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Development and Validation of UPLC-MS/MS Analysis for Sphingolipids Isolated from Velvet Antlers of *Cervus elaphus*

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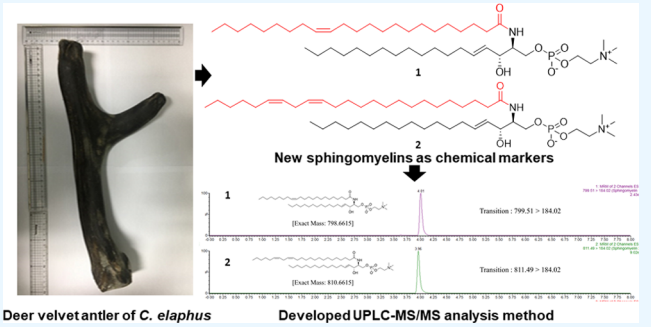
ABSTRACT: Deer velvet antlers, known as tonics, have created a large market as dietary supplements and have been consumed worldwide. Despite the high consumption of velvet antlers as dietary supplements, analytical methods for their identification and standardization remain limited. Quantitative analysis for gangliosides, considered quality indexes for velvet antlers, was developed to indirectly analyze the sialic acid obtained from chemical degradation. Owing to the complex and time-consuming chemical derivatization of gangliosides, a simple and rapid quality evaluation method for velvet antlers must be developed. For the first time, this study reports the isolation and structural elucidation of two new sphingomyelins (**1** and **2**), two known sphingomyelins (**3** and **4**), and four ceramides (**5**−**8**) as chemical markers from the velvet antlers of *Cervus elaphus*. To expedite and simplify the quality control of velvet antlers, advanced quantitative analysis of sphingolipids has been developed using ultra-performance liquid chromatography−mass spectroscopy.



1. INTRODUCTION

According to a recent market research report, the global dietary supplement market size was approximately $155.2 billion in 2022 and is expected to reach $220.8 billion in 2027.[1] Specifically, the dietary supplement market size in Korea has exceeded approximately $4.5 billion and continues to grow annually.[2]

Deer velvet antlers are most often used as dietary supplement materials for health promotion as tonics along with ginseng. They are the main ingredients in Chinese, Japanese, and Korean pharmacopeias and are consumed worldwide as dietary supplements. Velvet antlers are dried deer velvet antlers made of cartilage that has not yet matured and hardened and are native to New Zealand, China, Australia, and Canada. In Korea, approximately 214 tons of velvet antlers (as of 2019) are imported from New Zealand and Russia and used annually.[3]

Recent studies on the bioactivities of velvet antlers have reported the immunomodulatory,[4] cancer-metastasis suppression,[5] antibacterial,[6] antioxidant,[7] antiosteoporosis,[8] antifatigue,[9] and wound healing effects[10] of velvet antlers. By having various biological activities, deer velvet antlers are utilized as important dietary supplement materials and considered potential pharmaceutical agents. In Korea, it is generally used as a tonic for children and older adults for immune system and body strengthening, blood cell production, and cardiovascular health improvement.[11] Many studies have examined their nutritious ingredients comprising mineral elements, polypeptides, proteins, amino acids, and fatty acids.[12] Other chemical investigations have resulted in the isolation of nucleosides and sterols from red deer (*Cervus elaphus*)[11] and gangliosides from sika deer (*Cervus nippon*).[13]

Sphingomyelins and ceramides, generally classified as sphingolipids, play important roles in immunity, antioxidant, growth, and tumor suppression.[14] Sphingomyelins and ceramides have wide applications in the functional food and pharmaceutical industry. However, analytical methods to precisely characterize and quantify these compounds are limited.

Although the general constituents of velvet antlers are well-known to the public, chemical marker compounds that can represent their characteristics and biological effects and analytical methods for the quality control of velvet antlers remain inadequate. Several studies have developed an ultra-performance liquid chromatography time-of-flight with mass spectroscopy (UPLC-TOF-MS) method using peptide markers to distinguish velvet antlers of *C. nippon*, *C. elaphus*, and *Rangifer tarandus*[15] and gas chromatography−mass spectrometry (GC-MS) for the simultaneous determination of steroid hormones.[16] The quality control of velvet antlers has

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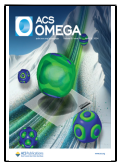

Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data (MeOD) of 1 and 2

1			2		
position	δ_H (J in Hz)	δ_C, type	position	δ_H (J in Hz)	δ_C, type
sphingosine			sphingosine		
1	3.98, m; 4.11, m	65.9, CH_2	1	3.98, m; 4.11, m	65.9, CH_2
2	3.95, m	55.2, CH	2	3.95, m	55.2, CH
3	4.04, dd (7.7, 9.1)	72.6, CH	3	4.04, dd (7.7, 9.1)	72.6, CH
4	5.45, dd (7.7, 15.3)	131.3, CH	4	5.45, dd (7.7, 15.3)	131.3, CH
5	5.71, td (6.5, 15.3)	135.1, CH	5	5.71, td (6.5, 15.3)	135.1, CH
6	2.03, m	33.5, CH_2	6	2.03, m	33.5, CH_2
7	1.38, m	30.5, CH_2	7	1.38, m	30.5, CH_2
alph. CH_2	1.29−1.38	23.8−30.9	alph. CH_2	1.29−1.38	23.7−30.9
18	0.90, t (6.9)	14.5, CH_3	18	0.90, t (6.9)	14.5, CH_3
fatty acyl			fatty acyl		
1′		175.9, C	1′		175.9, C
2′	2.18, m	37.4, CH_2	2′	2.18, m	37.4, CH_2
3′	1.58, m	27.2, CH_2	3′	1.58, m	27.2, CH_2
alph. CH_2	1.29−1.38	23.8−30.9	alph. CH_2	1.29−1.38	23.8−30.9
13′, 16′	2.03, m	28.1, CH_2	14′, 17′, 20′	2.03, m	28.2, CH_2
14′	5.34, m	130.8, CH	15′, 19′	5.35, m	131.0, CH
15′	5.34, m	130.9, CH	16′, 18′	5.35, m	130.6, CH
23′	0.90, t (6.9)	14.5, CH_3	24′	0.90, t (6.9)	14.5, CH_3
choline			choline		
1″	4.28, m	60.4, CH_2	1″	4.28, m	60.5, CH_2
2″	3.64, m	67.5, CH_2	2″	3.64, m	67.5, CH_2
N-CH_3	3.22, s	54.7, CH_3	N-CH_3	3.22, s	54.7, CH_3

often been evaluated by using gangliosides, which are known bioactive compounds of velvet antlers. Analytical methods for gangliosides have been developed to indirectly analyze the sialic acid derived from gangliosides.[17] However, these analytical methods are difficult and complex; thus, the potential application of these methods with the aforementioned markers for the routine quality control of velvet antlers and products derived from them is questionable. Owing to the time-consuming and complex process of these analysis methods, a simple and rapid improved analysis method is needed for the quality evaluation of velvet antlers.

In this study, we reported the isolation and structural elucidation of sphingosine-based components, sphingomyelins (**1**−**4**) and ceramides (**5**−**8**), as chemical markers from the velvet antlers of *C. elaphus*. Sphingomyelins **1** and **2** have never been described before, whereas ceramides **5**−**8** were reported for the first time from the deer velvet antlers of *C. elaphus*. In addition, a systematic study on the components of deer velvet antlers was conducted, and validation methods were developed for ceramides and sphingomyelins including new compounds. Consequently, the analysis method of sphingomyelins (**1**−**4**) and ceramides (**5**−**8**), active ingredients of velvet antlers from *C. elaphus*, was established and validated utilizing UPLC-MS/MS. This analytical method would contribute to the quality control development of velvet antlers from *C. elaphus*.

2. MATERIALS AND METHODS

2.1. General Experimental Procedures. Optical rotations were obtained on a JASCO DIP-1000 automatic digital polarimeter (Tokyo, Japan), and IR data were generated on a Thermo Electron US/Nicolet380 instrument (Madison, WI). Nuclear magnetic resonance (NMR) experiments were performed on a Bruker Avance III (600 MHz) spectrometer (Billerica, MA). High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) data were generated on a Waters SYNAPT G2 high-resolution mass spectrometer (Milford, MA). Thin layer chromatography (TLC) was performed on glass plates precoated with silica gel 60 F254 or RP-18 F254 (20 cm × 20 cm, 200 μm, 60 Å, Merck, Kenilworth, NJ). Vacuum-liquid chromatography (VLC) was conducted on Merck silica gel (70−230 mesh), and medium-pressure liquid chromatography (MPLC) was performed using a Biotage Isolera equipped with a reversed-phase C_{18} SNAP Cartridge KPC18-HS (120g, Uppsala, Sweden). Preparative high-performance liquid chromatography (HPLC) was performed on a Gilson system (Middleton, WI) with a Phenomenex Kinetex 5 μ C_{18} column (250 mm × 21.20 mm, 5 μm, Torrance, CA). For validation of the developed method, LC-MS/MS was performed on an Acquity UPLC system coupled with a Xevo TQD (Waters, Milford, MA) using an Acquity BEH C18 column (2.1 mm × 50 mm, 1.7 μm, Waters).

2.2. Deer Velvet Antler Materials. Dried velvet antlers (10 kg) of red deer (*C. elaphus*) from New Zealand were obtained and identified by Korea Ginseng Corporation (KGC, Daejeon, Korea). A voucher specimen (CNU-CPC, 2018.02.08) was deposited at the Laboratory of Pharmacognosy, College of Pharmacy, Chungnam National University, Daejeon, Korea.

2.3. Extraction and Isolation. Deer velvet antlers (8.8 kg) were cut into slices and extracted with EtOH (94%, v/v, 20 L) 4 times under reflux for 4 h. The crude extract was concentrated under a vacuum to yield a slurry (205.7 g). Then, a portion of the slurry (176.7 g) was subjected to silica gel VLC (high 20 cm × diameter 20 cm) and eluted with *n*-hexane/EtOAc (80:20, 60:40, 40:60, 30:70, 10:90, and 0:100, 6 L each), EtOAc-MeOH (80:20, 60:40, and 30:70, 6 L each), MeOH (22 L), and MeOH-H_2O (50:50, 3 L) to yield 10 fractions (CN-1 → CN-10). Fraction CN-4 (3.5 g), eluting with *n*-hexane/EtOAc (30:70 and 10:90), was divided into five subfractions (CN-4−1 → CN-4−5) using MPLC with a

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gradient of MeOH−H_2O (60:40, 350 mL, 90:10, 900 mL, and 100:0, 1700 mL) under a flow rate at 50 mL/min and UV detection at 205 nm. Compounds **5** (400 mg, t_R = 25.5 min) and **6** (35 mg, t_R = 22.0 min) were obtained from fraction CN-4−2 (670 mg) by HPLC eluting with MeOH (100%, 6 mL/min, UV 205 nm). Fractions CN-4−3 (218 mg) and CN-4−4 (128 mg) were purified by HPLC with the same conditions as those for CN-4−2 to afford compounds **7** (30 mg, t_R 32.0 min) and **8** (6 mg, t_R 47 min). Fraction CN-9 (32.8 g), which is eluted with 100% MeOH from the first VLC, was run over VLC (high [25 cm] × diameter [12 cm]) one more time and eluted with CH_2Cl_2−MeOH (19:1, 1 L; 10:1, 2 L), CH_2Cl_2−MeOH−H_2O (4:1:0.1, 2:1:0.1, and 2:1:0.15, 3 L each), $CHCl_3$−MeOH−9%NH_4OH (65:35:8, 5 L), CH_2Cl_2−MeOH−9%NH_4OH (2:1:0.2, 4 L), and MeOH (100%, 4 L) to yield six fractions (CN-9−1 → CN-9−6). Six fractions (CN-9−3−1 → CN-9−3−6) were collected from a portion of CN-9−3 (3 g out of total 6 g) using MPLC eluting with MeOH−H_2O (80:20, 300 mL, 100:0, 2700 mL, flow rate of 50 mL/min, UV 205 nm). Fraction CN-9−3−3 (480 mg) was purified by HPLC (100% MeOH, 6 mL/min, UV 205 nm) to afford compound **3** (280 mg, t_R 22.0 min). Compounds **2** (28 mg, t_R 42.5 min), **1** (10 mg, t_R 45.0 min), and **4** (150 mg, t_R 50.0 min) were obtained from CN-9−3−5 (260 mg) using HPLC eluting with 100% MeOH (6 mL/min, UV 205 nm).

*2.3.1. N-cis-14-Tricosenoylsphingomyelin (**1**).* White amorphous powder. ^1H and ^{13}C NMR data are shown in Table 1. ESI-MS/MS *m/z*: 833.60 $[M + Cl]^-$, 821.90 $[M + Na]^+$, 762.45 $[M - 2H_2O]^+$, 638.40 [M − phosphocholine−H_2O + $Na]^+$, 616.20 [M − phosphocholine−H_2O + $H]^+$, 239.10 [fragment at C2−C3 of sphingosine + $H]^+$, 184.05 [phosphocholine + $H]^+$, 146.70 [ethylene phosphate + $Na]^+$, 86.35 [choline]$^+$, 457.20 [M − phosphocholine − $2H_2O$ fragment at C13′−C14′−$H]^-$, and 484.35 [M − phosphocholine − $2H_2O$ fragment at C15′−C16′ + $H]^+$. HR-ESI-MS *m/z*: 799.6682 $[M + H]^+$ (calcd. for $C_{46}H_{92}N_2O_6P$, *m/z* 799.6693) and 821.6503 $[M + Na]^+$ (calcd. for $C_{46}H_{91}N_2NaO_6P$, *m/z* 821.6512).

*2.3.2. N-cis-15,18-Tetracosadienoylsphingomyelin (**2**).* White amorphous powder. ^1H and ^{13}C NMR data are shown in Table 1. ESI-MS/MS *m/z*: 833.70 $[M + Na]^+$, 774.55 $[M - 2H_2O]^+$, 650.45 [M − phosphocholine − H_2O + $Na]^+$, 264.50 [sphingosine − $2H_2O$ + $H]^+$, 239.10 [fragment at C2−C3 of sphingosine + $H]^+$, 183.75 [phosphocholine]$^+$, 146.80 [ethylene phosphate + $Na]^+$, 86.20 [choline]$^+$, and 498.55 [M − phosphocholine − $2H_2O$ fragment at C16′−C17′ + $H]^+$. HR-ESI-MS *m/z*: 811.6690 $[M + H]^+$ (calcd. for $C_{47}H_{92}N_2O_6P$, *m/z* 811.6693) and 833.6508 $[M + Na]^+$ (calcd. for $C_{47}H_{91}N_2NaO_6P$, *m/z* 833.6512).

*2.3.3. C16:0-Sphingomyelin (**3**).* White amorphous powder. One-dimensional (1D) NMR data are provided in Supporting Information. ESI-MS/MS *m/z*: 703.6 $[M + H]^+$, 725.6 $[M + Na]^+$, 737.5 $[M + Cl]^-$, and 236.9 [fragment at C2−C3−$H]^-$.

*2.3.4. C24:1-Sphingomyelin (**4**).* White amorphous powder. 1D NMR data are provided as Supporting Information. ESI-MS/MS *m/z*: 835.65 $[M + Na]^+$, 847.40 $[M + Cl]^-$, 776.40 $[M - 2H_2O]^+$, 652.55 [M − phosphocholine − H_2O + $Na]^+$, 612.35 [M − phosphocholine−$2H_2O$ + $H]^+$, and 239.10 [fragment at C2−C3 + $H]^+$.

*2.3.5. C16:0-Ceramide (**5**).* White amorphous powder. 1D NMR data are provided as Supporting Information. ESI-MS/MS *m/z*: 560.5 $[M + Na]^+$, 536.6 $[M - H]^-$, 572.4 $[M + Cl]^-$, 239.10 [fragment at C2−C3 of sphingosine + $H]^+$, and 280.50 [sphingosine − H_2O − $H]^-$.

*2.3.6. C16:1-Ceramide (**6**).* White amorphous powder. 1D NMR data are provided as Supporting Information. ESI-MS/MS *m/z*: 558.3 $[M + Na]^+$, 1094.9 $[2M + Na]^+$, 570.2 $[M + Cl]^-$, 1105.9 $[2M + Cl]^-$, and 469.60 [fragment at C10′−C11′ + $H]^+$, 280.10 [sphingosine − H_2O − $H]^-$, 237.25 [fragment at C2−C3 of sphingosine − $H]^-$.

*2.3.7. C18:0-Ceramide (**7**).* White amorphous powder. 1D NMR data are provided as Supporting Information. ESI-MS/MS *m/z*: 588.50 $[M + Na]^+$, 1154.05 $[2M + Na]^+$, 600.50 $[M + Cl]^-$, 263.35 [sphingosine − $2H_2O$ − $H]^-$, and 237.15 [fragment at C2−C3 of sphingosine − $H]^-$.

*2.3.8. C23:1-Ceramide (**8**).* White amorphous powder. 1D NMR data are provided as Supporting Information. ESI-MS/MS *m/z*: 656.6 $[M + Na]^+$, 1290.3 $[2 M + Na]^+$, 668.5 $[M + Cl]^-$, 239.10 [fragment at C2−C3 of sphingosine + $H]^+$, 488.60 [M − H_2O fragment at C14′-C15′−$H]^-$, and 263.30 [sphingosine − $2H_2O$ − $H]^-$.

2.4. ESI-MS/MS Analysis. An LCMS-8040 system (Shimadzu, Japan) with a scanning molecular mass of 100−1000 Da was utilized for the MS fragmentation of compounds. An interface voltage of −3.5 kV for the negative mode and 4.5 kV for the positive mode under a desolvation line temperature of 250 °C was used. A product-ion survey scan was performed in both positive and negative modes. Other conditions included a heating block temperature of 400 °C, a nebulizing gas flow rate of 3 L/min, and a drying gas flow rate of 15 L/min.

2.5. Validation of the Developed Method. Two new sphingomyelins (**1** and **2**), two known ones (**3** and **4**), and four ceramides (**5**−**8**) isolated from deer velvet antlers were validated in terms of linearity, precision, accuracy, and recovery by UPLC-MS/MS.

2.5.1. Materials and Reagents. The entire velvet antler sample was pulverized, passed through a 20-mesh filter to homogenize, and stored at −20 °C. The solvents used in the experiment were HPLC-grade methanol, acetonitrile, 2-propanol, and formic acid purchased from Merck (Darmstadt, Germany). Distilled water was purified by the Milli-Q Direct Water Purification system (Merck, Darmstadt, Germany), and resistance was measured to be over 18 MΩ before use.

2.5.2. Sample Preparation. To optimize the sample preparation, various solvents such as methanol, ethanol, acetonitrile, acetone, isopropanol, and hexane were used, and different extraction times (shaking, 15, 30 min, and 1 h) were applied for optimized sample extraction. As a result, 30 min of ultrasonic extraction in MeOH showed the highest yield of sphingomyelins and ceramides.

A deer velvet antler sample (1.0 g) was accurately weighed and aliquoted into a 50 mL volumetric flask. Then, 50 mL of 100% MeOH was added to the flask, and the flask was capped and shaken vigorously. Extraction was performed in an ultrasonic cleaner (60 Hz; Wiseclean, Seoul, Korea) for 30 min at room temperature. After ultrasonic extraction, the extract was moved to a 50 mL conical tube (50 mL, PP; Corning) and centrifuged for 10 min at 4000 rpm (Sorvall ST40R; Thermo Fisher Scientific). The MeOH layer was used as the analysis sample.

2.5.3. Preparation of Mixed Standard Solution and Stock Solution Preparation. A standard solution was prepared by accurately weighing sphingomyelins (**1**−**4**) and ceramides (**5**−**8**) and dissolving them in MeOH at a concentration of about 1

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mg/10 mL. The stock solution was diluted with MeOH to construct a calibration curve at 7 concentration levels, and the linear range of each compound is shown in Table 2. All solutions were stored at 4 °C.

2.5.4. UPLC-MS/MS. UPLC-MS/MS was performed on an Acquity UPLC system coupled to a Xevo TQD Mass Spectrometer (Waters) using an Acquity BEH C18 column (2.1 mm × 50 mm, 1.7 μm, Waters) maintained at 60 °C. The injection volume of the sample was 5 μL.

The gradient system consisted of solvent A [0.1% formic acid in acetonitrile/water (20:80, v/v)] and solvent B [0.1% formic acid in acetonitrile/2-propanol (20:80, v/v)] (Basit, Piomelli, & Armirotti, 2015) at a flow rate of 0.6 mL/min. The total run time was 7 min, and the gradients were as follows: 20% B (0.0−1.0 min), 20−70% B (1.0−2.5 min), 70−90% B (2.5−3.0 min), 90−90% B (3.0−5.0 min), 90−20% B (5.0−5.1 min), and 20−20% B (5.1−7.0 min). MS was performed with the positive ESI mode, and multiple-reaction monitoring (MRM) was used for the quantitative analysis. The MRM conditions of each compound are listed in Table 3. The capillary voltage was 3.0 kV, the source temperature was 120 °C, and the desolvation gas temperature was 450 °C. The flow rates of the desolvation gas and cone gas (N_2) were 800 and 20 L/h, respectively. MassLynx software (version 4.1) was used for the data analysis, and Target Lynx (version 4.1) was used for the quantitative analysis.

2.5.5. Determination of Linearity, Limit of Detection (LOD), and Limit of Quantitation (LOQ). For the linearity of the eight components including *N-cis*-14-tricosenoylsphingomyelin, we analyzed it using the prepared standard solution. A calibration curve was prepared with the peak area of each standard material, and linearity was confirmed by obtaining a correlation coefficient from the prepared calibration curve. The LOD and LOQ for each component were calculated according to Eurachem guidelines (Magnusson & Örnemark, 2014). After replicating the measurements of the reagent blanks and calculating the standard deviation of the results, each was multiplied by factors of 3 and 10.

2.5.6. Determination of Selectivity, Precision, and Accuracy. Selectivity was accurately analyzed by setting two MRM transitions for each component (Table 3). Intraday and interday precision variabilities were measured. For the intraday precision, extraction and analysis were performed on 1 day and three samples were measured 5 times each. Interday precision was measured in the same way on different dates. Relative standard deviations (RSDs) were used as a precision indicator. The acceptance criterion was a maximum of 5.0% deviation. Accuracy was performed as a recovery test. The recovery rate was measured by dispensing a mixed standard solution of three concentrations to the sample, analyzing it repeatedly 5 times for each concentration, and then comparing the added concentrations.

2.6. Analysis of Commercial Velvet Antlers. With the analysis method developed through validation, the results of four types of sphingomyelins and four types of ceramides were calculated by analyzing nine different lots of New Zealand velvet antler samples obtained from KGC in 2019.

3. RESULTS AND DISCUSSION

3.1. Isolation and Structural Elucidation. Compound **1** was isolated as a white, amorphous powder. The molecular formula of **1** was established as $C_{46}H_{91}N_2O_6P$ based on the HR-ESI-MS protonated ion at *m*/*z* 799.6682 and the sodiated

Table 2. Summary of Validation Results and Contents of Sphingomyelins and Ceramides in Deer Velvet

							intraday (n = 5)		interday (n = 5)	
no.	comp. name	linear range (ng/mL)	regression equation	R^2	LOD (ng/mL)	LOQ (ng/mL)	conc. (μg/g) ± SD.	precision (%)	accuracy (%)	precision (%)	accuracy (%)
1	*N-cis*-14-tricosenoylsphingomyelin	93.8−6000	y = 17.6870x − 2588.26	0.997	0.08	0.27	63.26 ± 0.78	1.14	102.79	2.58	95.35
2	*N-cis*-15,18-tetracosadienoylsphingomyelin	101.6−6500	y = 5.06084x − 1373.44	0.996	0.22	0.72	234.92 ± 3.01	2.86	102.28	3.17	101.04
3	C16:0-sphingomyelin	93.8−6000	y = 81.3834x − 5369.72	0.997	0.01	0.03	425.43 ± 2.61	1.07	100.55	2.03	102.93
4	C24:1-sphingomyelin	132.8−8500	y = 31.6825x − 5100.78	0.998	0.04	0.14	280.85 ± 5.18	1.51	104.01	3.48	98.63
5	C16:0-ceramide	101.6−6500	y = 7.35915x − 42.1590	0.999	0.22	0.74	295.08 ± 3.36	4.16	99.22	3.89	104.6
6	C16:1-ceramide	109.4−7000	y = 1.70723x − 26.3473	0.999	0.54	1.78	87.08 ± 2.52	4.23	107.15	4.78	108.92
7	C18:0-ceramide	93.8−6000	y = 5.05169x − 54.3468	0.999	0.36	1.21	55.02 ± 1.14	2.34	99.51	3.58	101.99
8	C23:1-ceramide	187.5−12000	y = 0.342011x − 46.8881	0.999	19.91	66.36	156.03 ± 6.70	3.08	98.89	2.63	99.94

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Table 3. Performance Characteristics of Eight Standards and UPLC-MS/MS Spectrometry Transitions for Acquisition in the MRM Mode

						MRM			
no.	comp. name	formula	exact mass	Rt (min)	ionization mode	precursor ion (m/z)	product ion (m/z)	cone voltage (V)	collision energy (ev)
1	*N-cis*-14-tricosenoylsphingomyelin	$C_{46}H_{91}N_2O_6P$	798.6615	4.01	$[M + H]^+$	799.51	184.02	50	30
					$[M + H]^+$	799.51	86.12	50	70
2	*N-cis*-15,18-tetracosadienoylsphingomyelin	$C_{47}H_{91}N_2O_6P$	810.6615	3.96	$[M + H]^+$	811.49	184.02	50	30
					$[M + H]^+$	811.49	86.12	50	70
3	C16:0-sphingomyelin	$C_{39}H_{79}N_2O_6P$	702.5676	3.75	$[M + H]^+$	703.43	184.02	50	30
					$[M + H]^+$	703.43	86.12	50	70
4	C24:1-sphingomyelin	$C_{47}H_{93}N_2O_6P$	812.6771	4.07	$[M + H]^+$	813.53	184.02	50	30
					$[M + H]^+$	813.53	86.12	50	70
5	C16:0-ceramide	$C_{34}H_{67}NO_3$	537.5121	3.84	$[M + H]^+$	538.39	264.21	25	25
					$[M + H]^+$	538.39	520.38	25	10
6	C16:1-ceramide	$C_{34}H_{65}NO_3$	535.4964	3.76	$[M + H]^+$	536.34	262.19	25	25
					$[M + H]^+$	536.34	518.36	25	10
7	C18:0-ceramide	$C_{36}H_{71}NO_3$	565.5434	3.96	$[M + H]^+$	566.41	264.21	25	25
					$[M + H]^+$	566.41	548.4	25	10
8	C23:1-ceramide	$C_{41}H_{79}NO_3$	633.606	4.12	$[M + H]^+$	634.46	616.44	25	10
					$[M + H]^+$	634.46	264.21	25	25

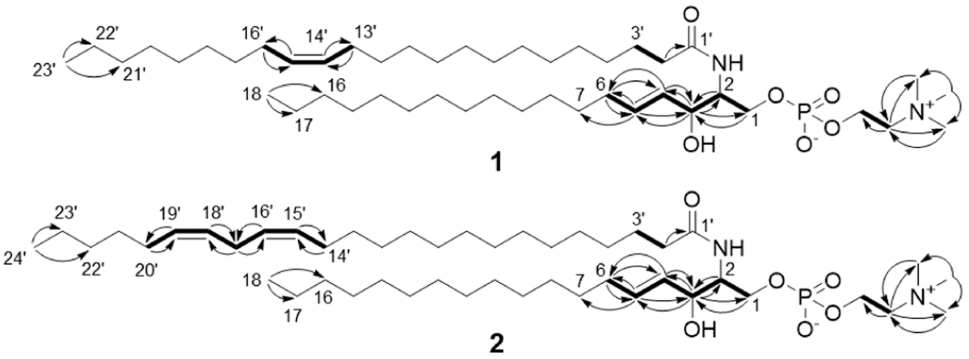

Figure 1. Key COSY (▬) and HMBC (⌒) cross-peaks for structural elucidation of **1** and **2**.

ion at m/z 821.6503 (Figure S9, Supporting Information) (calcd. $[M + H]^+$, m/z 799.6693; $[M + Na]^+$, m/z 821.6512). The ^{1}H NMR data (Table 1) of **1** displayed signals of two methyls (δ_H 0.90, 6H), aliphatic methylenes (δ_H 1.29−1.38, 1.58, 2.18), three allylic methylenes (δ_H 2.03, 6H), three *N*-methyls (δ_H 3.22, 9H), nitrogenated and oxygenated methylenes and methines (δ_H 3.64, 2H; 3.95, 1H; 3.98, 1H; 4.04, 1H; 4.11, 1H; 4.28, 2H), and two olefinic bonds (δ_H 5.34, m, 2H; 5.45, dd, J = 7.7, 15.3, 1H; 5.71, td, J = 6.5, 15.3, 1H). The ^{13}C and DEPT135 NMR data of **1** delineated resonances of two methyls (δ_C 14.5), aliphatic and allylic methylenes (δ_C 23.8−37.4), three *N*-methyls (δ_C 54.7), three nitrogenated and oxygenated methylenes (δ_C 60.4, 65.9, 67.5), two nitrogenated and oxygenated methines (δ_C 55.2, 72.6), two olefinic bonds (δ_C 130.8, 130.9, 131.3, 135.1), and an ester carbonyl (δ_C 175.9). The analysis of 1D and two-dimensional (2D) NMR data of **1** revealed clear information for a sphingosine (δ_H 3.98, 4.11, δ_C 65.9, CH_2; δ_H 3.95, δ_C 55.2, CH; δ_H 4.04, δ_C 72.6, CH; δ_H 5.45, 5.71, δ_C 131.3, 135.1, CH), a phosphocholine (δ_H 3.64, δ_C 67.5, CH_2; δ_H 4.28, δ_C 60.4, CH_2; δ_H 3.22, δ_C 54.7, CH_3), and a fatty acyl moiety (δ_C 175.9, C), which are characteristic components of a sphingomyelin. 2D correlations for structural elucidation of **1** are described in Figure 1. An olefinic bond was assigned for the fatty acyl chain of **1** based on NMR resonances (δ_H 5.34, δ_C 130.8, 130.9, CH), and its *cis*-geometry was determined from the ^{13}C chemical shifts of adjacent allylic carbons (δ_C 28.1, C13′, C16′). On the other hand, the C4−C5 double bond of the sphingosine moiety was characterized as *trans*-orientation given its coupling constants ($J_{H\text{-}4/JH\text{-}5}$ = 15.3), proton splitting patterns (δ_H 5.45, dd, J = 7.7, 15.3, 5.71, td, J = 6.5, 15.3), and correspondent allylic carbon NMR shifts (δ_C 33.5, C6).[18] The relative configurations of 2-NH and 3-OH in the sphingosine of **1** and **2** were deduced by comparing their $J_{H2,H3}$ and $J_{H3,H4}$ coupling constants with those from the stereoisomers of sphingosine in the stereochemical study.[19] In brief, the $J_{H2,H3}$ and $J_{H3,H4}$ coupling constants of D-erythro sphingosine and L-erythro sphingosine derivatives were reported at 6.8 and 7.5 Hz, respectively, while those of D-threo sphingosine and L-threo sphingosine derivatives were recorded at 2.8 and 7.1 Hz. Thus, the $J_{H2,H3}$ and $J_{H3,H4}$ coupling constants of **1** and **2** at 9.1 and 7.7 Hz proposed the erythro relative configurations for the 2-NH and the 3-OH which were consistent with the most common D-erythro (2*S*,3*R*,4*E*) sphingosine base biosynthesized *de novo* from serine and palmitoyl-CoA.[19]

The HR-ESI-MS data of **2**, acquired as a white amorphous powder, displayed the protonated ion at m/z 811.6690 $[M + H]^+$ (calcd. $[M + H]^+$, m/z 811.6693) and the sodium-adduct ion at m/z 833.6508 (calcd. $[M + Na]^+$, m/z 833.6512), which assigned its molecular formula as $C_{47}H_{91}N_2O_6P$ (Figure S18,

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Supporting Information). The 1D and 2D NMR data of **2** resembled closely to those of **1**, except for the difference in the fatty acyl chain (*cis*-15,18-tetracosadienoyl vs *cis*-14-tricosenoyl in **1**).

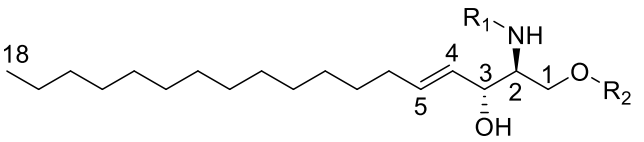
Accurate determination of the double-bond positions of unsaturated fatty acyl chains of **1** and **2** is challenging because of the highly overlapped signals of aliphatic methylenes in their NMR spectroscopic data. Therefore, MS fragmentation was utilized for the in-depth analysis of their long chains and whole structures (Figure S1). In the full-scan MS spectra, the sodiated ion at m/z 821.90 had the most prominent mass-to-charge ratio for **1**; thus, this ion was chosen as the precursor ion. In its MS^2 spectrum, product ions exhibited fragments at m/z 762.45, 638.40, 616.20, 239.10, 184.05, 146.70, and 86.35, which corresponded to $[M - 2H_2O]^+$, [M − phosphocholine − H_2O + Na]$^+$, [M − phosphocholine − H_2O + H]$^+$, [fragment at C2−C3 of sphingosine + H]$^+$, [phosphocholine]$^+$, [sodiated ethylene phosphate]$^+$, and [choline]$^+$, respectively.[20−22]

Furthermore, during the ionization process, the β-cleavage of **1** led to two fragmented ions at m/z 457.20 [M − phosphocholine − $2H_2O$ fragment at C13′−C14′−H]$^-$ and m/z 484.35 [M − phosphocholine − $2H_2O$ fragment at C15′−C16′ + H]$^+$, which proposed the double-bond position of its fatty acyl chain at C14′−C15′.[23] Similarly, the sodium-adduct ion at m/z 833.70 was utilized as the precursor ion for **2** and consequently created corresponding fragments for [M − $2H_2O$]$^+$, [M − phosphocholine − H_2O + Na]$^+$, [sphingosine − $2H_2O$ + H]$^+$, [fragment at C2−C3 of sphingosine + H]$^+$, [phosphocholine]$^+$, [sodiated ethylene phosphate]$^+$, and [choline]$^+$ at m/z 774.55, 650.45, 264.50, 239.10, 183.75, 146.80, and 86.20, respectively. The fragmented ion at m/z 498.55 for [M phosphocholine − $2H_2O$ fragment at C16′−C17′ + H]$^+$ also indicated the C15′−C16′ olefinic bond of **2**. The remaining double bond at C18′−C19′ was unveiled through COSY correlations between H-16′ (δ_H 5.35), H-17′ (δ_H 2.03), and H-18′ (δ_H 5.35) and HMBC cross-peaks of H-17′ to C-16′ (δ_C 130.6) and C-18′ (δ_C 130.6).

In addition to MS fragmentation, olefin cross-metathesis reaction using a second-generation Hoveyda−Grubbs catalyst was utilized to reaffirm the structures of fatty acyl chains in **1** and **2**.[22] As shown in Figures S11 and S20 (Supporting Information), molecular ions at m/z 745.50 [M + H]$^+$ and 767.50 [M + Na]$^+$ for **1** and 759.50 [M + H]$^+$and 781.50 [M + Na]$^+$ for **2** in the ESI-positive ionization mode determined their molecular mass products as 744 and 758, respectively.

The obtained mass of these cross-metathesis products matched well with the theoretical values for the reaction products of **1** and **2**, which clearly indicated that the double-bond positions are located correspondently at C14′ and C15′ of **1** and **2** (Figure S2). To validate the chemical method, it was also applied for confirming the structures of **6** and **8** whose fatty acyl chains were identified as palmitoleyl and *cis*-14-tricosenoyl, respectively, by MS fragmentation. Consistent results confirming the position of the double bonds are summarized in Figures S34 and S39 (Supporting Information). From the above evidence, structures of two new sphingomyelins **1** and **2** were established as shown in Figure 2.

Two known sphingomyelins (**3** and **4**) and four known ceramides (**5**−**8**) were isolated and identified. Their structures were supported by 1D NMR and MS fragmentation, and detailed data are described in the Supporting Information.



1 R_1 = *cis*-14-tricosenoyl R_2 = phosphocholine
2 R_1 = *cis*-15,18-tetracosadienoyl R_2 = phosphocholine
3 R_1 = palmitoyl R_2 = phosphocholine
4 R_1 = *cis*-15-tetracosenoyl R_2 = phosphocholine
5 R_1 = palmitoyl R_2 = H
6 R_1 = palmitoleyl R_2 = H
7 R_1 = stearoyl R_2 = H
8 R_1 = *cis*-14-tricosenoyl R_2 = H

Figure 2. Structures of sphingosine-based and choline-based metabolites **1**−**8** isolated from *C. elaphus*.

3.2. Method Validation. *3.2.1. Linearity, LOD, and LOQ.* The linear range and regression equations of the calibration curves of the four sphingomyelins and four ceramides are shown in Table 2. The linearity of all components showed correlation coefficients (R^2) > 0.99. Furthermore, the LOD and LOQ of each component were determined by the method described in Section 2.5.1 and are shown in Table 2. The LOD and LOQ were 0.01 and 19.91 and 0.03 and 66.36 ng/mL, respectively.

3.2.2. Selectivity, Precision, and Accuracy. The MRM transitions and conditions of each component are shown in Figure 3 and Table 3, and the precision and accuracy of each component are summarized in Table 2. Precision was measured for intra- and interday variations. As a result, RSD (%) was in the ranges of 1.07−4.23 and 2.03−4.78%, respectively. Accuracy was also measured for intra- and interday variations, and the recovery ranges were 99.22−107.15 and 95.35−108.92%, respectively. Precision was measured by preparing three samples (0.75 1.00, and 1.25 g), which were repeated 5 times to obtain the average and RSD (%) values. As a result of measuring precision variation, the intra- and interday RSD (%) ranges from 1.07 to 4.23% and from 2.03 to 4.78%, respectively, which were within 5% of RSD (%) acceptance range. For the accuracy test, 1.00 g of the sample was spiked with the mixed standard solution that is made of three different concentrations of each compound. Then, the reaction was repeated 5 times to obtain the recovery (%) value. As a result of measuring accuracy variation, recovery ranges of intraday and interday were from 99.22 to 107.15% and from 95.35 to 108.92%, respectively, confirming that recoveries of all components were within the range of 90−110%.

3.2.3. Quantitative Analytical Results of Sphingomyelins and Ceramides in Deer Velvet Antler. Validation confirmed that the UPLC-MS/MS analytical method of sphingomyelins and ceramides can be used for the quantification of deer velvet antlers. Table 2 shows the contents of each component. On average, the contents of *N*-cis-14-tricosenoylsphingomyelin and *N*-cis-15,18-tetracosadienoylsphingomyelin were 63.26 and 234.92 μg/g, respectively. The contents of other compounds were as follows: 425.43 μg/g for C16:0-sphingomyelin, 280.85 μg/g for C24:1-sphingomyelin, 295.08 μg/g for C16:0-ceramide, 87.08 μg/g for C16:1-ceramide, 55.02 μg/g for C18:0-ceramide, and 156.03 μg/g for C23:1-ceramide.

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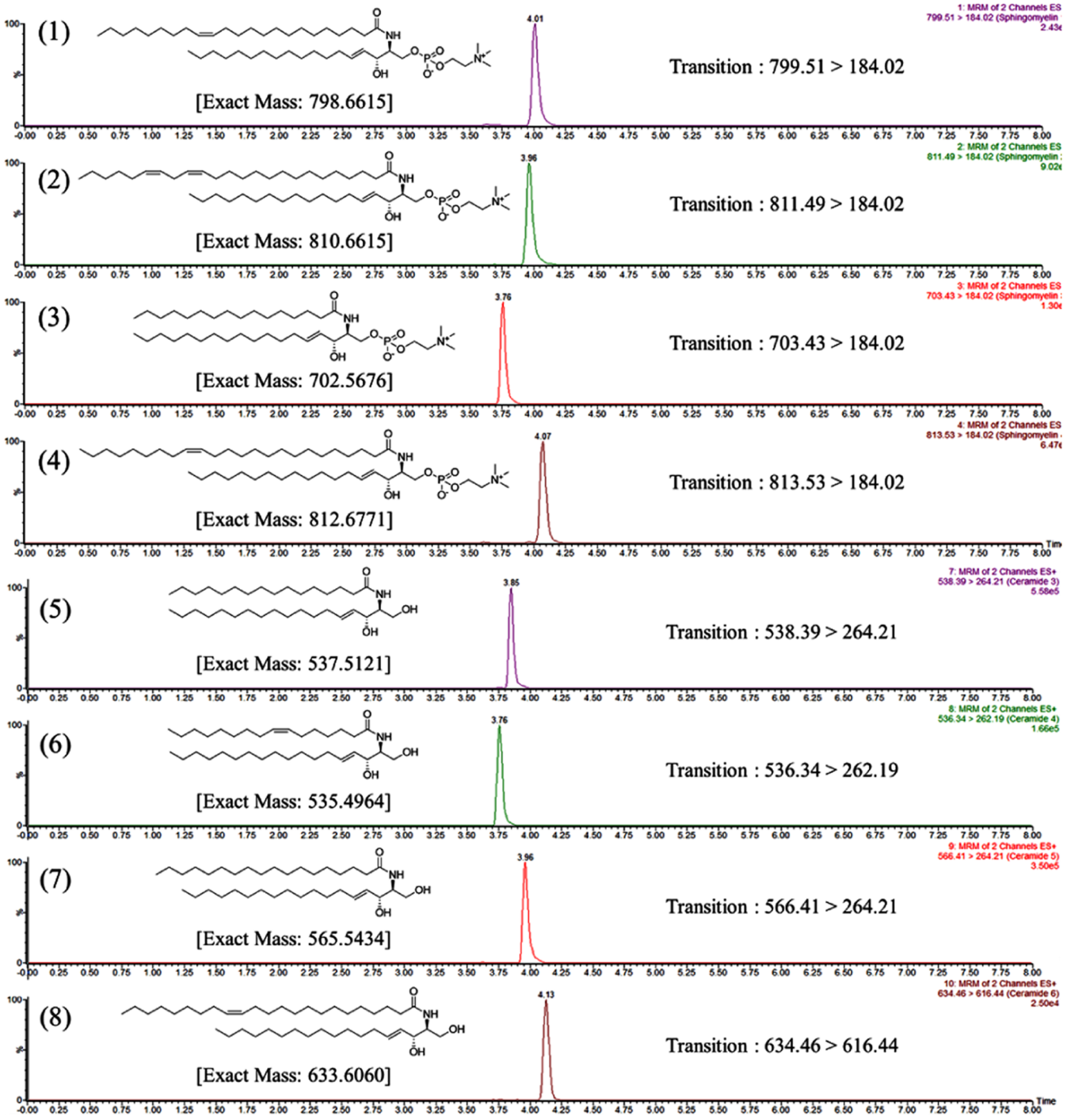

Figure 3. Chemical structures, exact masses, and MRM transitions (quantitation ion) of (**1**) *N-cis*-14-tricosenoylsphingomyelin, (**2**) *N-cis*-15,18-tetracosadienoylsphingomyelin, (**3**) C16:0-sphingomyelin, (**4**) C24:1-sphingomyelin, (**5**) C16:0-ceramide, (**6**) C16:1-ceramide, (**7**) C18:0-ceramide, and (**8**) C23:1-ceramide.

3.3. Analysis of Commercial Velvet Antlers. Four sphingomyelins and four ceramides of nine New Zealand velvet antler samples were investigated by the analytical method developed, which was confirmed by validation. Consequently, the content of *N-cis*-14-tricosenoylsphingomyelin ranged from 35.30 to 54.27 μg/g; *N-cis*-15,18-tetracosadienoylsphingomyelin, from 145.17 to 268.26 μg/g; C16:0-sphingomyelin, from 258.64 to 329.54 μg/g; C24:1-sphingomyelin, from 168.23 to 266.80 μg/g; C16:0-ceramide, from 193.58 to 256.90 μg/g; C16:1-ceramide, from 64.15 to 82.74 μg/g; C18:0-ceramide, from 44.37 to 57.14 μg/g; and C23:1-ceramide, from 156.88 to 190.22 μg/g. Although there are variations for each sample, it was similar to that of the sample used for validation. The analysis results are found in Table 4.

4. CONCLUSIONS

Four sphingomyelins and four ceramides were isolated from the velvet antlers of *C. elaphus*, and two new sphingomyelins (**1** and **2**) were determined to be amides of sphingosine phosphorylcholine with *cis*-14-tricosenoic (**1**) and *cis*-15,18-

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Table 4. Quantitative Analytical Results of Commercial Deer Velvet Antlers

		content (μg/g)								
no.	comp. name	deer velvet 1	deer velvet 2	deer velvet 3	deer velvet 4	deer velvet 5	deer velvet 6	deer velvet 7	deer velvet 8	deer velvet 9
1	*N-cis*-14-tricosenoylsphingomyelin	35.57	35.30	40.21	54.27	39.36	49.05	46.82	53.34	43.24
2	*N-cis*-15,18-tetracosadienoylsphingomyelin	150.55	145.17	163.49	240.30	170.12	248.99	219.77	268.26	198.58
3	C16:0-sphingomyelin	280.61	281.27	284.54	329.54	274.63	280.09	264.30	281.99	258.64
4	C24:1-sphingomyelin	177.44	168.23	186.06	266.80	180.65	229.88	213.70	249.31	202.55
5	C16:0-ceramide	256.90	254.43	226.92	217.12	232.74	205.01	215.67	193.58	246.02
6	C16:1-ceramide	82.74	80.80	73.10	69.47	76.77	70.16	71.17	64.15	82.55
7	C18:0-ceramide	44.37	47.12	46.33	54.38	45.68	51.31	51.80	52.23	57.14
8	C23:1-ceramide	163.52	157.36	159.02	190.22	158.11	157.95	156.88	162.86	178.23

tetracosadienoic acids (**2**). To the best of our knowledge, this is the first report of the isolation and structure determination of two new compounds (**1** and **2**). We believe that these two new sphingomyelins can be applied as bioactive marker compounds for deer velvet antlers.

Existing methods for analyzing velvet antler components such as analysis using TLC-based analysis, derivatization for ganglioside analysis, and sialic acid analysis are difficult and complex to perform quality control. Thus, the analysis method must be enhanced for the quality evaluation of velvet antlers. In this study, we developed a UPLC-MS/MS method for the verification of isolated compounds that can overcome the limitations of existing analytical methods. As a result of validation, the correlation coefficients (R^2) of all tested components were ≥0.99. The precision was estimated using the RSD (%) for both intra- and interdays. The RSDs of all tested samples were <5%. The accuracy range was within 100 ± 10%. This result is anticipated to be useful as basic research data for the quality control of velvet antlers in the development of velvet antlers as functional food and pharmaceutical materials.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c10118.

1D, 2D NMR, high-resolution electrospray ionization mass spectrometry (HRESIMS) data of **1**−**2**, and ESI-MS/MS spectra of **1**−**8** (PDF)

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Notes

The authors declare no competing financial interest.

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