Analysis of Nonvolatile Lipid Oxidation Products in Vegetable Oils by Normal-Phase High-Performance Liquid Chromatography with Mass Spectrometric Detection[1]

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ABSTRACT: Nonvolatile triacylglyceride (TAG) oxidation products play an important role in the oxidative degradation of lipids. They serve as a reservoir of oxygen-containing species and hence can act as off-flavor precursors or as initiators for further oxidation reactions. Possible nonvolatile lipid oxidation products are TAG with a hydroperoxy, hydroxy, epoxy, or oxo (ketone or aldehyde) group or combination of these groups. The breakdown of TAG hydroperoxides yields nonvolatile glyceride species with two intact fatty acid chains and one short chain mostly ending in an aldehyde or hydroxy group (2½ glycerides). By means of normal-phase high-performance liquid chromatography (HPLC) with mass spectrometric (MS) detection, nonvolatile lipid oxidation products can be separated according to polarity. This results in separation into classes of TAG oxidation products, such as epoxy-TAG, oxo-TAG, hydroperoxy-TAG, hydroxy-TAG and 2½ glycerides, which can be identified using selected ion chromatograms. The retention times of TAG oxidation products on the normal-phase HPLC system and the signal intensity of the MS detector are stable enough to enable quantitative analysis based on external calibration. The normal-phase HPLC–MS method is very suitable for the characterization and quantitation of nonvolatile TAG oxidation products in oxidized TAG reference compounds as well as in real oils or oil phases isolated from emulsions, spreads, or other fat- based food products. This method can give detailed information for the study of lipid oxidation mechanisms.

Paper no. J9480 in *JAOCS 77*, 837–845 (August 2000).

KEY WORDS: Autoxidation, HPLC–MS, lipid oxidation, nonvolatile lipid oxidation products, NP-HPLC, triacylglyceride hydroperoxides.

During recent years the consumption of healthy vegetable oils, i.e., oils containing considerable levels of polyunsaturated lipids, has increased significantly. With that, the oxidation of polyunsaturated lipids has also received great attention. Polyunsaturated lipids are much more susceptible to oxidation than saturated and monounsaturated triacylglycerides (TAG). Following a free radical chain mechanism, TAG hydroperoxides (OOH-TAG) are formed as primary products of TAG oxidation. It is well established that OOH-TAG readily decompose into a wide range of volatile components, encompassing off-flavors, as well as nonvolatile lipid oxidation products (NONVOLLOP). The increasing importance of polyunsaturated lipids calls for a better understanding of the mechanism of lipid oxidation in order to be able to develop technologies to prevent the formation of off-flavors in vegetable oil-based products. Reliable, detailed analytical methods are key to understanding the oxidation processes active in such products.

Volatile lipid oxidation products, many of which are potent off-flavors, have been intensively studied due to their direct impact on the keeping quality of many food products (1–3). Nonvolatile TAG oxidation products, although not perceived, can play an important role in the oxidative degradation of lipids. They serve as a reservoir of oxygen-containing species and hence can act as off-flavor precursors or as initiators for further oxidation reactions. Despite the importance of the NONVOLLOP, there currently is no analytical method available that can provide detailed structural and quantitative information on NONVOLLOP in real oil samples.

Analytical studies of lipid oxidation have so far focused almost exclusively on the volatile products formed (4–6). The complexity of nonvolatile TAG oxidation products is far beyond that of the already highly complex volatiles. This renders the analytical assessment of the products a difficult task. In the lipid oxidation process, numerous reaction routes are possible even for a single unsaturated TAG. Nonvolatile TAG oxidation products hence exist in a huge variety of species belonging to several compound classes. Oxidized TAG can contain one or more hydroperoxy, oxo (ketone or aldehyde functionality), hydroxy, and epoxy groups or combinations of them.

Owing to the high molecular weight and the limited thermal stability of NONVOLLOP, gas chromatography, which is the preferred tool for the analysis of volatile compounds, is not an appropriate technique for the analysis of the nonvolatile products. High-performance liquid chromatography (HPLC), on the other hand, can be used to separate intact NONVOLLOP (7,8).

As most of these NONVOLLOP do not have specific chromophores or fluorophors, a nonselective universal detection

[1]Presented at the 15th Montreux Symposium on Liquid Chromatography/Mass Spectrometry, Montreux, Switzerland, November 9–10, 1998.

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technique has to be applied. Selective detection is only possible for the TAG hydroperoxides and hydroxides that contain conjugated double bonds (8). These species have a specific ultraviolet (UV) absorption band at 235 nm.

NONVOLLOP can be easily detected by means of mass spectrometric (MS) detection. MS detection provides universal and very sensitive detection, which is of great importance in real oils, because NONVOLLOP are usually present at low concentrations. In addition to this, MS detection has the advantage that it provides structural information about the analytes detected. This greatly facilitates the interpretation of the complex chromatograms obtained.

Ravandi *et al.* (9) analyzed synthetic lipid ester ozonides and core aldehydes, or oxo-2½ glycerides in our nomenclature, by reversed-phase HPLC with negative thermospray MS and normal-phase HPLC (NP-HPLC) with positive electrospray. By analyzing oxidized synthetic TAG, Sjövall *et al.* (10) could establish relative elution times of some TAG oxidation products in a reversed-phase HPLC system. More recently, model TAG autoxidation products were characterized by Neff and Byrdwell (11) *via* reversed-phase HPLC coupled with atmospheric pressure chemical ionization MS. When NONVOLLOP are analyzed by reversed-phase HPLC, the various species present are separated according to a combination of class [OOH-TAG, hydroxy-TAG (OH-TAG), epoxy-TAG, etc.] and size (e.g., OOH-LLL, OOH-LOL, OOH-OOL, etc., where L is linoleic acid and O is oleic acid) (8). For the analysis of oxidation products originating from one parent TAG, this yields an excellent separation. If, however, NONVOLLOP are analyzed in oxidized TAG mixtures or real oils, the additional size separation results in highly complex chromatograms, which are difficult to interpret. If a separation of compounds according to class or polarity is desired, NP-HPLC is the logical choice.

In this paper the analysis of nonvolatile TAG oxidation products is described. An NP-HPLC method was developed that allowed separation of the different groups of oxidation products formed in model oxidation studies as well as in real oils. MS detection was used to identify the various classes of products present. The quantitative performance of the method was evaluated. Finally, the method was applied to the quantitative analysis of lipid oxidation products in fat-based products.

EXPERIMENTAL PROCEDURES

Chemicals and reagents. Chromatography-grade *n*-hexane, methyl *tert*-butyl ether (MTBE), isopropyl alcohol (IPA), methanol, and ethanol were obtained from Merck BV (Amsterdam, The Netherlands). The TAG 1,3-dipalmitoyl-2-linoleoyl-glycerol (PLP),1-stearoyl-2,3-diricinoleoyl-glycerol (SRR), and triricinoleoyl-glycerol (RRR) and the nonvolatile TAG oxidation products 1-palmitoyl-2-(13-hydroperoxy-linoleoyl)-3-stearoyl-glycerol [P(13-OOH-L)S], 1-palmitoyl-2-(12-oxo-stearoyl)-3-stearoyl-glycerol P(12-oxo-S)S, 1-stearoyl-2-stearoyl-3-(epoxy-stearoyl)-glycerol [(epoxy-S)SS] and 1-palmitoyl-2-palmitoyl-3-(9-oxo)-nonanoyl-glycerol [PP(9:0,oxo)] were synthesized in-house. The OOH-TAG were synthesized according to the procedure published previously (8). The other oxidized reference TAG were also made *via* lipoxygenase from fatty acid oxidized prior to the linkage to the diacylglyceride. Starting fatty acids were oleic, ricinoleic, and 9-hydroxynonanoic acid (Acros Chimica, ’s Hertogenbosch, The Netherlands). The oleic acid was converted into the corresponding oxo and epoxy fatty acids. During the coupling to the desired diacylglyceride the oxidized reference TAG were formed. The purity of these compounds as established by ^{1}H nuclear magnetic resonance (NMR) was between 95 and 99%. Rapeseed oil and the linseed/safflower oil were obtained from Mildona oil mill (Kirkkonummi, Finland). These oils were analyzed as received and were not subjected to artificial oxidation. To improve the ionization of the components in MS detection, a sodium iodide solution (Merck) was added to the eluant.

Preparation of calibration and sample solutions. External calibration with the reference compounds P(13-OOH-L)S, P(12-oxo-S)S, (epoxy-S)SS, PP(9:0,oxo), SRR, and RRR was used for quantitative analysis. The concentrations of the reference compounds in the calibration solutions ranged from 0.02 to 100 μg/mL in *n*-hexane. For P(12-oxo-S)S, (epoxy-S)SS, PP(9:0,oxo), SRR, and RRR, solutions containing all five compounds could be prepared, as these compounds are sufficiently separated on the HPLC column. For P(13-OOH-L)S, a separate series of calibration solutions was prepared because this compound coelutes with PP(9:0,oxo). Moreover, the OOH-TAG is less stable in solution, and fresh solutions have to be prepared frequently.

For the analysis of oxidized TAG reference compounds and oxidized oil samples, the concentrations of the sample solutions were adjusted in such a way that the individual NONVOLLOP had concentrations between 0.02 and 100 μg/mL.

HPLC–MS. All analyses were performed on an HP1100 LC-MSD (Hewlett-Packard, Palo Alto, CA). The HPLC system consisted of a binary high-pressure gradient pump, an auto injector, a thermostated column, and a diode-array detector (DAD). Downstream of the DAD, 0.15 mM sodium iodide (NaI) in ethanol/methanol (1:1, vol/vol; flow 100 μL/min) was added to the eluant flow by means of an HPLC pump (Gilson 305, Villier-le-Bel, France) and a low-dead-volume T-piece.

During the optimization of the HPLC separation, three columns were evaluated, a Waters S3W column (150 × 2.1 mm, particle diameter (dp) = 3 μm; Waters Chromatography B.V., Etten-Leur, The Netherlands), a Waters Diol column (200 × 3 mm, dp = 5 μm), and a Waters S5W column (250 × 2.1 mm, dp = 5 μm). The DAD detector was operated at 235 nm with a bandwidth of 8 nm. Different mobile phase gradients starting with pure *n*-hexane to mixtures of *n*-hexane/MTBE or *n*-hexane/IPA were evaluated. The analytes were eluted at a flow rate of 300 μL/min. The injection volume was 5 μL.

The MS was operated in the full-scan mode (mass range 650–1100 amu) using the orthogonal positive electrospray source. The scan cycle time was 1.95 s/cycle. The operating

capillary voltage was 3500 V, the fragmentor voltage 80 V. The nebulizer pressure was kept at 20 psig, the gas temperature at 350°C, and the drying gas flow at 3 L/min.

Between 2 and 6 min after HPLC injection, the capillary voltage was set to zero in order to prevent the nonoxidized TAG, which represented the bulk of the sample, from entering the ion source. In this way, the nonoxidized TAG were directed to a waste container. By doing so, the cleaning and maintenance effort of the ion source was kept to a minimum.

The equipment was controlled by Chemstation software, revision number 5.02 (Hewlett-Packard).

RESULTS AND DISCUSSION

Nonvolatile TAG oxidation products accumulate in the fat phase and can provide an indication of the history of oxidation for the sample. The first step in lipid oxidation is the formation of a hydroperoxide. Degradation of OOH-TAG then yields nonvolatile glyceride species with two intact fatty acid chains and one short chain, mostly containing an aldehyde or hydroxy group, which we call two-and-a-half glycerides (2½ glycerides). The molecular structures of the most important classes of nonvolatile TAG oxidation products are shown in Scheme 1.

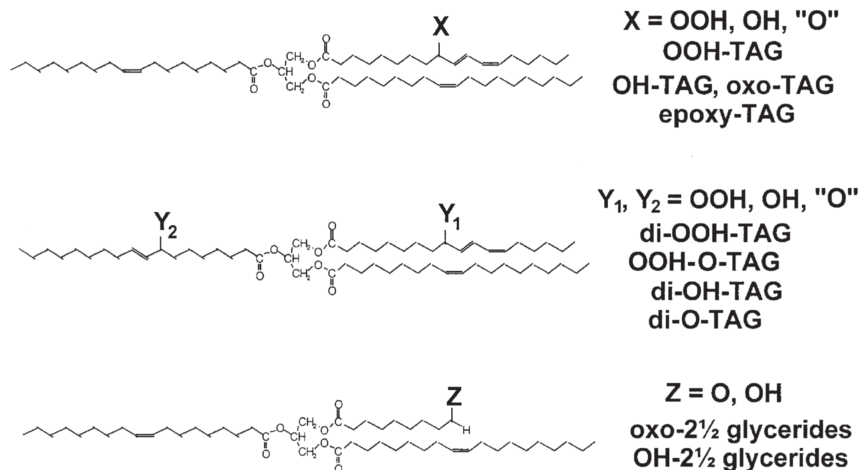
Optimization of HPLC conditions. The aim of the present work was to identify and quantitate the various classes of NONVOLLOP in partly oxidized oil and fat samples. As stated before, for such a polarity-type separation, NP-HPLC is the logical choice. In NP-HPLC, the elution order is based on polarity or ability to interact with silanol groups. Hence, the expected elution order is: TAG, epoxy-TAG, oxo-TAG, OOH-TAG, OH-TAG, bis(di)-hydroperoxy TAG, bis(di)-hydroxy TAG. The higher the degree of oxidation of a TAG, the more polar the oxidation products usually are.

HPLC columns with different dimensions, particle sizes, and stationary phase types were evaluated in order to obtain sufficient separation of the main classes of NONVOLLOP. This evaluation study was performed using model compounds and an oxidized PLP TAG sample. From the retention times and mass spectra, the various classes of oxidation products in the model TAG sample could be identified. Comparison of the chromatograms obtained on the three columns showed that the 250-mm silica column gave the best separation. By using this column, in the PLP sample OOH-, epoxy-, and oxo-groups, as well as 2½-glycerides, could be identified next to a cluster of peaks eluting at a higher retention time. Most likely, this cluster consists of multiple oxidized molecules, for example, bis(di)-hydroxy- and bis(di)-hydroperoxy-PLP. The 150-mm silica column could separate the epoxy-, oxo-, OOH-PLP, and 2½-glycerides. On this column, no peaks eluting at higher retention times were seen. The diol column was capable of separating all components according to chemical class with almost no separation within a group. The 2½-glyceride peak obtained was, unfortunately, very broad. Moreover, the OOH-PLP eluted very late, and some of the peaks seen on the 250-mm silica columns could not be recovered from the diol columns. Of the three columns evaluated, the 250-mm silica column was chosen for further work.

For the sequential elution of NONVOLLOP from a silica HPLC column according to polarity, a gradient of *n*-hexane/IPA was chosen. Elution of the test compounds with a hexane/IPA gradient resulted in good peak shapes, a stable baseline, and a low noise level. Also with MTBE as the polar mobile phase additive, a good separation was obtained. With this solvent system, however, baseline stability was poor.

For the 250-mm silica column, the hexane/IPA gradient was further optimized in order to obtain maximal separation according to compound class, minimal separation within a class, and rapid elution of highly oxidized components.

The final mobile phase program used a linear gradient of *n*-hexane (solvent A) and *n*-hexane/IPA 9:1, vol/vol (solvent B) programmed as follows: 5 min 3% B, in 25 min to 8% B,



SCHEME 1

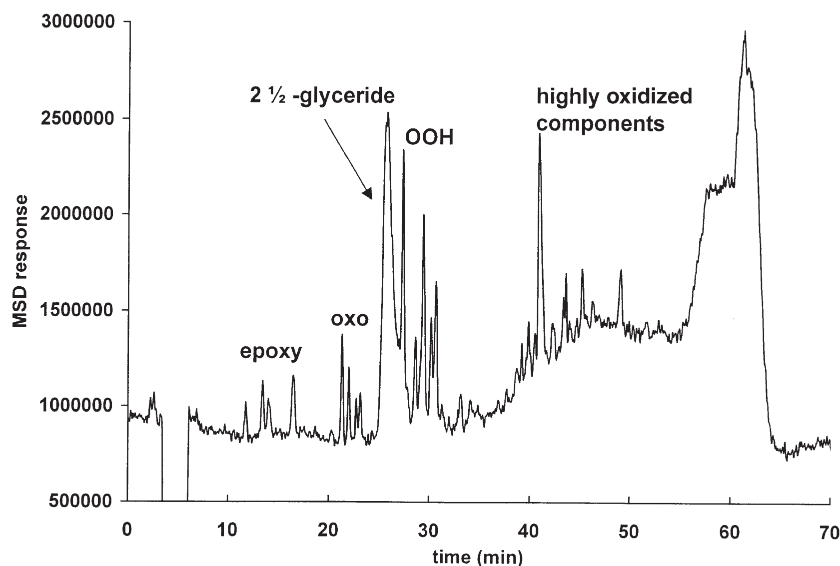


FIG. 1. Total ion current chromatogram (TIC) of oxidized triacylglyceride (TAG) 1,3-dipalmitoyl-2-linoleoyl-glycerol (PLP) under optimized normal-phase high-performance liquid chromatography (NP-HPLC) conditions. MSD, mass spectrometric detector.

in 10 min to 25% B, 10 min 25% B, in 2 min to 50% B, 5 min 50% B, in 1 min to 3% B, and 15 min 3% B. A total ion current (TIC) chromatogram of oxidized PLP under the final conditions is shown in Figure 1.

Optimization of MS conditions. The various classes of NONVOLLOP separated using HPLC can be identified from their molecular weights. Because of the complexity of the NONVOLLOP present in oxidized oil samples, fragmentation of individual NONVOLLOP species is highly undesirable. Fragmentation would further increase the complexity of the spectra obtained. The MS spectrum of a single peak, which represents a compound class in the HPLC chromatogram, already consists of the quasi-molecular ions of all the individual TAG of that chemical class (e.g., OOH-LLL, OOH-LOL, OOH-OOL, OOH-PLL). Fragmentation of parent ions would make the spectra nearly impossible to interpret. For that reason, the MS fragmentor voltage should be adjusted in a way that it gives minimal fragmentation, even if this results in some loss of sensitivity. The influence of the fragmentor voltage on the MS spectra obtained was studied for the hydroperoxide TAG, the components with the lowest stabilities.

The OOH-TAG are unstable components that easily lose an oxygen atom, resulting in the formation of the corresponding OH-TAG. Careful optimization of the MS ionization conditions is required to maximize the yield of intact OOH-TAG molecular ions. The sensitivity of the MS system to OOH-TAG was optimized by flow injection experiments with a solution of the model component P(13-OOH-L)S (50 ng/10 μL) at different fragmentor voltages and nebulizer gas temperatures. The fragmentor voltage varied from 80 to 140 V in steps of 20 V. In order to facilitate ionization of the analyte molecules in the electrospray interface, a solution of NaI in methanol/ethanol was added postcolumn just prior to the inlet of the ion source of the MS. Consequently, true molecular ions (M^+) are not formed, but sodium adduct quasi-molecular ions are formed with sodium attached to the original parent molecular ion [$(M + Na)^+$]. The signal intensity of P(13-OOH-L)S (*m/z* of 913.7) was found to increase with increasing fragmentor voltage. From the TIC chromatogram at different fragmentor voltages, however, it could clearly be seen that at higher voltages undesired fragmentation of the OOH-TAG occurred. The loss of an oxygen atom results in the formation of a fragment ion with *m/z* of 897.6 in addition to the molecular ion. This mass fragment is most likely the result of the formation of P(13-OH-L)S. The results of the fragmentor voltage optimization are shown in Figure 2. As can be seen from this figure, the fragment ion reaches approximately 60% of the intensity of the parent ion at a fragmentor voltage of 140 V compared to only 10% at 80 V. In all further experiments a fragmentor voltage of 80 V was used.

In addition to the fragmentor voltage, the nebulizer gas temperature also can affect the ionization behavior of the solutes in the ion source. In a separate series of experiments, the nebulizer gas temperature was ranged from 150 to 350°C at intervals of 50°C. The optimal nebulizer gas temperature for P(13-OOH-L)S was found to be 350°C. At this temperature no additional fragmentation of P(13-OOH-L)S was observed, and sensitivity was maximized.

Analysis of oxidized TAG. Before applying the newly developed method to real oil samples, a series of oxidized model TAG was studied. The TAG reference compound PLP was autoxidized to an undefined extent, and the resulting mixture was analyzed by HPLC–MS using the optimized conditions identified above. The TIC chromatograms and the 235 nm UV trace are shown in Figure 3. This figure also shows the mass

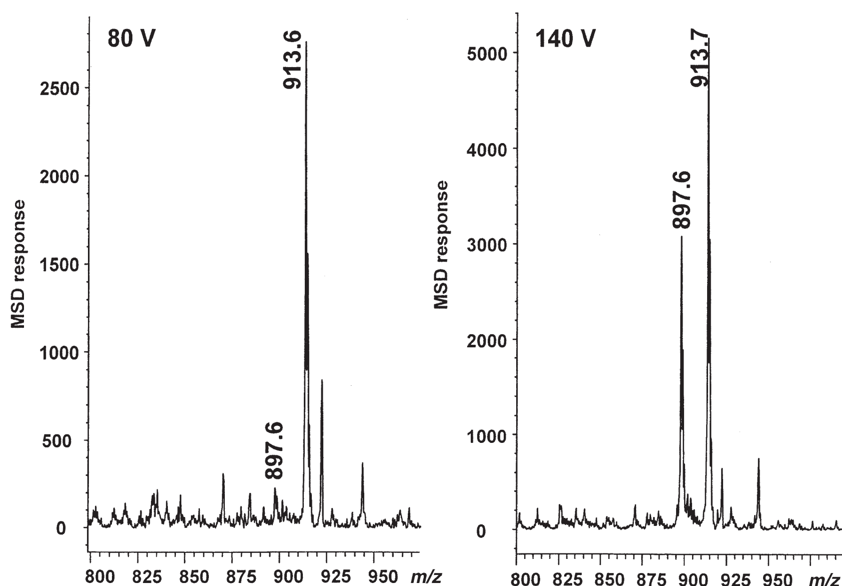


FIG. 2. Mass spectra of 1-palmitoyl-2-(13-hydroperoxy-linoleoyl)-3-stearoyl-glycerol [P(13-OOH-L)S] obtained in flow injection experiments at fragmentor voltages of 80 and 140 V. The mass of P(13-OOH-L)S is 913.7 *m/z* (sodium adduct).

spectrum of the peak corresponding to the oxo-2½-glycerides. UV detection at the specified wavelength of 235 nm selectively detects oxidized species with conjugated double bonds, such as OOH- and OH-TAG. Epoxy-TAG, oxo-TAG, and 2½-glycerides as observed in the TIC chromatogram do not show up in the UV trace.

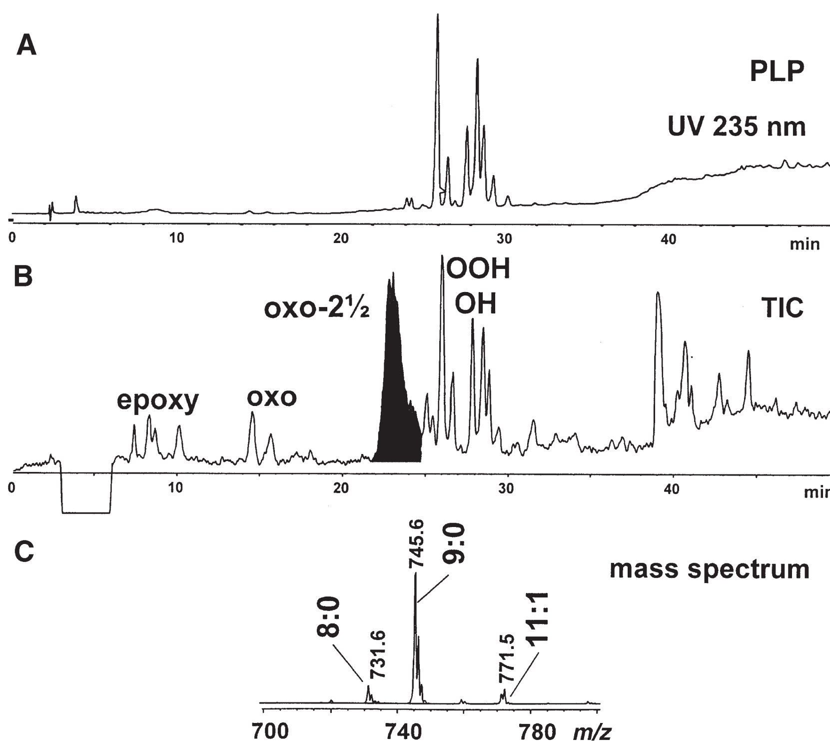


FIG. 3. NP-HPLC–mass spectrometric (MS) separation of nonvolatile lipid oxidation products formed during autoxidation of the model TAG PLP. From top to bottom: (A) Ultraviolet chromatogram, (B) TIC chromatogram of oxidized PLP, and (C) the mass spectrum of the oxo-2½-glyceride peak. See Figure 1 for other abbreviations.

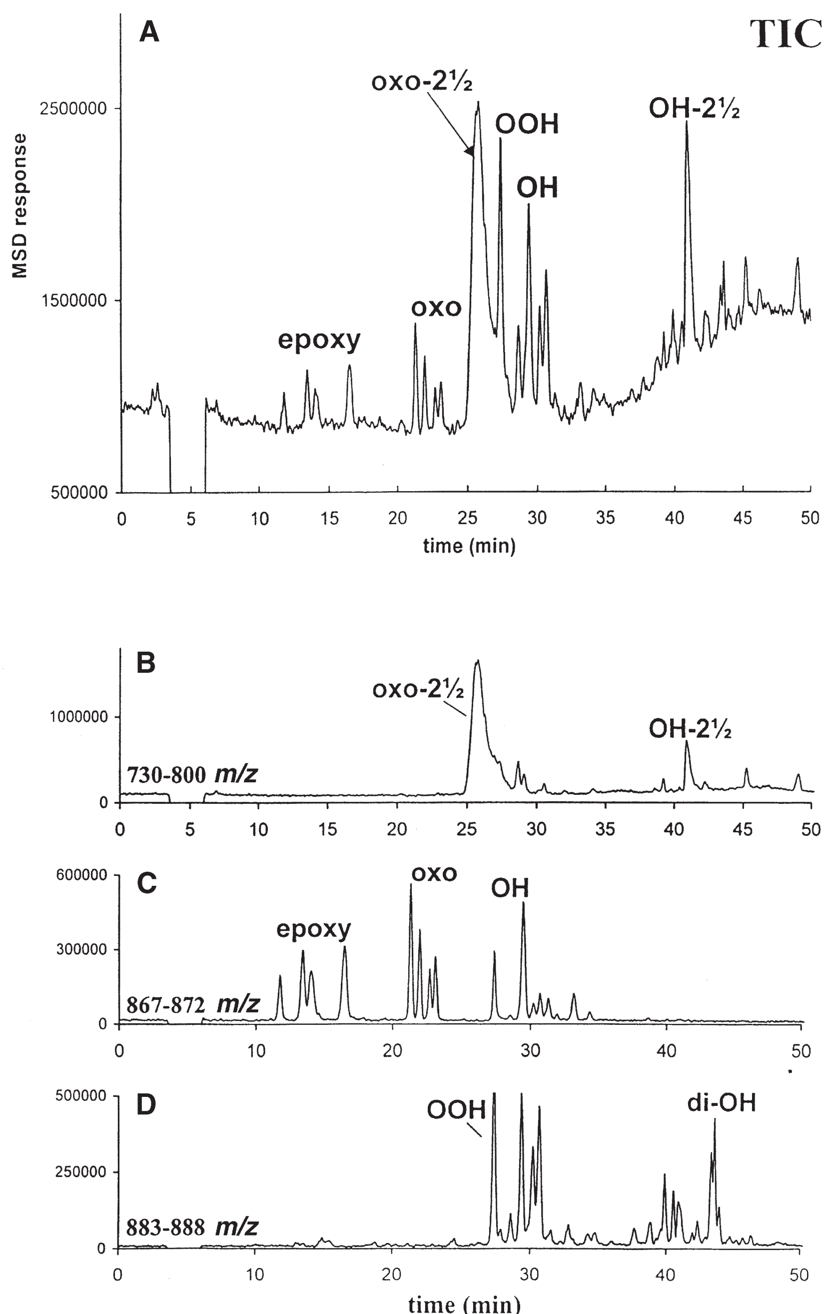


FIG. 4. HPLC–MS analysis of oxidized PLP. (A) TIC chromatogram, (B) extracted ion chromatogram of oxo- and OH-2$^1/_2$-glycerides, (C) single oxygen TAG, and (D) double oxygen TAG. See Figures 1 and 3 for abbreviations.

The identity of the NONVOLLOP observed in the HPLC–TIC chromatogram of oxidized TAG can be elucidated using extracted ion chromatograms. The TIC and several extracted ion chromatograms obtained for oxidized PLP are shown in Figure 4. The extracted ion chromatogram covering the *m/z* range 730–800 shows the peaks of oxo- and OH-2$^1/_2$-glycerides. In the *m/z* range of 867–872, peaks corresponding to the oxidation products of PLP functionalized with one oxygen atom, such as epoxy-PLP, oxo-PLP and OH-PLP, can be observed. The distinction between epoxy-PLP, oxo-PLP, and OH-PLP is based on their difference in polarity, and consequently retention time, in the NP-HPLC separation. This assignment of peaks has been confirmed by comparison with reference compounds. Oxidation products containing two oxygen atoms, such as OOH-PLP and di-OH-PLP, show up in the extracted ion chromatogram at an *m/z* range of 883–888. From the extracted ion chromatograms of targeted *m/z* ranges, most of the NONVOLLOP can be

identified. Retention times and full mass spectra offer further means of classification and confirmation.

If a mass spectrum is taken from the peak corresponding to the oxo-2½-glycerides, detailed information about the identity of the individual species present in the peak can be obtained. In the case of oxidized PLP (Fig. 3), the most abundant oxo-2½-glycerides are the saturated P(8:0,oxo)P and P(9:0,oxo)P and the monounsaturated P(11:1,oxo)P.

Analysis of oxidized oils. Oxidized real oil samples were analyzed and processed in the same way as the oxidized TAG. A major difference was that the mass spectrum obtained for a particular peak from the chromatogram was much more complex. In an oil or fat, a large number of different TAG are present, which all can be oxidized. A peak in the NP-HPLC chromatogram consequently does not correspond to one oxidation product of one parent TAG, but to a class of chemically identical oxidation products originating from different parent TAG (e.g., the peak designated as OOH-TAG could correspond to the hydroperoxides of LLL, LOL, OLO, PLO, etc.). Hence, for a real oil sample, the extracted ion chromatogram showing the peaks of all oxidized TAG containing, e.g., one oxygen atom (epoxy-, oxo-, and hydroxy-TAG), must cover a broader *m/z* range than a single oxidized TAG.

In Figure 5 the TIC and several extracted ion chromatograms obtained for oxidized rapeseed oil are shown. The extracted ion chromatogram for the *m/z* range 750–860 shows the peaks of oxo- and OH-2½-glycerides. In the *m/z* range 911–928, TAG with one oxygen atom, such as epoxy-TAG, oxo-TAG and OH-TAG, can be seen. In contrast to situations with a pure TAG, in the real oil sample oxo and epoxy components can no longer be distinguished. TAG oxidation products containing two oxygen atoms, e.g., the OOH-TAG and di-OH-TAG, are found in the extracted ion chromatogram of 929–942 *m/z*. Again, peak identification is based on the combination of molecular mass and retention time in NP-HPLC. Most of the peaks observed in the TIC chromatogram of oxidized rapeseed oil could be unambiguously assigned to a class of NONVOLLOP.

Quantitative aspects. Stable signal intensities are prerequisites for quantitative chromatographic analysis. In order to validate the short-term stability of the MS signal intensity, a mixture containing P(12-oxo-S)S, (epoxy-S)SS, PP(9:0,oxo), SRR, and RRR was analyzed five times on the same day. Furthermore, the same mixture was analyzed on three subsequent days. The relative within-day repeatabilities (r_{within}) and relative between-day reproducibilities (r_{between}) of the peak areas of the individual compounds obtained from the TIC chromatograms are between 2 and 7%. The r_{within} and the r_{between} values of the peak areas as obtained from the extracted ion chromatograms are also between 2 and 7%. The variation in peak areas in the extracted ion chromatograms is similar and low enough to allow quantitative determination based on external standard calibration.

For the quantitative analysis of lipid oxidation products, the relation between the MS signal and the concentration of the compounds in the sample needs to be established. Unfortunately, most of the target solutes are not available at sufficient purity to allow direct external standard calibration. Hence, we had to resort to using calibration components that resemble the different NONVOLLOP in real samples as closely as possible. Calibration was carried out by analyzing solutions containing the reference substances P(13-OOH-L)S, P(12-oxo-S)S, (epoxy-S)SS, PP(9:0,oxo), SRR, and RRR in the concentration range between 0.02 and 100 μg/mL. The amounts injected into the HPLC–MS ranged from 0 to 500 ng. The peak areas obtained from the extracted ion chromatograms are plotted against the mass injected. For all reference compounds, the data points could be fitted well with quadratic equations. In Figure 6, the calibration curves obtained from the extracted ion chromatograms are given for all reference components. The curves for P(12-oxo-S)S, (epoxy-S)SS, SRR, and RRR show approximately the same slope. This means that the MS response is relatively independent of the compound structure. The slope of the curve for P(13-OOH-L)S is significantly lower. Most likely this is the result of the low stability of the compound resulting in partial fragmentation of the quasi-molecular ion. The calibration curve for PP(9:0,oxo) is steeper than the curves of the other reference components. Apparently the PP(9:0,oxo) is more readily ionized.

To determine the limit of detection of the present method, solutions containing low concentrations of the reference components P(13-OOH-L)S, P(12-oxo-S)S, (epoxy-S)SS, PP(9:0, oxo), SRR, and RRR were analyzed. From the peak areas obtained and the baseline noise level, the limit of detection was estimated (signal/noise ratio of 3:1). The limit of detection for all compounds investigated is 5 ng in the TIC chromatograms and 0.1 to 0.5 ng for the extracted ion chromatograms. This corresponds with approximately 1–3 ppm of NONVOLLOP in an oil sample. These detection limits were measured at a fragmentor voltage of 80 V. By increasing the fragmentor voltage the limit of detection can be further increased. However, this would result in fragmentation of OOH-TAG.

In the current study, typical NONVOLLOP levels ranging from less than 3 ppm in the highly saturated palm oil to more than 5,000 ppm in polyunsaturated oils such as linseed/safflower oil were found. A detailed overview of some of the NONVOLLOP found in these samples is given in Table 1. It is important here to emphasize that all these samples were not artificially oxidized. The NONVOLLOP levels found were caused by normal oxidation during storage. This clearly illustrated that the current method can be used to analyze NONVOLLOP levels in a wide variety of oils and fats.

By using NP-HPLC, nonvolatile TAG oxidation products can be separated according to polarity. This results in a separation into classes of TAG oxidation products, such as epoxy-TAG, oxo-TAG, hydroperoxy-TAG, hydroxy-TAG, 2½ glycerides, etc. Within one class of TAG oxidation products, no further separation into individual molecular glyceride species is obtained.

The nonvolatile oxidation products of TAG, separated by means of NP-HPLC, can be detected and identified by MS in the positive electrospray mode. MS coupled to HPLC allows for very sensitive detection and provides structural information of the analytes at the same time.

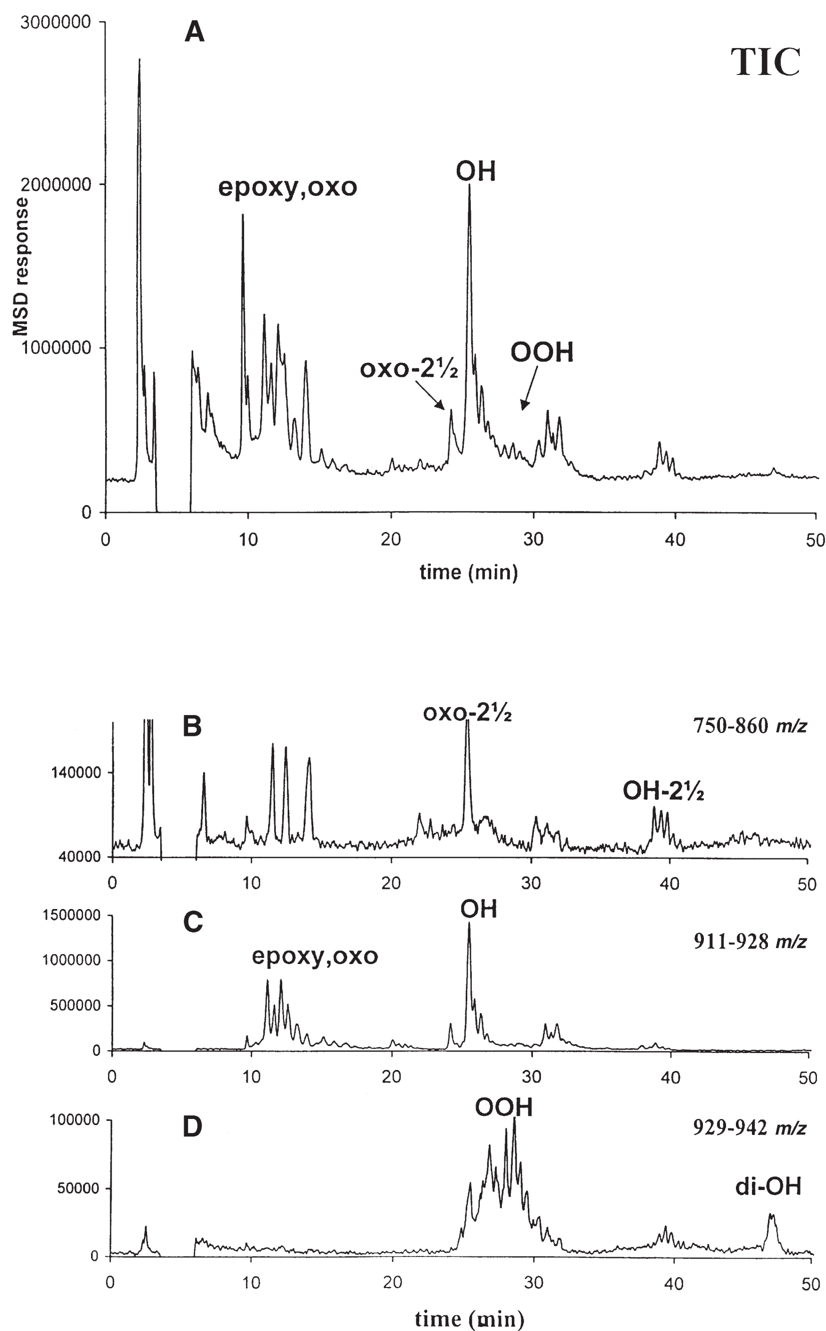


FIG. 5. HPLC–MS analysis of oxidized rapeseed oil. (A) TIC chromatogram, (B) extracted ion chromatogram of oxo- and OH-2½-glycerides, (C) single oxygen TAG, and (D) double oxygen TAG. See Figures 1 and 3 for abbreviations.

The analysis of an oxidized TAG of reference compound, PLP, and of oxidized rapeseed oil by means of the newly developed HPLC–MS proved the existence of a large variety of nonvolatile oxidation products. TAG that contain one or more epoxy, oxo, hydroperoxy, and hydroxy groups have been detected next to oxo- and hydroxy-2½ glycerides.

The retention times of the TAG oxidation products in the NP-HPLC system and the signal intensity of the MS detector are stable enough to enable quantitative analysis based on external calibration. Quantitation can be based on peak areas obtained from either TIC chromatograms or extracted ion chromatograms. The MS detector shows a similar response for the different classes of TAG oxidation products. Hence, calibration with a limited set of reference compounds is sufficient to obtain accurate quantitative results even for oxidation products where no reference compounds are available or for unidentified oxidation products.

The NP-HPLC–MS method is very suitable for the char-

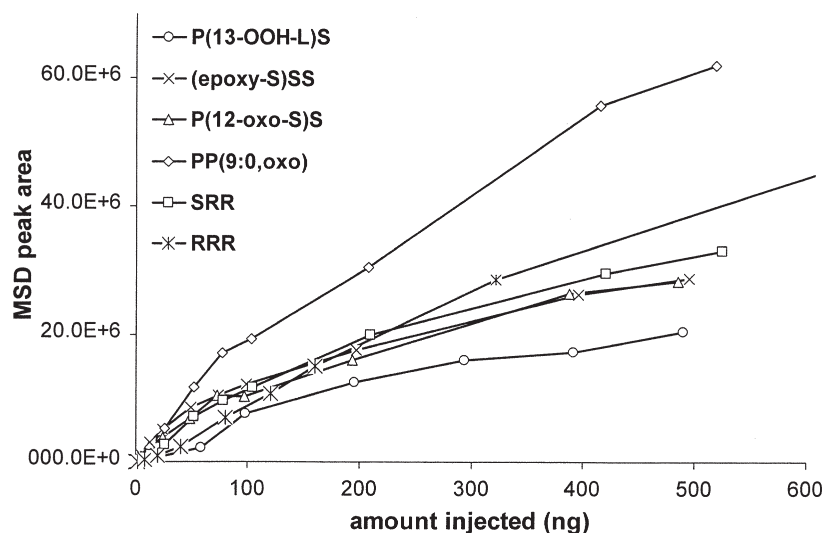


FIG. 6. Calibration curves of oxidized TAG reference compounds obtained from extracted ion chromatograms. (epoxy-S)SS, 1-stearoyl-2-stearoyl-3-(epoxy-stearoyl)-glycerol; P(12-oxo-S)S, 1-palmitoyl-2(12-oxo-stearoyl)-3-stearoyl-glycerol; PP(9:0,oxo)1-palmitoyl-2-palmitoyl-3-(9-oxo)-nonanoyl-glycerol; SRR, 1-stearoyl-2,3-diricinoleoyl-glycerol; RRR, triricinoleoyl-glycerol; for other abbreviations see Figures 1 and 2.

TABLE 1
Concentration of Nonvolatile Lipid Oxidation Products (NONVOLLOP) in Oxidized Rapeseed Oil and Linseed/Safflower Oil

NONVOLLOP	Concentration in rapeseed oil (ppm)	Concentration in linseed/safflower (ppm)
OOH-TAG	490	1,103
Epoxy- and oxo-TAG	2,734	28,701
Oxo-2½-glycerides	506	2,146
OH-TAG	8,759	3,549
diOH-TAG	1,049	1,823

[a]OOH-TAG, hydroperoxy triacylglycerides (TAG); OH-TAG, hydroxy TAG; and diOH-TAG, di-hydroxy TAG.

acterization and quantitation of nonvolatile TAG oxidation products in oxidized TAG reference compounds as well as in real oils or oil phases isolated from emulsions, spreads, or other fat-based food products. The method can also give detailed information for the study of lipid oxidation. The typical levels of NONVOLLOP in real oil samples range from 3 to 5,000 ppm.

ACKNOWLEDGMENT

The authors want to express their thanks to J.H.M. van den Broek for the synthesis of the nonvolatile lipid oxidation reference compounds.

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[Received December 6, 1999; accepted June 25, 2000]