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Characterization of model triacylglycerol (triolein, trilinolein and trilinolenin) autoxidation products via high-performance liquid chromatography coupled with atmospheric pressure chemical ionization mass spectrometry

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Abstract

Oxidation products from the autoxidation of three triacylglycerol standards have been analyzed using reversed-phase high-performance liquid chromatography (RP-HPLC) coupled to mass spectrometry via an atmospheric pressure chemical ionization (APCI) source. Triolein, trilinolein and trilinolenin were autoxidized in the dark at 50–60°C until the oxidation products represented approximately 30% of the starting material. These oxidation product mixtures were then analyzed using RP-HPLC–APCI-MS. Several classes of oxidation products were directly detected and identified. Monohydroperoxides were present in the largest amounts in the oxidation products mixtures. The hydroperoxides were found to provide several structurally useful fragments: epoxide intermediates were formed which then underwent further fragmentation, and other fragments were formed from concerted loss of the hydroperoxide group to form a site of unsaturation. Fragments formed by intra-annular cleavage of epoxide intermediates allowed identification of several hydroperoxide isomers. Bishydroperoxides were observed which underwent similar fragmentation pathways. Mono- and diepoxides were also formed by the autoxidation reaction. Two classes of epoxides were observed: those in which an epoxide formed in place of an existing double bond, and those in which an epoxide formed away from a double bond. Two distinct fragmentation mechanisms were observed for epoxides which were not formed across a double bond. Other oxidation products which were observed included hydroxy trilinolenin, epidioxy trilinolenin and hydroperoxy, epidioxy trilinolenin.

Keywords: Fatty acids; Triacylglycerol oxidation products; Triolein; Trilinolein; Trilinolenin

1. Introduction

Autoxidation is a chemical reaction by which oxygen is added via a free radical mechanism to unsaturated fatty acids in vegetable oils like corn, canola and soybean oils. The initial compounds produced by autoxidation are hydroperoxides and hydroperoxide cyclic peroxides. The mechanisms of the reactions and the implications of the autoxidation reactions with vegetable oil unsaturated fatty acids have been thoroughly reviewed by Frankel [1], Porter et al. [2] and Hamilton et al. [3]. While the

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hydroperoxide compounds formed by autoxidation are odorless and tasteless, their decomposition products are responsible, in part, for the deterioration of lipid-containing foods and products [4]. Also, hydroperoxide decomposition products may have negative health implications regarding cancer, heart disease and aging. Much research has been conducted on the utilization of antioxidants to prevent the formation or decomposition of hydroperoxide products [5,6].

Investigation of the mechanism of hydroperoxide formation in lipids involved first the identification and characterization of triacylglycerol hydroperoxides in model systems, such as pure triolein (trioleoylglycerol) [7,8], trilinolein (trilinoleoylglycerol) [7,9,10] and trilinolenin (trilinolenoylglycerol) [7,11]. Then, pure triacylglycerols (TAGs) with mixed fatty acids such as linoleic and linolenic [12], linoleic and palmitic [13], eicosapentaenoic and docosahexaenoic [14] and vegetable oil TAGs [7,10,14,15] were examined. The advancement of technology has greatly advanced the investigation of lipid hydroperoxide formation mechanisms by allowing the application of new analytical techniques for detection and identification or characterization of TAG oxidation products [16]. The new analytical techniques have included gas chromatography (GC), high-performance liquid chromatography (HPLC), proton and carbon nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) [16].

Due to their thermal instability, early MS characterization of TAG hydroperoxides required the reduction of the hydroperoxy group to a hydroxy group followed by transmethylation of the triacylglycerol to a mixture of methyl esters and hydroxy methyl esters. This mixture was then reacted with a silylating agent to convert the hydroxy methyl esters to silyl ethers. The derivatized mixture was analyzed by GC–MS to allow elucidation of the original TAG hydroperoxide structure [8,9,11,12]. In another study, TAG hydroperoxides were isolated, reduced with sodium borohydride and hydrogen and analyzed by fast atom bombardment (FAB) MS [7]. Recently, new MS techniques have become available for characterization of intact TAG hydroperoxides without the need for derivatization. Isolated intact triolein oxidation product fractions were characterized by methane chemical ionization MS [8]. However, it would be more convenient to perform the previously demonstrated separation of TAG hydroperoxides by HPLC [9,11,12] and couple the HPLC column directly to a mass spectrometer. Thermospray [17,18] and chemical ionization [18,19] MS procedures have been reported previously for TAG hydroperoxides. However, Sjovall et al. reported that these procedures are not entirely successful due to the poor stability of TAG hydroperoxides [20]. Successful analysis of synthetic isomers of TAG hydroperoxides of eicosapentaenoic acid by HPLC–electrospray ionization MS was reported by Endo et al. [14]. Sjovall et al. also reported a successful method of analysis in which HPLC was coupled with electrospray ionization MS for analysis of many TAG hydroperoxides, hydroxides, epoxides and core aldehydes [20]. However, the electrospray methodology yielded molecular ions without fragment ions (unless ionization voltage was greatly increased), which were not definitive for direct confirmation of the TAG hydroperoxide structure. Thus, mixtures of TAG oxidation products, which contain many isomers with similar chromatographic properties and identical masses, are difficult to characterize by the electrospray ionization MS procedure. TAG hydroperoxide standards have to be prepared and their HPLC retention times established to assist TAG hydroperoxide identification [20].

Previously, we developed a methodology using reversed-phase HPLC coupled with atmospheric-pressure chemical ionization mass spectrometry (APCI-MS) which allowed us to perform qualitative and quantitative analysis of non-oxidized TAG [21]. This procedure gave a combination of protonated molecular ions and diacylglycerol fragment ions for TAGs which proved useful for identification of individual molecular species, even in complex mixtures of vegetable oil TAGs. We report here the extension of our reversed-phase HPLC–APCI-MS method to the characterization and identification of TAG hydroperoxides and other TAG oxidation products in model autoxidized triolein, trilinolein and trilinolenin oxidation systems. Conclusive identification of TAG hydroperoxides was possible because the APCI source produced protonated molecular ions, diagnostic near-molecular fragments, molecular ion adducts and also characteristic diacylglycerol fragment ions.

Thus, the method reported here did not require the synthesis of pure TAG oxidation products for HPLC retention data to assist the use of mass spectrometric data, nor did it require derivatization to identify various classes of oxidation products. To our knowledge, there is only one report on the use of HPLC–APCI-MS analysis of TAG hydroperoxides. Kusaka et al. reported analysis of one TAG hydroperoxide: hydroperoxidized stearoyloleoyllinoleoyl glycerol [22] (although the masses for all TAG reported therein were 2 u higher than reported elsewhere).

2. Experimental

2.1. Materials

Triolein, trilinolein and trilinolenin (99+% purity) were purchased from (NuCheck Prep, Elysian, MN, USA). Thin-layer chromatography (TLC) was performed using Polygram SIL G/UV 254 polar phase plates, 4×8 cm plates coated with 0.25 mm silica gel with fluorescent indicator (Alltech Associates, Deerfield, IL, USA).

2.2. TAG autoxidation method

Before oxidation, TAGs were verified free of initial oxidation products by confirmation that they had peroxide values of zero by the ferric thiocyanate method [23] and by polar phase TLC (procedure given below). For samples which showed initial oxidation products, purification was conducted by a previously reported silica column procedure [15]. The TAGs (1.0 g) were autoxidized neat under a static oxygen head space in a 12.5×2.0 cm sealed test tube. Triolein was heated in the dark for three weeks at 60°C. Trilinolein and trilinolenin were heated in the dark at 50°C for 96 and 24 h, respectively. Oxidation progress of the TAGs was monitored by TLC with diethyl ether–hexane (20:80, v/v) as solvent. For trilinolein and trilinolenin, TAG oxidation products contained a conjugated diene functionality and were located by UV light on the TLC plate. Also, visualization of unreacted TAGs and all TAG oxidation mixture components resolved by TLC was obtained by exposure of the TLC plate to iodine vapor. When TLC (iodine vapor) indicated the production of about 30% oxidation products compared to unreacted TAG, the autoxidation was stopped. The oxidized samples were frozen in the dark under nitrogen head space at −20°C, until sample solutions were prepared for RP-HPLC–APCI-MS.

2.3. Mass spectrometry

A Finnigan MAT (San Jose, CA, USA) SSQ 710C mass spectrometer fitted with an APCI source was used to acquire mass spectral data. The vaporizer was operated a 400°C and the inlet capillary was operated at 265°C. The corona discharge needle was set to 6.0 μA. High purity nitrogen was used for the sheath and auxiliary gases, which were set to 35 p.s.i. and 5 ml/min, respectively (1 p.s.i.=6894.76 Pa). The scan range was from m/z 300 to 1100 in 2.75 s for triolein and trilinolein oxidation product mixtures, and m/z 400 to 1100 in 2.67 s for the trilinolenin oxidation product mixture. Mass spectra shown were averaged across the breadth of a chromatographic peak.

2.4. Liquid chromatography

The HPLC pump was an LDC 4100 MS (Thermo Separation Products, Shaumburg, IL, USA) quaternary pump with membrane degasser. Two columns in series were used: Inertsil ODS-2, 25 cm×4.6 mm, 5 μm (GL Sciences, Keystone Scientific, Bellefonte, PA, USA). Gradient solvent programs with acetonitrile (ACN) and dichloromethane (DCM) were used. The gradient used for triolein and trilinolein oxidation products was as follows: initial ACN–DCM (85:15); linear from 0 to 40 min to ACN–DCM (70:30), then linear from 40 to 80 min to ACN–DCM (30:70), held until 85 min; the column was recycled to starting conditions linear from 85 to 99 min. The gradient used for trilinolenin oxidation products was the same as above except that the starting composition was ACN–DCM (95:5). A higher initial content of ACN was used for the separation of trilinolenin oxidation products to lengthen the retention times. Otherwise, these products eluted within a very short time period. A flow-rate of 0.8 ml/min was used throughout. The column effluent was split so that ~680 μl/min went to an

evaporative light scattering detection (ELSD) system and ~120 μl/min went to the APCI interface. 10 μl of each sample was injected. The ELSD system was an ELSD MKIII (Varex, Burtonsville, MD, USA). The drift tube was set to 140°C, the gas flow was 2.0 standard liters per minute. High purity N_2 was used as the nebulizer gas.

3. Results and discussion

Previously, we have identified oxidation products in fractions collected of autoxidized triolein [8], trilinolein [9] and trilinolenin [11] Model TAGs isolated by reversed-phase HPLC followed by analysis using spectrometric techniques, such as ultraviolet and infrared spectrometry and proton and carbon NMR spectrometry. For most of the previous mass spectrometric work on TAG oxidation products, it was necessary to do GC–MS analysis of the isolated products after their conversion to silylated hydroxy methyl esters. We identified TAG hydroperoxides in the triolein, trilinolein and trilinolenin oxidation product mixtures and TAG hydroperoxyepidoxides in the trilinolenin autoxidized sample. In the work described below we have coupled the reversed-phase HPLC columns directly to a mass spectrometer via an APCI source to identify the intact TAG oxidation products as they eluted. This procedure eliminated the need for collection of the TAG oxidation products fractions for spectrometric analysis and later derivatization for mass spectrometric confirmation of structure. We were able to use LC–MS not only to identify known products such as hydroperoxides, but also to identify TAG oxidation products which have not been described in the previous work on model triolein, trilinolein and trilinolenin systems.

The oxidation products of the three triacylglycerol standards were substantially more polar than the normal TAGs, so the chromatographic separation had to incorporate a much higher initial proportion of ACN than is used in normal TAG separations to elute the components over a sufficiently broad time period. The chromatographic system employed here was similar to that used previously for hydroxy-containing seed oils [24]. Trilinolenin oxidation products required an even higher initial proportion of ACN to produce satisfactory resolution of all components.

3.1. Monohydroperoxides

Fig. 1 shows reconstructed ion chromatograms (RICs) of the triolein, trilinolein and trilinolenin oxidation products mixtures obtained using RP-HPLC–APCI-MS. In all three cases, the primary oxidation products were TAGs containing monohydroperoxy functional groups. In addition to these primary products, many other products present in smaller amounts were directly detected, as well as unreacted TAG starting material. Each of the classes of oxidation products yielded characteristic mass spectra which were differentiable based on relative proportions of fragments produced from several different, but similar, fragmentation pathways. Fig. 2 shows the averaged mass spectra obtained across each of the monohydroperoxy TAG peaks. Fig. 2A, which shows the mass spectrum obtained for triolein monohydroperoxide, demonstrates most of the fragmentation pathways observed for all other samples. In this mass spectrum, only a small amount of protonated molecule is observed, with the primary high mass fragments being produced by sequential loss of portions of the hydroperoxy group. The first primary fragment formed was loss of the outer –OH from the hydroperoxy group followed by cyclization of the remaining oxygen to form an epoxide, resulting in loss of another hydrogen at the site of cyclization, for a net loss of 18 u. This epoxide appears to be a stable, long-lived intermediate, as evidenced by the number of fragments which resulted from this ion, discussed below. The second primary high mass fragment was formed by complete loss of the hydroperoxy group along with a neighboring hydrogen to form an additional site of unsaturation. This loss of the hydroperoxy group to form additional unsaturation was very similar to the fragmentation observed for hydroxy-containing TAGs during APCI-MS, which was recently reported [24]. The net result was a fragment ion which was isobaric with OOL. Since normal triolein has few sites of unsaturation, it usually produces only very small abundances of high mass ions (protonated molecular ion), producing instead primarily diacylglycerol fragments, as has been reported extensively

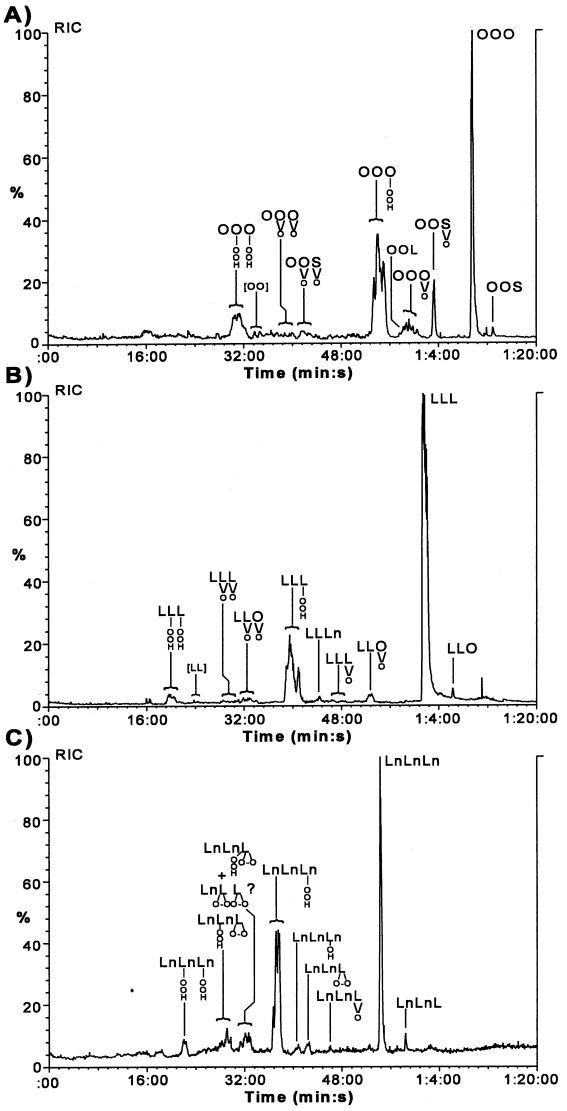

Fig. 1. RICs of (A) triolein oxidation products mixture autoxidized in the dark at 60°C for three weeks; (B) trilinolein oxidation products mixture autoxidized in the dark at 50°C for 96 h; (C) trilinolenin oxidation products mixture autoxidized at 50°C for 24 h. HPLC conditions as in Section 2.4. Abbreviations: O=Oleic acid or oleoyl acyl chain; OOO=triolein; [OO]=intact normal dioleoyl diacylglycerol; S=stearic acid or acyl chain; L=linoleic acid or acyl chain; LLL=trilinoleoyl triacylglycerol (TAG); Ln=linolenic acid or acyl chain; LnLnLn=trilinolenoyl TAG; hydroperoxides denoted by –OOH; epoxides denoted by >O; epidioxides denoted by $O^{/}-{}^{\backslash}O$.

[21,25]. Here it was seen that the proportion of $[(M+H)-H_2O_2]^+$ ion was much larger than might be expected for a TAG with so little unsaturation. The increased amount of high mass peaks was valuable for identification of the oxidation products. Also observed for all hydroperoxides was formation of an ion having even one less site of unsaturation. These were present in substantial proportions, and the exact mechanism of this fragment's formation will be discussed below. The primary two fragmentation pathways (formation of the epoxide and loss of the hydroperoxide to give unsaturation) were also found to occur in the diacylglycerol fragments. Epoxy diacylglycerol fragment ions were formed by a net loss of H_2O and diacylglycerol fragments representing loss of the hydroperoxy to form additional unsaturation were observed, as well as normal diacylglycerol fragment ions. Only very small abundances of diacylglycerol fragment ions containing intact hydroperoxy groups were observed.

Numerous other fragment ions were formed from the hydroperoxides. Most of these arose from fragmentation of the stable epoxy intermediates. We previously reported results from vernolic acid-containing TAGs which allowed us to identify the general mechanism of epoxide fragmentation which occurred during APCI-MS [26]. The overall mechanism was intra-annular cleavage of the bond between the two carbons of the epoxide ring to form an additional site of unsaturation and loss of the oxygen-containing fragment, as presented therein. The specific stepwise mechanism (not shown) likely involved protonation of the epoxy oxygen followed by ring opening and bond cleavage. Some of the assignments of peaks in the mass spectra shown for vernolic-acid containing TAGs were mislabelled; Table 2 in the previous publication contained the correct assignments. In the case of vernolic acid-containing TAG, the epoxide ring was always β to the double bond on the distal side of the acyl chain so the mechanism favored loss of the oxygen with the leaving group. However, in the heterogenous mixture of oxidation product isomers studied here, fragments were observed in which the oxygen stayed with the larger backbone fragments (loss of a hydrocarbon fragment), as well as fragments in which the oxygen was lost with the leaving group. In the case of the epoxide formed from triolein hy-

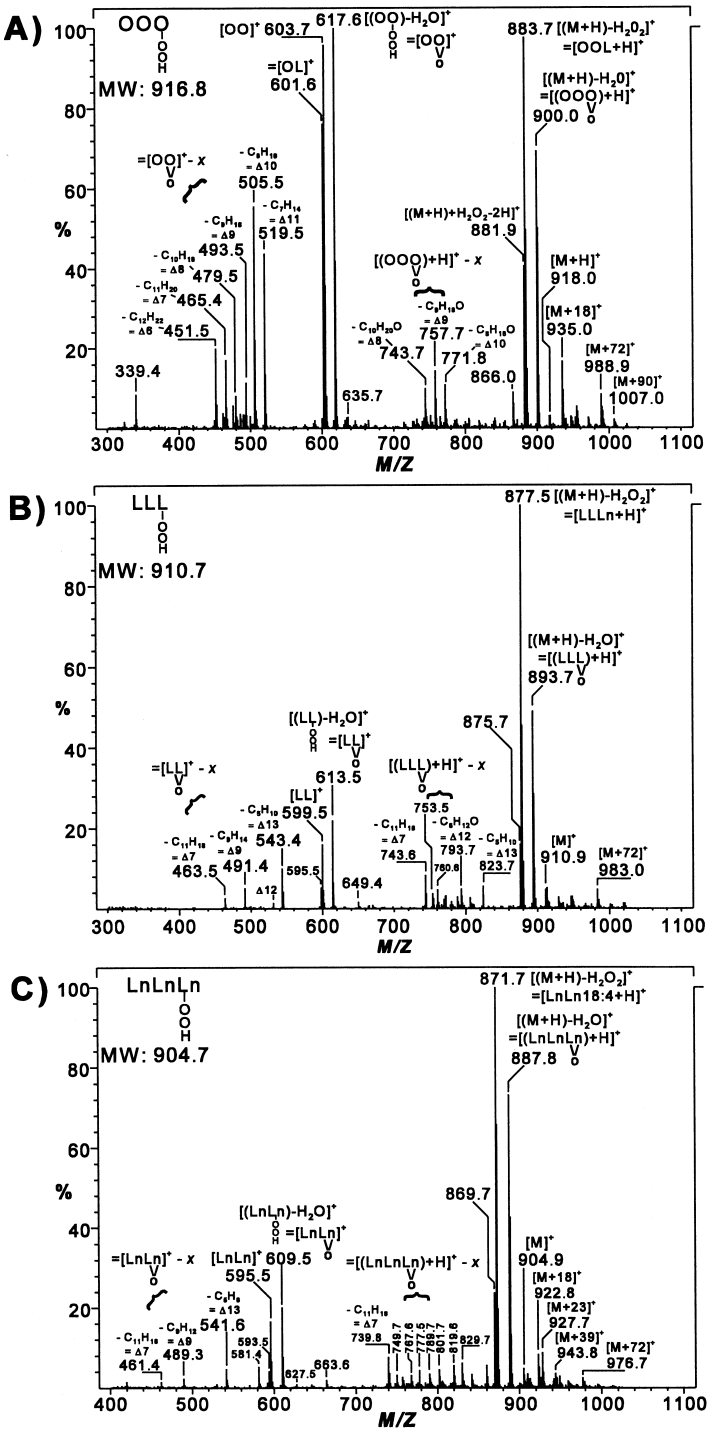

Fig. 2. Mass spectra averaged over the widths of the triacylglycerol monohydroperoxide peaks: (A) triolein hydroperoxide, (B) trilinolein hydroperoxide, (C) trilinolenin hydroperoxide. Abbreviations: $[OO]^+$ = diacylglycerol fragment ion, same as $[M-RCOO]^+$ = intact normal diacylglycerol minus OH. Other abbreviations as in Fig. 1. MW = Molecular mass.

droperoxide, retention of the oxygen and loss of a hydrocarbon group was favored in the diacylglycerol fragments, while loss of the oxygen in the leaving fragment was favored by fragmentation of the pseudo-molecular epoxide (see Fig. 2A). In fact, in most cases for all oxidation products, the loss of a hydrocarbon fragment with retention of the oxygen was favored by the diacylglycerol fragments. Nevertheless, peaks of differing abundances representing both possibilities were observed for nearly all oxidation products. The fragmentation of trioleoyl hydroperoxide to form the epoxide and loss of a specific hydrocarbon length allowed identification of the position of the epoxide ring. The position of the epoxide ring then localized the hydroperoxide to have originated from one of the two ring carbons. In the cases of the upper and lower extremes, the position of the hydroperoxide could be more specifically localized. For instance, the fragment at m/z 451.5 in Fig. 2A indicated that a $C_{12}H_{22}$ fragment was lost, so the epoxide was at the Δ_6 position (between carbons 6 and 7). The hydroperoxide which produced this epoxide would have been at the 7 position, because if it were at carbon 6, it could have epoxidized either to carbon 5 or carbon 7. If it had epoxidized to carbon 5, a Δ_5 fragment would have been observed, which was not the case. Thus, fragments representing losses from Δ_6 to Δ_{11} in Fig. 2A meant that the hydroperoxides were initially formed on carbons 7 through 11. Also, the hydrocarbon fragments which were lost from the trioleoyl hydroperoxide changed from C_nH_{2n} to C_nH_{2n-2} as they changed from Δ_{10} to Δ_9, confirming the original location of the double bond at the Δ_9 position.

In addition to the fragments mentioned above, important and diagnostic adducts were formed in the APCI source. Across all oxidation products, the most important adducts which were formed were $[M+18]^+$, $[M+23]^+$ and $[M+39]^+$ adducts. The identities of the $[M+23]^+$ and $[M+39]^+$ adducts have been previously described [24]. These two adducts were derived from acetonitrile in the column effluent. The fact that the HPLC runs used for these separation were higher in ACN than normal TAG separations accounts for the similarity between these data and the data reported for hydroxy seed oils, which used a similar separation. The presence of all of these adducts together acted as a valuable tool for identification of the molecular masses of the various oxidation products, especially in cases where the abundance of the protonated molecular ion was small. Another adduct which was common for the hydroperoxides, as seen in Fig. 2, was the $[M+72]^+$ ion, the identity of which has not been determined. An adduct which was common to many of the various oxidation products was an $[M+90]^+$ adduct, which has similarly not been conclusively identified, but which added confirmation for the molecular masses determined for many of the species.

The mass spectra of trilinolein hydroperoxide and trilinolenin hydroperoxide exhibited the same fragmentation pathways as triolein hydroperoxide discussed above. These oxidation products also demonstrated large epoxide fragments which underwent further fragmentation to give losses of hydrocarbon chains or oxygen-containing hydrocarbon chains. The longest fragments lost from these species indicated that the epoxides occurred at carbons 7, 8 and further down the acyl chain, so the hydroperoxides originated from carbons no lower in number than carbon 8.

3.2. Epoxides

In addition to the major oxidation products (the hydroperoxides), the three TAG standards produced a variety of other oxygen-containing compounds. Among these were stable epoxides formed by at least two distinct processes resulting in two type of epoxides. The first process was formation of the epoxide at the site of a double bond in the TAG molecule, while the second was formation of the epoxide not across, but rather nearby a double bond. Mass spectra of epoxides of the first type are shown in Fig. 3, while mass spectra of the second type are shown in Fig. 4. In the case of triolein, the formation of an epoxide at the double bond resulted in a single sharp chromatographic peak (see Fig. 1A). The mass spectrum of the first type of triolein epoxide is given in Fig. 3A. This mass spectrum exhibited a substantial protonated molecular ion, along with several of the important adducts described above which conclusively identified the molecular mass of the molecule as 900.8. This molecular mass was 16 u larger than normal triolein, indicating that an oxygen was added without loss of two hydrogens at the site of the

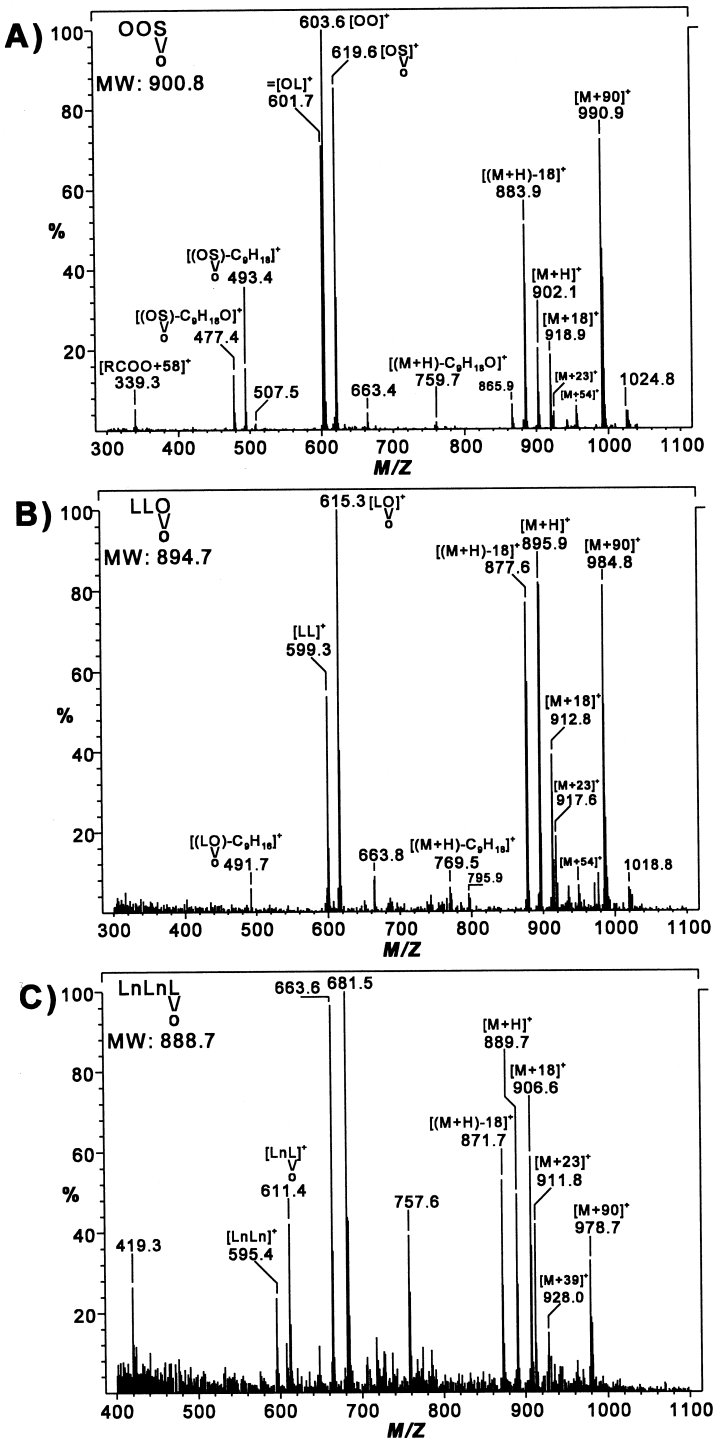

Fig. 3. Mass spectra averaged over the widths of TAG monoepoxide chromatographic peaks, in which the epoxide formed with loss of a site of unsaturation: (A) epoxidized triolein; (B) epoxidized trilinolein; (C) epoxidized trilinolenin. Abbreviations as in Figs. 1 and 2.

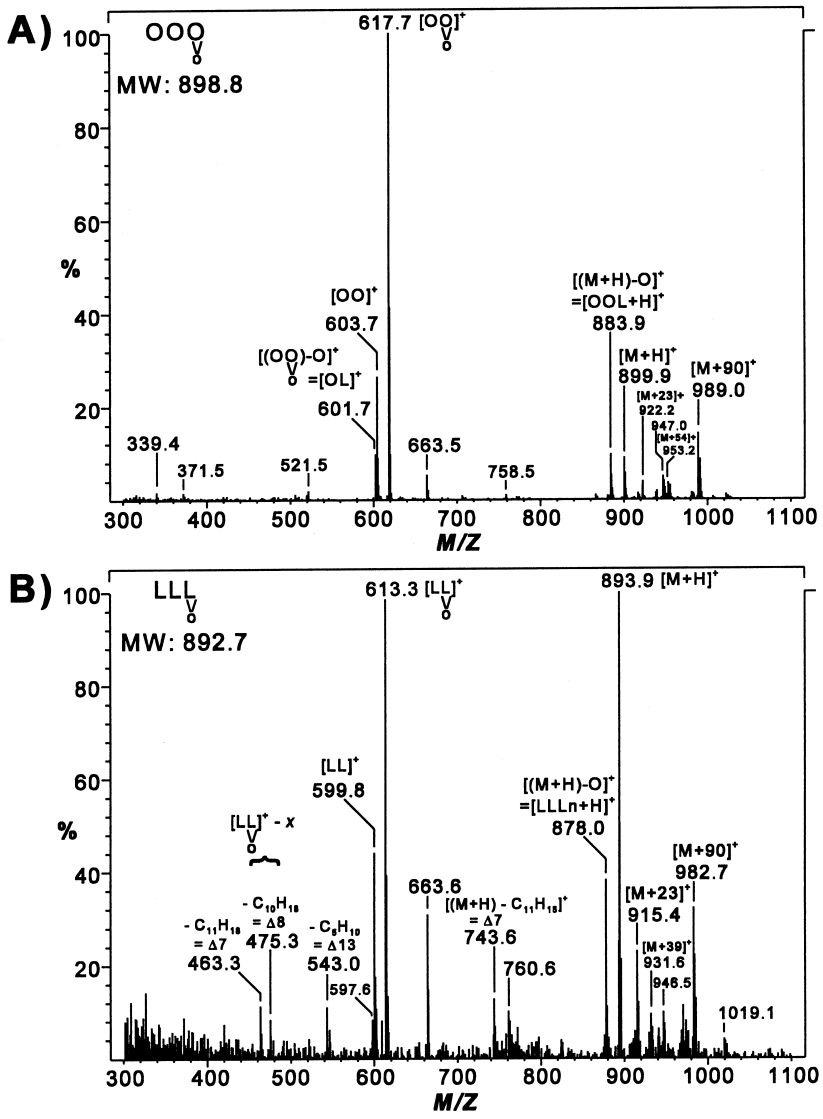

Fig. 4. Mass spectra averaged over the widths of TAG monoepoxide chromatographic peaks, in which the epoxide formed without the loss of a site of unsaturation: (A) epoxidized triolein; (B) epoxidized trilinolein. Abbreviations as in Figs. 1 and 2.

epoxide ring, indicating that it was formed by replacing a site of unsaturation. The base peak at m/z 619.6 confirmed that when the epoxide ring formed on the acyl chain, the acyl chain no longer contained any unsaturation. Furthermore, the fragments at m/z 477.4 and m/z 493.4 represented cleavage of the Δ_9 epoxide from the diacylglycerol epoxide such that the lower mass fragment indicated that the leaving fragment contained the oxygen, while the higher mass fragment indicated that the leaving fragment did not contain the oxygen. The combination of these fragments clearly identified this epoxide. An interesting observation arose from this mass spectrum. The large fragment at m/z 883.9 in Fig. 3 indicated that a different mechanism was involved in loss of the epoxy group in this molecule than was involved in the loss when the epoxy was next to a double bond. The mechanism resulted in formation of two double

bonds, rather than just one. Two possibilities are likely which may explain formation of two double bonds when the epoxide was not next to an existing double bond. The first likely possibility was simply the acid-catalyzed hydrolysis to form the *vic*-diol, followed by dehydration to form unsaturation, shown in Fig. 5A. This mechanism is classical epoxide chemistry, so is assumed to be occurring to some extent under the atmospheric pressure conditions in the source. The other possibility for the first type of mechanism is shown in Fig. 5B. This involves a higher energy reaction which is believed to take place in the APCI source. In this case, the epoxide ring is protonated, followed by ring opening, which leads to a charge on the acyl chain. Loss of a proton from the high energy intermediate allows formation of an enol, which quickly loses the hydroxy group (as shown previously for hydroxy-containing TAGs) to form a second site of unsaturation. The critical and defining difference between this mechanism (Fig. 5B) and another mechanism, observed when the epoxide was adjacent to a double bond (Fig. 5C),

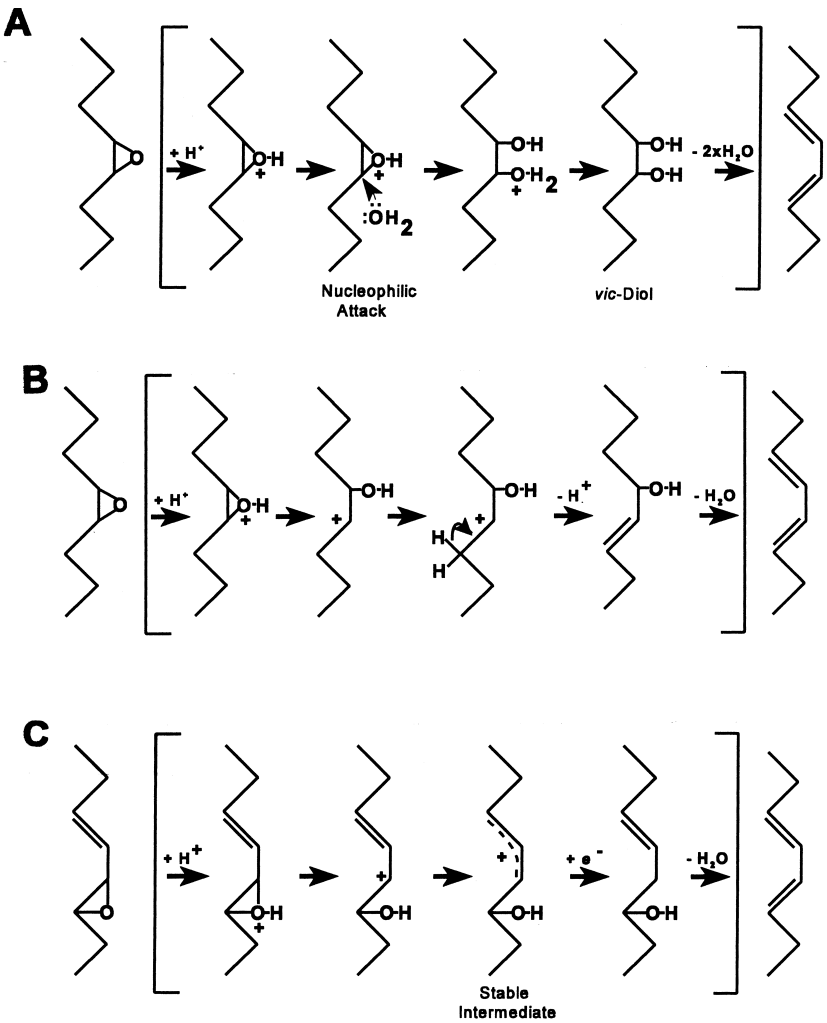

Fig. 5. Possible mechanisms for the formation of two double bonds (see Section 3.2). (A and B): Epoxide not next to an existing double bond: (A) acid-catalyzed hydrolysis; (B) protonation of epoxide ring, followed by ring opening. (C) Epoxide adjacent to existing double bond

was that the charged intermediate had no possibility for resonance stabilization from a neighboring double bond, so had to lose a proton. On the other hand, when the epoxide was next to a double bond, a resonance-stabilized intermediate was formed which has commonly been reported to be a stable oxidation by-product which is involved in epoxide solvolysis [27]. This resonance-stabilized intermediate was sufficiently long-lived to acquire an electron in the atmospheric pressure region to produce the enol, which immediately formed a site of unsaturation by loss of the hydroxy group through dehydration. These two distinct mechanisms account for the fact that epoxides which replaced a site of unsaturation had a protonated molecular ion and adduct ions which were 2 u larger than the epoxides formed next to double bonds, but both of these epoxides led to the same fragment ions having identical masses. The stereochemistry shown in Fig. 5 is arbitrary. Bond rotation leading to *trans* isomers is likely. The mechanisms of oxidation reviewed by Gardner [27] indicated that oxidative attack usually occurs at an allylic carbon, which causes a shift of a double bond, leading to conjugation (in cases of polyunsaturated fatty acyl chains). This can then lead to an allylic epoxide, which undergoes resonance stabilization during fragmentation, as shown in Fig. 5C. An epoxide formed at the next carbon away from the double bond cannot undergo resonance stabilization.

The previous results for vernolic acid [26] demonstrated that it followed the former mechanism (Fig. 5A,B) rather than the latter (Fig. 5C), giving a net loss of 18 u. This is expected based on our proposed mechanism, because the epoxide ring (Δ_{12}) of vernolic acid was not next to the double bond (Δ_9). These two different mechanisms also successfully explain why trilinolein (with methylene-interrupted double bonds) which was epoxidized across a double bond obeyed the first mechanism and produced a major fragment from loss of 18 u in Fig. 3B. On the other hand, if it was not epoxidized at a double bond but rather at one of the methylene groups next to the double bonds, then it resulted in the epoxide being next to a double bond, so it obeyed the second mechanism and produced a major fragment from loss of only 16 u, seen in Fig. 4B. Trilinolenin (also with methylene-interrupted double bonds) which was epoxidized across a double bond similarly followed the first mechanism (see Fig. 3C), as expected. Trilinolenin epoxidized next to a double bond was not identified in these runs, if present. The other fragments and adducts in Fig. 3B,C, Fig. 4B confirm the identities of the proposed structures. Re-examination of Fig. 2 shows that some of the stable epoxide intermediates, $[(M+H)-H_2O]^+$, formed from the hydroperoxides underwent the first fragmentation mechanism to form the equivalent of $[(M+H)-H_2O_2-2H]^+$ fragments. Another point to note about the epoxides of all types was that the cyclization of the epoxide produced much higher abundances of adduct ions, especially the $[M+90]^+$ ion than did non-cyclic oxidation products. In some cases, the abundance of the $[M+90]^+$ peak was as high as, or higher than any other near-molecular ion. As discussed below, this became useful for identifying some of the other cyclic oxidation products. Finally, it is worthwhile to note that the chromatographic retention time of the epoxides was longer than the monohydroperoxides. This is expected on the reversed-phase column, since the epoxides were less polar than the hydroperoxides.

3.3. Bishydroperoxides

Another set of major oxidation products which was produced by these three standard TAGs were the bishydroperoxides. As seen in Fig. 6, the fragmentation pathways were the same as those for the monohydroperoxides shown in Fig. 2, except that two hydroperoxide groups were available to exhibit such fragmentation. As with the monohydroperoxides, the stable epoxide intermediates were the primary fragments formed, and these acted as precursors to other fragments which were formed. In the case of the bishydroperoxides, two epoxides could be formed, which then underwent further fragmentation. As seen from the sizes of the bishydroperoxide peaks in the chromatograms in Fig. 1, these were produced in smaller amounts than the monohydroperoxides. This smaller amount of sample passing into the mass spectrometer, along with the increased number of fragments arising from an equimolar amount of molecules resulted in a poorer signal-to-noise ratio for the mass spectra of the bishydroperoxides (as well as other multiple-functional group oxidation products). Nevertheless, the combination of the

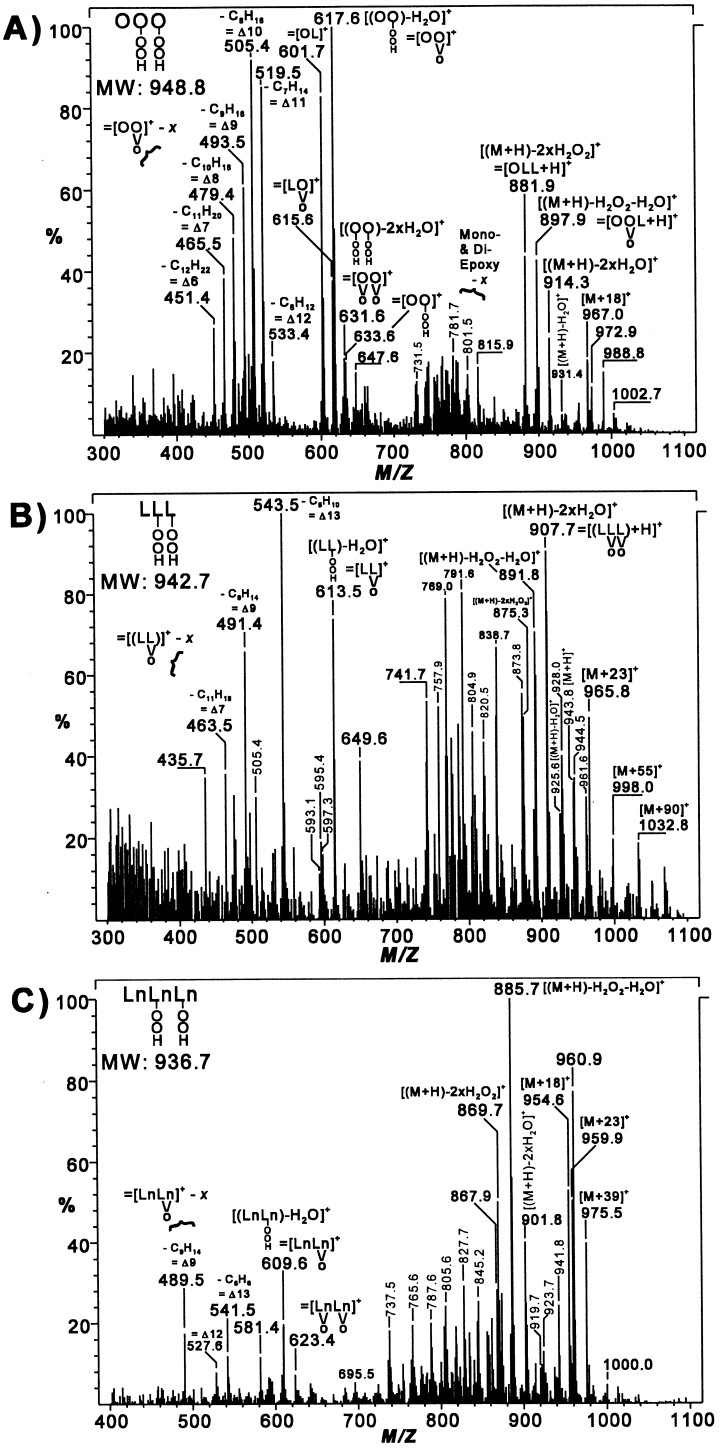

Fig. 6. Mass spectra averaged over the widths of bishydroperoxy TAG chromatographic peaks: (A) triolein bishydroperoxide; (B) trilinolein bishydroperoxide; (C) trilinolenin bishydroperoxide. Abbreviations as in Figs. 1 and 2.

valuable set of adducts formed along with other high mass ions resulting from the expected fragmentation pathways did allow identification of the bishydroperoxide species. As with the monohydroperoxides, the loss of hydrocarbon units from the epoxy-diacylglycerol ions, as well as from the high mass epoxide fragments indicated the presence of several isomers. Because of the similarities to the monohydroperoxy species, which were discussed in depth above, additional discussion of bishydroperoxides is not presented.

3.4. Other oxidation products from trilinolenin

3.4.1. Hydroxy trilinolenin

Trilinolenin formed several oxidation products which were not observed for the other triacylglycerols. Fig. 7 presents averaged mass spectra for three such species. Fig. 7A shows an averaged mass spectrum for the peak in Fig. 1 which eluted just after the monohydroperoxides, indicating that these species were slightly less polar than the monohydroperoxides. The protonated molecular ion and the set of adducts formed from acetonitrile and other adducts indicated a molecular mass of 888.7 u. The single primary pseudo-molecular fragment at m/z 871.8 and lack of acyl chain cleavage fragments (especially for the diacylglycerol ions) indicated that this molecule contained only a single hydroxy group. The spectrum was very similar to the spectra reported previously for hydroxy-containing TAGs containing one hydroxy group [24]. The peak a m/z 663.6 in this (and other) spectra did not derive from this molecule, but was a background peak which increased over the length of the run (inverse to the concentration of ACN, increasing with the percentage of DCM).

3.4.2. Trilinolenin epidioxide

Fig. 7B shows a mass spectrum for the peak which eluted after the hydroxy TAG, but before the epoxy TAG discussed above. This indicated a polarity which was intermediate between these two classes. The very abundant adduct ions, along with some protonated molecular ion, gave a molecular mass of 904.7. This indicated the presence of two oxygens without the loss of any hydrogens from the original trilinolenin. The difference of 16 u between the protonated molecular ion and the first near-molecular fragment, followed by loss of another 16 u to form the second near-molecular ion, were in contrast to the losses of H_2O observed for hydroperoxy-containing oxidation products discussed above. The protonated molecular ion produced a fragment from concerted loss of the two oxygens for a change of 32 u. The epoxy near-molecular ion formed from loss of the first oxygen was also observed to undergo fragmentation according to the first epoxide mechanism described above for epoxides formed across a double bond. This formed the ion at m/z 871.8. The molecular mass, the fragmentation pathways followed by these molecules and the chromatography allowed us to identify this as an epidioxide which was formed across an existing double bond. As expected, the diacylglycerol fragments helped to confirm this identification. Also, as mentioned above, oxidation products containing one cyclized oxygen group yielded much larger abundances of the adducts than did non-cyclic compounds.

3.4.3. Trilinolenin hydroperoxide epidioxide

The next oxidation product formed by trilinolenin which could be identified was the hydroperoxide epidioxide, with a molecular mass of 936.9. As with most of the compounds discussed above, the presence of several adducts allowed identification of the molecular mass of this class of molecule. The large sizes of the $[M+18]^+$ and $[M+23]^+$ adduct peaks was similar to the mass spectrum of the epidioxide molecule shown in Fig. 7B. Again, the large adducts were observed from molecules which contained one cyclized oxygen functional group (epoxides and epidioxides). As with the monohydroperoxides, the primary fragment formed from the hydroperoxide functional group was an epoxide formed by loss of an OH group from the hydroperoxide followed by cyclization with loss of an acyl chain hydrogen for a net loss of 18 u, or H_2O. As shown above for the monohydroperoxides, another fragmentation pathway was that the intact molecule also lost the hydroperoxy group along with an acyl chain hydrogen to form an additional site of unsaturation, for a net loss of H_2O_2, or 34 u, giving rise to a fragment at m/z 903.9. Additionally, the peak at m/z 903.9 had a contribution from fragmentation of the peak at m/z

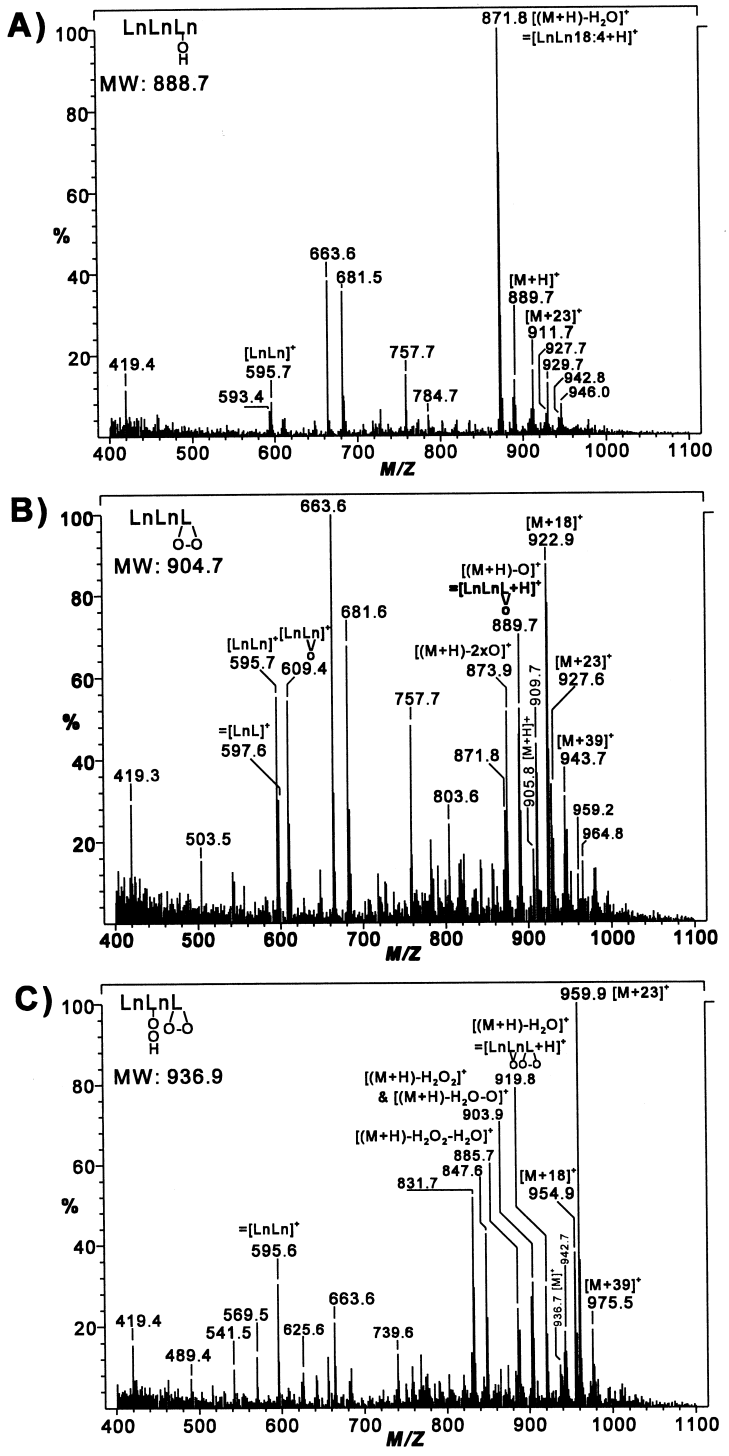

Fig. 7. Average mass spectra across peaks in the RIC of trilinolenin oxidation products: (A) hydroxy trilinolenin; (B) dilinolenoyl, linoleoyl glycerol epidioxide; (C) dilinolenoyl, linoleoyl glycerol hydroperoxy epidioxide.

919.8. The epoxy epidioxy intermediate at m/z 919.8 underwent loss of an oxygen from the epidioxy group to form another epoxy group, giving a diepoxy intermediate, also at m/z 903.9. The fragment giving the peak at m/z 919.8 was sufficiently long-lived that it also formed an $[x+23]^+$ adduct at m/z 942.7. The diepoxy intermediate at m/z 903.9 had one epoxy group which came from the epidioxide at the position where a double bond had been, so this epoxide underwent fragmentation according to the first epoxide mechanism discussed above for epoxides, for a net loss of 18 u. The intermediate diepoxide at m/z 903.9 also had an epoxide formed from the hydroperoxide, some isomers of which were located next to a double bond, so these obeyed the second epoxide fragmentation pathway for a net loss of only 16 u. This gave rise to the peak at m/z 887.9 which accompanied the peak at m/z 885.7. Diacylglycerol ions helped to confirm the presence of the two functional groups identified in this class of molecules. The peak at m/z 641.5 came from the epoxy, epidioxy fragment arising from loss of 18 amu from the hydroperoxy group. The peak at 625.5 arose from the diepoxide formed by loss of 18 u from the hydroperoxy group and 16 u from the epidioxy group. Finally, although hydroperoxy, epidioxy TAGs were isobaric with dihydroperoxy molecules, shown in Fig. 6C, the chromatographic retention times added valuable information about the relative polarities of these different classes. The hydroperoxy, epidioxy TAGs were less polar than their dihydroperoxy homologs.

Other classes of oxidation products from trilinolenin were also observed, but the signal-to-noise ratios and fragmentation patterns produced were not sufficiently unambiguous to allow identification of the classes. Diepidioxy molecules and hydroperoxy, epidioxy molecules in which both functional groups were localized on one acyl chain were believed to be present. These classes were both also isobaric with dihydroperoxides, with molecular masses of 936.7. Extracted ion chromatograms (EICs) of the $[M+18]^+$, $[M+23]^+$ and $[M+39]^+$ adduct masses are shown in Fig. 8. These EICs show the elution of the dihydroperoxy and hydroperoxy, epidioxy classes discussed above, but also show that the peak which eluted after the hydroperoxy, epidioxy molecules had the same adduct peaks, but the chromatography indicated that these were less polar. The chromatography is consistent with the diepidioxy class following the hydroperoxy, epidioxy class, but since this cannot be demonstrated with confidence, no further discussion of these other classes will be presented. Given the variety of classes which could be identified using the RP-HPLC–APCI-MS, compared to previous reports, the failure to conclusively identify

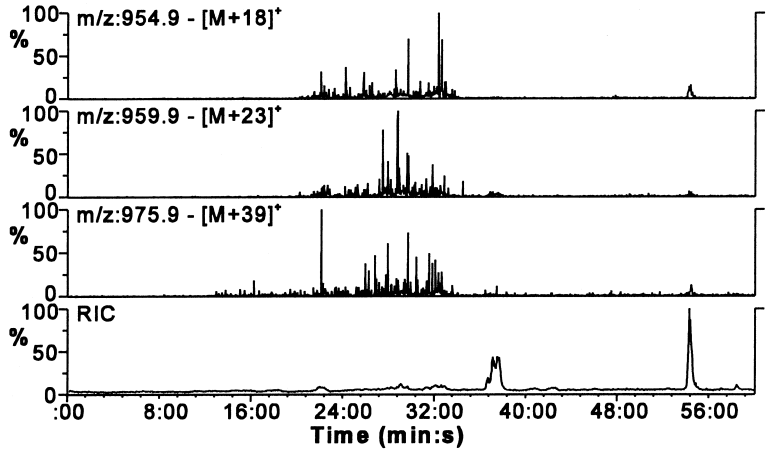

Fig. 8. Reconstructed ion chromatogram and extracted ion chromatograms of $[M+18]^+$, $[M+23]^+$ and $[M+39]^+$ adducts of trilinolenin oxidation products having the same mass as trilinolenin bishydroperoxide and dilinolenoyl, linoleoyl glycerol hydroperoxy epidioxide (see Fig. 6C, Fig. 7C). $[M+23]^+$ acetonitrile adducts given by $-(-OH)+(-N=C=CH_2)$, for $-17+40=+23$ and $[M+39]^+$ acetonitrile adducts given by $-(H)+(-N=C=CH_2)$, for $-1+40=+39$.

these and other remaining minor components does not detract from the high degree of analytical utility which the methodology offers.

3.4.4. Triolein and trilinolein diepoxides

The last class of oxidation products which is discussed is the diepoxy molecules which resulted from triolein and trilinolein. Analogous to the two types of epoxy molecules discussed above (next to a site of unsaturation or not next to a site of unsaturation), and the two mechanisms of their fragmentation, diepoxy molecules were found in which either one of the epoxy groups formed where a site of unsaturation had been (so was not next to another double bond), or in which the epoxy was not formed across a site of unsaturation (so might or might not be next to double bond, depending on the isomer). Fig. 9A,B show mass spectra for triolein diepoxide in which the epoxide was not formed at a site of unsaturation and in which the epoxide was formed at a site of unsaturation, respectively. The formation of the epoxide across a site of unsaturation, without loss of two acyl chain hydrogens, led to a molecular mass for this class which was 2 u larger than the other diepoxide. In the first two mass spectra of Fig. 9, the molecular mass is given both by protonated molecular ion and by several adducts, and clearly reflects this 2 u difference. Although these two classes differed by 2 u in their molecular masses, they both formed fragments having the same masses. The first diepoxide (not across an existing double bond site) primarily obeyed the second mechanism, losing only 16 u for each epoxide lost. However, the two epoxide groups led to several isomers, so that a small peak at m/z 879.8 in Fig. 9A did arise from loss of 18 u from the monoepoxide intermediate at m/z 898.0 (897.8). Conversely, the difference of 18 u between the fragments at m/z 899.9 and 881.7 in Fig. 9B showed that this class obeyed the first mechanism. The diacylglycerol ions provided unambiguous confirmation of the difference of two mass units between these two classes. The peaks at m/z 631.5, 617.6 and 615.7 in Fig. 9A were characteristic of the epoxide formed in addition to, not in place of, a double bond. Conversely, the peaks at m/z 633.7 and 619.5 in Fig. 9B clearly indicated that an epoxide had been formed which replaced one of the double bonds. The chromatographic retention times added further evidence to these identifications.

Similar to the triolein diepoxides, trilinolein diepoxides could also be identified. Fig. 9C shows a mass spectrum for the diepoxide in which one of the epoxide groups formed at the site of a double bond. Just as with the analogous triolein diepoxide shown in Fig. 9B, this trilinolein diepoxide showed mostly loss of 16 u from the protonated molecular ion and then loss of an additional 18 u upon loss of the second epoxide, for a total difference of 34 u between these ions. As expected, the diacylglycerol ions added strong confirmation to the identification of this class of oxidation product. The corresponding diepoxide in which neither of the epoxides formed across a double bond was observed by the use of EICs, but the presence of numerous isomers made the signal-to-noise ratio of the averaged mass spectrum sufficiently poor that identification could not be made unambiguously, even though the chromatographic retention times acted as supporting evidence.

4. Conclusions

Overall, we have been able to identify several classes of oxidation products by direct detection following liquid chromatographic separation without fraction collection or prior class separation. In fact, RP-HPLC–APCI-MS runs of samples of monohydroperoxides which were pre-separated using TLC showed chromatograms which were actually quite similar to the unseparated mixtures. This indicated that the silicic acid TLC caused degradation similar to normal oxidation.

The dramatic differences in the relative proportions of fragments arising from APCI of molecules of different classes having identical masses highlighted the utility of this soft ionization technique. The wealth of mass spectral data offered by online LC detection along with the traditional retention time information made the combination of APCI-MS with LC separation possibly the most effective single methodology yet demonstrated for analysis of complex mixtures of oxidation products.

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The advice and assistance from Harold W. Gardner and Ronald D. Plattner in elucidating the specific

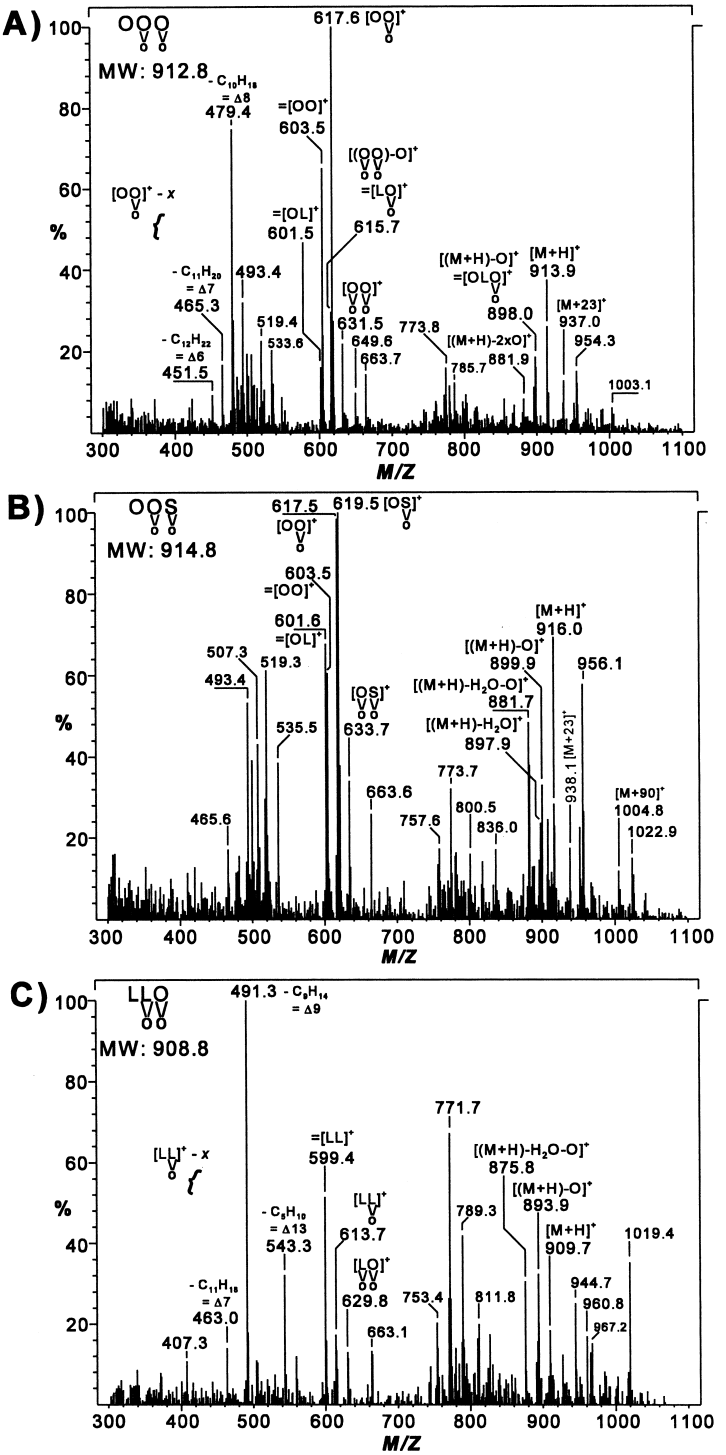

Fig. 9. Average mass spectra across peaks of trioleoyl and trilinoleoyl diepoxides: (A) triolein diepoxide (neither epoxide formed with loss of a site of unsaturation); (B) dioleoyl, stearoyl glycerol diepoxide (one of the epoxide groups formed with loss of a site of unsaturation, the other epoxide formed without loss of a site of unsaturation); (C) dilinoleoyl, oleoyl glycerol diepoxide (one epoxide formed with loss of a site of unsaturation, the other epoxide formed without loss of a site of unsaturation).

fragmentation mechanisms was greatly appreciated and is gratefully acknowledged.

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