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SIMULTANEOUS DETERMINATION OF BENZOIC ACID AND PARABENS (METHYL-PARABEN AND N-BUTYL PARABEN) IN SOY SAUCE BY GC-MS

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***Abstract*— Soy sauce, especially the sweet one, is one of favorite condiment that used in Indonesian cuisine. The commercial soy sauce normally contains several amount of preservatives so it can be stored for longer time and for preventing from spoilage. Chemical preservatives such as sodium benzoate and parabens are now widely used as food preservation. The uses of chemical preservatives above the allowable limit could probably impact to the human health.. Therefore, accurate determination of those preservatives in soy sauce becomes important. In this study, the GCMS method employing non polar column and external calibration quantitation technique, had been developed for simultaneously determination of benzoic acid, methyl paraben and n-butyl paraben in soy sauce at ppm level. The analytical method was validated by using appropriate matrix Certified Reference Material (CRM). The accuracy and precision studies showed that the method has good performance. The estimation of measurement uncertainty was evaluated by using bottom up approach. The overall relative expanded uncertainty (k=2 at 95% confidence level) of the analytical method used for analyzing the matrix CRM was in range of 8 – 12 % at the 95% level confidence for those three target analytes. This high accuracy method with its reasonable measurement uncertainty produced a good and reliable result, indicating that the method is suitable for using in a routine analysis.**

***Keywords*— Soy sauce; benzoic acid; methyl paraben; n-butyl paraben; GCMS**

I. INTRODUCTION

Preservation is a common method in food industries to prevent food from damage or spoilage caused by chemical, physical or biological factors. A 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 lead to minimize the risk to become toxic food. Thus, the preservative allows the food to be stored or available for longer time. Among the available commercial food preservatives, benzoic acid and its ester derivates are the most widely used. Benzoic acid is one of weak organic acid preservative agent that inhibits the growth of both bacterial and fungal cell [1-2]. It has been previously reported that the concentration of benzoic acid and sodium benzoate used as a food preservatives was ranging up to 2000 mg kg^{-1} [2]. Although there are many claims regarding to the preservative effect of benzoate acid ; however, the fact that the bezoic acid has been examined for its potentiual helath riks of human such as worse asthma, urticaria and angeoedema, allergic rhinitis and flushing . They also reported have directly linked with childhood hyperactive [2-3]. Moreover, K.Seetaramaiah et.al (2011) reported that the esters of benzoic acid so called as parabens are class of antimicrobial agents. The parabens are possble to be used as a single or combination anti microbial agents to exert the intended affects against mold and yeasts. The parabens are concidered safe antimicrobial agents with relatively non-irritating and non-sensitizing effects as well as low toxicity to the human [4].

In Indonesia, sweet soy sauce is become one of favorite condiments and very often used as food flavour enhancer. The commercial sweet soy sauce contains some amount of preservatives to prevent it from spoiled and to make it possible to be stored for a longer time. Sodium benzoate and methyl paraben, also known as nipagin, are the two very common preservatives added in the commercial soy sauce. Based on the Indonesian National Standard (Standard National Indonesia), the maximum allowable amount of benzoic acid and parabens added in soy souce are 600 mg kg^{-1} and 250 mg kg^{-1}, respectively [5].

In recent years, some anaytical method have been developed for the determination of presrevatives in food. The simple and accurate method 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

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or diode array detector [6-9]. Benzoic acid is slightly soluble in water but highly solublity property in inpolar organic solvent such as ethanol. The melting and boiling point of benzoic acid are 122 ^{o}C and 249 ^{o}C respectively. A solubility propertie of paraben in polar organic solvent such as ethanol is reported to be the same with benzoic acid, where its solubility may increase by incerasing the number of atom in its ester gorups. Both benzoic acid and paraben are concidered as polar compounds and also have relatively high boling point. With regard to their properties as polar and low volatile compounds, therefore, the determinatuon of benzoic acid and parabens can be suitably counducted by teh LC method. Although, the benzoic acid and the parabens can also be determined by GC and GCMS method with and without derivatization step [10-13].

Practically, the most common sample preparation methods for the analysis of preservative in food including water based condiment befire analysis by the HPLC are direct dilution, extraction with organic solvent followed bythe filtration, and extraction by using SPE (solid hase extraction) and clean-up process to minimize the matrix effect [8,12,14]. In addition, a derivatization procedure is reguired when GC method is used. The derivatization agent may increase the volatility of the compounds by converting the hydroxyl group or/and the carboxylic acid to their derivatzied form, leading to more appropriate for GC analysis by using non-polar column. For obtaining good quantitation results, the following parameterts of the derivatization process should be optimized such as solvent, amount of derivatization agent, time and temperature of derivatization The derivation process may take longer time compared to the direct analysis without derivatization. In a normal condition, analysis of benzoic acid and parabens by a GC method can be conducted without any derivatization by employing polar column.

This study was aimed to develop simple and accurate method for the determination of preservatives (benzoic acid, methyl-paraben and n-butyl paraben) in soy sauce by using GC-MS. The separation of preservatives component was conducted in a non polar column.

II. EXPERIMENTAL

A. Reagent and Chemicals

Certified Reference Material (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. The CRM matrix (HRM-1005A) for preservatives in soy sauce containing benzoic acid (871.1 ± 21.6 mg kg^{-1}), methyl paraben (237.6 ± 13.5 mg kg^{-1}) and n-butyl paraben (93.7 ± 6.0 mg kg-1) was purchased from the Health Science Authority (HSA), Singapore. Phosphoric acid (85% puritiy) for the eluent preparation for solid phase extraction (SPE) and methanol (LC grade) for solvent in the preparation of both standard solution and sample were obtained from Merck, Germany. Solid phase extraction C18 Bond Elut (500 mg, 6 ml) was obtained from Agilent, USA. distilled water was used in all experimental runs for sample dilution and eluent preparation for SPE.

B. Preparation of Standard Solution

Stock solution of each compounds (denoted as SS) were in-house prepared gravimetrically at level concentration of 5000 µg g^{-1}. The intermediate standard solution (denoted as ISS) was prepared by mixing an appropriate amount of all target compounds and diluted gravimetrically by using methanol to produce final solution having concentration of 200 µg g-1. From the ISS, a series of working standard solutions (denoted as WSS) containing different concentration level of the target compounds were prepared gravimetrically. The WSS's were then utilized to establish the calibration curves for a quantitative analysis of the sample.

C. Sample Preparation

The sample of this study was prepared by using a method adopted from from Tzu-Yun C., et. al., 2003, with a slight modification[14]. Typically, a certain amount (2 g) of soy sauce sample was accurately weighed in a centrifuge tube and about eight ml of water was then immediately added into a centrifuge tube containing sample, and the final mass of the solution was recorded. After that, one ml of the diluted soy sauce sample, which known the mass, was passed through into the conditioned C18 cartridge, and followed by washing the cartridge with four ml of 10% (v/v) methanol in 1% phosphoric 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 0.45 mm disk filter and was gravimetrically diluted to about 5-fold with methanol for direct analysis by GCMS without any derivatization.

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D. GCMS Analysis

An Agilent 7890B GC System interfaced with a single quadrupole Agilent 5977A MSD was used to determine the benzoic acid, methyl paraben and n-butyl paraben in soy sauce. A series of standard solutions and the extract solutions of soy sauce samples were injected into the GCMS system by using Gerstel Multipurpose sampler that has been optimized for the rinsing method prior to use. The target preservative compounds were separated on a DB-5MS column (30m x 0.25mm x 0.25um) under the following analytical condition of the GCMS system. Helium gas (GCMS grade from Air Liquid Indonesia) was used as mobile phase/carrier gas. The flow rate of the carrier gas was maintained at 1 ml/min. Temperature of injector (Tinjector) was set at 250 ^{o}C. The oven temperature was set at 60 ^{o}C as initial temperature and without holding, the temperature was increased to 280 ^{o}C at 15^{o}C/min, and then the temperature was hold at 280 ^{o}C for 6 minutes. The injection volume was 1 uL with split injection mode (split ratio of 1:10).

The temperatures of the transfer line (Taux), ion source (Tion source) and the quadrupole (Tquad) were maintained at 280 ^{o}C, 230 ^{o}C and 150 ^{o}C respectively. The solvent delay was set at 3.5 min. For the qualitative analysis, the mass spectrometer was operated in scan mode (scan range: m/z 35 to m/z 350), with electron impact ionization (EI) at 70 eV. The National Institute of Standards and Technology (NIST) MS library was used to confirm the identity of the target compounds observed in the chromatogram of standard and sample solutions. The quantitative analysis was done by using external calibration technique.

III. RESULT AND DISCUSSION

A. Separation and Identity confirmation of target compounds

The standard mixture solution of benzoic acid, methyl paraben and n-butyl paraben at concentration level of 4 µg/g with and without derivatization were analyzed in the GCMS system in scan mode, in order to get separation profile, the data of retention time and the full spectra of each target compounds. Figure 1 depicts a total ion chromatogram (TIC) of pure substance (benzoic acid, methyl paraben and n-butyl paraben) in the CRM standard mixture, showing a good separation profile. From Fig. 1, it was clearly observed that the TIC of the benzoic acid, methyl paraben and n-butyl paraben is identified at retention time of 5.36 min, 8.06 min and 10.20 min, repectively. Good separation profile of the TIC having sharp and symmetrical peaks for those peaks indicates that the methods used under study is worth for quantitative purpose. Moreover, in order to confirm the identity of each analyte, then their full spectra obtained by using scan mode were compared to the NIST library software and the results are presented in Fig. 2. In addition, selected quantifying (Q) and qualifying (q) ions for the quantitative analysis purpose are tabulated in Table 1. For the quantitative identification, the ion having the highest abundance (base peak) was selected as quantifier ion. In this reagrds, therefore, it was found that the ion with m/z 105 was selected to be the quantifier ion for benzoic acid, while ion with m/z of 121 was selected to be the quantifier ion for both methyl and n-butyl paraben.

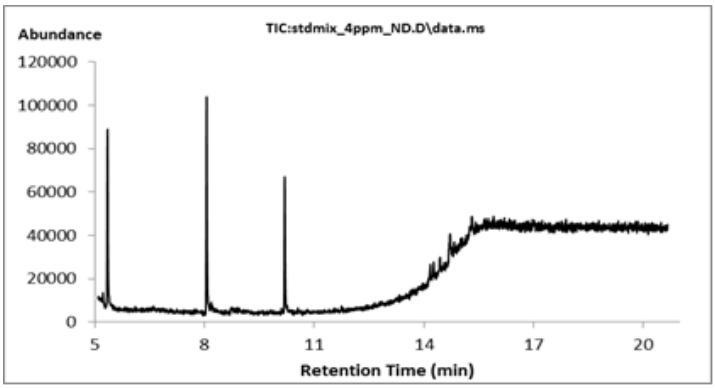

Fig. 1 TIC of target compounds at level concentration of 4 µg g^{-1} in Scan mode

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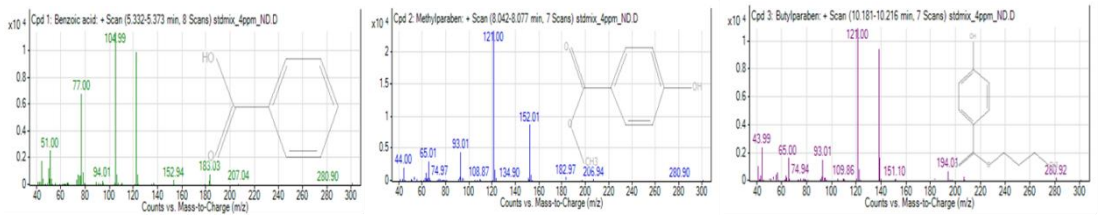

Fig. 2 The NIST library result of those three target compounds in standard mixture solutions.

TABLE I
THE QUANTIFYING AND QUALIFYING IONS FOR TARGET COMPOUNDS

Compound	Molecular formulae	Retention time (min)	Q,q,q (m/z)
Benzoic Acid	$C_7H_6O_2$	5.36	105, 77, 122
Methyl paraben	$C_8H_8O_3$	8.06	121, 93, 152
n-Butyl paraben	$C_{11}H_{14}O_3$	10.20	121, 93, 138

B. Quantitative analysis

For the quantitative analysis, the final extract solution of sample and CRM were analyzed by using Selective Ion Monitoring (SIM) mode, taking into account the selected of quantifying and the qualifying ions (Table 1). Typical chromatogram of the pure substance in the CRM standard mixture and CRM matrix resulted from analysis with SIM mode are shown in Fig. 3 and Fig. 4, respectively.

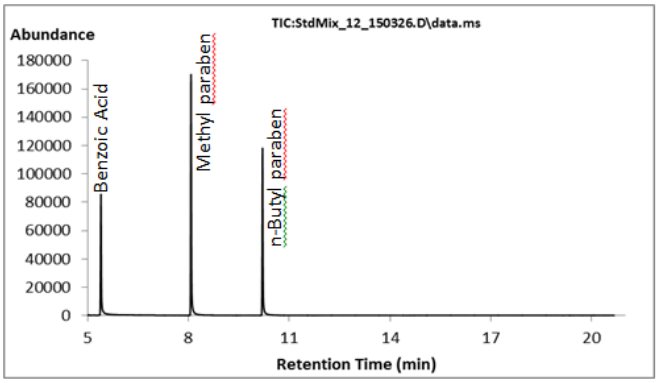

Fig. 3 Chromatogram of standard mixture at level concentration of 9 mg kg^{-1} in SIM mode.

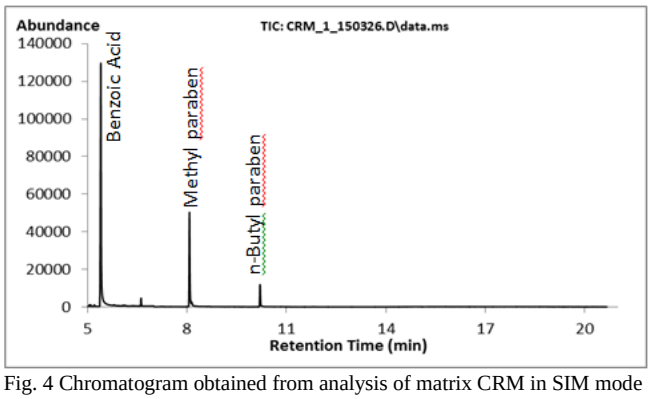

Fig. 4 Chromatogram obtained from analysis of matrix CRM in SIM mode

A quantitation technique used in this study was external calibration with multiple point calibration and the mass fraction of all target analytes were calculated based on Eq. 1.

$$C = \frac{C_{GC} \times m_2 \times m_4}{m_1 \times m_3} \times \frac{m_6}{m_5} \times \frac{1}{\mathrm{Re}\,c} \qquad (1)$$

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Where:

C : mass fraction of analyte (mg/kg)
C_{GC} : measured concentration in GCMS (mg/kg)
m_1 : mass of 2 mL of sample (g)
m_2 : mass of solution after addition of distilled water (g)
m_3 : mass of 1 ml aliquot taken to be passed through the SPE cartridge (g)
m_4 : Mass of methanol extract solution after clean up with SPE (g)
m_5 : Mass of 200 µl of methanol extract solution (g)
m_6 : Mass of final solution after dilution (g)
Rec : Recovery of the method

For quantitative analysis, performance of the GCMS system was firstly checked by evaluating the precision of the GCMS response. The mixture of standard solution was injected into the GCMS system in seven replications. The %RSD of peak area for each target compound were found to be 1.85%, 1.29% and 2.08% for benzoic acid, methyl paraben and n-butyl paraben, respectively. Since all the %RSD values of the target compounds are less than 3%, thus, the system was considered to acquire a stable detection response. The series of standard solution at different concentration level ranging from 1 to 10 µg g^{-1} were injected to conform the linearity of the detector response. It was found that the correlation coefficient (R2-value) (Table 2) of all target compounds were greater than 0.990.

TABLE II
SUMMARY OF THE PRECISION (%RSD) AND THE CORRELATION COEFICIENT (R2) OF THE GCMS METHOD

Compound	Precision (%RSD[a])	R^2
Benzoic Acid	1.85	0.998
Methyl paraben	1.29	0.991
n-Butyl paraben	2.08	0.992

a: n=7

In this study, the bias of the analytical method is expressed as analytical recovery where value observed is divided by a reference value or an expected value. It is usually determined by studying of relevant reference materials or by using spiking technique [15]. In this study, the recovery of the method was evaluated based on the result of matrix CRM analysis (HRM-1005A) in total of 9 replications in three different days. The recoveries for all target analytes were found to be 96.04, 93.54 and 101.70 % for benzoic acid, methyl paraben and n-butyl paraben, respectively, as listed in Table 3.

TABLE III
THE RECOVERY OF THE METHOD

Compound	Certified value ± U (mg kg^{-1})	Mean recovery (%)	RSD (%)
Benzoic acid	871.1 ±21.6	96.04	5.67
Methyl paraben	237.6 ± 13.5	91.15	5.95
n-Butyl paraben	93.7 ± 6.0	101.70	4.01

In practice, the recovery factor can be neglected or be used for the correction of the results, meaning that the recovery factor is depending on the results of the statistical significant test. The significant test (t-test) was used to determine whether the mean recovery is significantly different from 1.0. The statistical t value was calculated using Eq. 2 and the result then was compared with the 2-tailed critical value tcrit for n-1 degrees of freedom at 95% confidence level.

$$t = \left| \frac{(\bar{x} - \mu)\sqrt{n}}{s} \right| \quad (2)$$

The recovery is significantly different from 1 when t is greater or equal than the tcrit. The tcrit value in this study was 2.306 and the t value for benzoic acid, methyl paraben and n-butyl paraben were 2.18, 4.89 and 1.47, respectively. From the statistical test, the recovery of methyl paraben was found to be significantly different from 1, implying that a correction factor (1/Rec) was explicitly included in the calculation of the result of methyl paraben.

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The precision of the method in term of method repeatability and intermediate precision was also evaluated (Table 4). From Table 4, it can be observed that the %RSD for the target analytes in the same day were less than 10% and the %RSD from different day analysis were in range of 6.78-10.94%. These precision results indicated that the method used in this study is precise.

TABLE IV
REPEATABILITY AND INTERMEDIATE PRECISION OF THE METHOD

Compound	%RSD	
	Repeatability[a]	Intermediate precision[b]
Benzoic acid	8.93	9.33
Methyl paraben	7.89	10.94
n-Butyl paraben	9.08	6.78

a results from 6 replications of analysis in the same day
b results from 13 replications of analysis in the different day

C. Estimation of Measurement Uncertainty

The estimation of measurement uncertainty was evaluated by using Bottom-Up approach in accordance to the ISO/IEC Guide to the Expression of Uncertainty in Measurement and EURACHEM / CITAC Guide [15, 16]. For the estimation of the measurement uncertainty, the contribution of the uncertainty components such as method precision, weighing process, GCMS calibration, calibration standard solutions and recovery (Table 5) were considered.

TABLE V
COMPONENT OF UNCERTAINTY AND SOURCE OF UNCERTAINTY DATA

Component of uncertainty	Source of uncertainty data
m_1 m_2 m_3 m_4 m_5 m_6	▪ Uncertainty associated with the mass of sample and solution was estimated by using the data from the calibration certificate. (Type B).
C_{GC}	▪ Uncertainty associated with the C_{GC} obtained from the linear least square fitting procedure of the calibration curve. (Type A).
Rec	▪ Uncertainty of the concentration of target analyte in CRM matrix used in the accuracy study (Type A) ▪ SD of mean of nine independent analysis of CRM matrix. (Type A)
Precision	▪ Uncertainty based on value of RSD of the mean of nine independent analysis of CRM matrix. (Type A)
Calibration standard solution	▪ Uncertainty of purity value of the pure substance CRM used from the certificate of CRM. (Type B) ▪ Uncertainty value from the calibration certificate of the analytical balance used in preparing the calibration solution. (Type B)

All those sources of uncertainty were then converted into the standard and combined uncertainty using the following equation (Eq. 3).

$$u_C = C\sqrt{\left(\frac{u_{C_{GC}}}{C_{GC}}\right)^2+\left(\frac{u_{m_1}}{m_1}\right)^2+\left(\frac{u_{m_2}}{m_2}\right)^2+\left(\frac{u_{m_3}}{m_3}\right)^2+\left(\frac{u_{m_4}}{m_4}\right)^2+\left(\frac{u_{m_5}}{m_5}\right)^2+\left(\frac{u_{m_6}}{m_6}\right)^2+\left(\frac{u_{\mathrm{Rec}}}{\mathrm{Rec}}\right)^2+\left(\frac{u_P}{1}\right)^2+\left(\frac{u_{C_{cal}}}{C_{cal}}\right)^2} \quad (3)$$

The contribution values of each uncertainty component to the measurement result for benzoic acid, methyl paraben, and n-buthyl paraben are tabulated in Table 6, Table 7 and Table 8, respectively.

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TABLE VI
UNCERTAINTY BUDGET FOR MEASUREMENT OF BENZOIC ACID IN THE MATRIX CRM BY GCMS

Parameter	Value *x*	Standard uncertainty *u(x)*	Unit	*u(x)/x (%)*
▪ m_1	2.3149	7.07107E-05	g	0.0031
▪ m_2	10.3987	7.07107E-05	g	0.00068
▪ m_3	1.02678	2.12132E-05	g	0.0021
▪ m_4	2.70737	2.12132E-05	g	0.00078
▪ m_5	0.15960	2.12132E-05	g	0.013
▪ m_6	0.80057	2.12132E-05	g	0.0026
▪ Precision	1	0.0189		1.89
▪ C_{GC}	14.080	0.4117	mg/kg	2.92
▪ Recovery	0.9604	0.02170		2.26
▪ Calibration standard solution	6.8136	0.002081	mg/kg	0.031
Combined uncertainty (u_c)		35	mg/kg	
Expanded uncertainty, Coverage factor *k*=2 (corresponding to a confidence level of approximately 95%)		70	mg/kg	
% uncertainty		8.04	%	

TABLE VII
UNCERTAINTY BUDGET FOR MEASUREMENT OF METHYL PARABEN IN THE MATRIX CRM BY GCMS

Description	Value *x*	Standard uncertainty *u(x)*	Unit	*u(x)/x (%)*
▪ m_1	2.3149	7.07107E-05	g	0.0031
▪ m_2	10.3987	7.07107E-05	g	0.00067
▪ m_3	1.02678	2.12132E-05	g	0.0021
▪ m_4	2.70737	2.12132E-05	g	0.00078
▪ m_5	0.15960	2.12132E-05	g	0.013
▪ m_6	0.80057	2.12132E-05	g	0.0026
▪ Precision	1	0.0198		1.98
▪ C_{GC}	3.323	0.170	mg/kg	5.12
▪ Recovery	0.9115	0.03158		3.47
▪ Calibration standard solution	3.1918	0.005884	mg/kg	0.18
Combined uncertainty (u_c)		14.1	mg/kg	
Expanded uncertainty Coverage factor *k*=2 (corresponding to a confidence level of approximately 95%)		28.1	mg/kg	
% uncertainty		12.97	%	

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TABLE VIII
UNCERTAINTY BUDGET FOR MEASUREMENT OF n-BUTYL PARABEN IN THE MATRIX CRM BY GCMS

Description	Value *x*	Standard uncertainty *u(x)*	Unit	*u(x)/x (%)*
▪ m_1	2.3149	7.07107E-05	g	0.0031
▪ m_2	10.3987	7.07107E-05	g	0.00067
▪ m_3	1.02678	2.12132E-05	g	0.0021
▪ m_4	2.70737	2.12132E-05	g	0.00078
▪ m_5	0.15960	2.12132E-05	g	0.013
▪ m_6	0.80057	2.12132E-05	g	0.0026
▪ Precision	1	0.01337		1.34
▪ C_{GC}	1.60	0.0598	mg/kg	3.74
▪ Recovery	1.017	0.03527		3.47
▪ Calibration standard solution	3.14426	0.006104	mg/kg	0.194
Combined uncertainty (u_c)		5.04	mg/kg	
Expanded uncertainty Coverage factor *k*=2 (corresponding to a confidence level of approximately 95%)		10.08	mg/kg	
% uncertainty		10.7	%	

The overall relative expanded uncertainty of the analytical method used for analyzing the CRM matrix was in range of 8 – 13 % at the 95% confidence level for those three target compounds. The main contribution to the uncertainty budget was from the predicted concentration of the compound (CGC) that obtained from the calibration curve.

IV. CONCLUSIONS

The GCMS method employing non polar column and external calibration quantitation technique have been developed for simultaneously determination of benzoic acid, methyl paraben and n-butyl paraben in soy sauce. The performance of the method was evaluated by using appropriate CRM matrix and the result showed that the method had good accuracy and precision. The method also produced reasonable measurement uncertainty estimation for those three target analytes. Finding of this study demonstrates that the developed method is suitable for achieving good and reliable result in routine analysis.

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