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Improved gas chromatography-flame ionization detector analytical method for the analysis of epoxy fatty acids

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ABSTRACT

In this study an improved method for analysis of epoxy fatty acids is reported. Data obtained from analysis of polar fatty acids has previously been presented, but due to the high number of compounds that co-elute in the polar fraction, the resultant chromatograms are complex which may lead to compromising the accuracy of the data. A three steps separation of fatty acid methyl esters (FAMEs) by solid-phase extraction (SPE) on a silica gel column to remove hydroxy fatty acid interferences was proposed. This approach is opposed to a two step separation procedure that has been often used to prevent analytical interferences caused by non-altered fatty acids. A gas chromatograph with a flame ionization detector (GC-FID) equipped with a polar CP-Sil 88^{TM} column was used. Quantification was based on the use of methyl nonadecanoate (C19:0), as an internal standard. Individual mono epoxy fatty acids were well separated without co-eluting compounds. The optimized method was finally applied to screen epoxy fatty acids in 37 fresh oil samples. Results obtained for the total epoxy fatty acids were in the range 0.03–2 mg g^{-1} of oil with repeatability coefficient of variation (CV) ranging from 2.8 to 9.9% for duplicate analysis showing that the results obtained are repeatable.

1. Introduction

Lipid oxidation is one of the most important chemical reactions in food which results in food deterioration. A number of toxic compounds such as hydroxylated alkenals and malonaldehyde formed during lipid oxidation have been well studied [1–3]. In addition, polar fatty acids with one or more oxygenated functional groups such as epoxy, keto and hydroxyl can be formed in at least one of the fatty acyl chains of the triacylglycerols. These compounds are believed to be stable final products that result from the decomposition of hydroperoxides [4]. The major precursors of these oxygenated fatty acids are polyunsaturated fatty acids (PUFAs) [5,6]. Since the drive today is to enrich oils with polyunsaturated fatty acids thus making them more prone to oxidation, the need for sensitive methods for analysis of these compounds is timely.

The analysis of oxygenated fatty acids has received less attention and yet they are reported to form in high amounts especially in thermally oxidized foods [7–9]. Generally these oxygenated fatty acids have been reported to have toxic effects [10,11], particularly the long chain epoxy compounds have been reported to be protoxins [11]. A protoxin is a chemical compound that only becomes a toxin after it is altered typically during metabolism. Studies conducted on women have revealed that epoxy and hydroxy fatty acids are absorbed in healthy women, a property which makes them a food safety issue [12,13].

The analysis of oxygenated polar fatty acids is generally based on the pre-separation of polar and non polar fatty acid methyl esters (FAMEs) in a silica column followed by the gas chromatographic separation coupled with a flame ionization detector (GC-FID). Solid-phase extraction (SPE) on a silica column by use of a two step separation technique which involves the use of an apolar solvent mixture (e.g. hexane–diethyl ether, 98:2, v/v) to elute the non polar FAMEs followed by the elution with a polar solvent (e.g. diethyl ether) to collect all the polar FAMEs in one fraction has been described previously.

However, this analysis is challenging as revealed by the complex gas chromatograms reported earlier [7,9,14,15] due to co-elution and interference from other compounds especially the hydroxy fatty acids. Several researchers have tried to overcome this gas chromatographic separation challenge by use of different techniques such as use of different GC columns and hydrogenation. Though the use of non polar GC columns has an advantage that the hydroxy fatty acids can be determined without silylation, their capacity to resolve isomers is limited [16]. Hydrogenation also has some setbacks as it is not possible to identify and quantify the oxygenated unsaturated fatty acid isomers individually and also leads

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to co-elution of peaks. Epoxy FAME studies done on some polar GC columns like SGE BPX70 could not identify all the six isomers of C18:1 and C18:2 due to co-elution [15]. In another study done using a SP 2560 without silylation, authors complained of chromatographic distortion by the hydroxy compounds and co-elution due to the high number of compounds that form during lipid oxidation [14].

A more polar high resolution GC capillary column CP-Sil 88^{TM} has been highly recommended for the analysis of FAMEs because of its capacity to resolve cis and trans isomers. Moreover, in recent times improved analytical approaches have been necessary to meet increasingly stringent requirements for food safety standards. Though there has not been any attempt to set tolerable daily intakes (TDIs) for the polar fatty acids especially the epoxy and hydroxy fatty acids by international bodies involved in risk assessment and management, studies towards effective and sensitive methods that can enable accurate quantification of these compounds is a move in the right direction. However, the European Food Safety Authority (EFSA) has only set a TDI for the Epoxidised Soya Bean Oil (ESBO) at 1 mg kg^{-1} body weight based on a minimum 7% – maximum 8% oxirane oxygen content [17]. But since ESBO comprises of 25% epoxy oleate fatty acids, this value can indirectly be used to infer about the toxic contribution of epoxy compounds in the diet [14].

The objective of the study was to improve the method of analysis of epoxy fatty acids. Emphasis was put on the epoxy fatty acids containing eighteen carbon atoms. To achieve this, a simple three steps SPE of the methyl ester on 10% moisture silica gel column has been proposed. The three steps SPE involved use of three solvent systems of different polarity to partition the FAMEs. This SPE separation step and the use of a polar GC column CP-Sil 88^{TM} proved to be strong analytical techniques in this method. To demonstrate the suitability of the method, results obtained after analysing different fresh oil samples on Belgian market are presented.

2. Materials and methods

2.1. Chemicals and materials

Tert-butyl methyl ether (tBME) and 3-chloroperoxybenzoic acid (70–75%) were purchased from Acros Organics (Geel, Belgium). Sodium methoxide solution (25%), silica gel 60 for column chromatography (particle size = 0.063–0.100 mm), methyl 12-oxostearate and methyl 12-hydroxystearate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methyl oleate (C18:1), methyl linoleate (C18:2), methyl linolenate (C18:3), methyl nonadecanoate (C19:0) and a mixed GLC 68D standard were obtained from Nu-Chek-Prep., Inc. (USA). All other chemicals and reagents were of analytical grade and obtained from the local suppliers.

2.2. Samples

2.2.1. Oils

A total of 37 fresh oil samples were purchased from five different local markets in Belgium. They were brought to the laboratory and analyzed immediately.

2.3. Standards

2.3.1. Qualitative standards

Standards that were used for qualitative identification of epoxy fatty acids on GC-FID were prepared by thermoxidising methyl oleate, linoleate, α-linolenate and sunflower oil in a Muffle furnace (Heraeus Instruments, Germany) for 10 h at 180 ± 2 °C. About 1 g of the FAMEs and sunflower oil was placed in a Duran test tube (GL 14) without a cap and put in a beaker containing glycerol to aid uniform transfer of heat to the samples.

2.3.2. Synthesis of cis-9,10-epoxystearate

The epoxy analyte was synthesized based on a method described by Gunstone and Jacobsberg as reported by Christie [18]. The methyl oleate was reacted with 3-chloroperoxybenzoic acid in chloroform at room temperature for 4 h. The epoxy ester was purified on silica gel column (25 g) by eluting the unreacted fatty acids with 200 mL hexane–diethyl ether (98:2, v/v) as the mobile phase and the epoxy fatty acid with 300 mL of hexane–diethyl ether (90:10, v/v). Confirmation of the identity and purity of the synthesized compound was done by use of GC–MS.

2.4. Sample preparation

2.4.1. Base-catalyzed-transmethylation with sodium methoxide at room temperature

Transmethylation followed Berdeaux et al. method [19] with modifications: Briefly 500 mg oil sample was accurately weighed into 25 mL glass centrifuge tube and a volume of 5 mL of tert-butyl methyl ether (tBME) was added. Then, 2.5 mL volume of 0.2 M sodium methoxide solution in methanol was added and vortexed for 1 min, and allowed to stand at room temperature for 2 min. For neutralization purposes and prevent saponification, a 0.17 mL volume of 0.5 M sulphuric acid was added and the mixture was vortexed for a few seconds. Finally, 5 mL of water was added, vortexed for 30 s and centrifuged at 3600 × *g* for 1 min. The organic layer was collected over a layer of sodium sulphate and the extraction was repeated two times with 5 mL of tBME. The organic layer was evaporated on a rotavap and finally dried under nitrogen. The resultant FAME was dissolved into 5 mL of n-hexane–diethyl ether (98:2, v/v) and accurately 2 mL was loaded onto the silica column.

2.5. SPE column preparation and packing

2.5.1. Activation of the silica gel

Silica gel was dried in a muffle furnace at 450 °C for 12 h and later cooled in a desiccator. Finally the moisture content was adjusted to 10% and equilibrated on a shaker for 1 h before use.

2.5.2. Preparation of the silica column

An empty SPE cartridge column (6 mL, 6.5 cm × 1.3 cm) was filled with 2 mL of the elution solvent; n-hexane–diethyl ether (98:2, v/v). Silica gel slurry was prepared by mixing 1 g in 3 mL of the elution solvent and poured into the column; care was taken to avoid trapping of air by tapping the column slightly to ensure uniform packing and finally a small amount of sand was added to protect the column.

2.5.3. Optimization of the column chromatography-partitioning

An aliquot of 2 mL FAMEs was loaded onto the prepared silica column and the non-polar fraction which comprises of the non-altered FAMEs was eluted with 15 mL of n-hexane–diethyl ether (98:2, v/v). The remaining polar compounds in the silica were eluted in two steps: polar fraction 1 comprising mainly of epoxy compounds was eluted with 15 mL of n-hexane–diethyl ether (90:10, v/v) and 10 μg of methyl nonadecanoate (C19:0) prepared in iso-octane, was added as internal standard. This was later completely dried under nitrogen and dissolved in 200 μL of iso-octane ready for GC analysis. The second fraction, polar fraction 2 mainly comprising of the hydroxy fatty acids was eluted with 25 mL of hexane–diethyl ether (70:30, v/v).

Separation of the fractions was confirmed by thin-layer chromatography (TLC) according to Marmesat [7], using a plate of silica gel 60 (5 cm × 10 cm plates, 0.25 mm thickness). The

plate was deliberately overloaded, developed with hexane–diethyl ether–acetic acid (80:20:1, v/v/v) and visualized with iodine vapour. Results from TLC showed that the non polar, the polar fraction 1 and the polar fraction 2 were clearly separated.

2.5.4. SPE recoveries

Recoveries of the analytes were determined on a model system consisting of a mixture of methyl *trans*-10-heptadecanoate, methyl *cis*-9,10-epoxystearate, methyl 12-oxostearate and methyl 12-hydroxystearate in iso-octane at the same concentration level of 100 μg mL^{-1}. The mixture (1 mL) was separated on the silica columns in triplicate and the recoveries were calculated. The quantification was based on methyl nonadecanoate as an internal standard. The recovery was calculated as the ratio of the concentration of analyte remaining after the separation divided by the analyte's initial concentration in the mixture before partitioning [20].

2.6. Silylation of the FAMEs

The fractions were silylated using 10% trimethylchlorosilane (TMCS) in N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA). The FAMEs to be silylated were dried under nitrogen and the silylating reagent was added in excess (100 μL). The reaction was allowed to proceed at room temperature for 20 min, the reagent was evaporated under nitrogen and then the analytes were dissolved in iso-octane prior to GC injection.

2.7. Instrumentation

2.7.1. Gas chromatography-FID conditions

The FAMEs were analyzed by GC-FID using an Agilent 6890 N series gas chromatograph (Agilent, USA). The samples were dissolved in iso-octane, and 0.1 μL was injected directly into the column using a cold on column injector (COC); separation was performed in a CP-Sil 88^{TM} for FAME (60 m × 0.25 mm i.d.) capillary column coated with a 0.2 μm film. Deactivated fused silica precolumn 3 m × 0.25 mm i.d. (Agilent, Belgium) was fitted to protect the column. The oven temperature programme was set as follows; 50 °C hold for 4 min, then ramp to 225 °C at 12 °C min^{-1}, and hold for 25 min. The flame ionization detector temperature was set at 300 °C. The detector flow rates for hydrogen, air and helium (makeup) was 40, 400 and 20 mL min^{-1} respectively. The column flow rate of helium as a carrier gas was 1 mL min^{-1}. Identification of individual FAMEs was carried out by comparison of retention times with those of authentic standards (GLC 68D, Nu-Chek Prep., Inc., and USA). The epoxy FAMEs were confirmed by use of GC–MS and for quantification the response factor based on epoxystearate was used.

2.7.2. Gas chromatography–mass spectrometer conditions

A GC–MS was used for the qualitative identification of the polar FAMEs. The FAMEs were injected in an Agilent 7890A GC equipped with a 5975C Mass Spectrometer (Agilent Technologies, Palo Alto, CA). Chromatographic conditions and column were the same as those applied on GC-FID. The injection volume was 1 μL and the PTV injector was kept at 53 °C for 0.2 min then ramped to 200 °C at a rate of 700 °C min^{-1}. The MSD conditions were: capillary direct interface temperature, 250 °C; ionization energy, 70 eV; operating in a scan mode between *m*/*z* 30 and *m*/*z* 600; scan rate 3.64 cycles s^{-1}.

2.8. Fatty acid composition and peroxide value (PV) analysis

Fatty acid composition was determined after preparation of FAMEs according to American Oil Chemists' Society (AOCS) Official Method (Ce 1b-89) [21]. The peroxide values was determined according to AOCS official (Cd 8b-90) [22].

2.9. Statistical analyses

Analytical determinations were carried out in duplicate unless otherwise indicated. Evaluation of the standard deviation from duplicate results for reliability testing was performed according to Synek [23]. All the other statistical analysis was carried out using TIBCO Spotfire S+® 8.2 for Windows statistics package.

3. Results and discussion

3.1. Optimization of the SPE separation and spectral peak identification

FAMEs obtained after transesterification of the thermally oxidized sunflower oil were separated into polar and non polar FAMEs using the extensively applied two steps SPE method [4,7–9,15]. The gas chromatograms of these fractions analyzed on a CP-Sil 88^{TM} GC column are shown in (Fig. 1). As the hydroxy compounds do not elute easily from this column, resulting in the formation of broad peaks, the polar fraction was analyzed only after silylation. Identification of the peaks was based on the results obtained from mass spectrometry data and are shown in (Table 1). Spectral data interpretation in this table was done by consulting study reports [16,24–27], reference to the NIST library and evaluating the fragmentation pattern of these compounds. Normally it is difficult to identify cis or trans isomers by mass spectrometry, but a CP-Sil 88^{TM} GC column used has a known elution order of trans isomers first. Correct elution order and peak identification corresponded well with the previous studies [7,9,15] except for [8] who had a different elution order on an Innowax fused-silica capillary column.

In Table 1, two peaks of saturated epoxy fatty acid isomers from oleic acid namely methyl *trans*-9,10-epoxystearate (*trans*-9,10-ES, peak 1) and methyl *cis*-9,10-epoxystearate (*cis*-9,10-ES, peak 2) were confirmed. Four unsaturated epoxy fatty acids peaks (3–6) from linoleic acid namely methyl *trans*-12,13-epoxyoleate (*trans*-12,13-EO, peak 3), methyl *cis*-12,13-epoxyoleate (*cis*-12,13-EO, peak 5), methyl *trans*-9,10-epoxyoleate (*trans*-9,10-EO, peak 4) and methyl *cis*-9,10-epoxyoleate (*cis*-9,10-EO, peak 6) were confirmed as previously reported [7,9,28].

In Fig. 1, a peak corresponding to *cis*-9,10-ES is visibly distorted, while there are interfering compounds eluting together with *trans*-9,10-ES. These interfering compounds were not observed when the polar fraction was injected without silylation, inferring that they were due to hydroxy compounds. The interference due to silylated hydroxy compounds was further confirmed in GC–MS by studying the characteristics ions (*m*/*z* 73 and 75) due to silylation. It is not recommended to inject polar FAMEs containing such hydroxy compounds without silylation in this column, as these hydroxy compounds either stay in the column and shorten the column life or emerge very late as broad tailing peaks which would increase the analysis time. Furthermore, silylating agents can also quickly foul and eventually plug the GC injector inserts. In order to prolong column life, Christie [29] recommends a cleanup step after silylation by liquid–liquid extraction with hexane and water and then drying the organic phase over sodium sulphate. This extra step increases the analysis time and may also affect recoveries. In this way, such chromatographic interferences due to hydroxy compounds could present both qualitative and quantitative analytical problems for epoxy compounds and reduce the reliability of the results obtained.

In an attempt to reduce the interferences, hydrogenation of the polar fatty acids so as to saturate them and reduce the number of peaks has been preferred by many researchers. Although it can partially solve the problem, quantification of the individual unsaturated polar fatty acids becomes impossible, as they would be converted into saturated ones. During this study, when the

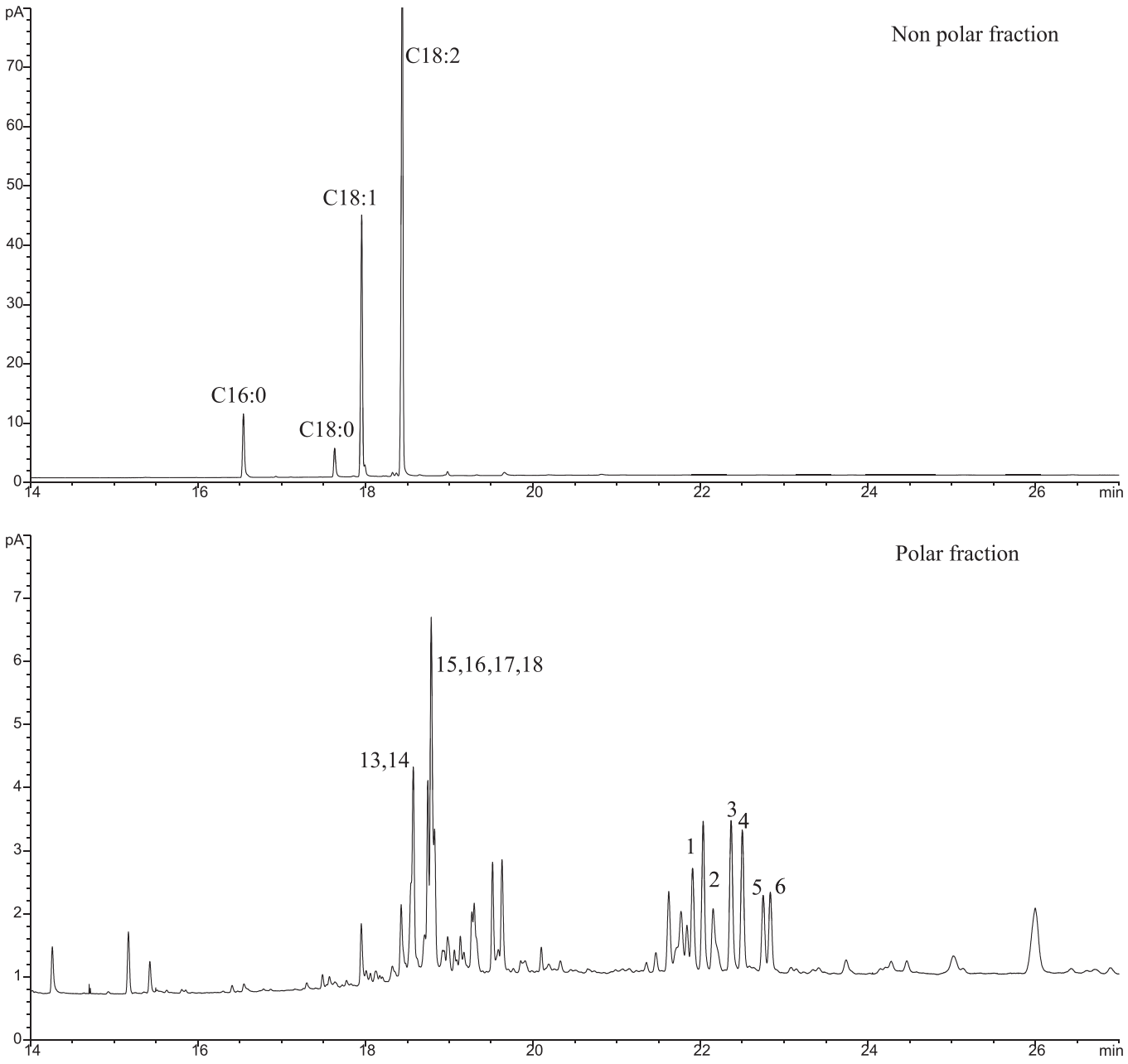

Fig. 1. Chromatograms of the non polar and silylated polar fraction of sunflower oil FAMEs after two steps SPE showing distorted peaks on GC-FID coupled with a CP Sil 88 column. Peaks 1–18 see Table 1.

polar FAMEs from the thermoxidised methyl linoleate were hydrogenated, methyl *trans*-9,10-ES and methyl *trans*-12,13-ES co-eluted as a single peak, while methyl *cis*-9,10-ES and methyl *cis*-12,13-ES were separated individually. In this way, hydrogenation was not a solution for a better analytical method of epoxy fatty acids as we would lose information not only about unsaturation, but also on the positional isomer distribution.

In order to remove the interferences due to hydroxy and oxo fatty acids, a three steps separation of FAMEs in a silica SPE column so as to collect the non polar fatty acids, epoxy fatty acids and hydroxy fatty acids in three different fractions was developed and has been described in the method. The developed method is based on the differences in polarity of the oxygenated fatty acids as described previously by different researchers [7–9,19,28,30]. The method was optimized using the mixture of thermally oxidized methyl oleate, methyl linoleate and methyl linolenate. Results of the GC-FID chromatograms obtained after a three steps SPE separation on silica gel column are shown in (Fig. 2). The GLC 68D standard has also been presented on the same figure (Fig. 2A) to evaluate the interference that could be expected from the neutral fatty acids.

Six mono epoxy FAMEs (peaks 1–6) from methyl oleate and methyl linoleate were observed in polar fraction 1 as shown in (Fig. 2B). The identification of these peaks using GC–MS has already been discussed. Additionally, six mono epoxy FAMEs (peak 7–12, Table 1) from methyl linolenate were also observed in the same fraction, out of which co-elution of peak 9 and 10 was confirmed by GC–MS. These epoxy FAMEs eluted according to their polarity with the less polar one first [5] in the order 12,13-epoxide, 15,16-epoxide and 9,10-epoxide. It is already reported that it is not easy to explain the fragmentation pattern of the methyl epoxy octadienoates on positive EI-mode [16,31], however, some characteristic ions

Table 1
Mass spectral ions of some of the polar fatty acid methyl esters identified in the different fractions after SPE separation.

Name of compound	Peak	Spectral ions; *m/z* (relative abundance %)
Methyl 9,10-epoxyoctadecanoate	1 (trans) 2 (cis)	*312* (*ND*), *281* (*1*), **155 (70), 199 (10)**, 294 (7), 171 (11), 109 (29), 97 (41), 83 (44), 55 (100)
Methyl 12,13-epoxy-octadec-9-enoate	3 (trans) 5 (cis)	*310* (*1*), *279* (*3*), **164 (20), 207 (4)**, 167 (18), 149 (11), 136 (21), 123 (23), 99 (45), 95 (57), 81 (89), 74 (25), 55 (100)
Methyl 9,10-epoxy-octadec-12-enoate	4 (trans) 6 (cis)	*310* (*1*), *279* (*2*), *200* (*4*), **155 (26), 185 (12)**, 168 (9), 135 (10), 109 (25), 95 (44), 81 (70), 67 (70), 55 (100)
Methyl 12,13-epoxy-9,15-octadecadienoate	7 (trans) 9 (cis)	*308* (*1*), *277* (*2*), *189* (*5*), *171* (*6*), *151* (*6*), *239* (*2*), **207 (11), 111 (38)**, 161 (9), 189 (5),211 (1), 147 (18), 123 (17), 108 (10), 95 (47), 83 (61), 81 (77), 67 (100)
Methyl 15,16-epoxy-9,12-octadecadienoate	8 (trans) 11 (cis)	*308* (*1*), *279* (*1*), *189* (*2*), *250* (*1*), **236 (10), 247 (1)**, 207 (2), 189 (2), 121 (20), 107 (37), 79 (100), 67 (49)
Methyl 9,10-epoxy-12,15-octadecadienoate	10 (trans) 12 (cis)	*308* (*ND*), **185 (4), 155 (17), 108 (52)**, 167 (1), 93 (42), 79 (100), 67 (23)
Methyl 9-hydroxyoctadecanoate trimethylsilyl ether	13	*386* (*ND*), *371* (*1*), *355* (*2*), *339* (*4*), **229 (89), 259 (100)**, 155 (16), 129 (28), 103 (14), 75 (56), 73 (85)
Methyl 10-hydroxyoctadecanoate trimethylsilyl ether	14	*386* (*ND*), *372* (*1*), *355* (*3*), *339* (*7*), **215 (100), 273 (95)**, 129 (3), 103 (13), 75 (33), 73 (55)
Methyl 12-hydroxy-octadec-9-enoate trimethylsilyl ether	15	*384* (*ND*), *369* (*1*), *353* (*1*), *337* (*2*), **187 (100), 299 (3)**, 270 (11), 129 (4), 103 (15), 97 (7), 81 (6), 75 (14), 73 (52)
Methyl 10-hydroxy-octadec-12-enoate trimethylsilyl ether	16	*384* (*ND*), *369* (*1*), *353* (*1*), *337* (*2*), **213 (5), 273 (100)**, 185 (23), 173 (9), 169 (17), 129 (18), 103 (8), 97 (5), 81 (25), 75 (28), 73 (82)
Methyl 9-hydroxy-octadec-12-enoate trimethylsilyl ether	17	*384* (*ND*), *369* (*3*), *353* (*1*), *337* (*5*), **227 (45), 259 (42)**, 294 (19), 155 (27), 130 (32), 103 (11), 97 (12), 81 (56), 75 (57), 73 (100)
Methyl 13-hydroxy-octadec-9-enoate trimethylsilyl ether	18	*384* (*ND*), *369* (*2*), *353* (*1*), *337* (*6*), **173 (75), 313 (17)**, 130 (28), 103 (22), 97 (10),81 (45), 75 (61), 73 (100)

ND, not detected.
Italics: the common fragment M; M-15, M-31, M-43, M-47 for all silylated methyl esters; M-31 for epoxy fatty acids.
Bold: The characteristic or identifier ions and the other most abundant fragments are presented in normal font.

were present to confirm the identity of the different epoxy fatty acids. Methyl *trans*-12,13-epoxy-9,15-octadecadienoate (peak 7) and methyl *cis*-12,13-epoxy-9,15-octadecadienoate (peak 9) were confirmed by use of the molecular ion (*m/z* = 308) and the characteristic ions with *m/z* values 207 after loss of mass *m/z* = 69 and methanol on one side, and 111 which forms after loss of mass *m/z* = 197 on the other side of the epoxy group. Methyl *trans*-15,16-epoxy-9,12-octadecadienoate (peak 8) and methyl *cis*-15,16-epoxy-9,12-octadecadienoate (peak 11) were confirmed by use of the molecular ion (*m/z* = 308) and the characteristic ions with *m/z* values 247 after loss of methanol and 236 which form after the cleavage on both sides of the epoxy group. Methyl *trans*-9,10-epoxy-12,15-octadecadienoate (peak 10) and methyl *cis*-9,10-epoxy-12,15-octadecadienoate (peak 12) were confirmed by use of the molecular ion (*m/z* = 308) and the characteristic ions with *m/z* values 108 and 155 which form after the cleavage on both sides of the epoxy group. Also ion *m/z* = 185 due to cleavage inside the epoxy ring was characteristic to these isomers [31].

When the polar fraction 1 was injected in GC-FID after silylation, the interfering hydroxy FAMEs were not detected in the chromatogram, confirming their removal from this fraction. The polar fraction 2 was injected in GC-FID after silylation to reveal the hydroxy FAMEs and has been shown in (Fig. 2C). In Table 1, two hydroxy trimethylsily ether derivatives which were partially separated on GC were identified as monohydroxyoctadecanoates (peak 13 and 14). Two isomers, 9-OH (peak 13) and10-OH (peak 14) were confirmed by the base peak at *m/z* = 259. Four peaks, (15–18) eluting very closely were found to be the positional isomers of hydroxyoctadecenoates. These were confirmed to be 12-hydroxyoactadecenoate (peak 15), 10-hydroxyoctadecenoate (peak 16), 9-hydroxyoctadecenoate (peak 17) and 13-hydroxyoctadecenoate (peak 18) as previously reported [25].

The results displayed in Fig. 2 demonstrate that the interferences observed after the two steps SPE separation Fig. 1 in the epoxy region can be removed by application of the proposed separation method. Use of three steps separation on silica column separated the hydroxy and other polar FAMEs, which led to obtaining high resolution chromatograms with well separated peaks of epoxy compounds. The interferences caused by some of the unmodified FAMEs like C22:0, C24:0 and C24:1 as reported by different authors [9,15,32] were not observed since these unmodified FAMEs eluted earlier than the epoxy FAMEs. The only possible interference would come from methyl ester of docosahexaenoic acid (DHA) 22:6 a common fatty acid on fish oil which was not present in vegetable oils used in this study. Another advantage of the three steps SPE separation is that minor quantities of the unmodified FAMEs (C18:0, C18:1 and C18:2; Fig. 2B) remaining in silica column after elution of non polar FAME fraction, would be completely removed during elution of polar fraction 1. Those FAMEs could otherwise interfere with quantification of hydroxy FAMEs if all the polar compounds would have been collected together in one fraction as reported previously in a two steps SPE separation.

The use of a three steps SPE separation and a polar FAME column CP-Sil 88^{TM} enabled individual separation of each of the six different epoxy fatty acid isomers obtained from C18:1 and C18:2 fatty acids that are common in most oils. Such well resolved peaks can be used in studies to determine isomeric distribution of epoxy fatty acids in oxidized fats. It was found out that at temperatures >180 °C (data not shown), the trans isomers were dominating and in methyl oleate their percentage was 65%. The two trans isomers in methyl linoleate were both present at 30% each. Methyl linolenate though had two peaks co-eluting, their percentages were: 21% for *trans* 12,13-epoxide and 22% for *trans* 15,16-epoxide. The two peaks that co-eluted namely *trans* 9,10-epoxide and *cis* 12,13-epoxide were present at 31%. The two cis peaks namely *cis* 15,16-epoxide and *cis* 9,10-epoxide were present at 13% each.

3.2. Calibration curves, linearity, LOD, LOQ and recoveries

As commercial epoxy standards are not readily available, the analyte used in the recovery study was synthesized. The response factor used to quantify all the isomers was based on C19:0 as an internal standard and the synthesized *cis*-9,10-epoxystearate since

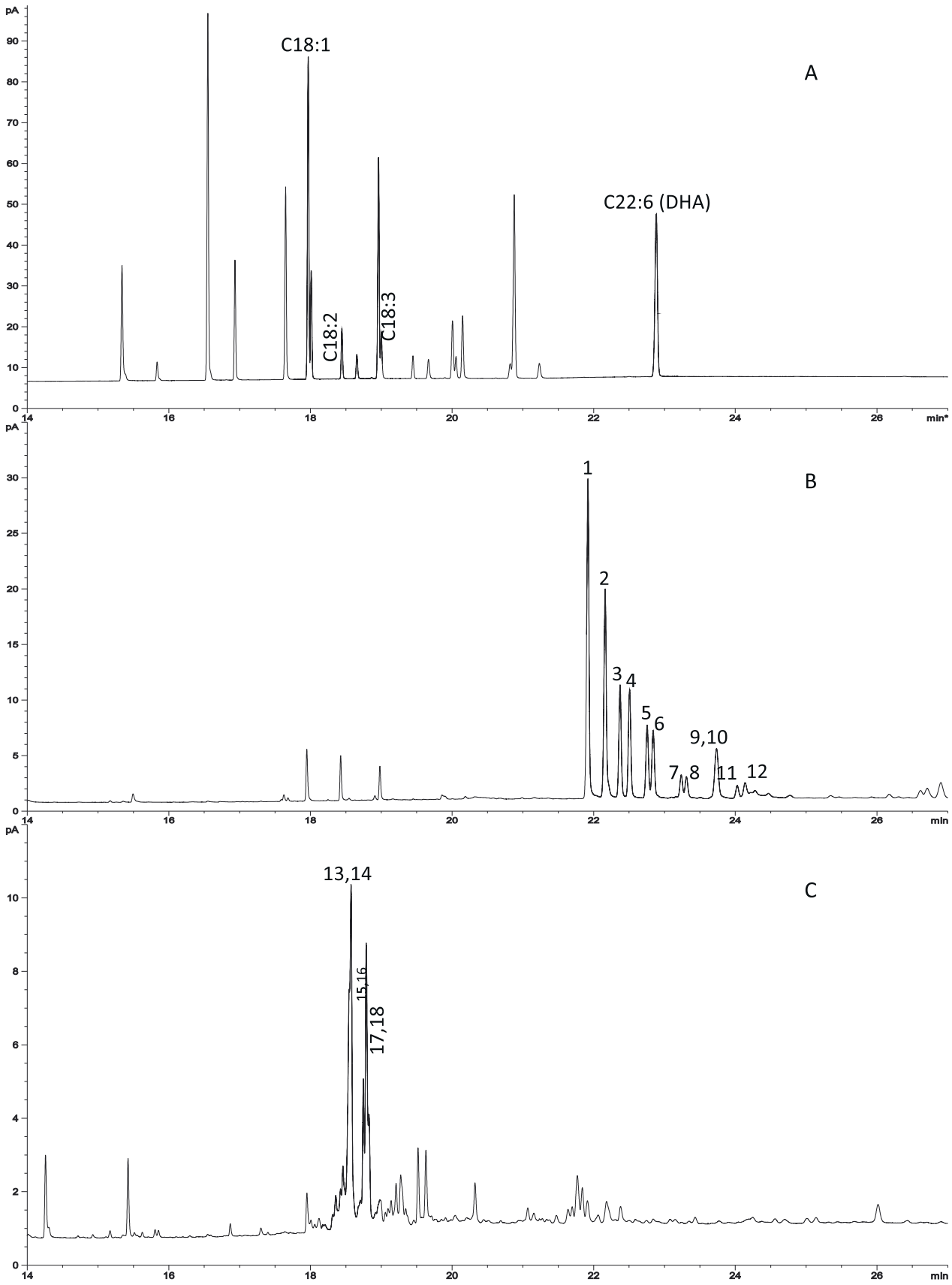

Fig. 2. Chromatograms of a GLC 68D standard and a mixture of C18:1, C18:2 and C18:3 thermoxidised FAMEs after three steps SPE showing well resolved peaks on GC-FID coupled with a CP Sil 88 column. (A) GLC 68D standard, (B) polar fraction 1 and (C) the silylated polar fraction 2. Peaks 1–18 see Table 1.

Table 2
Epoxy fatty acids (μg g^{-1} of oil), PV (mequiv. O_2 kg^{-1} of oil) and fatty acid composition (g 100 g^{-1} of oil) in fresh oils obtained from Belgian markets.

Type of oil	PV[a]	Epoxy fatty acid composition (μg g^{-1} of oil)[a]						Fatty acid composition (g 100 g^{-1} of oil)			
		trans- 9,10-ES	cis-9,10-ES	trans-12,13-EO	trans-9,10-EO	cis-12,13-EO	cis-9,10-EO	C18:1	C18:2	C18:3	Others
Arachid	1.5	22.8	685.0	10.3	6.9	65.7	350.1	43.7	7.5	0.0	10.4
Arachid	2.4	13.1	172.1	7.2	4.6	32.0	125.9	44.9	16.0	0.2	14.0
Colza	4.4	15.1	25.7	9.5	0.8	7.3	6.5	43.9	14.8	7.7	6.6
Colza	2.5	8.7	19.5	6.5	0.4	4.5	6.9	39.1	13.2	6.4	5.5
Colza	2.3	21.9	64.7	0.9	0.9	24.8	67.2	45.6	14.6	7.2	6.0
Colza	4.0	7.7	19.0	2.6	2.1	7.5	7.7	54.2	16.9	8.9	7.7
Colza	3.6	6.7	12.2	6.1	0.4	3.5	2.5	59.1	19.1	9.4	8.8
Corn	1.8	12.1	59.8	19.3	9.1	48.6	79.7	28.1	46.8	0.9	12.4
Corn	0.9	7.4	75.6	13.1	3.8	59.1	107.7	28.9	54.0	0.9	13.0
Corn	0.0	9.1	61.0	14.0	3.9	46.8	92.3	24.5	46.1	0.8	11.2
Corn	3.1	9.4	118.1	15.8	16.6	98.1	161.9	18.7	32.5	0.6	8.3
Corn	1.8	12.9	92.0	18.6	10.3	67.9	123.1	29.7	49.9	1.2	13.2
Frying	3.7	9.2	179.4	7.5	7.2	44.5	570.1	36.0	41.0	0.9	15.4
Frying	3.3	7.4	78.2	6.7	5.3	24.1	173.8	37.9	38.9	2.5	14.4
Frying	5.3	8.9	60.4	6.6	3.4	23.6	161.4	33.7	48.0	2.7	11.8
Frying	2.2	14.8	315.8	2.2	7.8	66.4	485.8	33.0	34.1	1.4	10.7
Frying	3.7	9.6	177.3	4.0	7.9	54.7	576.1	24.5	38.4	0.8	11.9
Frying	5.2	6.9	34.1	1.7	1.9	9.5	52.6	50.6	22.0	3.7	12.8
Frying	5.2	6.5	125.3	1.6	3.8	31.2	391.1	27.3	43.7	0.1	8.5
Frying	3.3	11.2	60.7	8.8	2.8	24.0	138.6	42.7	37.6	3.8	9.8
Mixture	4.5	13.7	95.3	9.3	2.9	24.5	109.6	43.5	23.3	3.8	7.8
Mixture	2.6	9.8	56.5	5.1	2.0	16.0	109.3	42.7	19.9	3.6	6.7
Olive	5.2	9.5	218.3	5.3	0.0	7.6	10.7	72.8	4.3	0.6	15.2
Olive	6.9	9.9	221.5	4.5	0.0	8.6	16.7	75.8	4.5	0.7	15.5
Olive	8.8	9.0	165.3	7.5	0.8	13.5	20.3	51.4	7.4	0.5	13.4
Olive	6.0	6.8	218.3	0.0	0.3	9.3	12.0	55.9	3.4	0.5	12.1
Olive	12.4	6.8	214.6	0.0	0.4	13.1	18.2	52.7	5.5	0.5	10.8
Olive	12.6	7.9	134.7	6.8	0.7	11.4	16.1	47.3	6.3	0.5	12.6
Olive	5.8	4.3	76.5	4.1	0.0	3.3	6.4	75.1	8.1	0.8	12.5
Plant oil	8.2	5.6	7.5	4.3	3.4	4.7	4.8	47.8	14.4	7.5	8.5
Salad	1.7	8.0	38.5	5.2	5.3	16.7	99.8	33.6	33.5	3.5	8.0
Soya	5.9	7.6	3.7	12.5	3.1	3.3	19.5	15.1	31.3	3.2	10.2
Sunflower	2.1	10.9	231.0	8.4	8.5	54.7	747.5	34.4	50.7	0.1	10.7
Sunflower	2.2	33.2	414.3	15.7	10.2	102.2	1434.0	23.8	60.8	0.1	10.7
Sunflower	1.8	12.8	397.7	5.7	13.4	110.4	1393.3	20.3	51.7	0.1	9.1
Sunflower	4.8	8.2	223.9	4.2	9.6	68.8	759.3	18.9	47.7	0.1	8.4
Sunflower	3.1	8.8	239.3	9.5	10.1	69.6	826.0	22.6	58.8	0.1	10.6

Abbreviations: ES, epoxystearate; EO, epoxyoleate; PV, peroxide value.
[a] Values are means of duplicate analyses.

similarity of the analytical response of saturated and unsaturated epoxy fatty acids on GC-FID has been reported [9]. The working range was 0, 20, 40, 60, 80 and 100 μg mL^{-1} and the internal standard was kept at 50 μg mL^{-1} in all cases. The response factor was calculated by linear regression of area ratio of the analyte to the internal standard (y axis) versus the concentration ratio of the analyte to the internal standard in iso-octane. The linearity obtained was satisfactory at this range with $R^2 = 0.9992$. The response factor obtained was 1.04 and this is what was used in all the calculations. The LODs and LOQs were determined by a method based on two variable regression of the calibration curve, LOD = 3 (SE/slope) and LOQ = 2 × LOD; where SE is the standard error of the intercept. The LOD and LOQ were found to be 1.45 and 2.9 μg g^{-1} of oil respectively.

In order to access the efficiency of the separation step, and to ascertain that there were no losses of the analytes of interest during separation, the developed method was evaluated for recoveries as this is critical in quantification. Good recoveries for all the analytes were obtained and with each compound being recovered in the fraction as expected (not shown). The percentage recoveries were in the range of 98% and above in all the three analytes (epoxy, oxo and hydroxy FAMEs) showing that the SPE conditions used were optimal. Silylation was done to confirm that there were no hydroxy compounds remaining in the epoxy fraction and the chromatograms obtained did not show any new peaks (data not shown).

3.3. Application of the developed method on fresh oil samples

The developed method was used to analyze 37 different fresh oil samples on the Belgian market. The results of the analyses are summarized in (Table 2), where only unsaturated fatty acids relevant in the production of epoxy fatty acids are shown. Repeatability was checked for the duplicate analysis and the CV was found to be 9.9% for epoxy fatty acids in the range 0–10, 6.9% for 11–49, 4.8% for 50–249 and 2.8% for oils with epoxy fatty acid content of more than 250 μg g^{-1} of oil. To measure the extent of oxidation in the samples, the peroxide value (PV) was determined. The fatty acid composition and the epoxy fatty acid content were also determined. Results showed that all the oils analyzed were fresh as indicated by the low to very low peroxide values ranging from 0 to 12.55 mequiv. O_2 kg^{-1}. Fatty acid composition results showed that most of the oils were rich in C18:1 and C18:2, with the exception of colza and the plant oil which had significant amount of C18:3 fatty acids. Generally all the oils had higher amounts of unsaturated epoxy fatty acids corresponding to the original high amounts of unsaturated fatty acids and in all cases the cis isomers dominated. However, more trans fatty acids have been reported to form in thermally oxidized samples [7,8,15] mainly due to the conversion of the cis isomers to more stable trans isomers at elevated temperatures that are normally used during deodorization of oils. On average, sunflower oil had the highest amount of epoxy fatty acids while colza oil had the lowest, despite the comparable PV values. Results

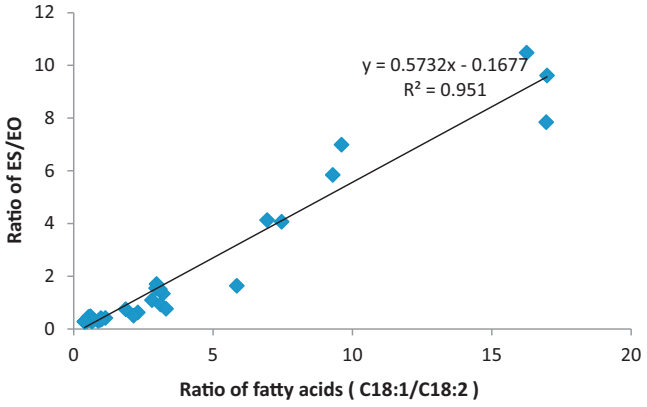

Fig. 3. Regression of the ratio of epoxy fatty acids and the ratio of major 18C fatty acid composition of fresh oils, ($n = 37$). (ES) epoxystearate; (EO), epoxyoleate.

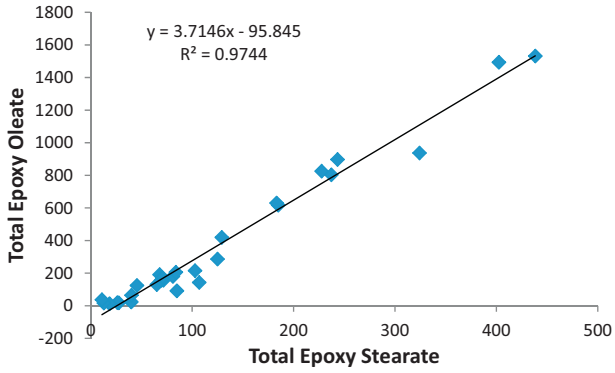

Fig. 4. Regression of total saturated and unsaturated epoxy fatty acids for oils (colza, corn, frying, mixture, plant, salad, soya and sunflower) with comparable amounts of C18:1 and C18:2 fatty acids ($n = 28$).

of higher amounts of unsaturated epoxy fatty acids in fresh oil samples are in a similar range as those obtained by Fankhauser-Noti et al. [14]. It was interesting to note that sunflower oil had comparable amounts of saturated epoxy fatty acids to olive oil even when the two oils had differing amounts of C18:1 fatty acids. Since it is known that fatty acids are the precursors of secondary lipid oxidation products and hydroperoxides are the initial reaction products with oxygen during lipid oxidation [3], it would be expected that virgin olive oil in absence of other factors like antioxidants, should have the highest amount of the saturated epoxy fatty acids which was not the case. From the results obtained, the amount of epoxy fatty acids formed seem to be related to the amount of unsaturated fatty acids present but not to the relative oxidizability rates, where C18:1 has the lowest rate.

In order to explain the observed trend of epoxy fatty acid content, correlations were performed and results are presented in (Figs. 3 and 4). There was a strong correlation ($p < 0.001$; $r = 0.97$; $n = 37$) between the fatty acids ratio (C18:1/C18:2) and the ratio of the amount of epoxy fatty acids (ES/EO) formed. Also a strong correlation ($p < 0.001$; $r = 0.99$; $n = 28$) exists between the total saturated and unsaturated epoxy fatty acids of colza, corn, frying, mixture, plant, salad, soya and sunflower oils which had comparable amounts of C18:1 and C18:2. The strong correlation observed indicate that the rate of formation of the epoxy fatty acids may be related to the amount of unsaturated fatty acids present in oil. However, there was no correlation ($p > 0.05$; $r = -0.310$; $n = 37$) between the epoxy fatty acids formed with the peroxide value.

The occurrence of high amount of epoxy fatty acids in fresh oil samples and the lack of correlation between PV and the epoxy fatty acids may be due to multiple reasons: (1) PV is an unspecific method and it does not give any information about the concentration of the different peroxides present. (2) Probably it is an indication that there is another more specific route of formation for epoxy fatty acids apart from the breakdown of the hydroperoxides [31,33,34]. However, the known processes of formation of epoxy fatty acids are through direct oxygen addition either at the site of the double bond or nearby it [35]. Also during thermal oxidation at 80 °C it is reported that 9,10-epoxyoctadecanote can be formed by the reaction between oleate and oleate hydroperoxide [3,25]. Epoxidation can as well occur when a peracid reacts with hydroperoxides but in all these routes of formation, epoxidation happens by replacing the point of unsaturation in the fatty acid [6,10]. The total amounts of epoxy fatty acids present in fresh oils varied between 0.03–2 mg g^{-1} of oil. Based on these amounts detected in the fresh oils, it is clear that the safety issue of epoxy fatty acids is being underestimated. However, due to lack of toxicity and intake data, the risk posed by these fatty acids cannot be inferred.

Though it has not been possible to determine from this study how these fatty acids form in the oils that have been analyzed, the study has been able to separate the co eluting hydroxy fatty acids thus accurately quantify the epoxy fatty acids in oils.

4. Conclusions

The innovation of this method is the three steps SPE separation technique of the different fatty acids on silica gel which has improved the analysis of the epoxy fatty acids. This purification step separates the co eluting hydroxy fatty acids and thus better peaks are obtained on a polar FAME CP Sil 88^{TM} GC column. After this step it is possible to determine polar fatty acids in less oxidized samples due to increased sensitivity. The need to hydrogenate the FAMEs so as to avoid the co-elution of hydroxy and fatty acids has been overcome thus determine each compound in its original form. Strong correlations were illustrated, between the ratios of fatty acids to the ratio of epoxy fatty acid formed and between saturated and unsaturated epoxy fatty acids. The new approach exhibits excellent chromatographic performance with well resolved peaks and low detection limits.

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