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Simultaneous Quantitative Determination of Four Kinds of Parabens in Soy Sauce by UPLC-MS/MS

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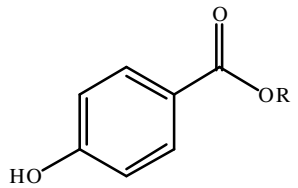
A new method for simultaneous determination and quantitation of four kinds of parabens (methyl paraben, ethyl paraben, propyl paraben and butyl paraben) in soy sauce has been developed by using solid-phase extraction (SPE) combined with ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Two sample type (blended and brewed) were used for method evaluation. Recoveries were mostly higher than 90 %, method detection limits ranged from 0.55 to 2.08 ng mL^{-1} and method quantification limits were included between 1.84-6.92 ng mL^{-1}. Due to matrix effect, quantitation was performed by referring to a matrix matched calibration curve, for each soy sauce typology. This method was also applied to commercial soy sauce samples, with good results.

Keywords: Parabens, Soy sauce, Solid-phase extraction, UPLC-MS/MS.

INTRODUCTION

Esters of *p*-hydroxy benzoic acid (Fig. 1), commonly known as parabens, include methyl paraben (MP), ethyl paraben (EP), propyl paraben (PP) and butyl paraben (BP). They are widely used as preservatives for food, cosmetics and pharmaceuticals in restricted concentration levels because of their relatively low toxicity profile, non-volatility, neutrality and broad-spectrum antimicrobial activity[1-3]. The antimicrobial activity of parabens increases with an increase in the length of the alkyl chain of the ester group[1], but in practice, shorter esters are commonly employed because of their higher solubility in water[4]. Combinations of two or more parabens are often used together since they have synergistic effects[4].



Methyl paraben R = $-CH_3$
Ethyl paraben R = $-CH_2CH_3$
Eropyl paraben R = $-CH_2CH_2CH_3$
Butyl paraben R = $-CH_2CH_2CH_2CH_3$

Fig. 1. Chemical structures of paraben compounds

Recently, parabens in cosmetic products have received more attention, because the elevated amounts of parabens in topical products have been shown to induce allergic contact dermatitis[1]. Routledge *et al.*[5] described the oestrogenic activity of parabens and they have been recently reported to have oestrogenic activity in yeast cells and animal models[1,6-8]. Oestrogen is a major etiological factor in the growth and development of the majority of human breast cancers. It has been reported that the mean concentration of parabens in 20 human breast tumors was found to be 20.6 ± 4.2 ng g^{-1} tissue[6]. In addition, butyl paraben even has an epigenetic effect on spermatogenic germ cells in the adult rat testis[8]. The above studies indicated that even though long-term, low-dose consumption maybe has side effects on humans and wildlife. Therefore, it is necessary to establish rapid and effective methods for the determination of parabens in different kinds of food to ensure food safety and human health.

In the last few years several methods have been developed for the determination of parabens, mainly using high-performance liquid chromatography (HPLC)[9,10], gas chromatography (GC)[11], capillary electrophoresis[12-15], thin-layer chromatography[6] and microemulsion electrokinetic chromatography[16]. A few methods have been published based on the use of LC-MS/MS[16,17] and GC-MS[18-21].

In this study, we have developed a sensitive, dependable and simple method, based on solid-phase extraction (SPE) of the samples and reversed-phase UPLC-MS/MS to simultaneously determine four parabens including methyl paraben, ethyl paraben, propyl paraben and butyl paraben in soy sauce, which is often used in cooking as a condiment. Two typologies of soy sauce were spiked with the parabens and then analyzed, to optimize the entire method and determine accuracy (recovery), precision, method detection limit (MDL), method quantification limit (MQL) and linearity range. The method was finally tested on commercial soy sauce samples.

EXPERIMENTAL

HPLC-grade methanol were purchased from TEDIA (Ohio, USA). Standards, including methyl paraben, ethyl paraben, propyl paraben, butyl paraben, were purchased from AccuStandard Inc (New Haven, CT). All other chemicals were analytical-reagent grade. Deionized water was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA) and was used throughout the study.

For solid phase extraction, Sep-pak Vac 6 cc (1 g) C18 Cartridges (Waters, Milford, MA, USA) were used.

Standard stock solutions of each paraben were prepared at 100 μg mL^{-1} level in methanol and stored at 4 °C in glass vials.

Extraction procedure: Test samples including blended soy sauce and brewed soy sauce were purchased from local supermarkets in Kunming, P.R. China and stored at 4 °C.

2 mL sample was diluted to 100 mL with water and then 5 mL of the diluted sample was loaded into an C18 cartridge activated prior to use by passing through 5 mL of methanol followed by 5 mL water. The cartridge was then sequentially rinsed with 3 mL 90 % aqueous methanol. The eluate was made up to 5 mL with 90 % aqueous methanol before filtration (0.22 μm microporous membrane) into injection bottles. The final extract was analyzed by UPLC-MS/MS.

UPLC-MS/MS instrumentation and conditions: The liquid chromatography tandem mass spectrometry system was comprised of an API 4000 MS/MS System equipped with an electrospray ionization (ESI) probe and a syringe pump (AB Sciex, Foster City, CA, USA) and an Ultra Performance LC system was equipped with a binary pump and an autosampler (Waters, Milford, MA, USA). The system was connected by PEEK tubing (1/16 in. o.d. × 0.01 in. i.d.). Data was acquired and processed using AB Sciex Analyst software (version 1.5.1). Samples (5 μL) of the final extracts were separated on an ACQUITY UPLC BEH C18 column (2.1 × 100 mm; 1.7 μm particles) at a flow rate of 0.4 mL min^{-1} and eluted with a linear binary gradient of 0.05 % formic acid in water (A) and methanol (B) (Table-1). The temperature of the analytical column was maintained at 40 °C.

TABLE-1
MOBILE PHASE GRADIENT PROGRAM OF UPLC-MS/MS
(A: 0.05 % FORMIC ACID IN WATER AND B: METHANOL)

Time (min)	Methanol (%)	0.05 % Formic acid in water (%)
0	10	90
3	90	10
4	90	10
5	10	90
6	10	90

Detection of analytes were operated in the negative ion mode. Optimization of the operation conditions, infusing diluted stock solutions of each analyte into the mass spectrometer were as follows: source temperature 600 °C, curtain gas 30 psi (83 kPa of max. 99.5% nitrogen), ion source gas 1 (nebulizer gas) 60 psi (414 kPa of nitrogen), ion source gas 2 (auxiliary gas) of 60 psi (276 kPa of nitrogen), spray voltage -4.5 kV. Other MS parameters are shown in Table-2.

Method validation: A standard calibration line was constructed by analyzing mix solutions at six concentration levels in the ranges of 10-500 ng mL^{-1}. Two different matrix matched calibration curves were also performed by spiking the extracts of blended and brewed soy sauce, respectively, in order to cover two main typologies of soy sauce. Each curve was constructed by addition of appropriate volumes of the standard mix working solution at blank soy sauce sample extracts in order to have the same concentration levels of the standard working solution. The paraben peak area *versus* paraben concentration in soy sauce samples were plotted to get the calibration curves.

Signal suppression or enhancement on ESI-MS/MS response due to matrix effect was evaluated, for each analyte, by comparing the slope of the standard calibration curve with the slope of the matrix matched calibration curve.

Accuracy was evaluated in terms of percentage of recovery on the two soy sauce typologies earlier described. For recovery studies blank soy sauces were spiked prior to the extraction step. A volumn-measured aliquot of the sample was added of a small and suitable volume of working solutions of the analytes. After a few minutes extraction was carried out, as previously described.

For each analyte, five replicates of three levels of concentration, corresponding to 20, 100 and 500 ng mL^{-1}, were investigated. The averaged recovery, for each soy sauce typology, the relative standard deviations (RSD) and the relative errors (RE) were calculated.

To calculate the method detection limit and method quantification limit of each analyte, seven repliacates of blank soy sauce sample extracts spiked with an appropriate volume of the standard mix working solution in order to have the same concentration level of the lowest level of the calibration curve were analyzed and the method detection limit and method

TABLE-2
OPTIMIZED MS PARAMETERS OF PARABENS

Analyte	Precursor ion (*m/z*)	Product ion (*m/z*)	Declustering potential (U/V)	Collision energy (U/eV)
Methyl paraben	151.6 $[M-H]^-$	92.0*/136.0	55/55	29/19
Ethyl paraben	165.5 $[M-H]^-$	92.0*/136.0	51/55	30/21
Propyl paraben	179.8 $[M-H]^-$	92.0*/136.0	62/61	31/23
Butyl paraben	193.8 $[M-H]^-$	92.0*/136.0	55/55	29/22

*Quantitative ion.

quantification limit were expressed as 3 × SD and 10 × SD, respectively.

RESULTS AND DISCUSSION

Extraction: Matrix effect can be reduced with dilution and samples were diluted 50-fold in this research for sufficient recoveries of four kinds of parabens were obtained with that.

For solid phase extraction clean-up, the effect of various concentrations of eluent solution on the recoveries of the analytes were investigated. Soy sauce matrix spiked with parabens was used and quantitation was calculated by using the standard calibration curve. Fig. 2, it can be seen that the recoveries of parabens in 90% methanol solution was high, especially the recovery of butyl paraben. So 90 % methanol was selected as the eluent for the clean-up of parabens.

Optimization of chromatographic and MS/MS conditions: Analytes were mass-selected and fragmented. For each compound two ion pairs were chosen for acquisition in multiple reaction monitoring (MRM) mode. Precursor ions of methyl paraben, ethyl paraben, propyl paraben and butyl paraben were *m/z* 151.6 $[M-H]^-$, *m/z* 165.5 $[M-H]^-$, *m/z* 179.8 $[M-H]^-$ and *m/z* 193.8 $[M-H]^-$. Product ions were *m/z* 136.0 $[M-CH_3]^-$ and *m/z* 92.0 $[M-CH_3CO_2]^-$ for methyl paraben, *m/z* 136.0 $[M-C_2H_5]^-$ and *m/z* 92.0 $[M-C_2H_5CO_2]^-$ for ethyl paraben, *m/z* 136.0 $[M-C_3H_7]^-$ and *m/z* 92.0 $[M-C_3H_7CO_2]^-$ for propyl paraben, *m/z* 136.0 $[M-C_4H_9]^-$ and *m/z* 92.0 $[M-C_4H_9CO_2]^-$ for butyl paraben, respectively. Tuning parameters are summarized in Table-2.

UPLC separation was performed using reversed phase chromatography and satisfactory separation was obtained with methanol and formic acid in water as mobile phases. Typical UPLC-ESI-MS/MS chromatogram of four parabens was shown in Fig. 3. The retention time of methyl paraben, ethyl paraben, propyl paraben and butyl paraben are 3.03, 3.37, 3.66 and 3.90 min, respectively.

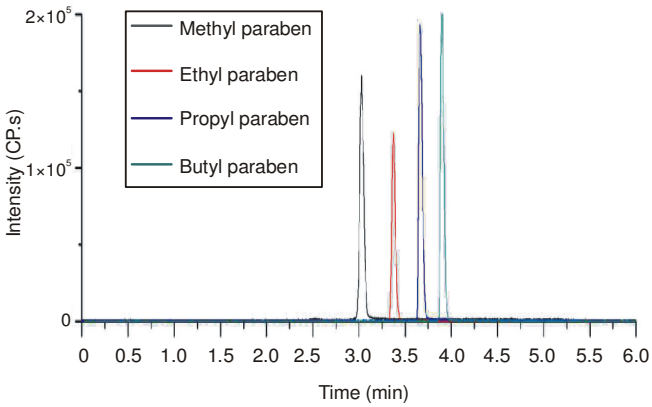


Fig. 3. Typical extracted ion chromatogram (XIC) of four prarabens

Method validation: As illustrated above, linear calibration curves were obtained both by standard calibration and by matrix matched procedures. The linearity ranges of all the analytes, in the two different soy sauce typologies, were evaluated. For each analyte the calibration curves and their linear regression analysis are shown in Table-3. All calibration curves showed good linear regression ($R \geq 0.9990$) within linear range.

The matrix effect was calculated and shown in Table-3. The average ratio between slopes ($b_{matrix}/b_{standard}$) is strongly dependent on soy sauce typology and parabens. For blended soy sauce it was less than or equal to 1, showing signal suppression. For brewed soy it was more than 1, showing signal enhancement. Due to these obvious differences between standard and matrix matched calibration, we chose to carry

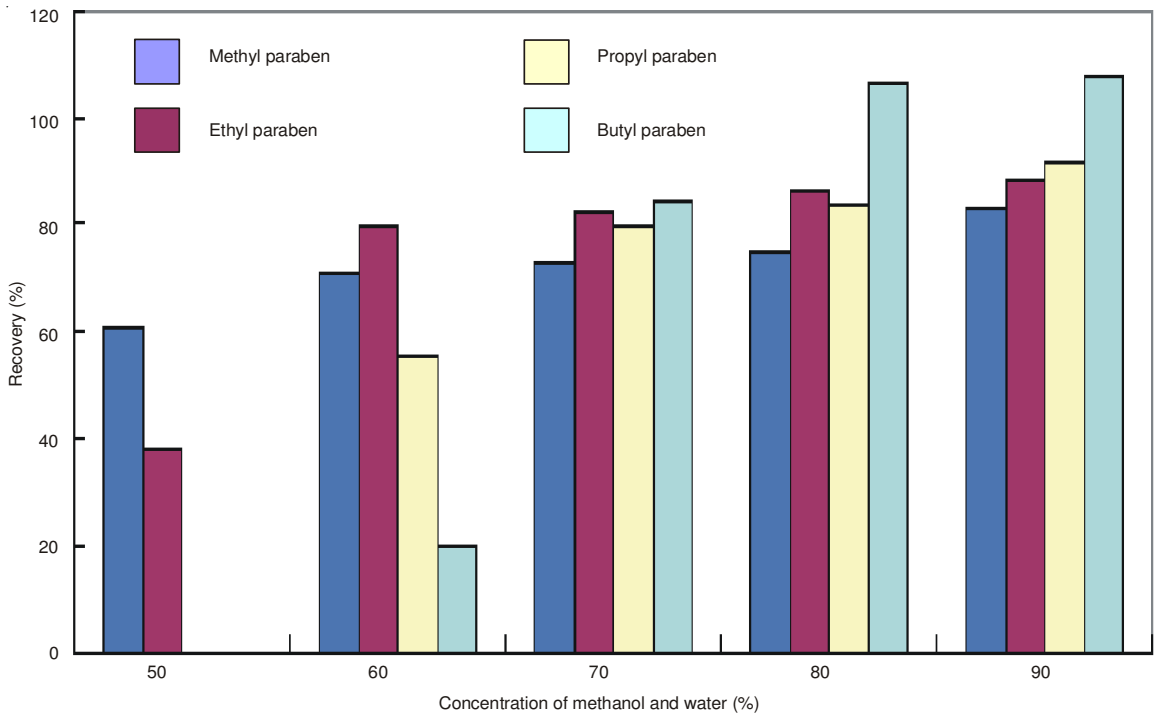


Fig. 2. Recovery of parabens (methyl paraben, ethyl paraben, propyl paraben and butyl paraben) using dfferent concentrations of methanol (from 50 % water to 90 % methanol) as washing solution

TABLE-3
CALIBRATION CURVES

Paraben	Standard equation[a]	Matrix-matched equation[b]		Matrix effect[c]
Methyl paraben	$y = 2.89e^4x+3.41e^5$ (R = 0.9992)	Blended	$y = 1.69e^4x + 1.24e^5$ (R = 0.1000)	0.58
		Brewed	$y = 3.08e^4x + 4.18e^5$ (R = 0.9992)	1.07
Ethyl paraben	$y = 2.28e^4x+2.1e^5$ (R = 0.9996)	Blended	$y = 1.92e^4x + 1.56e^5$ (R = 0.9995)	0.84
		Brewed	$y = 2.67e^4x + 2.72e^5$ (R = 0.9993)	1.17
Propyl paraben	$y = 3.27e^4x+4.02e^5$ (R = 0.9995)	Blended	$y = 2.97e^4x + 5.25e^5$ (R = 0.9992)	0.91
		Brewed	$y = 3.59e^4x + 6.39e^5$ (R = 0.9990)	1.10
Butyl paraben	$y = 3.36e^4x+4.4e^5$ (R = 0.9993)	Blended	$y = 3.35e^4x + 3.94e^5$ (R = 0.9994)	1.00
		Brewed	$y = 4.03e^4x + 6.41e^5$ (R = 0.9992)	1.20

[a]y = preservative peak area and x = concentration of paraben expressed as ng mL^{-1}. Standard calibration lines were constructed by analyzing mix standard solutions at six concentration levels in the ranges of 10-500 ng mL^{-1}.
[b]y = preservative peak area and x = concentration of paraben expressed as ng mL^{-1}. Matrix matched calibration lines were constructed by addiction of appropriate volumes of the standard mix working solution at blank soy sauce sample extracts of blended soy sauce and brewed soy sauce, respectively, in order to have the same concentration levels of the standard working solution.
[c]Matrix effect was evaluated for each analyte by comparing the slope of the standard calibration curve with the slope of the matrix-matched calibration curve.

TABLE-4
ACCURAY AND PRECISION

Paraben	Soy sauce typology	Spiking level (ng mL^{-1})	Average recovery (%)	RSD (%)[a]	RE[b]
Methyl paraben	Blended	20	94.40	2.23	-5.60
		100	93.00	3.40	-7.00
		500	102.80	1.47	2.80
	Brewed	20	94.30	2.45	-5.70
		100	101.70	8.78	1.70
		500	98.92	4.34	-1.08
Ethyl paraben	Blended	20	83.50	2.51	-16.10
		100	100.40	8.72	0.400
		500	107.64	1.29	7.64
	Brewed	20	102.80	3.98	2.80
		100	93.80	1.39	-6.20
		500	106.56	1.98	6.56
Propyl paraben	Blended	20	92.00	6.15	-8.00
		100	98.30	4.74	-1.70
		500	106.04	2.49	6.04
	Brewed	20	86.68	1.36	-13.32
		100	99.40	3.80	-0.60
		500	102.24	4.19	2.24
Butyl paraben	Blended	20	96.30	2.10	-3.70
		100	113.80	3.80	13.80
		500	105.68	2.00	5.68
	Brewed	20	94.85	6.90	5.15
		100	102.40	4.51	2.40
		500	109.12	2.13	9.12

[a]Relative standard deviation; [b]Relative error

out the evaluation of method performances on the matrix curve, in order to improve the accuracy of the evaluation.

The matrix effect seems to be related both to the soy sauce typology and the parabens. Differences in the chemical structure of the parabens, in the composition of the soy sauce matrices, due to the different microbial fermentations, different chemical-physical structures, different chemical conditions (water activity, pH, salt concentration, *etc.*) may give rise to really different chemical behaviours, that explain the observed differences between standard and matrix matched calibration curves and among the matrix matched calibration curves.

The evaluation of accuracy, expressed as percentage of recovery was carried out on blank sample extracts, spiked with a known amount of the analytes. In order to test the method suitability, accuracy was investigated in the two soy sauce typologies (blended and brewed). Recoveries (Table-4) were evaluated at three different levels of concentration for each analyte, corresponding to a low,a high and an intermediate value of the evaluated range. Experimental data showed the overall good accuracy of the method for the four kinds of parabens.

Method detection limit and method quantification limit were evaluated as described above and data are listed in Table-5. Results showed that method detection limit was 0.55-2.08 ng mL^{-1} and method quantification limit was 1.84-6.92 ng mL^{-1}.

Real sample analysis: The method was finally applied to analyse the four parabens in commercial samples of soy sauce. Each sample was three times analyzed and in order to

TABLE-5
METHOD DETECTION LIMIT (MDL) AND METHOD QUANTITATION LIMIT (MQL)

Paraben	Soy sauce typology	MDL (ng mL^{-1})[a]	MQL (ng mL^{-1})[b]
Methyl paraben	Blended	0.70	2.34
	Brewed	0.55	1.84
Ethyl paraben	Blended	1.43	4.77
	Brewed	0.69	2.31
Propyl paraben	Blended	1.24	4.13
	Brewed	1.19	3.95
Butyl paraben	Blended	2.08	6.92
	Brewed	1.60	5.34

[a]Method detection limit was calculated as 3 × SD of 7 replicates of blank soy sauce sample extracts spiked with standard mix working solution to have the concentration of 10 ng mL^{-1} of each paraben.
[b]Method detection limit was calculated as 10 × SD of 7 replicates of blank soy sauce sample extracts spiked with standard mix working solution to have the concentration of 10 ng mL^{-1} of each paraben.

assure an accurate determination, quantitation was calculated by using the matrix matched calibration curve depending on soy sauce typology. All the four kinds of parabens were not found in those real samples,as it is declared on the label of the samples.

Conclusion

In this work, combining solid-phase extraction with UPLC-MS/MS, a new method for simultaneous determination and quantitation of four kinds of parabens (methyl paraben, ethyl paraben, propyl paraben and butyl paraben) in soy sauce has been developed by using two different typologies of soy sauce (blended and brewed). A relevant matrix effect in both of the two typologies was observed. By applying the matrix matched calibration curves, the method showed good recoveries of parabens added to soy sauce of two different typologies, always above 83 % and RSDs were less than 8.78 %. Method detection limit and method quantification limit were 0.55-2.08 ng mL^{-1} and 1.84-6.92 ng mL^{-1}, respectively.

The method was tested against commercial samples, to confirm its reliability, with results in line with their respective labels.

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