artificial preservatives at the same time. Soy sauce is a flavorful condiment and one of the main components in Asian cuisine. First produced in China 2000 years ago, soy sauce is made from soybeans with fermentation process or chemical process. Soy sauce can be varied from a salty light-liquid to a sweet thick-liquid[19].

As a consequence of the preservative regulation and the presence of preservatives in condiments, the analytical method to accurately detect preservatives in soy sauce is particularly important to support quality assurance and consumer safety[20].

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Liquid chromatography (LC) with diode array detector (DAD) is a typical method to determine benzoic acid and parabens due to their UV characteristic. Many works have been published to show the successful analysis in various foods and pharmaceutical products[20]–[24]. In this study, the analytical procedure for simultaneous determination of benzoic acid, methylparaben, and n-butylparaben in soy sauce is developed by using LC-DAD. The report describes its method validation result and uncertainty evaluation to show the reliability and analytical performance of the quantification.

Experimental

Materials

Ultrapure water (18 Megaohm) was produced by a Milli-Q Plus 185 by Millipore (MA, United States). LC grade acetonitrile, methanol, and formic acid were supplied by Merck (NJ, United States). Ammonium formate was supplied by Sigma Aldrich (MA, United States). A solid phase extraction (SPE) with C18 was used in this experiment for clean-up process and purchased from Agilent, Bond Elut (PA, United States).

Analytical standards

Pure Certified Reference Material (CRM) of benzoic acid (BA), methylparaben (MP), and n-butylparaben (BP) were purchased from HSA (Singapore) with declared purities 99.9%, 99.5%, and 99.2% respectively. These solid compounds were dissolved in methanol to produce 5000 mg/kg stock solutions for each compound. Further, standard solution 75 and 150 mg/kg were prepared by diluting stock solution with methanol. As calibration, various standard mix solutions were prepared by diluting single standard solution of BA, MP, and BP in methanol to have approximately 1, 2, 5, 10, 25, 60, 90, and 120 mg/kg concentrations. Matrix CRM purchased from HSA Singapore and HRM-1005A Preservatives in Soy Sauce were employed for accuracy and recovery study.

Instruments

For simultaneous determination of BA, MP, and BP, a liquid chromatography (LC) method was developed for their separation and detection. LC was performed using Agilent 1200 system (United States) coupled to diode array detector. The chromatographic separation was carried out using an Agilent (PA, United States) Poroshell 120 EC-C18 2.7 µm (3.0 x 100 mm) column, held at 30 °C. Elution A consisted of a 10 mM formate buffer (pH 4.4) while elution B was acetonitrile containing 0.005% formic acid. The linear gradient elution program was as follows: 0-5 min, 80-50% A; 5-7 min 50% A; 7-8 min, 50%-20% A; 8-10 min, 20% A; 10-10.2 min, 20%-80% A; 10.2-17 min, held at 80% A to give re-equilibration. The elution flow rate was set at 600 µL/min and 5 µL sample solution was injected into LC instrument. DAD performance as detector for quantification was obtained at 230 nm for BA while for both BP and MP were at 254 nm.

Sample preparation

This sample preparation is adopted from Chu et. al.[25] with minor modification. Two grams soy sauce sample was diluted 5 fold with water in centrifuge tube. Mixture was vortexed for 1 minute followed by 10 minute centrifugation at 2500 rpm. A weighted 1 mL of the aliquot was taken and then passed through to the conditioned SPE C18 cartridge. SPE was conditioned using 4 mL of methanol and 3 mL of water. After placing 1 mL of sample, clean-up process was followed immediately by flowing 4 mL of 10% methanol solution (in phosphoric acid 1%) and the analyte was then eluted with 3 mL of methanol. The collected methanol phase was filtered by using 0.45 µm PTFE syringe filter and injected into LC-DAD instrument.

Validation method

Linearity for all compounds was obtained by plotting the peak area against the concentration of the corresponding calibration standards (in pure solvent) at nine calibration levels ranging between 1 to 150 mg/kg.

Limit of quantification (LOQ) was estimated by performing serial dilution method of standard mix solution from the lowest calibration standard with signal to noise (S/N) of 10 and observed in 7 times experiment. Precision was evaluated in two levels: (a) repeatability: in the same day analysis and (b) intermediate precision: in different days analysis, at three-level concentrations (1, 15, and 100 mg/kg).

Accuracy/recovery studies were carried out by evaluating CRM measurement (HRM-1005A) with five replicates on three different days. The recovery is defined by the mass fraction comparison (in percentage) between the experimental results and the CRM value from certificate.

The developed method in this study was applied to measure the concentration of BA, MP, and BP in soy sauce sample. The mass fraction of analyte (C_{sample}) in sample was determined from the validated LC-DAD analysis, as follow (Eq.1):

$$C_{sample} = \frac{C_{LC} \times W_{LC} \times W_{centrifuge}}{W_{SPE} \times W_{sample}} \times \frac{Rec}{100} \quad \text{(Eq.1)}$$

where C_{sample} is the mass fraction of the preservative analyte (mg/kg); C_{LC} corresponds to the concentration of analyte from calibration curve in the LC system (mg/kg); W_{LC} corresponds to the mass of final methanol solution after clean-up with SPE (g); $W_{centrifuge}$ corresponds to the mass of sample solution in water (extraction) (g); W_{SPE} corresponds to the mass of 1 ml aliquot of sample solution passed through into the SPE cartridge (g); W_{sample} corresponds to the mass of 2 mL of soy sauce sample (g); and Rec corresponds to the value from recovery study.

Estimation of measurement uncertainty

A measurement uncertainty was derived from the definition of a measurement model and calculated according to the GUM[26]. Sources of uncertainty that could possibly contribute to measurement results are considered, including those arising from balances, studies of precision, certified reference material, standard solution, linear calibration curve, and data on the performance of the analytical process, as described in Figure 1. The results for each analyte were expressed as the mean and its expanded uncertainty at the 95% confidence level.

Results and Disscusion

Chromatographic separation

Many published studies employed C18 column chromatography for benzoic acid determination in various matrices such as fruit, vegetables, and derived beverages[27], products based on cocaine[28], liquid pharmaceutical[20], and hard and pasta-filata cheese[29].

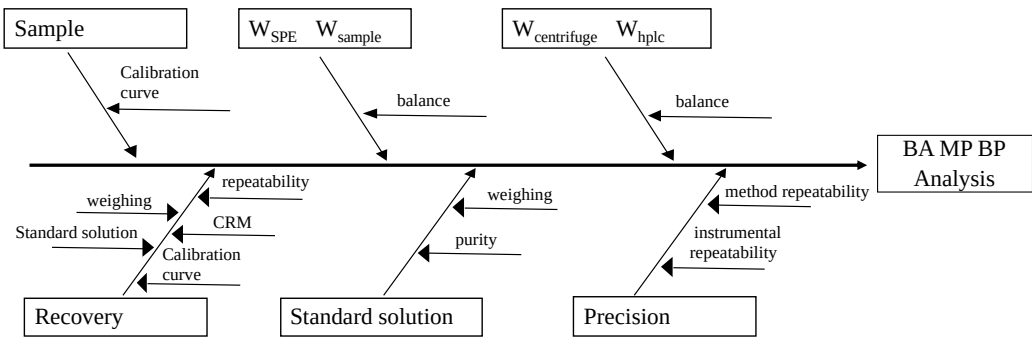

Figure 1. The cause-effect diagram for BA, MP and BP measurement by LC-DAD.

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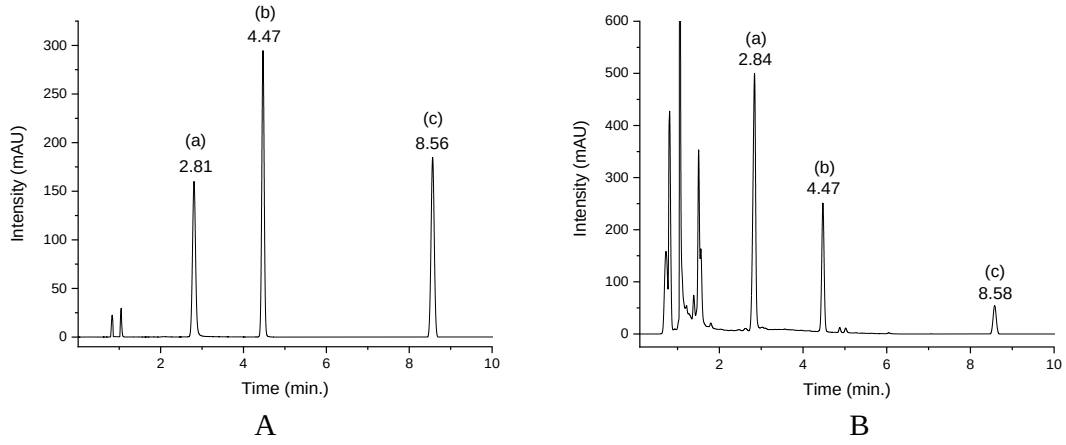

Gambar 2. Chromatogram of benzoic acid (a), methylparaben (b), and n-butylparaben (c) in pure CRM (A) and matrix CRM (B).

All these studies showed good result with satisfactory quantification. This study also occupied the C18 column to separate and quantify benzoic acid (BA), methylparaben (MP), and n-butylparaben (BP) in soy sauce. Figure 2 shows the chromatogram of the analytes in standard solution from pure CRM and matrix CRM, at optimized condition. The peaks of the target analytes were appeared with the absence of peak tailing and clear baseline separation.

The gradient elution was selected as chromatographic separation due to the difference of solubility in water of three analytes. BP is known having lower solubility in water because of longer alkyl chain. By initiating the higher water content (formate buffer) in early elution and then followed by the increment of acidified organic solvent, the separation of preservatives showed good performance where more polar compounds were eluted earlier. In this study, BA, MP, and BP were found at retention time of 2.8 minutes, 4.5 minutes, and 8.5 minutes, respectively. This gradient system also was proved by comparing with the isocratic elution, eluent arranged by 70% format buffer pH 4.4 and 30% acidified methanol, which showed split peaks and no single BP peak appeared in the chromatogram until time analysis reached to 15 minutes (data not shown).

Method validation

The results of linearity study are shown in Table 1. The values obtained in the linearity study, using calibration data, denote that the model is adequate, by showing the Pearson coefficient of determination (R^2) of the curves were greater than 0.90 as a proof of a fit of the data to the regression line. The linearity was observed at range 0.60–140 mg/kg for all analytes.

The instrumental LOQ was found to be in a good response that complies with the defined LOQ requirement, as shown in Table 1. In 7 replicate measurements, BA showed higher LOQ than parabens, at 0.409 mg/kg because of its lower response factor.

Precision was evaluated in term of system precision (intra-day repeatability) and intermediate precision (inter-day repeatability) by performing eight consecutive injections (n=8) of a standard mixture solution containing BA, MP, and BP in three level concentrations (1, 15, and 100 mg/kg). The %RSD of peak area response was calculated for both intra-day and inter-day repeatability, as shown in Table 2. The measurement was found to be precise with %RSD values ranging within 0.15-1.89%, where acceptable values for repeatability based on AOAC Guideline[30] at 1 mg/kg level is 8%.

To further evaluate the validation of SPE-LC-DAD method, recovery study was conducted by analyzing CRM from HSA Singapore, HRM-1005A, which possess assigned value for BA, MP, BP in soy sauce at 871.1 ± 21.6 mg/kg, 237.6 ± 13.5 mg/kg, 93.7 ± 6.0 mg/kg, respectively, using the validated method. It was found that the recovery (described at Table 3) for all analytes agrees to AOAC Guideline[30] which note recovery limit for concentration at 1000 mg/kg is 90-108% whereas 100 and 200 mg/kg limit are at 85-110% range. To confirm statistically whether the recovery is significantly different from 1, a student's t test is applied in this study[31]. The test statistic t is calculated using the following equation:

$$t = \frac{|1\text{-Rec}|}{u_{Rec}}$$

where *Rec* is the recovery value (ratio) between the mean observed value and the certified value from certificate and u_{Rec} is the measurement uncertainty from *Rec*, associated with bias estimate. This *t* value is compared with the 2-tailed critical value t_{crit}, for n–1 degrees of freedom at 95% confidence, where n denotes the number of experiments used to estimate *Rec*.

Table 1. Linearity evaluation and estimated limits of quantification of instrument for targeted analytes

Analyte	Linearity range (mg/kg)	R^2	Limit of quantification	
			(mg/kg)	(%RSD)
Benzoic Acid	0.65 – 147.5	0.9999	0.409	3.63
Methylparaben	0.61 – 142.4	0.9999	0.101	1.40
Butylparaben	0.61 – 144.6	0.9999	0.114	4.61

Table 2. Relative standard deviation of peak areas for benzoic acid, methylparaben, and n-butylparaben obtained in the analysis of repeatability and intermediate precision

Concentration (mg/kg)	%RSD								
	Benzoic Acid			Methylparaben			n-Butylparaben		
	Day 1	Day 2	Intermediate Precision	Day 1	Day 2	Intermediate Precision	Day 1	Day 2	Intermediate Precision
1	1.89	0.77	2.60	0.15	0.34	0.48	1.55	0.64	1.16
5	0.27	0.16	0.21	0.36	0.31	0.34	0.34	0.23	0.42
100	0.32	1.35	1.09	0.28	0.64	0.69	0.33	0.70	0.83

Table 3. Evaluation of recovery

Analyte	BA	MP	BP
Certified value ± U (mg/kg)	871.1 ± 21.6	237.6 ± 13.5	93.7 ± 6.0
Mean observed value (mg/kg)	880.4	245.5	94.2
n	15	15	15
Mean Rec (%)	101.1	103.3	100.5
u_{Rec}	0.013	0.033	0.033
t	0.846	1.000	0.152
t_{crit}	2.144	2.144	2.144

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If t is greater or equal than t_{crit}, *Rec* is significantly different from 1 and could show the evidence of systematic errors. Although *Rec* is found to be not significantly different from 1 in this study, *Rec* is still included in the following analysis, as a correction factor for the calculation of sample results. The recovery study in this case was aimed to check the possibility of systematic errors in the developed method and the method will be avoided to use if the recovery is significantly different from 1, not only corrected.

Uncertainty of measurement

In this study, the uncertainty of measurement for each analyte was estimated based on bottom-up approach. The estimation of uncertainty was focused on those supplying the significant contributions to the result. All sources of uncertainty were then combined according to the law of propagation of uncertainties, giving the combined standard uncertainty ($u_{Csample}$). The final result was reported as expanded uncertainty ($U_{Csample}$), by multiplying the combined standard uncertainty ($u_{Csample}$) by a coverage factor, k=2, which gives a level of confidence of approximately 95%.

The combined uncertainty is calculated as follow (Eq.2):

where C_{sample} and $u_{Csample}$ are the mass fraction of the preservative analyte and its uncertainty; C_{LC} and u_{CLC} are the concentration of analyte from calibration curve in the LC system and its uncertainty; W_{LC} and u_{WLC} are the mass of final methanol solution after clean up with SPE and its uncertainty; $W_{centrifuge}$ and $u_{Wcentrifuge}$ are the mass of sample solution in water (extraction) and its uncertainty; W_{SPE} and u_{WSPE} are the mass of 1 ml aliquot of sample solution passed through into the SPE cartridge and its uncertainty; W_{sample} and $u_{Wsample}$ are the mass of 2 mL of soy sauce sample and its uncertainty; Rec and u_{Rec} are the value from recovery study and its uncertainty; Rep is the repeability of measurement; and Std and u_{std} are the middle standard solution of calibration curve and its uncertainty. Table 4, 5, and 6 show the contributions of the individual uncertainty components for BA, MP, and BP, respectively.

It is clear from Table 4, 5, and 6 that Rec contributes the highest value of the overall uncertainty for all analytes. Sample measurement from calibration curve (C_{LC}) is the second major sources to the uncertainty for BA while Rep is found as the second major sources in MP and BP measurements.

$$u_{Csample}=C_{sample}\sqrt{\left(\frac{u_{CLC}}{C_{LC}}\right)^2+\left(\frac{u_{WLC}}{W_{LC}}\right)^2+\left(\frac{u_{Wcentrifuge}}{W_{centrifuge}}\right)^2+\left(\frac{u_{WSPE}}{W_{SPE}}\right)^2+\left(\frac{u_{Wsample}}{W_{sample}}\right)^2+\left(\frac{u_{Rec}}{Rec}\right)^2+Rep^2+\left(\frac{u_{std}}{Std}\right)^2} \quad \text{(Eq.2)}$$

Table 4. Uncertainty budget of benzoic acid determination by using LC-DAD

Sources of Uncertainty	Value (xi)	Unit	$u_{(xi)}$	$u_{(xi)}$/xi
C_{LC}	16.81	mg/kg	1.7×10^{-1}	1.0×10^{-2}
W_{LC}	2.38	g	7.1×10^{-6}	2.9×10^{-7}
$W_{centrifuge}$	10.44	g	1.4×10^{-4}	1.4×10^{-5}
W_{SPE}	1.02	g	7.1×10^{-6}	7.0×10^{-6}
W_{sample}	2.40	g	1.4×10^{-4}	5.9×10^{-5}
Rec	101.07	%	1.31	1.3×10^{-2}
Rep	1		3.7×10^{-3}	3.7×10^{-3}
Std	10.93	mg/kg	2.0×10^{-2}	1.8×10^{-3}

Table 5. Uncertainty budget of methylparaben determination by using LC-DAD

Sources of Uncertainty	Value (xi)	Unit	$u_{(xi)}$	$u_{(xi)}$/xi
C_{LC}	10.63	mg/kg	1.7×10^{-2}	1.6×10^{-3}
W_{LC}	2.38	g	7.1×10^{-6}	2.9×10^{-7}
$W_{centrifuge}$	10.44	g	1.4×10^{-4}	1.4×10^{-5}
W_{SPE}	1.02	g	7.1×10^{-6}	7.0×10^{-6}
W_{sample}	2.40	g	1.4×10^{-4}	5.9×10^{-5}
Rec	103.34	%	3.28	3.2×10^{-2}
Rep	1		7.3×10^{-3}	7.3×10^{-3}
Std	4.91	mg/kg	9.0×10^{-3}	1.8×10^{-3}

Table 6. Uncertainty budget of n-butylparaben determination by using LC-DAD

Sources of Uncertainty	Value (xi)	Unit	$u_{(xi)}$	$u_{(xi)}$/xi
C_{LC}	10.46	mg/kg	2.9×10^{-2}	2.7×10^{-3}
W_{LC}	2.38	g	7.1×10^{-6}	2.9×10^{-7}
$W_{centrifuge}$	10.44	g	1.4×10^{-4}	1.4×10^{-5}
W_{SPE}	1.02	g	7.1×10^{-6}	7.0×10^{-6}
W_{sample}	2.40	g	1.4×10^{-4}	5.9×10^{-5}
Rec	100.53	%	3.28	3.2×10^{-2}
Rep	1		6.9×10^{-3}	6.9×10^{-3}
Std	10.93	mg/kg	2.0×10^{-2}	1.9×10^{-3}

Table 7. Mass fraction and the expanded uncertainty of benzoic acid (BA), methylparaben (MP), and n-butylparaben (BP) in sample determination

		BA	MP	BP
		mg/kg	mg/kg	mg/kg
Sample mass fraction	C_{sample}	162.8	101.1	99.8
Combined uncertainty	$u_{Csample}$	2.8	3.3	3.3
Expanded uncertainty	$U_{Csample}$	5.6	6.6	6.7

For all analytes, the same value of uncertainty relating to the amount of weighed sample and weighing in preparation was applied because the quantity of them did not change significantly in the experiments as simultaneous analysis. This contribution could be considered negligible. However, great attention should still be paid when preparing standard solution and conducting measurements, with weighing is the basic process to Std and C_{LC} contributions, the other significant sources of uncertainty.

The contributions of uncertainty proportional to analyte concentration were combined by calculating the root sum of squares as given by Equation (2). As recommended in GUM[26], a level of confidence of about 95 % was obtained by multiplying of the combined uncertainty with a coverage factor of k = 2. The mass fraction of determined soy sauce sample and the individual uncertainties are presented in Table 7. Our results showed the expanded uncertainty of sample measurement ranging from 3.4% to about 6.5% for each analyte, which is smaller than the expected relative standard

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deviation (RSD) for food sector application, calculated by the Horwitz equation[32],[33]. In other words, the established LC method with DAD for determination of preservatives in soy sauce does fit-for-purpose.

Conclusions

An analytical method for simultaneous determination of benzoic acid, methylparaben, and n-butylparaben in soy sauce matrice was well developed by using LC-DAD method on a C18 column. Using the proposed separation method, benzoic acid, methylparaben, and n-butylparaben were determined within 17 minutes with a simple, low-cost clean-up procedure and a high-throughput method.

The method was also completely validated, including an evaluation of measurement uncertainty and the traceability establishment. The measurement uncertainty was found to be reasonable for the purpose to the LC method and the studied concentration, obtained in a complete single laboratory validation study compliant with international guidelines.

The method applicability was verified using matrix certified reference material from HSA Singapore and showed good analytical performance that makes it suitable for implementation in food testing laboratories for routine analysis, especially soy sauce commodity.

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