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Simultaneous quantification of epoxy and hydroxy fatty acids as oxidation products of triacylglycerols in edible oils

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ABSTRACT

Epoxy and hydroxy fatty acids are important intermediates during lipid oxidation; quantification of both structures may help evaluate the extent of competition among various lipid oxidation pathways. This article describes a method to simultaneously determine saturated- and unsaturated- epoxy and hydroxy fatty acids derived from oxidation of vegetable oils. The experimental procedures employed transesterification with sodium methoxide, separation of epoxy and hydroxy fatty acid methyl esters (FAME) using solid-phase extraction (SPE), and trimethylsilyl (TMS) derivatization of hydroxy groups. GC–MS was used to identify the epoxy and hydroxy FAME in two different SPE fractions, while GC-flame ionization detection (GC-FID) was used to determine their quantities. Epoxy-octadecanoate/octadecenoate and hydroxy-octadecanoate/octadecenoate/octadecadienoate were determined as lipid oxidation products generated from oxidation of sunflower and canola oils. An isomer of methyl 13-hydroxyoctadeca-9,11-dienoate (13-HODE) TMS ether co-eluted with methyl 15-hydroxyoctadeca-9,12-dienoate TMS ether which was only present in canola oil; thus, GC–MS-selected ion monitoring (GC–MS–SIM) was used to determine the concentration of 13-HODE. The proposed method has been successfully applied to monitor epoxy and hydroxy fatty acids in sunflower oil and canola oil oxidized at 40 °C.

1. Introduction

Lipid oxidation is an important reaction that limits the shelf-life of fat-containing foods [1]. Oxidative deterioration of triacylglycerols, the major lipid class present in foods, leads to a variety of oxidation products. The most commonly measured products are hydroperoxides, known as primary oxidation products, and conventionally determined by a titration method, named peroxide value (PV) [2]. In addition to hydroperoxides, conjugated dienes are considered primary products and are measured by a spectrophotometric method [3]. Secondary oxidation products, including aldehydes and other volatile compounds derived from the decomposition of hydroperoxides, are also measured using a number of quantitative approaches [4].

Although epoxy and hydroxy fatty acids have long been discussed as secondary lipid oxidation products [5], the formation of these oxygenated fatty acids in oils and foods has gained interest in recent years [6,7]. These oxygenated fatty acids are normally present at low levels (e.g., mg/g levels in used frying oils and μg/g in fresh oils), so GC and HPLC have become the most suitable methods due to the sensitive detectors they employ. HPLC-UV methods enabled the quantification of hydroxydienes by measurement of absorption at 234 nm due to the conjugated double bonds [8], but for epoxy fatty acids, an evaporative light scattering detector (ELSD) was necessary [9]. When coupled with GC, flame ionization detection (FID) has been the major detector for the determination of epoxy fatty acids alone [10,11] or together with hydroxy fatty acids [12]. The major experimental setup in the GC approach was to prepare fatty acid methyl esters (FAME) from triacylglycerols and then extract oxygenated FAME from non-polar (non-oxygenated) FAME for GC analysis utilizing a solid phase extraction (SPE) step. When simultaneously determining these two types of compounds, hydrogenation was used to simplify the chromatograms for peak identification [12], which resulted in the loss of information regarding double bond position.

Here, we improve on that earlier work by avoiding the use of hydrogenation and retaining the unsaturated structure. A three-step SPE separation was employed to eliminate co-elution of hydroxy compounds with epoxy FAME in chromatograms [11]; thus, epoxy and hydroxy FAME were separated into two SPE fractions. The hydroxy groups were then converted into their trimethylsilyl (TMS) ethers so GC–MS enabled identifications of both the epoxy and hydroxy FAME. With confirmation of the

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peak identities, the two SPE fractions were analyzed by GC-FID and GC–MS-selected ion monitoring (SIM) for quantification. This method was then applied to determine both saturated and unsaturated, epoxy and hydroxy fatty acids in oxidized canola oil and sunflower oil.

2. Materials and methods

2.1. Materials

2.1.1. Standards and oils

N, O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) (>99%, for GC derivatization), sodium methoxide solution (0.5 M CH_3ONa in methanol), methyl 12-hydroxystearate (>99%), and methyl ricinoleate (>99%, methyl 12-hydroxyoctadec-9-enoate) were purchased from Sigma-Aldrich Inc (St. Louis, MO, US). 13(S)-HODE (13(S)-hydroxyoctadeca-9,11-dienoic acid) methyl ester (>98%) was obtained from Cayman Chemical Company (Ann Arbor, Michigan, US). Methyl nonadecanoate (C19:0) and methyl octadec-9-enoate (C18:1) was purchased from Nu-chek Prep, Inc. (Elysian, MN, US). Si gel 40 (35–70 mesh) from Fluka Analytical (Switzerland) was used for solid-phase extraction (SPE). Sunflower oil and canola oil were obtained from local markets. Their fatty acid compositions were determined after transesterification (Table 1). The sunflower and canola oils were selected because of their distinctly different concentrations of oleic and linoleic acids.

2.1.2. Synthesis of methyl 9,10-epoxyoctadecanoate

Methyl 9,10-epoxyoctadecanoate was chemically synthesized from methyl octadec-9-enoate (methyl oleate) using a hydrogen peroxide-formic acid method [13]. Specifically, the molar ratio of methyl oleate: formic acid: hydrogen peroxide used for synthesis was 2:1:3. Methyl oleate, formic acid, and hydrogen peroxide were mixed and heated at 38 °C for 6 h. The epoxidized methyl oleate was then extracted by ethyl acetate following procedures reported by Anuar et al. [14]. The epoxidized material was purified using the SPE conditions described in Section 2.4. The purity of methyl 9,10-epoxyoctadecanoate was determined to be >95% by GC-FID.

2.2. Transesterification

Transesterification with sodium methoxide was carried out following the procedures described by Berdeaux, Márquez-Ruiz and Dobarganes [15]. Aliquots of 50 mg oil were weighed into 10 mL test tubes, to which were then added 1 mL of *tert*-butyl methyl ether and 0.5 mL of 0.2 M sodium methoxide in methanol. The test tubes were shaken for 1 min and kept at room temperature for 2 min. Then, 0.1 mL of 0.5 M sulfuric acid solution was added, followed by addition of another 1.5 mL of water. After shaking and centrifugation, the organic layer containing FAME was collected in another test tube and then diluted to 5 mL with *tert*-butyl methyl ether.

2.3. SPE conditions

Aliquots of 1 mL FAME solution, containing ~10 mg FAME, were dried under nitrogen and re-dissolved in 0.5 mL of 98:2 hexane: diethyl ether for SPE. The silica column was prepared by filling a Pasteur pipette with 1 g of activated Si gel. A three-step SPE was employed for extraction of epoxy and hydroxy FAME [11]. First, the non-polar fraction, consisting of unaltered FAME (non-polar FAME) was eluted using 15 mL of 98:2 hexane: diethyl ether. Then, Polar Fraction 1, which contained epoxy FAME, was eluted from the silica column using 15 mL of 90:10 hexane: diethyl ether. The third fraction, Polar Fraction 2, mainly containing hydroxy FAME, was eluted using 30 mL of diethyl ether.

2.4. TMS derivatization

Methyl nonadecanoate (C19:0) was added to Polar Fractions 1 (5 μg) and 2 (1 μg) as the internal standard. Both fractions were evaporated to dryness under nitrogen and derivatized with 50 μL BSTFA and 50 μL pyridine for 30 min at room temperature. After the reaction, the samples were again evaporated to dryness. Polar Fraction 1 was dissolved in 1 mL hexane and Polar Fraction 2 in 200 μL hexane for GC analysis. The final concentration of the internal standard was 5 μg/mL in both fractions.

2.5. GC analysis

2.5.1. GC-FID

After TMS derivatization, GC-FID (Scion 436-GC, Bruker) was used to analyze all the FAME samples. All samples were introduced by a Bruker CP-8400 autosampler with an injection volume of 1 μL. Polar Fraction 1 was analyzed using an Rtx-2330 capillary column (90% biscyanopropyl/10% phenylcyanopropyl polysiloxane, 105 m × 0.25 mm i.d., 0.2 μm). The temperature program started at 50 °C and was held for 2 min, increased to 174 °C at 15 °C/min and was held for 10 min, and finally reached 250 °C at 20 °C/min and was held for 31 min. The total analysis time was 55.07 min. The carrier gas was helium at 1.2 mL/min, with injector at 250 °C in splitless mode. The FID detector temperature was set at 270 °C, with argon (make-up gas), hydrogen, and air flow rates at 50, 30, 300 mL/min.

Polar Fraction 2 was analyzed using a DB-23 capillary column (50%-cyanopropyl-methylpolysiloxane, 30 m × 0.25 mm i.d., 0.25 μm). The temperature program started at 50 °C and was held for 2 min, increased to 165 °C at 15 °C/min and was held for 15 min, then increased to 205 °C at 10 °C/min and was held for 10 min, increased to 230 °C at 10 °C/min and was held for 10 min with a total time of 51.17 min. The carrier gas was helium at 1.2 mL/min, with injector at 250 °C in splitless mode. The FID detector parameters remained the same.

2.5.2. GC–MS

A Trace GC Ultra gas chromatography coupled with a PolarisQ ion trap mass spectrometer was used to identify the epoxy and hydroxy FAME. The GC parameters remained the same for each type of column as applied in GC-FID. In the full scan mode, the ion source was kept at 200 °C, using an ionization potential of 70 eV with a mass scan range of m/z 60–600. In addition, GC–MS-SIM was employed to analyze Polar Fraction 2 derived from canola oil. In SIM mode, m/z 312 was measured from 22 to 26 min for C19:0 (internal standard) and m/z 382 was measured from 26 min to the end of the run to determine 13-HODE.

2.6. Quantitative studies

2.6.1. Response factors

Methyl nonadecanoate (C19:0) was the internal standard for GC-FID quantifications. The response factors for hydroxy-C18:0, hydroxy-C18:1, and hydroxy-C18:2 FAME were obtained using methyl 12-hydroxystearate, methyl ricinoleate, and 13-HODE methyl ester, respectively. Since 9,10-epoxyoctadecanoate was not commercially available, synthetic 9,10-epoxyoctadecanoate obtained from Section 2.1.2 was used to acquire the GC-FID response factor. The response factor obtained for 9,10-epoxyoctadecanoate was applied to quantify all epoxy FAME analyzed by GC-FID. Such application has been reported in a number of previous studies [10,11,16]. Calibration curves were established by plotting the area ratio of analyte standard/internal standard versus the concentration ratio of analyte standard/internal standard. In addition to GC-FID, a calibration

Table 1
Fatty acid composition (, %, w/w total FAME) of the oils.

Oil	C16:0	C18:0	C18:1n-9	C18:2n-6	C18:3n-3	others[a]
Canola oil	3.65 ± 0.02	1.75 ± 0.00[b]	59.76 ± 0.02	18.39 ± 0.01	9.35 ± 0.00[b]	7.10
Sunflower oil	5.89 ± 0.02	3.28 ± 0.00[b]	34.34 ± 0.02	53.87 ± 0.01	0.08 ± 0.00[b]	2.54

[a] 'Others' include 16:1n-9, 18:1n-7, 18:3n-6, 20:0, 20:1n-9/20:1n-11, 22:0.
[b] The SD values were less than 0.01.

curve for 13-HODE methyl ester was obtained in GC–MS-SIM, taking methyl nonadecanoate as the internal standard. In both, GC-FID and GC–MS-SIM, the working ranges of the calibration curves were 0, 2, 4, 6, 8, and 10 μg/mL, with the internal standard at 5 μg/mL. For the calibration curves established by GC-FID, the instrumental limit of detection (LOD) and limit of quantification (LOQ) were calculated using the standard deviation (σ) of the intercepts and the slope (s) of the calibration curves (LOD = 3σ/s and LOQ = 10σ/s) [17]. The method LOQ, taking into account the sample analyzed here, was then converted from the instrumental LOQ using the sample mass carried through the experimental procedures.

2.6.2. Spiking and recovery

The SPE recovery tests of epoxy fatty acids in edible oils have been previously carried out using the three-step SPE approach [18]. In order to test the recovery of hydroxy FAME here, saturated and unsaturated hydroxy FAME standards were used for the spiking and recovery test. Specifically, the test was conducted by adding known amounts of methyl 9, 10-epoxyoctadecanoate, methyl 12-hydroxystearate, methyl ricinoleate, and 13(S)-HODE methyl ester to the FAME derived from canola and sunflower oil. The amount of recovered standard was calculated by subtracting the amount of standard found in the unspiked matrix, if present, from the spiked matrix. The apparent recovery (%) was obtained by the mass ratio of recovered standard/spiked standard. The recoveries of epoxy and hydroxy FAME were tested in Polar Fraction 1 and Polar Fraction 2, respectively. The same data was used to construct standard addition curves. These were plotted for each spiked standard using the area ratio of analyte standard/internal standard versus the concentration ratio of analyte standard/internal standard. Matrix effects were evaluated for each standard material using analysis of covariance (ANCOVA) to examine the difference between the slopes of the calibration curve and the standard addition curve. A p-value >0.05 indicated that there was no general matrix effect.

2.6.3. Thermo-oxidation of oils

For identification of epoxy and hydroxy FAME, 30 g of sunflower oil and canola oil in 100 mL beakers were kept in the dark at 70 °C for 7 days. For method application, a stability study was conducted by applying the above method to monitor the levels of epoxy and hydroxy fatty acids in sunflower oil and canola oil during oxidation. Sunflower oil and canola oil (30 g) were weighed into 100 mL beakers and oxidized in the dark at 40 °C for 40 days. The oils were analyzed every 10 days for concentrations of epoxy and hydroxy fatty acids as well as for PV (American Oil Chemists' Society Method Cd 8-53) [2].

3. Results and discussion

3.1. Identification of epoxy and hydroxy fatty acids in oils

The chromatograms associated with the epoxy and hydroxy fatty acids quantified in oxidized oils are shown in Fig. 1, with peak identities listed in Table 2. Epoxy fatty acids (Peak 1–6 in Fig. 1) were identified by reference to the mass spectra and GC chromatograms reported by Mubiru et al. [11]. The structures of TMS derived hydroxy fatty acids were deduced from their mass spectra by referring to the fragmentation patterns reported by Kleiman and Spencer [19] and Xia and Budge [20]. The interpretation of their mass spectra can be summarized as follows: 1) α-cleavage at the carbon carrying the TMS group generated intense ions in saturated structures (Peak 15,16 in Fig. 1); 2) in allylic and conjugated structures (Peak 7–14, 19–22 in Fig. 1), cleavages on both sides of the allylic/conjugated system resulted in diagnostic ions, with the base ion formed from the cleavage α to the carbon carrying the TMS group; and 3) identities of non-allylic structures (Peak 17,18 in Fig. 1) were confirmed by their mass spectral data reported in previous studies [19,21].

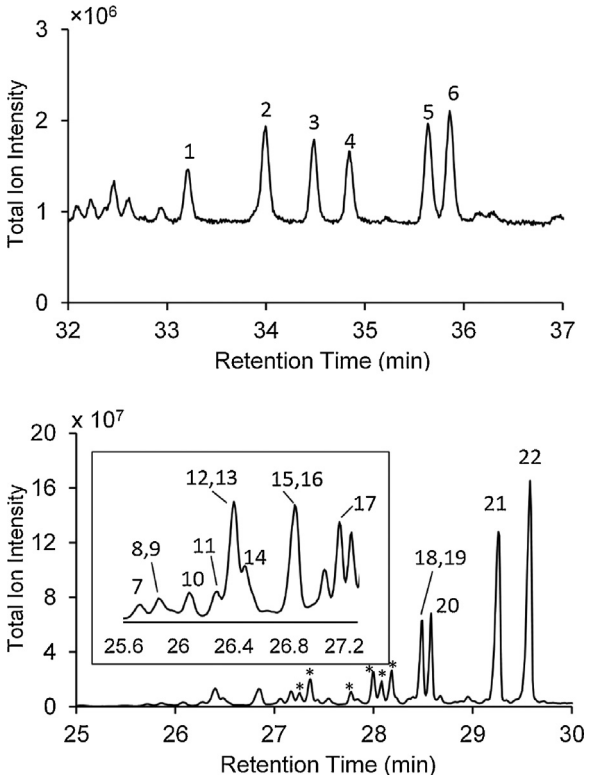

Fig. 1. GC–MS total ion chromatograms of epoxy FAME in Polar Fraction 1 (top) and TMS ethers of hydroxy FAME in Polar Fraction 2 (bottom) derived from sunflower oil oxidized at 70 °C for 7 days, with peak identities listed in Table 2. *indicates unidentified peaks with m/z 73 in the mass spectra, suggesting that they were TMS derivatives.

The occurrences of epoxy fatty acids in Polar Fraction 1 were the same as those reported in the model systems [15] and thermo-oxidized oils [10,16]; specifically, two epoxy-C18:0 and four epoxy-C18:1 were found in oxidized oils. The formation of the epoxy group was expected to occur by replacement of an original double bond in the unsaturated fatty acids [15]. Therefore, 9,10-epoxyoctadecanoate (9,10-epoxy-C18:0) formed from oxidation of oleic acid, with 12,13-epoxyoctadec-9-enoate (12,13-epoxy-C18:1) and 9,10-epoxyoctadec-12-enoate (9,10-epoxy-C18:1) derived from oxidized linoleic acid. In addition, each positional isomer appeared as two isomers of *cis/trans* epoxides. The Rtx-2330 capillary column (105 m × 0.25 mm i.d.) used here employed a biscyanopropyl cyanopropylphenyl polysiloxane

Table 2
Identification of epoxy and hydroxy FAME in Polar Fraction 1 and 2, corresponding to the peaks labelled in Fig. 1.

Peak No.[a]	Compound	M^+	Base peak	High mass ions $m/z > 100$ (Relative abundance%)
Epoxy FAME				
1,2	Methyl 9,10-epoxyoctadecanoate	312	67	199 (11), 171 (11), 167 (19), 155 (55), 153 (33), 139 (28), 127 (35), 121 (33), 109 (50)
3,5	Methyl 12,13-epoxyoctadec-9-enoate	310	79	217 (12), 189 (10), 164 (14), 149 (22), 135 (30), 121 (44), 107 (24), 105 (21)
4,6	Methyl 9,10-epoxyoctadec-12-enoate	310	67	155 (20), 150 (26), 125 (23), 121 (24), 109 (36)
Hydroxy FAME (in TMS ether form)				
7,11	Methyl 8-hydroxyoctadec-9-enoate	384	241	369 (2), 337 (10), 241 (100), 129 (52)
8,12	Methyl 9-hydroxyoctadec-10-enoate	384	227	369 (2), 337 (8), 227 (100), 129 (62)
9,13	Methyl 10-hydroxyoctadec-8-enoate	384	271	369 (2), 337 (12), 271 (100), 149 (80)
10,14	Methyl 11-hydroxyoctadec-9-enoate	384	285	369 (2), 337 (7), 285 (100), 163 (50), 135 (35)
15	Methyl 9-hydroxyoctadecanoate	386	259	371 (4), 339 (22), 259 (100), 229 (29), 155 (68)
16	Methyl 10-hydroxyoctadecanoate	386	273	371 (4), 339 (12), 273 (100), 215 (28), 169 (30)
17	Methyl 12-hydroxyoctadec-9-enoate (methyl ricinoleate)	384	187	369 (6), 337 (20), 299 (14), 270 (54), 187 (100), 159 (18), 146 (10), 103 (46)
18	Methyl 15-hydroxyoctadeca-9,12-dienoate	382	73	335 (10), 310 (67), 145 (70), 103 (49)
19,22	Methyl 13-hydroxyoctadeca-9,11-dienoate (13-HODE)	382	311	382 (40), 367 (4), 335 (7), 311(100), 269 (14), 259 (22), 225 (40), 207 (60),189 (40), 171 (84), 161 (50), 130 (67)
20,21	Methyl 9-hydroxyoctadeca-10,12-dienoate (9-HODE)	382	225	382 (42), 367 (2), 311(30), 272 (15), 225 (100), 215 (14), 155 (19), 135 (34), 130 (26), 107 (24)

[a] The compounds identified with two peak numbers were geometric isomers, giving identical mass spectra but different retention times.

phase. The cyano-dipole associated with this phase has a stronger interaction with *cis*-isomers (both *cis*-double bonds and *cis*-epoxy groups) than with *trans*-isomers, due to the differences in the distribution of isomeric hydrogens, resulting in longer retention times for *cis*-isomers than their *trans*-isomers [22]. Peak 1, 3, 4 in Fig. 1 were *trans* epoxy isomers and Peak 2, 5, 6 were *cis* isomers. The assignments of the epoxy fatty acids in this study were consistent with the findings reported by Mubiru, Shrestha, Papastergiadis and De Meulenaer [11], who employed a CP-Sil 88 (60 m × 0.25 mm i.d.) column with a highly substituted cyanopropyl phase, for separation of epoxy FAME isomers.

The occurrences of unsaturated hydroxy fatty acids in oxidized oils have rarely been reported in the literature, except for the hydroxydienes determined by the HPLC-UV method [8,23,24]. In this study, TMS derivatives of saturated, monounsaturated, and diunsaturated hydroxy FAME were identified in Polar Fraction 2, which represented hydroxy-C18:0, hydroxy-C18:1, and hydroxy-C18:2 in triacylglycerols in oils. The only saturated hydroxy FAME were 9- and 10-hydroxyoctadecanoate (9- and 10-hydroxy-C18:0), appearing as a co-eluting peak in GC. Four positional isomers of allylic hydroxy-C18:1 eluted before hydroxy-C18:0, with hydroxy groups at the 8, 9, 10, and 11 positions. A pair of *cis/trans* isomers were associated with each positional isomer; therefore, each of 8-, 9-, 10-, and 11-hydroxy-C18:1 was associated with two GC peaks in Table 2. A non-allylic hydroxy-C18:1, ricinoleate (12-hydroxyoctadec-9-enoate), was also quantified in this study. The conjugated hydroxydienes were identified as 13-hydroxyoctadeca-9,11-dienoate (13-HODE) and 9-hydroxyoctadeca-10,12-dienoate (9-HODE) and both occurred as two geometric isomers.

In canola oil, a non-conjugated hydroxy-C18:2, 15-hydroxyoctadeca-9,12-dienoate, co-eluted with an isomer of 13-HODE in Polar Fraction 2 (Peak 18 and 19 in Fig. 1). Such co-elution did not occur with sunflower oil since 15-hydroxyoctadeca-9,12-dienoate was not found in sunflower oil even by monitoring its diagnostic ion m/z 310. Due to the co-elution problem, GC-FID was not sufficient to determine the hydroxydiene concentrations in canola oil so GC–MS-SIM was employed to determine 13-HODE by monitoring m/z 382. Ideally, intense ions are used for MS-SIM determination. However, the most intense ions in the mass spectrum of 13-HODE, including m/z 311, 207, 171, 161, 130, and 73, were all present in the spectrum of 15-hydroxyoctadeca-9,12-dienoate so they were not suitable for quantifying 13-HODE. The molecular ion (M^+, m/z 382) of 15-hydroxyoctadeca-9,12-dienoate was not detected in its spectrum, allowing m/z 382 to be used in SIM mode to quantify 13-HODE without interference (Supplementary Fig. 1) [21].

3.2. Quantification of epoxy and hydroxy fatty acids in oils

3.2.1. Response factors

Calibration curves were plotted for epoxy and hydroxy FAME standards and their response factors are listed in Table 3. Using GC-FID, LOQ varied between 0.4-1.0 μg/mL, which was comparable to the LOQ reported previously for saturated epoxy (1.6 μg/mL) and hydroxy FAME (2.1 μg/mL) using GC-FID [12] and for hydroxydienes (0.6 μg/mL) using HPLC-UV [8]. Using GC–MS-SIM, the calibration curve of 13-HODE methyl ester was $y = 0.3183x - 0.0722$, where x was the concentration ratio of 13-HODE/C19:0 and y was the area ratio of 13-HODE/C19:0. The intercept was a negative value, and was significantly different from 0 (one sample *t*-test *p*-value < 0.001). Thus, the regression equation was only applicable for quantification of 13-HODE methyl ester within the linear range of 2–10 μg/mL in SIM mode.

3.2.2. Recoveries

The SPE recoveries for epoxy and hydroxy standards were between 91 and 112% with RSD of 0.3-9% (Table 4). The matrix effect was then examined by comparing the slopes of the calibration curve and the standard addition curve for each type of standard (Table 5). P-values >0.05 for all standards indicated that the two slopes were not different and that a matrix effect did not exist for sunflower or canola oil.

3.2.3. Stability study

The LOD for epoxy fatty acids in oils was 30 μg/g, much higher than the LOD (1.45 μg/g) reported by Mubiru, Shrestha, Papastergiadis and De Meulenaer [11]. The lower LOD of their method was likely due to the larger sample load (~200 mg) applied to the SPE column, compared to ~10 mg employed in our study. LOD obtained for saturated and unsaturated hydroxy fatty acids in our study ranged between 3 and 4 μg/g, better than those reported for hydroxydienes using HPLC-UV and HPLC-ELSD without a concentration step like SPE [8,9]. However, we are unable to compare our results for unsaturated hydroxy fatty acids using SPE procedures to literature values since such data do not exist. Although the LOQ for epoxy and hydroxy fatty acids can be interpreted as the lowest

Table 3
Determination of response factors with C19:0 as the internal standard.

Standard	Detection	Response factor	Correlation coefficient (R^2)	LOD (μg/mL)	LOQ (μg/mL)
methyl 9,10-epoxyoctadecanoate	FID	0.8170	0.9958	0.3	1.0
methyl 12-hydroxyoctadecanoate[a]	FID	1.0650	0.9994	0.1	0.4
methyl ricinoleate[a]	FID	0.9763	0.9991	0.2	0.5
13-HODE methyl ester[a]	FID	0.9630	0.9991	0.1	0.5
13-HODE methyl ester[a]	SIM-MS	0.3183	0.9954	–[b]	2.0[c]

[a] The hydroxy FAME were analyzed in their TMS ether forms.
[b] LOD was not determined for 13-HODE in SIM-MS because the intercept of the response curve was significantly different from 0 (one sample *t*-test *p*-value<0.001), which may indicate a non-linear response curve between 0 and 2 μg/mL.
[c] LOQ for 13-HODE in SIM-MS was determined as the lower limit of the working range, 2–10 μg/mL.

Table 4
SPE recoveries of epoxy and hydroxy FAME standards spiked in sunflower oil and canola oil.

Standard	Spiked mass (μg)	Sunflower oil		Canola oil	
		Apparent recovery mean ± SD (%)	RSD (%)	Apparent recovery mean ± SD (%)	RSD (%)
methyl 9,10-epoxyoctadecanoate	2	105 ± 4	4	105 ± 4	4
	4	91 ± 5	5	101 ± 4	4
	6	96 ± 3	3	93 ± 6	7
	8	108 ± 7	7	105 ± 2	2
	10	99 ± 3	2	104 ± 2	2
methyl 12-hydroxyoctadecanoate	0.4	103 ± 1	1	110 ± 0.35	0.3
	0.8	93 ± 2	2	107 ± 2	2
	1.2	94 ± 2	2	99 ± 4	4
	1.6	104 ± 2	2	107 ± 1	1
	2.0	95 ± 1	1	101 ± 2	2
methyl ricinoleate	0.4	100 ± 6	6	106 ± 4	4
	0.8	107 ± 1	1	101 ± 9	9
	1.2	95 ± 5	5	94 ± 6	7
	1.6	101 ± 5	5	102 ± 2	2
	2.0	106 ± 3	3	95 ± 5	5
13-HODE methyl ester	0.4	102 ± 7	7	100 ± 3	3
	0.8	97 ± 3	4	97 ± 5	6
	1.2	104 ± 3	3	95 ± 7	7
	1.6	101 ± 6	6	112 ± 7	6
	2.0	97 ± 1	1	96 ± 3	3

Table 5
The slopes and correlation coefficient (R^2) of the standard addition curves obtained for sunflower and canola oils, with matrix effects evaluated by the *p*-values of the ANCOVA tests.

Standard	Sunflower oil			Canola oil		
	slope	R^2	p-value	slope	R^2	p-value
methyl 9,10-epoxyoctadecanoate	0.8291	0.9877	0.651	0.8472	0.9930	0.181
methyl 12-hydroxyoctadecanoate	1.0387	0.9936	0.237	1.0426	0.9944	0.286
methyl ricinoleate	1.0099	0.9928	0.149	0.9324	0.9857	0.140
13-HODE methyl ester	0.9505	0.9946	0.517	0.9866	0.9737	0.569

limit for quantification, the use of LOQ has been controversial and measurements below a LOQ may not be devoid of information [25]. Therefore, in this paper, quantitative data above LOD but below LOQ is presented.

Upon artificial oxidation at 40 °C, a number of epoxy and hydroxy fatty acids were apparent and measured in sunflower oil and canola oil (Tables 6 and 7). Using GC-FID, RSDs for epoxy fatty acids in the oils were 0.6–13.7%, while RSDs obtained for hydroxy fatty acids were 1.2–15.1%. In MS-SIM, the RSDs for quantification of 13-HODE were 10.0–18.8%. The RSDs in this study were comparable to other studies which determined epoxy fatty acids in food samples [18], and epoxy and hydroxy fatty acids in oils [12].

Among the epoxy fatty acids present in the fresh sunflower oil (Day 0), *cis*-9,10-epoxy-C18:1 was the highest in quantity. The levels of individual epoxy fatty acids in the sunflower oil were slightly lower than those found in five sunflower oils in a previous study [11] (223–414 μg/g for *cis*-9,10-epoxy-C18:0, 55–110 μg/g for *cis*-12,13-epoxy-C18:1, and 748–1434 μg/g for *cis*-9,10-epoxy-C18:1), but within the range of those found in 13 sunflower oil samples tested in another work [6] (10–500 μg/g for *cis*-9,10-epoxy-C18:0, 20–1200 μg/g for *cis*-12,13-epoxy-C18:1, and 20–1140 μg/g for *cis*-9,10-epoxy-C18:1). During the stability study, the peaks associated with *cis*-12,13-epoxy-C18:1 and *cis*-9,10-epoxy-C18:1 were quantified in sunflower oil but they were present below 30 μg/g in canola oil throughout 40 days. No quantitative data of epoxy fatty acids has been reported for canola oil in literature to allow comparison. However, Mubiru, Shrestha, Papastergiadis and De Meulenaer [11] suggested a strong correlation between the fatty acid ratios of C18:1/C18:2 and epoxy-C18:0/epoxy-C18:1, so the lower content of epoxy-C18:1 in canola oil could be due to its lower concentration of linoleic acid (C18:2) compared to sunflower oil.

Peaks associated with *trans*-epoxy fatty acids were not observed in sunflower oil or canola oil over the period of 40 days, likely due to the mild oxidation temperature of 40 °C. *trans*-epoxy fatty acids are produced fast during frying of oils and thus are present in large amounts in used frying oils [6,16]. In non-fried oils, the levels of *trans*-epoxy fatty acids are normally ~10-50 times lower than those of *cis*-epoxy fatty acids [6]. Therefore, in order to determine *trans*-epoxy fatty acids in fresh or mildly oxidized oils, the LOD needs to be pushed to a much lower level, e.g. <1 μg/g.

Table 6
Quantification of epoxy and hydroxy fatty acids in sunflower oil oxidized at 40 °C.

Day	PV (meq/kg)	Epoxy fatty acid concentration (μg/g)			Hydroxy fatty acid concentration (μg/g)						
		cis−9,10-epoxy-C18:0[a]	*cis*−12,13-epoxy-C18:1[a]	*cis*−9,10-epoxy-C18:1[a]	9- and 10-hydroxy-C18:0	9- and 10-hydroxy-C18:1[b]	ricinoleate	13-HODE1[c]	9-HODE1[c]	9-HODE2[c]	13-HODE2[c]
0	13.7 ± 0.1	111.1 ± 7.3	38.8 ± 2.5	194.1 ± 16.8	32.8 ± 2.4	35.4 ± 3.0	134.6 ± 8.0	63.2 ± 5.7	76.7 ± 6.7	58.5 ± 4.4	52.4 ± 4.1
10	31.8 ± 0.3	122.7 ± 8.7	44.8 ± 6.1	205.4 ± 11.0	33.6 ± 0.6	35.7 ± 1.7	123.5 ± 5.8	78.3 ± 11.0	103.0 ± 15.9	67.1 ± 4.3	58.4 ± 4.5
20	57.9 ± 0.6	117.5 ± 4.4	50.2 ± 4.3	195.9 ± 7.6	31.5 ± 0.4	33.5 ± 0.6	128.6 ± 1.9	111.2 ± 2.6	132.1 ± 3.0	63.7 ± 1.2	55.9 ± 1.3
30	88.9 ± 0.3	150.2 ± 7.0	74.1 ± 3.4	238.1 ± 12.6	34.7 ± 2.6	37.1 ± 3.0	140.9 ± 11.0	140.4 ± 6.7	171.7 ± 7.9	74.3 ± 2.7	65.2 ± 1.9
40	122.8 ± 0.3	146.1 ± 10.8	59.1 ± 0.3	214.8 ± 9.2	33.0 ± 2.6	35.7 ± 2.5	134.2 ± 12.5	176.8 ± 7.7	208.6 ± 9.5	78.9 ± 3.4	67.6 ± 2.6

[a] *cis*-9,10-epoxyoctadecanoate, *cis*-12,13-epoxyoctadec-9-enoate, and *cis*-9,10-epoxyoctadec-12-enoate were abbreviated as *cis*-9,10-epoxy-C18:0, *cis*-12,13-epoxy-C18:1, and *cis*-9,10-epoxy-C18:1, respectively.
[b] 9-hydroxyoctadec-10-enoate and 10-hydroxyoctadec-8-enoate were abbreviated as 9- and 10-hydroxy-C18:1, respectively.
[c] 13-hydroxyoctadeca-9,11-dienoate and 9-hydroxyoctadeca-10,12-dienoate were abbreviated as 13-HODE and 9-HODE, respectively. 13-HODE1, 9-HODE1, 9-HODE2, and 13-HODE2 corresponded to Peak 19, 20, 21, and 22 in Fig. 1, following ascending order of their retention times.

Table 7
Quantification of epoxy and hydroxy fatty acids in canola oil oxidized at 40 °C.

Day	PV (meq/kg)	Epoxy fatty acid concentration (μg/g)			Hydroxy fatty acid concentration (μg/g)						
		cis−9,10-epoxy-C18:0[a]	*cis*−12,13-epoxy-C18:1[a]	*cis*−9,10-epoxy-C18:1[a]	9- and 10-hydroxy-C18:0	9- and 10-hydroxy-C18:1[b]	ricinoleate	13-HODE1[c,d]	9-HODE1[c]	9-HODE2[c]	13-HODE2[c]
0	2.9 ± 0.1	<30	<30	<30	239.7 ± 3.2	<5	258.5 ± 5.8	<40	12.2 ± 1.3	<5	<5
10	9.7 ± 0.1	43.6 ± 5.7	<30	<30	247.3 ± 6.7	<5	248.3 ± 6.4	<40	13.1 ± 1.7	<5	<5
20	37.3 ± 0.6	32.5 ± 3.2	<30	<30	231.7 ± 22.3	<5	244.3 ± 26.0	41.9 ± 4.2	34.8 ± 3.5	8.4 ± 0.5	8.2 ± 0.8
30	71.2 ± 1.0	89.2 ± 2.7	<30	<30	230.5 ± 13.1	<5	246.5 ± 13.6	55.1 ± 10.3	56.6 ± 3.4	14.6 ± 1.6	13.9 ± 1.7
40	99.7 ± 0.9	94.0 ± 9.4	<30	<30	246.3 ± 13.0	8.9 ± 0.5	261.7 ± 14.5	95.6 ± 9.5	94.0 ± 9.0	34.2 ± 5.1	31.3 ± 4.2

[a] *cis*-9,10-epoxyoctadecanoate, *cis*-12,13-epoxyoctadec-9-enoate, and *cis*-9,10-epoxyoctadec-12-enoate were abbreviated as *cis*-9,10-epoxy-C18:0, *cis*-12,13-epoxy-C18:1, and *cis*-9,10-epoxy-C18:1, respectively.
[b] 9-hydroxyoctadec-10-enoate and 10-hydroxyoctadec-8-enoate were abbreviated as 9- and 10-hydroxy-C18:1, respectively.
[c] 13-hydroxyoctadeca-9,11-dienoate and 9-hydroxyoctadeca-10,12-dienoate were abbreviated as 13-HODE and 9-HODE, respectively. 13-HODE1, 9-HODE1, 9-HODE2, and 13-HODE2 corresponded to Peak 19, 20, 21, and 22 in Fig. 1, following ascending order of their retention times.
[d] Quantification of 13-HODE1 in canola oil was carried out using GC–MS-SIM.

During the stability study at 40 °C, the only observable allylic hydroxy-C18:1 were 9- and 10-hydroxy-C18:1 (Peak 12 and 13 in Fig. 1), which were measurable in sunflower oil from Day 0 but only detectable in canola oil on Day 40. The other isomers of allylic hydroxy-C18:1 (Peak 7–14 in Fig. 1) were not seen in sunflower oil or canola oil at 40 °C, although the signals were obvious in the oils oxidized at 70 °C for 7 days. Therefore, neither 8- nor 11-hydroxy-C18:1 were quantified during the stability study at 40 °C. Conjugated hydroxy-C18:2 (HODEs) were found during oxidation of sunflower oil and canola oil, with all four isomers presented as 13-HODE1, 9-HODE1, 9-HODE2, and 13-HODE2, based on the ascending order of their retention times (corresponding to Peak 19–22 in Fig. 1, respectively). Again, the detection of 13-HODE1 in canola oil was limited to the linear range of the calibration curve (2–10 μg/mL) in SIM mode; therefore, the occurrence of 13-HODE1 was only quantified from Day 20. The concentration of 15-hydroxyoctadeca-9,12-dienoate (Peak 18 in Fig. 1) was not determined in this study, although its concentration could have been calculated by subtracting the amount of 13-HODE1 from the mass of hydroxy-C18:2 determined by the area count of the co-eluting peak; however, this approach could have introduced large errors in the measurements so was not pursued.

The exclusion of hydrogenation suggests new findings about the formation of unsaturated hydroxy fatty acids in edible oils. First, the differences in the concentrations of hydroxy-C18:0, hydroxy-C18:1, and hydroxy-C18:2 would not be observed with hydrogenation applied, as they would all have been measured as saturated structures. The method introduced here allowed determinations of allylic hydroxy-C18:1 and conjugated hydroxy-C18:2, which would be considered the main products of hydrogen abstraction by oleic and linoleic alkoxy radicals. Although linoleic acid only comprised <20% of total fatty acids in canola oil, the total concentration of conjugated hydroxy-C18:2 was always higher than that of allylic hydroxy-C18:1 in canola oil over 40 days, which may be due to the higher oxidation rate of linoleic acid compared to oleic acid. Second, the concentration of an individual HODE isomer was quite consistent relative to the total content of HODE isomers. Specifically, in sunflower oil, 9-HODE1, 9-HODE2, 13-HODE1, and 13-HODE2 comprised 36 ± 4%, 19 ± 4%, 29 ± 4%, and 16 ± 3% of the total HODE in each sample over 40 days. In canola oil, since most HODEs were only detectable from Day 20, the percentages of 9-HODE1, 9-HODE2, 13-HODE1, and 13-HODE2 in total HODE from Day 20 to Day 40 were: 38 ± 2%, 11 ± 2%, 41 ± 4%, and 10 ± 2%. Third, significant changes were observed for the concentrations of allylic hydroxy-C18:1 and conjugated hydroxy-C18:2 during the stability study at 40 °C, while hydroxy-C18:0 and ricinoleate remained constant over the 40 day period, which suggested that the saturated and methylene-interrupted structures were not arising from autoxidation at 40 °C. Last, this method allowed comparison of the epoxy and hydroxy products formed from the same fatty acid, which can be a potential approach to compare the lipid oxidation pathways. For example, epoxy-C18:0 and hydroxy-C18:1 are both considered oxidation products of oleic acid, and epoxy-C18:1 and hydroxy-C18:2 are derived from linoleic acid. In both oils examined in this study, the concentration of epoxy-C18:0 was always higher than hydroxy-C18:1 but total epoxy-C18:1 were less than total hydroxy-C18:2. Further investigations of these findings can be carried out using the proposed method for the study of lipid oxidation mechanisms.

In summary, the method presented here allowed simultaneous determinations of both saturated and unsaturated, epoxy and hydroxy fatty acids in vegetable oils. The method has been successfully applied to monitor these oxidation products during stability studies of oils conducted at 40 °C. The focus of this paper was given to method development including the identification of the oxygenated FAME, validation of SPE conditions, and application to quantitative studies of these oxygenated products derived from lipid oxidation. Modifications can be made to increase the method LOD by, for example, increasing the SPE sample load, since this would result in more analyte collected for GC analysis. With appropriate validation of the SPE recoveries, the experimental procedures employed in this study will serve as a foundation for future studies on oxygenated fatty acids for applications to food lipids and oils.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at https://doi.org/10.1016/j.chroma.2017.12.066.

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