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A rapid method for the determination of alkenylbenzenes in spices by gas chromatography - high resolution mass spectrometry (GC-HRMS)

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Abstract

Alkenylbenzene is a group of natural toxins that can cause genetic damage and cancer in rodents. These toxins are found in high amounts in common spices such as pepper, basil, cinnamon, dill, and others. This study aimed to develop a method for measuring eight alkenylbenzenes (eugenol, methyl eugenol, acetyl eugenol, trans-isoeugenol, safrole, estragole, myristicin and trans-anethole) in various spices using gas chromatography high-resolution mass spectrometry (GC-HRMS) with a simple ultrasonic vibration sample extraction method. The method was validated according to AOAC standards. The method had detection and quantification limits of 0.05 mg/kg and 0.15 mg/kg, respectively. The recovery ranged from 90.1% to 107% and the repeatability (RSD) ranged from 1.00% to 6.60% for all eight compounds, meeting the AOAC requirements. The analysis of 90 different dried and fresh spice samples revealed that eugenol (1.86 - 3581 mg/kg), trans-anethole (0.89 - 45.6 × 103 mg/kg) and estragole (0.19 - 248 mg/kg) were the main alkenylbenzenes, while trans-isoeugenol (0.69 - 7.86 mg/kg) and safrole (0.15 - 0.42 mg/kg) were the minor ones.

Keywords: *Alkenylbenzene, GC-HRMS, Spices, ultrasonic extraction.*

1. INTRODUCTION

Nowadays, natural spices and herbs with aromatic scents are increasingly used as fragrances, as consumers believe that natural products are healthy and good for their well-being. Different parts of spice and herb plants that have aromatic scents (such as leaves, fruits, seeds and flowers) are often added to food as seasonings to enhance the sensory qualities and aroma of the dishes. However, herb and spice plants may also contain harmful contaminants (such as heavy metals, pesticide residues and azo dyes...) [1], as well as

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endogenous compounds (produced by the plants themselves) that can be genotoxic and carcinogenic to rodents, such as alkenylbenzenes, coumarin or pyrrolizidine alkaloids [2 - 4].

Alkenylbenzene is a volatile plant toxin that is commonly found in many spices and aromatic herbs that are frequently used, such as pepper, basil, cinnamon, dill and anise [2]. The European Rapid Alert System for Food and Feed (RASFF) recently indicated that five alkenylbenzenes (estragole, methyl eugenol, safrole, trans-anethole and myristicin) are a chemical hazard due to their natural occurrence in plants and their toxicity to humans [4]. Moreover, according to the European Commission regulation (EC) No. 1334/2008, the addition of three alkenylbenzenes (estragole, methyl eugenol and safrole) has been prohibited in food since 2008 [5]. The International Agency for Research on Cancer (IARC) has listed three alkenylbenzenes (methyleugenol, isoeugenol, safrole) in group II (possible carcinogens), in which safrole and isoeugenol are in group IIB and methyleugenol is in group IIA [6-7]. Therefore, the analysis of the content of alkenylbenzenes in natural spices is necessary.

Various methods are commonly used to analyze alkenylbenzene, such as: liquid chromatography (LC) coupled with optical detectors (UV-Vis, PDA), mass spectrometry (MS/MS, HRMS), capillary electrophoresis (CE), gas chromatography (GC) coupled with flame ionization detector (FID), mass spectrometry (MS/MS, HRMS) [4],... Among them, GC-HRMS is a modern analytical method, with high resolution and mass accuracy, which can effectively screen and quantify alkenylbenzene compounds in spice samples.

Sample preparation is an important step in the analytical process to obtain the maximum amount of analytes in the sample matrix. Currently, the trend of using sample preparation methods with high efficiency, automation and environmental friendliness is increasingly concerned and selected. Therefore, the methods commonly used to extract alkenylbenzene from plant materials are: QuEChERS extraction, Soxhlet, solid phase microextraction (SPME), simultaneous extraction-distillation (SDE) and ultrasonic extraction (UAE) [4]. In particular, UAE is a solid-liquid extraction (SLE) method that does not require complex equipment and has a simple, time-saving procedure. In this study, a simple sample preparation procedure based on UAE was combined with the GC-HRMS method to determine eight alkenylbenzene compounds in spice products.

2. MATERIAL AND METHOD

2.1. Objective

The study selected eight alkenylbenzene compounds, including: estragol, trans-anethole, safrole, eugenol, methyleugenol, isoeugenol, acetyleugenol, myristicin due to their health risks and prevalence in various spices [2]. The samples in this study were spices and herbs commonly used in Vietnamese meals, both fresh (basil, cinnamon basil, peppermint, lemon balm, ginger, galangal) and dried (pepper, cinnamon, star anise, amomum fruit, nutmeg, zanthoxylum) collected randomly in Hanoi area.

2.2. Chemical and reagents

The chemicals used in the study were all analytical grade, including:

Standard substances: estragol, trans-anethol, eugenol, methyleugenol, isoeugenol, acetyleugenol, myristicin (Sigma Aldrich, purity > 97.0%); safrole (Chemservice, purity 99.5%); internal standard caffeine (Dr. Ehrenstorfer, purity > 99.0%); dicyclohexylmethanol as companion substance (TRC, purity > 99.0%). Ethyl acetate (AcOEt), n-hexane, acetonitrile (ACN), NaCl, anhydrous $MgSO_4$, disodium hydrocitrate sesquihydrate (Merck).

2.3. Equipment

A gas chromatography (GC) Trace GC 1300 series with an automatic sample injection unit TriPlus RSH, a sample injection port couple with high-resolution mass spectrometry system (HRMS-Q-Orbitrap) detector with a combined EI/CI ionization source and a TG-35MS separation column (30 m × 0.25 mm × 0.25 µm) from Thermo scientific and other common instrument in the laboratory were used.

2.4. Analytical method

2.4.1. Sample collection and treatment

Sample collection: 90 spice samples including black pepper (Piper nigrum) (n=15), cassia bark (Cinamomum cassia) (n=13), star anise (Illicium verum) (n=18), coriander stems and leaves (Coriandrum sativum) (n=2), dill stems and leaves (Anethum graveolens) (n=5), basil stems and leaves (Ocimum bacilicum) (n=4), water mint stems and leaves (Mentha aquatica) (n=6), Mexican mint leaves (Coleus amboinicus) (n=3), clove buds (Syzigium aromaticum) (n=2), ginger rhizomes (Zingiber officinale) (n=1), galangal rhizomes (Alpinia officinarum) (n=2), michelia seeds (Michelia tonkinensis) (n=2), prickly ash seeds (Zanthoxylum rhetsa) (n=2), tsao-ko seeds (Amomum tsao-ko) (n=1) and powdered samples of pepper (n=5), cinnamon (n=7), anise (n=2) were randomly purchased in Hanoi.

Sample treatment: Weigh accurately an appropriate amount of each homogenized sample (about 1g for dry samples, 3g for fresh samples) into a 50 mL centrifuge tube, and add 10 µl of the surrogate dicyclohexylmethanol 100 ppm. The methods selected for investigation were ultrasonic extraction with 25 mL of solvent (type of solvent, extraction time) and QuEChERS to achieve the highest extraction efficiency for 8 alkenylbenzenes. Then, the sample was centrifuged at 6000 rpm for 5 minutes, filtered through a 0.2 µm filter membrane and transferred exactly 990 µL of the filtrate into a sample vial. Add 10 µl of the internal standard caffeine 100 ppm and analyze on the GC-HRMS system.

2.4.2. Analytical condition and method validation

According to the reference [4], the GC-HRMS analysis conditions were: The mobile phase was high purity N_2 gas (99.999%) with a constant flow rate of 1 mL/min; the temperature program was: 50ºC for 3 minutes; increase 10ºC/min to 150ºC, increase 20ºC/min to 200ºC, and then 50ºC/min to 280ºC, hold for 3 minutes. The total analysis time was 25 minutes. The sample injection mode was splitless with a splitless time of 6 minutes, the injector temperature was 250oC and the injection volume was 1 µl with full scan mode (m/z 50 - 700 Da), the scan time was 200ms, the resolution was 60000 (FWHM), the mass accuracy was ≤ 5 ppm.

The method was evaluated by specificity (through IP points; chromatograms of blank, test and spiked samples), calibration curve, method detection limit (MDL) and quantification limit (MQL), repeatability (n = 6), recovery at two different concentration levels (n = 6). The evaluation was performed on two sample matrices: dry spice sample (1 g of black pepper) and fresh spice sample (3 g of mint).

2.4.3. Data processing method

The TraceFinder 5.1 in GC-HRMS sofware was used to investigate the amount of alkenylbenzenes in the sample. The results of the method validation were processed by Microsoft Excel 2016 software. The amount of alkenylbenzenes was calculated using the following formula:

$$X = \frac{C \text{ x V x } k}{m}$$

In which: V: volume of extraction solvent (mL); C: concentration of sample extract solution calculated according to standard curve (mg/L); k: dilution factor; m: mass of the analyzed sample (g); X: alkenylbenzene content in the test sample (mg/kg).

3. RESULTS AND DISCUSSION

3.1. Investigation on GC-HRMS analytical conditions

The HRMS analysis conditions were automatically optimized by injecting standard solutions of each alkenylbenzene, internal standard, companion substance at a concentration of 0.5 ppm into the Q-Orbitrap-HRMS system to select characteristic ions for each analyte (Table 1). The full scan mode (m/z 50–700 Da) was used to give the total ion chromatogram (TIC) (Figure 1). The identification of the analytes was performed by comparing the retention times of the analytes in EI mode at 70 eV with the mass spectrum library provided and updated with the GC-HRMS system (NIST MS Search 2.2). Accordingly, at least two daughter ions with mass accuracy ≤5 ppm were selected for each analyte, with corresponding retention time to perform the identification requirements for the HRMS-Q-Orbitrap technique.

***Table 1**. Alkenylbenzene analytical condition on HRMS*

Compound	***Parent ion (m/z)***	***Mass accuracy (ppm)***	***Fomular structure***	***Daughter ion (m/z)***	***Mass accuracy (ppm)***	***Fomular structure***	***Retention time (min)***
Estragol	148,08788	-2,610	$C_{10}H_{12}O$	121,06470	-0,756	C_8H_9O	11,33
				117,06976	-0,998	C_9H_9	
Trans-anethol	148,08788	-2,610	$C_{10}H_{12}O$	133,06471	-0,612	C_9H_9O	12,64
				117,06976	-0,998	C_9H_9	
Safrol	162,06728	-1,549	$C_{10}H_{10}O_2$	131,04907	-0,545	C_9H_7O	12,71
				104,06185	-1,940	C_8H_8	

Compound	***Parent ion (m/z)***	***Mass accuracy (ppm)***	***Fomular structure***	***Daughter ion (m/z)***	***Mass accuracy (ppm)***	***Fomular structure***	***Retention time (min)***
Eugenol	164,08293	-1,530	$C_{10}H_{12}O_2$	149,05960	-0,007	$C_9H_9O_2$	13,52
				131,04907	-0,545	C_9H_7O	
Methyl eugenol	178,09862	-1,186	$C_{11}H_{14}O_2$	163,07517	-1,141	$C_{10}H_{11}O_2$	14,03
				147,08032	-0,826	$C_{10}H_{11}O$	
Isoeugenol	164,08293	-1,530	$C_{10}H_{12}O_2$	149,05960	-0,007	$C_9H_9O_2$	14,56
				133,06471	-0,612	C_9H_9O	
Acetyl eugenol	164,08293	-1,530	$C_{10}H_{12}O_2$	149,05960	-0,007	$C_9H_9O_2$	15,14
				121,06470	-0,756	C_8H_9O	
Myristicin	192,07790	0,021	$C_{11}H_{12}O_3$	161,05954	-1,031	$C_{10}H_9O_2$	15,23
				119,04904	-0,852	C_8H_7O	
Dicyclohexyl-methanol	112,08818	-0,772	$C_7H_{12}O$	95,08533	-2,071	C_7H_{11}	15,91
				67,05418	-0,698	C_5H_7	
Caffein	194,07950	-1,685	$C_8H_{10}N_4O_2$	109,06334	-0,998	$C_5H_7N_3$	17,04
				67,02904	-0,518	$C_3H_3N_2$	

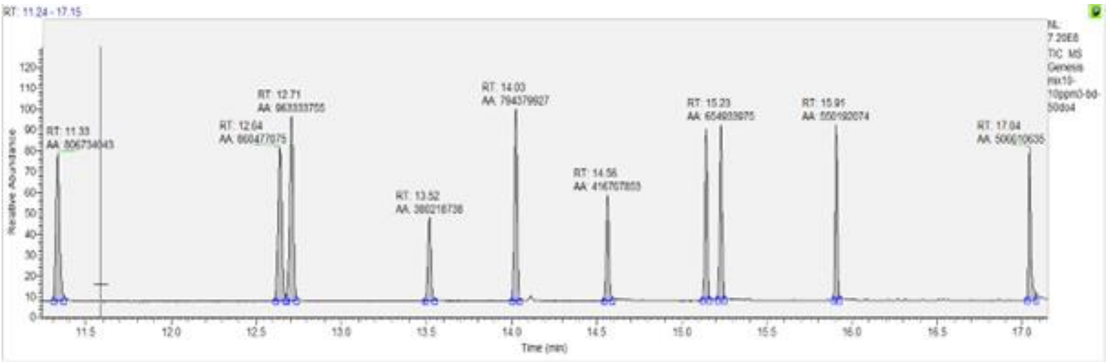


***Figure 1**. Total ion chromatogram (TIC) of the mixed 8 alkenylbenzene standard solution and internal standards and companion substances*

Some alkenylbenzenes in this study are isomers of each other and therefore have the same quantitation ion, for example trans-anethole and estragol have the molecular formula C10H12O and the quantitation ion with m/z of 148.08788. In addition, some alkenylbenzenes have similar ion fragments due to having similar chemical structures and fragmentation modes such as m/z 149.05960 common for eugenol, trans-isoeugenol and acetyl eugenol, corresponding to the loss of a methyl group. Therefore, good chromatographic separation for isomers or those with the same molecular weight or similar fragmentation is essential, in order to accurately quantify these groups of substances. Besides the separation column, the temperature program, which includes the starting temperature, also plays an important role in the separation of substances in gas chromatography. Three starting temperatures of 50°C, 60°C and 80°C were selected to investigate with a mixture of standard solutions of 8 alkenylbenzenes at a concentration of 0.5 mg/L. The results obtained showed that, when the starting temperature increased, the resolution between the two peaks of safrole and trans-anethole decreased significantly, tended to stick together, and the peaks were truncated. Therefore, the starting temperature of 50°C was chosen in this study.

3.1.2. Optimize sample treatment

Based on the reference [4], two sample preparation methods were investigated the analysis of alkenylbenzene compounds in spices by gas chromatography, which are ethyl acetate ultrasonic extraction and QuEChERS extraction (dry spice samples were added 5 mL H_2O, then 25 mL ACN, vortexed for 5 minutes, then added 4 g $MgSO_4$, 1 g NaCl, 1 g trisodium citrate trihydrate, 0.5 g disodium hydrocitrate sequihydrate to the sample, shaken vigorously by hand and vortexed for 5 minutes. Centrifuged at 6000 rpm/5 minutes, transferred 5 mL of the supernatant to a 15 mL centrifuge tube containing 4 g $MgSO_4$ and 1 g NaCl, shaken vigorously. Transferred 2 mL of the extract, dried under N_2 gas flow, reconstituted with 2 mL ethyl acetate). Some alkenylbenzenes (such as eugenol, aceteugenol...) are not stable at high temperatures especially in aqueous environments, thus, fresh spice samples were ultrasonic extracted at room temperature. Two types of dry (black pepper) and fresh (mint) spice samples were selected to evaluate the recovery efficiency at the spiked level of 62.5 µg for each alkenylbenzene standard. The results are shown in Figures 2 and 3.

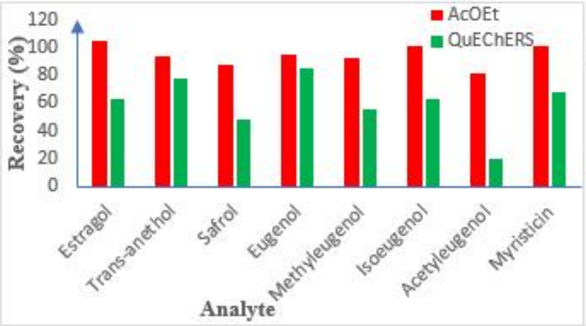


Figure 2. *Recovery of 8 investigated alkenylbenzenes with ultrasonic extraction method and QuEChERS in mint sample*

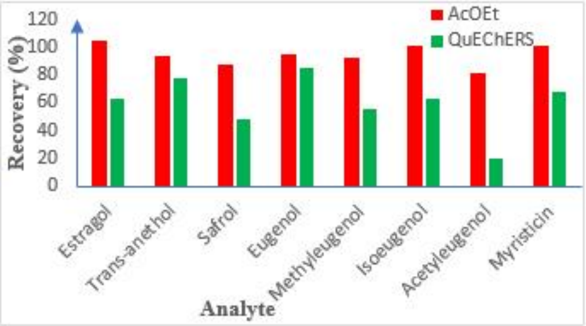
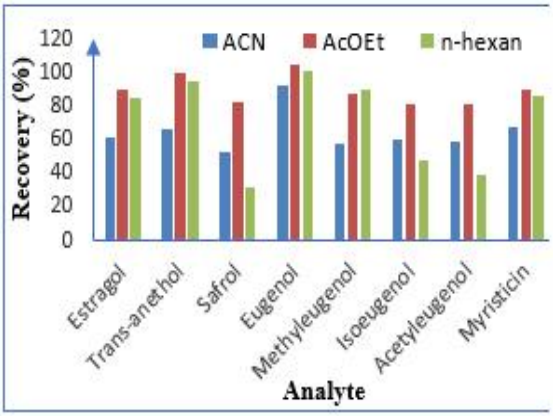


Figure 3. *Recovery of 8 alkenylbenzenes investigated with ultrasonic extraction and QuEChERS on black pepper samples*

The obtained results showed that the ultrasonic extraction method at room temperature gave higher recovery efficiency than the QuEChERS method on both sample matrices with all 8 alkenylbenzenes. Therefore, the ultrasonic extraction method at room temperature was selected for further investigation.

The solvent is also an important factor on the extraction efficiency of the analytes. Based on the study [8], 25 mL of three extraction solvents, ACN, AcOEt and n-hexane, were selected to investigate the extraction efficiency of 8 alkenylbenzenes in spice samples. The research was conducted with two representative sample matrices, dry spice (black pepper) and fresh spice (mint), then evaluate the recovery efficiency of the spiked samples. The results are shown in Figures 4 and Figure 5.



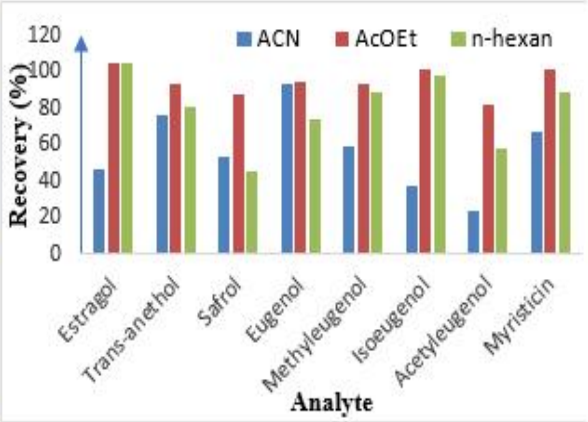


***Figure 4**. Results of investigating the influence of extraction solvent on the recovery efficiency of 8 alkenylbenzenes on mint samples*

***Figure 5**. Results of investigating the influence of extraction solvent on the recovery efficiency of 8 alkenylbenzenes on black pepper*

According to the results in Figures 4 and 5, among the three investigated solvents, ACN, EtOAc and n-hexane, the ACN and n-hexane gave lower recovery efficiency results than EtOAc. When extracted with ACN, the recovery efficiency of 8 alkenylbenzenes was the lowest almost less than 50%. The recovery efficiency of 8 alkenylbenzenes was highest when using EtOAc as the extraction solvent. EtOAc is a common solvent used for GC-MS analysis and also suitable for extracting alkenylbenzenes because their log Kow values range from 2.30 to 3.53 [4]. In addition, the cost and toxicity of EtOAc are lower than those of ACN and n-hexane. Therefore, EtOAc solvent was selected to extract 8 alkenylbenzenes in this study.

In the ultrasonic extraction process, the extraction time affects the analytical results significantly. Therefore, it is necessary to investigate the appropriate extraction time to obtain the highest extraction efficiency, while minimizing the degradation and evaporation of some alkenylbenzene compounds that are easily decomposed and volatile during the extraction. Five extraction times were selected for study: 15, 30, 45, 60 and 75 minutes on two sample matrices of dried spices (black pepper) and fresh spices (mint) at the 62.5 µg for each alkenylbenzene spiked level. As the increas of the extraction time from 15 minutes to 45 minutes, the recovery efficiency increased. However, the extraction time turned to 60 and 75 minutes, the recovery rate tended to decrease. The reason was when the extraction time increases, the extraction ability also increases, however, if it took too long, it can lead to the temperature decomposition or evaporation of some analytes. The extraction time of 45 minutes gave the best extraction efficiency for the alkenylbenzene group. Therefore, the extraction time of 45 minutes was chosen in this study.

In summary, the ultrasonic extraction with AcOEt for 45 minutes at room temperature was choosed in the sample processing procedure. This is a simple and effective sample preparation method for 8 alkenylbenzene compounds (estragol, trans-anethole, safrole, eugenol, methyleugenol, isoeugenol, acetyleugenol, myristicin) and can be applied to other alkenylbenzenes such as apiol, chavicol, elemicin,... based on the similarity in chemical structure among them. This result is similar to the publications of Rivera-Pérez et al. [4] and Zhao et al. [8] in the study of alkenylbenzene determination. However, this is the first time this method has been successfully applied to fresh spice samples.

3.2. Method validation

The method was validated with parameters such as specificity, calibration curve, method detection limit (MDL), method quantification limit (MQL), repeatability and recovery.

Firstly, in the chromatographic mass spectrometry (MS) method, the number of IP points (identification points) is an important value to evaluate the specificity. The calculation of IP points in the GC-HRMS method is as follows: 1 point for GC separation technique, 1.5 points for each parent ion and 2.5 points for each daughter ion. According to the results in Table 1, each analyte has 1 parent ion and 2 daughter ions. Therefore, all analytes have IP points ≥ 7.5 meeting the requirement of IP points ($IP \geq 5$) [9]. In addition, the specificity of the method was also confirmed by analyzing blank samples, standard samples and spiked samples. The results indicated that the blank sample did not show any signal of the analytes, the retention time of 8 analytes in the standard and spiked samples did not differ significantly (< 1%). Therefore, the method has good specificity, meeting the requirements.

To determine the method detection limit (MDL) and quantification limit (MQL), the signal-to-noise ratio (S/N) were used. The MDL was determined by adding decreasing concentrations of the alkenylbenzene mixture to the blank sample (the non-analyte sample) until the signal-to-noise ratio (S/N = 3) was obtained. The MQL was determined according to the statistical theory in analytical chemistry MQL = 10 x MDL/3. The results of MDL, MQL of the analytes showed that MDL= 0.05 mg/kg, MQL= 0.15 mg/kg for the alkenylbenzene group. This detection limit is small, suitable for determining the content of the substance in the actual sample, so the method can be applied successfully to determine the alkenylbenzene group.

Based on the selected analytical conditions, the calibration curves of 8 alkenylbenzenes were constructed with a linear dependence between the peak area ratio of the analyte and the internal standard with the concentration of the analytes in the range from 0.02 to 5.0 ppm. The results of the calibration curves are shown in Table 2.

The repeatability (RSD, n = 6) and recovery (R, n = 6) at two different concentration levels were also evaluated on dry spice matrix (1 g black pepper sample) and fresh spice matrix (3 g mint sample). The results are shown in Table 2.

***Table 2**. Calibration curve, repeatability and recovery on some sample matrices*

No.	*Compound*	*Calibration curve*	*R^2*	*MDL (mg/kg)*	*MQL (mg/kg)*	*RSD (%)*	*R (%)*
1	Estragol	y= 2.0255x + 0.0266	1.0	0.05	0.15	4.10-6.30	90.6-106.9
2	Trans-anethol	y= 2.0463x + 0.0054	0.9999			2.60-4.80	90.1-106.6
3	Safrol	y= 4.8720x + 0.1314	0.9995			2.50-3.70	91.2-106.2
4	Eugenol	y= 1.1754x + 0.0164	0.9998			5.20-6.60	90.7-104.6
5	Methyleugenol	y= 1.3346x – 0.0114	1.0			1.00-1.40	91.3-105.9
6	Isoeugenol	y= 0.9270x – 0.0323	0.9999			2.60-4.70	95.5-107
7	Acetyleugenol	y= 3.5476x – 0.0204	1.0			2.50-3.00	90.6-106.3
8	Myristicin	y= 1.0139x + 0.0004	0.9997			1.80-2.00	93.3-106.1

The results in Table 2 show that all the calibration curve with correlation coefficients $R^2 > 0.99$, repeatability (RSD), and recovery (R) all meet the requirements according to AOAC [9]. The method can be applied to analyze the content of 8 alkenylbenzene in real samples (dry and fresh spices).

3.3. Evaluation results on real sample

The validated method was applied to determine the content of alkenylbenzene group in 90 spice samples randomly collected in Hanoi. The summary results are presented in Table 3.

The results obtained in Table 3 demonstrate that the main alkenylbenzenes obtained in pepper seeds are trans-anethole (83.2 - 1.30 x 103 mg/kg) and eugenol (27.0 - 552 mg/kg), a low amount of acetyleugenol (<LOQ-29.7 mg/kg), other alkenylbenzenes was detected with smaller amounts. Previously, in the study of Rivera-Pérez et al. on black pepper and peppercorn samples of Piper nigrum species, the main alkenylbenzenes were trans-anethole (20 - 50 mg/kg) and eugenol (10 - 30 mg/kg), especially, there was a black pepper sample with a methyleugenol content of up to 20.1 mg/kg [4]. The pepper samples in this study in Vietnam have higher alkenylbenzene content than those of Rivera-Pérez et al., specifically eugenol, trans-anethole and acetyleugenol. This difference may be due to different varieties, geographical regions, post-harvest processing, etc.

Similar to the pepper sample, the cassia cinnamon samples also had high concentrations of trans-anethole (264 - 5.01 x 103 mg/kg) and eugenol (93.3 - 3.58 x 103 mg/kg). In addition, 07/13 samples had acetyl eugenol levels greater than 10 mg/kg, 03/13 samples with isoeugenol contents greater than 10 mg/kg (a substance belonging to group 2B of IARC - possibly carcinogenic) and 3/13 samples with estragol levels greater than 10 mg/kg.

The anise samples contained trans-anethole (531 - 45.6 x 103 mg/kg), eugenol (36.1 - 840 mg/kg), acetyl eugenol (10.6 - 105 mg/kg), especially high levels of highly toxic alkenylbenzenes such as estragol with 10/18 samples having levels higher than 100 mg/kg, 3/18 samples with levels of 10 - 100 mg/kg. Previous studies have shown that the main component in anise oil is trans-anethole (72 - 92%), in addition, some other alkenylbenzenes also account for a large proportion such as estragol (about 2%) and cis-anethole (about 0.5%), eugenol accounts for a small proportion. This study showed similar results when most of the samples had high to very high levels of estragol and trans-anethole.

Table 3. *Summary results of 8 alkenylbenzenes in 90 spice samples*

Sample	***Content (mg/kg)***							
	Estragol	***Trans-anethol***	***Safrol***	***Eugenol***	***Methyl-eugenol***	***Iso-eugenol***	***Acetyl-eugenol***	***Myristicin***
Mint basil (n=6)	0.74-1.74	0.55-1.64	<LOD	25.0-36.4	<LOQ-0.17	0.26-0.73	0.72-1.35	<LOQ-0.31
Basil (n=4)	705-1.04 × 10^3	1.77-9.32	<LOD-0.93	0.67-5.66	19.2-37.6	<LOQ-0.48	<LOD-1.01	<LOD-<LOQ
Lemon basil (n=3)	<LOD	<LOQ-0.20	<LOQ-0.28	0.72-2.04	<LOD	<LOQ-0.46	<LOD	<LOD
Dill (n=5)	0.16-6.06	0.57-2.32	<LOD	1.06-26.7	<LOQ-1.73	0.15-0.33	0.54-1.22	13.9-24.7
Coriander (n=2)	0.66-0.85	2.35-2.35	<LOD	48.1-50.2	0.38-0.62	0.58-0.64	0.71-0.74	<LOQ
Ginger (n=1)	0.46	81.9	<LOD	6.13	<LOD	0.17	3.49	0.24
Galangal (n=2)	0.47-1.71	<LOD-0.72	<LOD	1.92-4.14	1.67-6.47	0.62-1.58	0.58-5.51	<LOD-0.20
Cinnamon (n=13)	1.44-24.8	264-5.01 × 10^3	<LOQ-0.24	93.3-3.58 × 10^3	<LOQ-1.40	0.40-16.0	2.23-160	<LOQ-0.71
Anise (n=18)	1.10-298	531-45.6 × 10^3	<LOD	36.1-840	0.23-1.81	1.95-8.12	10.6-105	0.24-1.63
Pepper (n=15)	<LOQ-8.95	83.2-1.30× 10^3	<LOD-0.42	27.0-552	<LOQ-5.24	0.24-2.96	<LOQ-29.7	<LOQ-0.43
Clove (n=2)	2.73-19.4	472-2.19 × 10^3	<LOQ-0.79	2.12 × 10^3-55.5 × 10^3	2.98-9.45	22.2-59.6	290-15.6 × 10^3	0.71-1.51
Cadarmom (n=1)	2.42	675	<LOD	42.1	<LOQ	1.68	22.9	0.41
Indian Prickly Ash(n=2)	2.89-8.24	18.9-145	13,4 × 10^3-35.0 × 10^3	91.6-104	290-452	28.0-30.4	<LOQ-6.20	3.49-4.68
Sichuan pepper (n=2)	2.60-3.60	4.27-18.7	57.5-3.24 × 10^3	5.18-17.5	0.22-9.84	5.21-10.5	2.17-5.39	1.35-1.73
Anise powder (n=2)	56.3-612	17.1 × 10^3-37.4 × 10^3	<LOD	6.3-38.6	1.77-1.94	5.85-9.53	3.79-4.70	0.83-2.61
Cinnamon powder (n=7)	<LOQ-1.24	20.3-85.6	<LOD	5.59-37.6	<LOQ-1.78	1.20-7.66	1.92-<LOQ	<LOQ-24.7
Pepper powder (n=5)	<LOQ-0.70	0.64-10.1	<LOD	<LOQ-0.18	<LOD-<LOQ	2.19-7.86	<LOD-<LOQ	<LOD-<LOQ

For the spice powder samples: pepper powder (5 samples), cinnamon powder (7 samples), anise powder (2 samples), the main alkenylbenzenes are similar as the corresponding whole spice samples but in significantly lower levels. The activities involved in processing the spice powders such as chopping, drying those created conditions for the evaporation and decomposition of the essential oils. This suggests that consuming ready-made dry spice powders will expose less to alkenylbenzenes.

In the fresh herbs, the content of alkenylbenzenes is lower than the dried spices, possibly due to the high water content in the fresh samples. Except for lemon basil containing an insignificant amount of alkenylbenzenes, the most of other studied fresh herbs only have one alkenylbenzene compound with a content greater than 10 mg/kg (mint basil with 25.0 - 36.4 mg/kg eugenol; coriander with 48.1 - 50.2 mg/kg eugenol). In addition, 03/5 samples of dill have eugenol content greater than 10 mg/kg and 5/5 samples of dill have myristicin content greater than 10 mg/kg. Especially, the cinnamon basil sample in this study has a high content of two alkenylbenzenes, which are methyleugenol with a content of 19.2 - 37.6 mg/kg and estragol up to 705 - 1.04 × 103 mg/kg. The analytical data demonstrate that myristicin can appear with mg/kg content in dill. In addition, estragole, methyleugenol and eugenol are alkenylbenzenes with high content in cinnamon basil essential oil samples, the ratio and content of estragol and methyleugenol are very different among different growing regions, between different varieties and depend on the season and solar radiation [2]. The results of this study are consistent with previous studies and show that the cinnamon basil in Vietnam under weather conditions in mid-summer in the north (the time when the sample was collected) gave a very high estragol content (over 1000 mg/kg).

The clove samples collected in this study had very high levels of eugenol (2.12×10^3 - 55.5×10^3 mg/kg) and large amounts of acetyleugenol (280 – 15.6×10^3 mg/kg), trans-anethol (472 - 2.19×10^3 mg/kg) and half of the samples had estragol levels up to 19.4 mg/kg. Indian Prickly Ash samples contained large amounts of safrole 13.4×10^3 - 35.0×10^3 mg/kg. In addition, anise seeds also had high levels of trans-anethol (18.9 - 149 mg/kg), eugenol (91.6 - 104 mg/kg), methyleugenol (290 - 452 mg/kg) and isoeugenol (28 - 30.4 mg/kg). Sichuan pepper also had high levels of safrole (57.5-3.24×10^3 mg/kg), half of the samples had high levels of trans-anethol, eugenol and isoeugenol greater than 10 mg/kg. Notably, the chemical composition of Sichuan pepper seeds and especially alkenylbenzenes has not been well studied, possibly due to the low popularity of using Sichuan pepper seeds as a spice and the low proportion of alkenylbenzenes in the essential oil. The cadarmom sample in this study had high levels of trans-anethol (675 mg/kg), eugenol (42.1 mg/kg) and acetyleugenol (22.9 mg/kg).

Based on the data analysis, most types of spices contain high levels of different alkenylbenzenes. The highest total levels were found in cloves, anise, and Indian Prickly Ash. The most common alkenylbenzenes were eugenol and trans-anethole. For the alkenylbenzenes with more toxicological concerns such as estragol, safrole, methyleugenol and isoeugenol, the results showed that they were abundant in anise, cinnamon basil, Indian Prickly Ash, sichuan pepper and at lower levels in cloves and cinnamon. However, further studies are necessary to determine the risk from exposure to alkenylbenzenes through spices used in meals, especially the spices with high toxic alkenylbenzenes identified in this study, in order to provide recommendations and control measures to protect public health.

4. CONCLUSION

The study successfully developed a high-resolution gas chromatography-mass spectrometry (GC-HRMS) method combined with ultrasonic extraction to determine the content of 8 alkenylbenzene compounds in spices commonly used in Vietnam. The advantage of the method is fast and simple sample processing procedure, and applicable to many sample matrices, including both dry and fresh spices. The method was validated to meet the requirements of AOAC. The method was applied to determine the content of 8 alkenylbenzenes in 90 spice samples popular in Vietnam. The results showed that some concern for the safety level when using regularly for anise, cinnamon, cardamom, Sichuan pepper, cloves and cinnamon. Further studies on the consumption level, content and risk of these substances in spices in Vietnam are necessary to propose management solutions, ensure quality and protect the health of consumers.

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Phát triển phương pháp sắc ký khí khối phổ phân giải cao (GC-HRMS) nhằm xác định hàm lượng một số hợp chất nhóm alkenylbenzene trong gia vị

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Tóm tắt

Alkenylbenzene là nhóm chất độc tự nhiên có thể gây đột biến gen và ung thư ở loài gặm nhấm. Alkenylbenzene có nhiều trong các loại gia vị thường xuyên được sử dụng tại Việt Nam như tiêu, húng quế, quế, thì là…. Trong nghiên cứu này, phương pháp sắc ký khí khối phổ phân giải cao (GC-HRMS) kết hợp với xử lý mẫu bằng chiết siêu âm (UAE) đã được phát triển để xác định tám alkenylbenzene (gồm: eugenol, methyl eugenol, acetyl eugenol, trans-isoeugenol, safrol, estragol, myristicin và trans-anethol) trong các loại gia vị tự nhiên. Phương pháp đã được thẩm định theo các tiêu chí của AOAC. Giới hạn phát hiện và định lượng của phương pháp tương ứng là 0,05 mg/kg và 0,15 mg/kg, độ lặp lại RSD trong khoảng 1,00 - 6,60 % và độ thu hồi trong khoảng 90,1 - 107% với 8 chất nghiên cứu, đều đạt theo yêu cầu của AOAC. Áp dụng phương pháp để xác định 8 alkenylbenzene trong 90 mẫu gia vị khô và tươi khác nhau. Kết quả cho thấy đã phát hiện cả 8 alkenylbenzene trong các mẫu. Trong đó, hàm lượng eugenol (1,86 - 3581 mg/kg), trans-anethole (0,89 - 45,6 × 10^3 mg/kg) và estragole (0,19 - 248 mg/kg) chiếm tỷ lệ lớn hơn, trong khi trans-isoeugenol (0,69 - 7,86 mg/kg) và safrole (0,15 - 0,42 mg/kg) chiếm tỷ lệ nhỏ hơn.

Từ khóa: *Alkenylbenzene, GC-HRMS, gia vị, chiết siêu âm.*