Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of lipids: ionization and prompt fragmentation patterns

Khalid A. Al-Saad¹, Vladimir Zabrouskov², William F. Siems¹, N. Richard Knowles², Richard M. Hannan³ and Herbert H. Hill Jr.¹*

¹Department ofChemistry, Washington State University, Pullman, WA 99164-4630, USA
²Department ofHorticulture and Landscape Architecture, Washington State University, Pullman, WA 99164-4630, USA
³USDA-ARS Regional Plant Introduction Station, Washington State University, Pullman, WA 99164-4630, USA

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Ionization and prompt fragmentation patterns of triacylglycerols, phospholipids (PLs) and galactolipids were investigated using matrix-assisted laser desorption/ionization (MALDI). Positive ions of non-nitrogen-containing lipids appeared only in the sodiated form, while nitrogen-containing lipids were detected as both sodiated and protonated adducts. Lipids containing acidic hydroxyls were detected as multiple sodium adducts or deprotonated ions in the positive and negative modes, respectively, with the exception of phosphatidylcholines. The positive MALDI spectra of triacylglycerols contained prompt fragments equivalent to the loss of $\text{RCOO}^-$ from the neutral molecules. Prompt fragment ions [PL–polar head]$^-$ were observed in the positive MALDI spectra of all phospholipids except phosphatidylcholines. The phosphatidylcholines produced only a minor positive fragment corresponding to the head group itself ($m/z$ 184). Galactolipids did not undergo prompt fragmentation. Post-source decay (PSD) was used to examine the source of prompt fragments. PSD fragment patterns indicated that the lipid prompt fragment ions did not originate from the observed molecular ions (sodiated or protonated), and suggested that the prompt fragmentation followed the formation of highly unstable, probably protonated, precursor ions. Pathways leading to the formation of prompt fragment ions are proposed. Copyright © 2002 John Wiley & Sons, Ltd.

Lipids are major constituents of living cells. Phospholipids (PLs) are structural components of biological membranes, regulating their biophysical properties, protein sorting and cell signaling pathways. Structurally, PLs are diacyl(alkyl) glycerols esterified to a polar head. According to the nature of the head group, they are divided into several classes, including phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), and cardiolipin (CL). The chemical nature of the polar head and acyl (alkyl) chains, as well as the distribution of the acyl chains within the lipid molecule, define the unique molecular species of PLs. Phospholipid composition of cellular membranes contributes to their biochemical and biophysical properties. Alterations in the degree of unsaturation of acyl side chains, for instance, can affect membrane fluidity. In many organisms, such changes are significant for adaptation to changes in temperature and to the presence of xenobiotics.¹² Triacylglycerols (TAGs) are not membrane struc-
tural lipids, but accumulate in some tissues as a storage form of carbon.

Mass spectrometric analysis of intact lipid molecules has been performed using chemical ionization,⁴⁻⁸ fast atom bombardment (FAB),⁹⁻¹³ electrospray,¹⁴⁻²² and MALDI.²³⁻³² Electrospray, FAB and MALDI have been the most widely used soft ionization methods. ESI and FAB with tandem mass analysis provide compositional and structural information of lipid molecular species in complex biological matrices¹⁰,¹⁹,²¹ and are indispensable tools for the analysis of novel lipid compounds. The routine application of these techniques to large numbers of samples is still complicated, due to their intolerance to impurities (ESI) and varied instrumental response to different lipid classes/molecular species (ESI, FAB).¹⁴,¹⁶,²²,²³ MALDI analysis of lipid molecules is generally more sensitive and less affected by impurities and polarities of the analytes than ESI.²⁵,²⁶,²⁸,³³ Although seldom used to provide detailed structural information for particular molecular species,²⁸,³¹,³⁸ MALDI is potentially an excellent analytical method for rapidly screening lipid components in biological matrices.³⁵

Using MALDI, all lipid classes have been detected in the
positive mode, while PA, PI and PS have also been observed in the negative mode.\textsuperscript{23,28,30} In the positive mode, lipids differed in their affinity for sodium ions. For instance, TAGs and diacylglycerols (DAGs) were only detected as sodium adducts and not as protonated molecules, when 2,5-dihydroxybenzoic acid (DHB) was used as matrix.\textsuperscript{23,26,28,31} For phospholipids such as PE and PS, protonation and the multiple additions of sodium ions were simultaneously observed.\textsuperscript{23,24,30} Potassium adducts have also been observed but to a relatively minor extent. In many cases, ionization of lipid molecules was accompanied by prompt (in-source) fragmentation.\textsuperscript{23,28} Harvey\textsuperscript{23} suggested that prompt fragmentation of lipids in MALDI is a result of gaseous hydrolysis, but did not identify the precursors of the prompt fragment ions. The generation of multiple signals by association with different cations and by prompt fragmentation as a function of lipid classes is not well described. Multiple signals lead to ambiguity in determining the relative amounts of individual lipid molecular species in biological mixtures.

In this study, we investigated factors that determine the tendency of lipid molecules to be sodiated, protonated or negatively charged. The susceptibility of lipids to undergo prompt fragmentation as a function of their molecular structures was studied, and pathways leading to the formation of prompt fragment ions were proposed.
**EXPERIMENTAL**

**Materials and sample preparation**

Solvants were reagent grade and purchased from J. T. Baker (Phillipsburgh, NJ, USA). 2,5-Dihydroxybenzoic acid (DHB) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). TAG, monogalactosyldiacylglycerol (MGDG), and digalactosyldiacylglycerols (DGDGs) were obtained from Sigma (St. Louis, MO, USA). Phospholipid standards were from Avanti Polar Lipid Inc (Alabaster, AL, USA). DHB in acetone was used as a matrix for all standards. All lipids in chloroform were premixed (1:1) with the matrix solution except TAGs and PE, which gave better signals when they were mixed on the MALDI plate surface. Mixing on the plate was performed by spotting 1 μL of the matrix solution followed by the addition of 1 μL of lipid sample. The samples were air-dried for 1 min before MALDI analysis. The MALDI plate wells each contained 50–100 ng of lipids. Addition of KCl or NaCl was performed by mixing 1 μL of salt solution (1 M) with 100 μL of the matrix solution (DHB).

**Instrumentation**

MALDI spectra were obtained using a PerSpective Biosystems (Framingham, MA, USA) Voyager DE-RP time-of-flight mass spectrometer equipped with a nitrogen laser (337 nm). All spectra were averages of 256 individual laser shots. The laser was adjusted to about one-third of its maximum power, depositing approximately 100 μJ per laser pulse. The applied potential was 25000 V, and the focusing guide wire was held at a potential of 0.16% of the accelerating voltage. A delay time of 140 ns was used between the time of the laser pulse and the application of the accelerating voltage. For all lipid standards, MALDI spectra were acquired in the positive and negative reflector modes, from m/z 0–5000. The matrix (DHB) did not saturate the detector in the lower mass region. Spectra were externally calibrated using standard PC(16:0/18:2) and PC(18:2/18:2) (The positive MALDI-TOF spectrum of PC contains peaks corresponding to [PC + H]⁺ and [PC + Na]⁺ 26,27,31). The resolution in the reflector mode was sufficient to obtain isotopic patterns of all analytes. In the PSD experiment, the precursor ions (sodium adducts and protonated molecules) were isolated using a timed ion selector. The laser intensity for PSD was maintained the same in the reflector mode, unless noted otherwise. The fragment ions were refocused onto the detector by stepping the voltage applied to the reflector in appropriate increments.

**RESULTS AND DISCUSSION**

**Triacylglycerols**

The types of ions observed from TAGs and their chemical structures are shown in Table 1. TAGs were detected in the positive mode as sodium ion adducts only. Fragment ions equivalent to the loss of one of the fatty acid anions from neutral TAGs (R₁COO⁻, where n can be 1, 2 or 3, referring to the carbon on the glycerol backbone) were also present. Positive ion MALDI spectra of TAG(12:0/14:0/14:0) and TAG(14:0/14:0/16:0) are shown in Figs 1(a) and 1(b), respectively. The peaks at m/z 717.6 and 773.7 correspond to the sodium ion adducts of the TAGs. No protonated TAGs were detected, in agreement with previous work on the analysis of TAGs by MALDI. Ions reflecting the loss of one of the carbohydrate groups were also observed. The signals at m/z 467.4 and 495.4 are equivalent to the loss of the carbohydrate groups (14:0) and (12:0), respectively, from TAG(12:0/14:0/14:0) (Fig. 1(a)). The signals at m/z 495.5...
Figure 2. (a) Positive ion MALDI spectrum of TAG(14:0/14:0/14:0); the precursor ion was [TAG + Na]⁺. (b) PSD-MALDI spectrum of TAG(14:0/14:0/14:0); 523.5 and 523.5 are equivalent to the loss of (16:0) and (14:0) anions, respectively, from TAG(14:0/14:0/16:0) (Fig. 1(b)). These observations are in agreement with previously published studies.²⁸

Six different TAGs with one side chain (at the sn-1 position) differing from the other two were studied. The relative intensities of the fragment ions due to the loss of the carboxylate groups are shown in Fig. 1(c). The intensities of the fragment ions corresponding to the loss of carboxylate group ‘A’ (R₃CO₂⁻) from TAG(A/A/B)s were normalized. The intensities of the fragment ions due to the loss of R₃CO₂⁻ were observed to be 0.5 (half the intensities due to the loss of group ‘A’). The consistency of the relative abundances of fatty acid substituents with the signal intensities (due to their loss) suggests that there was no preferential loss that could be attributed to size or position of the carboxylate groups. In contrast, a previous ESI-MS/MS-CID study²¹ indicated that the loss of the sn-2 fatty acid moiety from lithiated TAGs is the least favorable. However, there is no reason to suppose that CID of lithiated TAGs and prompt fragmentation of TAGs in the MALDI experiment should show the same behavior.

In all MALDI spectra of TAGs, the prompt fragments were not sodiated and the lost carboxylate groups were not detected as ions. Based on this observation, two possibilities were considered: (1) the sodium ion was associated with one of the side chains of TAGs promoting partial loss of RCOO⁻ as a neutral sodium salt; (2) the fragment ions were originally generated from extremely unstable protonated TAGs, which readily lost RCOOH, and therefore were not detected as precursor ions. On examining the spectra obtained using the continuous (non-delayed extraction) mode, no protonated molecules were observed. This does not necessarily eliminate the possibility of protonated ions being formed but suggests that, if TAGs indeed undergo protonation, the protonated ions are extremely unstable and may be fragmented before transfer to the gas phase. Based on these non-delayed extraction results, however, the possibility still remained that sodiated TAGs lost RCOONa to generate the prompt fragments observed.

PSD of TAGs was utilized to investigate the source of the prompt generated fragment ions. Figure 2(a) shows the MALDI spectrum of TAG(14:0/14:0/14:0) in the positive reflector mode, and Fig. 2(b) shows the PSD-MALDI spectrum obtained when the sodium adduct (m/z 745.5) of the TAG was isolated by the timed ion selector. Based on the intensities of the signals, the loss of RCOOH from [TAG + Na]⁺ is approximately as frequent as the loss of RCOONa. If this is generally true, our failure to observe a peak corresponding to the fragment ion [(TAG + Na)-

Scheme 1. Proposed fragmentation pathways of TAG. The solid arrow (a) shows the pathway leading to the prompt fragment ions and the dotted arrows (b, c) show the pathways leading to fragments observed in the PSD experiments. Both sn-1 and sn-2 substituents can also be expelled following similar pathways.
Table 2. Lipid ions observed in the MALDI spectra (N-containing lipids)

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Positive ions</th>
<th>Negative ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>PC + H⁺, PC + Na⁺</td>
<td>×</td>
</tr>
<tr>
<td>PE</td>
<td>PE + H⁺, PE + Na⁺, [PE + 2Na-H]⁺</td>
<td>√</td>
</tr>
<tr>
<td>PS</td>
<td>PS + H⁺, PS + Na⁺, [PS + 2Na-H]⁺, [PS + 3Na-2H]⁺</td>
<td>√</td>
</tr>
</tbody>
</table>

* The m/z values for the metastable ions belong to PE(18:0/18:1) and PS(18:0/18:1).

RCOOH⁺ in the reflector mode, and the appearance of a peak at m/z equivalent to the fragment ion [(TAG + Na)-RCOONa]⁺, suggests that this promptly generated fragment ion was derived from a TAG molecular ion other than the sodiated adduct actually detected.

At extreme laser intensity (near one-half of maximum power) a pair of metastable ions (m/z 526 and 544) was observed in the reflector mode (Fig. 2(a)). These ions are identical to those observed in the PSD spectrum (m/z 496 and 518, Fig. 2(b)) but, due to their reduced kinetic energy, do not exhibit their correct m/z values. [TAG + Na⁺] was confirmed to be the origin of these metastable ions (m/z 526 and 544) by using the timed ion selector to isolate the sodiated TAG precursor formed at high laser power.

Based on this evidence, the prompt TAG fragments were different from those produced from [TAG + Na⁺], it is likely that [TAG + H⁺] was formed and immediately (promptly) fragmented. This agrees with the speculation of Siuzdak, ⁴⁴ that “charge delocalization”, caused by the covalent nature of proton binding, “can destabilize the molecular ion, resulting in fragmentation.” The proposed pathways leading to the prompt fragment [(TAG + H)-RCOOH]⁺ (m/z 495.5 in Fig. 2(a)), as well as [(TAG + Na)-RCOOH]⁺ and [(TAG + Na)-RCOONa]⁺ observed as PSD products of [TAG + Na⁺] (m/z 496 and 518 in Fig. 2(b)), are illustrated in Scheme 1. In the protonated TAGs, the covalent bonding of the proton to any of the oxygens of the carboxylic groups reduces electron density in the C—O bond leading to the loss of RCOOH from [TAG + H⁺]. This fragmentation may be assisted by nucleophilic attack by an oxygen belonging to one of the other acyl side chains. The ionic bonding of sodium, on the other hand, forms a more stable [TAG + Na⁺] species. Sodium may initiate a fragmentation pathway (arrow ‘b’ in Scheme 1) similar to that initiated by the proton (arrow ‘a’), but not rapidly enough to produce prompt fragments. Another pathway (arrow ‘c’) involves removing a proton from the glycerol backbone. Previous work, using ESI coupled with high-energy collisional activation, proposed similar products of [TAG + Na⁺] fragmentation. ⁴⁵

Phospholipids

Precursor ions

Observed molecular ions and prompt fragments of non-N-containing lipids and N-containing lipids are summarized in Tables 1 and 2, respectively. As previously demonstrated, ²³,²⁴ all phospholipids (PL) were detected as molecular ions in both the positive and negative ion modes except PC, which could only be detected as a positive ion. Nitrogen-containing phospholipids (PC, PE, PS) were detected as both protonated and sodiated molecules. Non-N-containing phospholipids, on the other hand, were detected only as sodium adducts. PE, PG and PI exhibited the addition of two sodium ions, whereas PA, PS and CL exhibited the addition of three sodium ions (where one of the sodium ions contributes the positive charge and the other ions replace acidic protons). Only monosodiated PC was observed. In all PL spectra, dimers were occasionally observed ([2M + nNa]-mH⁺, where m = n – 1). The ion [2PC + H⁺] was also observed in addition to the sodiated PC dimer. A discussion of the formation and behavior of the dimers is beyond the scope of this study.

Positive ion MALDI spectra of PLs are shown in Figs 3(a)-3(g). The signals corresponding to the protonated and sodiated molecules are indicated in the spectra. As a neutral compound, PC contains a quaternary nitrogen, which is positively charged, and the counter negative charge is located on a phosphate oxygen. Thus, neutral PC (zwitter-ion) is already deprotonated. Further deprotonation of the phosphate group is not possible, and thus PC was not detected in the negative ion mode. The protonated molecules in the spectra of PE and PS were apparently stabilized by the
Figure 3. Positive ion MALDI spectra of PLs. (a) PC(18:0/16:0), MW 762.09; (b) PE(18:0/18:1), MW 746.05; (c) PS(18:0/18:1), MW 790.06; (d) PA(16:0/18:1), MW 674.93; (e) PG(16:0/16:0); MW 722.97. (f) Positive ion MALDI spectrum of soybean PI indicating the presence of mainly two molecular species: P1 = PI(16:0/18:2) and P2 = PI(18:0/18:2). (g) CL(14:0/14:0/14:0/14:0), MW 1240.09. * Metastable ions. The MALDI plate wells each contained approx. 20 ng of PC and 100 ng of the other PLs. The m/z values shown in all spectra refer to the monoisotopic mass.

presence of nitrogen. No protonated molecules were detected in the spectra of non-N-containing phospholipids (PA, PG, PI, CL) (Figs 3(d)-(g)) or TAGs.

Major peaks in the MALDI spectrum of soybean PI (Fig. 3(f)) may be assigned to singly and doubly sodiated PI(16:0/18:2) (P1) and PI(18:0/18:2) (P2), and to the fragments resulting from the prompt losses of polar head groups from these species (equivalent to the loss of inositol phosphate anion from PI). The negative spectrum of soybean PI (Fig. 4(a)) clearly shows peaks corresponding to deprotonation of P1 and P2, while addition of K⁺ (Fig. 4(b)) causes the main peaks in Fig. 3(f) to shift by 16 Da, confirming the postulated sodiation of PI species in this figure. The identification of P1 and P2 as PI(16:0/18:2) and PI(18:0/18:2) is consistent with that previously demonstrated using a MALDI-FITIR instrument. The fatty acid composition of PI provided by Avanti Polar Lipid Inc.: [33.2% (16:0); 7.1% (18:0), 5.5% (18:1); 46.8% (18:2) and 7.1% (18:3)] further supports the proposed assignment of molecular species.
Figure 4. (a) Negative ion MALDI spectrum of soybean PI. (b) Positive ion MALDI spectrum of soybean PI after addition of KCl to the matrix.

The apparent addition of two sodium ions in PE, PG and PI (Figs 3(b), 3(e), and 3(f)) to form a singly charged molecular ion was due to the presence of one acidic hydroxyl group in which the proton was replaced by a sodium ion, additional to that contributing the positive charge.23 PS, PA and CL contain two acidic hydroxyls and thus the addition of three sodium ions was possible (Figs 3(c), 3(d), and 3(g)). Also, due to the presence of acidic hydroxyls, all PLs (except PC) were detected in the negative ion mode (unlike TAGs).

In order to check the possibility that the extent of addition of sodium ion is dependent on the amount of sodium contaminants and not exclusively determined by the lipid type, sodium chloride was added to the matrix (10 mM NaCl). The number of sodium ions added to all lipid molecules investigated did not exceed that observed when no sodium salt was used in the matrix solution, indicating that only molecular structure determined the number of added sodium ions observed in the MALDI spectra.

Fragment ions
Unlike TAGs, the loss of an acyl group was not observed in the MALDI spectra of PLs. Using DHB as matrix, a prompt fragment ion equivalent to [PL–polar head]− was observed in the spectra of all PLs except PC (Tables 1 and 2). According to the proposal of Harvey,23 the prompt fragments were produced via hydrolysis (bimolecular decomposition) in the gas phase. This mechanism seems unlikely in our case, since prompt fragments were present when non-aqueous matrix solutions were used. In addition, the prompt fragments observed in this study were not hydrolysis products, but the products of unimolecular decomposition (water molecules were not added). The formation of the prompt fragments (equivalent to the loss of neutral phosphate moieties from positively charged PLs) was evidently caused by a positive charge localized on the phosphate groups, a situation that is not feasible for PC since it contains a positively charged quaternary nitrogen atom. Distinct fragmentation behavior of PC vs. other PLs was also observed in ESI-CID experiments.33 However, the formation of the fragment (not necessarily prompt) equivalent to [PC−polar head]− is still feasible, and indeed was described previously when using a more energetic matrix (e.g. α-cyano-4-hydroxycinnamic acid) dissolved in water and/or higher laser fluence.23 Interestingly, Marto et al.24 utilizing MALDI-FTICR, observed several fragments including [PC + Na−–polar head]−. In our experiments, these same fragments were observed only in MALDI-TOF-PSD spectra with [PC + Na]− as a precursor using high laser intensity. Possibly ions which are observed when using a TOF analyzer as metastable (fragmented during flight, and thus not focused, Fig. 3) can potentially become trapped in an FTICR cell when long relaxation times are used.24 Thus, the fragments observed in FTICR experiments are not likely to
Table 3. Polar head fragments observed in PSD experiment

<table>
<thead>
<tr>
<th>Phospholipids (PL)</th>
<th>Precursor ions</th>
<th>(m/z)</th>
<th>Equivalent to</th>
<th>[PL-HG]⁺ m/z</th>
<th>Equivalent to</th>
<th>[PL-HG + Na⁺H⁺] m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>PG(16:0/16:0)</td>
<td>[PG + Na⁺]⁺</td>
<td>(746)</td>
<td>552</td>
<td>574</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[PG + 2Na⁺H⁺]⁺</td>
<td>(768)</td>
<td>Not observed</td>
<td>NO</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[PG + Na⁺]⁺</td>
<td>(888)</td>
<td>576</td>
<td>598</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[PG + Na⁺]⁺</td>
<td>(698)</td>
<td></td>
<td>NO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA(16:0/18:1)</td>
<td>[PA + Na⁺]⁺</td>
<td>(720)</td>
<td>NO</td>
<td>NO</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[PA + Na⁺]⁺</td>
<td>(742)</td>
<td>NO</td>
<td>NO</td>
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<tr>
<td>PE(18:0/18:1)</td>
<td>[PE + Na⁺]⁺</td>
<td>(769)</td>
<td>606</td>
<td>628</td>
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<tr>
<td></td>
<td>[PE + 2Na⁺H⁺]⁺</td>
<td>(791)</td>
<td>NO</td>
<td>NO</td>
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<td></td>
<td>[PE + Na⁺]⁺</td>
<td>(813)</td>
<td>606</td>
<td>628</td>
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<tr>
<td></td>
<td>[PE + 2Na⁺H⁺]⁺</td>
<td>(835)</td>
<td>NO</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[PE + 3Na⁺H⁺]⁺</td>
<td>(857)</td>
<td>NO</td>
<td>NO</td>
<td></td>
<td></td>
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<tr>
<td>PS(18:0/18:1)</td>
<td>[PS + Na⁺]⁺</td>
<td>(763)</td>
<td>NO</td>
<td>NO</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>[PS + Na⁺]⁺</td>
<td>(785)</td>
<td>NO</td>
<td>NO</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

be equivalent to the prompt fragments observed using a MALDI-TOF instrument.

The prompt fragments observed here were not sodiated. Therefore, analogous to TAGs, two possibilities were considered: PLs were either fragmentated as extremely unstable protonated ions, or, alternatively, fragments resulted from sodiated ions that lost their head groups as sodium salts. It was suggested above that the loss of an acyl group from a TAG in MALDI was caused by the presence of a proton on an oxygen belonging to an acyl group. Thus, if PLs did fragment as protonated ions, failure to observe the loss of acyl groups from PLs, but rather the loss of head groups, would suggest that the proton could only be associated with the head group. However, if the prompt fragments originated from sodiated ions, our observation could be accounted for by two coexisting types of ions: (1) ions in which sodium is associated with the polar heads leading to [(PL + Na⁺)-(head group - Na⁺)]⁺; and (2) ions in which the sodium is associated with the acyl groups and which do not fragment promptly.

PSD was utilized to identify the source of the PL prompt fragments. PSD spectra of PE and PG are shown in Figs 5(a) and 5(b), respectively, for which singly sodiated precursors [PE + Na⁺]⁺ and [PG + Na⁺]⁺ were selected. PSD spectra were also obtained from singly sodiated PA and PS (results are summarized in Table 3). A signal at m/z 725 in the PSD spectrum of PE (Fig. 5(a)) corresponds to the loss of ethanolamine, but otherwise the PSD spectra of singly sodiated PE, PG, PA, and PS were similar, showing a pair of fragments: [(PL + Na⁺)-(HNaPO₄-R₃)]⁺ and [(PL + Na⁺)-(H₂PO₄-R₃)]⁺ (Table 3), where R₃ refers to the group attached to the phosphate moiety in the polar head. For example, a pair of fragment ions (m/z 606 and 628) is shown in Fig. 5(a), which correspond to [(PE + Na⁺)-(HNaPO₄-R₃)]⁺ and [(PE + Na⁺)-(H₂PO₄-R₃)]⁺, respectively. On the other hand, the positive spectra of PLs (except PC) showed a single prompt fragment at m/z equivalent to [PL-(HPO₄-R₃)]⁺ (Figs 3(b)–(g)). The fact that a pair of ions is observed in the PSD spectrum, in comparison to a single prompt fragment in the reflector mode, suggests that the prompt fragmentation pathway is different from that pertinent to PSD fragmentation.

The prompt fragments may originate either from protonated precursors (similar to TAGs), or from a population of sodiated ions structurally distinct from the population that yields the PSD fragments. The latter explanation seems less likely since PSD of doubly sodiated precursor ions ([PL + 2Na⁺H⁺]⁺) did not produce any fragments (Table 3) unless the laser fluence was increased far above the level used to generate the reflector mode spectra. For the purpose of determining the source of prompt fragment ions, the laser intensity in PSD experiments was kept the same as in the reflector experiments. It should be mentioned, however, that at increased laser intensities, several PSD fragment ions could be observed. These fragments indicated preferential loss of the sn-1 acyl chain, and thus were useful for determining the size and position of the fatty acyl groups on the glycerol backbone. A manuscript describing post-source decay behavior of lipid molecules is in preparation.

The susceptibility of protonated PLs to prompt fragmentation, and the formation of the fragments distinct from those produced via PSD using singly sodiated PL (s) as precursor ion(s), can be rationalized based on the nature of proton binding as opposed to the ionic binding of sodium as described by Szudak. The possible fragmentation pathways of phospholipids are presented in Scheme 2. The cleavage of a C–O bond was evidently initiated by a pathway similar to that proposed for TAGs (Scheme 1). However, failure to observe prompt fragments due to the loss of fatty acid groups suggests that the proton is localized on the oxygen belonging to the phosphate group.

The PSD spectrum of PE(18:0/18:1), when [PE + H⁺]⁺ was used as a precursor ion, produced a strong signal (m/z 606) corresponding to [(PE + H⁺)-(H₂PO₄-R₃)]⁺ (Fig. 5). Similarly, the PSD spectrum of the precursor ion [PS(18:0/18:1) + H⁺]⁺ produced a signal at m/z 606 which belonged to [PS + H⁺]-
Scheme 2. Proposed fragmentation pathways of PL (does not apply to PC). The solid arrow (a) shows the pathway leading to the observed prompt fragment ions and the dotted arrows (b, c, d) show the pathways leading to fragments observed in the PSD experiments. The PSD spectra of precursor ions [PL + 2Na+H]^+ and [PL + 3Na-2H]^+ did not show fragments due to the loss of the head group, when the laser intensity was equal to that used to generate prompt fragments.

Proposed fragmentation pathways of protonated PC.

(H_2PO_4-R_3)^+\^, where (H_2PO_4-R_3) represents the polar head group. Using the reflector mode in combination with the timed ion selector, the ions belonging to the loss of head group from the protonated precursors appeared as metastable ions and did not reflect their correct m/z values. [PE(18:0/18:1) + Na]^+ and [PS(18:0/18:1) + Na]^+ generated the metastable ions at m/z 617 and 623, respectively (same as those observed in Figs 3(b) and 3(c), labeled with asterisks). This indicates that the promptly fragmented protonated PE had originally a distinct structure from that isolated as a PSD precursor ion. Thus, two types of protonated ions were likely formed by MALDI of nitrogen-containing lipids (PE and PS): (1) ions in which the protons were attached to the oxygen atom, highly unstable and promptly fragmented (arrow ‘a’ in Scheme 2); and (2) ions in which the protons were attached to the nitrogen atom, more stable and detected mainly as protonated molecules. The metastable ions observed in the positive MALDI spectra of PE and PS (Figs 3(b) and 3(c)) were presumably generated from the second type of protonation (these pathways are illustrated by arrow ‘d’ in Scheme 2). The positively charged ammonium ion may initiate a remote fragmentation leading to a pathway (d) similar to that (a) driven by the proton.

A minor peak corresponding to the head group (m/z 184) was observed in the positive MALDI spectra of PC, but this disappeared when relatively low amounts of PC (20 ng) and lower laser intensity were used. This fragment (m/z 184) may be due to protonation followed by nucleophilic attack on a proton belonging to the glycerol backbone, as illustrated in Scheme 3. This electron rearrangement would lead to a net positive charge on the head group. Failure to observe a sodiated head group ion at m/z 206 was consistent with the speculation that protons localized on phosphate groups are more likely to cause fragmentation than the corresponding sodium ions.

Monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerols (DGDG)

Galactolipids (MGDG and DGDG) were detected only in the positive mode as single sodium adducts (Table 1). MGDG and DGDG do not contain acidic hydroxyls, and thus did not undergo deprotonation or multiple sodium addition. Also, galactolipids did not produce prompt fragments. Assuming that prompt fragmentations of TAGs and phospholipids

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were due to protonation, the absence of prompt fragments in the spectra of galactolipids suggests that galacto groups are sodiated and prevent acyl protonation, in turn eliminating prompt fragmentation.

CONCLUSIONS

Molecular structures of lipid molecules determine their ionization and fragmentation mechanisms. Only nitrogen-containing lipids are protonated to form stable \([M + H]^+\) ions. Our results suggest that phospholipids and triacylglycerols promptly fragment via the formation of highly unstable protonated molecules, which completely decompose within the timescale of the MALDI ion source.

REFERENCES