Printed in the Republic of Korea
ANALYTICAL SCIENCE & TECHNOLOGY
Vol. 35 No. 2, 82-91, 2022
https://doi.org/10.5806/AST.2022.35.2.82

Determination of Grayanotoxin I and Grayanotoxin III in mad honey from Nepal using liquid chromatography-tandem mass spectrometry

Su Youn Ahn[1], Suncheun Kim[1], and Hwangeui Cho[2,★]

[1]*National Forensic Service, Wonju 26460, Korea*
[2]*School of Pharmacy, Jeonbuk National University, Jeonju 54896 Korea*

(Received January 17, 2022; Revised January 26, 2022; Accepted January 28, 2022)

Abstract: Grayanotoxin-contaminated honey exhibits toxicity. In this study, a reliable and sensitive liquid-chromatography–tandem-mass-spectrometric method (LC–MS/MS) was developed and validated for the quantitation of grayanotoxin I and grayanotoxin III in honey. The grayanotoxins were extracted from honey via solid phase extraction and separated on a biphenyl column with a mobile phase consisting of 0.5 % acetic acid in water and methanol. Mass spectrometric detection was performed in the multiple-reaction monitoring mode with positive electrospray ionization. The calibration curve covered the range 0.25 to 100 μg/g. The intra- and inter-day deviations were less than 10.6 %, and the accuracy was between 94.3 and 114.0 %. The validated method was successfully applied to the determination of grayanotoxins in mad honey from Nepal. The concentrations of grayanotoxin I and grayanotoxin III in 33 out of 60 mad honey samples were 0.75 – 64.86 μg/g and 0.25 – 63.99 μg/g, respectively. The method established herein would help in preventing and confirming grayanotoxin poisoning.

Key words: grayanotoxin I, grayanotoxin III, LC-MS/MS, mad honey, solid phase extraction

1. Introduction

Grayanotoxins are diterpene toxins, which are found in the leaves, pollen, flowers and nectar of several plants from the family Ericaceae. It is reported that the major origins of gryanotoxins are *Rhododendron flavum, Rhododendron ponticum, Kalmia angustifolia*, *Kalmia latifolia*, *Agauria spp., Andromeda spp., Kalmia spp., Paullinia australis and Azelea pontica,* etc.[1] Over twenty-five grayanotoxin isoforms are isolated from *Rhododendron.*[2] Grayanotoxin I and III are known to be the most toxic with a mouse LD_{50} value of 1.3 and 0.8 mg/kg (i.p.), respectively.[3,4] Grayanotoxins bind to sodium channels in cell membranes, which modifies the channel configuration and prevents sodium channel inactivation. Thus, excitable tissues such as nerve and muscle cells remain in the depolarized state, during which calcium entering the cells may be increased. These toxic effects lead to the dysfunction of cardiac and skeletal muscles, and peripheral and the central nervous system.[5,6]

Grayanotoxins-containing honey known as “mad

★ Corresponding author
Phone : +82-(0)63-219-5653 Fax : +82-(0)63-219-5638
E-mail : hecho@jbnu.ac.kr

honey" has been widely used as the traditional medicine for treatment of gastric pains, hyperglycemia, bowel disease, sexual impotence, arthritis and hypertension in Asia, Europe and North America.[6,7] However, the intake of mad honey may lead to poisoning and the symptoms of intoxication are dose dependent. Low-dose grayanotoxins cause dizziness, excessive perspiration, hypersalivation, nausea, vomiting, blurred vision, convulsions, paresthesia, hypotension and bradycardia in mild form, while high doses cause impaired consciousness and cardiac disturbances such as brady-arrhythmia, and atrioventricular block.[8-10] Grayanotoxins intoxication occurs mainly in the Black Sea region of Turkey,[11] but intoxications are also reported from Germany,[12] Austria,[13] Switzerland,[14] China,[15] and USA.[16] In Korea, twenty-six patients have been diagnosed with mad honey poisoning at the single emergency department between January 2001 and December 2015.[17] Some people consider that the mad honey from Himalaya is special as a precious folk medicine. Purchasing the mad honey by the travelers or through the internet shopping mall is still continued in Korea. The ingestion may be caused by false beliefs that mad honey has the medicinal effects. The symptoms of grayanotoxin poisoning have even been considered as the sign of body's healing process. Because the intake of mad honey might lead to grayanotoxin poisoning, an importation of mad honey has been prohibited since 2005 in Korea, and it is enshrined in Korean Food Standards Codex by the Ministry of Food and Drug Safety that the grayanotoxin III should not be detected in the honey. But the mad honey is still brought into Korea by some tourist visiting Nepal. The mad honey samples being restricted items were confiscated at Airport Customs and sent to the National Forensic Service to identify grayanotoxins in their smuggled samples. Therefore, the development of reliable analytical method for the determination of grayanotoxins in the honey was required. For quantification of grayanotoxins, TLC,[18,19] GC with derivatization.[20] LC-MS[21-23] methods have been reported. Grayanotoxins cannot be detected by their UV absorbance due to the lack of a chromophore. Although gas chromatographic application following the derivatization is a technique that has been used earlier for determining the grayanotoxins, it is labor-intensive and time-consuming. Currently, the liquid chromatography mass spectrometry (LC-MS) method has become increasingly important to several toxicological analyses due to its advantages of the improved selectivity and sensitivity on complex matrices. The LC-MS/MS method was able to detect grayanotoxins in the honey after using SPE sample preparation. However, these methods have mainly focused on grayanotoxin III or have required large sample mount (~5 g) and large solvent volume (~30 mL) for extraction. Although the dilute-and-shoot LC-MS/MS method for the determination of grayanotoxins in honey was reported in a recent study, the method showed high ion suppression and frequently required instrument cleaning due to contamination of the LC column or MS in interface.[24] In the previous study, we reported LC-MS/MS method development for the determination of grayanotoxins in rat whole blood.[25] Because the analytical methods are dependent on the sample matrix, the matrix-specific method should be developed and validated.

This study aimed to establish a selective and specific LC-tandem MS method for quantification of ayanotoxin I and grayanotoxin III in the honey which is the main cause of grayanotoxins poisoning. The proposed method was successfully applied to the quantification of grayanotoxins in mad honey smuggled out of Nepal. The information obtained from this study would be useful to prevent grayanotoxins poisoning.

2. Experimental

2.1. Materials and reagents

Grayanotoxin I was kindly provided by Chungnam National University (Daejeon, Korea). Grayanotoxin III and clindamycin (internal standard) were supplied by Sigma-Aldrich (St. Louis, MO, USA). LC-MS-grade acetic acid, methanol and acetonitrile were obtained from Fischer Scientific (Fair Lawn, NJ, USA). Deionized water was produced by using a Milli-Q apparatus (Millipore, Bedford, MA, USA). Smuggled mad honey samples were provided by

Central Customs Laboratory and Scientific Service (Seoul, Korea). Blank honey produced by different manufacturers were purchased from local markets.

2.2. LC-MS/MS conditions

The HPLC system consisted of an Agilent 1200 series with a quaternary pump, an autosampler, a vacuum degasser and a column oven (Agilent Technologies Inc., Santa Clara, CA, USA). The compounds were separated on a Phenomenex Kinetex Biphenyl column (100 × 2.1 mm, 2.6 μm). The following stepwise gradient program was carried out using the mobile phases of 0.5 % acetic acid in water (A) and 0.5 % acetic acid in methanol (B): 0 – 13 min 5 – 90 % B, 13 - 20 min 90 % B at a flow rate of 0.25 mL/min. The autosampler and the column oven were maintained at 10 °C and 40 °C, respectively. The injection volume was 5 μL.

A Sciex 3200 QTRAP mass spectrometer (Sciex, Framingham, MA, USA) equipped with a turbo spray ion source was used for the mass spectrometric analysis in the positive ion mode. The ion spray voltage was 5500 V and the source temperature was 600 °C. Source parameters such as curtain gas (CUR), ion source gas 1 (GS1), gas 2 (GS2) and collision gas (CAD) were 10, 50, 55 and medium, respectively. The optimized declustering potential (DP) and collision energy (CE) were 90 V and 25 eV for grayanotoxin I, 41 V and 17 eV for grayanotoxin III, and 60 V and 41 eV for IS, respectively. The quantitative determination using multiple reaction monitoring (MRM) was performed at the transitions of m/z 435.1/375.1 for grayanotoxin I, m/z 335.1/299.1 for grayanotoxin III and m/z 425.1/126.1 for IS with a dwell time of 100 ms. Data processing was accomplished using the Analyst 1.6.2 software (AB Sciex).

2.3. Standards preparation

Stock solutions of grayanotoxin I and III were separately prepared in methanol at a concentration of 100 μg/mL, and then diluted with methanol for the preparation of working solutions. An aqueous stock solution of clindamycin (internal standard) at 100 μg/mL was diluted with water to concentration of 10 μg/mL. The calibration standards were prepared at the final concentrations of 0.25, 0.5, 1, 5, 10 and 100 μg/g for both analytes and 1.25 μg/g for internal standard (IS) by spiking the appropriate amounts of working solutions into 0.2 g of blank honey. Quality control (QC) samples were prepared in the same way at concentrations of 0.25, 1, 10 and 80 μg/g for two grayanotoxins. All stock solutions, working solutions, calibration standards and QC samples were stored at 4 °C before use.

2.4. Sample preparation

0.2 g honey samples were precisely weighed into a 10 mL volumetric flask. 25 μL of IS (10 μg/mL) was added to sample and the total volume was made up to 10 mL with water. The sample mixture was mixed by shaking for 3 min. 1 mL of well-mixed sample was loaded on a solid-phase extraction (SPE) cartridges (adande:l PEP, 60 mg/3 mL, Shiseido, Tokyo, Japan) preconditioned with 2 ml of methanol and 2 mL of water. The cartridge was rinsed with 2 mL of water, and the analytes were eluted with 2 mL of methanol. The eluate was evaporated to dryness under N_2 at 60 °C. The residue was reconstituted with 100 μL of 0.5 % acetic acid in methanol, and an aliquot of 5 μL were injected into the LC-MS/MS.

2.5. Method validation

The established method was validated for selectivity, lower limit of quantification (LLOQ), linearity, accuracy, precision, matrix effects, stability based upon the guideline of the International Council for Harmonization.[26]

3. Results and Discussion

3.1. Optimization of sample preparation

The major challenge encountered during the analysis of grayanotoxins in honey is the presence of very high sugar content, which may decrease the selectivity and sensitivity of analytes.[27,28] In fact, during analyzing honey samples it could be seen that the interface of MS or LC column is easily contaminated because of unremoved sugar in sample preparation process.

Therefore, the optimization of the extraction process was necessary in the method development. Different extraction methods of sample pretreatment were investigated to improve selectivity and sensitivity. At the beginning, liquid-liquid extraction (LLE) was evaluated as an extraction method using different organic solvents such as dichloromethane, hexane and ethyl acetate. But liquid-liquid extraction called for frequent cycle of the instrument cleaning. Salting-out method using ammonium sulfate was examined, but peak tailings of the analytes were shown in the chromatograms. In this study, solid-phase extraction (SPE) was used for sample preparation because it yielded clean chromatograms, effective removal of interferences and adequate extraction recoveries for analytes. The extraction was examined using adande:l PEP cartridges having adsorption and partitioning effect in the reverse phase mode. The dilution of honey sample with methanol increased the viscosity of the sample and made it difficult to load the sample onto cartridge. Thus, the honey sample was diluted with appropriate amounts of distilled water. It was also important to mix until honey could completely be dissolved in water. Shaking produced well-mixed samples rather than sonication or stirring with magnetic bar. After loading a portion of well-mixed sample, the cartridge was washed with 2 mL of water for withdrawal of polar substances, and then eluted with 2 mL of methanol. This extraction method was useful in the production of clean samples, the enhancement of sensitivity, and less introduction of highly polar sugars into the LC column or MS system.

3.2. Optimization of LC-MS/MS conditions

The analytes were dissolved in MeOH with 0.5 % acetic acid, and then introduced directly into mass spectrometer for full scans in the positive ESI mode to optimize the mass conditions for ionization. The analytes exhibited predominant Q1 ion; $[M+Na]^+$ ion at m/z 435 for grayanotoxin I, $[M+H-2H_2O]^+$ ion at m/z 335 for grayantoxin III and $[M+H]^+$ ion at m/z 425 for IS. Each precursor ion was subjected to collision-induced dissociation to obtain Q3 mass spectra to determine product ions (*Fig.* 1). The most stable and abundant product ions were produced $[M+Na-CH_3COOH]^+$ ion at m/z 375 for grayano- toxin I, $[M+H-4H_2O]^+$ ion at m/z 299 for grayanotoxin III and 3-propyl-N-methylpyrrolidine ion at m/z 126 for IS, respectively. Thus, the mass transitions for quantification of m/z 435 → 375, 335 → 299 and 425 → 126 were selected for grayanotoxin I, grayanotoxin III and IS, respectively. The second most abundant transitions for confirmation purposes of m/z 435 → 357 for grayanotoxin I, m/z 335 → 281 for grayanotoxin III, and 425 → 377 for IS were chosen. The proposed fragmentations of grayanotoxin I and III are shown in *Fig.* 1 and *Fig.* 2.

The chromatographic separations of grayanotoxins were assessed on the LC columns such as Kinetex Biphenyl (100 mm × 2.1 mm, 2.6 μm), SP C18 MG III

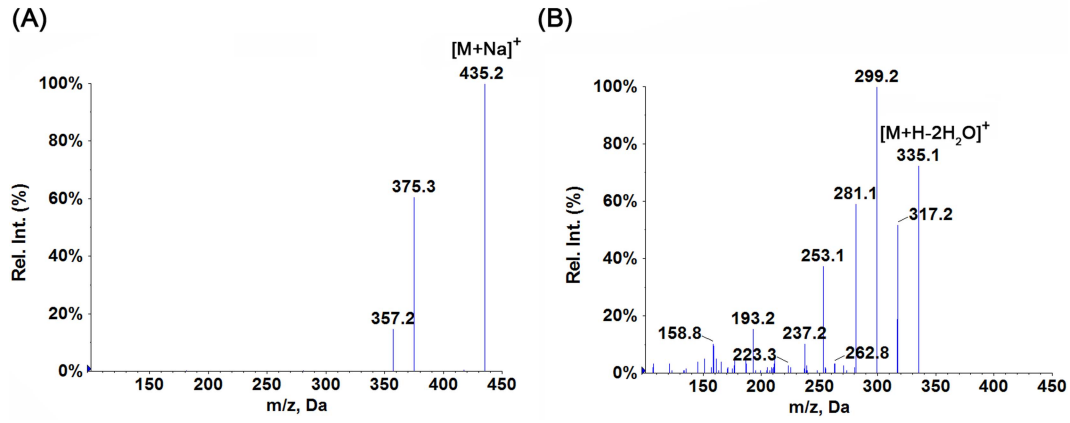

Fig. 1. Product ion mass spectra of (A) Grayanotoxin I and (B) Grayanotoxin III.

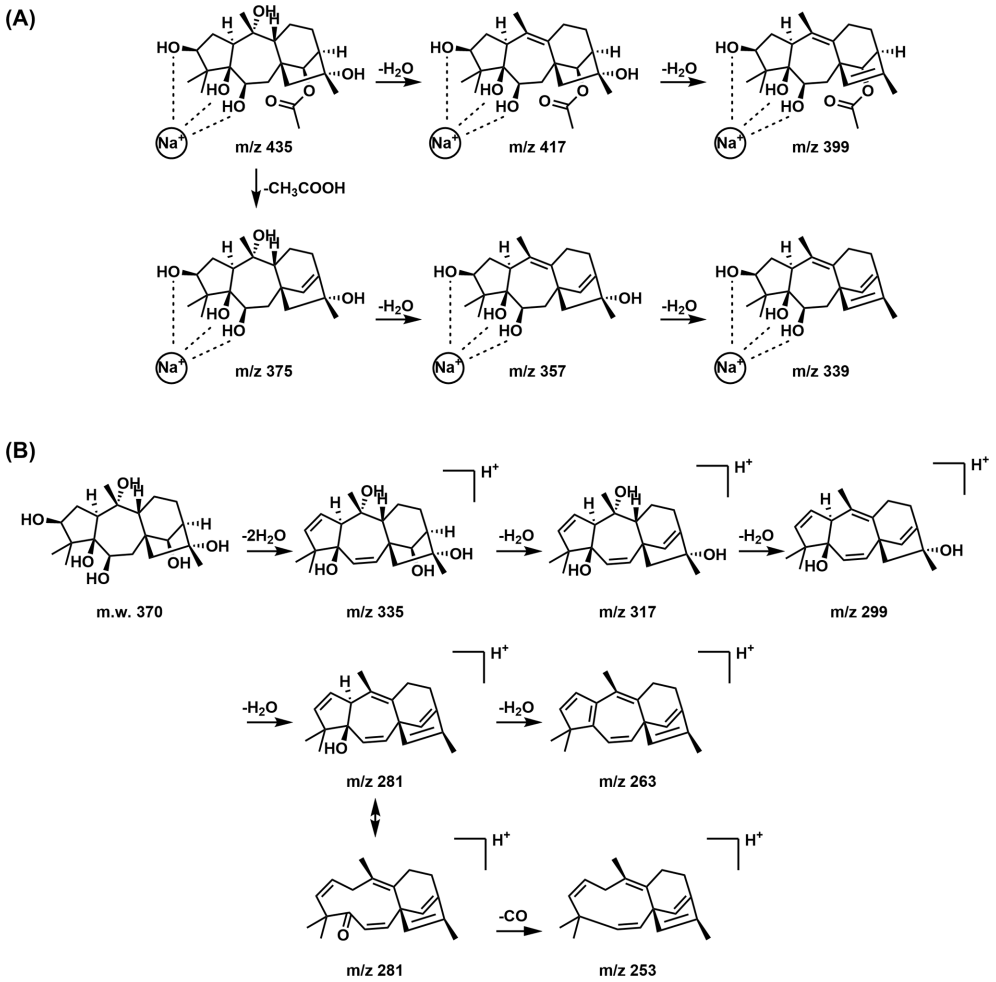

Fig. 2. Proposed fragmentation pathways of (A) Grayanotoxin I and (B) Grayanotoxin III.

(150 mm × 2.0 mm, 5 μm) and Kinetex C_{18} (100 mm × 2.1 mm, 2.6 μm). Both Kinetex Biphenyl and Kinetex C_{18} columns showed similar separation efficiency. Biphenyl column was selected as it produced slightly better peak intensities, peak shape and resolution.

The separation, ionization and sensitivity of grayanotoxin I and III were influenced by the types and composition of the mobile phase. Methanol was more efficient than acetonitrile as an organic phase for showing a higher signal response, better resolution and finer peak shape. The additives including formic acid, acetic acid, ammonium acetate and ammonium formate were tested in order to obtain the optimal chromatographic separation and the most effective ionizations of the analytes. The addition of 0.5 % acetic acid resulted in a significant increase in the intensities of the peaks and improvement in the peak symmetry. Therefore, 0.5 % acetic acid-water (v/v) and and 0.5 % acetic acid-methanol(v/v) were used as the mobile phases.

3.3. Method validation

The selectivity of this assay was examined through analyzing 6 independent blank honey samples produced by different manufacturers. No significant interference peaks from endogenous substances were observed at the elution times of analytes and IS. The typical chromatograms of the blank honey and blank honey

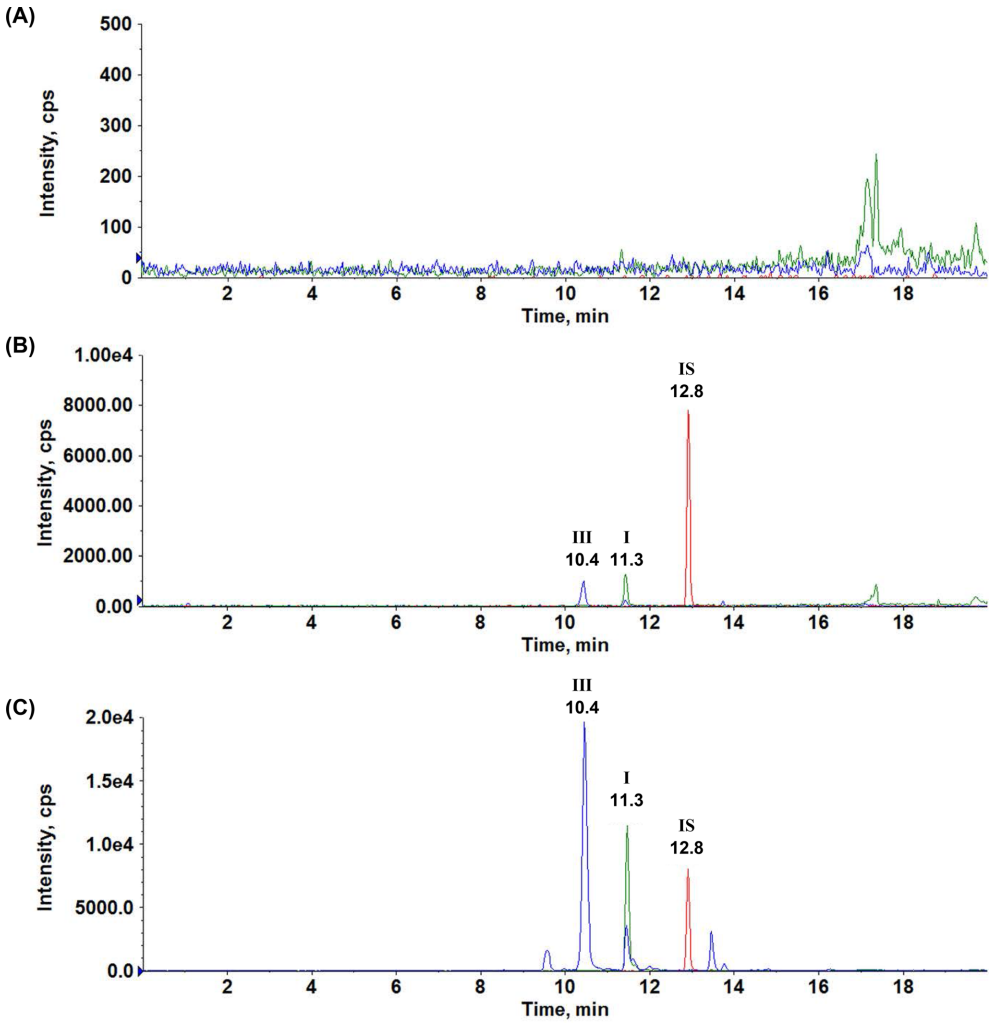

Fig. 3. Representative MRM chromatograms of Grayanotoxin I (11.3 min), Grayanotoxin III (10.4 min) and IS (12.8 min) in honey: (A) blank honey; (B) honey spiked with Grayanotoxin I and Grayanotoxin III at 0.25 μg/g (LLOQ), and IS at 1.25 μg/g; (C) mad honey sample (H28), I: Grayanotoxin I, III: Grayanotoxin I, IS: Clindamycin.

spiked at the LLOQ concentrations of grayanotoxins and IS are presented in *Fig.* 3A-B. The retention time of Grayanotoxin I, Grayanotoxin III and IS was roughly 11.3, 10.4 and 12.8 min, respectively.

The calibration curves of grayanotoxin I and III were established by the least-squared linear regression using ratio of peak area (Y) of each analyte to IS and the concentration (X), with weighting factor 1/x. The good linearity of this assay was showed over the range of 0.25 – 100 μg/g for grayanotoxin I and III with typical regression equations of $y = 0.0443x - 0.0027$ ($r^2 = 0.9995$) and $y = 0.0654x - 0.0024$ ($r^2 = 0.9997$), respectively. The LLOQ of grayanotoxin I and III in the honey were 0.25 μg/g with relative standard deviation (RSD) of not more than 12.38 % and accuracy ranging from 90.96 to 114.23 %.

The intra- and inter-day precisions and accuracies of the method for grayanotoxin I and III in honey are listed in *Table* 1. The intra- and inter-day precision and accuracy were examined at 0.25 (LLOQ), 1 (low QC), 10 (medium QC) and 80 (high QC) μg/g in six replicates on the same day and on three consecutive

Table 1. Precision and accuracy of Grayanotoxin I and Grayanotoxin III

Compounds	Nominal conc. (μg/g)	Intra-day (n=6)			Inter-day (n=3)		
		Observed conc. (Mean ± SD, μg/g)	RSD (%)	Accuracy (%)	Observed conc. (Mean ± SD, μg/g)	RSD (%)	Accuracy (%)
Grayanotoxin I	0.25	0.24 ± 0.02	8.55	97.33	0.24 ± 0.01	4.17	96.00
	1	0.94 ± 0.05	4.78	94.33	0.95 ± 0.04	3.80	95.00
	10	10.24 ± 0.35	3.44	102.39	9.91 ± 1.05	10.59	99.06
	80	85.58 ± 1.79	2.09	106.97	87.44 ± 6.88	7.87	109.30
Grayanotoxin III	0.25	0.24 ± 0.01	4.17	96.00	0.24 ± 0.02	6.28	97.33
	1	0.95 ± 0.02	2.23	95.43	0.99 ± 0.08	8.19	98.51
	10	11.00 ± 0.22	1.99	110.01	11.40 ± 0.31	2.72	114.01
	80	81.11 ± 1.54	1.90	101.39	80.84 ± 1.50	1.85	101.05

Table 2. Extraction recovery and matrix effect of Grayanotoxin I and Grayanotoxin III (n=5)

Compounds	Nominal conc. (μg/g)	Extraction recovery		Matrix effect	
		Mean ± SD (%)	RSD (%)	Mean ± SD (%)	RSD (%)
Grayanotoxin I	1	92.58 ± 6.71	7.25	87.84 ± 8.25	9.40
	10	87.44 ± 3.76	4.11	86.14 ± 3.87	4.49
	80	88.61 ± 4.69	5.03	91.81 ± 8.36	9.10
Grayanotoxin III	1	90.74 ± 7.67	8.55	85.57 ± 7.94	9.07
	10	87.34 ± 10.65	12.19	87.78 ± 7.75	8.83
	80	86.98 ± 5.26	6.60	92.53 ± 6.25	7.68
Clindamycin (IS)	1.25	95.05 ± 3.77	4.11	94.96 ± 2.76	3.28

days, respectively. The intra- and inter-day accuracies for grayanotoxin I and III ranged from 94.33 to 109.30 % and 95.43 – 114.01 %, respectively. And the intra- and inter-day precision (RSD, %) for grayanotoxin I and III were less than 10.59 % and 8.19 %, respectively. The results showed that this present method had sufficient precision and accuracy.

The extraction recoveries and matrix effects were summarized in *Table* 2. The extraction recoveries of grayanotoxin I ranged from 87.44 to 92.58 % and those of grayanotoxin III from 86.98 to 90.74 % at low, medium and high QC concentrations. The extraction recovery of the IS was 95.05 % at a concentration of 1.25 μg/g. The results indicated that the extraction method was reproducible and consistent.

The matrix effects result from co-eluting matrix substances on the ionization process, resulting in either ion suppression or ion enhancement. The matrix effects ranged from 86.14 to 91.81 %, for grayanotoxin I and from 85.57 to 92.53 % for grayanotoxin III over three QC concentrations, and the matrix effect of IS was 94.96 % at 1.25 μg/g. With these results, matrix effect from honey was negligible on the ionization of the analytes and IS in this method.

Dilution effect was evaluated to ensure that mad honey samples could be diluted with blank honey without affecting the analytical result. The accuracies of the samples diluted by 2- and 8-fold with blank honey were within 90.96 – 105.78 %. And the precisions (RSD, %) of the dilution samples were less than 9.12 %.

Evaluation of stability was carried out by analyzing QC samples at low, medium and high concentrations under the various conditions tested throughout the validation process (n=3). The RSDs of the mean test responses were within 15 % in all stability tests. The results listed in *Table* 3 suggested that the analytes in the honey were all stable after short-term storage, long-term storage, and post-preparation storage in the autosampler. Thus, the stability studies showed no significant degradation of the analytes and the

Table 3. Stability of Grayanotoxin I and Grayanotoxin III at different conditions (n=3)

Storage Conditions	Nominal conc. (μg/g)	Grayanotoxin I		Grayanotoxin III	
		Mean ± SD (%)	RSD (%)	Mean ± SD (%)	RSD (%)
Short-term (24 hr at room temperature)	1	102.10 ±3.95	3.87	94.23 ± 3.29	3.49
	10	94.99 ± 4.77	5.02	105.24 ± 8.19	7.78
	80	103.42 ± 3.84	3.71	101.54 ± 3.23	3.18
Long-term (1 month at room temperature)	1	96.61 ± 7.05	7.30	97.12 ± 5.05	5.20
	10	94.41 ± 5.57	5.90	93.33 ± 6.11	6.55
	80	103.09 ± 4.41	4.28	101.27 ± 3.68	3.63
Post-preparative (24 hr at 10 °C)	1	98.34 ± 4.08	4.15	96.46 ± 5.57	5.78
	10	91.34 ± 2.44	2.67	94.00 ± 5.29	5.63
	80	103.42 ± 3.84	3.71	101.61 ± 4.08	4.02

analytes could be processed and stored under routine laboratory conditions.

3.4. Application to the analysis of mad honey

The mad honey from Nepal being a restricted item were confiscated at Korea Airport Customs and sent to the National Forensic Service to confirm the presence of grayanotoxins in the samples. The method was successfully applied for the determination of grayanotoxins in 60 mad honey samples brought from Nepal. The chromatograms of mad honey samples are presented in *Fig.* 3C. Grayanotoxin I and III were detected in 33 samples out of 60 mad honey confiscated (*Table* 4). The amount of grayanotoxin I and III varied considerably among the mad honey samples. The concentrations of grayanotoxin I in 33 samples of mad honey were 0.75 – 64.86 μg/g with a mean content of 25.07 μg/g and the grayanotoxin III concentrations were 0.25 – 63.99 μg/g with a mean content of 17.05 μg/g. According to these results, the difference of the content of grayanotoxin I and III are up to 86 times and 255 times among the mad honey analyzed, respectively. There was no correlation between the concentrations of grayanotoxin I and grayanotoxin III. It was reported that the amount of mad honey causing grayanotoxins poisoning is between 5 and 30 g. The severity of the grayanotoxins poisoning generally depends on the amount ingested. In this study, it was found that grayanotoxins contents contained in the mad honey could be an important determinant of the cause and severity of the grayano-

Table 4. Concentrations of Grayanotoxin I and Grayanotoxin III in mad honey samples (n=5)

Sample	Grayanotoxin I	Grayanotoxin III
	Mean ± SD (μg/g)	Mean ± SD (μg/g)
H1	48.22 ± 0.35	16.45 ± 0.83
H2	54.53 ± 1.40	17.48 ± 0.70
H3	48.62 ± 2.03	15.50 ± 0.56
H4	47.73 ± 1.82	15.18 ± 0.64
H5	41.77 ± 1.80	12.22 ± 0.47
H6	30.62 ± 1.23	10.07 ± 0.41
H7	19.18 ± 1.22	42.09 ± 1.80
H8	57.53 ± 1.46	18.01 ± 0.49
H9	49.58 ± 1.15	17.72 ± 0.57
H10	64.86 ± 2.31	19.03 ± 0.91
H11	26.64 ± 6.70	42.97 ± 1.11
H12	26.52 ± 0.75	11.62 ± 0.23
H13	12.74 ± 0.40	9.10 ± 0.45
H14	26.64 ± 0.86	18.45 ± 1.10
H15	19.38 ± 2.76	21.83 ± 1.65
H16	13.87 ± 0.47	17.12 ± 0.72
H17	14.25 ± 0.34	20.38 ± 0.87
H18	15.55 ± 0.78	18.77 ± 0.85
H19	23.83 ± 5.39	14.28 ± 0.69
H20	19.03 ± 1.02	17.80 ± 0.73
H21	1.64 ± 0.29	0.30 ± 0.04
H22	1.32 ± 0.13	0.28 ± 0.06
H23	1.41 ± 0.11	0.28 ± 0.11
H24	1.60 ± 0.06	0.42 ± 0.05
H25	1.20 ± 0.07	0.26 ± 0.06
H26	19.03 ± 0.76	12.80 ± 0.92
H27	1.44 ± 0.08	0.41 ± 0.05
H28	36.05 ± 2.67	63.99 ± 1.45
H29	17.47 ± 1.16	27.69 ± 1.54
H30	20.97 ± 1.18	13.99 ± 0.66
H31	47.11 ± 2.77	39.60 ± 0.61
H32	0.75 ± 0.03	0.25 ± 0.02
H33	16.27 ± 0.84	26.36 ± 0.67

toxins poisoning. The huge variability of the grayanotoxins data among mad honey samples might occur due to production season, region or the procedure involved in processing of mad honey. The mad honey made in the spring is known to be more toxic and sometimes contain higher contents of grayanotoxins than that made in other seasons.[6,7] Although Korean honey does not contain grayanotoxins, grayanotoxins poisoning cases occur occasionally in Korea.[29,30] More careful examination and controls on mad honey should be needed because of the possibility of grayanotoxins poisoning. Also, this study might be helpful for further studies on the grayanotoxins poisoning, and useful for applications to grayanotoxins poisoning case. Recently in Korea, a 55-year-old male and a 31-year-old female hospitalized after intake of mad honey and after appropriate treatment their condition improved. By this proposed method, we confirmed that they consumed the mad honey containing grayanotoxin I and III (not reported). The determination of grayanotoxins in mad honey through a qualitative and quantitative analysis is meaningful to prevent and prove grayanotoxins poisoning.

4. Conclusions

There was a need for development of analytical methods for the determination of grayanotoxins in honey. Hence, an LC–tandem MS method was developed and validated for the determination of grayanotoxin I and III in the honey. This method provided good linearity, precision, accuracy and sensitivity for the quantitative measurement of the analytes in the honey. The method using solid phase extraction (SPE) as sample treatment could produce a clean sample, enhanced selectivity and high extraction recovery. The validated method can be used for identification and quantification of grayanotoxins in the honey suspected of containing grayanotoxins. Finally, the method was successfully applied to the determination of grayanotoxins in 60 mad honey samples brought from Nepal and finding out the grayanotoxins content present in the mad honey. Grayanotoxin I and III were detected together in 33 out of 60 mad honey samples. The only grayanotoxin III has been regulated in accordance with the related regulations. This result shows that nonexistence of both grayanotoxin I and III in honey should be evaluated for food safety. Further, this assay would help in preventing intake of mad honey as a potential poison and also confirm grayanotoxins poisoning for the forensic and clinical studies.

Conflict of Interest

The authors declare no competing financial interest.

References

1. I. Koca and A. F. Koca, *Food Chem Toxicol*, **45**(8), 1315-8 (2007).
2. Y. Qiang, B. Zhou and K. Gao, *Chem. Biodivers*., **8**(5), 792-815 (2011).
3. A. Gunduz, S. Turedi, R. M. Russell and F. A. Ayaz, *Clin. Toxicol. (Phila.),* **46**(5), 437-442 (2008).
4. H. Hikino, T. Ohta, M. Ogura, Y. Ohizumi, C. Konno and T. Takemoto, *Toxicol. Appl. Pharmacol*., **35**(2), 303-310 (1976).
5. J. Wong, E. Youde, B. Dickinson and M. Hale, 'Report of the Rhododendron feasibility study', University of Wales, Bangor, Gwynedd, LL57 2UW, UK (2002).
6. A. Gunduz, S. Turedi, H. Uzun and M. Topbas, *Am. J. Emerg. Med.,* **24**(5), 595-598 (2006).
7. E. Dilber, M. Kalyoncu, N. Yarıs and A. Ökten, *Turk. J. Med. Sci.,* **32**, 361-362(2002).
8. D. Dursunoglu, S. Gur and E. Semiz, *Ann. Emerg. Med.,* **50**(4), 484-485 (2007).
9. O. Yilmaz, M. Eser, A. Sahiner, L. Altintop and O. Yesildag, *Resuscitation*, **68**(3), 405-408 (2006).
10. K. Ergun, O. Tufekcioglu, D. Aras, S. Korkmaz and S. Pehlivan, *Int. J. Cardiol.,* **99**(2), 347-348 (2005).
11. K. E. Cagli, O. Tufekcioglu, N. Sen, D. Aras, S. Topaloglu, N. Basar and S. Pehlivan, *Tex. Heart Inst. J.,* **36**(4), 342-344 (2009).
12. H. Desel and H. Neurath, *Toxichem. Krimtech,* **65**, 63-64 (1998).
13. T. W. Weiss, P. Smetana, M. Nurnberg and K. Huber, *Int. J. Cardiol.,* **142**(1), e6-7 (2010).

14. S. Geroulanos, B. Attinger and M. Cakmakci, *Schweiz. Rundsch. Med. Prax.*, **81**(17), 535-540 (1992).
15. W. T. Poon, C. H. Ho, K. L. Yip, C. K. Lai, K. L. Cheung, R. Y. Sung, A. Y. Chan and T. W. Mak, *Hong Kong Med. J.,* **14**(5), 405-407 (2008).
16. EAPCCT, *Clin. Toxicol. (Phila.),* **46**(5), 351-421 (2008).
17. D. H. Lee, Y. H. Jin, J. C. Yoon, J. B. Lee, T. O. Jeong, S. E. Kim and T. W. Oh, *J. Korean Soc. Emerg. Med.,* **32**(3), 257-262 (2021).
18. T. Terai, *Nippon Nōgeikagaku Kaishi*, **58**(11), 1117-1122 (1984).
19. D. M. Holstege, T. Francis, B. Puschner, M. C. Booth and F. D. Galey, *J. Agric. Food Chem.,* **48**(1), 60-64 (2000).
20. T. Terai, T. Uda, J. Katakawa and T. Tetsumi, *Nippon Nōgeikagaku Kaishi,* **68**(5), 979-981 (1994).
21. S. Sibel, Y. M. Enis, S. Hüseyin, A. A. Timucin and O. Duran, *J. Ethnopharmacol.,* **156**, 155-161 (2014).
22. H. Sahin, E. A. Turumtay, O. Yildiz and S. Kolayli, *Int. J. Food Prop.,* **18**(12), 2665-2674 (2015).
23. A. B. Kurtoglu, R. Yavuz and G. A. Evrendilek, *Food Chem.,* **161**, 47-52 (2014).
24. M. Kaplan, E. O. Olgun and O. Karaoglu, *J. Agric. Food Chem.,* **62**(24), 5485-5491 (2014).
25. H. E. Cho, S. Y. Ahn, D.-W. Kim, S.-H. Woo, S.-H. Park, K. Hwang, D.-C. Moon and S. Kim, *Biomed. Chromatogr.,* **28**(12), 1624-1632 (2014).
26. ICH Guideline. Q2(R1): Validation of Analytical Procedure: Text and Methodology (Q2) (R1) in ICH Hamonised Tripartite Guideline (2005).
27. Y. E. Shim and S. W. Myung, *Anal. Sci. Technol.*, **21**(5), 424-431 (2008).
28. E. M. Lee and J. J. Ryoo, *Anal. Sci. Technol.*, **26**(1), 1-10 (2013).
29. Y. K. Choo, H. Y. Kang and S. H. Lim, *Circ. J.,* **72**(7), 1210-1211 (2008).
30. C. H. Sohn, D. W. Seo, S. M. Ryoo, J. H. Lee, W. Y. Kim, K. S. Lim and B. J. Oh, *Intern. Emerg. Med.,* **9**(2), 207-211 (2014).

Authors' Positions

Suyoun Ahn : Researcher
Suncheun Kim : Senior Researcher
Hwangeui Cho : Assistant Professor