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Headspace−Solid Phase Microextraction−Gas Chromatography−Tandem Mass Spectrometry (HS-SPME-GC-MS^2) Method for the Determination of Pyrazines in Perilla Seed Oils: Impact of Roasting on the Pyrazines in Perilla Seed Oils

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ABSTRACT: A new headspace (HS)−solid phase microextraction (SPME)−gas chromatography−tandem quadrupole mass spectrometry (GC-MS^2) was established for the simultaneous characterization and quantitation of pyrazines in perilla seed oils. HS-SPME conditions such as fiber choice, extraction temperature, and adsorption times were tested. The established GC-MS^2 showed low detection limit (LOD) and high specificity, recovery, and precision for analysis of pyrazines in perilla seed oils. The LODs for the pyrazines were in the range of 0.07−22.22 ng/g oil. The relative standard deviations (RSDs) for the intra- and interday repeated analyses of pyrazines were less than 9.49 and 9.76%, respectively. The mean recoveries for spiked pyrazines in perilla seed oil were in the range of 94.6−107.92%. Perilla seed oils were obtained by mechanical pressing from perilla seeds roasted to different degrees of roasting (mild, medium, medium dark, and dark roasting). Fourteen pyrazine compounds in perilla seed oils were isolated, identified, and quantitated. Among them, 2-methyl-3-propylpyrazine, tetramethylpyrazine, and 2,3-diethyl-5-methylpyrazine were the first identified in perilla seed oils. Degree of roasting influenced greatly the composition and contents of pyrazines in perilla seed oils. In light-roasted perilla seed oil, 2,5-dimethylpyrazine was the most predominant pyrazine. However, in dark-roasted perilla seed oil, 2-methylpyrazine was the most abundant pyrazine in the oil, representing 38.3% of its total pyrazine content. Dark-roasted perilla seed oil contains 16.78 times higher quantity of pyrazines than light-roasted perilla seed oil. This represents the first report on the quantity of pyrazines in perilla seed oils.

KEYWORDS: *pyrazines, roasting, perilla seed oil, identification, quantitation, SPME, GC-MS^2*

INTRODUCTION

Perilla seed oil is an excellent source of plant-derived ω-3 fatty acid (linolenic acid, ca. 60%).[1,2] Perilla seed oil has been reported to have a range of beneficiary activities, such as antitumor, anti-inflammatory, hypotension, antiatherosclerosis, and antianaphylactic shock activities and promotion of healthy aging and better learning performance.[3−6] Perilla seed oil is a widely used condiment oil along with sesame seed oil in Asia, mainly in Korea. Perilla seed oil is generally obtained by mechanical pressing after roasting of the perilla seeds for the development of a nutty flavor. Pyrazines have been reported to impart a nut-like flavor in a range of foods roasted and baked or in many other types of thermally processed foods resulting from Maillard reactions between reducing sugars and amino or amide groups.[7−11] It has been reported that pyrazines correlated highly with roasted peanut flavor and aroma and that 2,5-dimethylpyrazine may be the best overall pyrazine to measure as a predictor of roasted peanut flavor.[12] The nutty flavor of perilla seed oil is also believed to arise mainly from pyrazines formed during the roasting process.

Quantitative information on pyrazines in a few roasted seed oils (roasted peanut oil, soybean oil, red pepper seed oil, pumpkin seed oil, and sesame oil) has been available.[10,13−16] Liu et al.[10] reported that the total pyrazine content in roasted peanut oil was 7.32 mg/100 g oil. The authors reported that 2,5-dimethylpyrazine, 2-methylpyrazine, and 2,5-dimethyl-3-ethylpyrazine were the most abundant pyrazines in roasted peanut oil.[10] Park et al.[17] analyzed volatile compounds from roasted perilla seed oils by a headspace−solid phase microextraction−gas chromatography−mass spectrometry (HS-SPME-GC-MS) analysis. The authors identified only four pyrazines (2-methylpyrazine, 2,5-dimethylpyrazine, trimethylpyrazine, and 2-ethyl-3,6-dimethylpyrazine). Kim et al.[18] also studied the effects of roasting on the headspace volatile compounds in perilla seed oil by dynamic headspace GC-MS. The authors identified 12 pyrazines from perilla seed oils. However, no attempt has been made to carry out quantitative analysis on the pyrazines in perilla seed oils.[18]

The extraction of pyrazines from the food matrix has been performed by steam distillation, solvent extraction, or SPME.[10,13,14] Steam distillation and solvent extraction for the pyrazines are labor- and time-consuming processes and may lead to possible losses of target compounds during the extraction and/or purification procedures. SPME is a simple and easy technique for the extraction of volatile compounds from the complex sample matrix. SPME has been successfully applied to the extraction of many different flavor compounds in various foods for analytical purposes.[10,19−22]

Gas chromatography coupled with either FID or single-quadrupole mass spectrometry (MS) has been used for the identification and quantitation of pyrazines in foods.[10,13−16]

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The former technique does not provide unequivocal confirmation of the identity of pyrazines and is often subject to matrix interferences. To overcome this matrix interference problem, pyrazines should be purified or selectively extracted from the samples. However, it is not easy to purify or selectively extract pyrazines from perilla seed oils. GC-MS (single-quadrupole mass system) with a selective ion monitoring (SIM) mode has been known to be more robust than GC-FID. However, GC-MS with SIM may be also sometimes unsatisfactory under certain environments where matrix interferences having identical masses with target analysis exist. A GC-MS^2 with a multiple reaction monitoring (MRM) mode has been known to be a more reliable analytical method because this method can provide high selectivity even under environments with interferences.[23−25] To our knowledge, a GC-MS^2 with MRM mode for quantitative analysis of pyrazines in foods has not been reported previously.

The objectives of this research were (1) to develop a new HS-SPME-GC-MS^2 analytical method with a MRM mode for the simultaneous characterization and quantification of pyrazines present in roasted perilla seed oils and (2) to apply the established analytical method to monitor the impact of roasting (light, medium, medium-dark, and dark roasting) on the contents of pyrazine compounds in perilla seed oils. In addition to this, pyrazine contents in seven commercially obtained perilla seed oils were also monitored to check the common roasting practice for preparing commercial perilla seed oils.

MATERIALS AND METHODS

Materials. Pyrazine, 2-methylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2-ethylpyrazine, 2,3-dimethylpyrazine, 2-ethyl-5-methylpyrazine, 2-ethyl-6-methylpyrazine, 2-ethyl-3-methylpyrazine, trimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, 2-ethyl-3,6-dimethylpyrazine, 2,3-diethylpyrazine, tetramethylpyrazine, and 2,3-diethyl-5-methylpyrazine were purchased from Sigma-Aldrich (St. Louis, MO, USA). The pyrazine standards of 2-methyl-3-*n*-propylpyrazine, 2-isobutyl-3-methylpyrazine, and 2-butyl-3-methylpyrazine were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Perilla seeds were obtained from a local farm. Soybean oil and seven different brands of commercial perilla seed oils were obtained from local markets. Solid phase microextraction (SPME) fibers of polydimethylsiloxane (PDMS), polyacrylate (PA), carboxen/polydimethyl siloxane (Carboxen/PDMS), and polydimethyl siloxane/divinylbenzene (PDMS/DVB) were purchased from Supelco Co. (Bellefonte, PA, USA).

Roasting Conditions and Extraction Process for Perilla Seed Oils. Perilla seeds (4 kg) were first roasted at 240 °C for different roasting times (8, 10, 12, and 14 min) by using an automatic roasting machine (Poongnyun Inc., Jeonju, Republic of Korea) to obtain the roasted perilla seeds with different degrees of roasting (mild, medium, medium dark, and dark roasting, respectively). The roasted perilla seeds were cooled to ambient temperature. Then, the roasted perilla seeds were transferred into a hydraulic oil press machine (Poonggin Inc., Busan, Republic of Korea). Extractions were performed at a pressure of 600 kgf/cm^2 for 20 min. The obtained oils were filtered through filter cloths to remove any particles. The oils were kept overnight at −4 °C until two distinct layers formed (clear top oil layer and viscous gummy bottom layer). The clear top layers were collected and stored in a deep freezer at −70 °C until used.

SPME. Oil sample (2.00 g) was weighed into a 30 mL capacity serum bottle. Then the bottle was sealed air-tight with a Teflon-coated rubber septum and an aluminum cap. The sample vial was placed on a hot plate (laboratory stirrer/hot plate PC-420, Corning Inc., USA) for 10 min for the equilibrium, and then the SPME needle was inserted into the headspace of the serum bottle. Then, the SPME fiber was exposed to the headspace for the predetermined time to extract the volatile pyrazine compound. The fiber was retracted into a needle and transferred immediately to an injection port of a gas chromatograph, and the absorbed volatile compounds were desorbed at 280 °C for 5 min in the injection port. A direct SPME type deactivated glass inlet liner for capillary injection port (78.75 mm × 6.5 mm, 0.55 mm i.d., Sigma-Aldrich) was used in a GC injection port. To study the optimum SPME conditions, different parameters such as fiber choice, extraction time, and extraction temperature were studied. SPME was performed with four different fibers (Carboxen/PDMS, PA, PDMS, and PDMS/DVB). The tested extraction temperatures on the hot plate were 30, 55, 65, and 75 °C. An extraction temperature above 75 °C was not tested due to the possible thermal instability of perilla seed oils. The hot plate surface temperature was checked by an infrared thermometer (model 35629-20, Oakton Instruments, Vernon Hills, IL, USA). Refined, bleached, and deodorized (RBD) soybean oils spiked with various contents of authentic pyrazines were used to obtain the calibration curves. The SPME fiber was conditioned prior to analyses according to the manufacturer's recommendations. The fiber was exposed to a headspace of the sample for 30 min and after extraction time.

Gas Chromatography−Mass Spectrometry. The GC-MS and GC-MS^2 analyses were performed using an Agilent GC-MS system comprising a gas chromatograph (GC 7890A) equipped with a triple-quadruple mass spectrometer detector (triple quadrupole 7000B mass spectrometer, Agilent Technologies, Palo Alto, CA, USA). The column used was a polar capillary column (Suplecowax 10, 60 m × 0.25 mm, 0.25 μm film thickness, Supelco Co.). The oven temperature was programmed with an initial temperature of 70 °C for 5 min, then an increase at the rate of 3 °C/min to 190 °C with a 15 min holding at final oven temperature. Helium gas was used as a carrier gas with a flow rate of 1.0 mL/min. The injection split ratio was 2:1. The injector, mass detector transfer line, and ion source temperatures were 270, 280, and 240 °C, respectively. The collision cell was operated with nitrogen at 1.5 mL/min and helium quench gas at 30.0 mL/min. The content of each pyrazine in the oils was calculated with standard calibration curves obtained from each authentic pyrazine.

Statistical Analysis. Duncan's multiple-range test was performed to ascertain the roasting impact on the pyrazines in perilla seed oils at $\alpha = 0.05$ by using an SPSS statistical analysis program (SPSS 14.0K, SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Optimization of MS^2 Transitions for Pyrazines. Pyrazines in various agricultural products have been analyzed by a GC equipped with either FID or MS after the extraction of the target compounds. GC-FID does not provide unequivocal confirmation of identity. Thus, it could not be excluded that GC-FID is possibly subject to a matrix interference problem for the analysis of pyrazines. GC-MS (a single-quadrupole mass system) with a SIM mode has been known to be more robust than GC-FID. However, GC-MS with SIM may also be sometimes unsatisfactory under certain environments with matrix interferences. GC−tandem quadrupole mass spectrometry (a triple-quadrupole mass system) with a MRM mode may be a more robust analytical method than GC-MS with a SIM mode because this method can provide high selectivity of target compounds even under the environments with matrix interferences of identical masses with target analysts.[23−25] The extraction of pyrazines from the sample matrix has been performed by steam distillation, solvent extraction, or SPME. Steam distillation and solvent extraction for the pyrazines are labor- and time-consuming processes and may lead to possible losses of target compounds during the extraction and/or purification procedures. SPME is a simple and easy technique for the extraction of volatile compounds from complex sample matrices. SPME has been successfully applied to the extraction

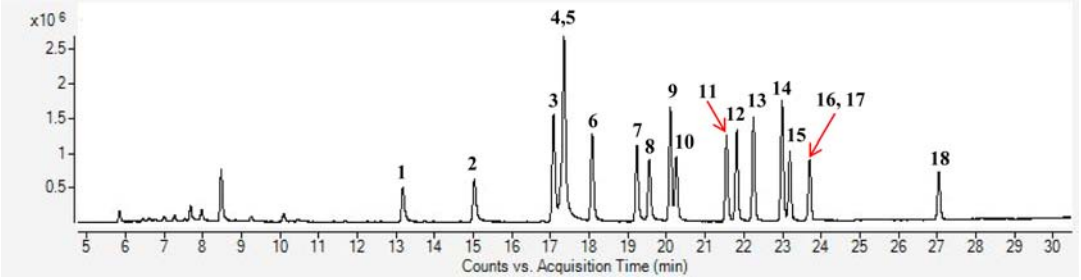

Figure 1. Total ion chromatogram (TIC) of 18 authentic pyrazaines analyzed by a HS-SPME-GC-MS. Peaks: 1, pyrazine; 2, 2-methylpyrazine; 3, 2,5-dimethylpyrazine; 4, 2,6-dimethylpyrazine; 5, 2-ethylpyrazine; 6, 2,3-dimethylpyrazine; 7, 2-ethyl-5-methylpyrazine; 8, 2-ethyl-6-methylpyrazine; 9, 2-ethyl-3-methylpyrazine; 10, trimethylpyrazine; 11, 2-ethyl-3,5-dimethylpyrazine; 12, 2-methyl-3-propylpyrazine; 13, 2,3-diethylpyrazine; 14, tetramethylpyrazine; 15, 2-ethyl-5-methylpyrazine; 16, 2-isobutyl-3-methylpyrazine; 17, 2-ethyl-3,6-dimethylpyrazine; 18, 2-butyl-3-methylpyrazine.

of many different flavor compounds in various foods for analytical purposes.[10,19−22] This study was carried out to check the usefulness of SPME and subsequent GC-MS^2 technique with a MRM mode for the isolation, identification, and quantitation of pyrazine compounds from perilla seed oils. We tried a polar wax column and a nonpolar 5% phenyl methylsiloxane column to obtain the full separation of all of these pyrazines. Full separation of all the pyrazines tested could not be achieved with either column. It was found that the polar wax column provided better separation of pyrazines than the nonpolar column (data not shown). Thus, a wax column was selected for the separation of pyrazines. Figure 1 shows a HS-SPME-GC-MS chromatogram of 18 authentic pyrazines spiked in RBD soybean oil. The 18 authentic pyrazines were pyrazine, 2-methylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2-ethylpyrazine, 2,3-dimethylpyrazine, 2-ethyl-5-methylpyrazine, 2-ethyl-6-methylpyrazine, 2-ethyl-3-methylpyrazine, trimethylpyrazine, 2,3-dimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, 2-methyl-3-propylpyrazine, tetramethylpyrazine, 2,3-diethyl-5-methylpyrazine, 2-isobutyl-3-methylpyrazine, 2-ethyl-3,6-dimethylpyrazine, and 2-butyl-3-methylpyrazine. As shown in Figure 1, 2,6-dimethylpyrazine (peak 4) and 2-ethylpyrazine (peak 5) as wll as and 2-isobutyl-3-methylpyrazine (peak 16) and 2-ethyl-3,6-dimethylpyrazine (peak 17) cannot be separated even with a 60 m polar wax column. Thus, we tried to check the possibility of separation of these coeluted pyrazines with GC-MS^2 with a MRM mode. We first selected the base peak (the most intensive ion peak) as precursor ions for each pyrazine compound on the basis of the full scan single mass spectrometry of authentic pyrazine standards to maximize their sensitivity. Thus, the precursor ion (base peak) could be either molecular ion or one of the fragment ions. Following the selection of these precursor ions for each pyrazine, their product ion scanning mass spectra were acquired by GC-MS^2 analysis with different collision-induced dissociation energies (CIDE) of 10, 20, 30, or 40 V with argon gas. The CIDE that gave the most intense product ion was chosen for each target ion of MRM transition. The most and second most intense product ions were selected for quantitative and qualitative ions at the chosen CIDE, respectively. For example, the precursor m/z 94 for 2-methylpyrazine gave the most and second most intense transition for m/z 94 → 53 at CIDE 20 V and m/z 94 → 67 at CIDE 10 V, respectively (Figure 2). Thus, the transition m/z 94 → 53 (CIDE 20 V) was selected as a quantitative ion and m/z 94 → 67 (CIDE 10 V) as a qualitative ion. With the GC-MS^2 instrument we used here, the simultaneous application of difference dissociation energies for qualitative ion and quantitative ion is possible. In a similar

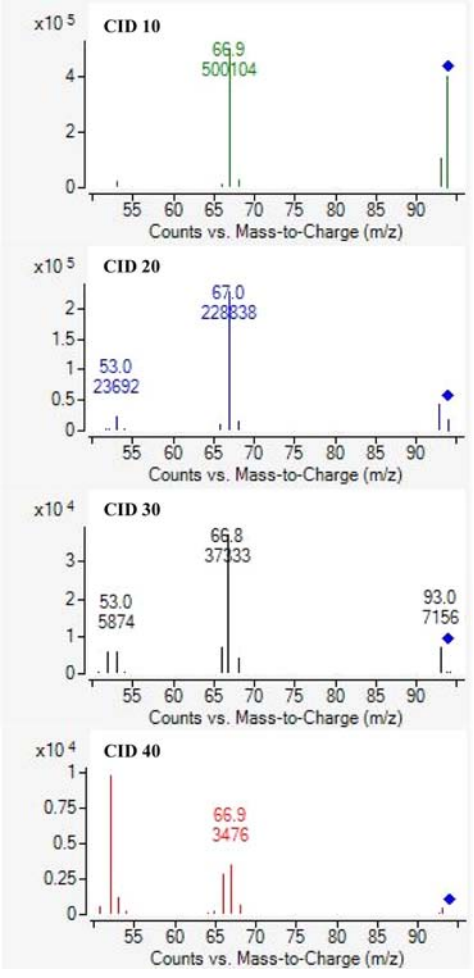

Figure 2. Fragment patterns of precursor ion for 2-methylpyrazine acquired by a gas chromatography−tadem mass spectrometry with a production ion scanning mode at the different collision-induced dissociation energies of 10, 20, 30, and 40 V.

manner, the quantitative ions and qualitative ions for all pyrazines were selected. The retention time, precursor ions, and quantitative and qualitative ions of individual pyrazines for the GC-MS^2 analysis with a MRM mode are shown in Table 1. On the basis of the elution profile, the MRM acquisition method was divided into as many time segments as possible in order to obtain the maximum signal for pyrazines that gave the lowest response. The number of transitions was restricted to maintain adequate sensitivity at the low concentrations of interest. Each

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Table 1. Retention Time and Mass Fragment Patterns of Individual Pyrazines for Gas Chromatography−Tandem Quadrupole Mass Spectrometric Analytical Conditions

		retention time and mass fragmentation						
peak	compound	retention time (min)	target ion (m/z)	qualitative ion (m/z)	quantitative ion (m/z)	qualitative collision energy (V)	quantitative collision energy (V)	scan time (cycle/s)
1	pyrazine	13.165	80	53	52	10	20	3.5
2	2-methylpyrazine	15.019	94	67	53	10	20	3.5
3	2,5-dimethylpyrazine	17.062	108	54	80	20	20	1.3
4	2,6-dimethylpyrazine	17.250	108	54	80	20	20	1.3
5	2-ethylpyrazine	17.338	107	52	79	30	20	1.3
6	2,3-dimethylpyrazine	18.070	108	67	93	30	20	1.3
7	2-ethyl-5-methylpyrazine	19.232	121	53	80	20	20	1.3
8	2-ethyl-6-methylpyrazine	19.554	121	53	80	20	20	1.3
9	2-ethyl-3-methylpyrazine	20.099	121	53	80	20	20	1.3
10	trimethylpyrazine	20.253	122	81	94	10	10	1.3
11	2-ethyl-3,5-dimethylpyrazine	21.551	135	107	80	10	20	3.2
12	2,3-diethylpyrazine	21.811	136	121	94	10	30	3.2
13	2-methyl-3-propylpyrazine	22.250	108	82	53	20	30	3.2
14	tetramethylpyrazine	22.992	136	54	95	20	10	3.2
15	2,3-diethyl-5-methylpyrazine	23.187	150	135	121	10	20	3.2
16	2-ethyl-3,6-dimethylpyrazine	23.698	135	107	66	10	20	2.5
17	2-isobutyl-3-methylpyrazine	23.699	108	53	67	20	30	2.5
18	2-butyl-3-methylpyrazine	27.046	108	80	82	30	10	2.5

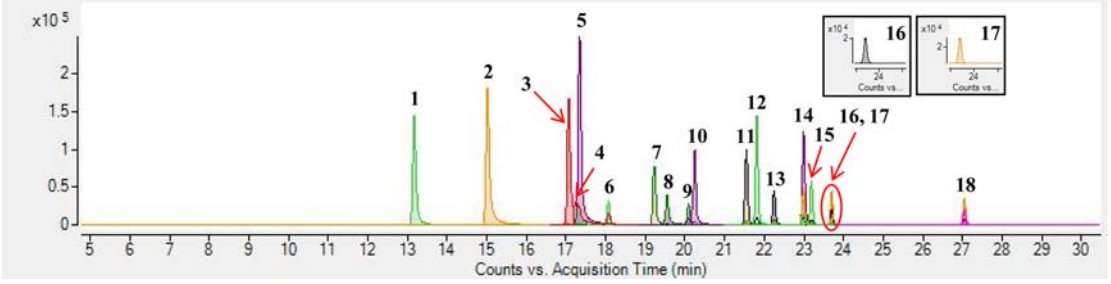

Figure 3. Extracted ion chromatogram (EIC) of 18 authentic pyrazines obtained by HS-SPME-GC-MS^2 with a MRM mode.

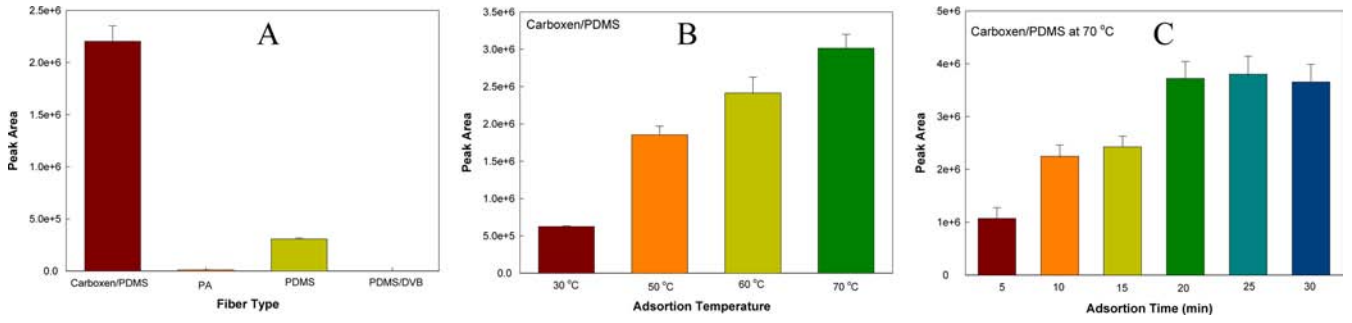

Figure 4. Total pyrazine peak areas obtained by a solid-phase microextraction (SPME) method with different fiber types (A), absorption temperatures (B), and extraction times (C).

segment contained a minimum of two and a maximum of five transitions. With this selected analytical condition, the GC-MS^2 analysis was conducted first to check the usefulness of this analytical method for the analysis of pyrazines. Figure 3 shows the extracted ion chromatograms (EIC) of GC-MS^2 with a MRM acquisition mode for the mixtures of 18 authentic pyrazines. The results clearly showed that all of the pyrazines were successfully separated by the established GC-MS^2 analysis. Note that the pyrazines (peaks 4 and 5; peaks 16 and 17) with the same retention times were separated by this MRM

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Table 2. Calibration Curves, Correlation Coefficients, Limits of Detection (LOD), and Limits of Quantification (LOQ) of 18 Authentic Pyrazines in the Tested Ranges

pyrazine	calibration curve (quadratic)	r^2	test range (ng/g)	LOD (ng/g)	LOQ (ng/g)
pyrazine	$Y = 6640.825777X + 14979.913950$	0.9993	10−500	0.64	2.14
2-methylpyrazine	$Y = 4134.722261X + 359167.774777$	0.9992	10−500	0.16	0.54
2,5-dimethylpyrazine	$Y = 2099.369293X + 52701.714995$	0.9990	10−500	0.38	1.25
2,6-dimethylpyrazine	$Y = 925.199579X + 26592.356280$	0.9995	10−500	0.38	1.25
2-ethylpyrazine	$Y = 1799.174812X + 7220.36893$	0.9996	10−500	0.11	0.36
2,3-dimethylpyrazine	$Y = 238.789022X + 310.338537$	0.9994	10−500	1.92	6.4
2-ethyl-5-methylpyrazine	$Y = 4378.233585X + 6158.541848$	0.9998	10−500	0.02	0.07
2-ethyl-6-methylpyrazine	$Y = 1873.438552X + 17379.788260$	0.9996	10−500	0.05	0.15
2-ethyl-3-methylpyrazine	$Y = 1102.098316X + 5873.354236$	0.9991	10−500	1.05	3.50
trimethylpyrazine	$Y = 593.583110X + 10831.913801$	0.9996	10−500	2.63	8.75
2-ethyl-3,5-dimethylpyrazine	$Y = 813.239882X + 3124.200259$	0.9998	10−500	3.60	12.00
2,3-diethylpyrazine	$Y = 229.484806X + 1472.896194$	0.9990	10−500	6.67	22.22
2-methyl-3-propylpyrazine	$Y = 293.655074X + 671.137109$	0.9996	10−500	1.33	4.44
tetramethylpyrazine	$Y = 790.985126X + 908.587041$	0.9996	10−500	1.03	3.43
2,3-diethyl-5-methylpyrazine	$Y = 364.060247X + 3678.014869$	0.9990	10−500	0.19	0.63
2-ethyl-3,6-dimethylpyrazine	$Y = 114.828843X + 748.150935$	0.9993	10−500	1.50	5.00
2-isobutyl-3-methylpyrazine	$Y = 189.966284X + 1040.359183$	0.9990	10−500	0.63	2.08
2-butyl-3-methylpyrazine	$Y = 178.004021X + 1196.598483$	0.9994	10−500	0.18	0.61

Table 3. Relative Standard Deviations (RSD) of Authentic Pyrazines Obtained from Six Repeated Analyses

	peak areas for the repeated analysis								
pyrazine	1st	2nd	3rd	4th	5th	6th	mean	STD	RSD (%)
pyrazine	356736	324836	294531	304939	326683	325122	322141	21409	6.65
2-methylpyrazine	474352	433123	401351	425531	473715	434528	440433	28622	6.50
2,5-dimethylpyrazine	400142	349805	339574	370644	394749	367881	370466	32422	8.75
2,6-dimethylpyrazine	101960	107572	92604	117700	131671	113408	110819	7562	6.82
2-ethylpyrazine	577139	507869	506556	549410	572646	528223	540307	40377	7.47
2,3-dimethylpyrazine	78773	71154	61162	71769	80569	71448	72479	6877	9.49
2-ethyl-5-methylpyrazine	154042	147724	143960	144764	159735	146779	149501	5095	3.41
2-ethyl-6-methylpyrazine	85298	82879	71036	78612	75291	78566	78614	5125	6.52
2-ethyl-3-methylpyrazine	59927	54736	58029	48734	51484	54357	54545	2627	4.82
trimethylpyrazine	221868	189920	194469	217614	219773	203821	207911	17282	8.31
2-ethyl-3,5-dimethylpyrazine	154464	156504	131934	145724	156594	138247	147245	10421	7.08
2,3-diethylpyrazine	240194	239967	202392	220191	235732	211274	224958	16079	7.15
2-methyl-3-propylpyrazine	81519	79126	74776	87920	89323	82625	82548	3419	4.14
tetramethylpyrazine	228873	203576	186946	516538	230097	201043	211179	17015	8.06
2,3-diethyl-5-methylpyrazine	90658	76952	77855	87183	91945	81022	84269	6534	7.75
2-ethyl-3,6-dimethylpyrazine	49199	49764	45766	50712	59639	49720	50800	4654	9.16
2-isobutyl-3-methylpyrazine	31344	31024	26217	29095	30499	27742	29320	2028	6.92
2-butyl-3-methylpyrazine	52304	46310	50036	56930	59275	52875	52955	4666	8.81

acquisition mode, suggesting the high selectivity of all tested pyrazines with the selected MRM condition of GC-MS^2 analysis.

SPME Conditions (Fiber Type, Heating Temperature, and Adsorption Time). For the optimal extraction of pyrazines, SPME was performed with different fiber types at different absorption temperatures and extraction times (Figure 4). The extractions of pyrazines were greatly dependent on the fiber type, as expected. Carboxen/PDMS fiber showed the highest peak areas for all pyrazines from the headspace of serum bottles containing soybean oil spiked with authentic pyrazines, followed by PDMS, PA, and PDMS/DVB in decreasing order (Figure 4A). Carboxen/PDMS fiber extracted about a 7 times higher quantity of pyrazines than PDMS. The extracting temperature on the hot plate (30, 50, 60, and 70 °C) also greatly influenced pyrazine extraction (Figure 4B). The higher the temperature, the higher the pyrazine peaks. We did not test extraction temperatures >70 °C for SPME due to the possible thermal instability of the perilla seed oils having high contents of thermal labile linolenic acid. The impact of different adsorption times (5, 10, 15, 20, 25, and 30 min) was also tested for the adsorption ability of pyrazines by the Carboxen/PDMS fiber (Figure 4C). The results showed that as the adsorption time increased from 5 to 20 min, the pyrazine peaks increased, but after 20 min, no further increases in pyrazine peaks were observed. Thus, the absorption time of 20 min at 70 °C with Carboxen/PDMS fiber was set as a SPME condition for pyrazines in perilla seed oils.

Calibration Curve, Limit of Detection (LOD), Limit of Quantification (LOQ), Recovery, and Reproducibility. Table 2 shows the calibration curve, correlation coefficient, and LOD and LOQ of each pyrazine over the range of interest. The correlation coefficients (r^2) of calibration curves of authentic pyrazines were >0.9998. The LOD and LOQ are defined as the

Table 4. Interday Repeatability of Analytical Method As Tested with 18 Authentic Pyrazines

pyrazine	peak areas					mean	STD	RSD (%)
	day 1	day 2	day 3	day 4	day 5			
pyrazine	357135	315103	315031	320540	317708	325103	18049	5.55
2-methylpyrazine	499352	434459	430030	441203	429509	446911	29688	6.64
2,5-dimethylpyrazine	412508	360016	369263	373518	377416	378544	20059	5.30
2,6-dimethylpyrazine	134437	119708	115554	103954	118527	118436	10905	9.20
2-ethylpyrazine	589322	506221	538817	523426	532807	538119	31160	5.79
2,3-dimethylpyrazine	77080	67066	69678	71987	72546	71671	3718	5.19
2-ethyl-5-methylpyrazine	165027	137807	145772	158470	150287	151473	10646	7.03
2-ethyl-6-methylpyrazine	78015	83903	78589	73315	79302	78625	3771	4.80
2-ethyl-3-methylpyrazine	64266	52361	51546	57650	53379	55840	5265	9.43
trimethylpyrazine	234602	195417	210718	194206	203017	207592	16490	7.94
2-ethyl-3,5-dimethylpyrazine	234740	209227	215733	262736	251672	234822	22818	9.76
2,3-diethylpyrazine	93433	73386	85273	86156	88219	85293	7371	8.64
2-methyl-3-propylpyrazine	168612	139276	141986	160467	161243	154317	12927	8.38
tetramethylpyrazine	241264	202246	208791	226206	223776	220457	15368	6.97
2,3-diethyl-5-methylpyrazine	94980	79552	84103	90012	91335	879966	6133	6.97
2-ethyl-3,6-dimethylpyrazine	56140	48544	50216	50474	56709	52417	3738	7.13
2-isobutyl-3-methylpyrazine	33082	30021	28419	32354	32106	31196	1925	6.17
2-butyl-3-methylpyrazine	61621	47648	54903	53055	56991	54844	5136	9.37

amount injected that gave a signal equivalent to 3 and 10 times the baseline noise, respectively. The LOD and LOQ were determined experimentally by injecting the serially diluted authentic samples. The LOD and LOQ for the pyrazines are in the ranges of 0.02−6.67 and 0.07−22.22 ng/g oil, respectively (Table 2). The intraday and interday repeatabilities of the pyrazine analysis by the established GC-MS^2 with a MRM mode were tested with authentic standards (Tables 3 and 4). The results showed that relative standard deviations (RSDs) for intraday and interday analyses were less than 9.49 and 9.76%, respectively, indicating the high precision of the analytical method even for the various different analytical dates. For the method validation, analysis of the perilla seed oil with authentic pyrazines spiked at the level of 2.5 μg/g oil was performed. The recovery for pyrazines from the sample matrix of perilla seed oil was in the range of 94.69−108.36% (Table 5), showing excellent recovery of pyrazines from the sample matrix.

Pyrazine Composition and Contents in Perilla Seed Oils. The totoal ion chromatogram (TIC) of GC-MS and extracted ion chromatogram (EIC) of GC-MS^2 with a MRM mode of volatile compounds in perilla seed oil are shown in Figure 5, panels A and B, respectively. The roasted perilla seed oils contained many nontarget compounds (interferences) along with pyrazines, which may interrupt the quantitation of pyrazines in oils by GC-MS analysis (Figure 5A). The pyrazines in perilla seed oils were first identified by comparing their full scan mass spectra and retention times with those of authentic samples. Fourteen pyrazines were identified in perilla seed oils by the GC-MS analysis. One (peak 10) of them is taken here as a typical example to demonstrate the identification of pyrazines found in the perilla seed oil. The full scan mass spectrum of peak 10 showed a molecular ion at m/z 122 (Figure 6C). Through the MS library search, it was found that 2-acetylpyrazine and trimethylpyrazine have the same molecular ion peak at m/z 122 (Figure 6A,B). However, these two pyrazines have different fragment ion patterns in the full scan mass spectra (Figure 6). The fragment ion pattern of peak 10 was identical to that of authentic trimethylpyrazine, but different from that of 2-acetylpyrazine. Thus, peak 10 was tentatively assigned as trimethylpyrazine. The identity of peak 10 was further confirmed by comparing the retention time of authentic trimethylpyrazine. The 14 pyrazines found in roasted perilla seed oils were pyrazine, 2-methylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2-ethylpyrazine, 2,3-dimethylpyrazine, 2-ethyl-5-methylpyrazine, 2-ethyl-6-methylpyrazine, 2-ethyl-3-methylpyrazine, trimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, 2-methyl-3-propylpyrazine, tetramethylpyrazine, and 2,3-diethyl-5-methylpyrazine. Park et al.[17] identified four pyrazines (2-methylpyrazine, 2,5-dimethylpyr-

Table 5. Recoveries of 18 Authentic Pyrazine Standards Spiked in Perilla Seed Oil

pyrazine	recovery			
	spike (μg/g)	recovered (μg/g)	recovery (%)	RSD (%)
pyrazine	2.5	2.37 ± 0.07	94.69	2.88
2-methylpyrazine	2.5	2.46 ± 0.06	98.27	2.45
2,5-dimethylpyrazine	2.5	2.37 ± 0.09	94.84	3.67
2,6-dimethylpyrazine	2.5	2.65 ± 0.39	105.92	3.07
2-ethylpyrazine	2.5	2.42 ± 0.16	96.83	6.70
2,3-dimethylpyrazine	2.5	2.39 ± 0.12	95.42	5.15
2-ethyl-5-methylpyrazine	2.5	2.42 ± 0.02	96.93	0.89
2-ethyl-6-methylpyrazine	2.5	2.61 ± 0.15	104.25	5.94
2-ethyl-3-methylpyrazine	2.5	2.52 ± 0.11	100.84	4.30
trimethylpyrazine	2.5	2.54 ± 0.07	101.70	2.75
2-ethyl-3,5-dimethylpyrazine	2.5	2.55 ± 0.17	101.87	6.49
2,3-diethylpyrazine	2.5	2.62 ± 0.16	104.98	6.13
2-methyl-3-propylpyrazine	2.5	2.63 ± 0.12	105.06	4.39
tetramethylpyrazine	2.5	2.60 ± 0.09	104.15	3.42
2,3-diethyl-5-methylpyrazine	2.5	2.70 ± 0.10	107.92	3.64
2-ethyl-3,6-dimethylpyrazine	2.5	2.64 ± 0.12	105.49	4.39
2-isobutyl-3-methylpyrazine	2.5	2.63 ± 0.10	105.21	3.73
2-butyl-3-methylpyrazine	2.5	2.71 ± 0.15	108.33	5.55

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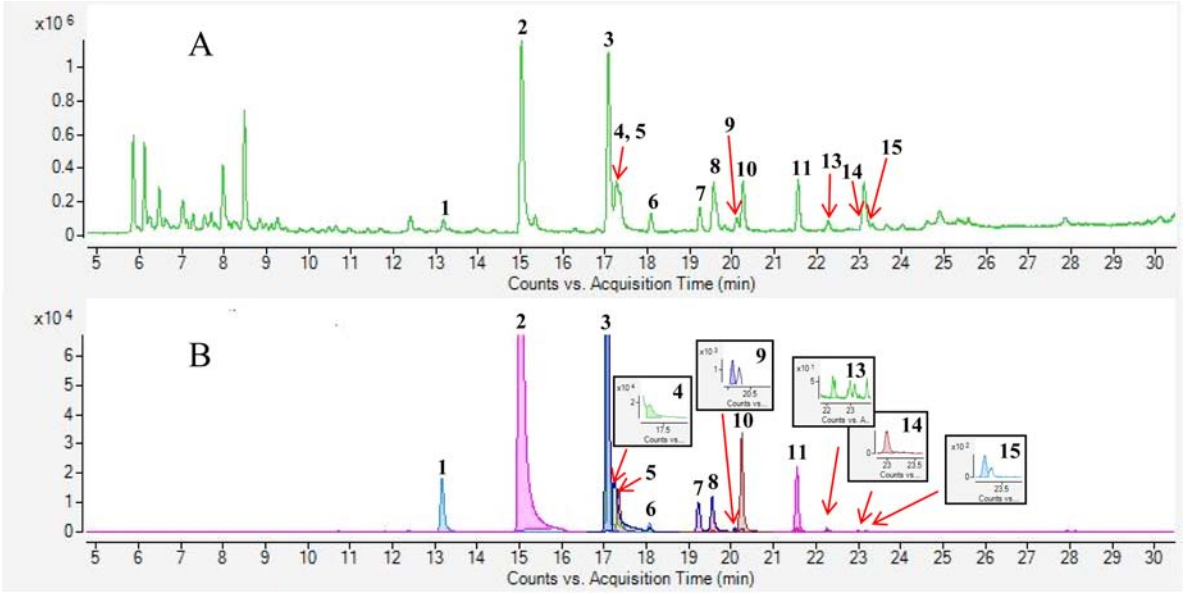

Figure 5. Total ion chromatogram (TIC) of HS-SPME-GC-MS (A) and extracted ion chromatogram (EIC) of HS-SPME-GC-MS^2 with a MRM mode (B) for the volatile compounds in perilla seed oil.

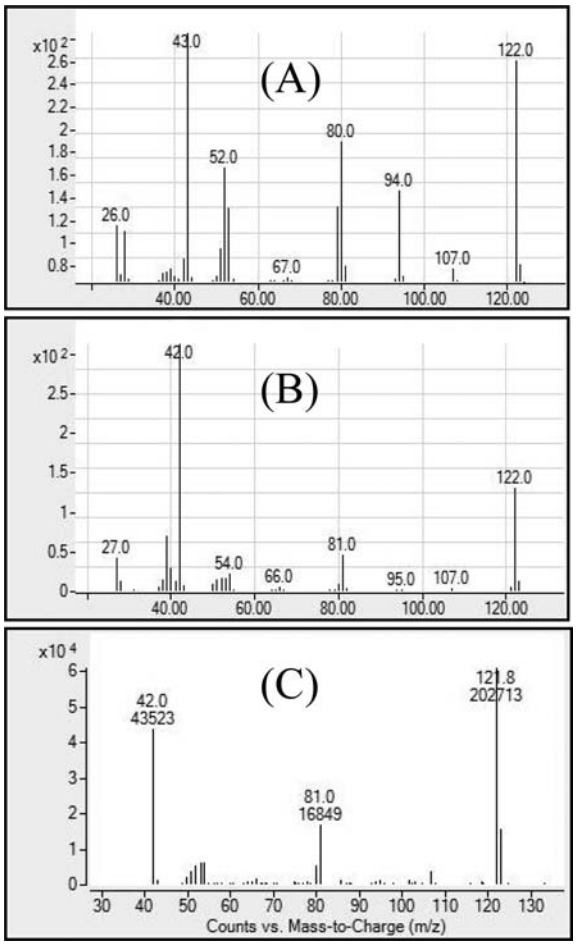

Figure 6. Full scan mass spectra of 2-acetylpyrazine (A) and trimethylpyrazine (B) from NIST library and peak 10 (C) from perilla seed oil.

azine, trimethylpyrazine, and 2-ethyl-3,6-dimethylpyrazine) in roasted perilla seed oil. Kim et al.[18] identified 12 pyrazines in the roasted perilla seed oils by a dynamic headspace gas chromatography−mass spectrometry. To our knowledge, 2-methyl-3-propylpyrazine, tetramethylpyrazine, and 2,3-diethyl-5-methylpyrazine have never been previously found in perilla seed oils. Most of the identified alkylpyrazines in the roasted perilla seed oil in the present research were also previously reported in roasted peanut volatiles.[11,12] Baker et al.[12] reported that pyrazines correlated highly with roasted peanut flavor and aroma and that 2,5-dimethylpyrazine may be the best overall pyrazine to measure as a predictor of roasted peanut flavor. Previous papers also showed that the presence of various pyrazines is a determining factor for the typical aroma of roasted pumpkin seed oils.[26−28]

The quantity of pyrazines in the perilla seed oils was analyzed by the established GC-MS^2 with a MRM mode after SPME from the headspace of the sample bottles. It was found that the pyrazine contents in perilla seed oils were far over the saturation level to the adsorption capability of Carboxen/PDMS fiber. Thus, perilla seed oil should be diluted with RBD soybean oil before analysis. In the present study, a $^1/_{20}$ dilution was required for the quantitation of pyrazines in roasted perilla seed oils. The RBD soybean (a dilution medium) did not contain any detectable quantity of pyrazines (data not shown). Table 6 shows the contents of pyrazines in perilla seed oils obtained from perilla seeds with different degrees of roasting (light, medium, medium dark, and dark). The higher the degree of roasting of the perilla seeds, the higher the pyrazine contents in the oils. The pyrazine content increased significantly as the roasting time increased, especially from medium-dark roasting to dark roasting ($p < 0.05$). The total pyrazine contents in perllia seed oils obtained from light-, medium-, medium-dark-, and dark-roasted perilla seeds were 15.45, 38.35, 75.67, and 259.48 μg pyrazines/g oil, respectively. The contents of pyrazines in a few roasted seed oils (roasted peanut oil, soybean oil, red pepper seed oil, pumpkin seed oil, and sesame oil) have been reported previously.[10,13−16] It is interesting to note that the total pyrazine contents in perilla seed oil were similar to the previously reported values of total pyrazine contents in roasted soybean oil, red pepper seed oil, and peanut

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Table 6. Pyrazine Compositions and Contents of Perilla Seed Oils Obtained from Roasted Perilla Seeds with Different Degrees of Roasting

	pyrazine contents in perilla seed oils (μg/g)			
pyrazine	light roast (265 °C, 8 min)	medium roast (265 °C, 10 min)	medium-dark roast (265 °C, 12 min)	dark roast (265 °C, 14 min)
pyrazine	0.05 ± 0.00	0.05 ± 0.00	0.27 ± 0.00	3.98 ± 0.09
2-methylpyrazine	1.11 ± 0.04	2.01 ± 0.02	7.64 ± 0.01	49.68 ± 0.37
2,5-dimethylpyrazine	2.15 ± 0.10	6.01 ± 0.19	10.32 ± 0.14	19.35 ± 0.23
2,6-dimethylpyrazine	0.81 ± 0.01	1.71 ± 0.12	4.07 ± 0.00	17.84 ± 0.03
2-ethylpyrazine	0.15 ± 0.01	0.28 ± 0.02	0.85 ± 0.03	4.38 ± 0.02
2,3-dimethylpyrazine	0.26 ± 0.01	0.52 ± 0.05	1.27 ± 0.01	4.34 ± 0.51
2-ethyl-5-methylpyrazine	0.17 ± 0.01	0.38 ± 0.00	0.98 ± 0.01	3.98 ± 0.03
2-ethyl-6-methylpyrazine	0.46 ± 0.02	1.39 ± 0.03	2.22 ± 0.08	5.23 ± 0.06
2-ethyl-3-methylpyrazine	0.17 ± 0.01	0.38 ± 0.03	0.83 ± 0.00	2.59 ± 0.18
trimethylpyrazine	1.40 ± 0.06	3.36 ± 0.01	5.27 ± 0.05	9.16 ± 0.09
2-ethyl-3,5-dimethylpyrazine	0.95 ± 0.04	2.96 ± 0.01	3.90 ± 0.07	9.01 ± 0.33
2,3-diethylpyrazine	nd	nd	nd	nd
2-methyl-3-propylpyrazine	0.01 ± 0.00	0.02 ± 0.00	0.04 ± 0.00	0.07 ± 0.00
tetramethylpyrazine	0.01 ± 0.00	0.02 ± 0.00	0.04 ± 0.00	0.09 ± 0.01
2,3-diethyl-5-methylpyrazine	0.03 ± 0.00	0.09 ± 0.00	0.14 ± 0.00	0.22 ± 0.00
2-ethyl-3,6-dimethylpyrazine	nd	nd	nd	nd
2-isobutyl-3-methylpyrazine	nd	nd	nd	nd
2-butyl-3-methylpyrazine	nd	nd	nd	nd
total pyrazines	15.45 ± 0.63	38.35 ± 0.96	75.67 ± 0.81	259.84 ± 3.90

Table 7. Pyrazine Composition and Contents in Commercial Roasted Perilla Seed Oils Obtained in Local Markets

	pyrazine contents in commercial perilla seed oils (μg/g)						
	A	B	C	D	E	F	G
pyrazine	0.43 ± 0.00	0.28 ± 0.00	0.31 ± 0.00	0.23 ± 0.00	1.20 ± 0.02	0.35 ± 0.00	0.24 ± 0.00
2-methylpyrazine	6.70 ± 0.00	5.71 ± 0.02	6.10 ± 0.05	4.76 ± 0.00	16.96 ± 0.36	6.63 ± 0.01	4.68 ± 0.01
2,5-dimethylpyrazine	7.30 ± 0.01	8.09 ± 0.01	4.75 ± 0.03	1.15 ± 0.01	9.73 ± 0.06	7.04 ± 0.03	5.95 ± 0.00
2,6-dimethylpyrazine	3.64 ± 0.03	2.92 ± 0.01	2.60 ± 0.04	2.75 ± 0.03	6.26 ± 0.01	3.36 ± 0.03	2.12 ± 0.01
2-ethylpyrazine	0.90 ± 0.00	0.74 ± 0.00	0.80 ± 0.00	0.63 ± 0.01	1.64 ± 0.00	0.67 ± 0.01	0.51 ± 0.00
2,3-dimethylpyrazine	1.30 ± 0.01	0.92 ± 0.00	1.10 ± 0.01	0.94 ± 0.00	1.75 ± 0.00	1.07 ± 0.00	0.86 ± 0.00
2-ethyl-5-methylpyrazine	1.00 ± 0.01	0.82 ± 0.00	0.82 ± 0.01	0.72 ± 0.00	1.79 ± 0.01	0.82 ± 0.00	0.63 ± 0.00
2-ethyl-6-methylpyrazine	1.73 ± 0.01	2.09 ± 0.03	1.50 ± 0.02	1.31 ± 0.00	2.09 ± 0.01	1.58 ± 0.01	1.27 ± 0.02
2-ethyl-3-methylpyrazine	0.91 ± 0.01	0.71 ± 0.00	0.72 ± 0.01	0.67 ± 0.01	1.32 ± 0.01	0.68 ± 0.05	0.58 ± 0.00
trimethylpyrazine	4.27 ± 0.01	3.18 ± 0.07	2.73 ± 0.04	3.19 ± 0.03	5.02 ± 0.00	3.88 ± 0.02	3.45 ± 0.02
2-ethyl-3,5-dimethylpyrazine	4.38 ± 0.03	3.29 ± 0.02	3.40 ± 0.01	3.92 ± 0.03	6.61 ± 0.00	3.94 ± 0.01	3.94 ± 0.01
2,3-diethylpyrazine	nd	nd	nd	nd	nd	nd	nd
2-methyl-3-propylpyrazine	nd	nd	nd	nd	nd	nd	nd
tetramethylpyrazine	0.06 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.04 ± 0.00	0.08 ± 0.00	0.04 ± 0.00	0.03 ± 0.00
2,3-diethyl-5-methylpyrazine	0.18 ± 0.00	0.13 ± 0.00	0.14 ± 0.00	0.14 ± 0.00	0.28 ± 0.00	0.12 ± 0.00	0.12 ± 0.00
2-ethyl-3,6-dimethylpyrazine	nd	nd	nd	nd	nd	nd	nd
2-isobutyl-3-methylpyrazine	nd	nd	nd	nd	nd	nd	nd
2-butyl-3-methylpyrazine	nd	nd	nd	nd	nd	nd	nd
total pyrazines	32.80 ± 0.13	28.92 ± 0.16	24.98 ± 0.21	20.47 ± 0.12	54.73 ± 0.49	30.19 ± 0.18	24.39 ± 0.08

oil. Liu et al.[10] reported that the total pyrazine content in roasted peanut oil was 73.2 μg/g oil by a HS-SPME-GC-MS analysis. Jung et al.[12] reported that the pyrazine content in red pepper seed oil increased markedly as the roasting time increased, showing 26.3, 50.1, 84.8, and 131.0 μg/g oil from red pepper seeds roasted at 210 °C for 6, 8, 10, and 12 min, respectively. The authors analyzed the pyrazine content in the oil by GC-FID after a serial extraction and purification of pyrazines from oil with solvents.

The present data showed that the pyrazine composition in perilla seed oils was greatly different with the degree of roasting. For example, in light-roasted perilla seed oil, 2,5-dimethylpyrazine was the most predominant pyrazine, followed by trimethylpyrazine and 2-methylpyrazine, in decreasing order. However, as the roasting degree increased, the contents of 2-methylpyrazine, 2,6-dimethylpyrazine, and trimethylpyrazine were greatly increased. As a result, in dark-roasted perilla seed oil, 2-methylpyrazine was the most prevalent pyrazine species, representing 38.26% of its total pyrazines (Table 6). Liu et al.[10] reported that 2,5-dimethylpyrazine, 2-methylpyrazine, and 2,5-dimethyl-3-ethylpyrazine were the most abundant pyrazines in roasted peanut oil. Our results also showed that the formations

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of pyrazine, 2,6-dimethylpyrazine, and 2-ethyl-3,5-dimethylpyrazine were also noticeable in the dark-roasted perilla seed oil. In the red pepper seed oil obtained from dark roasting, 2,5-dimethylpyrazine (34.8 μg/g of oil) was the most abundant pyrazine, followed by 2-methylpyrazine (24.5 μg/g of oil), trimethylpyrazine (14.4 μg/g of oil), and 2,6-dimethylpyrazine (13.3 μg/g of oil).[14]

In this present study, the contents of pyrazines in seven commercially obtained perilla seed oils were also analyzed (Table 7). Total pyrazine contents in seven commercial perilla seed oil were in the range of 20.47−54.73 μg/g oil. The results suggested that the commercial perilla seed oils were prepared from perilla seeds roasted to medium roasting. The identified pyrazine compounds are commonly found in numerous thermally processed foods[29] and are therefore not unique to perilla seed oil. Nevertheless, in the present research, a novel HS-SPME-GC-MS^2 analytical method has been developed for the simultaneous characterization and quantitation of pyrazines in perilla seed oils. The quantity of pyrazines in laboratory-prepared and commercially obtained perilla seed oils has been determined for the first time by this established analaytical method.

Conclusion. HS-SPME-GC-MS^2 was successfully established for the qualitative and quantitative analysis of pyrazines in perilla seed oils. The method provided high recovery, reproducibility, precision, and simultaneous confirmation of identity and quantification of pyrazines in perilla seed oils. Pyrazines in perilla seed oils with different degrees of roasting were analyzed by using the established analytical method. Fourteen pyrazines in perilla seed oils were identified and quantitated. Among them, tetramethylpyrazine and 2,3-diethyl-5-methylpyrazine were the first identified in perilla seed oils. Degree of roasting greatly influenced the composition and contents of pyrazines in perilla seed oils. In light-roasted perilla seed oil, 2,5-dimethylpyrazine was the most predominant pyrazine. However, in dark-roasted perilla seed oil, 2-methylpyrazine was the most abundant pyrazine, representing 38.3% of its total pyrazine content. The pyrazine content increased considerably as the roasting time increased, especially from medium-dark roasting to dark roasting. The analytical data of pyrazines in commercial perilla seed oils implied that all of the commercial perilla seed oils were obtained from perilla seeds with near medium roasting. The present analytical method would be useful for the quality control of the production of perilla seed oils for market purpose.

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Notes

The authors declare no competing financial interest.

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