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Non-volatile products of triolein produced at frying temperatures characterized using liquid chromatography with online mass spectrometric detection

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Abstract

Oxidation products from triolein under model heated frying conditions have been analyzed using liquid chromatography with an evaporative light scattering detector and atmospheric pressure chemical ionization (APCI) mass spectrometric detection. Triolein was heated at 190°C with 2% water added each hour, to simulate the moisture of a frozen product, until polar components reached approximately 30%. The samples were separated using reversed-phase high-performance liquid chromatography with APCI–MS detection. Triolein oxidation products included hydroperoxides, epoxides and a ketone. Other products were formed by shortening of an acyl chain on the intact triolein. Normal and oxygen-containing products formed by the dimerization of triolein were also observed. Other products included chain addition products formed by addition of acyl chain subunits to intact triolein to form higher molecular weight products. Published by Elsevier Science B.V.

Keywords: Vegetable oils; Triolein; Triacylglycerols; Hydroperoxides; Epoxides

1. Introduction

Chemical reactions in vegetable oils at heated frying temperatures have long been known to produce a complex mixture of volatile and non-volatile products. Volatile compounds have been shown to include small molecules such as short-chain hydrocarbons, aldehydes and ketones. Many volatile compounds are lost into the air above the frying vessel, but they may be captured and identified using gas chromatography (GC) [1]. Because these compounds are amenable to conventional GC analysis, numerous studies have been reported and and these have been thoroughly reviewed [2,3]. Other products formed by heated vegetable oils include non-volatile decomposition products (NVDPs). NVDPs remain in the oil and contribute to the discoloration and unpleasant taste of overused oils. A large number of NVDP are formed, and these can be difficult to analyze because they are not amenable to GC analysis, and some are not amenable to normal liquid chromatographic techniques. Generally, NVDPs fall into two categories: (i) dimers and

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other polymerization products, and (ii) oxidized products. Non-volatile decomposition products have been analyzed using thin-layer chromatograpy [2,3], size-exclusion chromatography [4–7] and more recently, supercritical fluid chromatography [8]. Unfortunately, polymerized products do not produce distinctly resolved chromatographic peaks using size-exclusion chromatography (SEC). Also, oxidized compounds are believed to coelute with normal triacylglycerols (TAGs), or elute just before them during SEC.

A common approach to analysis of the complex mixture produced by heated oils, as demonstrated by Paulose and Chang [2], among others, is conversion of the fatty acids in the oil to methyl or ethyl esters by transesterification and then treatment with urea to produce urea-adduct-forming (AF) esters and non-urea-adduct-forming (NAF) esters. The NAF esters are then fractionated by silica liquid chromatography. The fractions thus separated are cleaved using acid to break up polymers, and then are further separated using TLC. This is a labor-intensive, time consuming approach, but, because of the complexity of such reaction mixtures, few options were available.

These labor-intensive methods have proved effective at identifying numerous decomposition products from a variety of oil samples. However, it is desirable to identify these NVDPs in a single chromatographic run without the need for fractionation, derivatization and other chemical treatment. We recently demonstrated the utility of atmospheric pressure chemical ionization mass spectrometry (APCI–MS) for identification of numerous autoxidation products formed from triacylglycerol standards triolein, trilinolein and trilinolenin [9]. These autoxidation products consisted of hydroperoxides, bishydroperoxides, epoxides and bisepoxides, as well as hydroxy, epidioxy and other compounds. All of these autoxidation products were more polar than the starting TAGs, so chromatographic separation was focused on components eluted before the starting TAGs. In the case of decomposition products formed under model heated frying conditions, dimers and other compounds, which are less polar than the starting triolein, were expected. Thus a chromatographic separation was necessary which would provide separation of polar oxidation products such as those produced by autoxidation, as well as non-polar products with longer retention times. Extensive discussion of the polar and non-polar products formed from oxidation of unsaturated lipids, in which possible mechanisms for formation of the above classes are given, has been published elsewhere [10]. Presented here is study of NVDPs of heated triolein as a model frying system, analyzed using a reversed-phase high-performance liquid chromatographic (RP-HPLC) separation coupled to an evaporative light scattering detector and a quadrupole mass spectrometer via an APCI source. Numerous oxidation products which are more polar than the triolein starting material were directly detected and characterized, as well as oxidized and non-oxidized dimers and other addition products.

2. Experimental

2.1. Sample preparation

Six test tubes (2×12.5 cm) containing 5 g each of triolein were heated to 190°C by submersion in a temperature controlled silicone oil bath. 2% water (100 ml) was added each hour through a capillary submerged to the bottom of each test tube, to simulate the moisture introduced by frying a frozen food product. One sample was removed each hour for analysis. The sample removed was immediately purged with argon, and 25 mg of the sample was removed and dissolved in 2 ml of dichloromethane (DCM), and then kept at −15°C until analysis within 24 h. The remainder of the sample was frozen at −15°C. This reaction gave the desired polar component fraction of about 15% at 3 h and 30% at 6 h heating time. Polar components were analyzed by column chromatography using the AOAC official method [11]. Analysis was performed on the samples of the six hour reaction.

2.2. Liquid chromatography

Acetonitrile (ACN) (EM Science, Gibbstown, NJ, USA) and dichloromethane (Fisher, Fair Lawn, NJ, USA) were HPLC grade and were used without further purification.

Preparative reversed-phase separation was used for fractionation of the reaction products. A Dynamax (Rainin Instrument Co., Woburn, MA, USA) C_{18} column, 30×2.25 cm, 6 μm particle size was used with an isocratic flow of ACN–DCM (40:60) at 4.0 ml min^{-1}. A Waters 410 refractive index detector, with the cell at 50°C and a sensitivity of 32, was used for the preparative separation. 50 ml of a 500 mg ml^{-1} solution in DCM was injected. Fractions were collected manually and combined for further analysis of individual peaks.

The pump used for reversed-phase high-performance liquid chromatography (RP-HPLC)-atmospheric pressure chemical ionization mass spectrometry (APCI-MS) was an LDC 4100 MS (Thermo Separation Products, Schaumburg, IL, USA) quaternary pump with membrane degasser. Two Inertsil ODS-2, 25 cm×4.6 mm, 5 mm (GL Sciences, Keystone Scientific, Bellefonte, PA, USA) columns in series were used with a gradient solvent program of ACN and DCM. The gradient used for separation of the unfractionated mixture was as follows: initial ACN–DCM (70:30), held until 15 min; linear from 15 to 45 min to ACN–DCM (20:80), held until 85 min; the column was recycled to starting conditions linearly from 85 to 99 min. The flow-rate was 0.8 ml min^{-1} throughout. The gradient used for separation of the pre-fractionated components was as follows: initial ACN–DCM (75:25); linear from 0 to 20 min to ACN–DCM (70:30), then linear from 20 to 50 min to ACN–DCM (30:70), held until 85 min; the column was recycled to starting conditions linearly from 85 to 99 min. The flow-rate was 0.8 ml min^{-1} throughout. Flow was split using a tee so that ~680 μl min^{-1} went to an evaporative light scattering detection (ELSD) system and ~120 μl min^{-1} went to the mass spectrometer. A Varex MKIII ELSD system (Alltech Associates, Deerfield, IL, USA) was used as an auxiliary detector for RP-HPLC–APCI-MS. 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. ELSD output was simultaneously directed to a stand-alone data system with 24-bit resolution (EZ-Chrome Elite, Scientific Software, Pleasanton, CA, USA). Injections of 10 μl were made using a Series 1050 autosampler (Hewlett–Packard, Wilmington, DE, USA).

2.3. Mass spectrometry

A Finnigan MAT TSQ700 (San Jose, CA, USA) mass spectrometer operating in Q1 low-mass mode was used for acquisition of APCI-MS data. The vaporizer was operated at 400°C, the capillary heater was operated at 265°C, the corona voltage was set to 6.0 μA. Sheath and auxiliary gases were set to 35 psi and 5 ml min^{-1}, respectively (1 p.s.i.=6894.776 Pa). Spectra were obtained from 100 to 2000 u with a scan time of 2 s.

3. Results and discussion

3.1. Reversed-phase liquid chromatography

Increasing amounts of products were formed from the heated triolein over time, and the endpoint of the experiment, at which time polar components reached approximately 30%, was reached at 6 h. The point at which polar components reached ~30%, determined by column chromatography, was chosen as the end point of the experiment because this is accepted as the point at which an oil is considered excessively deteriorated and should be discarded. Therefore, chromatograms and mass spectra which allowed identification of numerous molecular classes are shown for the 6 h sample.

Initially, SEC columns were coupled directly to the mass spectrometer to perform online SEC–APCI-MS. Unfortunately, the APCI source failed to produce ions from the higher-molecular-masss components eluted before triolein using an isocratic THF tetrahydrofuran (THF) solvent system. Therefore, we opted to employ an acetonitrile/methylene chloride separation system which has previously proved successful for a range of TAG-related molecules [9].

Fig. 1 shows ELSD and RIC chromatograms obtained using the reversed-phase HPLC separation. We also acquired data for a more dilute sample in which the triolein peak remained onscale (data not shown), but the by-products were less obvious in those chromatograms than in Fig. 1. Using this separation, we observed peaks which eluted before triolein, indicating higher polarity. These components had retention times which were similar to oxidation products formed from autoxidation of

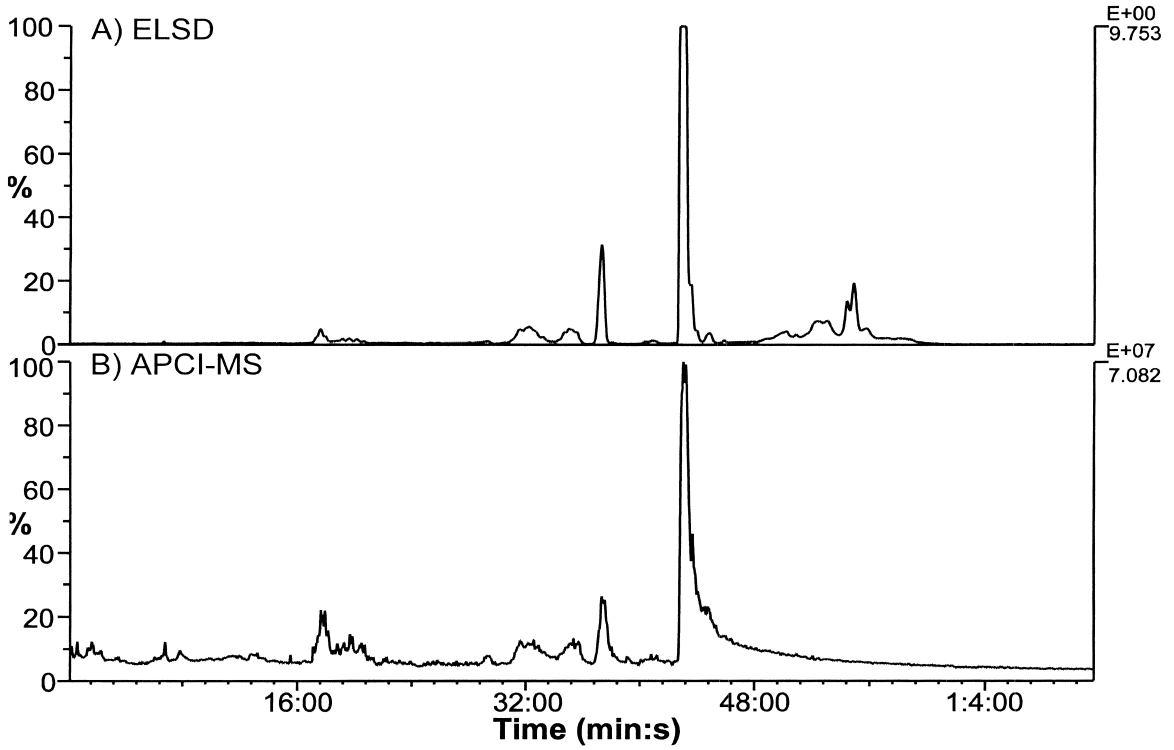

Fig. 1. Chromatograms of 6 h heated triolein samples obtained by reversed-phase high-performance liquid chromatography with (A) evaporative light scattering detection (ELSD), and (B) Atmospheric pressure chemical ionization mass spectrometric detection (APCI-MS).

triolein, which we recently characterized and reported by RP-HPLC–APCI-MS [9]. Extensive discussion of the broad range of products expected to be formed from oxidation of unsaturated lipids may be found elsewhere [10]. Fig. 1B shows that, like the SEC runs, the APCI source failed to produce ions from higher molecular weight components, which on this column eluted after triolein during RP-HPLC. Thus, we deemed it necessary to collect the fractions from preparative RP-HPLC–ELSD runs to obtain samples which were enriched in the higher molecular weight components, to increase the probability of detecting the components therein using APCI-MS. This approach proved successful, as indicated by results presented further below. First, however, discussion of the components which eluted before triolein is presented.

3.2. Oxidation products and chain-shortened TAG

The products which eluted before triolein gave mass spectra which indicated that some of these components were the same as those produced by autoxidation, reported earlier [9]. However, the presence of numerous species which were not present in the autoxidation mixture revealed that more extensive degradation occurred under model heated frying conditions. Fig. 2A shows a mass spectrum for the mono-epoxide formed when an oxygen added across an existing double bond, leading to OOS-epoxide, as was previously demonstrated for the autoxidation of triolein. The mass spectrum in Fig. 2A is virtually identical to that previously shown, except for the larger mass range shown here. All peaks in Fig. 2A have been described in detail previously, except the peak at m/z 265.1, which arose from the oleic acid acylium ion, RCO^+. The retention time, relative to triolein, and the mass spectrum allowed unambiguous identification of this mono-epoxide.

The peak which eluted before the OOS-epoxide in the mixture of autoxidation products, reported previ-

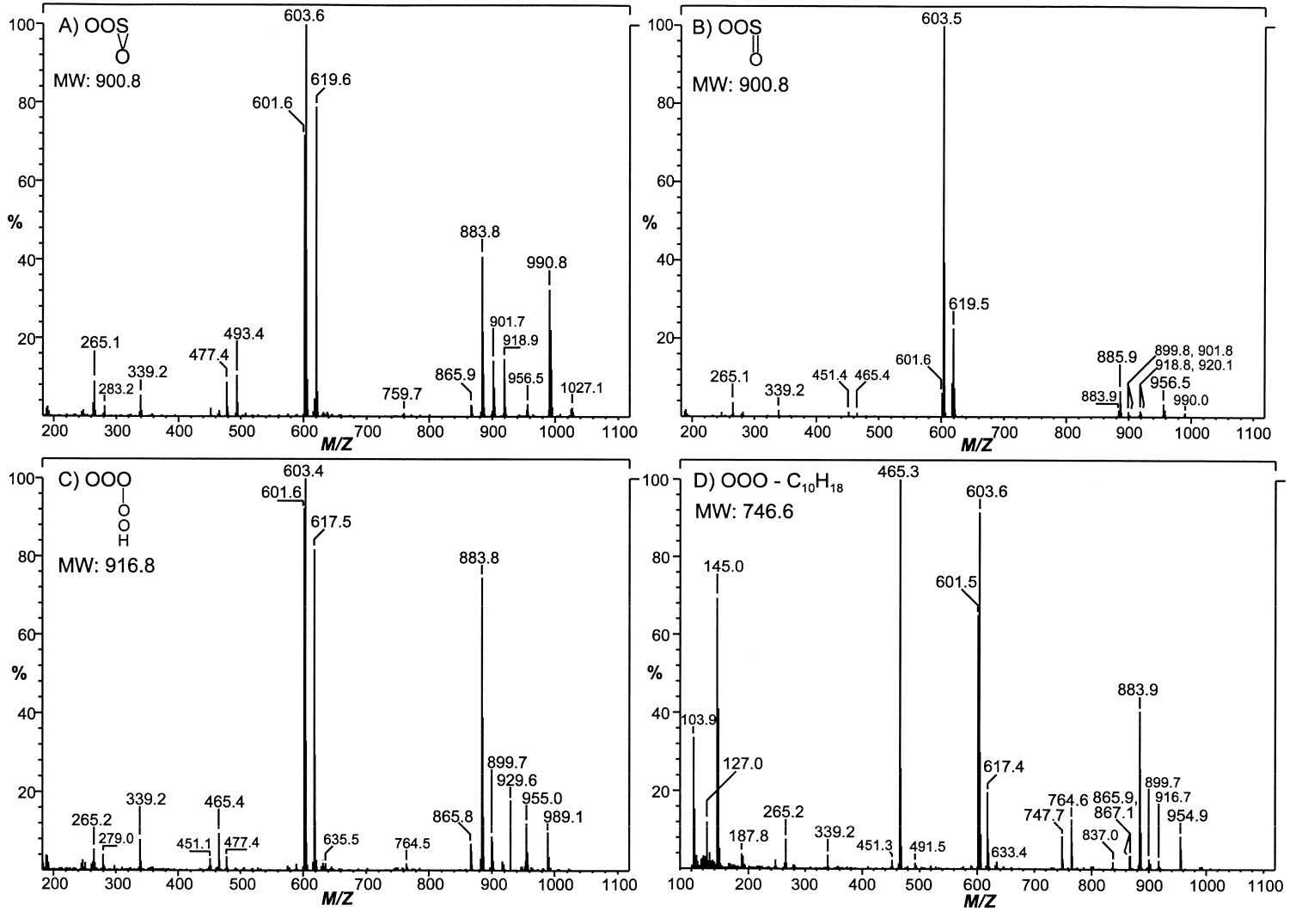

Fig. 2. APCI mass spectra of triacylglycerols in a triolein sample heated for 6 h. (A) Epoxy-triolein in which the epoxy was added across and existing double bond. (B) Keto-triolein in which the ketone oxygen was added at an existing site of unsaturation. (C) Hydroperoxy-triolein showing several isomers. (D) Chain-shortened triolein (overlapped with hydroperoxy-triolein).

ously, was identified as the mono-epoxide which formed nearby, not across, a triolein double bond, or OOO-epoxide. In the heated triolein products mixture studied here, the peak which eluted before the OOS-epoxide had a distinctly different mass spectrum, Fig. 2B, than that for the OOO-epoxide shown previously. The fragment at m/z 619.5 was in contrast to the primary fragment at m/z 617.5 expected for the OOO-epoxide. The distinct lack of abundant higher mass peaks also served to distinguish the mass spectrum in Fig. 2B from that given by OOO-epoxide. The peak at 619.5 in this Fig. indicated addition of an oxygen at the double bond location of triolein. A type of molecule which could give addition of an oxygen at the site of unsaturation is keto-triolein (actually dioleoyl,keto-stearoyl glycerol, since the ketone group formed by displacing a site of unsaturation in triolein). To assist in identification of the component giving the mass spectrum in Fig. 2B, keto-OOS was synthesized by the sodium methoxide-catalyzed interesterification of a mixture of 9- and 10-keto-stearate methyl ester with triolein. A partial chromatogram of the reaction products is shown in Fig. 3. As seen in this figure, the keto-OOS eluted before epoxy-OOS (which was also formed during the interesterification reaction) similar to the chromatogram in Fig. 1. Furthermore, the mass spectrum shown in Fig. 3B exhibited very small abundances of high-mass ions, similar to the mass spectrum in Fig. 2B. The primary difference the mass spectra in Figs. 3B and 2B is the relative abundances of the diacylglycerol fragments, though the masses are the same. One reason for this difference may be that different positional isomers give different relative proportions of diacylglycerol fragments [12,13]. The diacylglycerols (DAGs) re-

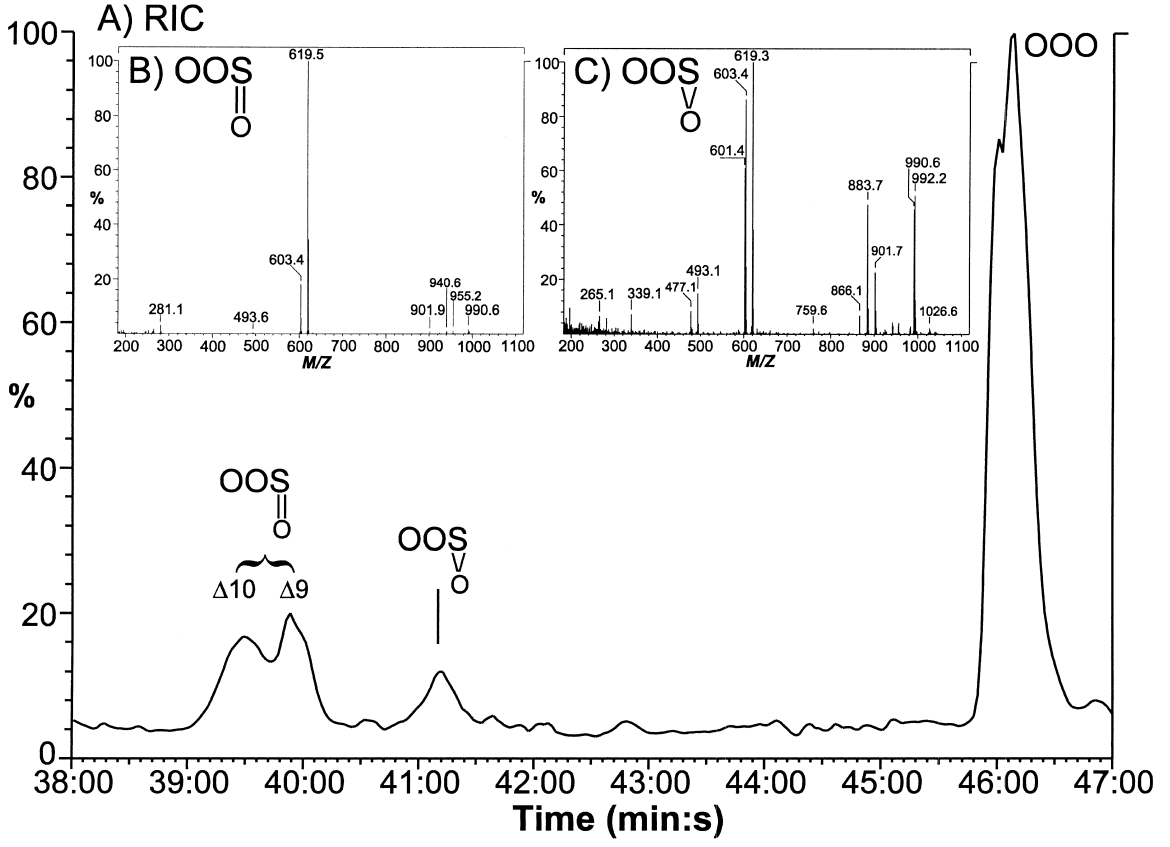

Fig. 3. (A) Partial chromatogram of products of the interesterification of keto-sterate with triolein. (B) Mass spectrum of dioleoyl,9-keto-stearoyl glycerol. (C) Mass spectrum of dioleoyl,9,10-epoxy stearoyl glycerol.

sulting from loss of the acyl chains in the *sn*-1 or -3 positions have higher relative abundances than the DAGs resulting from loss of the *sn*-2 acyl chain. The presence of different positional isomers may account for the differences in DAG abundances. Nevertheless, the masses, the lack of high mass ions and the retention time relative to triolein allowed us to make the tentative identification of keto-OOS. The mass spectrum shown in Fig. 3B is of the second half of the split peak in Fig. 3A. The fragment at 493.6, representing loss of C_9H_{18}, indicated that the Δ9 keto-OOS eluted in the second part of the peak, meaning that the Δ10 eluted in the first part. The mass spectrum of the first part of the peak was otherwise identical. The mass spectrum of epoxy-OOS, which was also formed as a by-product of the interesterification reaction, was nearly the same as that in Fig. 2A and shown previously. Since the keto-OOS occurred at nearly the same relative retention time as epoxy-OOO, in which the epoxy was not formed at the site of unsaturation, the mass spectrum was crucial to the identification of the keto-TAG. Use of a common two-dimensional detector could easily lead to misinterpretation based on retention behavior. Extracted ion chromatograms (EICs) indicated the likelihood that a small amount of the epoxy-OOO was also present, as would be expected by analogy to the autoxidation products, but because of the predominant overlapping keto-OOS, the mass spectra were not sufficiently unambiguous to make a definitive identification.

The mass spectrum of hydroperoxy-triolein in Fig. 2C was very similar to that presented previously for the hydroperoxy-containing TAG resulting from autoxidation, reported earlier. This mass spectrum, along with the relative retention time, allowed identification of this class of molecules. As was reported earlier, several isomers of hydroperoxy-triolein were also observed here. Normally, hydroperoxides are expected to be short-lived intermediates under elevated temperature frying conditions. It is possible that the hydroperoxides formed herein were 'trapped' during the cooling process as samples were removed from the heating bath and cooled to −15°C until the time of analysis.

The averaged mass spectrum at the front of the hydroperoxy peak exhibited some important differences to the latter portion of the peak. Further analysis using extracted ion chromatograms indicated that another class of molecules occurred partially overlapped with the hydroperoxy trioleins. There is a distinct 'bump' on the front of the hydroperoxy-OOO peak, which showed a strong fragment at *m*/*z* 465. This fragment is to be expected from the fragmentation of the stable epoxy intermediate which results from APCI ionization of hydroperoxy TAGs, as shown previously [9]. However, when extracted ion chromatograms of the autoxidation products were re-examined (data not shown), it was seen that all of the fragments described previously occurred at the same retention time, with only a little variation caused by the different hydroperoxy positional isomers. In the case of heated triolein oxidation products shown here, these same fragments were chromatographically separated by substantial retention times. This indicated that the products formed by chain fragmentation in the APCI source during ionization were the same as those formed as distinct intact TAGs during degradation of triolein upon heating. These TAGs represent chain-breakage products formed from the loss of volatile short chain fragments. Therefore, in the mass spectrum of the chain-breakage product shown in Fig. 2D, a protonated molecular ion, at *m*/*z* 747.7, and an acylium ion, RCO^+, at *m*/*z* 127.0, were present which were not present when the fragments were simply formed in the APCI source. This distinction is further clarified in Fig. 4, which shows extracted ion chromatograms for several components tentatively identified as chain-breakage products found in the heated trilolein mixture. The masses 451.3, 465.3, 479.4 and 493.4 represent the same species shown earlier as fragments formed from chain cleavage of the stable epoxy intermediate, but in Fig. 4 distinct chromatographically separated peaks are observed in contrast to the simultaneous appearance of all of these fragments which occurs during APCI ionization. Furthermore, peaks in the EICs of *m*/*z* 747.7 and *m*/*z* 127.0 show concurrent peaks representing the protonated molecular ion and the acylium ion, respectively, of the chain-shortened TAG caused by loss of $C_{10}H_{18}$. The EIC of *m*/*z* 493.4 shows only a barely distinguishable peak at the position chromatographically expected for the intact chain-shortened TAG, while at slightly longer retention time, this ion shows a strong peak at the location of epoxy-OOS,

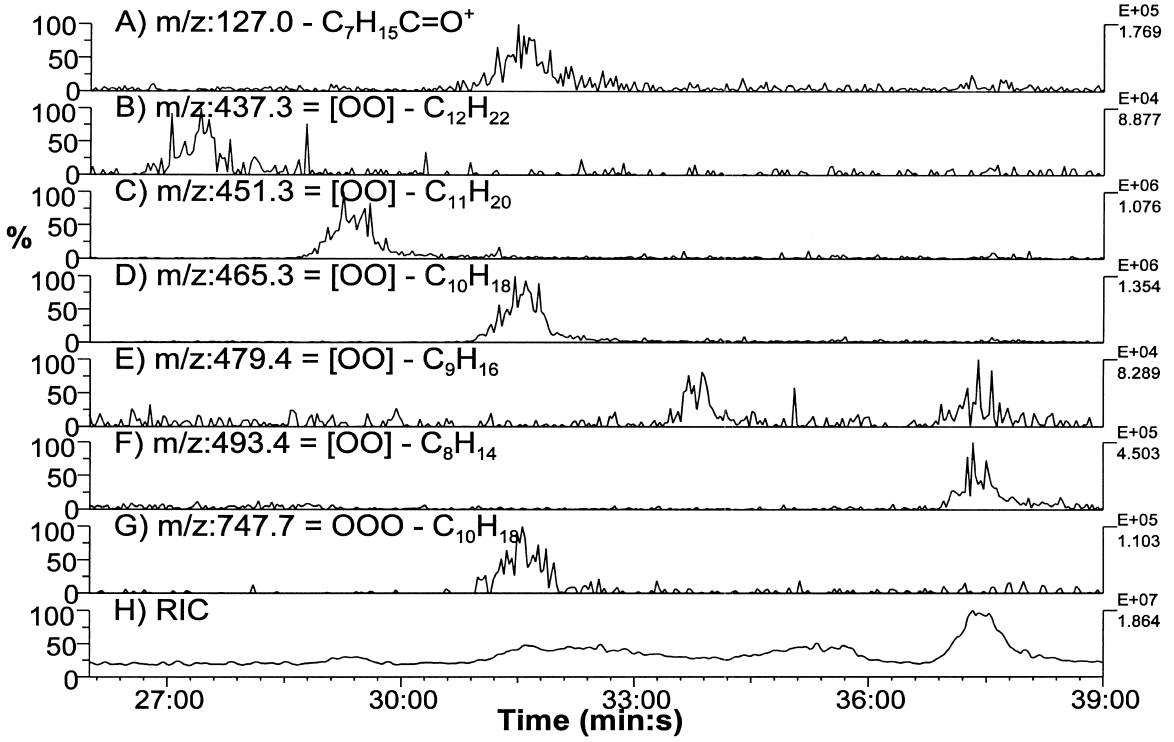

Fig. 4. Extracted ion chromatograms of masses corresponding to chain-shortened triolein species. (A) Acylium ion, RCO^+, of oleic acid chain with loss of $C_{10}H_{18}$, (B) dioleoyl fragment with further loss of $C_{12}H_{22}$, (C) dioleoyl fragment minus $C_{11}H_{20}$, (D) Dioleoyl fragment minus $C_{10}H_{18}$, (E) dioleoyl fragment minus C_9H_{16}, (F) dioleoyl fragment minus C_8H_{14} formed from epoxide during APCI, (G) protonated molecular ion of the chain-shortened triacylglycerol equal to triolein minus $C_{10}H_{18}$, (H) reconstructed (or total) ion chromatogram (RIC).

from which this ion is formed as a fragment in the APCI source. The EIC for m/z 479.4 shows peaks both at the location of the intact chain-shortened TAG and at the location where epoxy-OOS elutes. These ions may be seen in the mass spectrum of epoxy-OOS in Fig. 2A. The peak between 29 and 30 min in Fig. 1 and the RIC in Fig. 4 can be identified as the chain-shortened TAG resulting from loss of $C_{11}H_{20}$. Concurrent peaks in the EICs of m/z 113.0 and 734.6 (not shown) represent the acylium ion and protonated molecular ion of the TAG giving the DAG fragment at m/z 451.3 in Fig. 4C. Although the shortened DAG fragment and acylium ion fragment did not contain an oxygen, it is believed that the chain-shortened TAG resulted from oxidized species which lost the oxygen in the leaving chain fragment, giving rise to a volatile alkanal or similar species. The peak at m/z 764.6 in Fig. 2D was chromatographically concurrent with the peak at m/z 747.7 in Fig. 4. This appears to be an $[M+18]^+$ adduct of the intact chain-shortened TAG formed in the APCI source, as was common for autoxidation products and other TAGs reported earlier.

3.3. Diacylglycerols and chain-shortened oxidation products

The peaks appearing at shorter retention time, from ~17 to 21 min, are shown in the partial RIC and EICs shown in Fig. 5. The largest peak in this region arose from dioleoylglycerol, the intact diacylglycerol formed from loss of a fatty acyl chain of triolein. Dioleoyl glycerol, or diolein, produced the large peak in the EIC of m/z 603.5. Mass spectra such as those shown in Fig. 7A and B showed lower mass fragments, which might be mistaken for diacylglycerols of chain-shortened TAGs. However, these fragments were produced from species which

eluted with longer retention times than dioleoylglycerol, indicating that they are not normal or oxygenated diacylglycerols, which would elute before diolein. Although, small peaks corresponding to the normal chain-shortened diacylglycerols did also appear at shorter retention times than that of diolein. The mass spectra of those species eluted just after diolein, shown in Fig. 5A and B, also showed higher mass peaks accompanying the lower mass diacylglycerol peaks. EICs of some of these higher mass peaks indicate that they appear concurrently with the various diacylglycerol ions. For instance, concurrent with the peak in the EIC for m/z 505 between 19 and 20 min was a peak in the EIC for m/z 787.5. Also, peaks in the mass spectra at and near m/z 617.5 and m/z 633.5 tend to indicate the presence of oxygenated acyl chains in addition to the chain shortened diacylglycerol fragments. Just as the oxygenated triolein species eluted before normal triolein on these reversed-phase columns, so, too, would oxygenated chain-shortened TAGs be expected to elute before their non-oxygenated chain shortened analogs shown in Fig. 4. The presence of chain-shortened triolein TAGs having additional oxygen-containing functional groups which fragment to give chain-shortened diacylglycerol fragments could explain the retention behavior of the shortened DAGs in Fig. 5 which eluted near and after intact diolein. The mass spectra of these overlapped species are sufficiently complex that this set of peaks will require further investigation using tandem mass spectrometry before conclusive assignments may be made. Their presence would arise from molecules containing more than one site of oxidation, with chain shortening occurring at one site, leaving the other site intact.

Although we were able to identify several classes of oxidation products which were present, some of which were the same as those produced by autoxidation, it is highly likely that other oxidation products which eluted before triolein are present which were not identified. It is possible that almost every combination of aldehyde-, epoxy-, and hydroperoxy-containing TAGs are present at some level, in addition to the chain-shortened products in combination with these other functional groups. The chromatographic overlap of co-eluting species, and the appearance of the same masses resulting from APCI fragmentation as from intact chain-shortened and oxygenated species means that some species present at low levels may go unidentified. Nevertheless, the primary components in the chromatogram in Fig. 1 have been identified, and the APCI method has thus far been able to identify more oxidation products than are

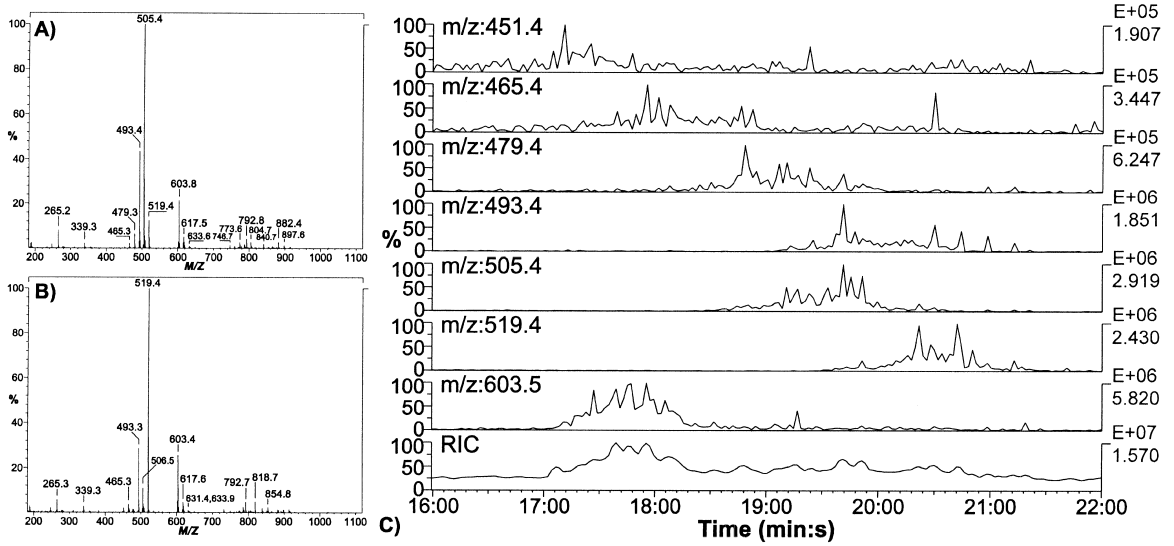

Fig. 5. (A) Mass spectrum of peak eluted around $19\frac{1}{2}$ to 20 min. (B) Mass spectrum of peak eluted around $20\frac{1}{4}$ to $20\frac{3}{4}$ min. (C) Extracted ion (EICCs) chromatograms of diacylglycerol fragments seen in mass spectra of components eluted in the region from 17 to 21 min.

possible to identify using a two-dimensional detector.

3.4. Dimers and addition products

In addition to the oxygenated and chain-shortened triolein species, a large number of addition products which may be formed by linkage of acyl chains to oxygen-containing functional groups, and also by polymerization, are possible. As mentioned above, and shown in Fig. 1, the products which eluted after triolein were not efficiently ionized during the normal chromatographic run. Therefore, the late-eluted species were collected from preparative RP-HPLC. Samples were combined from several preparative RP-HPLC runs to provide samples which were enriched in the late-eluting species. These enriched samples were then separated using the same analytical RP-HPLC gradient run which was used for the data presented in the preceding sections. A partial chromatogram of the separation of the enriched sample is shown in Fig. 6. Distinct differences can be seen between the responses of the evaporative light scattering detector and the ion chromatogram obtained by APCI–MS. Peaks marked (a.) and (b.) in Fig. 6B showed more response by APCI than did the peaks marked (c.), (d.) and (e.), which showed stronger response by ELSD. The sharpness of the peaks marked (a.) and (b.) indicated that these were specific molecular species, while the broader peaks were mixtures of homologous species.

Mass spectra of the labeled peaks are shown in Fig. 7. Fig. 7A corresponds to the peak labeled (a.) in Fig. 6B. This mass spectrum shows primary diacylglycerol fragments at m/z 603.6 and 703.7. The addition of 100 u to the peak at m/z 603.7 to produce the peak at m/z 703.7 was accompanied by addition of 100 u to the protonated molecular ion, normally at m/z 885.9, to produce a new protonated molecular ion at m/z 985.9, see Fig. 7A. Two

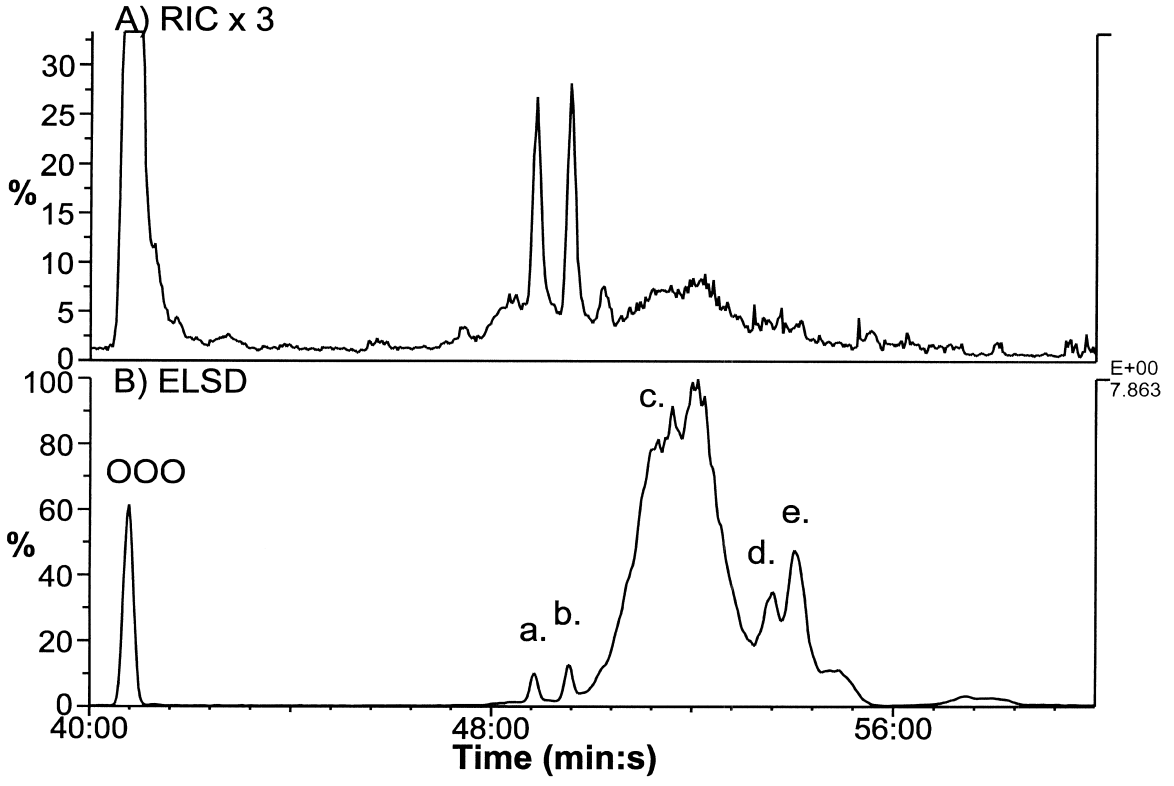

Fig. 6. (A) Reconstructed ion chromatogram and (B) evaporative light scattering detection chromatogram of components eluted after triolien by reversed-phase HPLC.

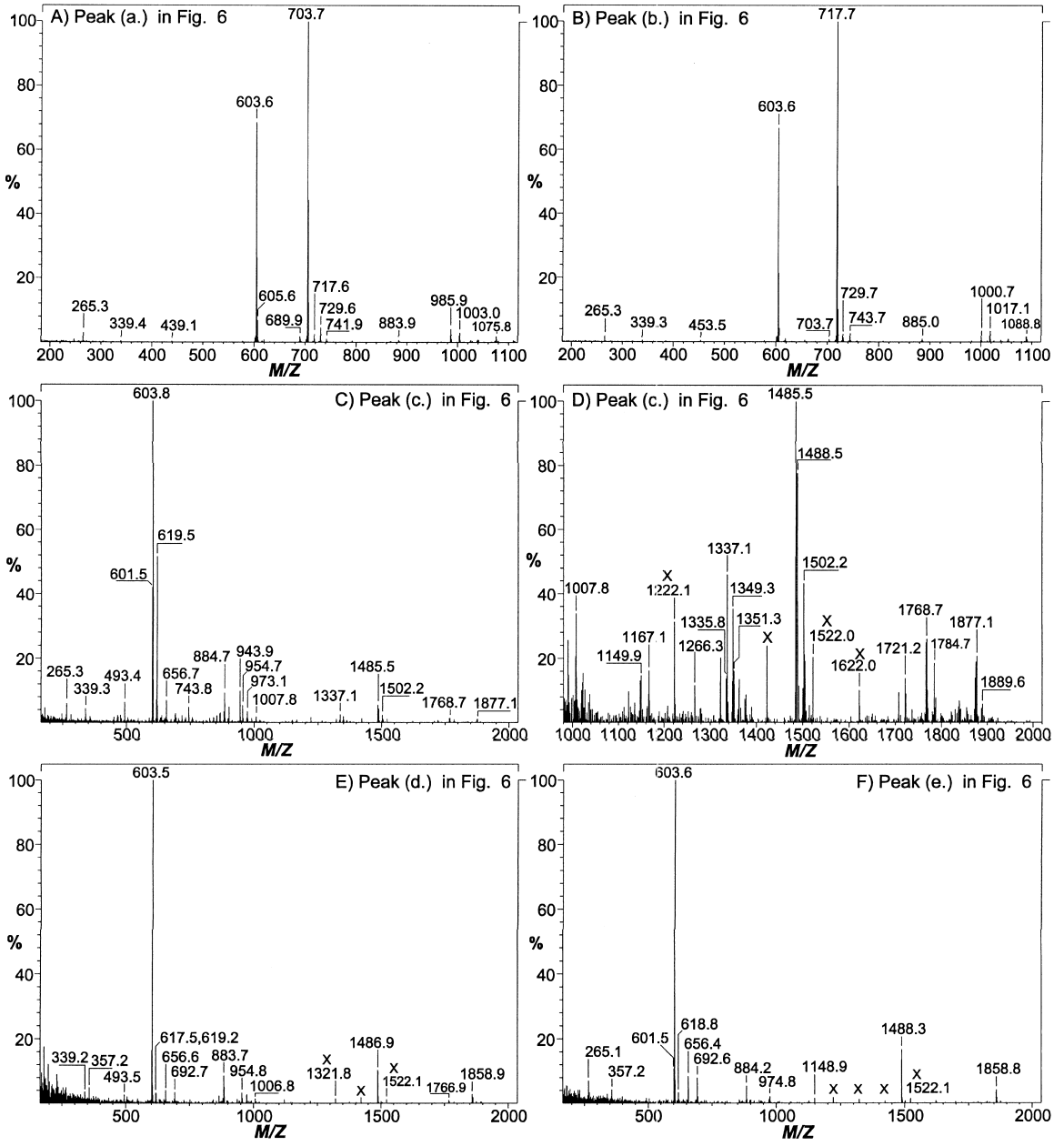

Fig. 7. APCI mass spectra of heated triolein addition products eluted after triolein by reversed-phase HPLC. (A) Ether-linked addition product corresponding to peak (a) in Fig. 8, (B) ether-linked addition product corresponding to peak (b) in Fig. 8, (C and D) full mass spectrum and high mass region of peak (c) in Fig. 8, (E) mass spectrum of peak (d) in Fig. 8, and (F) mass spectrum of peak (e) in Fig. 8. X=Masses arising from UltraMark 1621 tuning compound.

products are likely which would produce an increase of 100 u: (1) an ether-linked addition product which is equivalent to addition of $-OC_6H_{13}$ to normal triolein or (2) an ester-linked addition product (forming an estolide) which is equivalent to addition of $-OCOC_4H_9$ to normal triolein. In either case, the

added functional group would be in addition to, not replacing, the existing double bonds. Without comparison to the mass spectra of authentic standards of these two classes of molecules, which are not available, we cannot definitively determine which of these two possible classes of molecules are responsible for the peaks in Fig. 6. However, given that chain-shortened acyl chains were observed (described above) which could give rise to free short-chain aldehydes, and given that such groups have been demonstrated to form addition products (adducts) with epoxy-containing TAGs of *Vernonia galamensis* seed oil [14], there appears to be supporting evidence for the presence of the ether-linked addition products. Of course, the epoxy-containing TAGs previously reported for *Vernonia galamensis* formed adducts with masses increased by 102 u instead of 100 u because these adducts were formed either by ring opening of the epoxy group or by addition at a double bond, either of which required the TAG acyl chain to pick up one hydrogen and the hexanal to pick up one hydrogen to satisfy the requirements of the neighboring carbon atoms. These two additional hydrogens caused the increase of 2 u compared to the results seen here. If the chain addition products seen here were added at a site of unsaturation, the mass increase would similarly have been 102 u. It is to be remembered that the adducts described previously were formed in the APCI source, while the addition products described here were stable, chromatographically resolved species. Of course, as has been mentioned above, the pathways of fragment formation during atmospheric pressure chemical ionization have been demonstrated to lead to the same products as were formed by either autoxidation or by oxidation under model frying conditions.

Several of these short-chain addition products were observed. The mass spectrum shown in Fig. 7B shows masses corresponding to addition of a chain having a mass of 114 u, corresponding to peak (b.) in Fig. 6B. Although the peaks arising from the addition products of 100 u and 114 u were the largest of these products in Fig. 6, other species were also present. The extracted ion chromatograms in Fig. 8 show mass chromatograms for diacylglycerol fragment ions for addition products having mass increases of 72 u, 86 u, 100 u, 114 u and 128 u. Extracted ion chromatograms are also shown for the protonated molecular ions having masses of 985.7 and 999.7, corresponding to the two primary addition products of 100 u and 114 u, respectively.

The remainder of the mass spectra in Fig. 7 are for molecules representing dimers and related products of triolein. Fig. 7C represents an average mass spectrum of all components eluted across the peak labeled (c.) in Fig. 6. Fig. 7D represents only the high mass region of the averaged mass spectrum shown in Fig. 7C. Several ranges of masses are apparent which each contain a group of related fragments. The peak at m/z 1768.7 corresponds to the mass of the dimer formed by combination of two triolein molecules, minus two hydrogens at the linkage site, or $[(2M-2H)+H]^+$, where M is a triolein molecule. A peak representing this same dimer with two fewer hydrogens is also visible. A dimer containing an additional oxygen atom, $[(2M-2H+O)+H]^+$, gave rise to the peak at m/z 1784.7. This indicates that dimerization may have occurred through both carbon-to-carbon bonds and carbon–oxygen–carbon linkages, or that linkages may be formed with molecules which also contained oxygen functional groups at other locations (though this would be expected to lead to different retention times). These did not have sufficiently different polarities to be chromatographically resolved. The carbon-to-carbon dimer seemed to predominate, so the following discussion will use masses for these species, though it is understood that masses corresponding to the carbon–oxygen–carbon dimers were also present. Dimers having the carbon–oxygen–carbon linkage are also included in discussion of non-oxygenated dimers, because 'oxygenated dimers' is used below to refer to polar functional groups occurring in the middle of an acyl chain, such as epoxides, ketones and other oxygen functional groups.

The primary fragments arising from the dimers appear around m/z 1485.5. These represent loss of one acyl chain, RCOO, from the intact dimer. The peak at m/z 1485.5 specifically represents loss of an acyl chain plus loss of two additional hydrogens from the dimer, or $[(2M-4H-RCOO)+H]^+$. The peaks around m/z 1502 appear to represent loss of an acyl chain from the oxygen-linked dimer, $[(2M-4H+O-RCOO)+H]^+$. The peaks at 100 u intervals

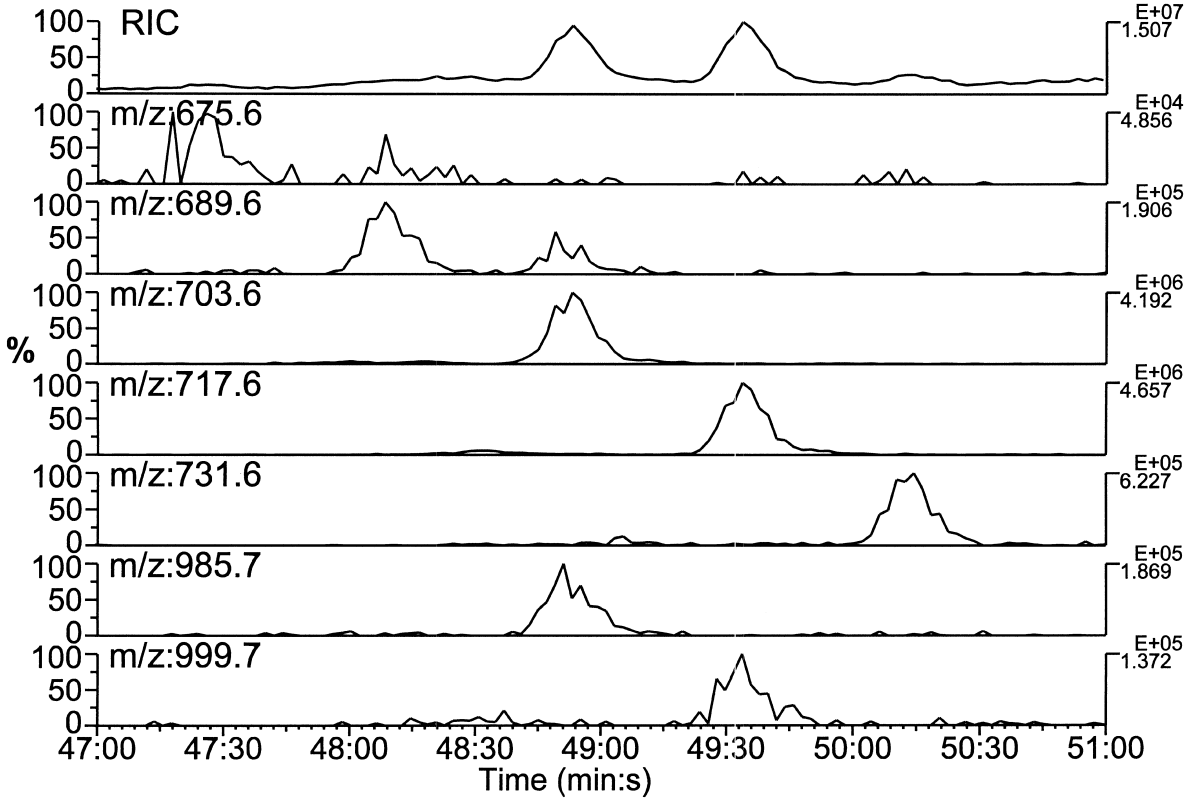

Fig. 8. Reconstructed ion chromatogram and extracted ion chromatograms of chain-branched addition products of heated triolein.

starting at m/z 1122 arose from Ultramark 1621 calibration compound which could not be eliminated from the system after tuning, and are not related to the triolein dimers. Extracted ion chromatograms of several of the mass regions discussed above and shown in Fig. 7C and D are shown in Fig. 9. The width of the peak given by the mass region of the 'dimer minus RCOO' fragment, from ~m/z 1484 to 1492, indicated that this fragment arose from numerous species across the breadth of the whole peak labeled (c.) in Fig. 8, as well as from the peaks labeled (e.) and (f.) in Fig. 8. The peak labeled (c.) contained different classes of species which coeluted. Across the major part of the peak, masses corresponding to the 'dimer protonated molecular ion', from ~m/z 1764 to 1772, eluted.

The latter part of the (c.) peak appeared to correspond to oxygenated chain-addition products of dimers, giving the protonated chain addition molecular ions in the range m/z 1870 to 1880 and the corresponding oxygenated fragment ion pattern in the range m/z 1332 to 1340 shown in Fig. 9. This class of molecules gave the full set of oxygenated fragments in the range m/z 1332 to 1400 seen in Fig. 7D. The peak sets around m/z 1337 which are separated by 14 u resemble the peak patterns given by oxygen functional group-containing acyl chains which fragment at the site of oxygenation, as seen in Fig. 2 and as shown previously for autoxidation products. Thus, these peaks most likely represent oxygenated, chain-addition dimer products after loss of one acyl chain, RCOO, followed by fragmentation at a site of oxygenation on a remaining acyl chain. As with other oxidation products, several isomers were possible corresponding to oxygenation at different positions in the acyl chain, giving rise to a set of fragments separated by 14 u. The peaks around m/z 493 which exhibit similar intervals between fragments represent the corresponding oxygenated diacylglycerol fragments fragmented at sites of oxygen-

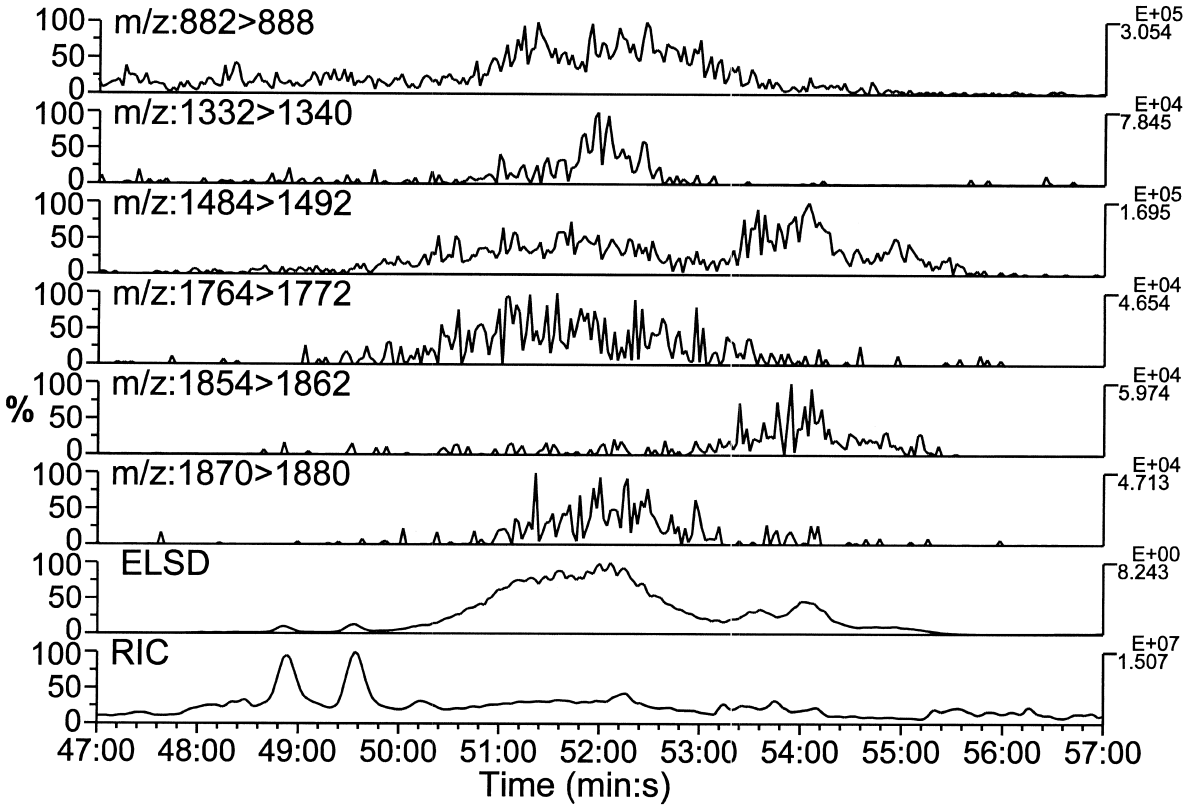

Fig. 9. Reconstructed ion chromatogram, evaporative light scattering detector chromatogram and extracted ion chromatograms of dimers and other addition products of heated triolein separated using reversed-phase HPLC.

ation, as in other mass spectra of oxidation products shown above and previously. This lower mass range also produced a mass chromatogram (not shown) with a peak which coeluted with the higher mass oxygenated fragments. Sets of peaks representing fragments from numerous isomers were also observed near the normal triolein molecular weight of 884.7. Because of the large number of oxygenated species possible, as demonstrated above for oxygenated normal triolein and demonstrated previously for autoxidation products, we cannot differentiate the variety of oxygenated dimer addition-product species which coeluted in the latter portion of peak (c.). However, we have demonstrated the ability to exceed the capabilities of two-dimensional detectors for direct identification of the types of molecules formed by model frying conditions and have directly detected and tentatively identified more oxidation products in a single chromatographic run than was previously possible.

The fragment representing the dimer minus one acyl chain (m/z 1484>1490) also arose from species eluted after the normal dimers and oxygenated dimer addition products discussed above. These other species, having the mass spectra shown in Fig. 7E and F, had higher masses, in the range m/z 1854 to 1862, than dimers, but slightly lower than the oxygenated chain-addition products. The lower masses of the species in Fig. 7E and F, compared to the higher mass species eluted at the end of peak (c.), and their later retention times leads us to suggest that these compounds may be non-oxygenated dimer addition products. These would be analogous to the chain addition products shown in Fig. 7A and B, but with the chain additions occurring on intact dimers. The reason that these are believed not to be oxy-

genated, so not having exposed polar functional groups, is that very similar species having slightly higher masses elute just before these dimer chain-addition products. This tentative identification would agree with the retention and mass trends of the other classes of compounds in which the oxygen functional group-containing species had slightly higher masses and eluted just before the less polar, non-oxygen-functional-group-containing species, as demonstrated by the oxygenated triolein products eluted before normal triolein, the oxygenated chain-shortened products eluted before the normal chain-shortened products, and the oxygenated diacylglycerols eluted before the normal diacylglycerol.

Because of the complexity of the mass spectra of the high mass species, complete characterization of all classes has not been possible. The remaining ambiguity highlights the need for further method development with the goal of better resolution of overlapped species eluted after the normal TAG. Previously, we have been successful at optimizing a gradient run to spread out the oxidation products eluted before normal TAGs so these were identified quite effectively as separate classes. This same progress needs to be made in the area of oxygenated and non-oxygenated dimers and dimer addition products. Nevertheless, the method described here has demonstrated the capability for the direct online detection of species which have not previously been identified, even using labor-intensive, time-consuming methods. Polymerization has always been discussed in terms of the dimers and trimers, etc., with little recognition of the other addition products which have been identified during this study. TAG addition products seem to represent many combinations of TAGs with TAG fragments and acyl chain subunits, both oxygenated and non-oxygenated. Although several addition products have been tentatively identified which have not been previously reported, the RP-HPLC/MS method did not produce ions from trimers and higher molecular weight species. These either did not elute under the conditions used or were not efficiently ionized in the APCI source. Still, the method shown here has plainly demonstrated the shortcomings of relying solely on a size exclusion chromatography separations.

The data given herein provides useful insight into the mechanisms of the formation of volatile oxidation products. Mass spectra such as those in Fig. 2 and Fig. 7C and 7D, as well as those shown previously for autoxidation products, show the non-volatile decomposition products left in the oil when numerous oxygenated positional isomers break down and lose volatile pieces from the intact TAGs and TAG addition products. These volatiles are usually detected using GC with flame ionization or mass spectrometric detection. In addition, the ionization exhibited in the APCI source appears to follow the same pathways as are followed during decomposition in the oil itself. It has been shown that the same molecules were formed as distinct species in the oil, and separated chromatographically, as were formed during ionization in the APCI source.

Although we have directly detected and tentatively identified many species which previously would have required fractionation, derivatization and then analysis, we believe there are still other species present which have not been directly identified. Cyclic fatty acids are an example of a class of compounds which has been shown to be present in heated oils at very low levels, but which we have not yet directly identified. However, one must keep in perspective the degree of information provided for the components and classes in the largest chromatographic peaks observed here compared to previous methods of analysis and compared to labor-intensive classical chemical methods of analysis. Though the demonstrated method is not yet the single, all inclusive methodology which identifies all heated oil products, the methodology demonstrated here does represent a significant step forward in the ability to identify many components in the largest chromatographic peaks resulting from the highly complex mixture obtained from heated frying oil.

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