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Analysis of Isomeric Forms of Oxidized Triacylglycerols Using Ultra-High-Performance Liquid Chromatography and Tandem Mass Spectrometry

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ABSTRACT: Detailed studies on the regioisomeric structures of oxidized species of triacylglycerols (TAG), formed in food during storage and processing, have not been published thus far. In this study, an analytical approach based on efficient ultra-high-performance liquid chromatographic (UHPLC) separation of different isomers of oxidized TAG species and their tandem mass spectrometric analysis was created. A linear solvent gradient based on acetonitrile and acetone was used in the UHPLC method. A novel method utilizing positive ion ESI using ammonia supplemented in the nebulizer gas was used to produce ammonium adduct ions for mass spectrometric analysis. With the UHPLC method used, different regioisomers of TAG species containing oxidized linoleic or oleic acid could be efficiently resolved. Differences in the fragmentation patterns of many of the oxidized TAG isomers could be demonstrated by the tandem mass spectrometric method. On the basis of the results, the approach enables regiospecific analysis of oxidized TAG molecules.

KEYWORDS: electrospray ionization, lipid oxidation, tandem mass spectrometry, ultra-high-performance liquid chromatography

INTRODUCTION

Lipids may be gradually oxidized during normal storage and processing of foods. With few exceptions, oxidation affects all lipid classes. Several studies have shown that oxidation of dietary lipids is reflected in the degree of oxidation of chylomicrons and very-low-density lipoproteins (VLDL).[1−4] During the past 20 years, evidence has accumulated on the contribution of oxidized low-density lipoproteins (LDL) to atherogenesis.[5−8] On the basis of the results of various research groups, also oxidized chylomicron remnants seem to be potentially atherogenic.[1]

Reversed-phase liquid chromatographic columns have been typically used in liquid chromatographic separation of different TAG species. However, mixtures of oxygenated triacylglycerols are difficult to analyze because of the presence of a large variety of homologues, regioisomers, and *cis*−*trans* isomers, which often overlap with each other and with homologues of unoxidized parent compounds.[9,10] The combination of a highly selective chromatographic method and a sensitive, regiospecific mass spectrometric detection would be valuable in the analysis of oxygenated triacylglycerol (TAG) species. This is of interest not only because of the structural information obtained but also because the stability of fatty acid residues to oxidation may depend on their position within the TAG molecule.[11]

Previously, mass spectrometric methods based on positive ion electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI)[12,13] as well as on ammonia negative ion chemical ionization in vacuum[14,15] or in atmospheric pressure[16] have been utilized in determination of the regioisomeric composition of TAG molecules. Byrdwell and Neff[12] and Giuffrida et al.[17] have also used various mass spectrometric methods to study fragmentation of oxidized TAG molecules but, to our knowledge, detailed studies on the regioisomeric structures of the oxidized species of TAGs have not been published.

In this study, an analytical approach based on efficient ultra-high-performance liquid chromatographic (UHPLC) separation of different isomers of oxidized TAG species with two reversed-phase columns and their tandem mass spectrometric (MS/MS) analysis is presented. A novel method[18] utilizing positive ion ESI using ammonia supplemented in the nebulizer gas was used to produce ammonium adduct ions. The aim of the study was to efficiently distinguish between different isomers of various oxidized TAG species by combining the chromatographic and mass spectrometric approaches.

EXPERIMENTAL PROCEDURES

Abbreviations and Nomenclature. *sn* regioisomers denote the isomeric forms of TAG molecule in which the oxidized fatty acid or its oxidized form is situated in either *sn*-1/3 or *sn*-2 position. No distinction is made between the *sn*-1 and *sn*-3 positions. $[AB]^+$ denotes a diacylglycerol (DAG) fragment ion in which A is palmitic acid and B is oxidized linoleic or oleic acid.

TAG 50:2 OOH and TAG 50:1 OOH denote TAGs containing two palmitic acid (16:0) residues and one hydroperoxy linoleic acid (18:2 OOH) or one hydroperoxy oleic acid (18:1 OOH) residue, respectively, in an undefined *sn* position (hydroperoxides synthesized by photosensitized oxidation). Likewise, TAG 50:2 OH/keto/diepoxy and TAG 50:1 OH/keto/epoxy denote TAG molecules with a hydroxy, keto, or epoxy group attached to the linoleic or oleic acid residue, respectively. TAG 50:2 tOOH denotes a TAG containing two palmitic acid residues and one hydoperoxy linoleic acid synthesized by *tert*-butyl hydroperoxide oxidation.

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Table 1. Reference Compounds Used in the Study[a]

no.	TAG[a]	no.	derivatized TAG
I	18:1−16:0−16:0	**Ia**	18:1 epoxy[b]−16:0−16:0
		Ib	18:1 OOH−16:0−16:0
		Ic	18:1 OH−16:0−16:0
		Id	18:1 keto−16:0−16:0
II	16:0−18:1−16:0	**IIa**	16:0−18:1 epoxy[b]−16:0
		IIb	16:0−18:1 OOH−16:0
		IIc	16:0−18:1 OH−16:0
		IId	16:0−18:1 keto−16:0
III	18:2−16:0−16:0	**IIIa**	18:2 diepoxy[b]−16:0−16:0
		IIIb	18:2 OOH−16:0−16:0
		IIIc	18:2 OH−16:0−16:0
		IIId	18:2 keto−16:0−16:0
IV	16:0−18:2−16:0	**IVa**	16:0−18:2 diepoxy[b]−16:0
		IVb	16:0−18:2 OOH−16:0
		IVc	16:0−18:2 OH−16:0
		IVd	16:0−18:2 keto−16:0

[a] Regioisomers (*sn*-1/3 and *sn*-2 positions distinguished from each other; *sn*-1 and *sn*-3 positions not distinguished from each other). [b] Underlined double bonds have been replaced by the epoxy groups.

Chemicals and Reagents. 3-Chloroperoxybenzoic acid, *tert*-butyl hydroperoxide solution (70 wt % in water), and triphenylphosphine were obtained from Sigma-Aldrich (St. Louis, MO). Dess−Martin periodinane (15 wt % in dichloromethane) was purchased from Acros Organics (Geel, Belgium). Reagents were of reagent grade or better quality. Reference TAGs (purity = 99%) *sn*-18:1(n-9)−16:0−16:0 + *sn*-16:0−16:0−18:1(n-9), 16:0−18:1(n-9)−16:0, *sn*-18:2(n-6)−16:0−16:0 + *sn*-16:0−16:0−18:2(n-6), and 16:0−18:2(n-6)−16:0 were purchased from Larodan Fine Chemicals (Malmö, Sweden). All solvents were of chromatography or reagent grade and were purchased from local suppliers.

Preparation of Reference Compounds. The synthetic TAGs along with their oxidized derivatives prepared in this study are listed in Table 1. Epoxides (**Ia**, **IIa**, **IIIa**, **IVa**) were prepared according to the method of Deffense.[19] A sample of 5 mg of TAG was oxidized with 8 mg of 3-chloroperoxybenzoic acid in 400 μL of dichloromethane at room temperature for 1.75 h followed by purification using TLC as described below. In the procedure, epoxy groups are substituted for double bonds.

Hydroperoxides (**Ib**, **IIb**, **IIIb**, **IVb**) were prepared by photosensitized oxidation.[20] Ten milligrams of TAG was added to 4 mL of methylene blue solution (0.1 mM methylene blue in dichloromethane) in a test tube that was placed in an ice bath under a 250 W photographer's lamp for 13 h (TAG containing linoleic acid) or for 19 h (TAG containing oleic acid). The distance between the sample solution and the lamp was 20 cm. Some hydroperoxides (**IIIb**, **IVb**) were also prepared by oxidation with *tert*-butyl hydroperoxide solution. Twelve milligrams of TAG was added to 1 mL of the 70 wt % solution, and the mixture was shaken at 37 °C for 60 min. Hydroperoxides were purified by TLC as described below.

For the preparation of hydroxides (**Ic**, **IIc**, **IIIc**, **IVc**), 3−4 mg of hydroperoxide TAG was dissolved in 1 mL of 9 mg/mL triphenylphosphine in chloroform. The mixture was shaken and held at room temperature for 1 h.[21] The hydroxy compounds were purified by TLC as described below.

Ketone standards (**Id**, **IId**, **IIId**, **IVd**) were prepared by oxidizing the corresponding hydroxides with Dess−Martin periodinane solution.[22] The hydroxides (1 mg) were dissolved in 0.4 mL of dichloromethane, and 40 μL of Dess−Martin periodinane solution was added. The mixture was shaken and held in an ice bath (0 °C) for 5 min. The keto compounds were purified by TLC as described below.

Purification of TAG Oxidation Products. Normal-phase TLC was used to purify the TAG derivatives.[23] A heptane/di-isopropyl ether/acetic acid (60:40:4, by vol) solution was used as the mobile eluent. The TLC system separates different classes of oxidized TAG from each other. Synthesized TAG derivatives were applied to silica G-plates. Resolved components were scraped off the plates and were recovered from the silica gel by extraction with chloroform/methanol (2:1, by vol). The extracts were washed with distilled water.

Ultra-High-Performance Liquid Chromatography and Mass Spectrometry. The UHPLC system consisted of two Kinetex C18 columns (100 mm × 2.1 mm i.d., 1.7 μm particle size) (Phenomenex, Torrence, CA) and Acquity Ultra Performance LC equipment (Waters Corp., Milford, MA). A binary solvent gradient consisted of acetonitrile (designated A) and acetone/acetonitrile (80:20, by vol) (designated B). The gradient program was as follows: initial A/B (100:0, v/v), linear from 0 to 25 min to A/B (24:76). The flow rate was 0.4 mL/min. The columns were kept at constant room temperature, 21 °C. An amount of 3−5 μL of each sample (concentration approximately 0.1 mg/mL) was injected into the UHPLC/ESI-MS/MS system.

MS(/MS) analyses were performed with a Quattro Premier tandem quadrupole mass spectrometer (Waters Corp.) using positive ESI. The capillary was set at 4.5 kV and the sample cone at 150 V. The source and the desolvation temperatures were set at 100 and 130 °C, respectively. Nitrogen was used as desolvation and cone gas, and the flows were set at 400 and 70 L/h, respectively. The collision gas (argon) flow was set at 0.25 mL/min and the collision energy at 25 eV. Ammonia gas (purity 5.0; Linde AG, Munich, Germany) was introduced to the nebulizer gas flow (nitrogen) to produce ammonium adducts of oxidized TAGs $[M + NH_4]^+$. The mass flow of the ammonia gas was optimized to generate a maximal intensity for $[M + NH_4]^+$ ions. The technique enabled convenient and continuous HPLC/ESI-MS/MS analyses, without introducing ammonia in water or ammonia salts in the postcolumn flow or mobile phases. (Note: To avoid degradation of material caused by ammonia gas, it is advisible to use O-rings made of perfluoroelastomer (Kalrez) in the ion source.) MassLynx v4.1 (Waters Corp.) was used for the collection and analysis of mass chromatograms and spectra. The proportions of different ions were calculated on the basis of the height of the centroid peaks of mass spectra.

RESULTS AND DISCUSSION

Different *sn* regioisomers of individual oxidized TAG species were at least partially resolved by the UHPLC method created (Figure 1). The TAG *sn* regioisomers in which the oxidized linoleic/oleic acid moiety was in the *sn*-2 position were eluted slightly earlier than the *sn*-1/3 isomers. When individual *sn* regioisomers of oxidized TAGs 50:2 OOH, 50:2 OH, 50:2 keto, 50:2 diepoxy, 50:1 OOH, 50:1 OH, and 50:1 keto were studied, two or more additional isomers (isomers in terms of the position of the oxygen group within the linoleic/oleic acid moiety and possibly some *cis*−*trans* isomers) could be at least partially separated by the UHPLC method. Examples of resolved peaks are shown in Figure 2. Individual *sn* regioisomers of TAGs 50:2 tOOH and 50:1 epoxy gave only one chromatographic peak each.

When using photosensitized oxidation with methylene blue, mostly 9- and 10-hydroperoxy oleic acids (in *trans* configuration) are expected to be formed from oleic acid and 9- and 13-hydroperoxy as well as some 10- and 12-hydroperoxy linoleic acids (mostly in *cis*−*trans* configuration) from linoleic acid.[24] In the case of pure *sn* regioisomers of the hydroperoxy linoleic

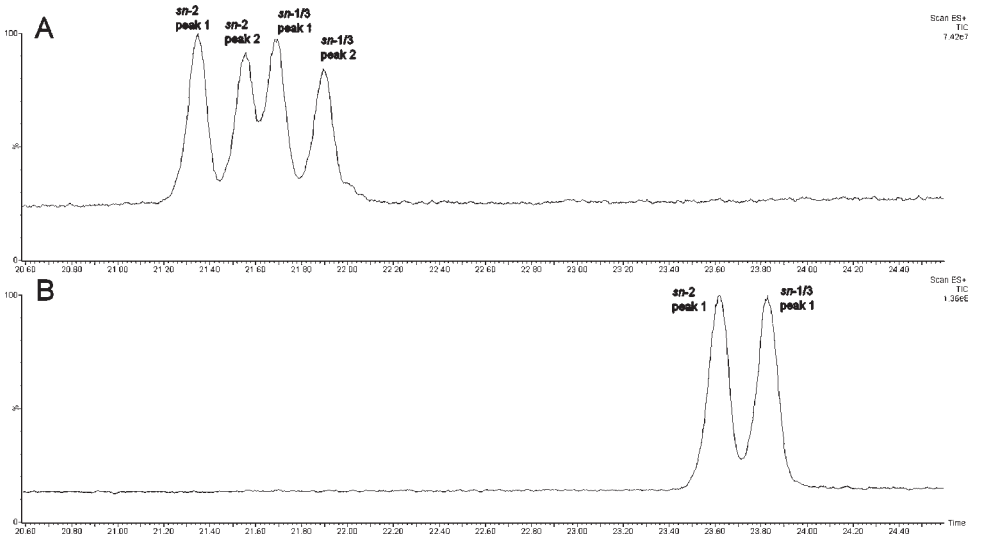

Figure 1. Mass chromatograms showing examples of separation of *sn* regioisomers of oxidized TAG species: (A) TAG 50:1 OH; (B) TAG 50:1 epoxy. See Table 2 for abbreviations. *sn*-1/3 and *sn*-2 denote the position of the oxidized oleic acid residue in the TAG molecule. Peaks 1 and 2 denote resolved, unidentified isomers within the particular *sn* regioisomeric oxidized TAG species.

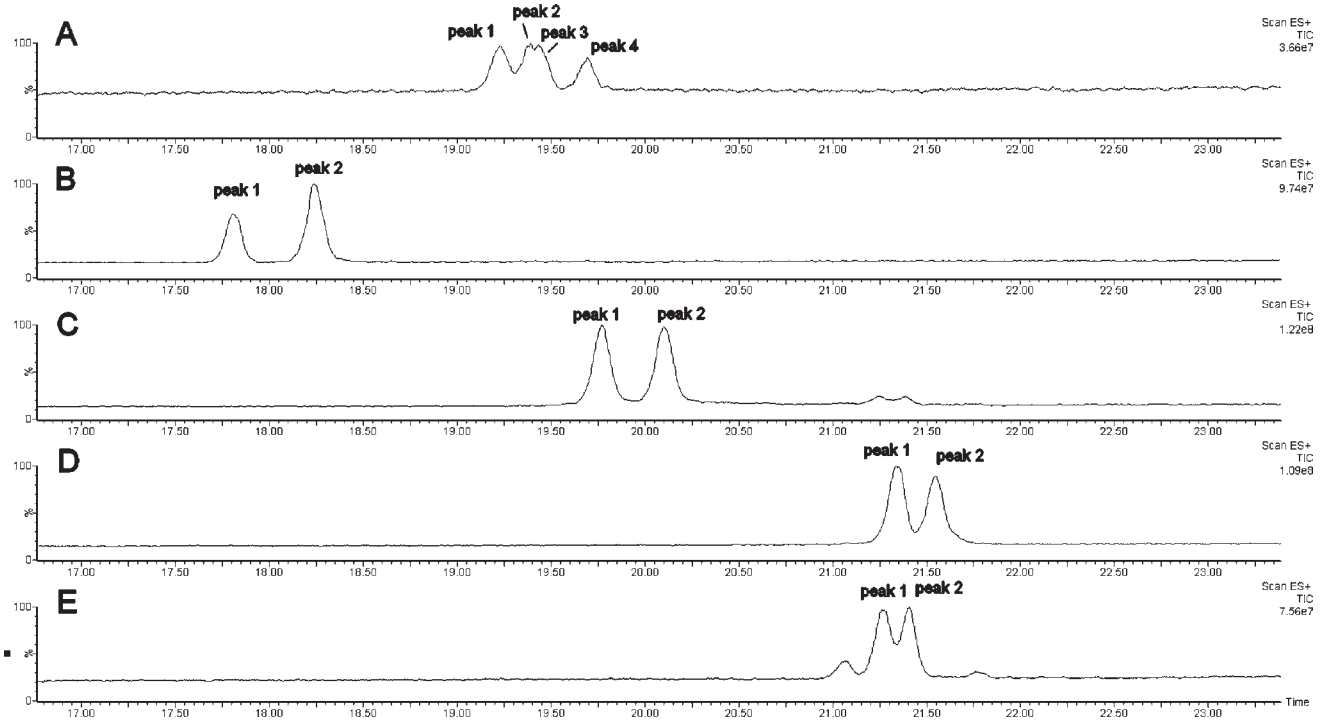

Figure 2. Mass chromatograms of different oxidized TAG species with the oxidized fatty acid in *sn*-2 position; ion profiles of *sn*-1/3 isomers are similar with slightly longer retention times: (A) TAG 50:2 OH; (B) TAG 50:2 diepoxy; (C) TAG 50:1 OOH; (D) TAG 50:1 OH; (E) TAG 50:1 keto. See Table 2 for abbreviations. Peaks 1−4 denote resolved, unidentified isomers within the *sn*-2 regioisomeric oxidized TAG species.

acid-containing TAG 50:2 OOH (synthesized by photosensitized oxidation), overlapping of chromatographic peaks was present, but in the mass chromatograms of TAG 50:2 OH (synthesized by reduction from TAG 50:2 OOH), four chromatographic peaks, although still slightly overlapping, were present accordingly (Figure 2A). The isomers in which the hydroperoxyl or other oxygen group is closer to the glycerol backbone are expected to interact more with the reversed-phase material and thus elute later. In TAG 50:2 keto (synthesized by oxidation from TAG 50:2 OH), more than four chromatographic peaks were present, possibly because of *cis*−*trans* isomerization. Interestingly, TAG 50:2 diepoxy, which was synthesized directly from TAG 50:2 with both of its double bonds replaced by epoxy groups, generated two chromatographic peaks (Figure 2B). Two isomers of TAG 50:1 OOH were efficiently separated (unlike TAGs 50:2 OOH and 50:2 tOOH), as were two isomers of TAG

Table 2. Proportions of Different $[AB]^+$ Ions Formed from Different *sn* Regioisomers (Oxidized Fatty Acid in *sn*-1/3 vs *sn*-2 Position) of Oxidized TAG Molecules[a]

	fragment ion[b]					
	$[AB_1]^+$		$[AB_2]^+$		$[AB_3]^+$	
original TAG[c]	*sn*-1/3	*sn*-2	*sn*-1/3	*sn*-2	*sn*-1/3	*sn*-2
50:2 tOOH			40.1 ± 0.6 a	50.6 ± 2.7 b	20.3 ± 0.6 a	29.9 ± 1.1 b
50:2 OOH			37.1 ± 0.9 a	47.7 ± 0.7 b	25.9 ± 1.4 a	39.7 ± 0.7 b
50:2 OH			49.9 ± 1.5a	67.1 ± 1.5b		
50:2 keto	69.3 ± 0.6 a	75.5 ± 1.1 b				
50:2 diepoxy	22.6 ± 1.0 a	28.5 ± 0.4 b	34.2 ± 0.5 a	41.5 ± 0.4 b		
50:1 OOH			26.8 ± 0.8 a	41.9 ± 2.3 b	32.7 ± 0.4 a	50.0 ± 3.1 b
50:1 OH			33.4 ± 0.6 a	60.9 ± 0.2 b		
50:1 keto	78.9 ± 1.0 a	80.6 ± 0.9 a				
50:1 epoxy	45.5 ± 0.4 a	54.1 ± 0.2 b	29.3 ± 0.5 a	43.2 ± 0.8 b		

[a] Proportions are calculated as percentages of the individual $[AB_x]^+$ ion of the sum $[AA]^+ + [AB_x]^+$, where A denotes palmitic acid and B denotes oxidized fatty acid. Different letters in a row indicate significant differences between the *sn*-1/3 and *sn*-2 isomers ($P < 0.05$). [b] $[AB_1]^+$ denotes ion $[M + NH_4 - RCOONH_4]^+$; $[AB_2]^+$ denotes ion $[M + NH_4 - RCOONH_4 - 18]^+$; $[AB_3]^+$ denotes ion $[M + NH_4 - RCOONH_4 - 18 - 16]^+$. $RCOONH_4$ denotes ammoniated palmitic acid. [c] TAG 50:2 OOH/OH/keto/diepoxy denote TAG molecules containing a hydroperoxy, hydroxy, keto, or epoxy group attached to a linoleic acid residue, respectively, as well as two palmitic acid (16:0) residues. TAG 50:2 tOOH denotes a hydroperoxyl TAG species synthesized by *tert*-butyl hydroperoxide oxidation. TAG 50:1 OOH/OH/keto/epoxy denote TAG molecules containing a hydroperoxy, hydroxy, keto, or epoxy group attached to an oleic acid residue, respectively, as well as two palmitic acid (16:0) residues.

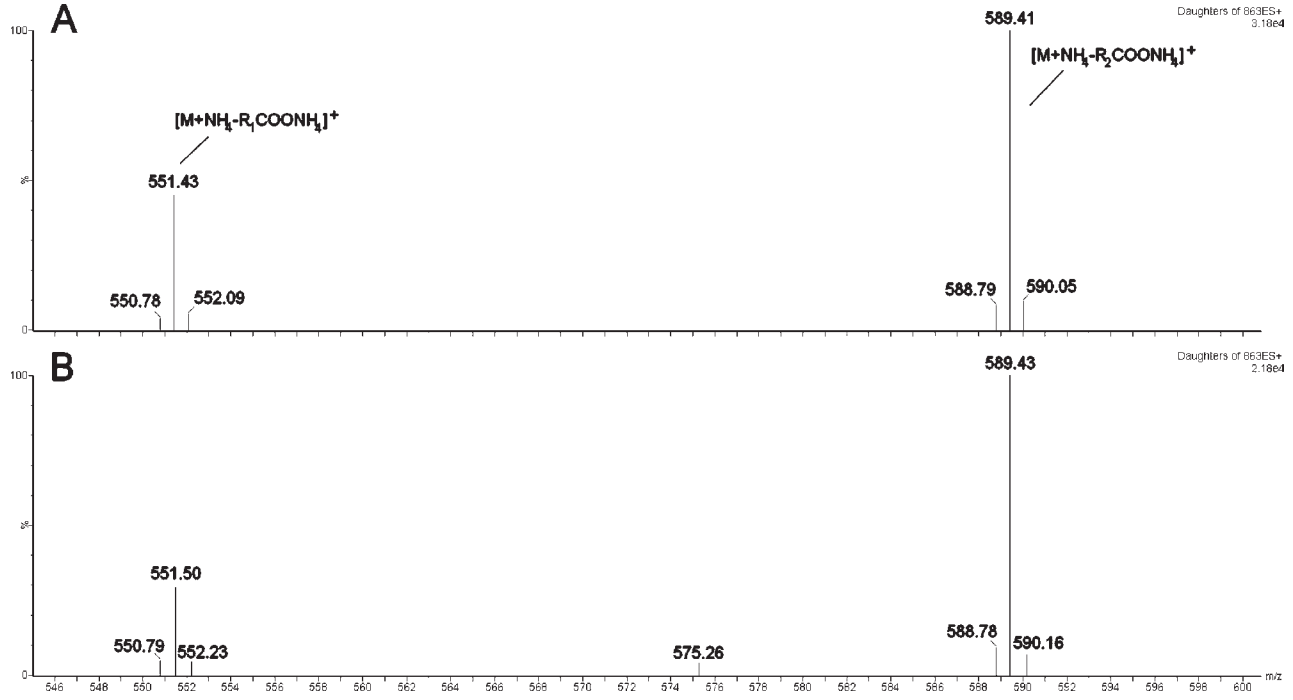

Figure 3. Examples of mass spectra showing the fragment ions formed from TAG molecular species containing a keto group: (A) TAG 50:2 keto, oxidized linoleic acid in *sn*-1/3 position; (B) TAG 50:2 keto, oxidized linoleic acid in *sn*-2 position. See Table 2 for abbreviations. R_1COONH_4 and R_2COONH_4 denote ammoniated linoleic and palmitic acids, respectively.

50:1 OH (Figure 2C,D, respectively). In addition to two major isomeric peaks, TAG 50:1 keto also gave rise to two minor peaks (Figure 2E).

As could be expected, fragmentation of the ammoniated ions of oxidized TAG species was efficient. In the case of TAGs 50:2 tOOH, 50:2 OOH, 50:2 OH, 50:2 keto, 50:2 diepoxy, 50:1 OOH, 50:1 OH, and 50:1 epoxy, there were differences in the fragmentation of different *sn* regioisomers of oxidized TAG species. This was investigated by comparing proportions of $[AB]^+$ ions formed from different *sn* regioisomers. As earlier demonstrated with nonoxidized fatty acids attached to TAG glycerol moiety,[16] oxidized fatty acids were cleaved statistically significantly more readily from *sn*-1/3 positions than from *sn*-2 position when the $[M + NH_4]^+$ ions were fragmented (Table 2).

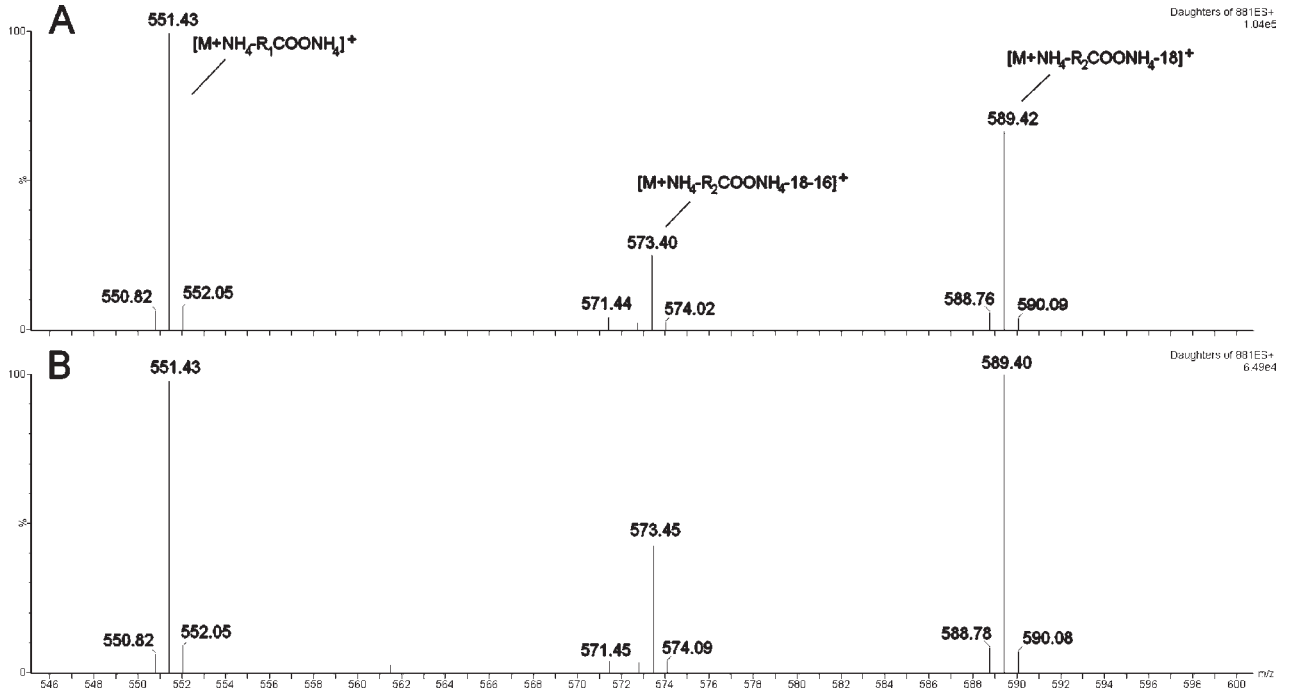

Figure 4. Examples of mass spectra showing fragment ions formed from TAG molecular species containing a hydroperoxyl group: (A) TAG 50:2 tOOH, oxidized linoleic acid in *sn*-1/3 position; (B) TAG 50:2 tOOH, oxidized linoleic acid in *sn*-2 position. See Table 2 for abbreviations. R_1COONH_4 and R_2COONH_4 denote ammoniated linoleic and palmitic acids, respectively.

Table 3. Proportions of Different $[AB]^+$ Ions Formed from Different Isomers (Peaks Resolved in Liquid Chromatography) of Oxidized TAG Molecules in Which the Oxidized Fatty Acid Is Present in the (A) *sn*-1/3 Position (*sn*-1/3 Regioisomers) or (B) *sn*-2 Position (*sn*-2 Regioisomers)[a]

	fragment ion[b]							
	$[AB_1]^+$		$[AB_2]^+$				$[AB_3]^+$	
original TAG[c]	peak 1	peak 2	peak 1	peak 2	peak 3	peak 4	peak 1	peak 2
(A) *sn*-1/3 Position (*sn*-1/3 Regioisomers)								
50:2 OH			50.6 ± 4.2 a	50.8 ± 6.1 a	50.6 ± 2.2 a	47.2 ± 3.3 a		
50:2 epoxy	31.7 ± 0.8 a	17.5 ± 0.8 b	33.4 ± 0.5 a	34.7 ± 0.8 b				
50:1 OOH			27.5 ± 0.8 a	25.7 ± 1.3 a			34.5 ± 1.1 a	31.1 ± 0.2 b
50:1 OH			35.7 ± 1.2 a	30.1 ± 0.4 b				
50:1 keto	82.4 ± 2.3 a	76.9 ± 0.4 b						
(B) *sn*-2 Position (*sn*-2 Regioisomers)								
50:2 OH			65.7 ± 2.5 ab	69.1 ± 3.9 a	69.2 ± 0.9 a	61.8 ± 1.4 b		
50:2 epoxy	38.0 ± 0.7 a	22.9 ± 0.7 b	40.5 ± 0.2 a	42.1 ± 0.8 b				
50:1 OOH			44.1 ± 2.0 a	40.7 ± 4.4 a			54.0 ± 3.6 a	47.6 ± 1.0 b
50:1 OH			63.8 ± 0.9 a	56.5 ± 1.2 b				
50:1 keto	84.5 ± 0.7 a	76.8 ± 1.8 b						

[a] Proportions are calculated as percentages of the individual $[AB_x]^+$ ion of the sum $[AA]^+ + [AB_x]^+$, where A denotes palmitic acid and B denotes oxidized fatty acid. Different letters in a row (within the same $[AB_x]^+$ ion) indicate significant differences between the isomeric peaks ($P < 0.05$). Data on those compounds for which chromatographic resolution allowed comparison of isomers is included. [b] $[AB_1]^+$ denotes ion $[M + NH_4 - RCOONH_4]^+$; $[AB_2]^+$ denotes ion $[M + NH_4 - RCOONH_4\text{-}18]^+$; $[AB_3]^+$ denotes ion $[M + NH_4 - RCOONH_4 - 18 - 16]^+$. $RCOONH_4$ denotes ammoniated palmitic acid. [c] See Table 2 for abbreviations.

In the $[AB]^+$ DAG fragment ions formed, the oxidized fatty acid moieties remained intact in the case of 18:2 keto (Figure 3), 18:1 keto, 18:2 diepoxy, and 18:1 epoxy only. The $[AB]^+$ DAG fragment ions of the molecules originally containing 18:2 OOH or 18:1 OOH consisted of the ions $[M + NH_4 - COONH_4 - 18]^+$ and $[M + NH_4 - COONH_4 - 18 - 16]^+$ (Figure 4), and the $[AB]^+$ DAG fragment ions of the molecules originally containing 18:2 OH or 18:1 OH consisted of the ion $[M + NH_4 - COONH_4 - 18]^+$,

$COONH_4$ representing a loss of an ammoniated palmitic acid residue. In addition to the $[AB]^+$ DAG fragment ions with intact oxidized fatty acid moieties ($[M + NH_4 - COONH_4]^+$ ion), ion $[M + NH_4 - COONH_4 - 18]^+$ was formed from the molecules originally containing 18:2 diepoxy or 18:1 epoxy.

It is also interesting that, within a particular *sn* regioisomer of most of the oxidized TAG species, differences in terms of the selectivity of cleavage from the glycerol backbone were found between molecular species containing the oxygen group in different positions within the linoleic or oleic acid moiety. The oxidized TAG species in which this selectivity was discovered are the following: TAG 50:2 OH (when oxidized linoleic acid is present in the *sn*-2 position), TAG 50:2 diepoxy (when oxidized linoleic acid is present in the *sn*-1/3 or *sn*-2 position), TAG 50:1 OOH (when oxidized oleic acid is present in the *sn*-1/3 or *sn*-2 position), TAG 50:1 OH (when oxidized oleic acid is present in the *sn*-1/3 or *sn*-2 position), and TAG 50:1 keto (when oxidized oleic acid is present in the *sn*-1/3 or *sn*-2 position) (Table 3). Differences between these isomers were not studied in case of TAGs 50:2 tOOH (only one chromatographic peak), 50:2 OOH, 50:2 keto (overlapping peaks), and 50:1 epoxy (only one chromatographic peak).

The novel UHPLC method proved to be an efficient approach in resolving different regioisomers of various oxidized TAG species, although some overlapping was still present. The tandem mass spectrometric approach utilizing modified nebulizer gas composition allowed regiospecific analysis of oxidized TAG isomers and demonstrated interesting differences in the fragmentation patterns of these isomers. The addition of ammonia directly into the nebulizer gas proved to be less labor intensive than the earlier utilized addition as postcolumn solvent flow; the ions formed were nonetheless similar. The chromatographic separation and regiospecific characteristics can be utilized in both qualitative and semiquantitative analyses of oxidized TAG molecules typically present in various foods.

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ABBREVIATIONS USED

APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; DAG, diacylglycerol; LDL, low-density lipoprotein; *sn*, stereospecific numbering; TAG, triacylglycerol; UHPLC, ultra-high-performance liquid chromatography; VLDL, very-low-density lipoprotein.

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