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Effects of Antioxidants on Rapeseed Oil Oxidation in an Artificial Digestion Model Analyzed by UHPLC–ESI–MS

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Supporting Information

ABSTRACT: A normal diet contains large quantities of oxidized fatty acids, glycerolipids, cholesterol, and their cytotoxic degradation products because many foods in the diet are fried, heated, or otherwise processed and consumed often after long periods of storage. There is also evidence that the acid medium of the stomach promotes lipid peroxidation and that the gastrointestinal tract is a major site of antioxidant action, as demonstrated by various colorimetric methods. The identity and yields of specific products of lipid transformation have seldom been determined. The present study describes the molecular species profiles of all major gastrointestinal lipids formed during digestion of autoxidized rapeseed oil in an artificial digestion model in the presence of L-ascorbic acid, 6-palmitoyl-*O*-L-ascorbic acid, 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT), DL-α-tocopherol, and DL-α-tocopheryl acetate. Differences in oxidized lipid profiles were detected in the samples digested in the presence of different antioxidants, but none of them could prevent the formation of oxidized lipids or promote their degradation in a gastric digestion model. The lack of effect is attributed to the inappropriate nature of the gastrointestinal medium for the antioxidant activity of these vitamins and BHT. A fast ultrahigh performance liquid chromatographic–electrospray ionization–mass spectrometric method was developed for the analysis of lipolysis products, including epoxy, hydroperoxy, and hydroxy fatty acids, and acylglycerols, utilizing lithium as ionization enhancer.

KEYWORDS: *antioxidant activity, digestion, lithiated adducts, oxidized lipids, ultrahigh performance liquid chromatography–mass spectrometry*

INTRODUCTION

Low erucic acid rapeseed oil, often referred to as Canola oil, is a widely used vegetable oil in Europe and North America, rich in unsaturated $n-3$ and $n-6$ family fatty acids (UFAs), especially the essential α-linolenic and linoleic acids. Unfortunately, double bonds in UFAs sensitize them also for autoxidation, which can eventually produce highly toxic compounds such as hydroxyalkenals, malondialdehyde, leukotoxins, and other oxygenated reactive compounds.[1,2] General mechanisms of lipid oxidation are well-known, but the oxidation of lipids during digestion has been less well investigated. It has been previously found, that the stomach acts as an oxidizing bioreactor[3] and that ingested hydroperoxides are broken down to aldehydes, alcohols, and ketones during digestion.[4,5] It is also known that epoxidized lipids are altered under acidic conditions of the gut, forming vicinal diol structures.[6] Furthermore, volatile oxidation products of heat treated oil in a simulated digestive system have been determined, and it was assessed that several toxic compounds could be available for absorption.[7]

A recent review points out that flavonoids and carotenoids exert antioxidant effects in the human body and that the place where they are most likely to do so is within the gastrointestinal (GI) tract.[8] However, in the presence of high concentrations of polyphenol antioxidants, hydroperoxides are decomposed mostly to hydroxyl compounds and not to genotoxic and cytotoxic aldehydes.[3,9,10]

Tocopherols can prevent the autoxidation of triacylglycerols (TAGs) in stored rapeseed oil,[11] but the true efficacy of these antioxidants during the digestion of lipids is largely unknown. A role for ascorbate and tocopherols as scavengers of reactive species is feasible in the GI tract, particularly in subjects consuming vitamin E supplements, when considerable amounts of tocopherols may remain unabsorbed and reach the colon.[12] Tocopherols can react not only with reactive oxygen species such as peroxyl radicals but also with reactive nitrogen species.[13] The actions and products of the above antioxidants have been assessed in the past mainly by colorimetric methods,[14,15] which frequently have given contradictory findings. In the absence of a standard assay, it is difficult to compare the results reported from different research groups.

Specialized liquid chromatography–mass spectrometric methods may provide better answers to the questions of the formation of oxidized lipids than generic colorimetric assays.[16,17] Suomela et al. have employed high performance liquid chromatography–electrospray ionization–mass spectrometry (HPLC–ESI–MS) in the quantitative analysis of oxidized triacylglycerols in lipoproteins and intestinal epithelial cells.[18,19] Recently, Tarvainen et al. have described an ultrahigh performance liquid chromatography (UHPLC)–ESI–MS method, which enables accurate and sensitive determination of oxidized and nonoxidized molecular species of lipids present in digested and processed samples with minimal sample preparation.[20]

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In the present study, this method has been further improved by including lithium ions as ionization enhancers in ESI−MS. Lithium has been previously used for tandem mass spectrometric analysis of glycerophosphocholine and triacylglycerols, but to our knowledge, this is the first time it has been applied to the simultaneous analysis of oxidized free fatty acids and acylglycerols by LC−MS.[21,22] It was the purpose of this study to apply the new method to an examination of the effects of some common antioxidants during the digestion of oxidized and unoxidized rapeseed oil in an artificial digestion model.

MATERIALS AND METHODS

Chemicals. All solvents were of HPLC grade or when adequate, *p.a.* grade. HPLC grade water was prepared with a Millipore Milli-Q water purification system (Millipore SA, Molsheim, France). Porcine pepsin, pancreatic lipase, pancreatin, α-amylase from *Aspergillus oryzae*, and bovine serum type II albumin were purchased from Sigma-Aldrich Co. (St. Louis, MO). Mucin was purchased from Carl Roth GmbH (Karslruhe, Germany). 1(3)-Monopalmitoyl-*sn*-glycerol (>99%), 1(3)-monostearoyl-*sn*-glycerol (>99%), 1(3)-monooleoyl-*sn*-glycerol (>99%), 1(3)-monolinoleoyl-*sn*-glycerol (>99%), 1(3)-monolinolenoyl-*sn*-glycerol (>98%), 1,2(2,3)-dipalmitoyl-*sn*-glycerol (>99%), 1,3-dipalmitoyl-*sn*-glycerol (>99%), 1,3-dioleoyl-*sn*-glycerol (>99%), 1,3-dilinoleoyl-*sn*-glycerol (>99%), and 1,3-dilinolenoyl-*sn*-glycerol (>99%) were purchased from Larodan Fine Chemicals AB (Malmö, Sweden). Saturated even carbon number free fatty acids (C10−C24), palmitoleic, oleic, linoleic, and α-linolenic acids, and trinonadecanoyl-*sn*-glycerol were purchased from Sigma-Aldrich Co. Triacylglycerol mixture (GLC HPLC # G-1) was purchased from Nu-Check Prep Inc. (Elysian, MN). The mixture was composed of saturated TAGs from tricapryloylglycerol to tristearoylglycerol, also including tripalmitoleoylglycerol, trioleoylglycerol, trilinoleoylglycerol, and trilinolenoylglycerol. L-Ascorbic acid, 6-palmitoyl-*O*-L-ascorbic acid, 3,5-di-*tert*-butyl-4-hydroxytoluene, DL-α-tocopherol, and DL-α-tocopheryl acetate were purchased from Sigma-Aldrich Co. Rapeseed oil (Kultasula, Ravintoraisio Oy, Raisio, Finland) was purchased from a local grocery store.

Oxidized Oils and Reference Compounds. Chemically and thermally oxidized rapeseed oils were used from previous experiments.[20] 12-Hydroxyoctadecanoic acid (>98%) was obtained from Larodan Fine Chemicals AB. Hydroperoxidized α-linolenic acid, linoleic acid, 1(3)-monooleoyl-*sn*-glycerol, 1(3)-monolinoleoyl-*sn*-glycerol, 1(3)-monolinolenoyl-*sn*-glycerol, 1,3-dioleoyl-*sn*-glycerol, and 1,3-dilinoleoyl-*sn*-glycerol were obtained by photosensitized oxidation with methylene blue as described earlier.[23] Epoxidized α-linolenic acid and linoleic acid were prepared according to Deffence[24] by reaction of pure fatty acid with 3-chloroperoxybenzoic acid (Sigma Aldrich Co.) as described earlier.[23] Hydroxy fatty acids and hydroxy monoacylglycerols (MAGs) were prepared by reduction of freshly synthesized hydroperoxidized fatty acids and MAGs with triphenylphosphine (Sigma Aldrich Co.)

Partial Hydrolysis of Rapeseed Oil. It was estimated that up to 20% of the TAGs in the stomach would be hydrolyzed by gastric lipase.[25] To simulate the effect of gastric lipase, which was unavailable to us, free fatty acids (FFAs) and diacylglycerols (DAGs) were hydrolyzed and purified from rapeseed oil and added into one set of samples to be subjected to an artificial digestion model. FFAs and DAGs were prepared as follows: fresh oil (100 mg) was subjected to hydrolysis by pancreatin, pancreatic lipase, and bile salts in purified water for 1 h (volume 11 mL). The mixture was filtered through a filter paper (Whatman No. 2, Sigma Aldrich Co.), and the filter paper and any residue was washed with methanol (15 mL) and chloroform (30 mL). The resulting liquid was transferred into a separatory funnel, and after separation of the two layers, the lower organic layer was collected. After evaporation of solvents with nitrogen stream, FFAs, DAGs, MAGs, and TAGs were separated by TLC (hexane/diethyl ether/formic acid, 80:20:2, by vol). FFAs and DAGs were detected by dyeing with fluoresceine, scraped off the plate, extracted with isopropanol, filtered with a 0.2 μm PTFE syringe filter (Whatman Spartan, Sigma Aldrich Co.), and stored under nitrogen for subsequent experiments

Artificial Digestion Model. The artificial digestion model that simulates the digestive processes in humans has been described previously in detail elsewhere.[23,26,27] Briefly, a lipid free standard meal (900 mg) was mixed with oil (40 mg), internal standard (2 mg of 12-hydroxyoctadeanoic acid), and different antioxidants, in a test tube. Tables 1 and 2 describe the amounts and different antioxidant combinations used in the two experiments. Three distinctive oils were used in the first experiment (triplicate samples): fresh rapeseed oil, chemically oxidized rapeseed oil, and thermally oxidized rapeseed oil. Also, to simulate the effects of gastric lipase, we added FFAs (2.5 mg) and DAGs (5.5 mg) into two sets of samples (32.0 mg of fresh rapeseed oil) with and without α-tocopherol in the first experiment (see Table 1). In the second experiment, only fresh rapeseed oil was used, but 10 parallel digestions were made of each antioxidant addition.

Table 1. Samples[a] Prepared in the Preliminary in Vitro Digestion Study for Mixing with the Standard Meal

I. Fresh rapeseed oil (40 mg)[b] II. Chemically oxidized rapeseed oil III. Thermally oxidized rapeseed oil	
antioxidant addition	amount of added antioxidant (% of oil)
1. no added antioxidant	
2. FFAs and DAGs without added antioxidant[c]	
3. FFAs and DAGs with DL-α-tocopherol[c]	0.0125
4. DL-α-tocopherol, high	1.2500
5. DL-α-tocopherol (T)	0.0125
6. DL-α-tocopheryl acetate (TA)	0.0125
7. 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT)	0.0125
8. 6-palmitoyl-*O*-L-ascorbic acid (PA)	0.0125
9. T + PA	0.0250[d]
10. T + TA	0.0250[d]
11. T + BHT	0.0250[d]
12. TA + BHT	0.0250[d]
13. TA + PA	0.0250[d]
14. PA + BHT	0.0250[d]

[a]Three replicates of each antioxidant combination. (12 × 3 + 2) × 3 separate digestions in total. [b]Natural vitamin E content in the rapeseed oil was 0.025%. [c]Free fatty acids (FFAs) and diacylglycerols (DAGs) added to fresh rapeseed oil (I) only. [d]Combined concentration of the added antioxidants.

Table 2. Samples[a] Prepared in the Second in Vitro Digestion Study with Fresh Rapeseed Oil for Mixing with the Standard Meal

antioxidant addition	amount of added antioxidant (% of oil)	
1. no added antioxidant[b]		
2. DL-α-tocopherol (T)	0.01	0.10
3. DL-α-tocopheryl acetate	0.01	0.10
4. 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT)	0.01	0.10
5. L-ascorbic acid (AA)	0.01	0.10
6. 6-palmitoyl-*O*-L-ascorbic acid (PA)	0.01	0.10
7. T + AA	0.01[c]	0.10[c]
8. T + BHT	0.01[c]	0.10[c]
9. T + PA	0.01[c]	0.10[c]

[a]Ten replicates of each antioxidant level. 17 × 10 digestions in total. [b]Natural vitamin E content in the rapeseed oil was 0.025%. [c]Combined concentration of the added antioxidants.

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In total, 114 digestions were made in the preliminary experiment, and a total of 170 digestions were made in the second experiment. Next, digestive fluids were added for the first phase of incubation, simulating the processes in the stomach. After 1.5 h of incubation in a shaker (600 U min^{-1} at 37 °C), the remaining digestive fluids were added, and incubation was continued for 2.5 h (V_{tot} = 4.7 mL). The digestive fluids are as follows: artificial saliva, gastric fluid, bile, and intestinal fluid, contained hydrochloric acid, pepsin, amylase, pancreatic lipase, pancreatin, bile salts, other organics, and inorganic compounds.[23,26,27]

Extraction of the Digested Samples. Two parallel samples (0.75 mL each) of one digestion tube were taken to a slightly modified Folch extraction in the second experiment. Only one extraction of single digestion was made in the preliminary experiment. Methanol (1 mL) and chloroform (2 mL) were added to the digested samples in disposable glass test tubes, which were flushed with nitrogen gas and capped. After thorough mixing, samples were centrifuged (1100*g*, 10 min), the lower phases were collected, and the extraction was repeated by adding pure lower phase (2 mL) to the remaining upper phases. The lower phases were combined, and after evaporation of solvents by nitrogen stream, the residues were dissolved in isopropanol (1 mL) and frozen (−80 °C) for a short period of time until UHPLC−ESI−MS analyses.

Ultrahigh Performance Liquid Chromatography−Electrospray Ionization−Mass Spectrometry. A Waters Acquity UPLC (Waters Corp., Milford, MA) was used as an LC inlet for a Waters Quattro Premier tandem mass spectrometer. MassLynx v4.1 and QuanLynx (Waters Corp.) were used for the collection and analysis of mass chromatograms and spectra. A reversed phase 2.1 × 100 mm (1.8 μm particle size) Phenomenex Kinetex C18 column (Phenomenex Inc., Torrence, CA) was used for the chromatographic separation of oxidized and unoxidized free fatty acids and acylglycerols. A column oven was utilized and set at 60 °C. Solvent A was composed of acetonitrile/H_2O/HCOOH (50:50:0.1, by vol) and solvent B of acetone/HCOOH (100:0.1, by vol). Both solvents contained also lithium formate (1 mM) as ionization enhancer. Initial gradient of 1% B was increased to 99% B in 14.00 min and back to 1% B in 14.05 min with a constant level of 1% B until 16.50 min. The flow rate was 0.90 mL min^{-1}, and the injection volume was 3 μL.

The gradient method developed was based on our earlier UHPLC−ESI−MS method for total lipid analysis.[20] The solvent gradient was adjusted for the new column type (previously Waters BEH C18, 2.1 × 100 mm, 1.7 μm particle size) and the different solvent B. In the previous gradient system, we used isopropanol as the strong solvent because it produced less noise with mass spectrometric detection compared to acetone. This was, however, changed when lithium formate was added into the LC solvents. Excessive noise was then detected also with isopropanol when scanning a large mass window (*m*/*z* 100−1500). Acetone, however, produced noise mostly in the lower *m*/*z* region. The problem was solved by splitting the MS program into two parts. The first part had lower cone voltage value, which increased the signal intensity of lithiated FFAs and MAGs. The second part had higher cone voltage and subsequently lower noise. Tuning was otherwise optimized for the simultaneous analysis of free fatty acids and acylglycerols. ESI capillary voltage was set at 4.00 kV. Cone voltage was initially 60 V and after 8.70 min 350 V. RF lens voltage was set at 0.2 V and extractor voltage at 8 V. Ion source temperature was set at 100 °C and desolvation temperature at 400 °C. Desolvation gas (N_2) flow was set at 900 L h^{-1} and cone gas (N_2) flow at 400 L h^{-1}. The mass spectrometer was mass calibrated with water clusters for *m*/*z* 50−1800 and scanning speeds of 300−5000 amu s^{-1}. Mass spectra was collected initially of ions with *m*/*z* 165−800 and after 8.70 min ions with *m*/*z* 350−1100.

Preliminary studies were conducted using the UHPLC−ESI−MS method we developed earlier.[20] All the samples in the preliminary studies were analyzed both in positive and negative ionization modes. Conditions for the negative ionization ESI−MS were as follows: scan range was *m*/*z* 150−400, capillary voltage was set at 3.00 kV, cone voltage at 40 V, extractor voltage at 4.0 V, and rf lens voltage at 0.6 V.

Quantitation. Oxidized compounds were quantitated from mass chromatograms obtained from selected ion extraction from the total ion profiles by the MassLynx software. Results of parallel digestions were averaged. A series of oxo-lipid standards served as an aid for identification and for calibration of the instrument response. Since complete purification of the sensitive oxidized compounds was difficult to obtain, absolute correction factors could not be obtained for all compounds. Instead, total areas of oxidized molecules were used for comparisons of the effects of the different antioxidants. The quantitative data were normalized by reference to palmitic or 12-hydroxyoctadecanoic acid to compensate for differences in degrees of hydrolysis of parallel digestions. Saturated fatty acids are unaffected by oxidation under mild conditions and therefore can be used to correct for increased molecular weight of oxidized hydrolysis products.

Statistical Analysis. Data from the experiments was analyzed by one-way analysis of variance (ANOVA) with posthoc Tukey's HSD for statistical differences between the groups. *P*-values of less than 0.05 were considered statistically significant. IBM SPSS Statistics version 19 (IBM Corporation, New York) was used for the statistical calculations.

RESULTS

Preliminary studies. Preliminary studies on the effect of different antioxidants on triacylglycerol degradation in the artificial digestion model were conducted using our earlier developed UHPLC−ESI−MS method of product analysis.[20] A total 114 of assays, excluding blanks and reference compound analyses, were performed with three oil preparations in the presence of the types and amounts of antioxidants listed in Tables 1 and 2. As an illustration, Figure 1 shows the total ion current (TIC) profiles of fresh rapeseed oil (A), thermally oxidized rapeseed oil (B), and chemically oxidized rapeseed oil (C). The TIC profiles of the oils digested (4 h) in presence of DL-α-tocopherol are shown as follows: digested fresh rapeseed oil (D), digested thermally oxidized rapeseed oil (E), and digested chemically oxidized rapeseed oil (F). The peaks were identified as discussed by Tarvainen et al.[20] In addition to positive ionization ESI−MS, the samples were also analyzed in negative ionization mode.

In addition to hydroperoxides, special attention was given to epoxides since it had been reported that epoxidized lipids are altered under acidic conditions of the gut, forming vicinal diol structures.[6] Figure 2 shows the detection of epoxidized free fatty acids in a sample of digested thermally oxidized rapeseed oil: enlarged section of positive ionization TIC profile (A), selected ion mass chromatogram and possible structures of the epoxidized fatty acids (B), negative ionization TIC profile (C), and negative ionization selected ion mass chromatogram (D). Thus, significant amounts of different constitutional isomers of saturated 18 carbon monoepoxy fatty acid (*m*/*z* 321.3 corresponding to $[M + Na]^+$ adduct and *m*/*z* 297.4, corresponding to $[M - H]^-$ ion) and 18 carbon monoepoxy fatty acid with one remaining double bond (*m*/*z* 319.3 corresponding to $[M + Na]^+$ adduct and *m*/*z* 295.4 corresponding to $[M - H]^-$ ion) were identified in the samples. Small amounts of monoepoxy octadecadienoic acid was also detected at *m*/*z* 293.4 and *m*/*z* 317.3. On the basis of both negative and positive ionization MS analyses, there were approximately equal amounts of isomers of monoepoxy octadecanoic and monoepoxy octadecenoic acids present. The combined amounts of the three epoxidized free fatty acids reflected the overall amounts of oxylipids in the digested thermally treated oil samples as seen in Figure 3 (primary data not shown). Only in samples with high tocopherol addition were the concentrations lower than in samples without any added antioxidants. In samples with DL-α-tocopherol and BHT,

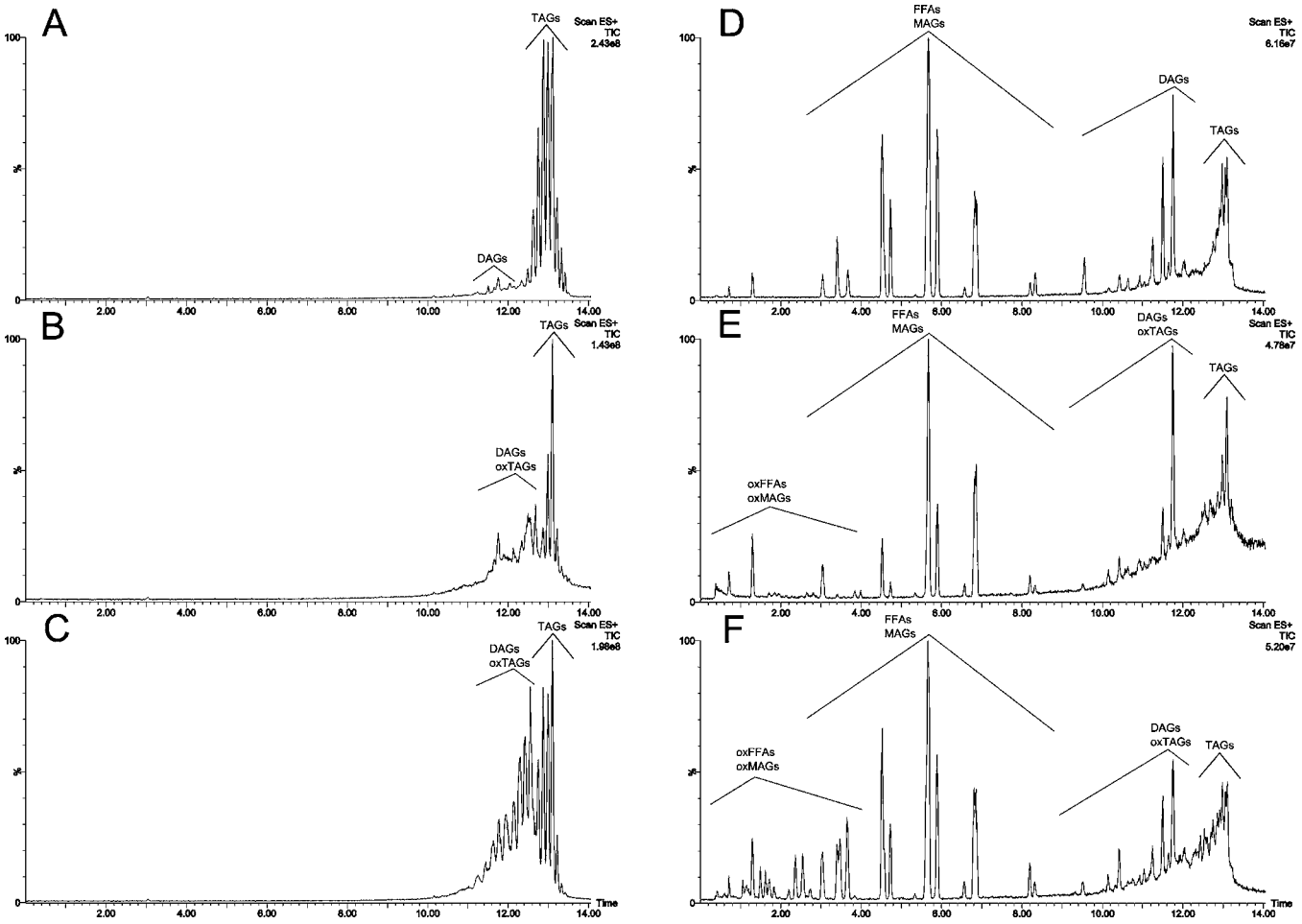

Figure 1. Total ion current (TIC) mass chromatograms of fresh rapeseed oil (A), thermally oxidized rapeseed oil (B), chemically oxidized rapeseed oil (C), digested fresh rapeseed oil (D), digested thermally oxidized rapeseed oil (E), and digested chemically oxidized rapeseed oil (F). FFAs, free fatty acids; DAGs, diacylglycerols; MAGs, monoacylglycerols; TAGs, triacylglycerols; ox, oxidized.

there were equal amounts of epoxidized FFAs compared to the digested samples without added antioxidants. The rest of the antioxidant additions increased the amounts of epoxidized FFAs 2- to 3-fold. The difference in the fate of epoxidized lipids compared to the previous report[6] may be attributed to the short exposure time to the acidic medium in our experiments.

Figure 3 shows the amounts of oxidized lipids in the digested oxidized rapeseed oils. Normalized amounts of detected oxidized MAGs in the digested thermally oxidized rapeseed oil are shown in A, oxidized FFAs in digested thermally oxidized rapeseed oil in B, oxidized MAGs in digested chemically oxidized rapeseed oil in C, and oxidized FFAs in chemically oxidized rapeseed oil in D. Addition of a high amount of DL-α-tocopherol (T, high) had varying results. In the case of thermally oxidized rapeseed oil, high amounts of oxidized MAGs were detected but little oxidized FFAs. In the case of chemically oxidized rapeseed oil, high amounts of oxidized FFAs were detected and low amounts of oxidized MAGs. High amount of antioxidants perhaps selectively stabilizes some oxidized molecules found in the oxidized oils. Other tested antioxidants had generally no positive (decreasing) effect on the amounts of oxidized lipids detected. The amounts of oxidized FFAs and MAGs were on a similar level or higher than that in the samples without any added antioxidant. Small addition of DL-α-tocopherol (T) increased the amount of oxidized lipids in all samples. An explanation of this phenomenon might be that primary oxidation products are rapidly decomposed to secondary oxidation products in the artificial digestion model but that the addition of antioxidants alters the decomposition pathways resulting in different end-products. Specifically, the action of tocopheryl acetate (TA) was interesting, as the acetyl group would need to be hydrolyzed before any antioxidative effects can be expected. Thermally oxidized digested rapeseed oil samples containing TA had, on average, more oxidized lipids than samples without any added antioxidants. However, chemically oxidized rapeseed oil samples had equal amounts of oxylipids in samples with and without TA. No oxidized lipids were detected after digestion of native rapeseed oil samples and samples with added FFAs and DAGs. Because the amounts of oxidized lipids generated during the digestion of rapeseed oil were small and challenging to determine, a more sensitive lithium adduct based detection method was developed.

High Resolution Lithium Adduct UHPLC–ESI–MS Analysis. Figure 4 shows the UHPLC–ESI–MS TIC profile of the Li adducts of the unoxidized reference compound mixture (A) and a digested sample of fresh rapeseed oil (B). Overlaid and vertically aligned scans are from 0.00–8.70 min and from 8.70–14.6 min. The peak identification is given in Table 3, which also contains the validation data for the various reference compounds, including hydroxyl fatty acids, partial acylglycerols, and triacylglycerols as the Li adducts. The use of

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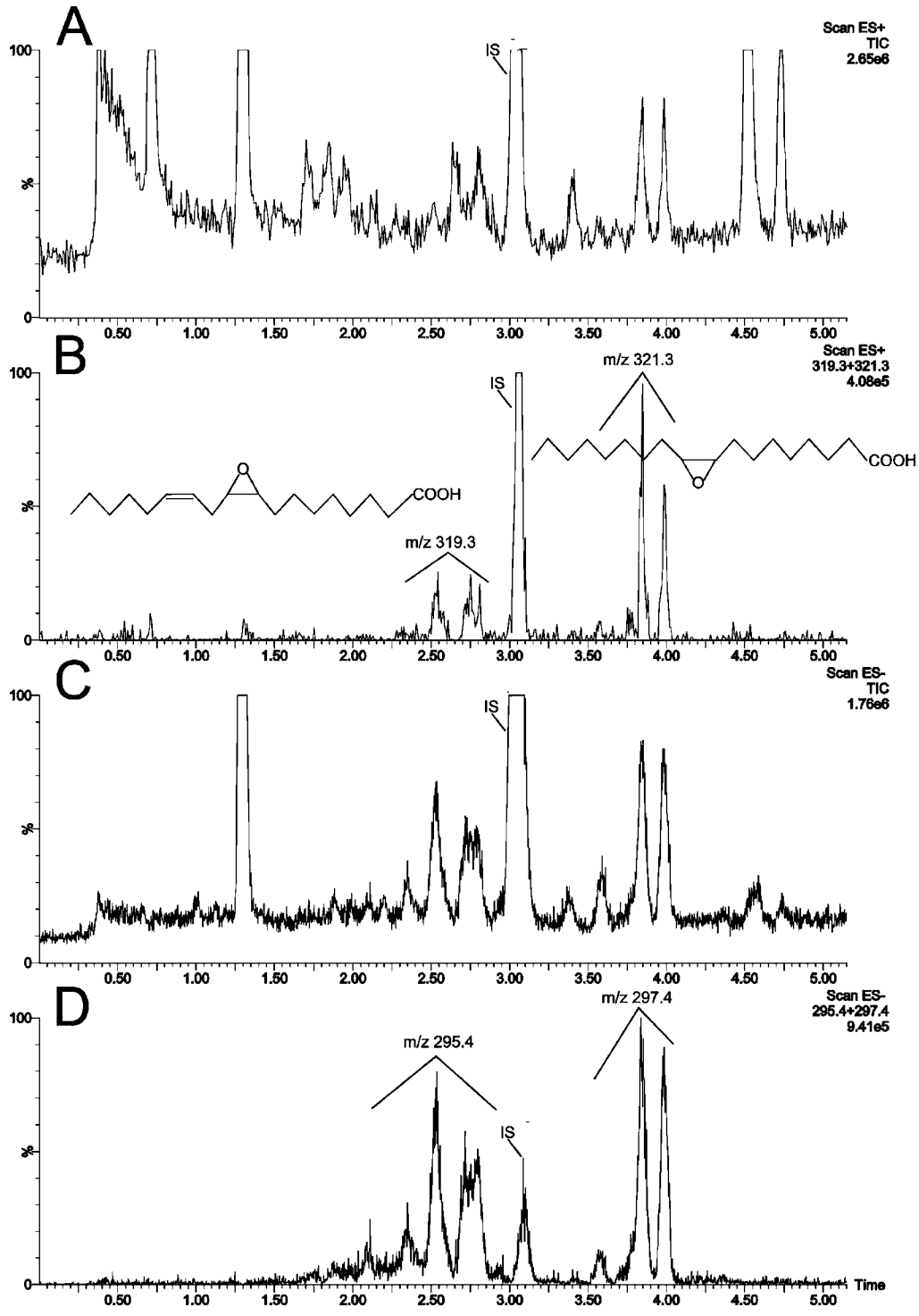

Figure 2. Enlargements of positive ionization total ion current (TIC) mass chromatogram (A), selected ion mass chromatogram and possible structures of epoxidized free fatty acids (B), negative ionization TIC mass chromatogram (C), and negative ionization selected ion mass chromatogram (D) of digested oven oxidized rapeseed oil. IS, internal standard.

fully linear gradient improved peak shapes and the chromatography as a whole over the previously used method.[20] The concentration of lithium formate in the solvents affected the intensity of the formed lithium adducts. Concentration of 1 mM was chosen over 0.1 mM as the ion intensity increased 5-fold with the higher lithium concentration. Sensitivity was assessed by injecting diminishing quantities of 12-hydroxyoctadecanoic acid whose detection limit was calculated. Less than 40 pg injection was clearly detectable. Sensitivity increase depended on the compound group and varied from 0 to up to 100-fold, when compared to detection limits of the sodiated adducts in the previous method.

FFAs produced mainly $[M + Li]^+$ and $[M + 2*Li - H]^+$ adducts with small amounts of $[M + Na]^+$ ions. When oxidized groups were present, also $[M - H_2O + Li]^+$ ions were formed, especially with hydroperoxides, for which the intensities of $[M - H_2O + Li]^+$ ions were higher than $[M + Li]^+$ ions. Hyrdoperoxyl groups are sensitive to in-source decomposition.

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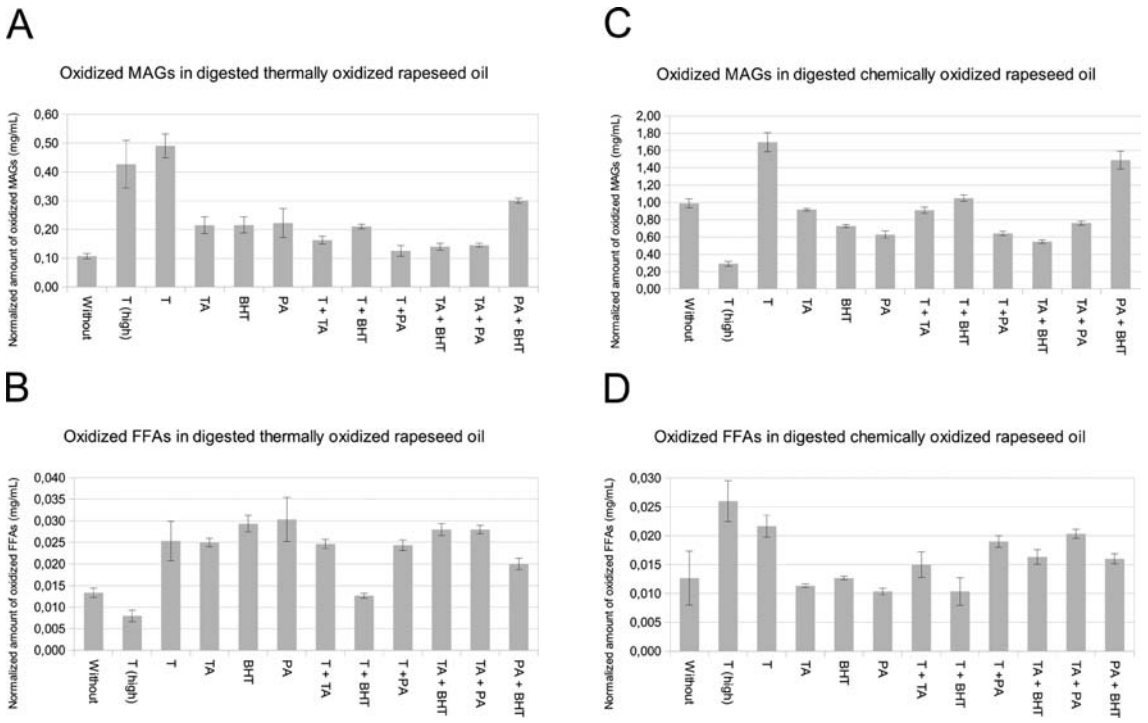

Figure 3. Normalized amounts of detected oxidized MAGs in digested thermally oxidized rapeseed oil (A), oxidized FFAs in digested thermally oxidized rapeseed oil (B), oxidized MAGs in digested chemically oxidized rapeseed oil (C), and oxidized FFAs in chemically oxidized rapeseed oil (D). T, DL-α-tocopherol; TA, DL-α-tocopheryl acetate; PA, 6-palmitoyl-*O*-L-ascorbic acid; BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene; FFAs, free fatty acids; MAGs, monoacylglycerols. Three parallel digestions and one analysis of each were made (n = 3).

Monoacylglycerols, diacylglycerols, and triacylglycerols formed mainly $[M + Li]^+$ adducts and $[M + 2*Li- H]^+$ adducts. MAGs and TAGs formed also $[M - FA + H]^+$ and $[M - FA + Li]^+$ adducts, where the FA describes the loss of one acyl group. This decomposition was not observed in earlier experiments with sodium adducts.

Ions formed from low energy collision-induced dissociation (CAD) of $[M + Na]^+$ and of $[M + NH_4]^+$ contain limited structural information, and structural details such as the position of the fatty acyl moieties on the glycerol backbone cannot be determined.[28] However, high energy CAD tandem MS of $[M + NH_4]^+$ and $[M + Na]^+$ adduct ions generated by ESI have been previously reported,[29] although the product ion spectra are rather complicated.[30] In contrast, low energy CAD product-ion spectra of the $[M + Li]^+$ ions of TAG contain abundant fragment ions that are applicable for structural identification and differentiation of regioisomers.[22] As a result, there is increased sensitivity of detection for the parent ions, although simultaneous analysis of Li adducts of oxidized fatty acids and acylglycerols has not been previously reported. The fatty acid is cleaved most easily from *sn*-1 or *sn*-3 positions of the glycerol backbone, which can help in the assignments of positional isomers of these molecules even without the use of true MS/MS. The cleavage of fatty acid groups from the glycerol backbone occurred readily with monoacylglycerols, and in the case of monoolein, *sn*-1 and *sn*-3 positions lost the fatty acid group 6.5 times more likely than the *sn*-2 position. The ion ratios of the cleaved fatty acids and the remaining intact monoacylglycerols ($[FA + Li]^+/[MAG + Li]^+$) were 3.7% for 2-monoolein-*sn*-glycerol and 24.0% for 1(3)-monoolein-*sn*-glycerol. Altogether, these ions helped in the identification of the molecular species present. See Supporting Information for figures of the spontaneous cleavage of acyl moieties from monoacylglycerol backbones.

The enlargement in Figure 4B shows a selected ion mass chromatogram of digested rapeseed oil without any added antioxidants. Peaks eluted between 1.70 and 2.00 min correspond to $[M + Li]^+$ adducts (m/z 379) and $[M - H_2O + Li]^+$ adducts (m/z 361) of constitutional isomers of monohydroxy monooleoylglycerol. Also, fully saturated monohydroxy monostearoylglycerol was detected in the digested rapeseed oil at m/z 381 and m/z 363 (chromatograms not shown).

Table 4 lists all the synthesized oxo-lipid reference standards along with their characteristic retention times and the most abundant ions detected. 12-Hydroxyoctadecanoic acid served as internal standard for the quantitation of unoxidized unsaturated fatty acids and monoacylglycerols.

Figure 5 shows the normalized amounts of oxidized lipids detected in digested fresh rapeseed oil with different antioxidant additions. Ten parallel digestions and two parallel extractions of each digestion were made. Samples were analyzed once (n = 20). No significant statistical differences were found among the groups.

In addition to epoxides, hydroxy fatty acids, and hydroxy monoacylglycerols, special attention was also given to the identification and quantitation of the fatty acid hydroperoxides. Figure 6 gives an example of the separation and ease of detection of hydroperoxidized linoleic acid as the Li adducts. Four different isomers could be partially resolved on the basis of selected ion mass chromatograms: TIC of hydroperoxidized linoleic acid (A), partial resolution of isomers, as revealed by selected ion mass chromatograms (B), and the mass spectra of selected chromatographic peaks (C). Hydroperoxides and hydroxides of FFAs decomposed in the ion source and formed mainly $[M + Li - H_2O]^+$ and $[M + 2*Li - H - H_2O]^+$ ions.

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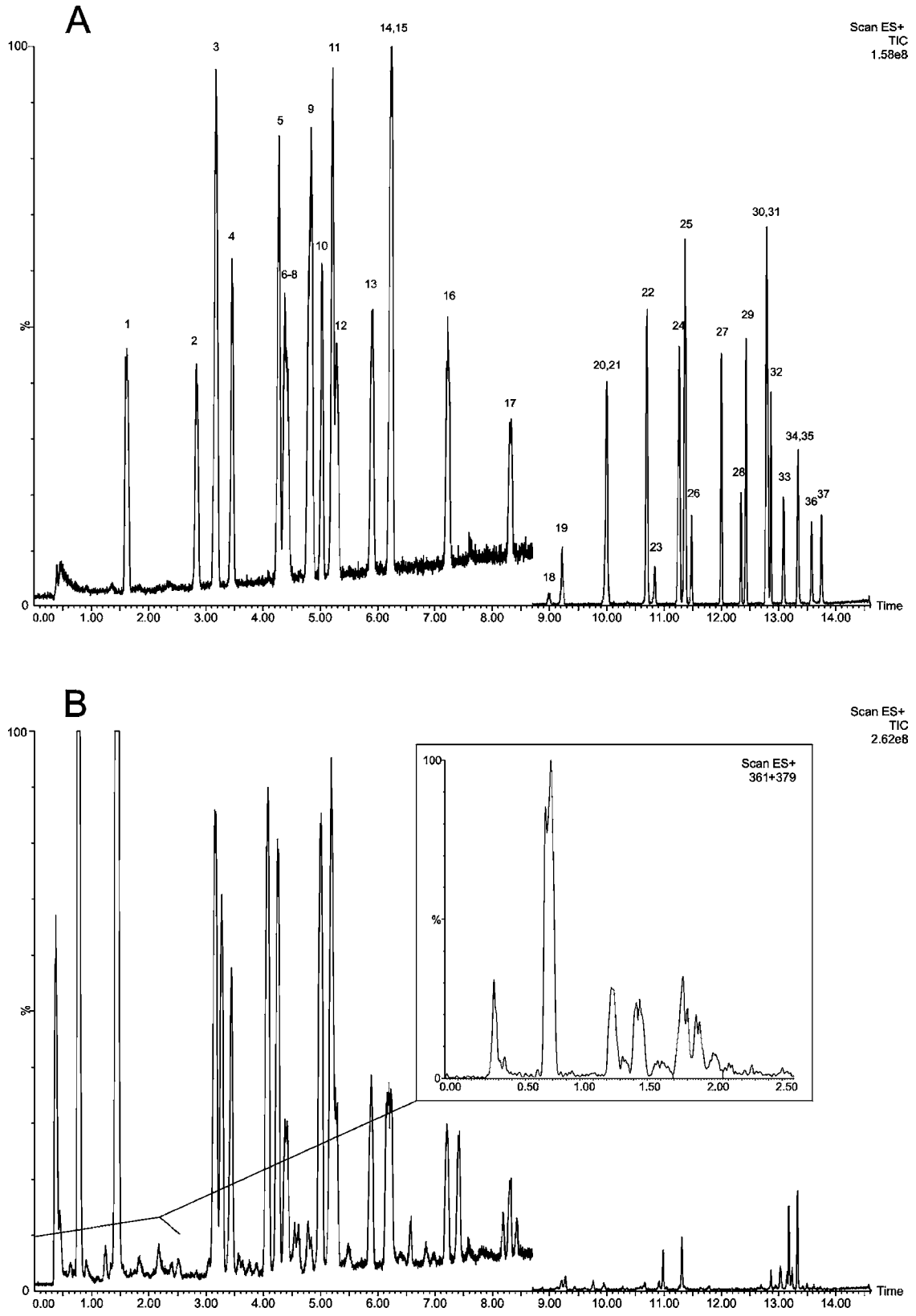

Figure 4. Total ion current (TIC) mass chromatograms of unoxidized reference compounds (A) and digested rapeseed oil without any added antioxidants (B). Enlarged selected ion mass chromatogram (m/z 361 + 379 correspond to hydroxy monoolein) is shown in panel B. Overlaid and vertically aligned scans of 0.00−8.70 min and 8.70−14.60 min. See Table 3 for peak listings. Lithium was used as ionization enhancer.

Previous experiments have shown that 9-hydroperoxy-*trans*-10-*cis*-12-octadecadienoic acid, 10-hydroperoxy-*trans*-8-*cis*-12-octadecadienoic acid, 12-hydroperoxy-*cis*-9-*trans*-13-octadecadienoic acid, and 13-hydroperoxy-*cis*-9-*trans*-11-octadecadienoic acid are the main products of photo-oxidation of linoleic acid.[30] Importantly, the small amounts of hydroxyoctadecadienoic acids

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Table 3. Validation Data and the Most Abundant Positive Ions of the Unoxidized Reference Compounds

peak no.	compound	ACN:DBN[a]	t_R (min)	RSD[b] (%)	RRT[c]	area[d] (av)	RSD[b] (%)	linear equation[e] (y = ng/μL, x = area)	R^2	LOD[f] (pg)	LOQ[f] (pg)	M_{mono}[g] (Da)	$[M + Li]^+$ $(m/z)^h$	$[M + 2 * Li - H]^+$ $(m/z)^h$	$[M - H_2O + H]^+$ $(m/z)^h$	$[M - H_2O + Li]^+$ $(m/z)^h$	$[M - FA + H]^+$ $(m/z)^h$	$[M - FA + Li]^+$ $(m/z)^h$
1	capric acid	10:0	1.62	0.08	0.51	31159	5.11	$y = 0.00100x - 3.9870$	0.9938	158.1	527.2	172.2	179.1	185.2	–	–	–	–
2	myristic acid	12:0	2.83	0.07	0.89	35983	4.24	$y = 0.00030x + 0.2411$	0.9905	67.1	223.7	200.2	207.2	213.2	–	–	–	–
3	12-hydroxystearic acid	18:0	3.17	0.11	1.00	616732	3.58	$y = 0.00002x - 1.1547$	0.9969	40.3	134.3	300.3	307.3	313.3	283.3	289.3	–	–
4	1(3)-monolinolenoyl-*sn*-glycerol	18:3	3.45	0.09	1.09	412008	4.38	$y = 0.00004x - 3.4438$	0.9957	33.8	112.8	352.3	359.3	365.3	–	–	–	–
5	1(3)-monolinoleoyl-*sn*-glycerol	18:2	4.28	0.12	1.35	526719	2.82	$y = 0.00003x - 3.5179$	0.9950	32.8	109.5	354.3	361.3	367.4	–	–	–	–
6	lauric acid	14:0	4.38	0.11	1.38	33304	4.77	$y = 0.00006x - 4.3960$	0.9947	114.1	380.6	228.2	235.2	241.2	–	–	–	–
7	α-linolenic acid	18:3	4.40	0.07	1.39	112088	2.58	$y = 0.00010x - 2.9315$	0.9935	53.0	176.6	278.2	285.2	291.3	–	–	–	–
8	palmitoleic acid	16:1	4.41	0.11	1.39	69295	3.97	$y = 0.00040x - 4.9138$	0.9860	31.9	106.3	254.2	261.1	267.2	–	–	–	–
9	1(3)-monopalmitoyl-*sn*-glycerol	16:0	4.85	0.05	1.53	68981	3.53	$y = 0.00020x - 3.9219$	0.9953	57.8	192.9	330.3	337.3	343.3	–	–	–	–
10	2-monooleoyl-*sn*-glycerol	18:1	5.04	0.10	1.59	389985	2.45	$y = 0.00004x - 3.8762$	0.9902	21.9	73.1	356.3	363.3	369.3	–	–	–	–
11	1(3)-monooleoyl-*sn*-glycerol	18:1	5.22	0.07	1.65	561270	3.50	$y = 0.00002x - 0.9320$	0.9923	20.2	67.4	356.3	363.3	369.3	–	–	–	–
12	linoleic acid	18:2	5.29	0.06	1.67	106004	1.82	$y = 0.00020x - 2.8530$	0.9958	33.2	110.7	280.2	287.2	293.3	–	–	–	–
13	palmitic acid	16:0	5.92	0.07	1.87	68981	3.31	$y = 0.00020x - 3.9219$	0.9953	57.8	192.9	256.2	263.2	269.3	–	–	–	–
14	oleic acid	18:1	6.25	0.10	1.97	70773	3.91	$y = 0.00070x - 0.7625$	0.9941	49.9	166.5	282.3	289.3	295.3	–	–	–	–
15	1(3)-monostearoyl-*sn*-glycerol	18:0	6.25	0.11	1.97	449136	4.87	$y = 0.00004x - 2.4782$	0.9958	41.2	137.3	358.3	365.3	371.3	–	–	–	–
16	stearic acid	18:0	7.25	0.07	2.29	100298	3.46	$y = 0.00020x - 2.3194$	0.9950	28.1	93.9	284.3	291.3	297.3	–	–	–	–
17	arachidic acid	20:0	8.30	0.06	2.62	59230	5.22	$y = 0.00020x - 0.0484$	0.9991	26.9	89.7	312.3	319.3	325.3	–	–	–	–
18	tricapryloyl-*sn*-glycerol	24:0	9.00	0.04	2.84	19369	4.12	$y = 0.00400x + 3.7648$	0.9995	8.89	29.6	470.4	477.4	–	–	–	–	–
19	behenic acid	22:0	9.23	0.08	2.91	–	–	–	–	14.5	48.5	340.3	347.4	353.4	–	–	–	–
20	trinonanoyl-*sn*-glycerol	27:0	10.00	0.10	3.15	7626	5.79	$y = 0.00500x - 0.2870$	0.9902	10.8	35.9	512.4	519.4	–	–	–	–	–
21	1,3-dilinolenoyl-*sn*-glycerol	36:6	10.00	0.08	3.15	595745	3.53	$y = 0.00002x - 0.3585$	0.9983	61.3	204.5	612.5	619.5	625.5	595.4	–	–	–
22	1,3-dilinoleoyl-*sn*-glycerol	36:4	10.70	0.06	3.38	874558	1.81	$y = 0.00002x - 1.7032$	0.9964	15.8	52.7	616.5	623.5	629.5	599.4	–	–	–
23	tridecanoyl-*sn*-glycerol	30:0	10.84	0.06	3.42	72570	3.36	$y = 0.00002x + 1.0112$	0.9946	8.05	26.8	554.5	561.5	–	–	–	383.5	389.5
24	1,3-dipalmitoyl-*sn*-glycerol	32:0	11.26	0.07	3.55	889713	2.54	$y = 0.00001x - 0.4639$	0.9953	22.7	75.7	568.5	575.5	581.5	551.5	–	–	–

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Table 3. continued

peak no.	compound	ACN:DBN[a]	t_R (min)	RSD[b] (%)	RRT[c]	area[d] (av)	RSD[b] (%)	linear equation[e] (y = ng/μL, x = area)	R^2	LOD[f] (pg)	LOQ[f] (pg)	M_{mono}[g] (Da)	$[M + Li]^+$ $(m/z)^h$	$[M + 2 * Li - H]^+$ $(m/z)^h$	$[M - H_2O + H]^+$ $(m/z)^h$	$[M - H_2O + Li]^+$ $(m/z)^h$	$[M - FA + H]^+$ $(m/z)^h$	$[M - FA + Li]^+$ $(m/z)^h$
25	1,3-dioleoyl-*sn*-glycerol	36:2	11.37	0.09	3.59	1228779	2.45	$y = 0.00001x - 0.3271$	0.9927	11.7	39.1	620.5	627.6	633.5	603.5	–	–	–
26	triundecanoyl-*sn*-glycerol	33:0	11.48	0.11	3.62	514595	2.26	$y = 0.000008x - 0.1943$	0.9996	0.72	2.40	596.5	603.5	–	–	–	411.5	417.4
27	trilauroyl-*sn*-glycerol	36:0	12.00	0.12	3.79	236889	2.99	$y = 0.000009x - 0.2326$	0.9916	3.71	12.4	638.5	645.5	–	–	–	439.5	445.5
28	trilinolenoyl-*sn*-glycerol	54:9	12.34	0.18	3.89	85169	1.98	$y = 0.000008x + 0.2566$	0.9910	0.97	3.24	872.7	879.7	–	–	–	595.5	601.5
29	tritridecanoyl-*sn*-glycerol	39:0	12.43	0.10	3.92	230066	2.43	$y = 0.000006x - 0.1781$	0.9925	0.62	2.07	680.6	687.6	–	–	–	467.6	473.6
30	trimyristoyl-*sn*-glycerol	42:0	12.79	0.10	4.03	473064	1.22	$y = 0.000009x - 0.6412$	0.9948	1.21	4.03	722.6	729.6	–	–	–	495.6	501.6
31	tripalmitoleoyl-*sn*-glycerol	48:3	12.81	0.15	4.04	191763	2.81	$y = 0.000006x - 0.1242$	0.9938	0.12	0.40	800.7	807.7	–	–	–	547.5	553.5
32	trilinoleoyl-*sn*-glycerol	54:6	12.87	0.18	4.05	127184	2.40	$y = 0.000006x + 0.0352$	0.9936	0.64	2.15	878.7	885.8	–	–	–	599.5	605.5
33	tripentadecanoyl-*sn*-glycerol	45:0	13.08	0.20	4.13	229747	2.35	$y = 0.00001x - 0.7896$	0.9821	0.59	1.98	764.7	771.7	–	–	–	523.6	529.6
34	trioleoyl-*sn*-glycerol	54:3	13.34	0.18	4.21	140990	3.11	$y = 0.00002x - 0.5092$	0.9900	0.44	1.47	884.8	891.8	–	–	–	603.5	609.5
35	tripalmitoyl-*sn*-glycerol	48:0	13.34	0.21	4.21	201385	2.71	$y = 0.00004x - 3.4618$	0.9885	2.70	9.00	806.7	813.8	–	–	–	551.6	557.6
36	triheptadecanoyl-*sn*-glycerol	51:0	13.57	0.25	4.28	129878	2.83	$y = 0.00003x - 0.4551$	0.9878	1.76	5.88	848.8	855.7	–	–	–	579.6	585.6
37	tristearoyl-*sn*-glycerol	54:0	13.75	0.33	4.34	178198	4.04	$y = 0.00004x - 0.1573$	0.9977	2.65	8.84	890.8	897.8	–	–	–	607.6	613.6

[a]Acyl carbon number: double bond number of fatty acid moieties. [b]Determined by injecting the level 2 mixture 10 times consecutively. [c]Relative retention time to internal standard (12-hydroxystearic acid). [d]Average areas of the most abundant ions extracted from total ion current chromatograms. [e]Determined by triplicate analysis. [f]LOD, limit of detection (3 × noise). LOQ, limit of quantification (10 × noise). [g]Monoisotopic mass (calculated from the most abundant atom isotope masses). [h]Measured mass of the ion.

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Table 4. Retention Times and Most Abundant Positive Ions of the Synthesized Oxidized Reference Compounds[a]

compound	ACN: DBN[b]	t_R (min)	RRT[c]	M_{mono} (Da)[d]	$[M+H]^+$ $(m/z)^e$	$[M+Li]^+$ $(m/z)^e$	$[M+2*Li-H]^+$ $(m/z)^e$	$[M+Li-H_2O]^+$ $(m/z)^e$	$[M+2*Li-H-H_2O]^+$ $(m/z)^e$	$[M+Li-2*H_2O]^+$ $(m/z)^e$	$[M+H-H_2O]^+$ $(m/z)^e$	$[M+H-2*H_2O]^+$ $(m/z)^e$	$[M+H-3*H_2O]^+$ $(m/z)^e$
triepoxyoctadecanoic acid	18:0	0.72	0.23	312.3	313.2	**319.2**	325.2	301.2	–	–	**295.2**	277.2	259.2
diepoxyoctadecanoic acid	18:0	1.62	0.51	326.3	327.2	**333.2**	339.1	315.2	–	–	309.2	**291.2**	273.2
trihydroperoxy-1(3)-octadecatrienoic acid-*sn*-glycerol	18:3	0.40[f]	–	448.3	–	**455.3**	–	**439.3**	–	–	–	–	–
dihydroperoxyoctadecatrienoic acid	18:3	0.74[f]	–	342.3	–	–	–	**331.3**	**337.3**	–	–	–	–
monohydroxyoctadecatrienoic acid	18:3	1.64	0.52	296.3	–	313.3	–	**283.3**	**289.3**	–	–	–	–
monohydroperoxyoctadecatrienoic acid	18:3	1.82	0.57	310.3	311.2	–	323.3	**299.3**	**305.3**	–	–	–	–
dihydroperoxy-1(3)-octadecatrienoic acid-*sn*-glycerol	18:3	0.56[f]	–	416.3	–	**423.3**	431.3	**407.3**	–	–	–	–	–
dihydroperoxy-1(3)-octadecadienoic acid-*sn*-glycerol	18:2	1.17[f]	–	418.3	–	**425.3**	431.3	**407.3**	–	–	–	–	–
monohydroxy-1(3)-octadecatrienoic acid-*sn*-glycerol	18:3	1.23	0.39	368.3	–	**375.3**	381.3	**357.3**	–	–	–	–	–
monohydroperoxy-1(3)-octadecatrienoic acid-*sn*-glycerol	18:3	1.35	0.43	384.3	–	**391.3**	397.3	**373.3**	379.3	–	–	–	–
monohydroperoxy-1(3)-octadecatrienoic acid-*sn*-glycerol	18:3	1.48	0.47	384.3	–	**391.3**	397.3	**373.3**	379.3	–	–	–	–
monohydroxy-1(3)-octadecadienoic acid-*sn*-glycerol	18:2	1.60	0.50	370.3	–	**377.3**	383.3	**359.3**	365.3	–	–	–	–
monohydroxy-1(3)-octadecadienoic acid-*sn*-glycerol	18:2	1.79	0.56	370.3	–	**377.3**	383.3	**359.3**	365.3	–	–	–	–
monohydroperoxy-1(3)-octadecadienoic acid-*sn*-glycerol	18:2	1.67	0.53	386.3	–	**393.3**	399.3	**375.3**	381.3	–	–	–	–
monohydroperoxy-1(3)-octadecadienoic acid-*sn*-glycerol	18:2	1.70	0.54	386.3	–	**393.3**	399.3	**375.3**	381.3	–	–	–	–
monohydroperoxy-1(3)-octadecadienoic acid-*sn*-glycerol	18:2	1.76	0.56	386.3	–	**393.3**	399.3	**375.3**	381.3	–	–	–	–
monohydroperoxy-1(3)-octadecadienoic acid-*sn*-glycerol	18:2	1.95	0.62	386.3	–	**393.3**	399.3	**375.3**	381.3	–	–	–	–
monohydroxyoctadecadienoic acid	18:2	2.03	0.64	296.3	–	–	309.2	**285.2**	**291.2**	–	–	–	–

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Table 4. continued

compound	ACN: DBN[b]	t_R (min)	RRT[c]	M_{mono} (Da)[d]	$[M+H]^+$ (m/z)[e]	$[M+Li]^+$ (m/z)[e]	$[M+2*Li-H]^+$ (m/z)[e]	$[M+Li-H_2O]^+$ (m/z)[e]	$[M+2*Li-H-H_2O]^+$ (m/z)[e]	$[M+Li-2*H_2O]^+$ (m/z)[e]	$[M+H-H_2O]^+$ (m/z)[e]	$[M+H-2*H_2O]^+$ (m/z)[e]	$[M+H-3*H_2O]^+$ (m/z)[e]
monohydroxyoctadecadienoic acid	18:2	2.10	0.66	296.3	–	–	309.2	**285.2**	**291.2**	–	–	–	–
monohydroxyoctadecadienoic acid	18:2	2.17	0.69	296.3	–	–	309.2	**285.2**	**291.2**	–	–	–	–
monohydroxyoctadecadienoic acid	18:2	2.35	0.74	296.3	–	–	309.2	**285.2**	**291.2**	–	–	–	–
monohydroxy-1(3)-octadecenoic acid-*sn*-glycerol	18:1	1.90	0.60	372.3	–	**379.3**	385.3	**361.3**	–	–	–	–	–
monohydroxy-1(3)-octadecenoic acid-*sn*-glycerol	18:1	2.00	0.63	372.3	–	**379.3**	385.3	**361.3**	–	–	–	–	–
monohydroxy-1(3)-octadecenoic acid-*sn*-glycerol	18:1	2.10	0.66	372.3	–	**379.3**	385.3	**361.3**	–	–	–	–	–
monohydroxy-1(3)-octadecenoic acid-*sn*-glycerol	18:1	2.21	0.70	372.3	–	**379.3**	385.3	**361.3**	–	–	–	–	–
monohydroperoxy-1(3)-octadecenoic acid-*sn*-glycerol	18:1	2.14	0.68	388.3	–	**395.3**	–	**377.3**	383.3	359.3	–	–	–
monohydroperoxy-1(3)-octadecenoic acid-*sn*-glycerol	18:1	2.24	0.71	388.3	–	**395.3**	–	**377.3**	383.3	359.3	–	–	–
monohydroperoxy-1(3)-octadecenoic acid-*sn*-glycerol	18:1	2.34	0.74	388.3	–	**395.3**	–	**377.3**	383.3	359.3	–	–	–
monohydroperoxy-1(3)-octadecenoic acid-*sn*-glycerol	18:1	2.39	0.75	388.3	–	**395.3**	–	**377.3**	383.3	359.3	–	–	–
monohydroperoxyoctadecadienoic acid	18:2	2.32	0.73	312.3	–	319.2	325.2	**301.2**	**307.2**	283.2	–	–	–
monohydroperoxyoctadecadienoic acid	18:2	2.45	0.77	312.3	–	319.2	325.2	**301.2**	**307.2**	283.2	–	–	–
monohydroperoxyoctadecadienoic acid	18:2	2.56	0.81	312.3	–	319.2	325.2	**301.2**	**307.2**	283.2	–	–	–
monohydroperoxyoctadecadienoic acid	18:2	2.69	0.85	312.3	–	319.2	325.2	**301.2**	**307.2**	283.2	–	–	–
monohydroperoxyoctadecenoic acid	18:1	3.01	0.95	314.3	–	–	–	**303.2**	**309.2**	285.2	–	–	–
monohydroperoxyoctadecenoic acid	18:1	3.10	0.98	314.3	–	–	–	**303.2**	**309.2**	285.2	–	–	–
12-hydroxyoctadecanoic acid	18:0	3.17	1.00	300.3	301.3	**307.3**	**313.2**	289.3	295.3	283.3	–	–	–
monohydroperoxyoctadecenoic acid	18:1	3.51	1.11	298.3	–	**305.2**	**311.2**	287.2	–	–	–	–	–

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Table 4. continued

compound	ACN: DBN[b]	t_R (min)	RRT[c]	M_{mono} (Da)[d]	$[M+H]^+$ (m/z)[e]	$[M+Li]^+$ (m/z)[e]	$[M+2*Li-H]^+$ (m/z)[e]	$[M+Li-H_2O]^+$ (m/z)[e]	$[M+2*Li-H-H_2O]^+$ (m/z)[e]	$[M+Li-2*H_2O]^+$ (m/z)[e]	$[M+H-H_2O]^+$ (m/z)[e]	$[M+H-2*H_2O]^+$ (m/z)[e]	$[M+H-3*H_2O]^+$ (m/z)[e]
monohydroperoxyoctadecenoic acid	18:1	3.87	1.22	314.3	–	321.3	**327.2**	303.2	**309.2**	285.2	–	–	–
dihydroperoxy-1,3-dioctadecadienoic acid-*sn*-glycerol	36:4	7.12	2.25	680.5	–	**687.5**	–	**669.5**	–	–	–	–	–
dihydroperoxy-1,3-dioctadecadienoic acid-*sn*-glycerol	36:4	7.17	2.26	680.5	–	**687.5**	–	**669.5**	–	–	–	–	–
dihydroperoxy-1,3-dioctadecadienoic acid-*sn*-glycerol	36:4	7.23	2.28	680.5	–	**687.5**	–	**669.5**	–	–	–	–	–
dihydroperoxy-1,3-dioctadecadienoic acid-*sn*-glycerol	36:4	7.30	2.30	680.5	–	**687.5**	–	**669.5**	–	–	–	–	–
dihydroperoxy-1,3-dioctadecadienoic acid-*sn*-glycerol	36:4	7.36	2.32	680.5	–	**687.5**	–	**669.5**	–	–	–	–	–
dihydroperoxy-1,3-dioctadecenoic acid-*sn*-glycerol	36:2	7.86	2.48	684.5	–	**691.5**	–	673.5	–	–	–	–	–
dihydroperoxy-1,3-dioctadecenoic acid-*sn*-glycerol	36:2	7.91	2.50	684.5	–	**691.5**	–	673.5	–	–	–	–	–
dihydroperoxy-1,3-dioctadecenoic acid-*sn*-glycerol	36:2	8.00	2.52	684.5	–	**691.5**	–	673.5	–	–	–	–	–
dihydroperoxy-1,3-dioctadecenoic acid-*sn*-glycerol	36:2	8.05	2.54	684.5	–	**691.5**	–	673.5	–	–	–	–	–
dihydroperoxy-1,3-dioctadecenoic acid-*sn*-glycerol	36:2	8.11	2.56	684.5	–	**691.5**	–	673.5	–	–	–	–	–
dihydroperoxy-1,3-dioctadecenoic acid-*sn*-glycerol	36:2	8.16	2.57	684.5	–	**691.5**	–	673.5	–	–	–	–	–
monohydroperoxy-1,3-dioctadecadienoic acid-*sn*-glycerol	36:4	9.06	2.86	648.5	–	**655.5**	–	**637.5**	–	–	–	–	–
monohydroperoxy-1,3-dioctadecadienoic acid-*sn*-glycerol	36:4	9.10	2.87	648.5	–	**655.5**	–	**637.5**	–	–	–	–	–
monohydroperoxy-1,3-dioctadecadienoic acid-*sn*-glycerol	36:4	9.14	2.88	648.5	–	**655.5**	–	**637.5**	–	–	–	–	–
monohydroperoxy-1,3-dioctadecadienoic acid-*sn*-glycerol	36:4	9.18	2.90	648.5	–	**655.5**	–	**637.5**	–	–	–	–	–

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Table 4. continued

compound	ACN:DBN[b]	t_R (min)	RRT[c]	M_{mono} (Da)[d]	$[M+H]^+$ $(m/z)^e$	$[M+Li]^+$ $(m/z)^e$	$[M+2*Li-H]^+$ $(m/z)^e$	$[M+Li+H_2O]^+$ $(m/z)^e$	$[M+2*Li-H-H_2O]^+$ $(m/z)^e$	$[M+Li-2*H_2O]^+$ $(m/z)^e$	$[M+H-H_2O]^+$ $(m/z)^e$	$[M+H-2*H_2O]^+$ $(m/z)^e$	$[M+H-3*H_2O]^+$ $(m/z)^e$
monohydroperoxy-1,3-dioctadecenoic acid-*sn*-glycerol	36:2	9.84	3.10	652.5	–	**659.5**	–	**641.5**	–	–	–	–	–
monohydroperoxy-1,3-dioctadecenoic acid-*sn*-glycerol	36:2	9.89	3.12	652.5	–	**659.5**	–	**641.5**	–	–	–	–	–

[a]Two of the most abundant ions in bold, and the most abundant ion is underlined. [b]Acyl carbon number: double bond number. [c]Relative retention time to internal standard (12-hydroxystearic acid). [d]Monoisotopic mass (calculated from the most abundant atom isotope masses). [e]Measured mass of the ion. [f]Several unresolved peaks.

Figure 5. Normalized amounts of detected oxidized lipids from digested fresh rapeseed oil with different antioxidant additions. T, DL-α-tocopherol; TA, DL-α-tocopheryl acetate; AA, L-ascorbic acid; PA, 6-palmitoyl-*O*-L-ascorbic acid; BHT, 3,5-di-*tert*-butyl-4-hydroxytoluene. Ten parallel digestions and two parallel extractions of each digestion were made. Samples were analyzed once (n = 20). No statistically significant differences were found between the groups.

generated in photo-oxidation, accompanying the hydroperoxides, were eluted before the corresponding hydroperoxidized octadecadienoic acids. This phenomenon was observed on both the oxidized free fatty acids and oxidized monoacylglycerols and was confirmed by studying the mass spectra and the disappearance of peaks after TPP reduction of the hydroperoxidized lipids. Previously, it had been reported that the hydroperoxidized TAGs are eluted slightly ahead of the corresponding hydroxyl TAGs.[23] Hydroperoxidized TAGs were indeed detected in the thermally treated oil, but no hydroperoxidized FFAs or MAGs were present after digestion.

DISCUSSION

Normally, all lipophilic antioxidants provide some protection against oxidation of lipids, as shown by numerous studies with individual and mixed unsaturated fatty acids, purified unsaturated triacylglycerols, or raw and refined oils under different experimental conditions.[31−35] The effectiveness of different antioxidants in the protection of polyunsaturates in the gastric environment has received only recent experimental attention,[9,10,15,36] and detailed analyses of the molecular species formed during the digestion of normal and oxidized glycerolipids have not been reported. In view of the obvious differences between the gastric (low pH) and nongastric (neutral pH) conditions of the media employed to investigate the effectiveness of antioxidants, we hypothesized that the results would differ between the two conditions and among different antioxidants. In order to ensure the validity of the comparisons, we estimated the overall extent of oxidation or degradation of the dietary lipids during digestion using UHPLC−ESI−MS to identify and quantitate all oxo-lipid species and estimated the overall effect of a specific antioxidant by summing the oxo-lipid species. Colorimetric and titrimetric methodologies were avoided in view of the erratic and contradictory results frequently observed.[14,36,37] In the present study, we verified this hypothesis by investigating the effectiveness of several common antioxidants as protectors of lipid oxidation in an artificial model of lipid digestion.[26,27] The results showed that none of the antioxidants under our experimental conditions could prevent the formation of oxidized lipids, although differences in oxidized lipid profiles were detected in the digested samples using different antioxidants.

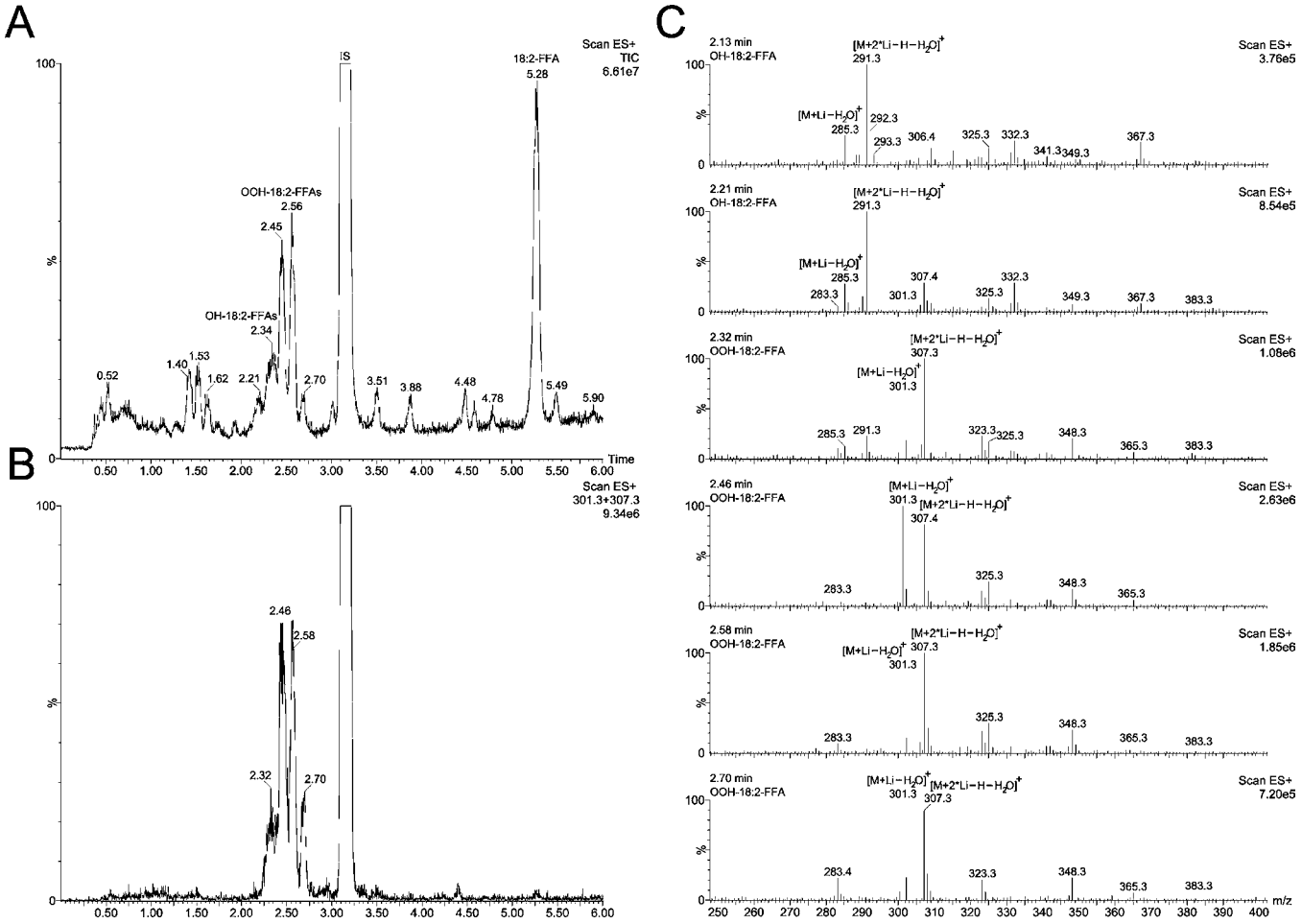

Figure 6. Total ion current (TIC) mass chromatogram of hydroperoxidized linoleic acid (A), selected ion mass chromatogram of hydroperoxidized linoleic acid (B), and mass spectra of selected peaks (C). Four constitutional isomers of hydroperoxidized linoleic acid were separated. Lithium was used as ionization enhancer.

The variations in the antioxidant activity could be attributed to their digestibility (tocopheryl acetate and palmitoyl ascorbate), destruction, and inactivation of our antioxidants in the digestion medium. There is evidence that the antioxidant activity of the vitamins used in this experiment could have been compromised by a number of factors. First, the low pH of the digestive model's first part (pH 1−2) could have inhibited the activity of vitamin E,[38] and the higher pH of the second part (pH 7) could have reduced the activity of vitamin C.[39] Second, ferric ions (Fe^{3+}) in food preparation and digestive fluids could be reduced by vitamin C to ferrous ions (Fe^{2+}), which can act as peroxidizing agents.[38] Thus, the cross-reaction between free radicals produced during this reaction co-oxidized vitamin E and vitamin C. Both lipid peroxidation and co-oxidation of the vitamins in the stomach medium could be inhibited by polyphenols.[9] Furthermore, saliva alone could not protect against the co-oxidation, and the presence of polyphenol antioxidants was required.[36]

It must be emphasized that our assays of antioxidant effects differ from traditional measurements of antioxidant activity. We assessed the effects of antioxidants by measuring the amount of oxidized lipid molecules actually formed, in contrast to traditional measurements of antioxidant activity and capacity methods, such as total radical trapping antioxidant parameter (TRAP), oxygen radical absorbance capacity (ORAC), or ferric ion reducing power (FRAP), which rely on generalized measurements of hydrogen atom or electron transfer.[37]

The significance of these observations for gastric lipid metabolism and fat absorption remains to be further assessed. Specifically, the postulated role for ascorbate and tocopherols as scavengers of reactive species in the GI tract[12] appears to be in doubt, even when considerable amounts of tocopherols remain unabsorbed and reach the colon.

In the present study, the gastric antioxidant activity of the plant polyphenols was not assessed. It is possible that the activity of these compounds, especially the red vine polyphenols, is greater than the effect of the commmon lipophilic antioxidants we used. Gorelik et al. have demonstrated that both lipid peroxidation and co-oxidation of vitamin E and β-carotene were inhibited at pH 3.0 by red vine polyphenols in simulated human gastric fluid.[9] Ascorbic acid, which on its own inhibited lipid peroxidation only slightly, did so significantly when combined with red vine polyphenols. Gorelik et al. measured hydroperoxides by means of the ferrous ion oxidation−xylenol orange method.[9] Siracusa et al. used a similar two-step in vitro digestion model that we have used to assess the antioxidative activity of phenolics from *Capparis spinosa* L. and *Crithmum maritimum* L. and found that the antioxidative activity, measured by the β-carotene bleaching method, was greatly diminished by the digestion processes.[40] We hope to reexamine the antioxidant effect of the red vine polyphenols or other phenolic compounds using the ultra-high performance UHPLC−ESI−MS method in the artificial digestion model.

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Overall, the method of assessing lipid oxidation by measuring the yield of the product proved to be fast and sensitive for the analysis of a variety of fresh and oxidized lipids. Lithium formate increased the sensitivity several times over our previous method based on naturally occurring sodium adducts. Chromatographic separation enabled the identification of structural isomers of oxidized lipids and their quantitative measurement. Small amounts of natural antioxidants (mainly different tocopherols) found in the native unoxidized rapeseed oil could not suppress the oxidation of unsaturated fatty acids in the artificial digestion model. Neither could the AA, BHT, PA, T, or TA, or their combinations, even when added in large amounts. The present in vitro results support the growing evidence of the limited usefulness of ascorbic acid and tocopherol supplementation as protection against lipid peroxidation.

SAFETY

Proper precautions must be followed when synthesizing peroxidized lipids because of their explosive nature. Only small quantities (few mg) should be synthesized and safety shields and goggles used.

ASSOCIATED CONTENT

Supporting Information

Spontaneous cleavage of the acyl moiety from the monoacylglycerol backbone of lithium adducts in positive ionization LC−ESI−MS. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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