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Investigation of Beam-induced Reactions Occurring Under Fast-atom Bombardment Conditions Between Triethanolamine and Various Phospholipids

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The generation of adduct ions when using a triethanolamine (TEA) matrix under conditions of fast-atom bombardment has been investigated. In particular, phospholipids containing a free amino group react in the TEA matrix to form abundant adduct ions 26 and 42 mass units higher than the analyte pseudomolecular ions. The identity of these adduct ions was investigated using specific phosphatidylethanolamines and phosphatidylserine as well as 2-aminoethyldihydrogenphosphate and 2-aminoethylmonomethylphosphate as model compounds. Evidence obtained from positive- and negative-ion high-energy collision-induced dissociation tandem mass spectra, high resolution mass spectra, deuterium labelling and experiments using diethanolamine/TEA mixtures revealed that the intensities of the adduct ions are dependent on the percentage of TEA in the matrix and on the polarity of the analyte. The nature of the adduct ions is best reflected by a structure in which a hydrogen atom of the free amino group is substituted by a vinyl (+26) or by an acetyl (+42) group. A mechanism for the adduct ion formation is proposed. © 1997 by John Wiley & Sons, Ltd.

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Since its introduction, over a decade ago, fast-atom bombardment (FAB) has proven to be a powerful ionization technique for the investigation of thermally labile, polar and non-volatile compounds by mass spectrometry.[1–3] Because of its extensive application in the analysis of a wide range of compounds, especially those of biological interest (peptides, carbohydrates, glycopeptides and phospholipids), there has been a continuous effort to further understand the fundamental processes involved in generating analytically relevant ions by using this technique.[4–6] One of the most critical parameters in FAB-MS is the utilization of an appropriate liquid matrix, the purpose of which is to facilitate the production of analyte pseudomolecular ions (protonated or deprotonated molecules), to assist in maintaining a persistent emission of such ions, and to dissipate a large portion of the energy originating from the primary beam. The choice of a suitable liquid matrix is made after consideration of several physical and chemical properties of the matrix and the analyte. Some of the more important characteristics of a FAB matrix include it being a non-volatile compound capable of dissolving the sample of interest. Also of extreme importance is that the analyte and its corresponding fragment ions are stable with respect to the matrix. In many cases, however, this requirement is not achieved, thus resulting in the formation of adduct or artefact ions. Also, in some cases the formation of adduct ions is believed to be promoted by the fast-atom beam itself.[7,8] Thus, for the successful utilization of FAB-MS it is extremely important to be aware of the presence of adduct or artefact ions, especially because they may complicate spectral interpretation or even lead to erroneous conclusions regarding the nature of the analyte.

Triethanolamine (TEA) is a widely used FAB matrix.[9,10] Because of its high proton affinity (approximately 233 kcal/mol),[11] it is an excellent matrix for producing abundant deprotonated molecules, especially suitable for negative-ion detection. Despite the popularity of TEA as a FAB matrix there have been reports regarding extensive adduct ion formation with phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingoethanolamine (SEA) and ceramide aminoethylphosphonate (CAEPn), all of which contain a free amino group, in the presence of TEA.[12–15] Adduct ions were found 26 and 42 mass units higher than the analyte $[M-H]^-$ or $[M+H]^+$ pseudomolecular ion. It was further suggested that these artefact ions were only formed when a free amino group was present on the phospholipid head group and when a TEA matrix was used; no adduct ions were observed when dithiothreitol, thioglycerol or diethanolamine (DEA) were used as the matrix. It was therefore assumed that the artefact ions formed between the analyte amino group and TEA originating species. Collisional-induced dissociation (CID) of the pseudomolecular and adduct ions confirmed this assumption. However, to our best knowledge, the exact nature of the adduct ions and the mechanism of their formation was never further investigated, neither was it suggested that the FAB beam played a role in their formation.

In the present study, the nature of the adduct ions formed under FAB conditions between various phospholipids (mainly PE and PS) and the TEA matrix were further investigated. FAB-MS measurements, using low and high mass resolution, in positive- and negative-ion detection modes, along with tandem mass spectrometry (MS/MS) measurements, were conducted in order to investigate the nature of the adduct ions. Experiments

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involving deuterium labelled compounds and TEA were used to further elucidate the structures of such ions. Furthermore, model compounds, viz. 2-aminoethyldihydrogenphosphate (AEP) and 2-aminoethylmonomethylphosphate (AEMP), were used to study the effect of analyte polarity on adduct ion formation. The data obtained throughout this study have allowed us to propose a mechanism by which artefact (adduct) ion formation occurs.

EXPERIMENTAL

Instrumentation

All FAB mass spectra were obtained using a JEOL JMS-SX/SX102A (JEOL, Tokyo, Japan) four sector mass spectrometer (B-E-B-E reversed geometry). The ion-accelerating voltage was 10 kV. The FAB gun was operated at a 10 mA emission current using xenon as the bombarding gas. CID spectra of selected ions were obtained using air as the collision gas at a pressure sufficient to reduce the precursor ion to half its original intensity. High resolution (HR) FAB-MS was performed at a resolution of approximately 10 000 ($m/\Delta m$, 10% valley definition) using polyethyleneglycol (PEG) as the calibrant. In all other cases, a resolution of approximately 1000 was used. TEA was used as the FAB matrix, unless indicated otherwise.

Chemicals

Triethanolamine (OPG Groothandel B.V., Utrecht, The Netherlands), diethanolamine (Fluka AG, Buchs SG, Switzerland), and 3-nitrobenzylalcohol [*m*-NBA] (Fluka AG) were used as FAB matrices. 2-Aminoethyldihydrogenphosphate was purchased from Aldrich (Milwaukee, WI, USA). Two phospholipid standards, PS (1,2-dihexadecanoyl-*rac*-glycerol-3-phospho-L-serine) and PE (1,2-ditetradecanoyl-*sn*-glycero-3-phosphoethanolamine), were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2-Aminoethylmonomethylphosphate was synthesized according to the procedures reported by Clerc *et al.*[16] TEA was labelled with deuterium by mixing 0.5 mL TEA with 0.5 mL of 99.75% deuterium oxide (D_2O) (Merck, Darmstadt, Germany), evaporating the deuterium oxide and repeating the procedure three more times. AEP was deuterium labelled by dissolving 60 mg of AEP in 1 mL of deuterium oxide, evaporating the solvent and repeating the procedure an additional three times.

RESULTS AND DISCUSSION

As mentioned in the introduction, numerous research groups have reported the formation of adduct ions during the FAB mass spectrometric analysis of free phospholipids which contain an amino group when using a TEA matrix. In order to explain this occurrence Jensen *et al.*[12] proposed that the observed adduct ions were formed between the matrix and the ethanolamine head group of PE. They also speculated that matrix species are H-bound to the amino-group hydrogen atoms. This assumption was supported by three pieces of evidence. First, adduct ions were not observed when a matrix other than TEA was used. Second, when the two adduct ions were collisionally activated, the resulting product ions associated with the carboxylate chains were identical to those produced by collisional activation of the $[M-H]^-$ ion. Third, the FAB mass spectrum of dipalmitoyl-*N,N*-dimethylphosphatidylethanolamine in TEA matrix did not contain any adduct ions.

In the present study we have conducted a series of mass spectrometric experiments which aim to further elucidate the nature of the adduct ions that form under FAB conditions in a TEA matrix. Initially, a series of phospholipids and model compounds were analysed using FAB-MS and a TEA matrix. Structures and monoisotopic masses of all compounds investigated in this study are presented in Fig. 1. The structures of the adduct ions were investigated by conducting a series of high-energy MS/MS experiments. The elemental composition of the adduct ions was further confirmed using HR-FAB-MS. Finally, deuterium labelled reagents were used to determine the number of exchangeable hydrogen atoms present on the adduct species and thus provide us with information regarding their structures.

FAB mass spectra of PE, PS, AEP and AEMP with a TEA matrix

In accordance with previous reports we too have observed adduct ion formation when using FAB-MS and a TEA matrix to analyse certain phospholipids. The FAB mass spectra of PE and PS obtained in a TEA matrix contain relatively intense adduct ions having *m/z* values that are 26 and 42 mass units higher than those of their corresponding $[M-H]^-$ pseudomolecular ions (Fig. 2). Of these adduct ions, the $[M-H+42]^-$ ion generally appears to be more abundant than the $[M-H+26]^-$ ion. From these spectra, it is also clearly observed that the adduct ions formed with PS are of lower relative intensity than those formed with PE. This indicates that the carboxylic group present on the PS head group influences the extent of adduct ion formation. This may be either because of an increase in polarity of the phospholipid or because of a decrease in the basicity of the amino group present in PS. It should be noted that we observed similar adduct ion formation when analysing small peptides in the negative-ion mode using TEA as the matrix (no experimental data presented). The abundances of the adduct ions, of which $[M-H+26]^-$ is generally more abundant than $[M-H+42]^-$, appear to be dependent on the nature of

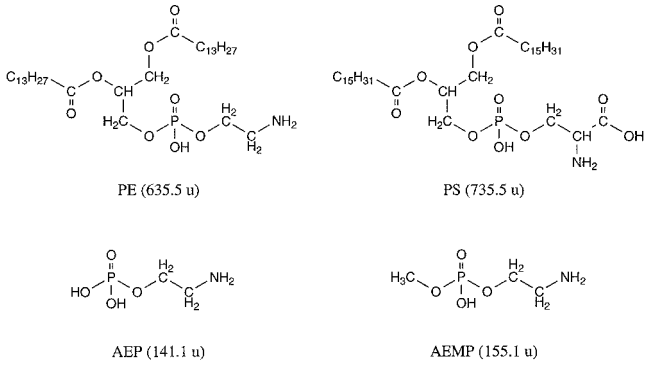


Figure 1. Structures and monoisotopic masses of compounds investigated.

the peptide but their relative intensities are often comparable to that of the $[M-H]^-$ ion.

In order to further understand the process of adduct ion formation, a simple model compound, AEP, was analysed under identical FAB conditions. In the negative-ion detection mode, artefact ions appeared at 26 and 42 u higher than the $[M-H]^-$ ion which originates from AEP (Fig. 3). Once again, the low relative intensity of the adduct ions suggests that the polarity of the analyte influences the formation of adduct ions to some extent. To further test this hypothesis another model compound, AEMP (the methyl ester of AEP), which is of intermediate polarity between AEP and PE, was analysed. Its FAB mass spectrum showed the presence of adduct ions with relative intensities ranging between those observed for PE and AEP (Table 1). From the data presented in Table 1 there is a strong indication that the relative abundance of the $[M-H+42]^-$ adduct ion is influenced by the polarity of the analyte. For the two compounds AEP and AEMP which do not contain fatty acid chains (as opposed to PE and PS which do) the relative abundance of the $[M-H+42]^-$ ion, compared with that of the $[M-H]^-$ ion, is quite low. In general, the more polar the compound, the lower the observed relative abundance of the $[M-H+42]^-$ ion (see Table 1). On the other hand, the relative abundances of the $[M-H+26]^-$ adduct ions do not seem to adhere to this trend, according to which the more polar analyte would have the highest relative abundance of $[M-H+26]^-$.

When an alternative matrix, *m*-NBA, is used, instead of TEA, for the analysis of PE or PS, the adduct ions $[M-H+26]^-$ and $[M-H+42]^-$ are no longer observed (Fig. 4(b)). Diethanolamine (DEA) which is structurally similar to TEA, and quite frequently used as a matrix in FAB-MS, was also tested for its potential to give rise to adduct ions. The resulting negative-ion FAB mass spectrum of PE in a DEA matrix did not show the presence of any such adduct ions (Fig. 4(a)). Also, when using mixtures of TEA and DEA as the matrix for the analysis of PE, it was observed that the intensity of the adduct ions depended on the relative amounts of the two matrix compounds (Fig. 5). Even when the TEA/DEA ratio was 50/50 w/w, the intensity of the ions corresponding to the adducts was already reduced to background levels. A similar experiment was conducted using a 50/50 w/w TEA + glycerol mixture in order to investigate the chance that the absence of adduct ions resulted from diluting the TEA matrix with the DEA. However, in this case, it was observed that adduct ions were formed having similar relative intensities to those observed when pure TEA was used. From these observations it can be proposed that DEA acts as a scavenging substance, removing

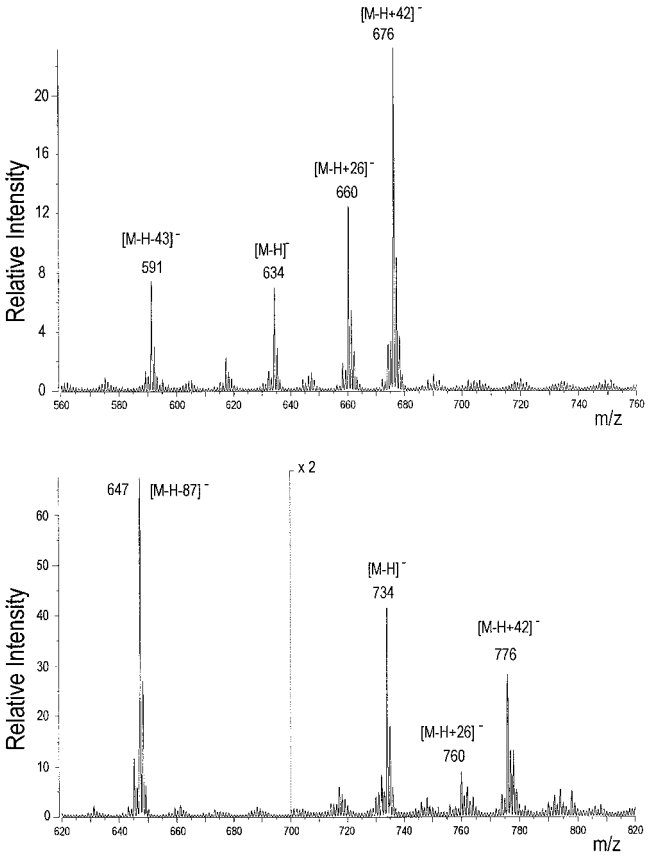


Figure 2. Negative-ion FAB mass spectra of (a) PE and (b) PS using TEA as the matrix.

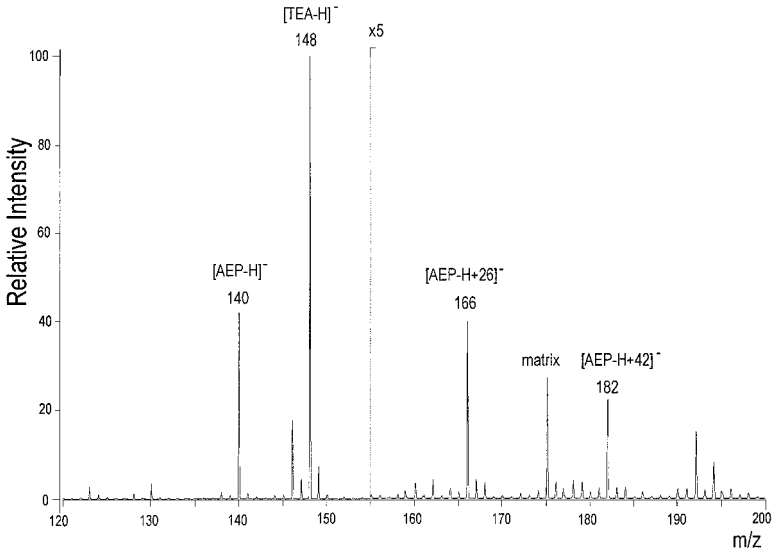


Figure 3. Negative-ion FAB mass spectrum of AEP using TEA as the matrix.

Table 1. Relative abundances of $[M–H+26]^-$ and $[M–H+42]^-$ ions, observed from PE, PS, AEMP and AEP[a]

Compounds investigated	$[M–H]^-$	$[M–H+26]^-$	$[M–H+42]^-$
PE	0.30	0.57	1.00
PS	1.00	0.22	0.68
AEMP	1.00	0.41	0.31
AEP	1.00	0.20	0.12

[a] For each compound relative abundance values have been normalized to the ion with the highest relative abundance.

matrix-originating species that would have otherwise promoted the formation of adduct ions. Further discussion concerning this hypothesis is presented later in this paper in the section 'Proposed mechanism for the formation of beam-induced reaction products'.

It should be noted that when analysing AEP in a TEA matrix using positive-ion FAB-MS no ions corresponding to AEP were observed. The reason for this is believed to be the high proton affinity of TEA. It has been reported that a high proton affinity for the matrix can reduce the intensity of the analyte ion in the positive-ion mode dramatically, even as far as complete absence of the ion.[11] TEA is reported to have a high PA (233 kcal/mol).[11] For this reason our subsequent measurements were executed only in the negative-ion mode.

MS/MS spectra of adduct ions

Negative-ion tandem mass spectra were obtained for the $[M-H]^-$ pseudomolecular ion and for each of the adduct ions, $[M-H+26]^-$ and $[M-H+42]^-$, of PE (Fig. 6) and PS (data not shown). After careful examination of these spectra it can be proposed that adduct attachment occurs at the free-amino group of the phospholipid. This is supported by the fact that, upon loss of the fatty-acid chains from the phospholipid, the resulting ions still contain the +26 or +42 u attachment. In Fig. 6(a), it is observed that, upon CID of the $[M-H+42]^-$ ion, product ions at *m/z* 448 and 466, corresponding to $[M-H-C_{13}H_{27}\ COOH+42]^-$ and $[M-H-C_{13}H_{26}\ CO+42]^-$ respectively, are present. This shows that, even though the fatty-acid chain has been removed from the phospholipid the +42 u attachment still remains. In Fig. 6(b), the CID tandem mass spectrum of $[M-H+26]^-$ contains ions at *m/z* 432 and 450 corresponding to $[M-H-C_{13}H_{27}\ COOH+26]^-$ and $[M-H-C_{13}H_{26}\ CO+26]^-$ respectively. Also in support of the hypothesis that attachment occurs via the phospholipid head-group is the fact that an ion (*m/z* 591), resulting from the loss of the phospholipid head-group plus the attachment, is present.

Furthermore, in the low-mass region of the CID spectrum of the $[M-H]^-$ ion (Fig. 6(c)), peaks are observed at *m/z* 140 and *m/z* 180, corresponding to $[NH_2CH_2OPO_2(OH)]^-$ and $[NH_2CH_2CH_2OPO_2(CH_2CHCH_2)]^-$ respectively. The CID spectra of $[M-H+42]^-$ and $[M-H+26]^-$ show similar product-ion pairs at *m/z* 182 and *m/z* 222 (Fig. 6(a)) as well as at *m/z* 166 and *m/z* 206 (Fig. 6(b)), again indicating the attachment of the adducts to the head group. It is also of importance to note that, upon high-energy CID of the phospholipid $[M-H+26]^-$ and $[M-H+42]^-$ adduct ions, loss of the 26 u or 42 u attached moieties is not observed. This finding suggests that a relatively strong chemical bond has formed between the matrix-originating attachment and the free-amino group present on the phospholipid.

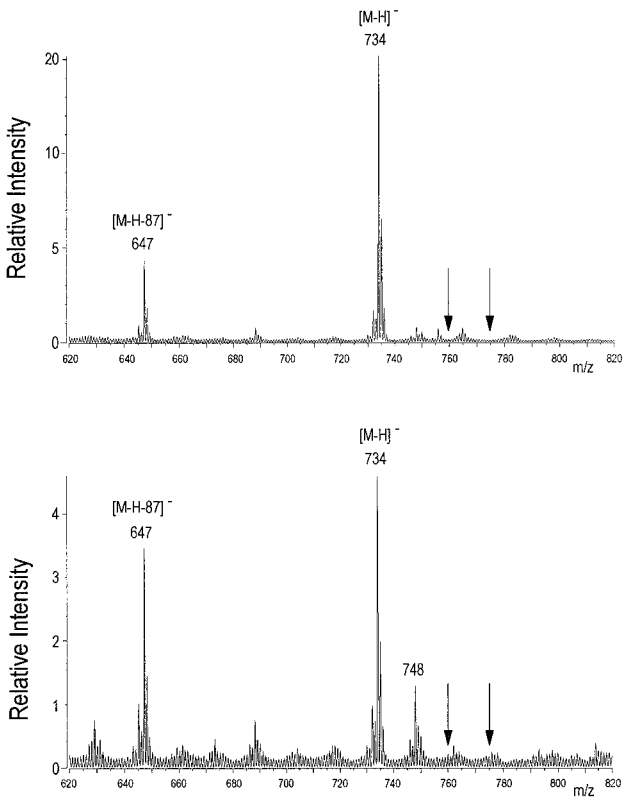


Figure 4. Negative-ion FAB mass spectra of PS, obtained using (a) DEA and (b) *m*-NBA as matrix substance. Arrows indicate positions of adduct ions if were present.

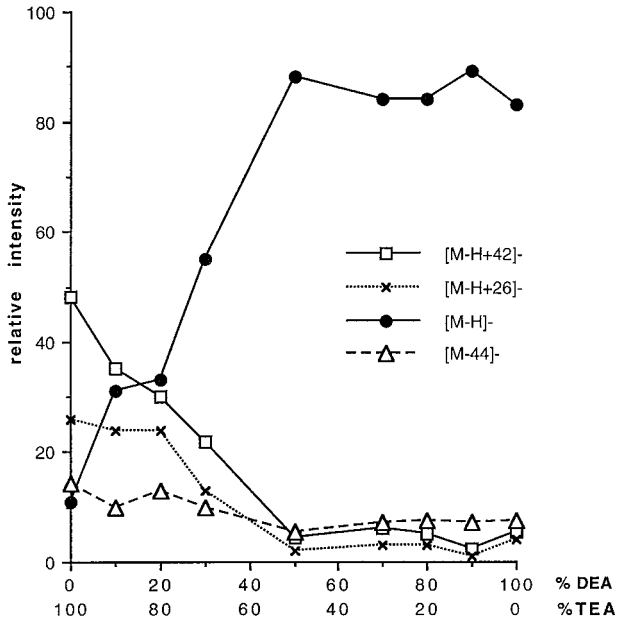


Figure 5. Relative intensities of some PE anions in TEA + DEA matrix mixtures.

Elemental composition of beam-induced reaction products

In order to determine the elemental composition of the adduct ions, HR-FAB-MS was performed on a mixture containing AEP, TEA and PEG (internal standard). Obtaining useful accurate masses under scanning conditions can be achieved with greater reliability for low mass rather than for high mass ions. We therefore selected AEP instead of PE for these measurements. The results obtained from the high resolution mass spectra (R = 10 000) are summarized in Table 2. From these data it can be proposed that the composition of the +26 adduct ion is $C_4H_9NO_4P$. This finding indicates that attachment of a C_2H_3 moiety occurs after the loss of a hydrogen atom from the AEP molecule. It was also shown that the $[M-H+42]^-$ adduct ion, for which *a priori* two elemental compositions are possible, has the composition $C_4H_9NO_5P$, which means that the attachment in this case has the composition C_2H_3O, and not of C_3H_7, and occurs after loss of hydrogen from AEP.

Determination of exchangeable hydrogen atoms

In order to determine the number of exchangeable hydrogen atoms in the adducts, AEP and TEA were labelled using deuterium oxide as described under Experimental. A molecule of AEP contains 4 exchangeable hydrogen atoms, so that the pseudomolecular ion $[M-D]^-$ is found at *m/z* 143 and contains 3 deuterium atoms (see Scheme 1(a)). The deuterated $[M-D+26]^-$ and $[M-D+42]^-$ adduct ions are found at *m/z* 168 and *m/z* 184 respectively and thus both contain only 2 deuterium atoms (see Scheme 1(b,c)). This observation reveals that the substituent groups do not contain any exchangeable hydrogen atom at all and that a deuterium atom of the AEP $[M-D]^-$ ion is replaced by substituent groups of 27 and 43 u respectively. These observations, combined with the data from the accurate mass determination, reveal that the adducts are most likely to correspond to the *N*-vinyl and *N*-acetyl derivatives of AEP (Scheme 1).

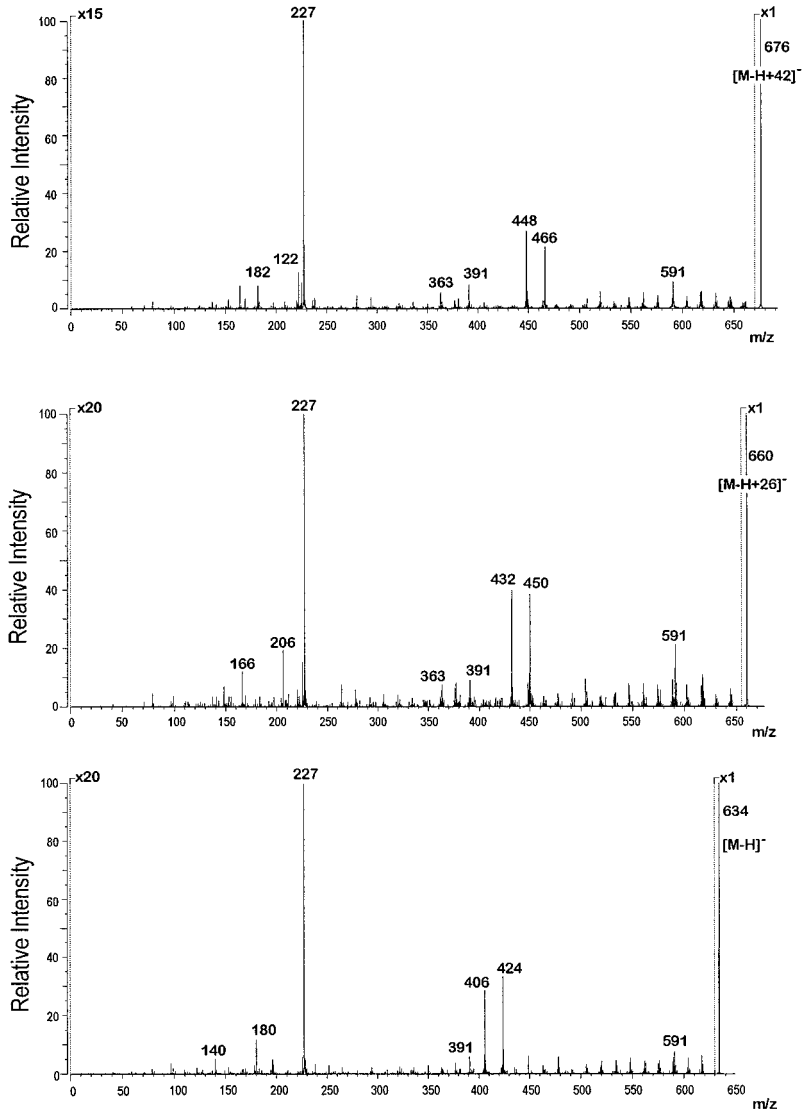


Figure 6. Negative-ion high-energy tandem mass spectra of PE, precursor ions selected: (a) $[M-H+42]^-$ (*m/z* 676), (b) $[M-H+26]^-$ (*m/z* 660) and (c) $[M-H]^-$ (*m/z* 634).

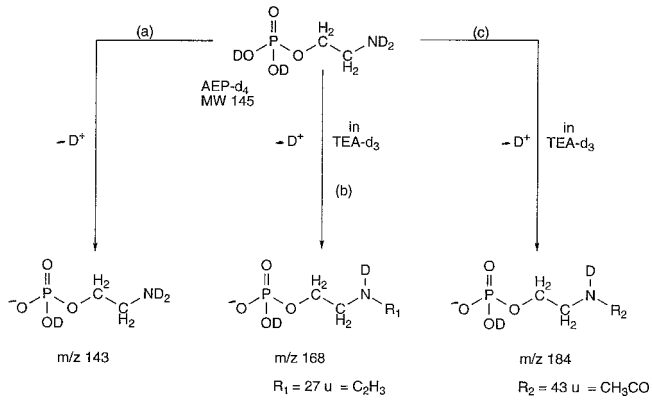
Table 2. Observed and calculated *m/z* values of some ions in a AEP+TEA mixture using PEG as an internal calibrant

Observed mass[a]	Calculated mass	Ion composition	Ion identity	Error (mmu)
140.0005	140.0113	$C_2H_7NO_4P^-$	AEP $[M–H]^-$	–10.8
146.0704	146.0817	$C_6H_{12}NO_3^-$	TEA $[M–3H]^-$	–11.3
148.0851	148.0974	$C_6H_{14}NO_3^-$	TEA $[M–H]^-$	–12.3
166.0152	166.0269	$C_4H_9NO_4P^-$[b]	adduct ion $[M–H]^-$	–11.7
182.0169	182.0218	$C_4H_9NO_5P^-$[b]	adduct ion $[M–H]^-$	–4.9
	182.0582	$C_5H_{13}NO_4P^-$	—	–41.3

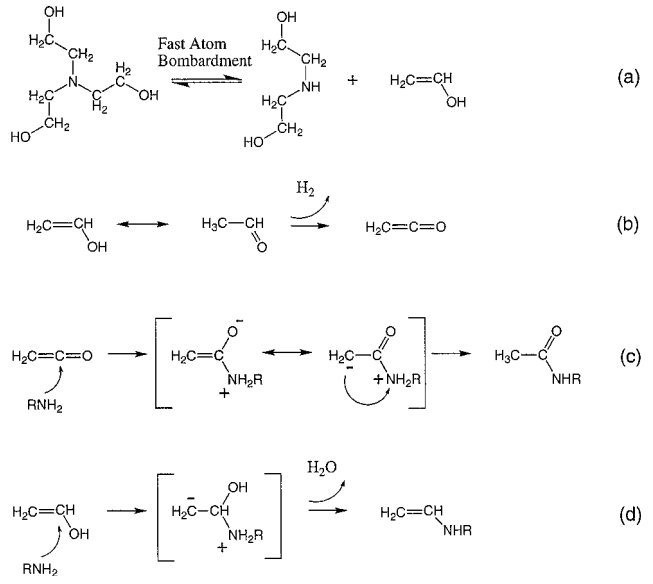
[a] Average of 4 runs.
[b] Proposed elemental composition.

Proposed mechanism for the formation of beam-induced reaction products

A plausible mechanism for the formation of the adducts is given in Scheme 2. This mechanism proposes the cleavage of TEA under FAB conditions to yield DEA and a vinyl alcohol molecule (**a**). This vinyl alcohol undergoes a keto–enol interconversion to acetaldehyde and loses a hydrogen molecule to yield ketene (**b**). This proposal is supported by the fact that ketene is formed from acetone upon losing a methane molecule under high temperature conditions in a similar reaction.[17] A ketene molecule thus formed may react with an amine to yield an *N*-acetyl adduct via the intermediates as shown (**c**). The vinyl alcohol may, on the other hand, react immediately with an amine group to yield the *N*-vinyl adduct after loss of water (**d**). This proposal is consistent with the observation that, when using a TEA-d_3 matrix, adduct ions of +26 and +42 u are still formed (Scheme 1). This is because the deuterium label from the OD group of TEA is lost during the adduct ion formation process. Also the fact that adduct ions are not observed in the presence of as little as 50% DEA in the matrix is in accordance with the proposed mechanism of adduct ion formation because the DEA would be expected to scavenge the vinyl alcohol molecules (Scheme 2(a)).



Scheme 1. Ions observed from AEP-d_4 in TEA-d_3.



Scheme 2. Proposed mechanism of adduct formation between an amino-group-containing compound and TEA.

CONCLUSIONS

From the data presented in this study it is apparent that certain precautions must be taken when using FAB-MS and a TEA matrix to analyse phospholipids containing free-amino groups. For example, in the case of a mixture of several PE and/or PS species, the formation of adduct ions can easily lead to the misinterpretation of the resulting mass spectrum. Alleviation of this problem, however, can be achieved through the addition of a relatively small amount of DEA. It has been demonstrated in the present study that even a small amount of DEA eliminates the presence of adduct ions. The FAB-mass spectrometric analyses of either pure phospholipids or their mixtures, using a TEA matrix, followed by the use a mixture of TEA + DEA, can further assist in making accurate assignments of pseudomolecular ions. Another attractive feature of the presence of adduct ions is that the triple ion pattern, $[M–H]^-$, $[M–H + 26]^-$, and $[M–H + 42]^-$, when present in a FAB mass spectrum, can be considered as an indication of the presence of a compound containing a free amino group.

Overall, TEA remains a very useful matrix for the FAB analysis of phospholipids containing free amino groups, providing that the spectral interpreter is aware of the specific behaviour of TEA towards such compounds. In this paper we have provided information regarding the identity of the adduct ions and have also demonstrated the potential for taking advantage of the spectral features resulting from the presence of adduct ions for making assignments of pseudomolecular ions.

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