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Extraction and Quantification of Sphingolipids from Hemiptera Insects by Ultra-Performance Liquid Chromatography Coupled to Tandem Mass Spectrometry

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[Abstract] Sphingolipids are major structural components of endomembranes and have also been described as an intracellular second messenger involved in various biological functions in all eukaryotes and a few prokaryotes. Ceramides (Cer), the central molecules of sphingolipids, have been depicted in cell growth arrest, cell differentiation, and apoptosis. With the development of lipidomics, the identification of ceramides has been analyzed in many species, mostly in model insects. However, there is still a lack of research in non-model organisms. Here we describe a relatively simple and sensitive method for the extraction, identification, and quantification of ceramides in Hemiptera Insects (brown planthooper), followed by Ultra-Performance Liquid Chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). C18 is used as the separation column for quantitative detection and analysis on the triple quadruple liquid mass spectrometer. In this protocol, the standard curve method is adopted to confirm the more accurate quantification of ceramides based on the optional detection conditions.

Keywords: Hemipetera Insects, *Nilaparvata lugens Stål*, Sphingolipids, Ceramides, UPLC-MS/MS, Extraction, Identification, Quantification, Standard curve

[Background] Sphingolipids are the second largest group of membrane lipids in living organisms and play an important role in many aspects of cell structure, metabolism, and regulation (Lahiri and Futerman, 2007). At first, it was thought that sphingolipids were a complex family of structurally related molecules, but more and more studies have shown that sphingolipids are involved in numerous cellular processes (Mao and Obeid, 2008). Ceramides (Cer) are essential bioactive lipids implicated in various cell biological processes ranging from cell growth regulation to cell death and senescence (Futerman and Hannun, 2004; Hannun and Obeid, 2008) through influencing of multiple signaling pathways. Although the physiological roles of ceramides are widely reported, few studies have described the extraction, identification, and quantification, thus, analysis of ceramides has gained significant interest in investigating the physiological functions of sphingolipid metabolism in Hemiptera Insects.

Currently, various methods have been described for this purpose, such as Diacylglycerol (DAG) Kinase assay (Preiss *et al.*, 1987), Thin-layer chromatography (TLC) (Gorska *et al.*, 2002), Gas chromatography mass spectrum (GC-MS) (Tserng *et al.*, 2003), High-performance liquid

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chromatography (HPLC) (Yano *et al*., 1998; Dobrzyn and Gorski, 2002). In the beginning, DAG kinase assay was commonly used for Cer quantitation, but the specificity has been questioned (Watts *et al*.,1997). Thin layer chromatography was the method of choice, but the resolution, sensibility, and separation were limited, resulting in inefficient separation of similar molecules (Bielawski *et al*., 2010). Despite the high sensitivity of chromatographic analysis, this method had some limitations, such as the need for standard substances and derivatization (Dobrzyn *et al*., 2004). High-performance liquid chromatography (HPLC) was introduced to obtain ceramides separation with higher resolution, but complex samples like tissue extracts, therefore, produced many unspecific signals that did not provide any information concerning the metabolism of molecular species by HPLC (Yano *et al*., 1998; Bode and Graler, 2012).

Given the ultra performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS), it is more efficient, rapid, and sensitive, improving the separation condition of extremely complex samples and reducing matrix interference (Cutignano *et al*., 2010). Therefore, the current choice method is the analysis of ceramides by Ultra Performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). This protocol provides a relatively rapid and reproducible method. Moreover, this method can be used to profile ceramides from the extraction of the plant sample. It has evolved as the method of choice to detect sphingolipids metabolites due to its high sensitivity and superior specificity. The content of ceramide is determined and a quantitative system of sphingolipids in Hemiptera Insects is established, which lay a foundation for understanding the metabolic process and elucidating the biological function of each component. This method was described and used successfully to extract other sphingolipids in previously published studies (Bielawski *et al*., 2010; Shi *et al*., 2018 and 2019).

Materials and Reagents

1. 1.5 ml Eppendorf tubes (Axygen, catalog number: MCT-150-C)
2. 1.0 mm Ceramic Beads (Nalgene, catalog number: 150010C)
3. 2 ml Micro tube (Sarstedt, catalog number: 72.609)
4. Pipette tips (Axygen, catalog numbers: T-300, T-200-Y, T-1000-B)
5. Glass Centrifuge Tubes (VWR International, catalog number: 734-4240)
6. Nitrogen gas (> 99% Purity) (any brand will suffice)
7. Isopropyl alcohol (Sangon Biotech, catalog number: A503069)
8. Ethyl acetate (Sangon Biotech, catalog number: A507048)
9. Liquid nitrogen (any brand will suffice)
10. HPLC-grade methanol (Sigma-Aldrich, catalog number: 34806)
11. Formic acid (Sangon Biotech, catalog number: A503066)
12. MilliQ Water (Millipore, catalog number: Direct-Q3)
13. Standards (see Table 1)

Note: All Ceramides standards list is shown in Table 1.

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14. Solvent extraction solution A (see Recipes)
15. Solvent extraction solution B (see Recipes)
16. Mobile phase A (see Recipes)
17. Mobile phase B (see Recipes)
18. Internal standard (Avanti company) (see Recipes)

Table 1. Example of ceramides standards list

Compound name	Standard	Source
Cer(d18:1/14:0)	Std	Avanti Lipids Polar, catalog number: 860514
Cer(d18:1/16:0)	Std	Avanti Lipids Polar, catalog number: 860516
Cer(d18:1/18:1)	Std	Avanti Lipids Polar, catalog number: 860519
Cer(d18:1/18:0)	Std	Avanti Lipids Polar, catalog number: 860518
Cer(d18:1/20:0)	Std	Avanti Lipids Polar, catalog number: 860520
Cer(d18:1/22:0)	Std	Avanti Lipids Polar, catalog number: 860501
Cer(d18:1/24:1)	Std	Avanti Lipids Polar, catalog number: 860525
Cer(d18:1/24:0)	Std	Avanti Lipids Polar, catalog number: 860524
Cer(d18:0/14:0)	Std	Avanti Lipids Polar, catalog number: 860632
Cer(d18:0/16:0)	Std	Avanti Lipids Polar, catalog number: 860634
Cer(d18:0/18:1)	Std	Avanti Lipids Polar, catalog number: 860624
Cer(d18:0/18:0)	Std	Avanti Lipids Polar, catalog number: 860627
Cer(d18:0/24:1)	Std	Avanti Lipids Polar, catalog number: 860629
Cer(d18:0/24:0)	Std	Avanti Lipids Polar, catalog number: 860628
Cer(d18:0/12:0)	IS	Avanti Lipids Polar, catalog number: 860635

Std: Standard; IS: Internal standard

Equipment

1. Nitrogen evaporator N-EVAP (Organomation, model: HGC-24A)
2. Centrifuge (Eppendorf, model: 5430R)
3. Autoclave (SANYO, model: MLS-3780)
4. UHPLC-Q-TOF-MS/MS system (AB SCIEX, Framingham, MA, USA)
5. UHPLC column (Zorbax sb-C8, 2.1 × 150 mm, 3.5 μm; Agilent, Palo Alto, CA, USA)
6. Ivory PTFE/red silicone rubber septa (Agilent Technologies, catalog number: 5182-0731)
7. 2 ml amber screw vial with patch USP 1 expansion (HAMAG Technologies, catalog number: HM-0716H)
8. Blue open-topped polypropylene cap and white PTFE/red Scilicone septa (HAMAG Technologies, catalog number: HM-0722)
9. 250 μl clear glass pulled conical-bottom (HAMAG Technologies, catalog number: HM-2085)
10. Analytical balance (METTLER TOLEDO, model: XS105)

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11. Tissue homogenizer (MP Biomedicals, USA, FastPrep-24)
12. Oven (Bluepard, model: BPG-9040A)
13. Vortexer (Germany, IKA, model: vortex 2)
14. -80 °C freezer

Software

1. PeakView (AB Sciex)
2. Excel software (Microsoft office 2010)
3. Data Processing software (DPS)

Procedure

A. Insect samples collection

The laboratory strain of *N. lugens* (brown planthopper) used in this study originated from a field population in the Huajiachi campus of Zhejiang University, Hangzhou, China. The BPHs (brown planthopper) were reared on susceptible rice seedlings cv.Taichung Native 1 (TN1) at 27 ± 1 °C, 70% relative humidity and a 16:8 h light:dark photoperiod.

About 2 g fresh weight of insects was determined and collected at different development stages (*e.g.*, eggs, first-fifth instar nymphs, female and male adults) in labeled 2 ml tissue grinding tubes. Samples were then stored at -80 °C after quickly freezing in liquid nitrogen. The sample was set for three biological repeats.

B. Total sphingolipids extraction (Figure 1)

Sphingolipids were extracted from insects according to Bielawski's method (Bielawski *et al*., 2010). Details were prepared as follows:

Note: All following steps were performed at room temperature if not stated otherwise.

1. The samples were taken out from the -80 °C freezer and dissolved the melted samples in 1 ml solvent extraction solution A (see Recipe 1), to which 10 µl Internal standard had been added (see Recipe 5).
2. Samples were next grounded twice in tissue homogenizer with grinding beads, each time for 20 s.

   Note: In the case of plant material, clean and pre-cooled mortars and pestles were used to grind the samples in liquid nitrogen, and the powder was then transferred to a 15 ml glass tube. The sample was homogenized into a fine powder and that powder was kept frozen at every homogenization step. The purpose of the griding bead is to facilitate insect tissue grinding, so it is okay just to cover the bottom layer for the amount of griding beads per mass of sample and total volume.
3. Powdered tissue was vortexed vigorously for 5 min and centrifuged for 5 min at 1,000 × *g*.

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4. After centrifugation, the solvent from the upper lipid-containing phase was transferred to a 15 ml sterilized glass bottle.
   Note: The vial label was protected with a clear tape to avoid being wiped off by the solution.
5. The extraction was repeated by adding 1 ml solvent extraction solution A (see Recipe 1) to the remaining aqueous phase, and the organic layers were combined and concentrated under a stream of nitrogen gas.
6. The dried lipid fractions were re-suspended into 500 µl solvent extraction solution B (see Recipe 2), and immediately sufficient liquid was transferred to glass vials and caped tightly.
7. The solvent was transferred into a labeled 1.5 ml microcentrifuge tube, centrifuge the samples at 12,000 × *g* for 5 min at room temperature.
   Note: The tube was caped tightly to avoid leakage of liquid during centrifugation.
8. The reconstitution solution was finally transferred to a mass spectrometer flask with an internal cannula and stored at -20 °C for machine test.

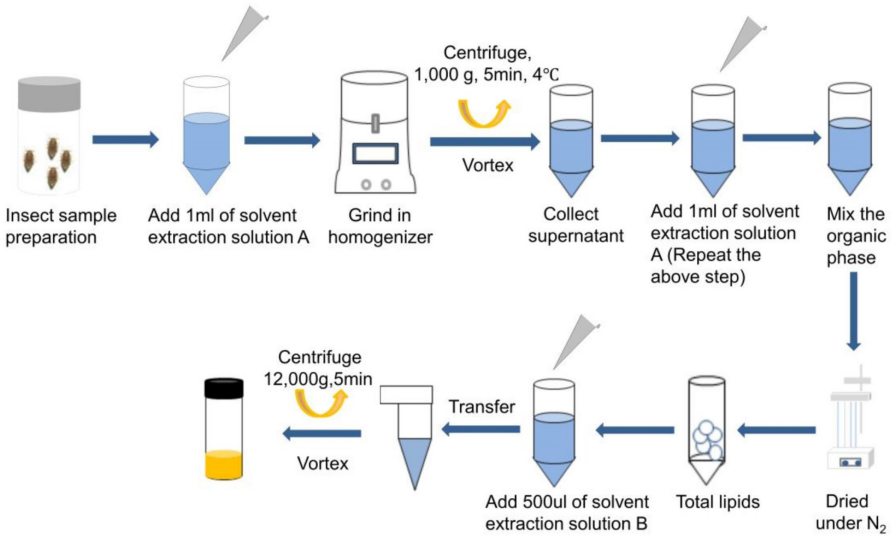

Figure 1. Workflow of sphingolipids extraction from Insect sample

C. Sphingolipids detection and identification

Sphingolipids were analysed on an AB Seriex 5600+quadrupole Time-of-Flight (Q-TOF) Premier mass spectrometer combined with a Water Acquity Ultra Performance liquid chromatography.

1. HPLC-program
   a. Solvent A (see Recipe 3)
   b. Solvent B (see Recipe 4)
   c. Constant flow at a rate of 0.3 ml/min stated by solvent A was running in a Waters UPLC (Waters Corp, Milford, MA, USA) coupled with an AB Triple TOF 5600 plus System (AB SCIEX, Framingham, MA, USA).
   d. The changes in gradient were comprised of an increase in solvent B (methanol) from 80 to 99% over 20 min and then from 99 to 100% over 15 min, followed by a reduction back to

80% over 1 min. The percentage of solvent B was then held at 80% for the last 9 min.

2. The reversed-phase analytical column (Zorbax sb-C8, 2.1 × 150 mm, 3.5 μm; Agilent, Palo Alto, CA, USA) was used to separate ceramides.
3. 10 μl of the samples were applied to the column.
4. The column was kept at 35 °C during the whole procedure.
5. The mass spectrum was acquired with an electrospray ionization (ESI) ion source in the positive ionization mode and following settings (Table 2).

Table 2. The instrument settings for sphingolipids analysis

Ion source voltage	5,500
Ion source heater temperature	600 °C
Ion source gas 1	50 psi
Ion source gas 2	50 psi
Curtain Gas	30 psi
Ion release delay	67
Ion release width	25
UV detector	245 nm
Declustering potential	100 V
Collision energy	10 V
MS/MS Capture collision energy	40±20 V
Ion release delay	67
Scan range	100-1,500 m/z

D. Sphingolipids profiling parameters for detection

The C18 column was used as the separation column on the Aligent 6460+ triple four-pole liquid mass spectrometer to explore the optimal detection conditions of each standard sample. The profiling parameters include precursor ion/targeted ion, retention time, fragmentor, and collision energy. Cer (d18:0/12:0) was used as the internal standard. The scanning parameters for each lipid class were listed in Table 3.

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Table 3. Scanning parameters for sphingolipids detection

Compound name	Standard	Mass of ion	Fragmentor	Collision energy	R_T[min]	Precursor ion m/z
Cer(d18:1/14:0)	Std	510.81	55	20	14.97	264.4
Cer(d18:1/16:0)	Std	538.91	60	20	16.55	264.4
Cer(d18:1/18:1)	Std	564.95	55	20	17.07	264.4
Cer(d18:1/18:0)	Std	566.96	60	4	17.93	548.6
Cer(d18:1/20:0)	Std	595.01	55	24	19.26	264.4
Cer(d18:1/22:0)	Std	623.01	55	24	20.39	264.4
Cer(d18:1/24:1)	Std	649.01	55	28	20.61	264.4
Cer(d18:1/24:0)	Std	651.01	223	20	20.94	632.7
Cer(d18:0/14:0)	Std	512.00	80	28	14.99	266.4
Cer(d18:0/16:0)	Std	541.01	60	16	17.04	522.6
Cer(d18:0/18:1)	Std	567.01	60	4	17.93	548.7
Cer(d18:0/18:0)	Std	568.98	182	16	18.40	550.6
Cer(d18:0/24:1)	Std	651.01	60	20	20.94	632.7
Cer(d18:0/24:0)	Std	653.13	213	20	21.75	634.7
Cer(d18:0/12:0)	IS	482.01	60	20	13.28	264.4

Method optimization notes: For samples containing large amounts of low volatility compounds, the HPLC-program can be made more stringent by increasing the rate flow and by increasing the sampling time. HPLC-program can be adjusted as appropriate for specific analytes and columns by altering holding times. For a few analytes which degrade at high temperatures, it may be desirable to reduce Ion source heater temperatures to 550-580 °C. The timed MS parameters may be used to reduce or eliminate signal from very abundant peaks or contaminants. Those sphingolipids that have different chain lengths, branching, or unsaturation will fragment to yield ions of different m/z. Thus, different precursor ion scans would be necessary to determine the corresponding molecular species. Furthermore, the scan range and collision energy will vary depending on the size and substitution of the various subspecies. All such method adjustments should only be undertaken with the assistance of an experienced MS user.

Data analysis

1. A standard curve with standards from 0.5 ng/ml to 200 ng/ml was generated for quantitative analysis. Curves consisted of triplicates of one blank sample and five calibration points at a concentration ranging from 0.5 ng/ml to 200 ng/ml (Table 4). The amount of each lipid species was calculated according to the sample peak area compared with the normalized internal standard peak area. We used the fitting curve to show the relationship between the concentration and peak area, followed by the fitting curve equation. Three or more biological repeats were recommended (Figure 2).

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Table 4. The data points of ceramide standard curve

Concentration (ng/ml)	0	0.5	5	10	100	200
Volume of C18-0 ceramide	0	0.0270	0.2308	0.5122	10.1798	13.7741

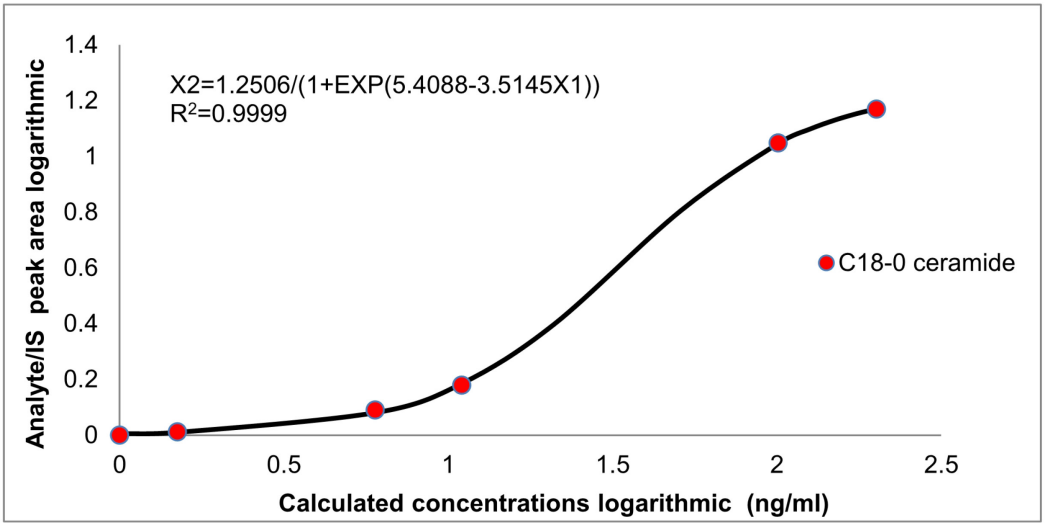

Figure 2. Example of ceramide standard curve. The following ceramides amounts were used: 0 ng/ml, 0.5 ng/ml, 5 ng/ml, 10 ng/ml, 100 ng/ml, 200 ng/ml. The x-coordinate is the concentration and the y-coordinate is the Analyte/IS peak area. For the generation of the standard curve, we converted the horizontal and vertical to logarithmic form.

2. C18 sphingoid bases are the major backbone of most sphingolipids in mammals (Jarne *et al*., 2018). So we chose the C18-0 as a representative to describe the standard curve (Figure 2). For calibration line measurement, equal amounts and 100 ng Cer (d18:0/12:0) as internal standard were added into different calibration points. The calibration lines from 0.5 ng/ml to 200 ng/ml were converted to a fitted curve, with r values consistently greater than 0.9999 during validation (Table 5). The logistic curve in DPS (Data Processing) software was used to fit the equation.

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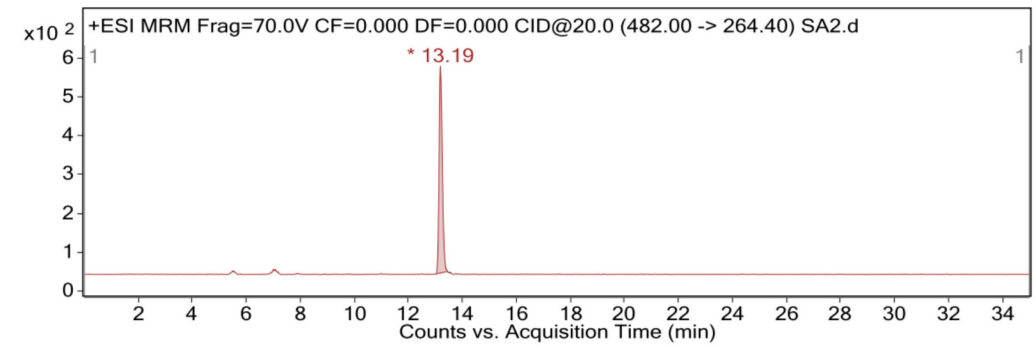
Table 5. The fitting value of ceramide standard curve

A	B	C	D	E
Concentration	C18-0 ceramide	Concentration (log10(A+1))	C18-0 ceramide (log10(B+1))	Fitted
0	0.0000	0.0000	0.0000	0.0056
0.5	0.0270	0.1761	0.0116	0.0103
5	0.2308	0.7782	0.0902	0.0807
10	0.5122	1.0414	0.1796	0.1853
20	/	1.3222	0.0000	0.3980
50	/	1.7076	0.0000	0.8053
100	10.1798	2.0043	1.0480	1.0467
125	/	2.1004	0.0000	1.0980
150	/	2.1790	0.0000	1.1313
175	/	2.2455	0.0000	1.1543
200	13.7741	2.3032	1.1695	1.1703

3. For acquisition, the multiple reaction monitoring (MRM) mode and the software PeakView were used. The internal standard with the mass transition 482.00 m/z -> 264.40 m/z, the retention time of the internal standard is 13.19 min. The Cer (d18:1/16:0) standard with the mass transition 538.91 m/z -> 264.40 m/z, the retention time of the Cer (d18:1/16:0) standard is 16.45 min. Since the retention times and compound-specific ionization and fragmentation values are highly dependent on the used instrumentation, the given values list in Table 2 may be used as a reference but should be individually determined for different instrument setups (Figure 3; Figure 4).



Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	13.03	13.19	13.45	532	4390	100

Figure 3. Example of internal standard mass spectrometry acquired with ESI ion source in positive mode from *Nilaparvata lugens*. Representative signal of internal standard Cer (d18:0/12:0) was plotted. Retention time of internal standard was slightly different from the table 2 list (13.19 min vs. 13.28 min).

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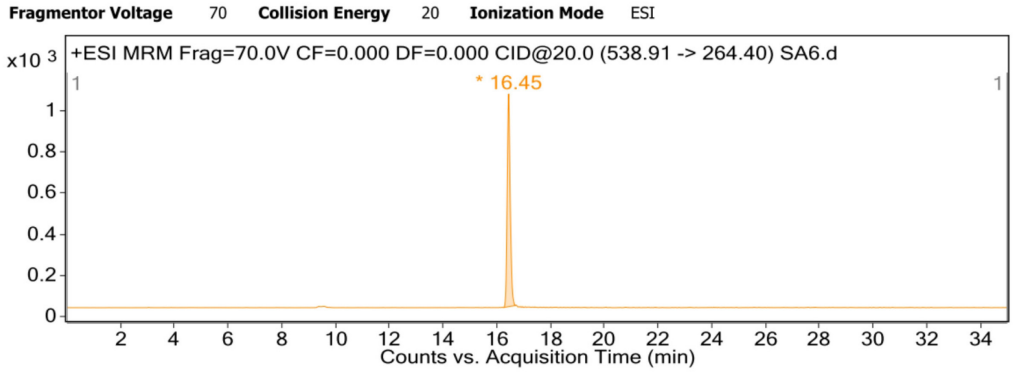

Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	16.25	16.45	16.74	1034	8113	100

Figure 4. Example of Cer (d18:1/16:0) standard mass spectrometry acquired with ESI ion source in positive mode from *Nilaparvata lugens*. Representative signal of Cer (d18:1/16:0) was plotted. Retention time of internal standard was slightly different from the table 2 list (16.45 min vs. 16.55 min).

4. Different ceramides were identified by comparing MS/MS ions of analysts with those of sphingolipid standards in ChemSpider base (http://www.chemspider.com/) through the software PeakView (Http://scie.com.cn/products/software/peakview-software). The maximum allowed error for the reliability was set to ± 10 ppm.

Recipes

1. Solvent extraction solution A
   Ethyl acetate:isopropanol:water, 60:30:10 (vol/vol/vol)
2. Solvent extraction solution B
   Methanol:0.1% formic acid, 9:1 (vol/vol)
3. Solution A
   MQ water containing 0.1% formic acid
4. Solution B
   100% Methanol
5. Internal standard
   100 μg/ml Cer (d18:0/12:0) dissolved in HPLC-grade MeOH

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analysis. The protocol was taken from the publication of Bielawski *et al*. 2010 with minor modified (Bielawski *et al.*, 2010).

Competing interests

The authors declare that no competing financial interest.

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