Autoxidation products of normal and genetically modified canola oil varieties determined using liquid chromatography with mass spectrometric detection

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Abstract

Normal, high stearic acid and high lauric acid canola oil varieties were heated in the presence of air to allow autoxidation to occur. After the reaction, the oils were analyzed using a non-aqueous reversed-phase high-performance liquid chromatographic separation followed by detection using atmospheric pressure chemical ionization mass spectrometry. Oxidized products were separated and identified. The major autoxidation products which remained intact were epoxides and hydroperoxides. Two classes of epoxy triacylglycerols (TAGs) were formed. One class with the epoxy group replacing a site of unsaturation and one class adjacent to a site of unsaturation, as was previously reported for model TAGs. Intact oxidation products resulted mostly from oxidation of oleic acid, while oxidation products of linoleic and linolenic acid chains decomposed to yield chain-shortened species. Both neutral and polar chain-shortened products were observed. Polar chain-shortened decomposition products eluted at very short retention times and required a different chromatographic gradient to separate the molecules. This class of molecules was tentatively identified as core aldehydes. The high stearic acid canola oil yielded more intact oxidation products containing stearic acid, as expected. The high lauric acid oil produced intact oxidation products which contained lauric acid. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

'Lipid oxidation' refers to the reaction of molecular oxygen with lipid molecules to add oxygen-containing functional groups. The oxygenated molecules formed during oxidation are referred to as the primary oxidation products. Secondary oxidation products are the molecules and fragments which result from the decomposition or further reaction of the primary oxidation products. Oxidation of lipids has been studied extensively before, starting with the oxidation of free fatty acids [1–15] (as their methyl esters) and, then oxidized triacylglycerols (TAGs) [16–21]. In some early studies, oxidation products fractions were collected following preparative HPLC. Fractions were subjected to NMR analysis to identify functional groups, and then in some cases, gas chromatography–mass spectrometry (GC–MS) was applied after derivatization. In almost all studies,

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either the fatty acid methyl esters (FAMEs) or the 'headspace volatiles', which resulted from fatty acid decomposition, were studied by GC–MS.

In the case of triacylglycerols, oxidized fats or oils were saponified (and usually derivatized) and the FAMEs were formed to allow analysis by GC or GC–MS. Detection of the intact TAGs following liquid chromatography was done using ultraviolet and/or refractive index detectors. Only more recently has a method for liquid chromatographic separation of intact (non-saponified) oxidized TAGs, followed by online mass spectrometric detection, been reported [20,21]. Atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) has now been shown, by a number of groups, to be very effective for analysis of normal triacylglycerols, as recently reviewed [22], and has also been applied to mixtures of oxidation products. We recently reported the analysis of the autoxidation products of triacylglycerol standards (OOO, LLL, LnLnLn) [20] and the analysis of oxidation products from triolein produced at frying temperature [21]. We confirmed, as has been reported since early studies, that hydroperoxides were the primary oxidation products formed from the autoxidation of triacylglycerols. Also, we confirmed the identities of dihydroperoxides, two classes of epoxides, epidioxides, and other products. Two advantages of the APCI-MS methodology that we used were that no saponification and derivatization was required, and that the triacylglycerols were determined intact, without modification. Thus, factors such as acyl migration during chemical reactions such as transesterification were not of concern. The APCI-MS methodology also produced different ionic fragments from different isomers of the oxidation products, and these were differentiable by mass. Thus, numerous positional isomers of the hydroperoxides, epoxides, and other classes were identified in one chromatographic separation.

The mixture of oxidation products which resulted from even the simple TAG standards was complex, and minor components were still present which require further study. But the majority of oxidation products have been identified, and new insight has been gained into the process of oxidation product formation, and the mechanism of fragmentation during APCI-MS analysis. We reported results which

showed that two distinct classes of epoxides were formed, those in which the oxygen atom was added at the site where a double bond had been, and those in which the epoxide group was added near to a double bond. For those epoxides in which the epoxide formed near a double bond, two possibilities were found. First, the epoxide could form directly adjacent to a double bond, or it could form two or more carbons away from the double bond. The mass spectra of the epoxides formed near double bonds showed two different types of fragment masses, depending on whether or not the epoxide was directly adjacent to a double bond. When the epoxide formed between two carbons neither of which was next to a double bond, the epoxide was lost during fragmentation in a way which left *two* additional sites of unsaturation. When the epoxide formed adjacent to a double bond, the epoxide was lost during fragmentation in a way which left *one* additional site of unsaturation. We proposed a mechanism by which resonance stabilization occurred, when the epoxide was next to a double bond, which stabilized the intermediate to allow only one additional double bond to form.

We also found that primary fragments formed from hydroperoxides in the APCI source were epoxides. The epoxides formed in the APCI source from hydroperoxides could have the epoxide directly next to a double bond, or not directly next to a double bond, depending on the position of the hydroperoxide. Thus, we found that the chemical reactions which occurred in the APCI source during fragmentation were similar to those which occurred over time during the process of autoxidation.

Numerous products containing one or two epoxides, or one or two hydroperoxides were formed from the single, simple model TAGs. As this method was extended to seed oils, it was expected that a prodigious number of oxidation products would result from the numerous TAGs present. While it was not feasible to identify all oxidation products from such complex TAG mixtures, our previous work has allowed most of the oxidation products from a wide variety of TAGs in three types of canola oils to be identified.

We report here the analysis, using a reversed-phase liquid chromatographic separation followed by detection with atmospheric pressure chemical ioniza-

tion mass spectrometry, of the mixtures of starting oils and products formed from the autoxidation of: (i) normal canola oil; (ii) high stearic acid canola oil; and (iii) high laurie canola oil.

2. Materials and methods

2.1. Materials

The canola oil samples were obtained from Calgene (Davis, CA, USA). A normal canola oil (NCO) (Calgene No. DS68494), a high stearic canola oil (HSCO) (Calgene No. DS68468), and a high lauric canola oil (HLCO) (Calgene No. DS19592) were analyzed. All solvents were HPLC grade or the highest quality available. The crude oils were extracted with hexane from seeds and then stripped of non triacylglycerol components by solid-phase extraction, both methods as previously described [23].

2.2. TAG autoxidation method

The canola oil samples were subjected to autoxidation according to the following procedure: 225 mg of each stripped oil neat was placed in a 2.5×5.5 cm, 20 ml scintillation vial. The vial was given an oxygen head space and then capped. The vials were heated in the dark at 60°C for 96 h in a forced air oven. At the end of the heating time the colorimetric peroxide values (procedure in Ref. [24]) were 60.5, 60.1, and 56.4 mEq peroxide/kg sample for the high laurie, high stearic, and normal canola oils, respectively. These oils were stored in the freezer in methylene chloride at −18°C until analysis by APCI-MS was performed.

2.3. Liquid chromatography

High-performance liquid chromatography was performed using an LDC 4100 quaternary pump (Thermo Separation Products, Shaumburg, IL, USA) that supplied a binary gradient of methylene chloride (DCM)–acetonitrile (ACN) to two reversed-phase columns in series. Two Inertsil ODS-2 columns, 25 cm×4.6 mm, 5 μm (GL Sciences, Keystone Scientific, Bellefonte, PA, USA) in series were used.

The gradient program used for the separation was as follows: initial ACN–DCM (70:30), held until 20 min; linear from 20 to 40 min to ACN–DCM (60:40), held until 50 min; linear from 50 to 70 min to ACN–DCM (40:60), held until 75 min; linear from 75 to 80 min to ACN–DCM (30:70), held until 85 min. Finally, the system was recycled to the initial conditions at 99 min. The flow-rate was 0.7 ml/min throughout. A separate chromatographic gradient was used to emphasize separation of the polar components. This gradient was previously used for hydroxy-containing seed oils, as previously reported [25]. Column effluent from both separations was split using a Valco Tee so that ~85% of flow went to an evaporative light scattering detection (ELSD) system and ~15% went to the APCI-MS source. 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 l/min. High purity N_2 was used as the nebulizer gas. ELSD was used to confirm proper separation and operation of the LC and MS systems. Quantification using the ELSD data was not performed and data obtained from this detector are not presented.

2.4. Mass spectrometry

A Finnigan MAT (San Jose, CA, USA) TSQ700 mass spectrometer, operating in Ql low mass mode, fitted with an APCI source was used to acquire mass spectral data. The vaporizer was operated at 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 used for these samples was m/z 200 to 1200 in 1.5 s. Mass spectra shown were averaged across the breadth of a chromatographic peak.

2.5. Gas chromatography

The fatty acid compositions were determined as the FAMEs using gas chromatography. FAMEs were made by acid transmethylation according to the method of Christie [26]. A Varian 3400 (Palo Alto, CA, USA) gas chromatograph was used with an SP2380 column (Supelco, Bellefonte, PA, USA), 30

m×0.25 mm I.D. with 0.2 μm film thickness. The following conditions for were used for FAME analysis: inlet temperature=240°C; detector temperature=280°C; initial temperature=150°C; initial time=15 min; ramp to 210°C at 2°C/min, held for 5 min; 0.5 μl of a 20 mg/ml sample was injected.

3. Results and discussion

3.1. Normal canola oil autoxidation products

Fig. 1 shows the reconstructed ion chromatograms (RICs) from the separation of normal canola oil (NCO), high stearic acid canola oil (HSCO), and high lauric acid canola oil (HLCO), obtained using RP-HPLC/APCI-MS. In all three chromatograms, numerous peaks that eluted at short retention times were present in addition to the normal TAG peaks. Also, many of the early eluting peaks appeared broader than those given by typical TAGs. This was similar to what was observed for the model TAGs, and will be explained below.

As was the case with the model TAG triolein, when an epoxide was formed across a double bond, that double bond was in a single, fixed location on the fatty acyl chain (Δ^9 for oleic acid), so only one epoxide could result (the chromatographic separation did not differentiate between epoxides formed on the sn-1, -2, or -3 position of the glycerol backbone). This epoxide gave a single sharp chromatographic peak, as seen in Fig. 1 of the previous report [20]. Similarly, any seed oil TAGs that contained an oleic acyl chain which was oxidized across the site of unsaturation yielded one specific epoxide. Conversely, when the epoxide did not form across an existing double bond, several isomers were possible, depending on where on the chain the epoxide formed. These isomers produced a broadened chromatographic peak containing all isomers present. These two types of chromatographic behavior, first described for model TAGs, were observed for seed oil TAGs. Fig. 2 represents extracted ion chromatograms (EICs) of masses associated with diglyceride fragments containing one epoxide group, formed in normal canola oil, as well as masses associated with normal diacylglycerol fragments. The EIC in Fig. 2F shows the mass associated with a dioleoyl diglyceride ('OO')

in which the epoxide formed across the site of unsaturation of one of the oleoyl acyl chains, converting it to a chain having no sites of unsaturation, but one epoxide group (equivalent to OS-epoxide). Since the site of unsaturation was in a single, discreet position (Δ^9), the peaks in the EIC of m/z 619.5 were sharp, well defined peaks. Any TAG that contained the 'OO' diglyceride in which the epoxide formed across the double bond had a peak in this EIC. Thus, peaks for OOO, POO, SOO and GOO (20:1,OO) which formed OOS-epoxide, POS-epoxide, SOS-epoxide, and GOS-epoxide, respectively, were observed. Each of these appeared as a single, sharp peak in the EIC for OS-epoxide.

Another epoxide had the same fragment mass as OS-epoxide. It was the epoxide formed by 'OS' with an epoxide formed on the oleoyl chain, but not across the double bond, or SO-epoxide. While this fragment was theorized as a possible product, it was not observed in NCO. It was, however, detected in substantial quantity in the HSCO, discussed below. TAGs containing OS-epoxide vs. SO-epoxide were chromatographically separable, with the SO-epoxide (having a normal, fully saturated chain) eluted after the OS-epoxide (which was functionally more polar).

The EIC of the fragment that corresponded to 'PS-epoxide', in which the epoxide formed across the double bond in 'PO', is shown in Fig. 2H. As expected, the peak that corresponded to the 'O' peak of 'PS-epoxide' occurred at the same retention time as the 'P' peak in the 'OS-epoxide' chromatogram, since both fragments arose from 'POS-epoxide'. Thus, multiple confirmatory fragments in EICs corresponding to different fragments arising from the same oxidized TAG molecules increased the confidence with which assignments were made.

As we reported previously, the process of ionization using APCI produced a fragment representing the loss of the epoxide group with concomitant formation of two double bonds when the epoxide was not directly adjacent to an existent double bond. If the seed oil oxidation products behaved like the model TAG triolein, OS-epoxide would produce a fragment equal to 'OL' in the APCI source. This was the case, with peaks appearing in the EIC associated with the mass of 'OL' diglyceride (m/z 601.5, Fig. 2A) at the same retention time as the peaks in the OS-epoxide EIC. Peaks were visible which repre-
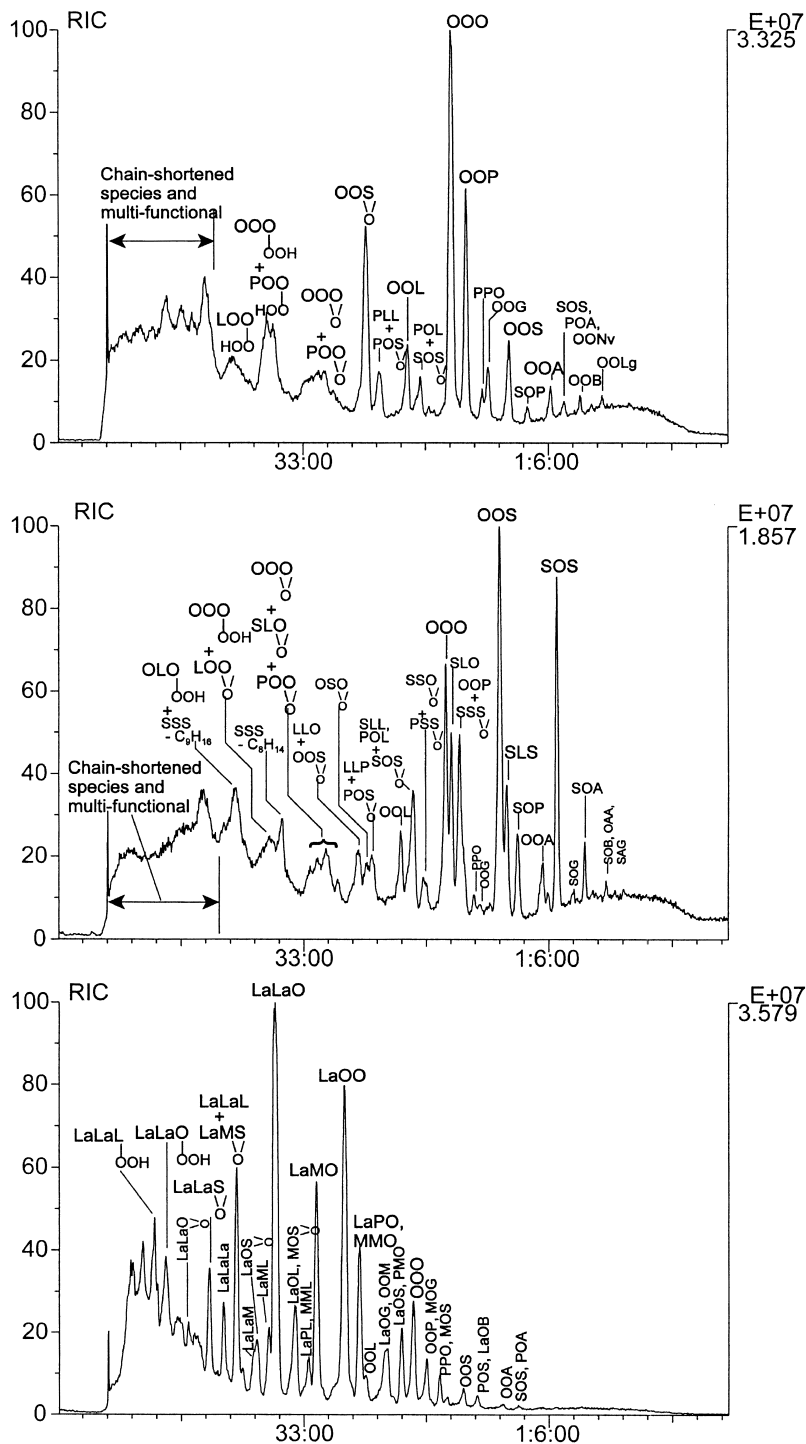
Fig. 1. Chromatograms of (A) normal canola oil autoxidation products, (B) high stearic canola oil autoxidation products, and (C) high lauric canola oil autoxidation products, obtained using reversed-phase high-performance liquid chromatography (RP-HPLC) with detection by atmospheric pressure chemical ionization-mass spectrometry (APCI-MS).
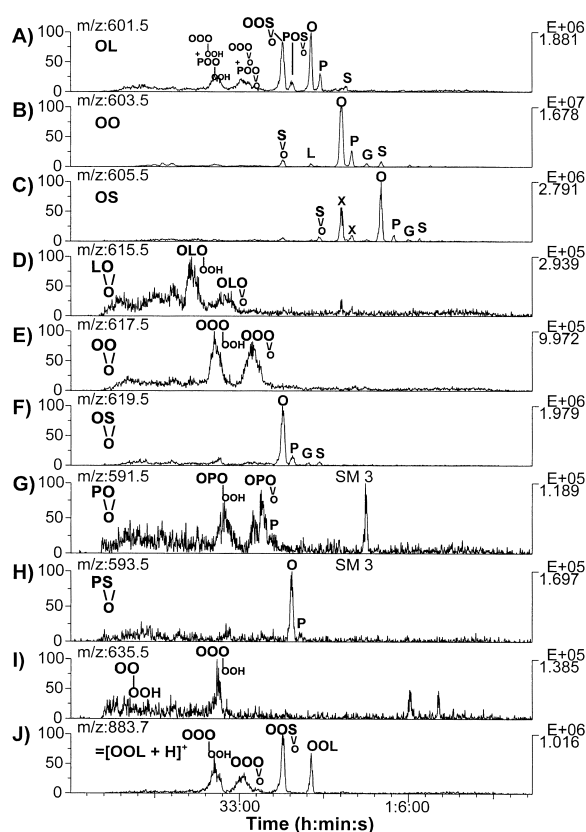
Fig. 2. Extracted ion chromatograms of important diacylglycerol fragments (A–G), and a molecular and near-molecular ion, (H) from normal canola oil autoxidation product mixture. (A–C) Normal diacylglycerol fragments; (D–H) mono-oxygenated diacylglycerol fragments; (I) a hydroperoxide; and (J) a protonated molecular ion for OOL, and a major APCI fragment for epoxides and hydroperoxides.

sented the fragmentation, in the APCI source, of OOS-epoxide to form OOL, and POS-epoxide to form POL. These OOL and POL fragments, formed from OOS-epoxide and POS-epoxide in the APCI source, were chromatographically very distinct from the same diglyceride fragments formed from normal 'OOL' and 'POL' TAGs. Fragments formed from SOS-epoxide and GOS-epoxide were expected, but were chromatographically overlapped with genuine TAGs containing the 'OL' diglyceride (GOS-epoxide occurred near the same time as OOL, while SOS-epoxide occurred near the same time as POL).

Peaks in EICs of other fragments formed from the OS-epoxide-containing TAGs confirmed the identifi-

cations. For instance, a peak representing S-epoxide appeared in the EIC for 'OO', Fig. 2B, at the same retention time as the peak representing oleic acid in the EIC for OS-epoxide. These fragments both arose from OOS-epoxide. Similarly, a peak representing S-epoxide appeared in the EIC for 'OS', Fig. 2C, at the same retention time as the peak representing stearic acid in the EIC for OS-epoxide. These fragments both arose from SOS-epoxide. The S-epoxide peaks in the 'OO' and 'OS' EICs occurred at nearly the same relative retention times that the linoleic acid (Ln) peaks would appear in each EIC, if it were present to an appreciable extent. This indicated that an epoxide group on a saturated 18-carbon chain had approximately the same effect on the chromatographic retention factor of the acyl chain as three sites of unsaturation. That is, S-epoxide in combination with a diglyceride, to form an epoxytriglyceride, had approximately the same retention time as a Ln acyl chain in combination with the same diglyceride. The fatty acid (FA) composition, determined as the FAME, is given in Table 1. The FA composition indicated that the percentage of linolenic acid had been reduced from 12.8% for the normal oil to 1.8% for the autoxidized oil.

Excellent agreement was seen between the mass spectrum (not shown) of OOS-epoxide obtained from the canola oxidation products and OOS-epoxide which resulted from the autoxidation of the model TAG triolein published previously. A similar oxidized TAG, not present in the triolein standard

Table 1
Canola oil fatty acid composition

Fatty Acid	NCO	HSCO	HLCO
6:0			0.1
8:0			0.0
10:0			0.2
12:0			46.7
14:0			5.2
16.0	9.3	5.9	3.6
16:1	4.9	1.7	1.5
18:0	3.0	44.0	1.3
18:1 cis, Δ^9	72.0	33.5	28.2
18:1 cis, Δ^{11}	5.7	3.7	2.7
18:2	1.7	4.0	3.3
18:3	1.8	3.4	0.7
Other	1.6	3.8	6.5
	100.0	100.0	100.0

mixture, was POS-epoxide, which eluted just after OOS-epoxide (see Fig. 2F and 2H). The mass spectrum of POS-epoxide is shown in Fig. 3A. POS-epoxide followed the same fragmentation pathways previously described for OOS-epoxide. POS-epoxide formed a substantial $[M+H]^+$ ion, which helped to identify its molecular mass. POS-epoxide also formed a very strong $[M+90]^+$ adduct, as observed previously, which further confirmed the molecular mass. This adduct has not yet been identified, but work is currently underway to identify it. The largest near-molecular fragment which formed was that formed by loss of 18 u. As previously observed, this fragment arose from loss of oxygen, leaving a double bond, followed by further loss of two hydrogens to form a second double bond. This fragment, at m/z 858.3 had the same m/z as 'POL' (but was chromatographically quite distinct from normal POL).

In the region of the mass spectrum associated with diacylglycerol fragments, the epoxy-diglyceride fragment OS-epoxide exhibited a large fragment at m/z 619.6. This fragment underwent loss of the epoxide oxygen, leaving a double bond, followed by further loss of two hydrogens to form a second double bond. This formed a fragment at m/z 601.4, which was equivalent to the diglyceride 'OL', but was chromatographically distinct from 'OL' produced by non-epoxy TAGs (see Fig. 2A). Similarly, the PS-epoxide fragment formed from POS-epoxide could be seen at m/z 593.5. This underwent fragmentation with loss of oxygen, described above, to form the fragment at m/z 575.5, which was equivalent to 'PL'. The other diacylglycerol fragment expected from POS-epoxide was 'PO'. This fragment was observed at m/z 577.5, as expected.

Other fragments that confirmed structural characteristics of POS-epoxide were present at lower masses. The fragment at m/z 493.3 corresponded to the fragment formed when OS-epoxide fragmented between the epoxide carbons to lose a C_9H_{18} fragment (619.5−126.2=m/z 493.3. Similarly, the fragment at m/z 467.5 corresponded to the fragment formed when PS-epoxide fragmented between the epoxide carbons to lose a C_9H_{18} fragment (593.5−126.2=m/z 467.5). Other peaks in the low mass region corresponded to fragments produced by individual acyl chains. The peak m/z 339.4 corresponded to the $[O+58]^+$ fragment reported

previously [20,21]. This peak is the same as that observed for phospholipids using APCI-MS [27]. The peak at m/z 265.3 corresponded to the acylium ion produced by the oleic acyl chain ($C_{17}H_{33}C=O^+$). The acylium ion produced by S-epoxide ($C_{17}OH_{33}C=O^+$) was seen at m/z 281.5. Lastly, the acylium ion produced by the palmitic acyl chain ($C_{15}H_{31}C=O^+$) was observed at m/z 239.2. The ions above were discussed in detail to demonstrate that the same behavior that was observed from model TAGs was also observed for epoxide TAGs produced from autoxidation of normal canola oil. These same fragmentation pathways were observed for all epoxides formed from TAGs in the three seed oils studied here, so additional detail will not be given for each TAG epoxide.

Excellent agreement was seen between the mass spectrum of triolein epoxide (OOO-Ep), Fig. 3B, obtained from NCO and that observed previously for the epoxide formed from the model TAG triolein. However, the mass spectrum of the epoxide obtained from the model TAG had a better S/N because no other TAGs were present which occurred at the same retention time. In the seed oil, other TAGs were present which were partially chromatographically overlapped with this component, resulting in additional fragments present in the mass spectrum. Similarly, excellent agreement was be seen between the mass spectrum of triolein hydroperoxide obtained from NCO, Fig. 3C, and that reported previously for the hydroperoxide obtained by autoxidation of triolein standard.

As mentioned above, the epoxides which formed near to, instead of across, an existing double had several isomers possible, leading to broader chromatographic peaks than the epoxides which formed across a former sight of unsaturation. Fig. 2F showed that, as with normal, non-oxidized TAGs, (See DG fragments in Fig. 2A, B and C) the 'O' and the 'P' peaks in the OS-epoxide EIC were completely chromatographically resolved. However, in the case of TAGs oxidized near, not across, to a double bond, the peak broadening caused by the presence of multiple isomers was sufficient to cause the 'O' and 'P' peaks of such oxidized TAGs to appear overlapped. Thus, the second of the broadened peaks in Fig. 2E represented OOO-epoxide and POO-epoxide which were not resolved. Therefore, mass spectra of
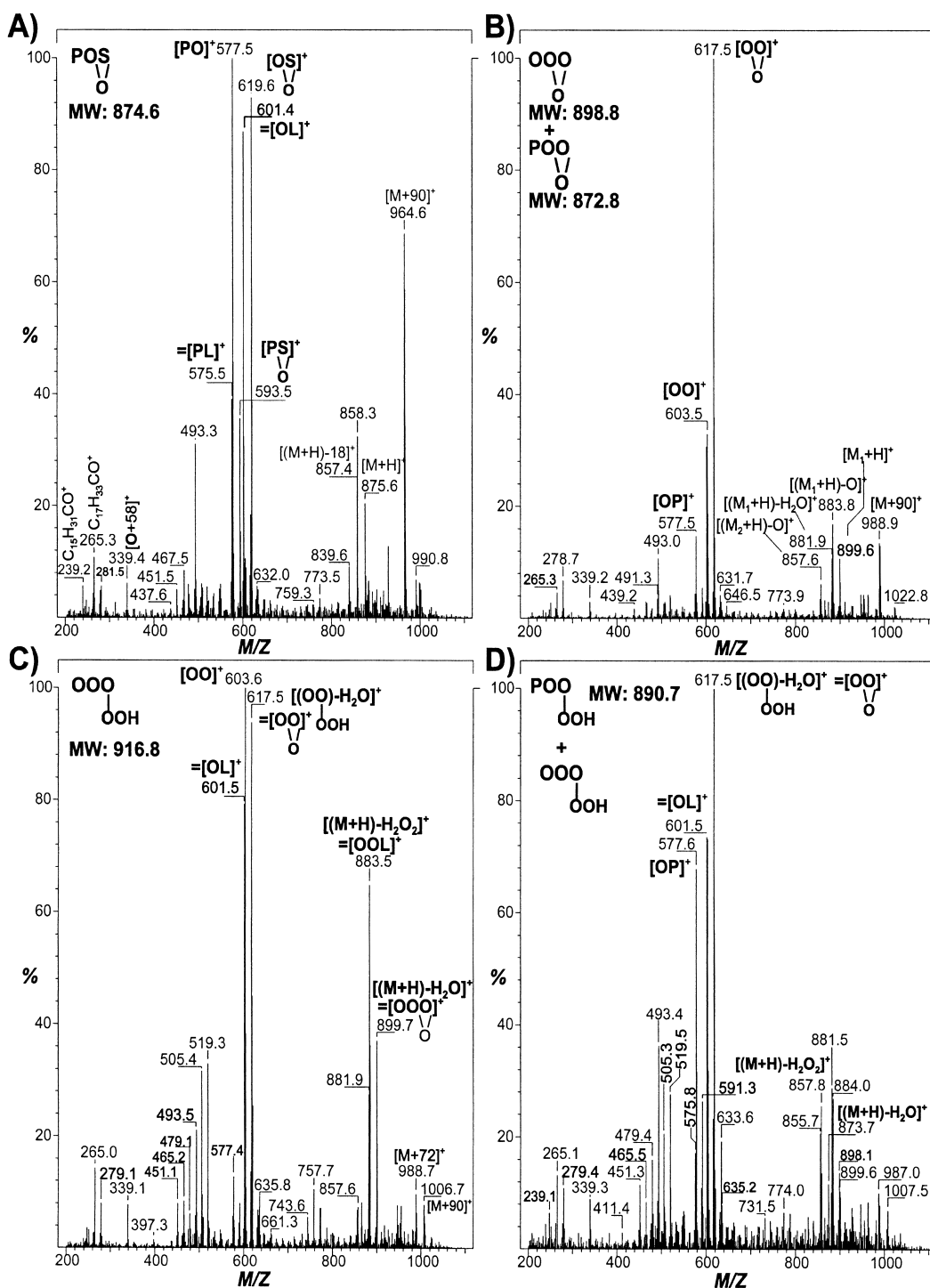
Fig. 3. Atmospheric pressure chemical ionization mass spectra of normal canola oil autoxidation products. MW=molecular mass.

OOO-epoxide which represent an average across the breadth of the peak, such as that shown in Fig. 3B, display a contribution from POO-epoxide. The peak at m/z 577.5 in Fig. 3B, representing the 'PO' fragment of POO-epoxide, is an example of a peak arising from this overlap. As would be expected, however, the first part of the broadened peak contained mostly OOO-epoxide while the latter part of the peak was enriched in POO-epoxide. Peaks in EICs associated with other fragments, such as the EIC for 'PO-epoxide' in Fig. 2G and 'PO' at m/z 577.5 (not shown), demonstrated the slightly later elution observed for POO-epoxide isomers which overlapped the OOO-epoxide isomers.

In fact, the EICs of distinct unique oxidation products, such as shown in Fig. 2F and H showed that oxidized TAGs were produced by combination of each possible fatty acid with the oxidized DAG fragment to produce an oxidized TAG. From this observation, it is predicted that almost all of the oxidized DAG fragments were formed from every statistically predicted oxidized TAG possible. While the most abundant oxidized TAGs were readily identified, those present in small amounts were difficult to conclusively identify because of the overlap of multiple broad peaks arising from the isomers of each of the species produced.

As mentioned above, hydroperoxides produced epoxide fragments during ionization in the APCI source. The first large peak in the EIC of OO-epoxide, Fig. 2E, corresponded to the OO-epoxide formed in the APCI source from the hydroperoxide-containing oxidized TAGs OOO-hydroperoxide and POO-hydroperoxide. As mentioned above, the second large peak in this EIC corresponded to the true epoxide-containing TAGs. The fragment which arose from the diacylglycerol containing the intact hydroperoxide appeared at m/z 635. As seen in Fig. 3C, the peak corresponding to the intact hydroperoxide did not have a large abundance. However, the EIC of the OO-hydroperoxide at m/z 635.5 (Fig. 2I) exhibited a distinct peak at the same retention time as that of the first peak in the EIC of m/z 617.5 (whereas no such peak occurred at the position of the second peak, where the actual epoxide eluted). Furthermore, it was already discussed in detail above that the epoxides (even those formed in the APCI source from hydroperoxides) fragmented in a manner

such that the epoxide was lost to form *two* sites of unsaturation. Thus, it was expected and observed that a peak having a mass equivalent to 'OL' (at m/z 601.5) resulted from the hydroperoxides (which formed epoxides, which then lost the epoxide, leaving unsaturation), as well as from the regular epoxides, which were discussed above. The EIC of the fragment equivalent to 'OL', Fig. 2A, exhibited the expected broadened peak at the retention time corresponding to elution of OOO-hydroperoxide and POO-hydroperoxide. The abundant peak at m/z 601.5 (equivalent to 'OL') was also clearly visible in the mass spectrum of OOO-hydroperoxide shown in Fig. 3C. The mass spectrum of POO-hydroperoxide, shown in Fig. 3D, exhibited peaks arising from not only POO-hydroperoxide, but also from the large amount of OOO-hydroperoxide, from which POO-hydroperoxide was unresolved.

The loss of an epoxide to form two sites of unsaturation was also made apparent by the distinct peaks in the EIC having the mass equivalent to the normal TAG protonated molecular ion $[OOL+H]^+$ in Fig. 2J. Based on the discussion above, it was expected and observed that OOO-hydroperoxide (which formed an epoxide, and then lost the epoxide to form two sites of unsaturation), OOO-epoxide, OOS-epoxide and normal OOL all formed an ion having a mass equivalent to $[OOL+H]^+$ in Fig. 2J.

The same elution behavior described for OOO-epoxide and OOO-hydroperoxide was also observed for OLO-epoxide and OLO-hydroperoxide, as well as others. The EIC at m/z 615.5 (Fig. 2D) corresponding to LO-epoxide showed two peaks which corresponded to: (i) OLO-epoxide+PLO-epoxide; and (ii) OLO-hydroperoxide+PLO-hydroperoxide, which eluted just prior to the analogous OOO-containing TAG oxidation products. Earlier peaks in the EIC for m/z 615.5 require additional analysis before assignments may be made with confidence.

It is important to mention that the areas under peaks of extracted ion chromatograms corresponding to oxygen-containing fragments do not provide a good measure of the relative amounts of the various oxidation products formed. To demonstrate this, it was necessary to re-examine the results for the model TAG standards, previously reported, in greater detail. Results of the integration of peaks under the total (or reconstructed) ion chromatogram (Fig. 1 of

the previous report) and also of the integration of the areas under peaks associated with the 'OO-epoxide' (m/z 617.5) and 'OO' (m/z 603.5) fragments from the model TAG triolein are given in Table 2. These results indicated that the peak sizes in the EIC for the 'OO-epoxide' fragment (m/z 617.5) did not give the same relative amounts of OOO-hydroperoxide and OOO-epoxide as were given by the reconstructed ion chromatogram (RIC). For instance, OOO-epoxide represented 26.7% of the integrated area under the EIC of m/z 617.5 (the 'OO-epoxide' fragment), while OOO-epoxide represented only 9.2% of the area under the reconstructed ion chromatogram. Examination of the other percentages in Table 2 further demonstrated that individual EICs could not be used effectively to estimate relative amounts of oxidation products formed, while the RIC may provide a better estimate of relative percentages. It can be seen that the oxidation products which contained more than one oxygen functional group could form a larger number of various fragments, so integration of the 'OO-epoxide' areas led to smaller percentages than the RIC, except in the case of the di-epoxide. The 'OO' fragment was also not useful for quantitative estimation. The 'OO' fragment was not formed at all from some oxidation products with two functional groups. The 'OO' fragment was formed in a higher proportion from OOS-epoxide than from OOO-epoxide, presumably because the OS-epoxide fragment was not as stable as the OO-epoxide fragment, so the 'OO' fragment was favored instead.

Applying the observations from the model TAG triolein to the normal canola oil data allows one to

conclude that, although it appears from the EIC of m/z 617.5 in Fig. 2E as though the epoxide (with a larger peak area) is present to a greater extent than the hydroperoxide, this was not the case. The OO-epoxide fragment peak arising from intact OOO-epoxide in Fig. 2E is larger than the OO-epoxide fragment formed from OOO-hydroperoxide because of the higher efficiency with which this fragment is formed from OOO-epoxide, not because OOO-epoxide is actually present to a larger extent.

Many of the peaks eluted before normal TAGs in the chromatogram in Fig. 1 have been identified by their mass spectra and by ion chromatograms such as those in Fig. 2. However, there was a group of unresolved peaks eluted at early retention times which exhibited spectra sufficiently complex that individual identifications could not be made using the chromatographic runs shown above. In our experience with TAG mixtures to date, we have encountered three primary sample types: (i) those which contained polar functional groups, such as hydroxy-containing seed oils [25], which required a gradient that spread polar components over a wide retention window; (ii) normal seed oils which contained non-polar TAGs (gradient described above); and (iii) highly oxidized samples (from heated oxidation) [21] which contained polar oxidation products, normal non-polar TAGs, and high-molecular-mass oligomers with long retention times. Thus, we have employed three primary chromatographic gradients, depending on the sample. To improve our ability to identify components in these autoxidized seed oil samples, samples were re-run using the same gradient as that used for hydroxy-containing seed oils, so the most polar components were spread over a very broad retention time frame. The resultant chromatograms from NCO and HSCO are shown in Fig. 4. As can be seen, the broadening of early eluted peaks was at the expense of resolution of the normal TAGs and moderately polar oxidation products, which eluted over a short span at longer retention time. As desired, however, this separation did result in resolution of multiple species which previously constituted unresolved early peaks. Fig. 5 shows extracted ion chromatograms associated with masses from several fragments. These fragments came from stable, distinct, chromatographically resolved species. Most of these species result from triolein

Table 2
TriOlein oxidation products composition

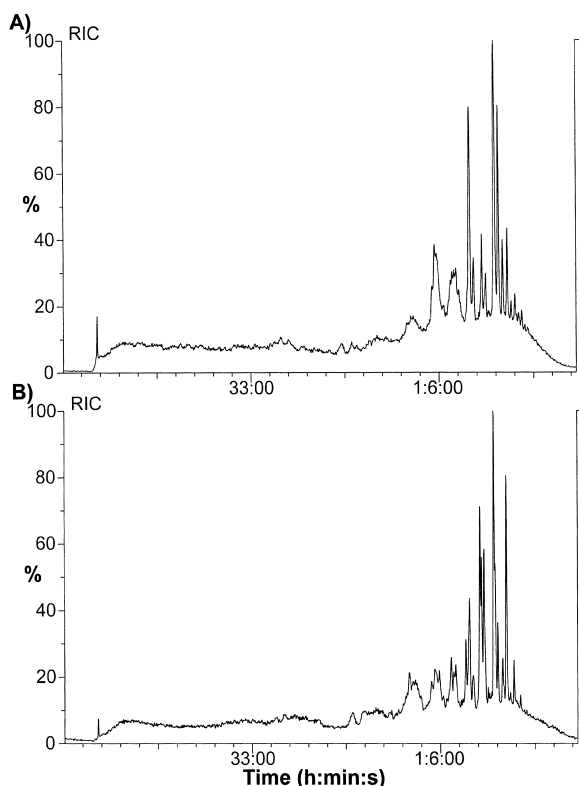| Tag Ox. Prod.	RIC	'OO-Ep EIC	'OO' EIC
Tris–OOH	4.4	1.2	
Peak 2	2.3	1.5	
Bis-OOH	15.0	8.1	
OOO-DiEp	4.4	5.0	7.0
OOS-DiEp	5.4	1.5	4.5
Mono-OOH	50.1	45.2	58.5
OOO-Ep	9.2	26.7	12.8
OOS-Ep	9.3	10.8	17.2
Sum	100.1	100.0	100.0

Fig. 4. Total (or reconstructed) ion chromatograms of (A) normal canola oil and (B) high stearic canola oil autoxidation products mixtures. Chromatographic gradient same as previously reported for hydroxy-containing seed oils (emphasizes polar components).
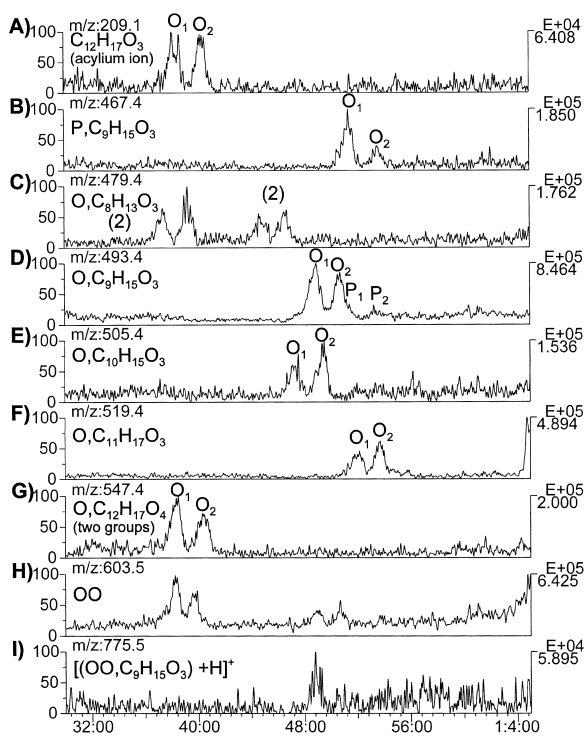

Fig. 5. Extracted ion chromatograms of short-chain oxidation products separated using the hydroxy oil gradient. (A) An acylium ion of a single fatty chain, (B–G) chain-shortened diacylglycerol ions, (H) dioleoyl fragment ion, and (I) protonated molecular ion of chain-shortened triacylglycerol.

because it was the most abundant TAG present in the oil [28]. They have the same masses as those reported earlier which came from two different classes of chain-shortened triolein species [21]. The first class was simple chain-shortened species in which any mid-chain oxygen functional group was lost with the leaving fragment, leaving a non-polar chain-shortened TAG. These species were shown in Fig. 4 in the previous publication [21]. Those species were were also present in autoxidized NCO, but were non-polar and did not elute in the time window shown in Fig. 5. Instead, they eluted later, for example the OOO-$C_{10}H_{18}$ species shown in the previous publication (fragment at m/z 465.4 and [M+H]$^+$ at m/z 747.7) eluted at 1:06:50 using this hydroxy chromatographic gradient. This same molecule eluted at 33 min in Fig. 1A (using the normal TAG gradient).

In contrast, many of the species shown in Fig. 5 are the same as those mentioned, but not fully described, in Fig. 5 of the previous report. We stated previously that we believed these species to be chain-shortened species which also still contained an oxygen functional group. Given the fact that we showed the presence of di-epoxides, etc., we suggested that the remaining functional group might be on one of the non-shortened (intact) chains, with one functional group lost during chain shortening, such as might arise from a di-epoxide which broke at one epoxy site, leaving the other. The current data has a better signal-to-noise ratio and exhibits better chromatographic resolution (because the gradient emphasizes polar components), and therefore clarifies the previous observations. The fragment at m/z 493.5 has a mass consistent with a shortened diacylglycerol fragment containing one oleoyl chain and one $C_9H_{15}O_3$ (nonaldehyde) chain, which would be

formed by cleavage at the epoxide site of the most abundant epoxide, OOS-epoxide. Concurrent with that fragment are peaks in the EIC of intact 'OO' at m/z 603.5. Also, a peak appears in the EIC for the oxidized TAG 'OO,$C_9H_{15}O_3$' at m/z 775.5. These three fragments together lead us to tentatively identify the component eluted at that time to be dioleoylnonaldehyde, 18:1,18:1,9:0-aldehyde, the core aldehyde formed by loss of a C_9 fragment, leaving the oxygen on the end of the shortened TAG. In other words, instead of the oxygen being lost with the leaving group to form volatile aldehydes, etc. as demonstrated previously, these species likely represent the core aldehydes which result when the aldehyde group is formed at the end of the remaining chain during breakage. Two isomers of each aldehyde are present in Fig. 5. Because of the excellent resolution of polar components provided by this gradient, these appear to be sn-1, 3 vs. sn-2 positional isomers. Peaks arising from oleoyl and palmitoyl chains which, when combined with the fragments shown in Fig. 5 make oxidized TAGs, are also seen in some EICs in Fig. 5. The 'O' peaks of 'P,$C_9H_{15}O_3$' eluted at the same times as the small 'P' peaks of 'O,$C_9H_{15}O_3$', which fragments both arose from the core aldehyde TAG 'OP,$C_9H_{15}O_3$'. While multiple confirmatory fragments provide good evidence to tentatively identify the core aldehydes, we cannot rely solely on mass spectrometry, and conclusive identification awaits confirmation by other techniques.

An additional set of peaks eluted just prior to those arising from molecules tentatively identified as core aldehydes, described above. This second set of peaks had similar, sometimes larger, masses, but earlier elution times. Shorter retention times than the core aldehydes, with similar or larger masses, would tend to indicate the presence of an additional oxidation functional group on the core aldehyde, rendering it slightly more polar. One such compound had an acylium ion which appeared above the m/z 200 cutoff used for these experiments, and gave a clue to the possibilities identities of these molecules. The acylium ion which EIC is shown in Fig. 5A was consistent with an acylium ion arising from a C_{12} chain-shortened fatty chain with one double bond, and two oxygen-containing functional groups (not one cyclic group, which would have a mass 2 u

larger), such as a C_{12} core aldehyde with an epoxy groups also still intact. This acylium ion would have the formula $C_{12}H_{17}O_3$, and would arise from the chain-shortened epoxy, aldehyde fatty chain $C_{12}H_{17}O_4$. This would be expected to elute prior to a similar core aldehyde without the additional epoxy group. If present, this chain would produce a diacylglycerol in combination with an oleoyl chain having a m/z of 547.4. As seen in Fig. 5, the EIC of m/z 547.4 did exhibit peaks which exactly coincide with the acylium ion. The EIC of the dioleoyl diacylglycerol (OO) also showed peaks corresponding to the same retention time. Furthermore, the protonated triacylglycerol ion which represented $[(OO,C_{12}H_{17}O_3)+H]^+$ at m/z 787.7 also showed a peak at that exact retention time (not shown). Thus, two diacylglycerol peaks, the acylium ion, and the protonated molecular ion all appear at the same retention time and indicate the likely presence of an epoxy-containing core aldehyde. Again, multiple confirmatory fragments provide evidence for identification of epoxy-containing core aldehydes, but we cannot rely solely on mass spectrometry, and conclusive identification awaits confirmation by additional techniques.

We also made the observation in the previous report on products of heated oxidation, that at least two types of high-molecular-mass components, which eluted very late in that chromatographic run, appeared to be present: neutral addition products such as dimers and other addition products, and addition products containing polar functional groups. By analogy to the results above, it may be the case that the previously observed polar high-MW components were chain addition products with additional aldehyde and/or epoxy groups remaining intact. Again, identification awaits application of additional techniques.

3.2. High stearic acid canola oil

The RIC of canola oil with a high stearic acid content is shown in Fig. 1B. The increased number and sizes of peaks eluted at long retention times was characteristic of an oil with a higher content of stearic acyl chains. Extracted ion chromatograms of many of the same masses as shown for normal canola oil are given for the high stearic canola oil in

Fig. 6. The EICs for diglycerides from TAGs in Fig. 6A, B and C displayed much larger peaks for the stearic acid–containing TAGs than did those from NCO. The EIC of m/z 603.5, which represented both 'OO' and 'SL' fragments, showed much larger peaks for the 'S' peaks of both the 'OO' and the 'SL' diacylglycerol fragments from HSCO TAGs than were observed in the NCO. This same EIC also showed a larger amount of all 'SL' containing TAGs than did the same EIC for NCO (Fig. 2B).

The EIC of fragments of HSCO TAGs having the mass of 'OL and SLn', Fig. 6A showed several important differences to the EIC of NCO, Fig. 2A. Despite a superficial close resemblance between Figs. 2A and 6A, the peaks in each chromatogram arose from entirely different TAG molecules. Were it not for multiple confirmatory diacylglycerol frag-

ments in other EICs, along with the retention times, it would be easy to conclude that the peaks in Fig. 6A were mislabeled. Fortunately, retention times were rather consistent between TAGs in the chromatograms of NCO vs. HSCO. The first primary difference between Figs. 2A and 6A was the presence of the large 'S' peak (from SOL) in Fig. 6A which was barely present in Fig. 2A. This large 'S' peak was expected for high stearic canola oil. The next important difference was the larger amounts of TAGs apparently containing the 'SLn' diacylglycerol, although there is some uncertainty in these species, as discussed below. Another important difference was the smaller appearance of the 'OL' peak which was formed from epoxides and hydroperoxides as a result of the mechanism previously described, in which loss of the epoxy group formed two double bonds, when the epoxy is not adjacent to an existing double bond.

Differences were seen in the other EICs of oxidized HSCO TAGs vs. those from oxidized NCO. Comparing the EIC of m/z 617.5, which arose from OO-epoxide, and from OO-hydroperoxide which formed an OO-epoxide fragment, we saw that Fig. 6E showed peaks arising from more stearic acid containing oxidized TAG than were visible in the EIC from NCO, Fig. 2E. A peak arising from SOO-epoxide was apparent in Fig. 6E which was not visible in Fig. 2E. Also, co-eluted with the broad peak for OOO-epoxide was an SO-epoxide fragment formed from SOO-hydroperoxide. Analogous peaks were similarly seen in the EIC for LO-epoxide arising from SOL-epoxide and the epoxide fragment from SOL-hydroperoxide.

Fig. 6F showed that an OS-epoxide fragment was isobaric with an SO-epoxide fragment, at m/z 619.5. In this EIC, an 'O' peak and an 'S' peak were seen in combination with the OS-epoxide and SO-epoxide, respectively, to give four TAG oxidation products shown in Fig. 6F. Fig. 7A, B and C show mass spectra of three of these TAGs. These mass spectra exhibited all of the fragmentation patterns and pathways described above, and described previously, so additional detail is not required.

We earlier discussed in great detail the fragmentation of an epoxide, when not next to a double bond, to form two sites of unsaturation, for a net loss of 18 u. This resulted in the S-epoxide giving a fragment
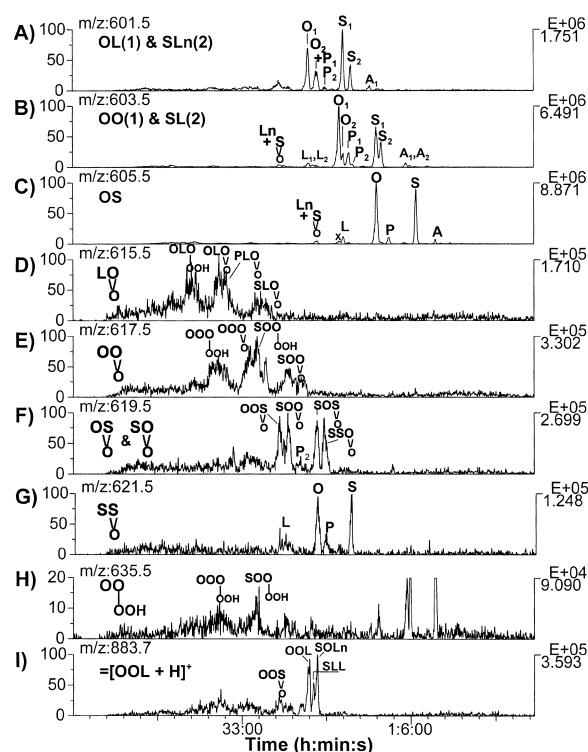

Fig. 6. Extracted ion chromatograms of important diacylglycerol fragments (A–H) and a molecular and near-molecular ion (I). (A–C) Normal diacylglycerol fragments; (D–G) mono-oxygenated diacylglycerol fragments; (H) a hydroperoxide; and (I) a molecular ion for OOL, and a major APCI fragment for epoxides and hydroperoxides.
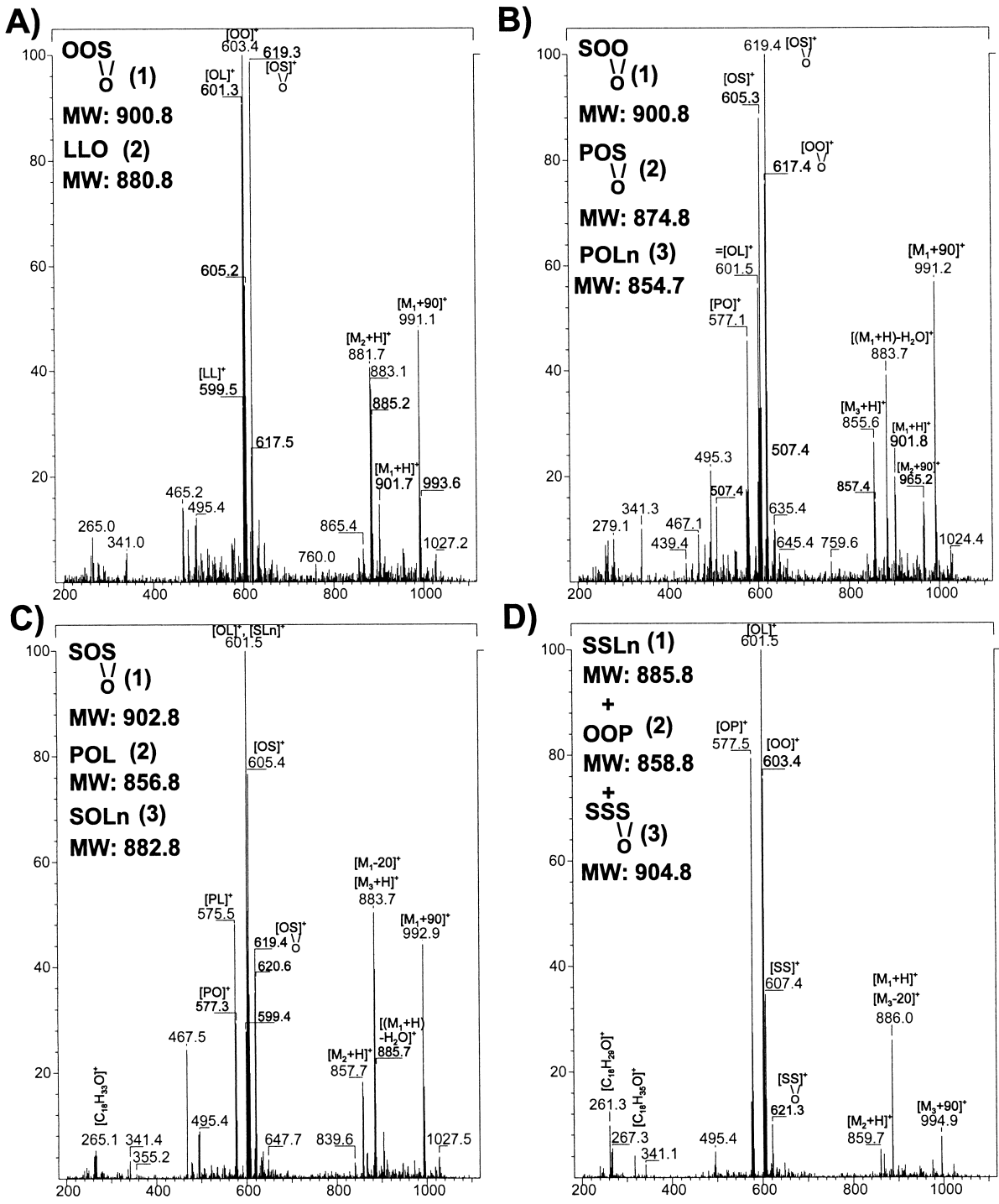
Fig. 7. APCI-MS mass spectra of high stearic acid canola oil normal triacylglycerols and epoxides. (A) OOS-epoxide and LLO, (B) SOO-epoxide, POS-epoxide, and POLn, (C) SOS-epoxide, POL, and SOLn and (D) SSLn, OOP, and SSS-epoxide.

having a mass isobaric with an 'L' chain. We previously proposed a mechanism by which such loss could occur. We observed in the HSCO (and HLCO) evidence of an even more perplexing process. In TAGs which contained an S-epoxide and one other saturated chain (S or P), an additional fragmentation process was observed which was not observed previously for S-epoxide-containing oxidized TAGs which contained two unsaturated acyl chains. In the mass spectra of the epoxides formed from high stearic acid canola oil, a fragment representing loss of 20 u from the epoxide was observed. This resulted the S-epoxide chain giving a mass which was isobaric with a 'Ln' chain. It would be expected that such a fragment would clearly stand out in the mass spectra of such highly saturated S-epoxide-containing TAGs. Unfortunately, though, as was mentioned above, the S-epoxide acyl chain affected the retention of TAGs into which it is incorporated to the same degree as a 'Ln' chain. Thus, SSS-epoxide eluted at the same retention time as SSLn. For this reason, we initially attributed the apparent loss of 20 u to the presence of 'Ln' chains. However, there appeared to be larger abundances of these fragments than could be accounted for solely from linolenic acyl chains. Then, examination of high lauric acid canola oil, which FAME analysis indicated very little 'Ln' present, also exhibited the fragments arising from loss of 20 u from the highly saturated epoxides. The fragments representing loss of 20 u (or H_2O plus two more H, leaving three sites of unsaturation) occurred from the protonated molecular ions and the diacylglycerol fragments, as did the '−18 u' fragment reported earlier. We are not prepared at this time to propose a mechanism by which such loss occurs. Isolation of highly saturated S-epoxide TAGs in the absence of linolenic fatty acyl chains will be carried out to demonstrate the process observed in the canola oil mixtures.

3.3. High lauric acid canola oil

Fig. 1C shows the reconstructed ion chromatogram of the TAG mixture resulting from autoxidation of HLCO. The larger number of peaks eluted at shorter retention times than were observed in NCO and HSCO indicated the presence of many lauric and other short fatty acid containing TAGs and TAG

oxidation products. Extracted ion chromatograms shown in Fig. 8 show oxidation products, as well as normal DAG fragments. Mass spectra of typical oxidation products are shown in Fig. 9. The normal fragmentation mechanisms reported earlier and discussed above are evident in the mass spectra shown in Fig. 9.

The EICs in Fig. 8 show the non-statistical distribution of fatty acids among TAGs. For instance, EICs in Fig. 8 showed that the 'LaM' and 'LaP' DAG fragments preferentially combined with oleic acid more than any other fatty acid, while the 'LaO' and 'MO' DAG fragments were incorporated into TAGs containing a (or another) lauric acid chain. Also, the 'LaLa', 'LaM' and 'LaP' fragments were
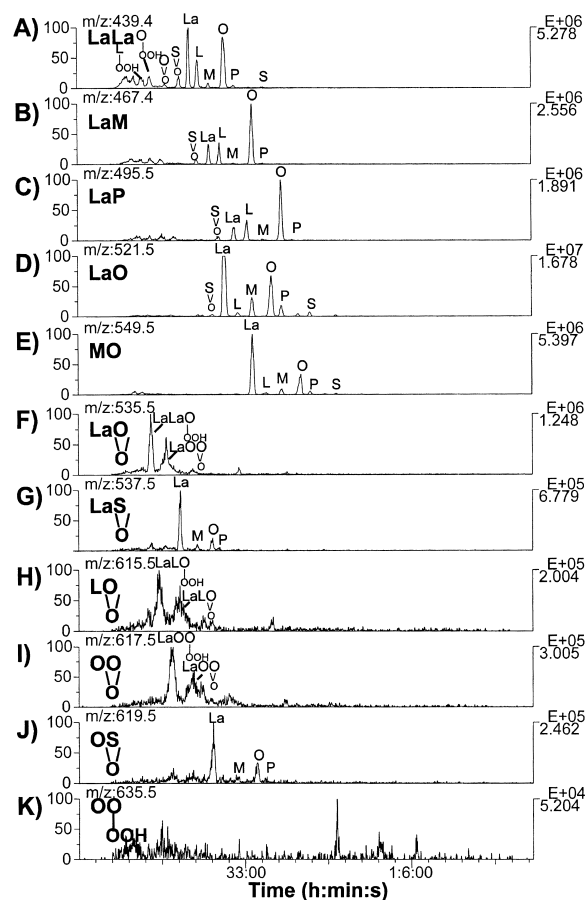

Fig. 8. Extracted ion chromatograms of important diacyiglycerol fragments. (A–E) Normal diacylglycerol fragments; (F–J) mono-oxygenated diacylglycerol fragments; (K) a hydroperoxide.
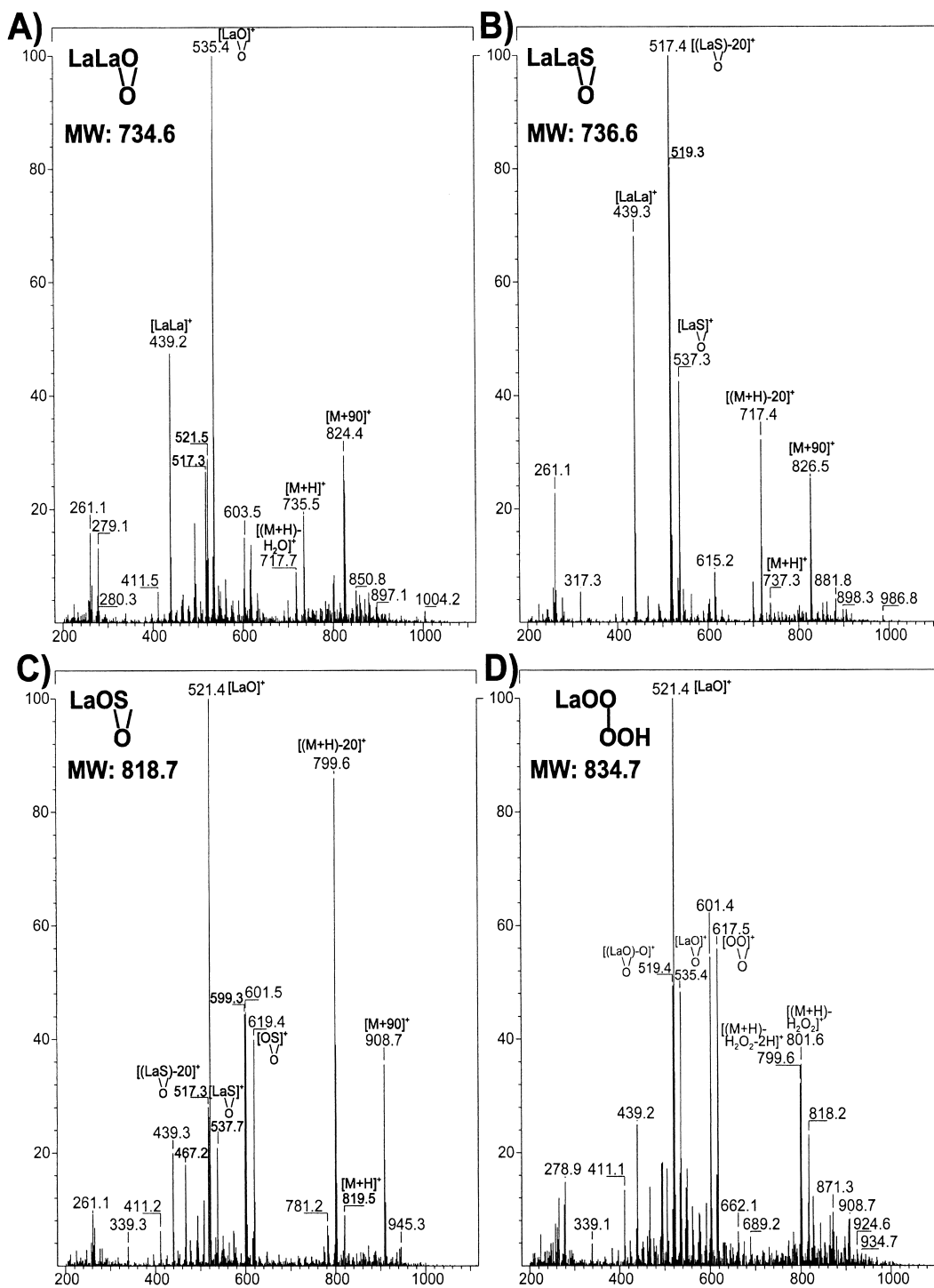
Fig. 9. Mass spectra of typical oxidation products from high lauric canola oil.

found in combination with linoleic acid more than any other DAGs. This type of non-statistical, preferential fatty acid distribution is not seen when only the fatty acid composition is determined (e.g. as the fatty acid methyl esters) for a triacyiglycerol mixture.

The oxidation products shown in the EICs in Fig. 8F–K are entirely consistent with the trends observed for the predominant oxidation products described in detail above. The oxidation products which formed across the site of a double bond produced sharp, distinct peaks, as in Fig. 8G and J. Those oxidation products in which multiple isomers were possible produced broad, indistinct peaks.

One fragmentation process was observed which cannot be adequately described. The mass spectrum of LaLaS-epoxide, Fig. 9B, exhibited a fragment having a m/z of 517.4, which represented a loss of 20 u from the intact LaS-epoxide diacylglycerol. This resulted in a diacylglycerol fragment isobaric with LaLn. However, the FAME analysis clearly showed a paucity of Ln present. This fragmentation by loss of 20 u to form three double bonds was also the primary fragment formed by the protonated molecular ion to go from m/z 737.3 to 717.4. Similarly, LaOS-epoxide formed a primary fragment ion having a m/z of 799.6 from the protonated molecular ion, which m/z was 819.7. The LaS-epoxide diacylglycerol fragment, which m/z was 537.7, formed a fragment ion at m/z 517.3, again exhibiting this unusual loss of 20 u form three sites of unsaturation with loss of the epoxide oxygen.

3.4. Conclusion

The most common oxidation products visible using APCI-MS were epoxides and hydroperoxides. Epoxides formed stable and long-lived species which eluted just before normal TAGs. This class of molecule formed abundant $[M+H]^+$ ions, $[M-18]^+$ ions, and $[M+90]^+$ adduct ions, and were readily identifiable by comparison to results from model TAGs obtained previously. Such epoxides resulted mostly from oxidation of the monounsaturated fatty acid, oleic acid. As was previously observed, two classes of epoxides were formed, those in which the epoxide formed across an existing double bond, and those in which the epoxide was not formed across an existing double bond. An epoxide formed across a linoleic double bond would be indistinguishable from an epoxide formed from oleic acid, not across a double bond. A new fragmentation process was observed for the most highly saturated epoxides. These produced an as yet unexplained loss of 20 u.

Intact hydroperoxides were observed in these samples, as was previously observed for TAG standards. Hydroperoxides formed only small amounts of protonated molecular ions, but instead formed primary fragments which were isobaric with epoxides resulting from loss of 18 u from the hydroperoxide, and these fragmented further, as did normal epoxides. Also, hydroperoxides formed only small abundances of diacylglycerol fragments which contained the intact hydroperoxide. However, sufficient abundances were formed that, in combination with chromatographic retention data, the hydroperoxides could be identified. The primary hydroperoxides which remained intact were those formed from oleic acid, and a lesser amount from linoleic acid. Hydroperoxides formed from linolenic acid and most of those from linoleic acid decomposed further to yield other product molecules.

The fatty acid compositions of the oils showed marked decreases in the content of linoleic and linolenic acids. Also, epoxides and hydroperoxides of these were observed to a lesser extent than those of oleic acid. However, breakdown products representing chain cleavage to yield chain-shortened neutral TAGs were observed, and chain-shortened core aldehydes were tentatively identified. Thus, it is believed that the hydroperoxides formed from linolenic acid, and to a great extent those from linoleic acid, went on to produce decomposition products, rather than remaining as stable, intact primary oxidation products.

As has been reported previously, aldehydes did not produce abundant protonated molecular ions by APCI-MS, so identification of these components required both mass and chromatographic data. Because of the paucity of molecular ions, even these data were not sufficient for unambiguous identification of the core aldehydes. Also as has been reported previously, hydroxy-containing oxidation products lost the hydroxy group very readily, giving species which were isobaric with normal unsaturated TAGs. Because of this and the large number of overlapping oxidation products, hydroxy-containing components

were not identified, although they were previously shown to be produced by autoxidation trilinolenin standard. Other oxidation products such as bis-epoxides, epidioxides, bis-hydroperoxides and epidioxide hydroperoxides which were reported for model TAGs were also likely to have been present, but due to the overlap of co-eluting species these were not identified. Because of the complexity of the spectra which such molecules produce, virtually complete chromatographic isolation of such compounds is required for their identification.

Despite the need for even greater improvement in the chromatographic separation and identification of oxidation products formed from the autoxidation of natural oil samples, we have been able to directly identify and characterize more intact oxidation products than have previously been reported to date. We have been able to confirm the formation of two distinct types of epoxides which form either across or near a double bond. No such observations were previously extent in the literature. Overall, APCI-MS has shown itself to be a very valuable tool for direct identification of oxidation products, which help one to gain a better understanding of the mechanisms of oxidation and the intermediate products formed during the oxidation process. Direct identification of the chain-shortened molecules which result from oxidation provides an excellent complement to the identification of short-chain volatiles which are most often used to assess the degree of oxidation of a sample.

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