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Complete List of Authors:	Xu, Chengdong; The University of Texas at Arlington, Pinto, Eduardo; Universidade Federal do Rio de Janeiro, Chemistry Armstrong, Daniel W; The University of Texas at Arlington,

Separation and Sensitive Determination of Sphingolipids at Low Femtomole Level by Using HPLC-PIESI-MS/MS

Chengdong Xu[a], Eduardo Costa Pinto[b] and Daniel W. Armstrong*[a]

[a]Department of Chemistry and Biochemistry, University of Texas at Arlington, Arlington, TX 76019, USA

[b]Department of Pharmaceutics, Faculty of Pharmacy, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil

Corresponding Author:

Daniel W. Armstrong

Department of Chemistry and Biochemistry, University of Texas at Arlington, Arlington, TX 76019, USA

E-mail: sec4dwa@uta.edu; Fax: +1-817-272-3808; Tel: +1-817-272-0632

Abstract

A highly sensitive paired ion electrospray ionization mass spectrometry (PIESI-MS) approach was developed for the trace determination of sphingolipids. Apart from their structure role, specific sphingolipids can play a role in cell signaling and as disease markers. With the optimal

pairing reagents, detection limits ranged from low femtomole to picomole levels for 14 selected sphingolipids. This improved the detection sensitivity of ESI-MS for many of these analytes up to ~4000 times.

Introduction

Sphingolipids (SLs) are a family of bioactive lipids with considerable functional and structural diversity. Beyond their role as a major class of structural lipids in cellular membranes of eukaryotes,[1,2] SLs and their metabolites also are involved in other important biological functions, such as signal transduction and the regulation of cell growth, differentiation, and apoptosis.[3-5] Both the levels of sphingolipids and the expression of their metabolizing enzymes has been shown to be altered in human diseases, such as Niemann-Pick disease[6,7] and Alzheimer's disease[8]. The biosynthetic pathways of sphingolipid metabolism begins with the condensation of palmitoyl CoA and serine to form 3-ketosphinganine, which is further reduced to produce the sphingoid base.[9] The sphingoid base backbone is subsequently acylated with a fatty acid, and the resulting ceramides produce sphingolipids via ester linkages to the hydrophilic headgroups. Of particular significance are phosphorycholine in case of sphingomyelin and oligosaccharide residues in case of gangliosides.[6,9-11] Various combinations of sphingoid bases, fatty acids, and hydrophilic headgroups result in numerous subspecies of sphingolipids.

Biological studies of sphingolipids require analytical approaches that can determine these entities with high specificity and sensitivity. Due to the structure similarity of many sphingolipid species, their often low concentrations, and the scarcity of their metabolites; both quantitative and qualitative analysis can be problematic. Mass spectrometry (MS) has been shown to be a useful

analytical technique for sphingolipid analysis given its outstanding specificity, sensitivity, and speed.[12] Methodologies based on several types of mass spectrometry has been developed for sphingolipid analysis, including fast atom bombardment mass spectrometry (FAB-MS),[13,14] matrix assisted laser desorption ionization mass spectrometry (MALDI-MS),[15] atmospheric pressure chemical ionization (APCI),[16,17] and electrospray ionization mass spectrometry (ESI-MS)[1,17-19]. ESI-MS has unique advantages for sphingolipid determination, because it is easily coupled with chromatographic separation techniques, such as high-performance liquid chromatography (HPLC),[20] and it is able to perform tandem MS for structure elucidation. In recent years, in-depth profiles of a large number of sphingolipids and their metabolites have been achieved with the use of HPLC-ESI-MS.[12,21]

Paired ion electrospray ionization (PIESI) mass spectrometry was developed as a technique that provides ultrasensitive detection for anions.[22-26] This technique involves introducing low concentrations of structurally optimized ion-pairing reagents (IPRs) into the sample stream, thereby allowing the anionic molecules and some zwitterions to be measured with high sensitivity in the positive ion mode ESI-MS as the anion/IPR associated complex. With the use of optimal IPRs, sub-picogram limits of detection (LOD) can be achieved for small organic anions and inorganic anions.[24,27] Further, it was shown that PIESI-MS is useful for both anions and zwitterions of moderate size molecules such as phospholipids.[28] This approach has been successfully employed in a number of actual applications involving more complex sample matrices.[24,29,30] The mechanism for the great sensitivity enhancement obtained by PIESI was recently investigated.[31,32] In the present study, methods for separation and ultrasensitive detection of sphingolipids utilizing HPLC-PIESI-MS are developed and discussed. The detection

limits for 14 sphingolipids, inlcuding sphingomyelins, phosphosphingolipids, gangliosides, and sulfatides, were evaluated by using dicationic ion pairing reagents and tetracationic ion pairing reagents. The best ion pairing reagent for sphingolipid determinations were optimized. HPLC was used with PIESI-MS detection for sphingolipid sample mixtures.

Experimental

Chemicals

Dicationic ion-pairing reagents 1,5-pentanediyl-bis(1-butylpyrrolidinium) difluoride (**Dicat I**) and 1,5-pentanediyl-bis(3-benzylimidazolium) difluoride (**Dicat II**) were originally developed in our laboratory, and are now commercially available from Sigma-Aldrich (St. Louis, MO). The synthetic procedures of the tetracationic ion-pairing reagents (**Tetcat I** and **Tetcat II**) were described in our previous publications.[33] They were initially synthesized with the bromide ion as the counterion and concerted to their fluoride salt form by using ion exchange resin prior to analysis. The structures of the four ion-pairing reagents are shown in Fig. 1. Natural sphingomyelins standards (SM(d18:1/18:0), SM(d18:1/16:0), and SM(d18:1/23:0)), synthetic sphingomyelins standards (SM(d18:1/0:0), SM(d18:1/2:0), SM(d18:1/6:0), and SM(d18:1/12:0)), phosphosphingolipids (PE-Cer(d18:1/0:0) and PE-Cer(d17:1/12:0)), gangliosides (GM1, GM3, and GD3), and sulfatides (I3SO3-GalCer(d18:1/24:0) and I3SO3-GalCer(d18:1/12:0)) were purchased from Avanti Polar Lipids (Alabaster, AL) and Matreya (Pleasant, PA). The structures of these sphingolipids are shown in Fig. 2. The standard solutions used for LOD determination were prepared with methanol/water (50:50, v/v) mixture. HPLC-grade water, methanol, and acetonitrile were purchased from Honeywell Burdick and Jackson (Morristown, NJ)

Instrumentation

The PIESI-MS analyses were performed using a Finnigan LXQ mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The mass spectrometry condition in the positive ion mode was set as follows: spray voltage, 3kV; capillary temperature, 350ºC; capillary voltage, 11V; sheath gas flow, 37 arbitrary units (AU); and the auxiliary gas flow, 6 AU. For the analysis in the negative ion mode, an opposite polarity was used while other instrumental parameters were the same. In the selected reaction monitoring (SRM) acquisition mode, the normalized collision energy was set at 30, the activation time was set at 30 ms, and the Q value was set at 0.25. A description and a schematic diagram of the instrumental configuration of PIESI-MS have been given in our previous publications.[24,26] The PIESI-MS instrumental configuration is similar to the operation principle of the flow injection analysis (FIA). Briefly, a continuous flow of a carrier solution (67% MeOH/33% H_2O, v/v) was provided by a Surveyor MS pump (Thermo Fisher Scientific, San Jose, CA) at a flow rate of 300 μL/min, and the ion-pairing reagent solution (40 μM IPR dissolved in H_2O) provided by a Shimadzu LC-6A pump (Shimadzu, Columbia, MD) was merged into the carrier solution at a flow rate of 100 μL/min through a Y-type mixing tee. This instrumental configuration results in a total flow rate of 400 μL/min and a final solvent composition of 50% MeOH/50% H_2O with 10 μM dicationic ion-pairing reagent flowing into the mass spectrometer. The sample was injected into the carrier solution through a six-port injection valve, and then reacted with the IPR in the mixing tee before reaching the mass spectrometer. For the HPLC-PIESI-MS analysis, a column was installed between the injection valve and the mixing tee, so that the post-column addition of the ion-paring reagent was achieved. The instrumental detection limits (LOD) were determined by serial dilutions of the standard solution until a signal-to-noise ratio of 3 was noted in 5 replicate injections of each sample. Mass-to-

charge ratios of sphingolipids monitored in the SIM and SRM mode were listed in the Supporting Information. The HPLC separation was achieved by using a Supelco Ascentis^{TM} C18 column (250 mm × 2.1 mm) with isocratic elution (40% MeOH/60% H_2O) in 15 min. A 5μL sample loop was used for both the LOD determination and the HPLC-PIESI-MS analysis. Xcalibur 2.0 software was used for the data analysis.

Results and discussion

The dicationic ion pairing reagents (**Dicat I** and **Dicat II**) and tetracationic ion pairing reagents (**Tetcat I** and **Tetcat II**) were selected as they provided the best performance for the singly charged anions and the zwitterions respectively in previously studies.[24,28] The charged moieties and alkyl linkage chain lengths of the IPRs determine the binding affinity between IPR and sphingolipid and also the surface activity of the IPR/sphingolipid associated complex. These are the essential structural properties that affect the observed detection limits of PIESI analyses. Utilizing structurally optimized ion-pairing reagents, all the sphingolipids are sensitively detected by using PIESI-MS in the positive single ion monitoring mode (SIM) (Table 1). It should be noted that the tetracation/sphingolipid complexes do not exist in only one charge state due to the deprotonation of the tetracations in the gas phase. The reported LODs were obtained by monitoring the complex ion that provides the highest ESI signal (Table S1). Fig. S1 in the Supporting Information shows the mass spectrum of **Tetcat I** and **Tetcat II** obtained in full scan, which shows the in-source deprotonation of the tetracationic ion-pairing reagents. The LODs obtained cover a broad range from 2 fmol to 30 pmol. The LODs for two sphingolipids in the SIM mode reached low fmol levels (see SM (d18:1/2:0) (2 fmol by using **Dicat I**) and SM (d18:1/16:0) (8 fmol by using **Dicat II**) in Table 1). It was shown that the LODs for the same

sphingolipids can be significantly different when using different IPRs. For example, the LOD of SM (d18:1/2:0) was 600 fmol when using **Tetcat II**, while it was 2 fmol with the use of **Dicat I**, indicating a 300 times difference in detection sensitivity. By using the optimal IPR, low fmol to pmol level LODs were obtained for all 14 sphingolipids. Interestingly, we found that the performances of the dication and tetracation IPRs on sphingolipid detection are complementary. Tetracationic IPRs show the best performance for phosphosphingolipids, gangliosides and sulfatides, while the dicationic IPRs were best for the determination of sphingomyelins (Table 1). Overall, **Tetcat I** was shown to be the best IPR for sphingolipid detection in the SIM mode. It was hypothesized that relatively higher detection sensitivity achieved by **Tetcat I** could be due to its longer alkyl linkage chain compared to other IPRs. This property may result in a higher affinity to the hydrophobic ceramide backbone of the sphingolipids, which would increase the presence of the IPR/sphingolipid associated complex, and consequently lead to a higher ESI response.

The LODs in the selected ion monitoring (SRM) mode were evaluated by using the IPR/sphingolipid complex ion as the precursor ion and the most abundant fragment as the daughter ion (Table 2 and Table S2). The SRM mode often provides better sensitivity than the SIM mode due to the enhancement in analyte specificity and background noise reduction. It was found that the LODs for most sphingolipids in the SRM mode were improved by 2 to 67 times compared to the SIM mode detection obtained using the same IPR. The LODs obtained for 13 out of 14 sphingolipids were below 1 pmol with the use of the optimal IPR in the SRM mode (Table 2). Compared to ESI-MS detection without using IPRs, PIESI-MS approach improved the LODs by 10 to 4000 times for most of sphingolipids analyzed (Table 3). It should be noted that

the PIESI-MS detection of SM (d18:1/0:0) shows comparable sensitivity to the detection without using IPRs. The high detection sensitivity obtained when no IPR was present could be attributed to the primary amine group of SM (d18:1/0:0), which facilitates the ionization of the specific analyte in the positive ion mode ESI-MS. Hence, there was no apparent advantage in using pairing reagents for SM (d18:1/0:0).

Fig. 3 shows proposed fragmentation pathways of complex [SM (d18:1/6:0) + **Dicat II**]$^{2+}$ during the collision induced dissociation (CID). The secondary ions [$C_{25}H_{30}N_4^{2+}$ (m/z = 193.1), $C_{15}H_{19}N_2^+$ (m/z = 227.1), $C_{18}H_{23}N_4^+$ (m/z = 295.2), and $C_{25}H_{29}N_4^+$ (m/z = 385.2)] were determined to be **Dicat II** and its fragments; while $C_{24}H_{46}NO_2^+$ (m/z = 380.3) and $C_5H_{15}NO_4P^+$ (m/z = 184.1) were the fragments generated from SM (d18:1/6:0) (Fig. 3). The formation of the **Dicat II** ion suggests that the non-covalent association between **Dicat II** and SM (d18:1/6:0) was disrupted during MS2. The generation of IPR as the major fragment ion in the CID process was found to be common in these SRM experiments while this is not the only transition pathway (Table S2). As examples of fragmentation patterns of the IPR/sphingolipid complex, the product ion spectra of the complex SM(d18:1/6:0)/**Dicat I**, SM(d18:1/6:0)/**Dicat II**, SM(d18:1/6:0)/**Tetcat I**, and SM(d18:1/6:0)/**Tetcat II** were shown in Fig. S2, S3, S4, and S5, respectively.

Fig. 4 is a comparison of the detection sensitivity of SM (d18:1/2:0), SM (d18:1/6:0), and PE-Cer (d17:1/12:0) by using HPLC-ESI-MS (Fig. 4A) and HPLC-PIESI-MS (Fig. 4B and 4C). It was shown in previous studies that the molecules processing phosphate moieties (i.e. sphingolipids) have inherently low ionization efficiencies in the positive ion mode ESI-MS,

which results in relatively poor detection sensitivity.[34-36] This is supported by the poor signal-to-noise ratio (S/N) of three sphingolipids observed in Fig. 4A (S/N = 7, 11, <3 for SM (d18:1/2:0), SM (d18:1/6:0), and PE-Cer (d17:1/12:0), respectively). When the same concentrations of sphingolipids were analyzed by HPLC-PIESI-MS, the S/N for SM (d18:1/2:0), SM (d18:1/6:0), and PE-Cer (d17:1/12:0) were increased to 137, 86, 11 in the SIM mode, and 161, 138, 63 in the SRM mode, respectively (Fig. 4B and 3C). Thus, the detection of these three sphingolipids using the HPLC-PIESI-MS approach was approximately 23 times more sensitive than HPLC-ESI-MS. Table 4 compares the detection of zwitterionic sphingolipids with the use of IPR, formic acid (FA) and trifluoroacetic acid (TFA) additives. As the most commonly used additives in the positive ion mode ESI-MS, FA and TFA provided decent sensitivity improvement in most cases when compared to the LODs obtained without using these acidic additives (improvement factor was 2 to 46 for FA and 8 to 170 for TFA, see Table 3). The IPR (PIESI) still produced better sensitivities compared to 0.1% FA and 0.1% TFA (Table 4). It was found that using the optimal IPR further improves the LODs of sphingolipids by 2 to 270 times compared to using FA and TFA as mobile phase additives (Table 4).

Conclusions

A highly sensitive methodology based on HPLC-PIESI-MS was developed for the efficient separation and detection of sphingolipids. Utilizing the optimal IPR, detection limits from low fmol to pmol were achieved for all 14 sphingolipids analyzed, showing 2 to 4100 times sensitivity improvement compared to the ESI-MS without using IPR. The SRM experiment improved the LODs by 2 to 62 times compared to the SIM mode detection, resulting in LODs for 13 out of 14 sphingolipids below 1 pmol. While **Tetcat I** was found overall to be the best ion

pairing reagent, the dications and the tetracations show complimentary performance for the determination of sphingolipids. Compared to most commonly used HPLC-ESI-MS additives, such as formic acid and trifluoroacetic acid, the PIESI approach produced better detection sensitivities of the sphingolipids. The PIESI-MS method is readily coupled with chromatographic separations (HPLC) to separate and sensitively determine the sphingolipids in a sample mixture. It can be anticipated that this method will be useful for the sphingolipids profiling at low concentration levels.

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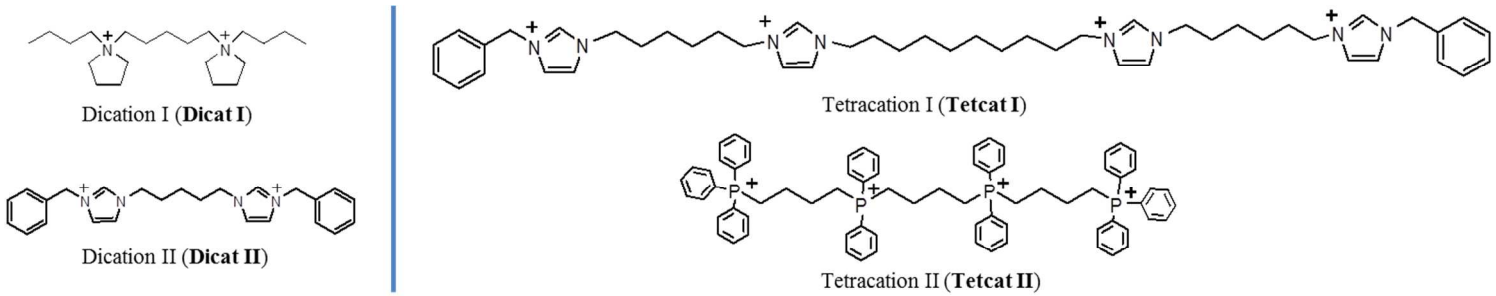

Fig. 1 Structures and abbreviations of the dicationic ion pairing reagents and tetracationic ion pairing reagents used in this study.

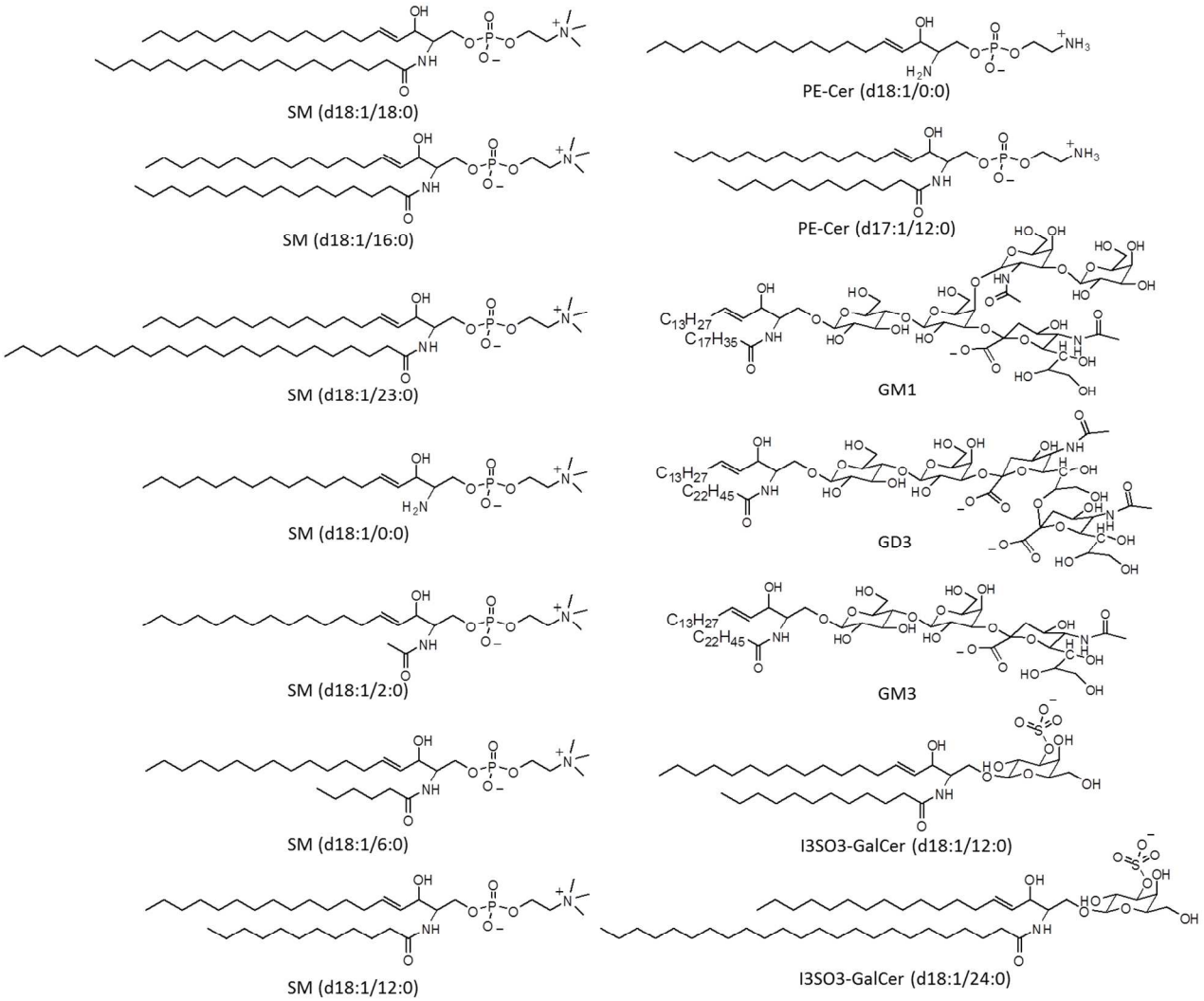

Fig. 2 Structures and abbreviations of the sphingolipids.

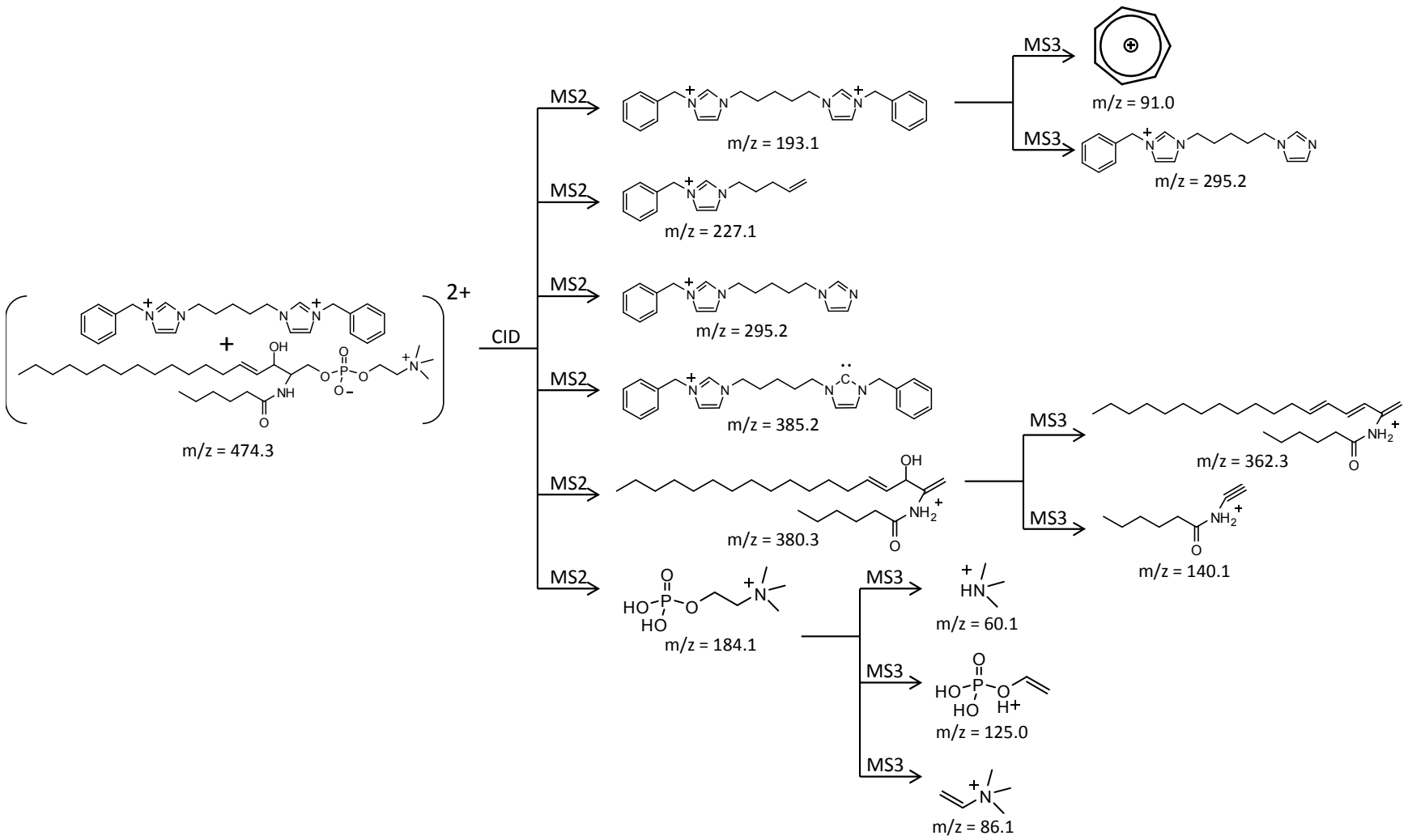

Fig. 3 Proposed CID fragmentation pathways of complex [SM(d18:1/6:0) + **Dicat II**]$^{2+}$ m/z: 474.3. See Fig. S3 in the Supporting Information for the corresponding mass spectrum.

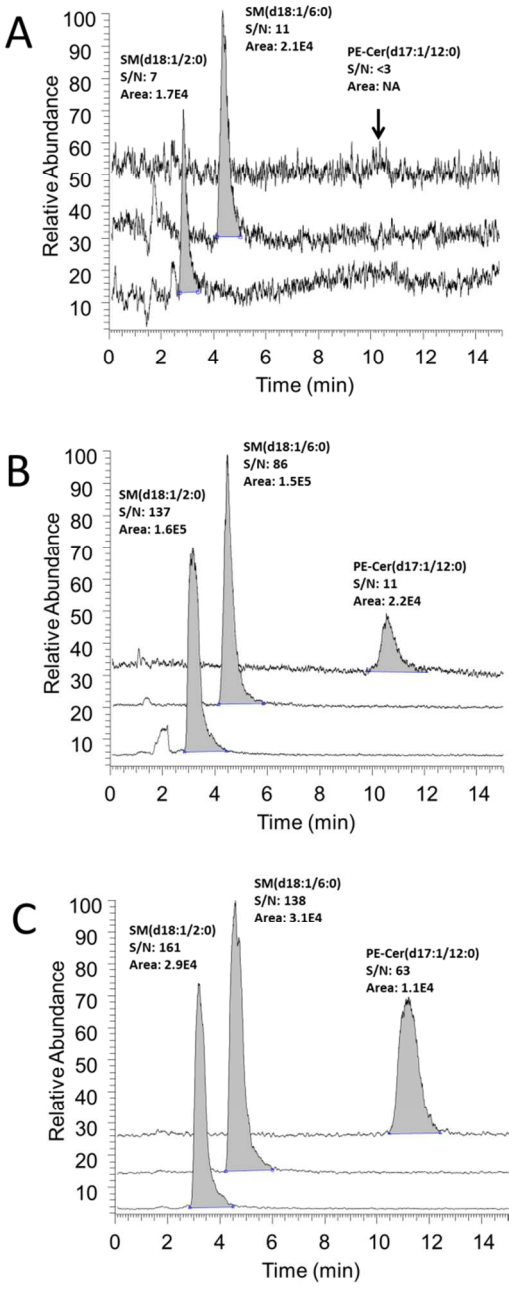

Fig. 4 A comparison of the HPLC-MS separation and sensitivity of three sphingolipids in the (A) regular positive SIM mode HPLC-MS without using IPR ($[SM(d18:1/2:0)]^+$ m/z: 507.4; $[SM(d18:1/6:0)]^+$ m/z: 563.4; $[PE\text{-}Cer(d17:1/12:0)]^+$ m/z: 591.4), (B) positive SIM mode by HPLC-PIESI-MS using the **Dicat I** ($[SM(d18:1/2:0) + \textbf{Dicat I}]^{2+}$ m/z: 415.4; $[SM(d18:1/6:0) + \textbf{Dicat I}]^{2+}$ m/z: 443.4; $[PE\text{-}Cer(d17:1/12:0) + \textbf{Dicat I}]^{2+}$ m/z: 457.4), and (C) positive SRM mode by HPLC-PIESI-MS using the **Dicat I** ($[SM(d18:1/2:0 + \textbf{Dicat I}]^{2+}$ SRM m/z: 415.4 → 162.3; $[SM(d18:1/6:0) + \textbf{Dicat I}]^{2+}$ SRM m/z: 443.4 → 162.3; $[PE\text{-}Cer(d17:1/12:0) + \textbf{Dicat I}]^{2+}$ SRM m/z: 457.4 → 162.3). The concentration of SM(d18:1/2:0), SM(d18:1/6:0), and PE-Cer(d17:1/12:0) was all equal to 500 ng/mL. Chromatographic separation condition was shown in the Experimental Section.

Table 1 Limits of detection of sphingolipid standard solutions obtained in the positive SIM mode using PIESI-MS.

Analyte	Dicat I	Dicat II	Tetcat I	Tetcat II	Best LOD	Best IPR[a]
	LOD (pmol)	LOD (pmol)	LOD (pmol)	LOD (pmol)	LOD (pmol)	
SM(d18:1/18:0)	0.2	1	1	5	**0.2**	Dicat I
SM(d18:1/16:0)	0.1	0.008	0.2	0.8	**0.008**	Dicat II
SM(d18:1/23:0)	30	2	1	30	**1**	Tetcat I
SM(d18:1/0:0)	1	0.2	0.6	2	**0.2**	Dicat II
SM(d18:1/2:0)	0.002	0.1	0.3	0.6	**0.002**	Dicat I
SM(d18:1/6:0)	0.08	0.09	0.2	0.4	**0.08**	Dicat I
SM(d18:1/12:0)	0.1	0.05	0.06	1	**0.05**	Dicat II
PE-Cer(d18:1/0:0)	1	0.4	0.01	0.1	**0.01**	Tetcat I
PE-Cer(d17:1/12:0)	0.2	2	0.03	0.4	**0.03**	Tetcat I
GM1	6	20	1	2	**1**	Tetcat I
GM3	20	10	0.4	3	**0.4**	Tetcat I
GD3	10	20	2	30	**2**	Tetcat I
I3SO3-GalCer(d18:1/24:0)	1	5	2	0.6	**0.6**	Tetcat II
I3SO3-GalCer(d18:1/12:0)	0.1	0.2	0.07	0.06	**0.06**	Tetcat II

[a]See Fig. 1 for the IPR structures and abbreviations.

Table 2 Limits of detection of sphingolipid standard solutions obtained in the positive SRM mode using PIESI-MS.

Analyte	Dicat I	Dicat II	Tetcat I	Tetcat II	Best LOD	Best IPR[a]
	LOD (pmol)	LOD (pmol)	LOD (pmol)	LOD (pmol)	LOD (pmol)	
SM(d18:1/18:0)	0.05	1	0.1	7	**0.05**	Dicat I
SM(d18:1/16:0)	0.1	0.3	0.06	0.3	**0.06**	Tetcat I
SM(d18:1/23:0)	5	2	1	3	**1**	Tetcat I
SM(d18:1/0:0)	1	0.2	0.06	0.2	**0.06**	Tetcat I
SM(d18:1/2:0)	0.04	0.08	0.3	0.6	**0.04**	Dicat I
SM(d18:1/6:0)	0.06	0.05	0.07	0.4	**0.05**	Dicat II
SM(d18:1/12:0)	0.02	0.05	0.05	0.5	**0.02**	Dicat I
PE-Cer(d18:1/0:0)	0.2	2	0.004	0.1	**0.004**	Tetcat I
PE-Cer(d17:1/12:0)	0.2	0.03	0.02	0.08	**0.02**	Tetcat I
GM1	1	—[b]	0.6	2	**0.6**	Tetcat I
GM3	0.4	—[b]	0.4	0.2	**0.2**	Tetcat II
GD3	—[b]	—[b]	0.2	6	**0.2**	Tetcat I
I3SO3-GalCer(d18:1/24:0)	1	—[b]	2	0.2	**0.2**	Tetcat II
I3SO3-GalCer(d18:1/12:0)	—[b]	7	—[b]	0.009	**0.009**	Tetcat II

[a]See Fig. 1 for the IPR structures and abbreviations.
[b]Not detected.

Table 3 Comparison of limits of detection of sphingolipid standard solutions obtained by using PIESI-MS and ESI-MS without using IPR.

Analyte	Best LOD by PIESI-MS	Without using IPR	Improvement factor[a]
	LOD (pmol)	LOD (pmol)	
SM(d18:1/18:0)	0.05[c]	30[d]	**600**
SM(d18:1/16:0)	0.008[b]	30[d]	**4000**
SM(d18:1/23:0)	1[b, c]	40[d]	**40**
SM(d18:1/0:0)	0.06[c]	0.02[d]	**0.3**
SM(d18:1/2:0)	0.002[b]	0.8[d]	**400**
SM(d18:1/6:0)	0.05[c]	0.8[d]	**16**
SM(d18:1/12:0)	0.02[c]	4[d]	**200**
PE-Cer(d18:1/0:0)	0.004[c]	0.04[d]	**10**
PE-Cer(d17:1/12:0)	0.02[c]	6[d]	**300**
GM1	0.6[c]	10[e]	**17**
GM3	0.2[c]	2[e]	**10**
GD3	0.2[c]	2[e]	**10**
I3SO3-GalCer(d18:1/24:0)	0.2[c]	0.3[e]	**1**
I3SO3-GalCer(d18:1/12:0)	0.009[c]	0.01[e]	**1**

[a] Times of improvement of LODs obtained using PIESI-MS vs. LODs obtained by ESI-MS without using IPR.
[b] Obtained in the SIM mode by PIESI-MS.
[c] Obtained in the SRM mode by PIESI-MS.
[d] Measured in the positive ion mode ESI-MS.
[e] Measured in the negative ion mode ESI-MS.

Table 4 Comparison of limits of detection of sphingolipid standards obtained by using PIESI-MS and formic acid (FA) and trifluoroacetic acid (TFA) as mobile phase additives.

Analyte	**PIESI-MS**[a]	**0.1% FA**[b]	**0.1% TFA**[c]
	LOD (pmol)	LOD (pmol)	LOD (pmol)
SM(d18:1/18:0)	0.05	10	2
SM(d18:1/16:0)	0.008	1	0.8
SM(d18:1/23:0)	1	10	6
SM(d18:1/0:0)	0.06	0.02	0.1
SM(d18:1/2:0)	0.002	0.06	0.1
SM(d18:1/6:0)	0.05	0.09	0.05
SM(d18:1/12:0)	0.02	0.1	0.2
PE-Cer(d18:1/0:0)	0.004	0.02	0.05
PE-Cer(d17:1/12:0)	0.02	0.1	0.03

[a] Data obtained from Table 3.
[b] LOD obtained using a mobile phase of methanol/water (50:50, v/v) mixture containing 0.1% formic acid.
[c] LOD obtained using a mobile phase of methanol/water (50:50, v/v) mixture containing 0.1% trifluoroacetic acid.

Graphic abstract