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Reversed-phase high-performance liquid chromatographic separation of *tert*.-butyl hydroperoxide oxidation products of unsaturated triacylglycerols

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Abstract

Triacylglycerols containing monounsaturated fatty acids are known to be relatively resistant to autoxidation and require long periods of exposure to dilute oxidants. Use of concentrated solutions of synthetic hydroperoxides, however, yields in addition to the hydroperoxides also unidentified oxidation by-products. In the present study we have employed synthetic triacylglycerols containing one (18:0/18:1/18:0 and 18:1/16:0/16:0) and two (18:0/18:0/18:2 and 18:1/18:1/18:0) double bonds per molecule to reinvestigate the formation of oxotriacylglycerols using *tert*.-butyl hydroperoxide as an oxidant. Reversed-phase HPLC was used to separate and tentatively identify the oxidation products based on relative retention times of standards and the estimated elution factors for functional groups and their positional distribution. Hydroperoxides, diepoxides and hydroxides were the major components of the oxidation mixtures (50–95% of total). Previously unidentified peroxide-bridged *tert*.-butyl adducts were present in significant amounts (5–50% of total oxidation products) in all preparations. In several instances more than one functional group was present on a single fatty chain. The tentative reversed-phase chromatographic identification of the adducts was confirmed by determination of the molecular mass of each component by on-line LC with electrospray MS. The oxidation products were quantified by HPLC with light scattering detection.

Keywords: Oxidation; Triacylglycerols; *tert*.-Butyl hydroperoxide

1. Introduction

tert.-Butyl hydroperoxide (TBHP) has been extensively utilized in the oxidation of unsaturated fatty acid methyl esters [1,2], unsaturated triacylglycerols [3,4], cholesteryl esters [5,6] and glycerophospholipids [7,8]. Previous chromatographic and mass spectrometric analyses [1,2] of the TBHP oxidation products have identified various hydroperoxides, epoxides and hydroxides as the only recognizable reaction products. The popularity of the oxidant has been due to the belief that it mimics autoxidation in natural systems [9]. The extent of oxidation depends on the nature of the unsaturation, the strength of the oxidant and the length of the exposure. Recently reversed-phase high-performance liquid chromatog-

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raphy–mass spectrometry (HPLC–MS) with electrospray (ES) ionization has provided characteristic retention times as well as molecular masses for the oxidation products of linoleate esters arising from TBHP [4], peroxynitrite [10] and copper [11] oxidation.

The monounsaturated fatty acids and triacylglycerols have been particularly resistant to peroxidation even in the presence of bile salts as emulsifiers [3,4]. In order to increase the yield of the oxygenated triacylglycerols concentrated reagents and elevated temperatures have been used [3,4], which have led to formation of unidentified by-products. The present study reports the separation and identification of most of the unknowns by reversed-phase HPLC in relation to the relative retention times of standards and of retention factors estimated for functional groups and their positional location. The study shows that peroxide-bridged *tert*.-butyl derivatives of the unsaturated triacylglycerols as well as of unsaturated oxotriacylglycerols are formed with great regularity in presence of excess TBHP. Furthermore, the concentrated reagent tends to promote desaturation by dehydration of the oxygenated triacylglycerols which undergo further peroxidation. The identities of the oxygenated triacylglycerols proposed from chromatographic analyses were consistent with the molecular mass determination by on-line LC–ES-MS.

2. Materials and methods

2.1. Materials

TBHP as 70% solution in water, sodium borohydride ($NaBH_4$), triphenylphosphine (TPP), and 2,4-dinitrophenylhydrazine (DNPH) were purchased from Sigma (St. Louis, MO, USA). The solvents were of chromatography grade or of reagent grade and were dried over anhydrous sodium sulfate. **HAZARD NOTE**: Workers new to the manipulation of organic peroxides should read appropriate literature prior to work [12]. Briefly, avoid contamination with strong acids, metals and their salts, and peroxidizable solvents, especially ethers. Work at ambient or subambient temperatures on a small scale. Avoid concentrating TBHP solutions to dryness, and use rotary evaporator at room temperature with **SAFETY SHIELD**.

Synthetic 1-stearoyl-2-oleoyl-3-stearoyl-*sn*-glycerol (18:0/18:1/18:0), 1,2-dipalmitoyl-3-oleoyl-*sn*-glycerol (16:0/16:0/18:1), 1,2-dioleoyl-3-stearoyl-*sn*-glycerol (18:1/18:1/8:0) and 1,2-distearoyl-3-linoleoyl-*sn*-glycerol (18:0/18:0/18:2) were available in the laboratory from a previous study [4] (where 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid and 18:2, linoleic acid). The triacylglycerols were purified by thin-layer chromatography (TLC) as previously described [4]. Reference monohydroperoxy, monohydroxy, and mono C_9 core aldehyde derivatives of the dipalmitoyloleoylglycerol, and of the mono- and dihydroperoxy, and mono- and dihydroxy and mono C_9 aldehyde derivatives of the dioleoylstearoyl and distearoyllinoleoylglycerols were also available from the previous study [4]. Reference monohydroperoxides were prepared by photosensitized oxidation [4,13], while the dihydroperoxides were prepared by oxidation with TBHP [4]. The corresponding hydroxy derivatives were obtained by reduction of the hydroperoxides with sodium borohydride [14] or triphenylphosphine [15]. Monoepoxides were prepared by the method of Defense [16]. Triacylglycerol core aldehydes were prepared by ozonization and triphenylphosphine reduction [17]. The oxotriacylglycerol standards were purified by TLC using heptane–isopropyl ether–acetic acid (60:40:4, v/v) [18] and their structures were verified by reversed-phase LC–ES-MS [19].

2.2. Oxidation

The oxidation was accelerated by adding 1 ml of 35 to 70% TBHP in water to 10 mg of purified triacylglycerols in the presence of 10 μM $FeSO_4$ and 100 μl of 0.2% taurocholic acid [4,6]. The reaction mixture was incubated on a mechanical agitator in dark for 30 min to 30 h at 37°C. Without an initiator the reaction was sometimes difficult to start [12]. The reaction was stopped by diluting with 5 ml of chloroform–methanol (2:1, v/v), 100 μl of 2% EDTA in water and 10 μl of 2% butylated hydroxy toluene in methanol. The extracts were washed three times with water (3×1 ml). The solvent was evaporated under nitrogen at 38°C and the lipid residue

saved for HPLC–light scattering detection (LSD) and LC–ES-MS.

2.3. Preparation of DNPH derivatives

The DNPH derivatives of triacylglycerol core aldehydes were synthesized by adding freshly prepared DNPH in 1 *M* HCl (3.6 mg/ml) to an aliquot of dry sample [4]. The mixture was shaken vigorously and kept in dark at room temperature for 4 h and overnight at 4°C. The lipids were extracted by 5 ml of chloroform–methanol (2:1, v/v), the chloroform phase was blown down under nitrogen, and the residue taken up in an appropriate solvent for chromatography and MS as described below.

2.4. Thin-layer chromatography

Normal-phase TLC was used to purify triacylglycerols and their oxidized derivatives. Silica gel H (Merck) plates were prepared in the laboratory and heptane–isopropyl ether–acetic acid (60:40:4, v/v) solution was used as a mobile phase [18]. The DNPH derivatives of the core aldehydes were seen as yellow bands on the chromatoplates (in daylight). The compounds were recovered from the silica gel scrapings by extraction with chloroform–methanol (2:1, v/v). Extracts were washed with distilled water, dried with anhydrous Na_2SO_4 and saved for subsequent HPLC–LSD and LC–ES-MS analysis.

2.5. HPLC–LSD and LC–ES-MS

Procedures for HPLC and LC–ES-MS were as described previously [4,19]. Triacylglycerols and oxidation products were resolved by reversed-phase HPLC on a Supelcosil LC-18 column (250 mm×4.6 mm I.D.) using a linear gradient of 20–80% 2-propanol in methanol (0.85 ml/min) in 30 min. A Hewlett-Packard Model 1050 liquid chromatograph was coupled to a Varex ELSD II light-scattering detector (Varex, MD, USA) using nitrogen as nebulization gas and an evaporation temperature of 85°C. LC–ES-MS was performed using a Hewlett-Packard Model 1090 liquid chromatograph interfaced with a nebulizer-assisted ES source connected to a Hewlett-Packard Model 5989A quadrupole mass spectrometer [20]. The ionization (capillary exit) voltage of this instrument was set at 170 V but could be increased to 300 V, to obtain fragment ions from any clearly resolved components (pseudo MS–MS) [20,21]. The HPLC conditions were the same as described above except that, ammonia–isopropanol (1%) was added post-column at a flow-rate of 0.15 ml/min in order to enhance ionization [20]. In a few instances (oxidation products of 18:0/18:1/18:0) the LC–ES-MS analyses were performed with normal-phase columns using a gradient of chloroform–methanol–30% ammonia as previously described for glycerophospholipids [19]. The triacylglycerol core aldehydes were identified as $[M-H]^-$ ions in the negative ion mode. Mass spectra were acquired in the 400–1600 mass range over the entire elution profile.

2.6. Peak identification

The chromatographic peaks of the oxotriacylglycerols were identified on basis of reversed-phase HPLC retention times (t_R) of standards and estimated elution factors for functional groups and their positional distribution, as previously described [4]. The identification of major peaks was confirmed by single ion mass chromatograms extracted by computer from the total positive ion current spectra.

2.7. Quantification

The yields of the various oxidation products were quantified by HPLC–LSD. The results were compared to those obtained with LC–ES-MS. The ammonia, sodium and potassium adducts were summed to obtain a total estimate for the mass of species in each mass spectrum. The TBHP adducts were estimated separately as they were clearly resolved from other species during the reversed-phase HPLC–LSD and LC–ES-MS. The weight response of HPLC–LSD was corrected for differences in molecular mass before comparing it to the molar response obtained by LC–ES-MS. No other correction factors were used for any remaining differences in response among molecular species or for the presence of other oxoacylglycerol species in the HPLC–LSD peaks.

The HPLC–LSD and LC–ES-MS peak area responses were compared on either the same samples or on samples that had been peroxidized to the same extent as indicated by their total HPLC profiles.

3. Results

3.1. Oxidation of 18:0/18:0/18:2

Fig. 1 shows the reversed-phase HPLC–LSD and the LC–ES-MS profiles of the products for the more extensively oxidized 18:0/18:0/18:2, along with the full mass spectrum averaged over the elution time (13.59–33.36 min) of the oxoacylglycerol derivatives. As anticipated, the elution profile detected by LSD (A) is rather similar to that (B) recorded for the total positive ion current by MS. The mass spectrum (C) averaged over the acylglycerol elution range is relatively simple when considering the presence of both ammonia and sodium adducts, and suggests that the complex chromatographic patterns result from resolution of isomeric oxoacylglycerols. It may be noted that peak 11 in A appears as a fully split peak 11 in B, and that peak 9 in both A and B possesses an advanced shoulder. The early peaks 1–4 represent free fatty acid and mono- and diacylglycerol derivatives as well as unidentified reaction products and contaminants with masses below those of the lowest molecular mass oxotriacylglycerols examined here.

Fig. 2A shows the single ion mass chromatograms for the major ions as ammonia adducts at m/z 1058, 952, 1024, 936 and 1114 arranged in order of increasing HPLC elution time. The ion at m/z 1058 (peak 6, Fig. 1A) represents the ammonia adduct of the most extensively oxidized derivative of 18:0/18:0/18:2. The mass and retention times are best accounted for by the presence of a hydroperoxide, a cyclic peroxide and a TBHP bridge in the molecule. The cyclic peroxide would be formed with a loss of the double bond. The ion at m/z 952 (peak 7, Fig. 1A) represents the ammonia adduct of the mixed hydroperoxide and epoxide. The ion at m/z 1024 (peak 8, Fig. 1A) represents the ammonia adduct of the hydroperoxide containing one TBHP bridge. The ion at m/z 936 (peak 9, Fig. 1A) represents the ammonia adduct of the monohydroperoxide, while the ion at m/z 1114 (peak 10, Fig. 1A) represents the ammonia adduct of the cyclic peroxide formed with a loss of one double bond but linked to two TBHP groups via the peroxide bridges. Fig. 2B shows the single ion mass chromatograms for the remaining major ions as the ammonia adducts (m/z 918, 1080, 992, 1170 and 904) in the total mass spectrum of the 18:0/18:0/18:2 oxidation products. The ion at m/z 918 (peak 11, Fig. 1A) represents the ammonia adduct of the epoxide formed with the introduction of an additional double bond. The ion at m/z 1080 (peak 14, Fig. 1A) represents the ammonia adduct of 18:0/18:0/18:2 containing two TBHP bridges and the ion at m/z 1170 (peak 15, Fig. 1A) three TBHP bridges, one of the three bridges being introduced with a loss of a double bond. The ion at m/z 992 (peak 15, Fig. 1A) represents an 18:0/18:0/18:2 molecule containing a single TBHP bridge, while the ion at m/z 904 (peak 16, Fig. 1A) represents the ammonia adduct of the residual 18:0/18:0/18:2. The split peaks observed in most instances are due to a resolution of the regioisomers generated by partial isomerization of the distearoylglycerol during acylation with the linoleoyl chloride at the time of synthesis of the triacylglycerol standard. The diTBHP adduct is eluted slightly ahead of the monoTBHP as anticipated, while the triTBHP adduct is eluted slightly behind the monoTBHP derivative because of the loss of a double bond. Some of the possible oxidative steps leading to the multifunctional oxidation products are represented in Scheme 1.

Sodium borohydride reduction of the oxidized 18:0/18:0/18:2 led to the conversion of the hydroperoxides and diepoxides to the corresponding monohydroxides (m/z 920, as ammonia adduct), dihydroxides (m/z 936, as ammonia adduct), and hydroxy epoxides (m/z 936, as ammonia adduct) of 18:0/18:0/18:2. The TBHP bridges were also reduced to the corresponding hydroxides as indicated by the increase in the proportion of the hydroxides, which exceeded the amount anticipated from a reduction of the available hydroperoxides alone. In addition to these ions, the total ion spectrum included those due to sodium (plus 23) and potassium (plus 39) adducts. These ions account for all the major ions detected and the proposed identities are consistent with the chromatographic retention times of the available standards and those calculated from previously determined retention factors [4], as well

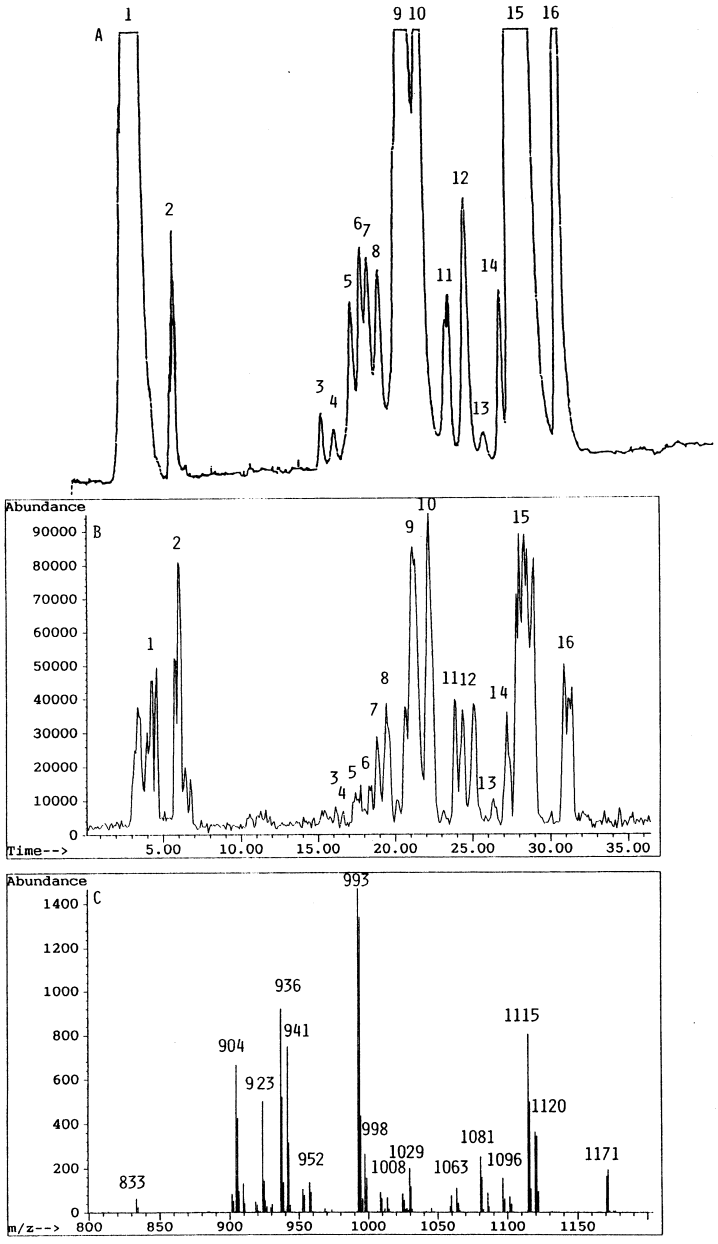


Fig. 1. Comparison of reversed-phase HPLC–LSD (A) and LC–ES-MS (B) profiles of the oxidation products of 18:0/18:0/18:2 following a 45-min exposure at 37°C to 7.8 *M* TBHP along with the full mass spectrum (C) averaged over the elution time (13.587–33.362 min) of the oxotriacylglycerol derivatives. Peak identification and quantification is given in Table 1. HPLC–LSD and LC–ES-MS conditions as described in Materials and methods.

as with the known transformation of methyl linoleate during peroxidation with excess TBHP [2]. There were no core aldehydes detected as the DNPH derivatives either by HPLC–LSD or by LC–ES-MS. The C9 aldehyde ester of distearoylglycerol (m/z 757) would have been eluted with a retention time of about 6 min [4]. Table 1 summarizes the quantitative results of the chromatographic and mass spectrometric

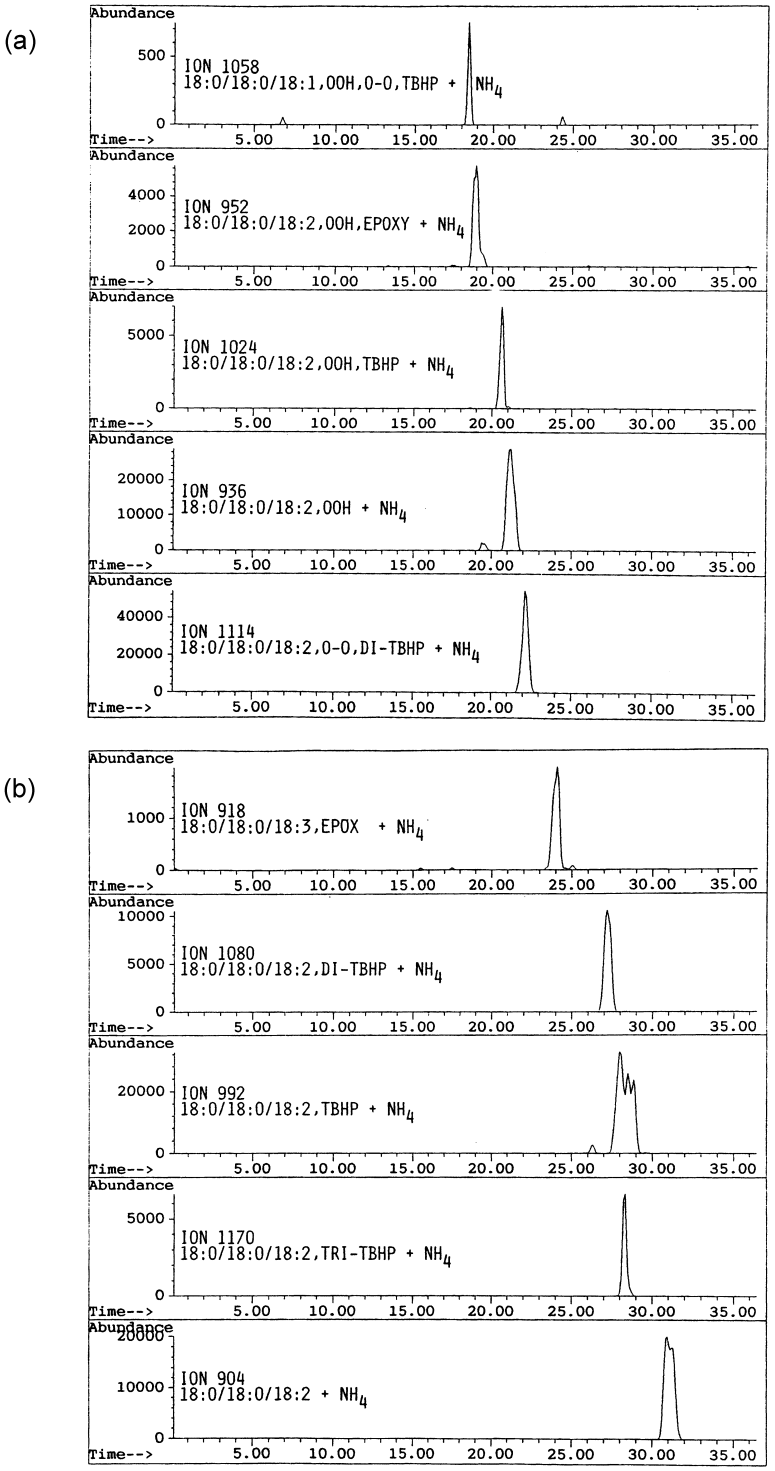
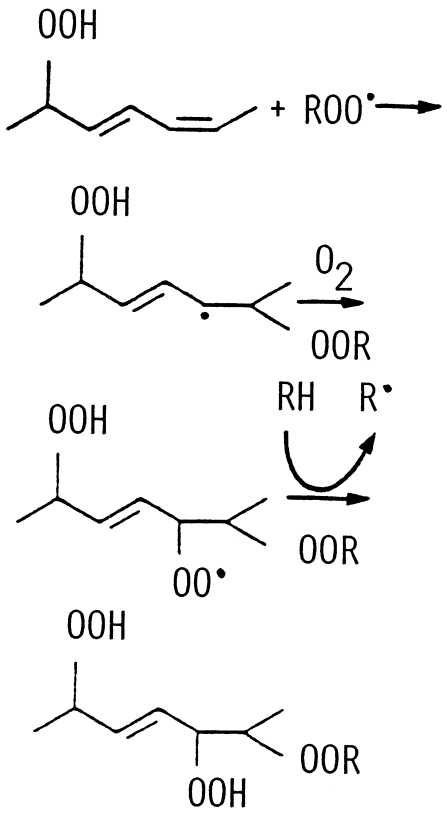


Fig. 2. Single ion mass chromatograms for the major ions m/z 1058, 952, 1024, 936 and 1114 (A) and 918, 1080, 992, 1170 and 904 (B) from the peroxidation of 18:0/18:0/18:2. Ion structural assignments are as given in figures. LC–ES-MS conditions as described in Materials and methods.



Scheme 1. Postulated formation of multioxygenated and peroxide-bridged products of linoleoyl hydroperoxide during oxidation with *tert*.-butyl (R) hydroperoxide. Reaction conditions are described in Materials and methods.

analyses of the products of TBHP treatment of 18:0/18:0/18:2.

3.2. Oxidation of 18:1/18:1/18:0

Fig. 3 shows the reversed-phase LC–ES-MS profile (A) of the oxidation products of 18:1/18:1/18:0, along with the full mass spectrum (B) averaged over the HPLC elution time of the oxoacylglycerols. Although this triacylglycerol is equivalent in carbon and double bond number to 18:0/18:0/18:2 just examined, it gave a markedly different elution profile. Likewise, differences were seen in the total mass spectra of the oxidation products of 18:0/18:0/18:2 and 18:1/18:1/18:0. Fig. 4 gives the single ion mass chromatograms for the major ions as the ammonia adducts at m/z 968, 952, 1024, 936, 918, 920, 992 and 904. The ion at m/z 968 represents the ammonia adduct of the dihydroperoxide of 18:1/18:1/18:0 (peak 1, Fig. 3). The ion at m/z 952 is accounted for by the ammonia adduct of the monohydroperoxy and monoepoxy derivative (peak 3, Fig. 3). This epoxide has been formed with retention of the double bond. The corresponding monohydroperoxy monoepoxide formed with the loss of the double bond (m/z 950, as ammonia adduct) is eluted earlier (peak 2, Fig. 3). The ion at m/z 1024 is accounted for as the ammonia adduct of the diepoxy derivative (all double bonds retained) containing one TBHP bridge (peak 4, Fig. 3). The corresponding

Table 1
Composition of major products of oxidation of 18:0/18:0/18:2 by *tert*.-butyl hydroperoxide

Peak No.[a]	$[M+NH_4]^{+}$[b]	TCN[c]	t_R	CN:DB[d]	Total peak area (%)		Proposed structure
					LC–ES-MS	HPLC–LSD	
6	1058	35.91	18.41	54:1	1.9	0.3	18:0/18:0/18:1, OOH,O-O,TBHP
7	952	36.8	18.95	54:2	4.2	0.3	18:0/18:0/18:2, OOH, epoxy
8	1024	39.88	20.70	54:2	5.8	2.0	18:0/18:0/18:2, OOH,TBHP
9	936	41.82	21.30	54:2	21.6	22.1	18:0/18:0/18:2, OOH
10	1114	44.07	22.20	54:1	18.9	19.3	18:0/18:0/18:1, O-O,diTBHP
11	918	44.02	24.14	54:3	8.8	3.5	18:0/18:0/18:3, epoxy
14	1080	47.56	27.22	54:2	4.2	1.0	18:0/18:0/18:2, diTBHP
15	992	48.28	28.06	54:2	18.3	28.5	18:0/18:0/18:2, TBHP
15	1170	48.11	28.35	54:1	2.9	2.0	18:0/18:0/18:1, triTBHP
16	904	50.22	30.88	54:2	13.1	21.0	18:0/18:0/18:2

[a] Peaks are numbered as in Fig. 1.
[b] Ions are given by their nominal masses.
[c] TCN, Theoretical carbon number.
[d] CN:DB, Total acyl carbon number:double bond number.

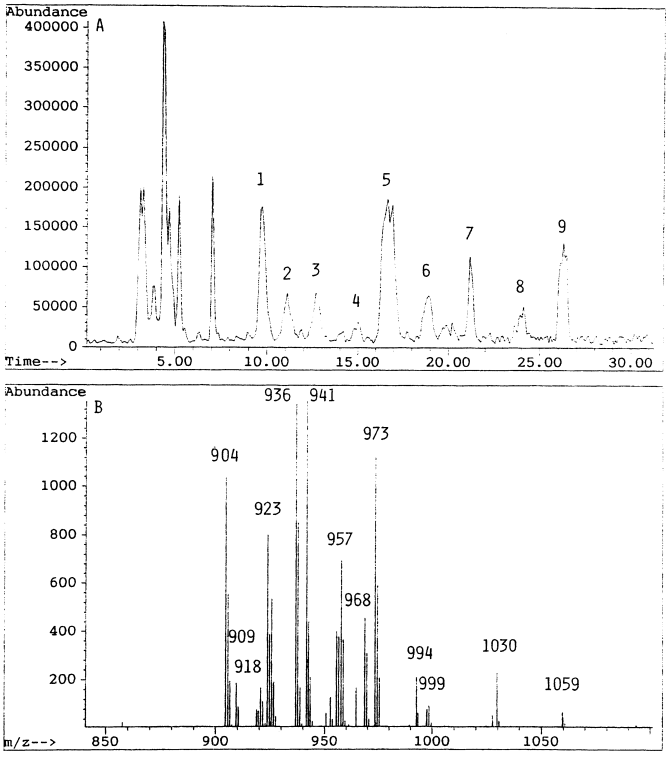


Fig. 3. Reversed-phase LC–ES-MS profile of the oxidation products of 18:1/18:1/18:0 following a 30-h exposure at room temperature to 7.8 *M* TBHP (A), along with the full mass spectrum (B) averaged over the elution time of the oxoacylglycerols (8.29–31.71 min). Peak identification and quantification is given in Table 2. LC–ES-MS conditions as described in Materials and methods.

diepoxides with one or both double bonds lost were not detected. The ion at *m/z* 936 represents the ammonia adduct of the simple hydroperoxide (peak 5, Fig. 3). The ion at *m/z* 918 represents the ammonia adduct of the epoxide that has been formed with introduction of a double bond (peak 6, Fig. 3), while the ion at *m/z* 920 represents the ammonia adduct of the epoxide formed with retention of the double bond (peak 7, Fig. 3). The ion at *m/z* 992 represents the ammonia adduct of 18:1/18:1/18:0 containing a TBHP bridge (peak 8, Fig. 3), while the ion at *m/z* 904 represents the ammonia adduct of the residual 18:1/18:1/18:0 (peak 9, Fig. 3). As a result of sodium borohydride reduction of the oxidized sample, new ions appeared at *m/z* 924 and 968 representing, respectively, the mono and dihydroxides (as the ammonia adducts) of the reduction products of the mono- and dihydroperoxides of 18:1/18:1/18:0, while the ions due to the ammonia adducts of the dihydroperoxide (*m/z* 968) and the monohydroperoxide (*m/z* 936) disappeared. The ions due to the ammonia adduct of the monohydroperoxy monoepoxide also disappeared, being replaced by that due to the hydroxy epoxide (*m/z* 936), which was eluted with characteristic retention time (data not shown). The oxidation products again were identified by a combination of the criteria based on reversed-phase HPLC separation and single ion mass chromatography. Table 2 summarizes the chromatographic and mass spectrometric properties of the oxidation products of 18:1/18:1/18:0 along with the quantitative estimates.

3.3. Oxidation of 18:1/16:0/16:0

Fig. 5 shows the reversed-phase LC–ES-MS profile (A) of the oxidation products of 18:1/16:0/16:0, along with the full mass spectrum (B) averaged over the elution range of the oxoacylglycerols. The chromatogram is complex and in contrast to the relatively

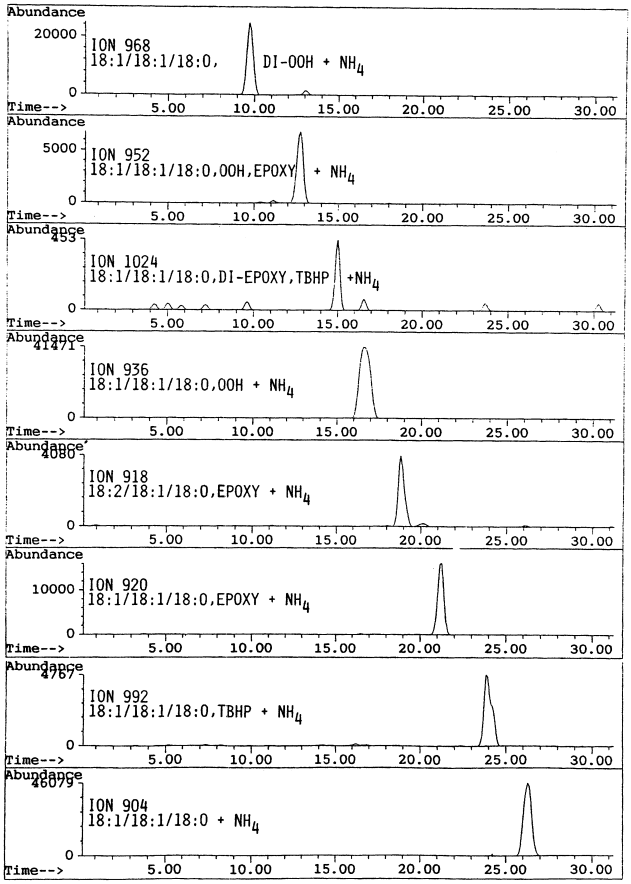


Fig. 4. Single ion mass chromatograms for the major ions m/z 968, 952, 1024, 936, 918, 920, 992 and 904. From the peroxidation of 18:1/18:1/18:0. Ion structural assignments are as given in figures. LC–ES-MS conditions as described in Materials and methods.

Table 2
Composition of major products of oxidation of 18:1/18:1/18:0 by *tert*.-butyl hydroperoxide[a]

Peak No.[b]	$[M+NH_4]^{+}$ [c]	TCN	t_R	CN:DB	Total peak area (%)		Proposed structure
					LC–ES-MS	HPLC–LSD	
1	968	32.96	9.76	54:2	18.2	20.0	18:1/18:1/18:0, diOOH,
2	950	32.96	11.30	54:1	3.8		18:0/18:1/18:0, OOH, epoxy
3	952	35.74	12.79	54:2	3.6	13.2	18:1/18:1/18:0, OOH, epoxy
4	1024	38.37	15.08	54:2	2.6	1.1	18:1/18:1/18:0, diepoxy, TBHP
5	936	40.39	16.76	54:2	34.5	40.0	18:1/18:1/18:0, OOH
6	918	42.46	18.90	54:3	8.7	6.6	18:2/18:1/18:0, epoxy
7	920	44.71	21.26	54:2	8.7	6.6	18:1/1/18:1/18:0, epoxy
8	992	47.1	24.09	54:2	4.1	3.3	18:1/18:1/18:0, TBHP
9	904	49.26	26.30	54:2	15.6	9.2	18:1/18:1/18:0

[a] Other abbreviations are as given in Table 1.
[b] Peaks are numbered as in Fig. 3.
[c] Ions are given by their nominal masses.

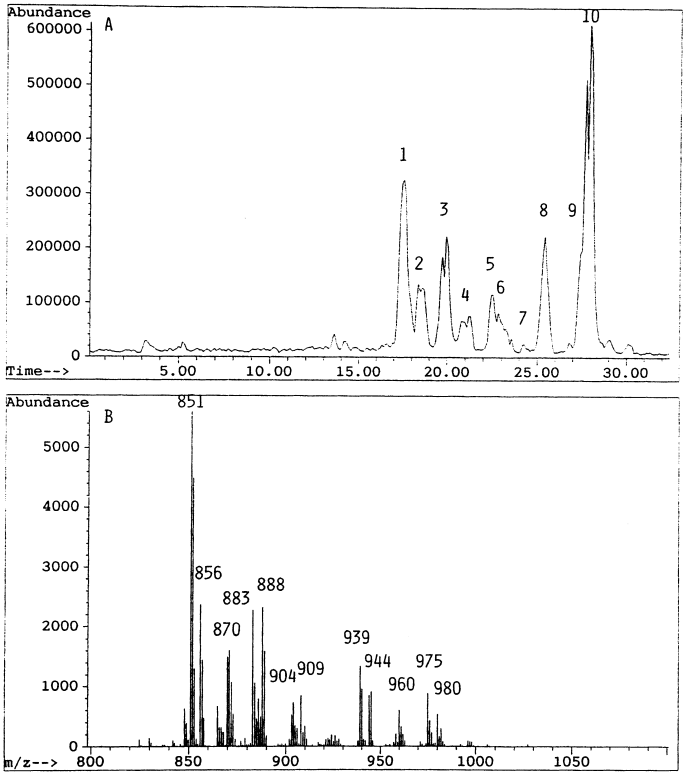


Fig. 5. Reversed-phase LC–ES-MS profile (A) of the oxidation products of 18:1/16:0/16:0 following a 30-h exposure at 37°C to 7.8 *M* TBHP along with the full mass spectrum (B) averaged over the elution time (13.314–30.720 min) of the oxotriacylglycerol derivatives. Peak identification and quantification as given in Table 3. LC–ES-MS conditions as described in Materials and methods.

simple mass spectrum, which includes both ammonia and sodium adducts. Fig. 6 shows the single ion mass chromatograms for the major ions (as ammonia adducts) at *m/z* 882, 902, 864, 1026, 938 and 850 arranged in order of increasing retention time. The ion at *m/z* 882 (peak 1, Fig. 5A) represents the ammonia adduct of the simple hydroperoxide. The ion at *m/z* 902 (peak 2, Fig. 5A) represents the ammonia adduct of 18:1/16:0/16:0 containing a hydroperoxy and an epoxy group introduced with a loss of the double bond. The ion at *m/z* 864 (peak 3, Fig. 5A) represents the epoxide formed with a retention of the double bond, while another ion at *m/z* 866 (not shown) represented the ammonia adduct of the epoxide of 18:1/16:0/16:0 formed with a loss of the double bond. The ion at *m/z* 1026 (peak 7, Fig. 5A) represents the ammonia adduct of the diTBHP derivative of 18:2/16:0/16:0 formed with the introduction of a double bond. The ion at *m/z* 938 (peaks 6 and 8, Fig. 5A) represents the ammonia adduct of the monoTBHP derivative of 18:1/16:0/16:0, while the ion at *m/z* 850 (peak 10, Fig. 5A) represents the ammonia adduct of the residual 18:1/16:0/16:0. In addition, the oxidation products of 18:1/16:0/16:0 also contained a minor ion at *m/z* 974 (peak 9, Fig. 5A), which apparently originated from the ammonia adduct of an unidentified derivative of 18:1/16:0/16:0 of increased mass and low polarity. Sodium borohydride reduction of the oxidation products of 18:1/16:0/16:0 resulted in the conversion of the monohydroperoxides into the monohydroxides (*m/z* 866) and of the mono and diTBHP derivatives into the corresponding mono- and dihydroxide derivatives (*m/z* 866 and 882, respectively). Table 3 summarizes the chromatographic and mass spectrometric properties of the oxidation products of 18:1/16:0/16:0 as well as compares the proportions of the products estimated

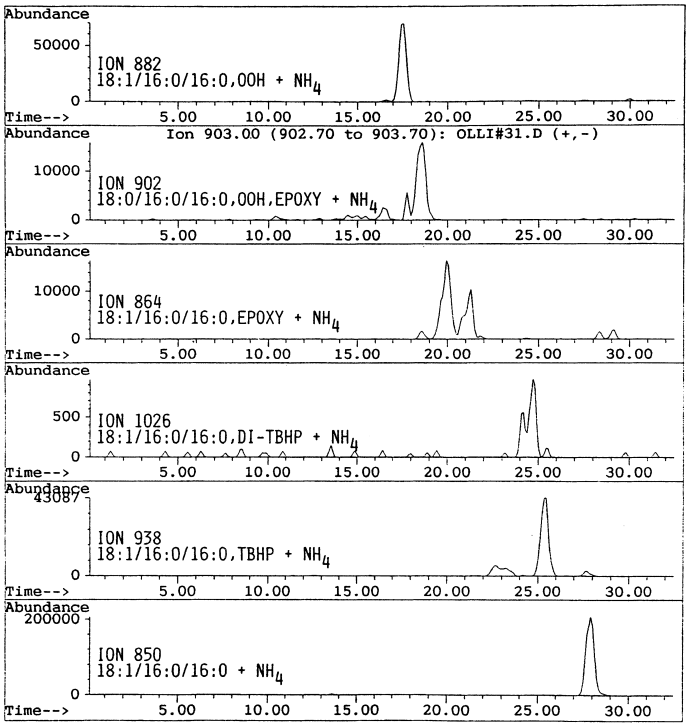


Fig. 6. Single ion mass chromatograms for the major ions m/z 882, 902, 864, 1026, 938 and 850 from the peroxidation of 18:1/16:0/16:0. Ion structural assignments are as given in figures. LC–ES-MS conditions as described in Materials and methods.

Table 3
Composition of major products of oxidation of 18:1/16:0/16:0 by *tert*.-butyl hydroperoxide[a]

Peak No.[b]	$[M+NH_4]^{+}$[c]	TCN	t_R	CN:DB	Total peak area (%)		Proposed structure
					LC–ES-MS	HPLC–LSD	
1	882	38.62	17.56	50:1	21.9	30	18:1/16:0/16:0, OOH
2	902		18.51	50:0	6.5	10	18:0/16:0/16:0, OOH, epoxy
3	864	40.69	20.00	50:1	13.0	20	18:1/16:0/16:0, epoxy
4	864	40.69	21.00	50:1			18:1/16:0/16:0, epoxy
5	867	40.69	22.50	50:0			18:0/16:0/16:0, epoxy
6	938	45.33	23.00	50:1	Trace	2	18:1/16:0/16:0, TBHP
7	1026	44.62	24.78	50:2	0.3	Trace	18:2/16:0/16:0, diTBHP
8	938	45.33	25.43	50:1	8.8	5	18:1/16:0/18:0, TBHP
9	974		27.44		2.7	Trace	18:1/16:0/18:0, TBHP
10	850	47.49	27.97	50:1	45.8	33	18:1/16:0/16:0

[a] Other abbreviations are as given in Table 1.
[b] Peaks are numbered as in Fig. 5.
[c] Ions are given by their nominal masses.

by HPLC–LSD and LC–ES-MS. Similar results were obtained for the TBHP oxidation products of 18:0/18:1/18:0, except that the masses of the corresponding ions were higher by 56 u (data not shown).

4. Discussion

In the past TBHP has been extensively used as the initiation and propagation reagent of the oxidation chain because it had been reported to mimic closely the natural autoxidation process [9]. In preliminary experiments with complex natural oils we had observed that concentrated solutions of this reagent yield in addition to the mono- and dihydroperoxides and epoxides also high proportions of unknown oxotriacylglycerols with molecular masses far exceeding those resulting from multiple additions of hydroperoxy, epoxy or hydroxy groups to the available unsaturation sites. In the present work we have characterized the unknown TBHP oxidation products of synthetic triacylglycerols on basis of the elution times of standard oxotriacylglycerols, the elution factors calculated from functional groups and their positional distribution, and the molecular masses determined by LC–ES-MS.

For our initial experiments we selected 1,2-distearoyl-3-linoleoyl-*sn*-glycerol (18:0/18:0/18:2). On the basis of extensive previous work with methyl linoleate we anticipated the conjugated 9-hydroperoxy and 13-hydroperoxy linoleates to be the major components, with both epoxides and hydroxides arising as degradation products of the hydroperoxides. Two of the hydroperoxides would have *cis*,*trans* geometry in the conjugated diene unit while the other two would display *trans*,*trans* geometry [2]. The 9-hydroperoxy and 13-hydroperoxy linoleates are resolved on normal-phase HPLC [22], while on short reversed-phase columns the hydroperoxides as well as the hydroxides migrate together [23]. The linoleate hydroperoxides are known to be degraded to the isomeric epoxyoxooctadecenoic and epoxyhydroxyoctadecenoic acids as major products [24]. If any high-molecular-mass chain cleavage products were formed they would be the 9-oxononanoyl and the 12:1-oxo-dodecenoyl derivatives as observed during autoxidation of the linoleates [25]. An examination of numerous reaction mixtures, however, revealed that in addition to the anticipated oxidation products several other high-molecular-mass oxygenated components were also resolved, although in somewhat variable proportions. Thus, the ion at m/z 992 possessed the correct mass for the ammonia adduct of 18:0/18:0/18:2 containing a peroxide-bridged *tert*.-butyl group, while the ion at m/z 1080 corresponded to the ammonia adduct of 18:0/18:0/18:2 containing two such peroxide bridges, but the ion at m/z 1170 correspond to the ammonia adduct of 18:0/18:0/18:1 containing three such peroxide-bridged *tert*.-butyl groups. The relative retention times of these molecules on the reversed-phase column were consistent with the limited increase in the polarity of the proposed structures. Since 18:0/18:0/18:2 contains only one unsaturated fatty chain, all the functional groups would have to be accommodated on the same fatty chain. These proposals are consistent with the observations of Courtneidge and Bush [12] who reported that during TBHP oxidation peroxy radicals can be diverted from hydroperoxide formation by addition reactions and that the latter reaction can for certain substances become an almost exclusive pathway. The introduction of two hydroperoxy groups or two peroxide bridged *tert*.-butyl groups is also consistent with the pathways proposed by Courtneidge [26] for the TBHP-loaded peroxidation of diunsaturated substrates. The introduction of a third TBHP group is proposed to arise as a result of an intermediate formation and opening of an epoxide group to yield an additional double bond [27], which can then direct further peroxide bridging. The peroxide bridging observed during TBHP oxidation is reminiscent of the dimerization of oxygenated methyl linoleate reported by Miyashita et al. [28], who also have proposed an oxidative pathway for the introduction of a third peroxide bridge into the linoleate chain. Scheme 1 illustrates the proposed structures, which are drawn to resemble the simple hydroperoxides already known to be formed during linoleate peroxidation [26,28].

The ion at m/z 1114 corresponds to the mass anticipated for the ammonia adduct of 18:0/18:0/18:2 containing two peroxide bridged *tert*.-butyl groups along with a cyclic peroxide, while the ion at m/z 1024 corresponds to the mass anticipated for an ammonia adduct of 18:0/18:/18:2 containing one

hydroperoxy group and one peroxide bridged *tert*.-butyl group, and the ion at *m/z* 1058 to the 18:0/18:0/18:2 derivative containing one cyclic peroxide, one hydroperoxide and one TBHP bridge, the formation of all of which can be accounted for by the mechanisms of polyfunctional peroxide formation proposed by Courtneidge [26].

The oleic acid-containing acylglycerols were much more resistant to peroxidation than the linoleic acid-containing ones. However, the anticipated hydroperoxides, hydroxides and epoxides were obtained along with the peroxide bridged adducts. Thus, the ion at *m/z* 992 could be attributed to the ammonia adduct of 18:1/18:1/18:0 containing one peroxide bridged *tert*.-butyl group, while the ion at *m/z* 1024 could be attributed to the ammonia adduct of 18:1/18:1/18:0 containing two epoxy groups along with one TBHP bridge. In the latter instance the epoxy group was assumed to have been introduced with a retention of the double bond [27] which would then have permitted the TBHP bridging.

The oxidation of the monooleoylacylglycerols gave the simplest mixture of products, yet hydroperoxide bridged *tert*.-butyl groups were recognized among them. Thus, the ion at *m/z* 938 corresponded to the ammonia adduct of 18:1/16:/16:0 containing one hydroperoxide bridge. Interestingly, a 18:0/18:1/18:0 species with the monounsaturated fatty acid at the *sn*-2 position of the triacylglycerol appeared to yield only limited peroxide bridging.

In addition, we encountered an unknown component. Thus, the ion at *m/z* 974 apparently represented the ammonia adduct of some unusual modification of the original 18:1/16:0/16:0, which migrated slightly ahead of it indicating a marginal increase in polarity. The ion at *m/z* 864 could be attributed to the ammonia adduct of 18:1/16:0/16:0 epoxide formed with retention of the double bond, while the ion at *m/z* 902 could be assigned to the ammonia adduct of 18:0/16:0/16:0 containing a hydroperoxy group and an epoxy group formed with loss of the double bond. The peak splitting observed in several instances was attributed to a partial resolution of the regioisomers of triacylglycerols [29], although the type of epoxide formed could also have contributed to the peak splitting.

The autoxidation of oleate in the presence of excess TBHP has been studied by Porter and coworkers [1,2]. Under cooxidation conditions of 4.0 *M tert*.-butyl hydroperoxide and 1.8 *M* oleate (30°C), only the 11-*cis*,9-*trans*,10-*trans*, and 8-*cis* oleate hydroperoxides were produced and no 11- or 8-*trans* hydroperoxides were obtained. The ratio of the 11-*cis*/9-*trans* products was 1:1.2, as was that of the 8-*cis*/10-*trans* compounds. It was assumed that the same isomers were produced in the present experiments using the glyceryl esters of oleate. Porter et al. [1] resolved the corresponding methyl oleate alcohols on a normal-phase HPLC column, but on our reversed-phase column the acylglycerol hydroperoxides migrated as a single peak.

We have previously demonstrated that the core aldehydes of triacylglycerols [3,4], cholesteryl esters [4,5], and glycerophospholipids [11] can be prepared in small amounts by TBHP oxidation in the presence of ferrous ions and effectively identified as the 2,4-dinitrophenylhydrazones by reversed-phase HPLC with on-line thermospray (TS) MS or ES-MS. In the present study the formation of small amounts of core aldehydes of acylglycerols was confirmed, with the 18:0/18:0/9:0ALD emerging at 3.8 and the 18:0/9:0ALD/9:0ALD at 6 min, showing the appropriate masses on LC–ES-MS.

5. Conclusions

The study shows that TBHP oxidation can be used to prepare milligram quantities of oxoacylglycerols but peroxide bridge formation constitutes an undesirable complication. Fortunately, the TBHP bridge adducts are readily resolved from the hydroperoxides and epoxides by reversed-phase HPLC. Since the peroxide bridge formation constitutes a part of the reaction mechanism it may not be entirely avoided, although milder reaction conditions minimize the bridge formation.

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