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Identification of lysophosphatidylcholine (LPC) and platelet activating factor (PAF) from PC12 cells and mouse cortex using liquid chromatography/multi-stage mass spectrometry (LC/MS^3)

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Lipids play essential roles in cellular structural support, energy storage and signal transduction. Recently, mass spectrometry (MS) has been used to produce three-dimensional maps that elucidate the lipid composition of complex cellular lysates. The identification of individual lipids within these maps is slow and requires the synthesis and spiking of each candidate lipid. We present a novel MS-based technique that rapidly elucidates the atomic connectivity of the fatty acid/alcohol substituent on the *sn*-1 position of several different families of glycerophosphocholine-containing lipids within the confines of a chromatographic separation. Sodiated lipid species were fragmented to produce radical cations which lost successive methylene groups upon further collisional activation to reveal the identity of the parent molecule. This approach was demonstrated to be effective on isobaric members of the lysophosphatidylcholine (LPC) and platelet activating factor (PAF) families of glycerophospholipids. We demonstrate the application of this technique to unambiguously identify these species within complex cellular lysates and tissue extracts. Copyright © 2008 John Wiley & Sons, Ltd.

Glycerophospholipids are often overlooked as immediate response elements yet they exert fundamental control over cell viability. They represent the largest lipid subclass by mass and are the major building blocks of biological membranes. They modulate membrane trafficking and their metabolites are powerful intracellular signalling molecules.[1–4] For example, platelet activating factors (PAFs, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) are members of the 1-alkyl-2-acyl-glycerophosphocholine subclass (GP0102)[5] of glycerophosphocholines (GP01)[5] demonstrated to elicit such diverse effects as aggregation and degranulation of platelets, activation of neutrophils, contraction of smooth muscle and stimulation of glycogenolysis.[6] Moreover, PAFs and other glycerophospholipid species have been implicated in the progression of Alzheimer's disease, Parkinson's disease, stroke and spinal cord injury.[7–10] Specific PAF species have been reported to be involved in neuronal differentiation.[11] Mechanistic insight has been complicated by the considerable technical challenges associated with the identification and quantification of individual glycerophospholipid species within complex lipid extracts, which may contain several thousand closely related isoforms. Consequently, a systematical rapid identification and quantification strategy would greatly enhance our ability to study glycerophospholipid families and the roles that individual isoforms play in the regulation of biological systems.

Comprehensive analysis of lipid extracts has been complicated by the vast structural diversity of lipid isoforms. Efforts to automate lipid identification from MS data are beginning to emerge; however, the fragmentation patterns of many lipid subclasses have not yet been explored. Electrospray ionization mass spectrometry (ESI-MS)-based approaches[12–14] have been successfully employed to identify polar lipids from cells and tissues,[11,15] including glycerophospholipids.[2] To date, these techniques have suffered from an inability to readily determine the structure of lipids and provide precise, rapid and unambiguous identification of individual isobaric lipid isoforms. Insight into lipid structure has also been achieved using fast atom bombardment,[16,17] MS^3,[18] and MS^4.[19] These strategies, however, do not completely elucidate the structure(s) of the fatty acid/alcohol group(s). Furthermore, the fatty acid/alcohol moieties of

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glycerophospholipid molecules have never been identified exclusively in the gas phase.

We describe a novel gas-phase method for rapidly and unambiguously identifying specific subclasses of glycerophospholipids, compatible with lipid extracts obtained from minute amounts of cultured cells or tissues. We focus on isobaric PAF and LPC family members to provide proof of principle. Our methodology utilizes sodium adduction (sodiation) to induce novel and unique fragmentation patterns, revealing the *sn*-1 hydrocarbon chain composed exclusively of carbon and hydrogen atoms. Moreover, the strategy is fully compatible with chromatographic separations, allowing high-throughput identification of lipid carbon chains by high-performance liquid chromatography (HPLC)/ESI-MS^3. Experiments were optimized on synthetic PAF and LPC samples and the methods were used for the online analysis of picograms of lipids extracted from PC12 cells and mouse cerebral cortex. The present strategy boasts a limit of detection comparable to the most sensitive results reported in this field,[20] with the added advantage of determining the carbon chain length and degree of unsaturation. Our initial analyses successfully identified known LPC species as well as two PAF species previously unreported as components of the lipid composition of these samples C16:1[a] PAF and C18:4 PAF.

EXPERIMENTAL

Materials

1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C16:0 PAF), 1-*O*-hexadecyl-2-hydroxy-*sn*-glycero-3-phosphocholine (C16:0 *lyso*-PAF), 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C18:0 PAF), 1-*O*-octadecenyl-2-acetyl-*sn*-glycero-3-phosphocholine (C18:1 PAF) and 1-*O*-octadecyl-2-hydroxy-*sn*-glycero-3-phosphocholine (C18:0 *lyso*-PAF) were purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). C13:0 lysophosphatidylcholine (C13:0 LPC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Stock chemicals were purchased from J.T. Baker (Phillipsburg, NJ, USA) with the exception of bovine serum albumin, 1-O-palmitol-2-(*N*-methylcarbamyl)-*sn*-glycero-3-phosphocholine (mcPAF), 1-O-hexadecyl-2-azelaoyl-*sn*-glycero-3-phosphocholine (azelaoyl PAF) and sodium acetate which were purchased from Sigma (St Louis, MO, USA). Lipid standards were prepared in 100 M sodium acetate at a concentration of 1 M.

Cell culture

Rat pheochromocytoma PC12 cells originally obtained from the American Type Tissue Collection were cultured in RPMI containing 10% horse serum and 5% newborn calf serum (complete media) at 37°C in a 5% CO_2 atmosphere. Cells were seeded at a density of 1×10^5 cells/dish and lipids extracted 72 h after plating. Plating controls seeded and cultured at the same time were counted immediately prior to lipid extraction to establish final cell number (PC12 cells – 2.34 e6). All culture reagents were obtained from Invitrogen (Burlington, ON, USA) except where indicated.

Mouse brain

One 6-month-old female C57Bl/6 mouse was sacrificed via lethal injection of euthanol. Following death, the mouse was decapitated and the brain was removed. The cerebral cortex was isolated on ice and weighed (114 mg).

Glycerophospholipid extraction

Glycerophospholipids were extracted according to a modified Bligh and Dyer procedure[21] that has been previously published.[22] Details of the procedure for PC12 cells have appeared elsewhere.[11] For mouse brain, the cerebral cortex was removed on ice and homogenized in 1 mL of ice-cold methanol acidified with 2% acetic acid using a Tissue Tearor (Biospec Products Inc., Bartlesville, OK, USA); lipids were extracted in the same manner as previously described.[11] Recovery was estimated at 94–96%.

LC/ESI-MS

Lipid standard solutions were infused into a 2000 Q TRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) using an ionspray source at a flow rate of 3 L/min. Collision energies in the q2 varied between 30 and 40 eV while the excitation energy setting in the linear ion trap varied between 50 and 125 mV. Pseudo-MS^4 was achieved by raising the orifice potential to 200 V. MS^3 experiments were repeated on the standard lipid solutions on a QSTAR quadrupole time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA, USA) using source collision conditions (orifice potential of 100–150 V) and collision energies ranging from 20–30 eV; the QSTAR was only used on PAF standards with a microionspray source and all other instrumental conditions were in the same ranges as those used for the Q TRAP. Following MS^3 optimization, the methods were optimized using online nano-HPLC/MS. To optimize the online methods, 10 ng injections of a 1:1:1 mixture of C16:0, C18:0 and C18:1 PAF were used; 10 L of lipid extract from either PC12 cells or mouse cortex was mixed with 30 L of 0.1% formic acid and analyzed. A model 1100 capillary HPLC system (Agilent, Palo Alto, CA, USA) was used for chromatographic separations. Samples were loaded onto a 200 m × 50 mm pre-column packed with 5 m YMC ODS-A C18 beads (Waters, Milford, MA, USA) into an isocratic flow of 0.1% formic acid and 5% acetonitrile at 20 L/min. The lipids were then separated by HPLC with solvents containing sodium acetate at a final concentration of 100 M. The HPLC flow rate was approximately 250 nL/min; final separation and ionization was achieved using a 75 m × 50 mm picotip emitter (New Objective, Woburn, MA, USA), packed with the YMC ODS-A beads. Data were collected on a 2000 Q TRAP mass spectrometer operated in positive ion mode with Analyst 1.4.1 (Applied Biosystems/MDS Sciex, Concord, ON, Canada). Lipid species were further analyzed using a precursor ion scan for 184.0 Th.[15] The capillary voltages for ionspray and micro/nanoflow experiments were 5.5 and 3.5 kV, respectively. Data were analyzed manually within Analyst 1.4.1 (Q TRAP) or Analyst QS 1.1 (QSTAR).

[a]For PAF and LPC species, CX:Y refers to the number of carbon atoms and double bonds in the *sn*-1 chain with a predicted acetyl (PAF) or hydroxyl (LPC and *lyso*-PAF) group at the *sn*-2 position.

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RESULTS AND DISCUSSION

Lipolysis (e.g. saponification) cleaves the volatile fatty acid/alcohol substituents from the glycerol backbone rendering them amenable to gas chromatography (GC)/MS analysis. This strategy effectively decreases the sensitivity of the analysis due to sample handling losses and eliminates the ability to decipher the *sn* positioning or pre-cleavage origin of the fatty acids/alcohols on the parent glycerolipid. The method reported here eliminates the need for chemical pre-processing and allows entire glycerophospholipids to be analyzed in the gas phase using LC/MS. Fatty alcohol/acid carbon chain identification of intact PAF and LPC lipids is achieved, respectively, retaining the lipid identity and *sn* group regiochemistry in each experiment. Figure 1 illustrates the preparatory and gas-phase workflow that was developed and employed to successfully identify these species from complex lipid extracts.

PAF standards

Initial experiments involved the separation and gas-phase sodiation of PAF standard species by HPLC/ESI-MS. Briefly, PAF (C16:0, C18:0 and C18:1) and *lyso*-PAF (C16:0 and C18:0) were separated by HPLC with a modified mobile phase containing 100 M sodium acetate. Under these conditions, approximately 95% of the PAF species observed by ESI-MS were sodiated. Conventional MS/MS analyses of protonated PAF and lyso-PAF did not produce fragments useful for the structural elucidation of the fatty alcohol group at the *sn*-1 position; all protonated species primarily produced an *sn*-3 group fragment at *m*/*z* 184 (i.e. protonated phosphocholine,

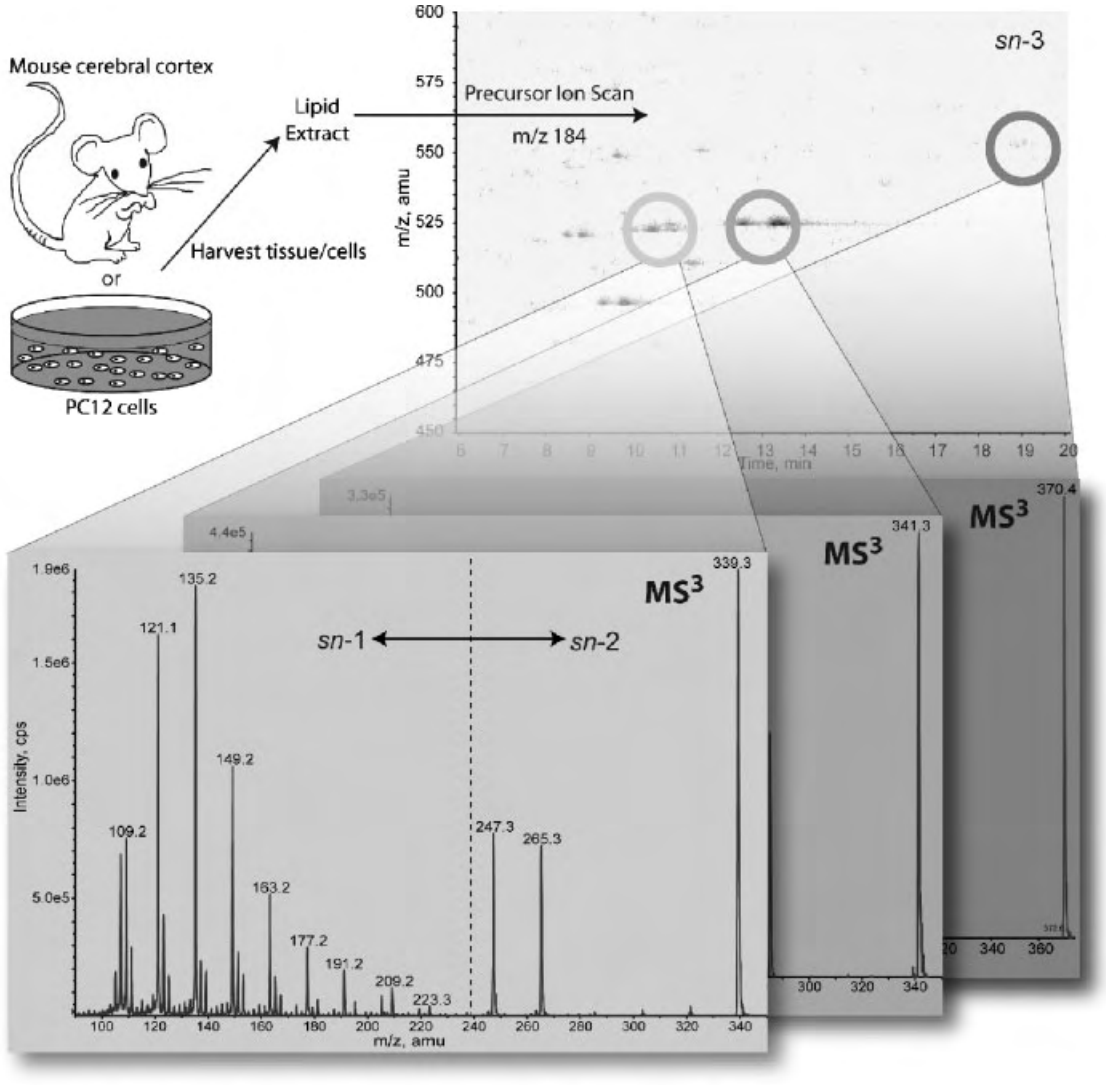

Figure 1. Summary of experimental workflow. PC12 cells and mouse cerebral cortex were harvested and analyzed independently. For each sample, the lipids were extracted and chromatographically separated and analyzed using a precursor ion scan for *m/z* 184. Three-dimensional plots of *m/z* versus chromatographic elution time versus abundance (indicated by the intensity of the spots) were produced for all lipid species containing a phosphocholine group at the *sn*-3 position. The plots served as a reference to select candidate lipid species for structural characterization in subsequent analyses using sodiated HPLC/ESI-MS^3. Three examples are illustrated (circled) whereby the *sn*-2 and *sn*-1 groups of specific lipid species were determined.

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structure highlighted in green in Fig. 2(a), data not shown). However, conducting MS/MS experiments on the sodiated molecules revealed alternate fragmentation patterns, an example of which is illustrated in Fig. 2(a). MS^3 analysis of the sodiated PAF fragment ions observed in Fig. 2(a) yielded minimal additional information with the exception of the 341 Th radical cation peak. Further fragmentation of this ion resulted in an information-rich spectrum revealing structural information pertaining to the fatty alcohol group (Fig. 2(b)).

We developed two MS-based strategies to identify the *sn*-1 chain in the gas phase. The first approach utilized pseudo-MS^4 to create the informative mass spectra, as illustrated in Fig. 2(b). For example, in the case of C16:0 PAF, the molecular ion (546 Th) was fragmented in the source region of the instrument by raising the orifice potential to 200 V to reveal the fragmentation observed in Fig. 2(a). The ion at *m/z* 487 was then selected in Q1, fragmented in q2 and *m/z* 341 was selected, fragmented and analyzed in the ion trap. The pseudo-MS^4 method decreased isobaric interference at 341 Th for simple samples; however, a second MS^3-based approach simplified the analysis, fragmenting *m/z* 546 in the q2 and directly selecting and fragmenting *m/z* 341 in the ion trap. Our results indicated that the MS^3 strategy was more sensitive than pseudo-MS^4.

The MS^3 fragmentation patterns of C16:0 and C18:0 PAF, revealed in Figs. 2(b) and 2(c), respectively, are not possible with the protonated species and demonstrate how the entire PAF molecule may be manipulated in the gas phase to reveal the atomic connectivity of the fatty alcohol group at the *sn*-1 position. The MS^3 spectra of PAF species can be divided into two regions, the blue region which reveals the *sn*-1 chain, and the red region which reveals the loss of the oxygen rich *sn*-2 chain (Fig. 2(b)). We determined that the ions in the lower mass (blue) regions represent saturated hydrocarbon radical cations that contain 17 and 19 carbon atoms for the C16:0 (Fig. 2(b)) and C18:0 (Fig. 2(c)) PAF species, respectively (*vide infra*). From these ions, a series of peaks are observable that correspond to successive losses of 14 Da, or CH_2 groups. In this way, *sn*-1 hydrocarbon chain fragments, corresponding to a structure of $[CH_3(CH_2)_n]^+$, are visible where $n = 7$ through 17 (C16:0 PAF) or 19 (C18:0 PAF). Also visible in the spectrum are peaks corresponding to the formation and migration of carbon–carbon double bonds (losses of H_2, 2 Da). This process occurs in the gas phase during the fragmentation process and has been previously reported.[12,14,23] These results represent the first time that a non-metallated radical cation has been observed to fragment in this manner in the gas phase in the context of a lipidomic study. Moreover, the novelty of this gas-phase chemistry has been demonstrated on the PAF family of lipids revealing the identity of the *sn*-1 chain in a specific manner.

Further experiments performed on a higher resolution mass spectrometer capable of a larger mass range (QSTAR utilizing pseudo-MS^3) demonstrated that the general fragmentation pattern of the radical cation hydrocarbon chain extends to a range of $[CH_3(CH_2)_2]^{\cdot+}$ (Supplementary Figure S1, see Supporting Information). All previous MS-based fatty acid identification reports have been based on oxygen-containing molecules, rather than a pure hydrocarbon radical cation (i.e. $[CH_3(CH_2)_n]^{\cdot+}$) framework.[24] Comparing the relative areas of the isotopic peaks for three fragment ions (two of which are illustrated in Supplementary Figure S2, see Supporting Information) confirmed the absence of oxygen and are in agreement with a molecule containing only carbon and hydrogen atoms. This observation further highlights the novelty of this strategy, utilizing fragmentation mechanisms previously unassociated with lipidomic methods. Unfortunately, inadequate pseudo-MS^3 fragmentation limited the utility of the QSTAR in this study; overall the Q TRAP provided higher sensitivity and better quality data.

Application to other lipid species

Investigation into the utility of this strategy beyond the PAF subfamily of lipids revealed its efficacy on lysophosphatidylcholine (LPC) molecules. Structural information regarding the *sn*-1 fatty acid of a synthetic LPC standard that is generally employed to standardize lipid extraction efficiency[11,25] was successfully obtained (C13:0 LPC, Fig. 3). A neutral loss of 205 Da from the sodiated LPC molecular ion (Fig. 3(a)) was more prominent than an equivalent loss from the sodiated PAF species (Fig. 2(a)), suggesting that this strategy may demonstrate a higher sensitivity towards LPC identification over PAF species. MS^3 analysis of *m/z* 271.2 (Fig. 3(b)) generated a strong peak corresponding to $[CH_3(CH_2)_{x+1}]^{\cdot+}$, where x equals the length of the hydrocarbon chain on the *sn*-1 group of the LPC molecule (*m/z* 197.3 for the C13:0 LPC molecule, Fig. 3(b)). Significantly, a neutral loss of 56 Da was observed from the 271.3 Th peak (Fig. 3(b)), which was not observed for the PAF species. Synthetic and oxidative analogs of PAF (mcPAF (1-*O*-palmitol-2-(*N*-methylcarbamyl)-*sn*-glycero-3-phosphocholine) and azelaoyl PAF (1-O-hexadecyl-2-azelaoyl-*sn*-glycero-3-phosphocholine)) also lost a neutral mass of 205 Th from the sodiated molecular ion and fragmented in a manner such that structural information pertaining to the *sn*-1 group was revealed (data not shown).

PC12 cell analysis

In order for a lipid identification strategy to be truly useful, it needs to be effective at discerning individual isoforms within complex samples. We have previously demonstrated that lipidomics can be used to quantitatively study changes in PAF species in PC12 cells differentiated to a neuronal phenotype.[11] Here we demonstrate that our novel gas-phase strategy can also be used to identify known and novel PAF species present in PC12 cells, including several that showed obvious abundance changes over the course of differentiation to a neuronal phenotype.[11] Following a full scan and a precursor ion scan for *m/z* 184.0 of the entire separation with a mass range of 450–600 Th, three ions were further selected for analysis by MS^3. Our previous report on PC12 differentiation[11] informed us when the C16:0 PAF and C18:0 PAF species eluted; MS^3 was attempted on each. We also knew that the concentration of the C18:0 PAF species was low in PC12 cells while the C16:0 PAF was well above the limit of detection.[11] Hence, we expected that, if detected, insufficient data would be accumulated for the C18:0 PAF while the C16:0 PAF should be amenable to this technique. MS^3 was also attempted on an unknown species

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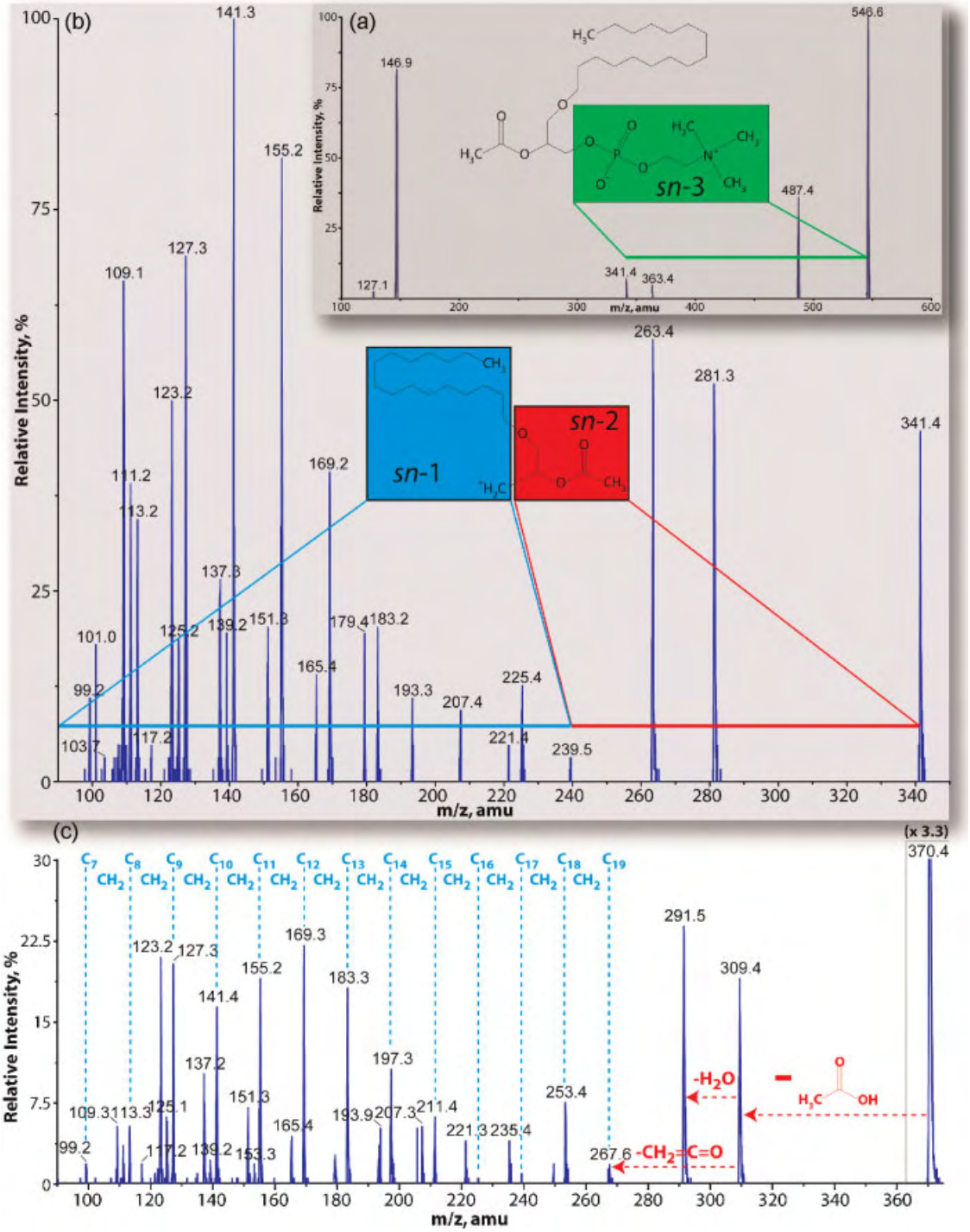

Figure 2. MS^2 and MS^3 analysis of PAF. (a) MS^2 spectrum of sodiated C16:0 PAF (structure indicated in inset) revealing a peak at *m/z* 341. The mass difference between *m/z* 341 and the molecular ion is consistent with the loss of neutral sodiated phosphocholine (highlighted in green). (b) Further fragmentation of the *m/z* 341 radical cation produced a spectrum revealing structural characteristics of the *sn*-1 and *sn*-2 groups. The lower mass ions correspond to fragments of the fatty alcohol chain of the *sn*-1 group while the higher mass fragments represent an intact *sn*-1 group with fragmentation occurring on the *sn*-2 group and glycerol backbone. (c) MS^3 spectrum of sodiated C18:0 PAF further illustrating how the low mass ions reveal the carbon chain connectivity of the *sn*-1 group fatty alcohol and the high mass ions reveal structural information pertaining to the *sn*-2 group and glycerol backbone. The lower mass peaks are separated by equal masses of 14 Da, representing successive losses of CH_2 from the *sn*-1 fatty alcohol, identifying the lipid subspecies with high confidence. Peaks corresponding to successive losses of 2 Da from each fragment peak arise from gas phase dehydrogenation, a commonly observed phenomenon.[12,14,23] This figure is available in colour online at www.interscience.wiley.com/journal/rcm.

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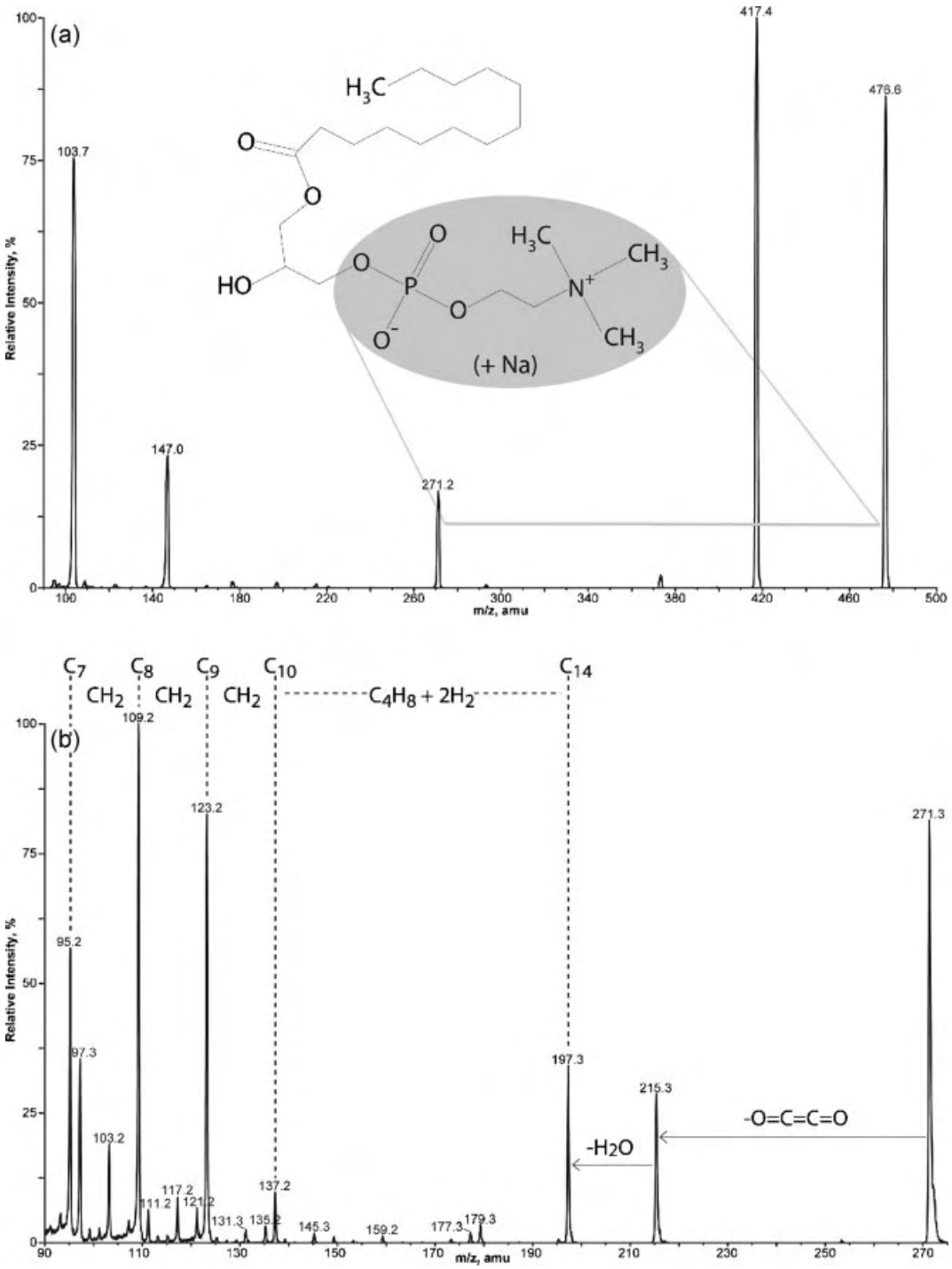

Figure 3. MS^2 and MS^3 analysis of LPC. (a) MS^2 spectrum of sodiated C13:0 LPC (structure shown in inset) revealing a fragment corresponding to the neutral loss of 205 Da from the molecular ion (neutral sodiated phosphocholine, highlighted in grey). (b) MS^3 of this fragment ion reveals the atomic connectivity of the *sn*-1 group through the lower mass ions (successive CH_2 losses are illustrated) as well as the *sn*-2 group and glycerol backbone at higher mass. With the exception of the C_{14} ion, all fatty acid radical cation fragments have undergone gas phase dehydrogenation.

at *m*/*z* 522 (protonated) with an intensity comparable to the C16:0 PAF species.

A second injection using mobile phases containing 100 M sodium acetate allowed the three phosphocholine-containing species to be dissociated using MS^3 and information regarding the carbon chain of the fatty acid/alcohol on their *sn*-1 positions to be obtained. The species corresponding to the mass of C18:0 PAF was present at very low concentration in accordance with previous findings[11] and could therefore not be identified. MS^3 on a second species produced the spectrum shown in Fig. 4(a). The fragmentation pattern is similar to Fig. 3(b); a large peak that represents a loss of 56 Da

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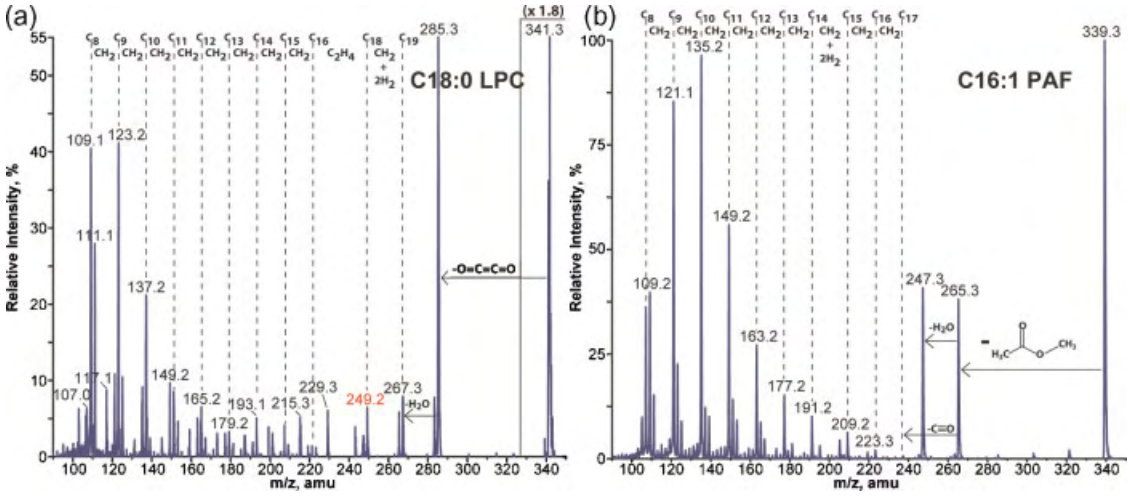

Figure 4. MS^3 elucidation of PAF species from PC12 cells. (a) MS^3 spectrum of C18:0 LPC; *m/z* 249.2 (in red) is unusually high in the ion series and represents the loss of water from *m/z* 267.3, demonstrating that the spectrum was produced from the additional fragmentation of a small amount of C16:0 PAF. (b) MS^3 spectrum of C16:1 PAF. The spectra reveal the atomic connectivity of the *sn*-1 fatty acid/alcohol chains, illustrated by differences of 14 Da (CH_2 groups), as well as fragments pertaining to the *sn*-2 group and glycerol backbone at higher masses within the spectra. Gas-phase dehydrogenation is evident as mass shifts of 2 Da. This figure is available in colour online at www.interscience.wiley.com/journal/rcm.

from the 341.3 Th ion is observed thus identifying C18:0 LPC in the PC12 extract. Several peaks in Fig. 4(a) are also consistent with the presence of C16:0 PAF (e.g. a relatively high abundance of 249.2 (in red) representing an additional loss of water from *m/z* 267.3); the spectrum was generated over a broad elution window and likely contains components of both C16:0 PAF and C18:0 LPC. The third species (*m/z* 522) eluted 5 min before the C18:0 LPC species, suggesting that C16:1 PAF and/or C18:1 LPC may be present in the extract. The sodiated version of this unidentified species at *m/z* 544 was analyzed by MS^3 (Fig. 4(b)). The resulting spectrum did not reveal a 56 Da neutral loss from the precursor ion (*m/z* 339.3) and produced diagnostic fragments up to $[CH_3(CH_2)_{16}]^{\cdot+}$, allowing us to readily identify the species as C16:1 PAF. To the best of our knowledge, the C16:1 PAF species has not been previously identified in PC12 cells. This example also demonstrates the ability of MS to elucidate the fatty acid/alcohol structure of intact phosphoglycerolipids separated from a complex lipid matrix within the temporal confines of a chromatographic separation. Furthermore, reanalysis of 3D phosphocholine profiles of PC12 and differentiated PC12 cells that we previously reported[11] indicate that the level of C16:1 PAF increases significantly during cell differentiation. The impact of the presence of C16:1 PAF and of its increase during cell differentiation remains unclear and requires further investigation but may suggest that naturally occurring 1-*O*-alkylglycerols present in diet[26] or *in vitro* fatty acid/alcohol supplementation are differentially incorporated into cells over the course of neuronal differentiation.

Mouse cortex analysis

Animal models are often used to study complex neurological diseases or disorders such as Alzheimer's disease, Parkinson's disease or stroke. In light of this, our gas-phase strategy was used to analyze and identify the PAF and LPC species present in mouse brain tissue. Briefly, lipids were extracted from the cerebral cortex of a single mouse and were analyzed using a precursor ion scan for *m/z* 184 as well as MS^3. Figure 5(a) illustrates the 3D profile of the protonated phosphocholine ion for this sample. Once again, the C18:0 PAF did not produce a significant signal nor a sufficiently detailed MS^3 spectrum (data not shown). Both C16:1 PAF and C18:0 LPC were readily identified from the mouse cortex sample (Figs. 5(c) and 5(e), respectively), revealing that the concentration of C18:0 LPC is sufficient for detection and that our newly identified C16:1 PAF is a prominent species within the mouse cerebral cortex lipid composition, possibly due to the presence of the 1-*O*-alkylglycerol precursor in mouse chow/diet. Figure 5(e) likely contains peaks from C16:0 PAF as well; the 285.2 Th ion was not produced throughout the entire acquisition and the relatively high *m/z* 249.3 peak (in yellow) indicates the loss of water from *m/z* 267.4.

In addition to lipids that have been previously identified, Fig. 5(a) reveals several other areas that produce high intensity precursor signals for the 184 Th fragment ion. Figure 5(b) reveals the MS^3 spectrum of the sodiated version of *m/z* 544.4. Although the spectrum that was attained is weak, enough information is revealed to demonstrate that a 56 Da neutral loss does not occur during MS^3 and therefore identify the species as C18:4 PAF. MS^3 on the sodiated version of another species at *m/z* 496.4 produced an intense loss of 56 Da, identifying the presence of C16:0 LPC (Fig. 5(d)). Again, an additional loss of water from *m/z* 239.2 (*m/z* 221.3 (in red)) would suggest that the spectrum was also generated by a small amount of C14:0 PAF that eluted during the spectral acquisition. An MS^3 investigation of the other ions visible in Fig. 5(a) is currently being pursued.

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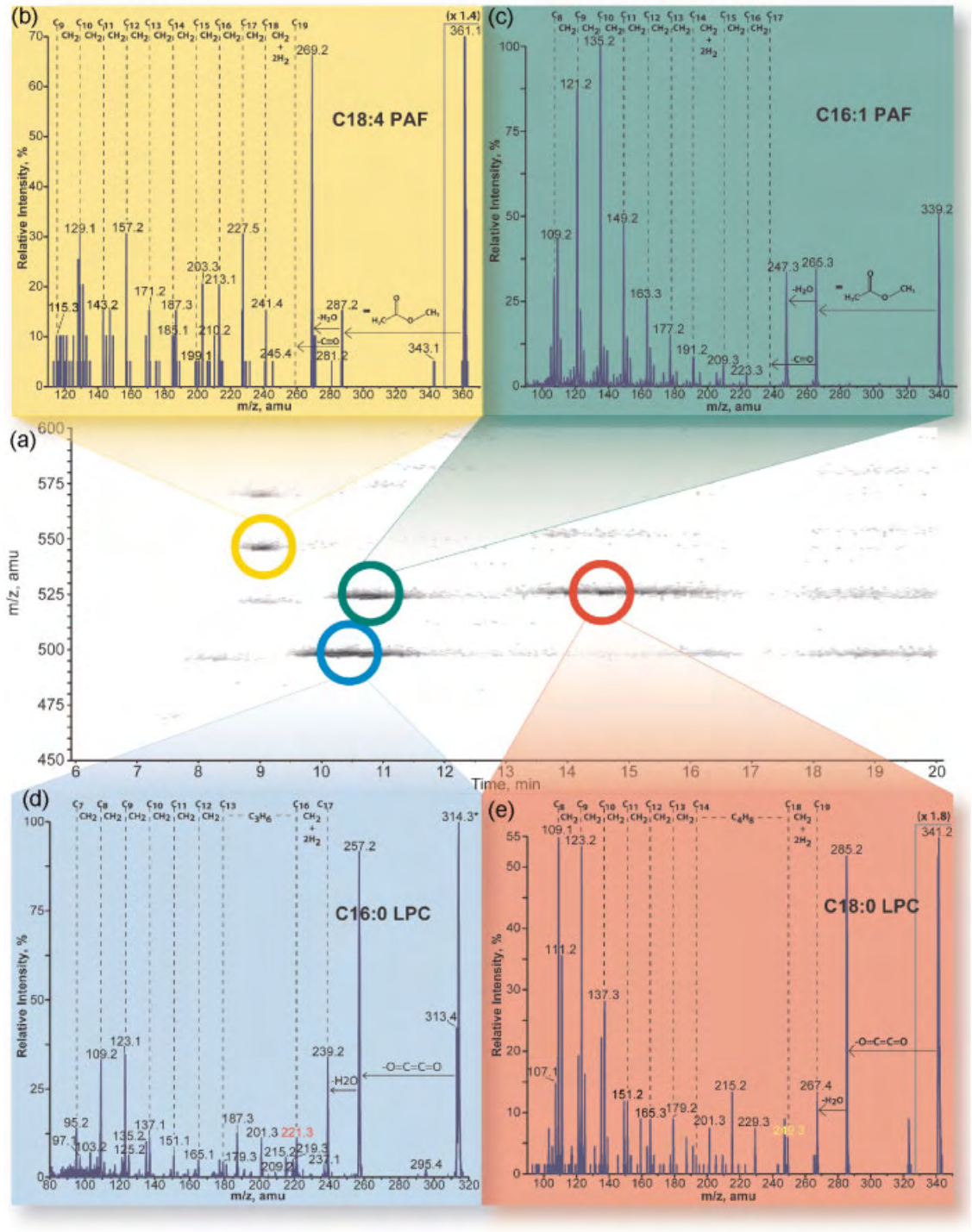

Figure 5. MS^3 elucidation of PAF species from mouse cerebral cortex. (a) Three-dimensional plot revealing phosphocholine-containing species in a lipid extract from mouse cerebral cortex, obtained through the use of a precursor ion scan for *m/z* 184. (b) Spectrum of a novel lipid species identified as C18:4 PAF using sodiated MS^3. (c) MS^3 spectrum of C16:1 PAF. (d) Spectrum of a novel lipid species identified as C16:0 LPC using sodiated MS^3; *m/z* 221.3 (in red) is unusually high in the ion series and represents the loss of water from *m/z* 239.2, demonstrating that the spectrum was produced from the additional fragmentation of a small amount of C14:0 PAF. (e) MS^3 spectrum of C18:0 LPC; *m/z* 249.3 (in yellow) is unusually high in the ion series and represents the loss of water from *m/z* 267.4, demonstrating that the spectrum was produced from the additional fragmentation of a small amount of C16:0 PAF. The MS^3 spectra reveal the atomic connectivity of the *sn*-1 fatty acid/alcohol chains, illustrated by differences of 14 Da (CH_2 groups), as well as fragments pertaining to the *sn*-2 group and glycerol backbone at higher masses within the spectra. Gas-phase dehydrogenation is evident as mass shifts of 2 Da. This figure is available in colour online at www.interscience.wiley.com/journal/rcm.

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CONCLUSIONS

Radical cations composed solely of carbon and hydrogen atoms were produced from intact sodiated PAF and LPC molecules and fragmented in the gas phase. Despite the structural similarity between isobaric PAF and LPC lipids, MS^3 analysis produced unique spectral differences for each species. In particular, the neutral loss of 56 Da is a signature of LPC and not PAF. Neutral losses of 56 Da were observed for some lipid species generated *in vivo*, and this criterion was the main element used to distinguish isobaric LPC and PAF species from each other.

Our proof-of-principle study demonstrates a unique approach to identify specific lipids extracted from biological samples exclusively using a gas-phase analytical technique. C18:0 LPC and C16:1 PAF were identified in both PC12 cells as well as the mouse cerebral cortex; C16:1 PAF being a novel component within each. The presence of C16:0 LPC was confirmed in mouse cerebral cortex while C18:4 PAF was, to the best of our knowledge, a novel discovery in this tissue. The biological implications of these discoveries are currently under investigation. This strategy is amenable to complicated samples even within the temporal confines of a chromatographic separation. Increasing the sensitivity of the approach (e.g. through the use of more modern instrumentation) and automating the data collection (e.g. developing information-dependent acquisition methods) will permit high-throughput identification on a global scale and will continue to aid research fields related to human health and disease or the regulation of biological systems.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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