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# Sensitive and accurate quantitation of monoepoxy fatty acids in thermoxidized oils by gas–liquid chromatography

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**Abstract**

A sensitive and accurate methodology for quantitation of monoepoxy fatty acid methyl esters (FAME) by gas–liquid chromatography is proposed. Analytical problems of interfering compounds, i.e, methyl monoester of azelaic acid and methyl docosanoate, were solved by a second methylation step with diazomethane and by elimination of nonpolar FAME by adsorption chromatography, respectively. Six monoepoxy FAME were identified and quantitated in olive and sunflower oils heated at 180 °C for 15 h: *trans*-9,10- and *cis*-9,10-epoxystearate coming from oleate and *trans*-12,13-, *trans*-9,10-, *cis*-12,13- and *cis*-9,10-epoxyoleate coming from linoleate. Results demonstrated total recovery of monoepoxy compounds after nonpolar FAME elimination with the additional advantage of sample concentration, which allowed quantitation of monoepoxy FAME in the initial oils. Also, repeatability was excellent as relative standard deviations ranged from 2.2 to 5.1% for on-column injection and from 0.1 to 2.0% for automatic split injection.


*Keywords:* Oils; Injection methods; Thermoxidation; Monoepoxy fatty acids

## 1. Introduction

Oxidation compounds are formed in substantial amounts during processes for food preparation involving high temperature [1] and are suspected of impairing the nutritional value of fats and oils [2]. Among these, formation of monoepoxy compounds has been studied in model systems of monoacid methyl esters and triacylglycerols heated at frying temperatures. As expected, two saturated epoxides, *trans*-9,10- and *cis*-9,10-epoxystearate, were formed in methyl oleate and triolein samples and four monounsaturated epoxides, referred as monoepoxyoleates, *trans*-12,13-, *trans*-9,10-, *cis*-12,13- and *cis*-9,10-epoxyoleate, were formed in methyl linoleate and trilinolein samples [3].

Nevertheless, when considering fats and oils, degradation depends on a large number of triacylglycerol species as well as on the minor compounds present modifying the susceptibility of oils to oxidation. As a consequence, the separation and quantitation of the new compounds formed require more complex analyses and interference by minor or major components naturally present in fats and oils can be expected when quantifying the alteration products. Thus, studies on complementary steps are necessary to define the possibilities of accurate quantitation of

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monoepoxy compounds in fats and oils heated at high temperature.

Previous studies on epoxy fatty acids are scarce and mainly refer to the structural analysis of the different isomers [4,5] or to their determination in oils from plants containing epoxyacids in high amounts [6,7]. Also, HPLC has been applied to detect a peak including epoxytriacylglycerols increasing with heating time [8], but no studies have been found on the detailed quantitation of the different isomers.

The main objective of this work was to establish the analytical conditions for a sensitive and accurate quantitation of monoepoxy fatty acids formed at high temperature in fats and oils. Towards this aim, analytical solutions to eliminate the two compounds interfering in their analysis by GC are proposed. First, the presence of the methyl monoester of azelaic acid, overlapping with monoepoxy compounds, in thermoxidized FAME model systems was recently reported [9]. The base-catalyzed transmethylation with sodium methoxide at room temperature used cannot methylate free carboxylic groups and a second methylation step with diazomethane was the best option to avoid any acid-catalyzed methylation method which would have resulted in epoxy ring opening and other undesirable reactions [10–12]. Second, interference of methyl docosanoate (C22:0), naturally present in fats and oils, was solved by elimination of nonpolar FAME by adsorption chromatography. Comparative results obtained by split and on-column injection systems are also presented.

## 2. Experimental

### 2.1. Chemicals

Methyl tridecanoate (C13:0), methyl pentadecanoate (C15:0), tridecanoic acid and pentadecanoic acid were purchased from Nu-Check-Prep (Elysian, MN, USA). Methyl *cis*-9,10-epoxystearate and *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide was purchased from Sigma–Aldrich (Steinheim, Germany). Silica gel 60 for column chromatography (particle size 0.063–0.100 mm) was obtained from Merck (Darmstad, Germany). All other chemicals and reagents were of analytical grade and were obtained from local suppliers.

### 2.2. Samples

Olive oil (OO) and conventional sunflower oil (SO) were purchased from local outlets.

### 2.3. Heating procedure

OO and SO samples ($2\pm0.01$ g) were weighed into standard glass tubes (20 cm$\times$12 mm I.D.). The tubes were introduced into Rancimat vessels containing 8 g of glycerol to facilitate heat transfer and inserted into the heating block of the apparatus previously heated at $180\pm1$ °C. The reaction vessels were left open to air during heating [13]. After 15 h of heating, samples were taken out, shaken and kept at $-20$ °C until analyses.

### 2.4. Methylation procedure

Methylation procedure was carried out in two consecutive steps: firstly, base-catalyzed transmethylation with sodium methoxide in *tert*-butyl methyl ether (TBME) at room temperature and, secondly, direct methylation of carboxylic groups with diazomethane.

A standard solution was prepared with a mixture of methyl tridecanoate (C13:0, 500 μg/ml) and methyl pentadecanoate (C15:0, 500 μg/ml) in TBME.

#### 2.4.1. Transmethylation with sodium methoxide at room temperature

Samples of 100 mg were accurately weighed into screwcapped centrifuge tubes and 1 ml of the standard solution was added. Then, a 0.5-ml volume of 0.2 *M* NaOMe solution in methanol was added, the vial closed, shaken for 1 min, and left at rest for 2 min at room temperature. For neutralization purposes, a 0.1 ml volume of 0.5 *M* $H_2SO_4$ solution was added and the mixture was shaken for a few seconds. Then, 1.5 ml of water was added, shaken for 10 s and centrifuged. The organic layer was separated, evaporated to dryness under nitrogen and redissolved in 2 ml of diethyl ether [3].

*2.4.2. Methylation with diazomethane*

Diazomethane was prepared by the action of sodium hydroxide on *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide using a device consisting of two connected tubes [14]. The first tube was half-filled with diethyl ether (approximately 5 ml) and the second one contained 3 ml of 2-(2-ethoxyethoxy)-ethanol, 3 ml of diethyl ether and 3 ml of 60% NaOH solution. Approximately 1 g of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide was added to the second tube. A stream of nitrogen (5–10 ml/min), saturated in ether vapour from passing through the first tube, was used to carry diazomethane generated in the second tube up to the vial containing the transmethylated sample dissolved in diethyl ether. Methylation was considered complete when the sample turned yellow.

*2.5. Separation of nonpolar and polar fatty acid methyl esters by adsorption chromatography*

Methyl esters were prepared in triplicate by base-catalyzed transmethylation with sodium methoxide in TBME as mentioned above except that a mixture of tridecanoic acid (500 μg/ml) and pentadecanoic acid (500 μg/ml) in TBME was used as a standard solution. After evaporation of TBME under nitrogen, FAME were dissolved in 2 ml of *n*-hexane–diethyl ether (95:5, v/v) and then separated into two fractions of different polarity by silica column chromatography. A 40-cm long×1-cm I.D. glass column filled with 6 g of silica adjusted to a water content of 5% (m/m) was used. The nonpolar fraction was firstly eluted with 50 ml of *n*-hexane–diethyl ether (95:5, v/v) and the polar fraction was then obtained using 50 ml of diethyl ether. Efficiency of the separation was checked by TLC using hexane–diethyl ether–acetic acid (80:20:1, v/v) for development of plates and exposure to iodine vapor to reveal the spots. After evaporation of diethyl ether, the polar fraction dissolved in 2 ml diethyl ether was subjected to methylation with diazomethane as mentioned above.

*2.6. Hydrogenation*

When required, oil samples were hydrogenated prior to methylation using platinum(IV) oxide as a metal catalyst (Adams'catalyst). Samples of 100 mg were accurately weighed into screwcapped tubes and 1 ml of the standard solution was added. After evaporation of TBME under nitrogen, 2 ml methanol was added followed by the addition of platinum oxide in catalytic amounts. Hydrogenation was carried out by bubbling with hydrogen for 15 min.

*2.7. Gas–liquid chromatography*

FAME were analyzed by gas–liquid chromatography using two HP 6890 Series chromatographs (Hewlett-Packard, Avondale, PA, USA) equipped with split and on-column injection systems.

*2.7.1. Split injection*

Samples (~50 mg/ml) were introduced into a split–splitless inlet operating in mode split with a 40:1 split ratio at 250 °C. Injections (2 μl) were carried out automatically using a 6890 Series injector (Agilent Technologies, Karlsruhe, Germany). For concentrated fraction of polar FAME, 1 μl of sample (~5 mg/ml) was injected.

*2.7.2. On-column injection*

Samples were introduced into a cool on-column inlet constituted by a fused-silica liner (10 cm×530 μm I.D.) connected directly to the capillary column by a straight press-tight connector. The inlet was operated in track oven mode, where the inlet temperature followed the oven temperature. One microliter of samples diluted in TBME (~5 mg/ml) were manually injected. For concentrated fraction of polar FAME, 1 μl of sample (~0.5 mg/ml) was injected.

The same J&W DB-Wax fused-silica capillary column, 30 m×0.25 mm I.D., film thickness 0.25 μm (J&W Scientific, USA), was used in both chromatographs. The analyses were run using hydrogen (1 ml/min) as carrier gas and with the following oven temperature programme: 90 °C held for 2 min, 4 °C/min to 240 °C held for 20 min. Flame ionization detectors were used at 250 °C with hydrogen at 40 ml/min and air at 450 ml/min, and nitrogen at 45 ml/min as auxiliary gas.

### *2.8. Gas–liquid chromatography–mass spectrometry (GC–MS)*

GC–MS analyses were performed with a Finnigan MAT 95 double focusing mass spectrometer (Finnigan, Bremen, Germany) operating in the electron ionization mode. Electron energy was 70 eV, multiplier voltage 1500 V, source temperature 200 °C and transfer line 250 °C. Spectral data were acquired over a mass range of 28–600 a.m.u. at a rate of 1 scan/s. Chromatographic conditions were the same as those used for GC analyses.

## 3. Results and discussion

Fig. 1 shows the complete gas chromatograms obtained for SO samples before (A) and after (B) heating for 15 h at 180 °C. As explained above, samples were derivatized to FAME in two steps using C13:0 and C15:0 as internal standards (a dilution factor of 1/25 was used for the initial sample) for quantitative purposes. First, transmethylation with NaOMe at room temperature was used and then in a second step the samples were methylated with diazomethane to eliminate any free carboxylic group present in the sample. As can be observed in Fig. 1, under the chromatographic conditions used monoepoxy compounds eluted in the zone of C22:0, occurring naturally in the oils. Due to the relative high concentration of C22:0 in the samples (0.1% in olive oil and 0.7% in sunflower oil), accurate quantitation of the compounds of interest is difficult. Even if this interference may not

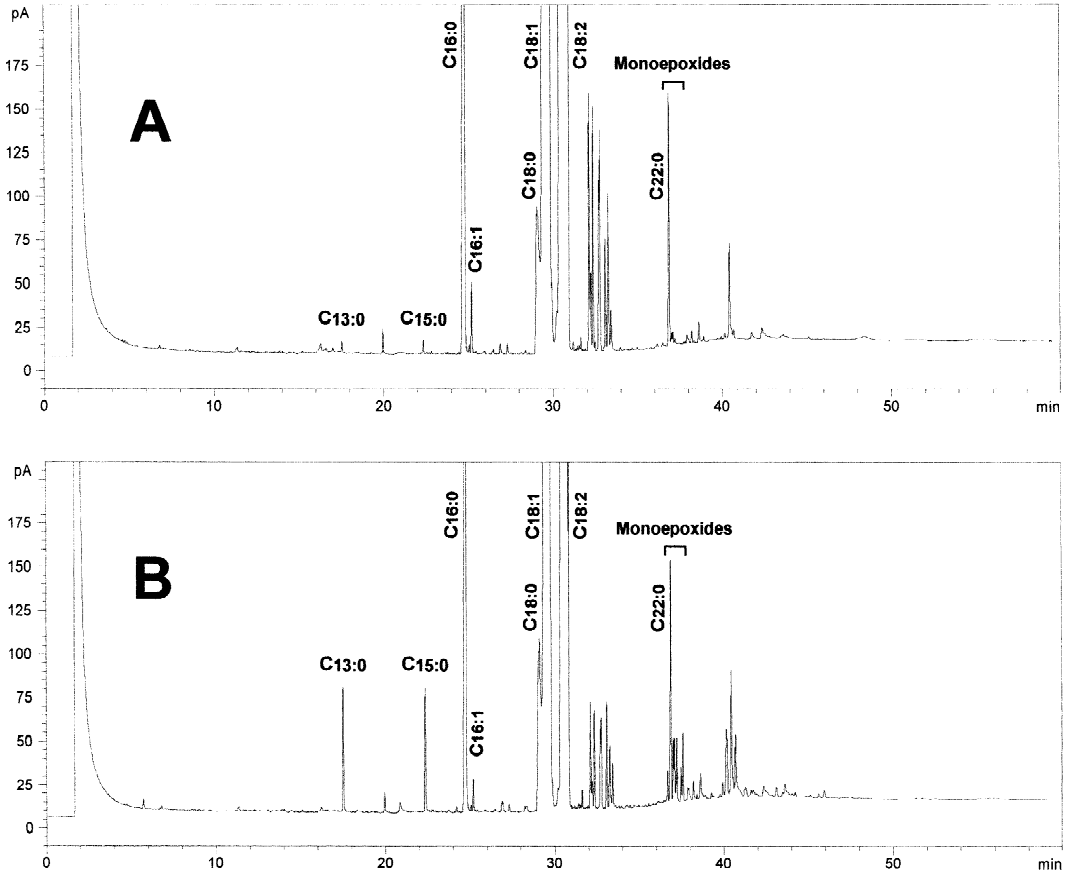


Fig. 1. Representative gas chromatograms of fatty acid methyl esters of sunflower oil before (A) and after heating at 180 °C for 15 h (B).

apply to other chromatographic phases, the absence of minor FAME in the zone of monoepoxy compounds should be checked and guaranteed before quantitation under other chromatographic conditions.

In order to avoid this interference on GC, the removal of nonpolar FAME by silica column chromatography is proposed. Thus, after transmethylation with NaOMe, methyl esters were separated on a silica column into two fractions of different polarity: the nonpolar fraction containing C22:0 and the rest of nonpolar FAME (FAME without extra oxygen), and the polar fraction containing monoepoxy compounds and the rest of FAME with at least one extra oxygen function (hydroperoxy, aldehyde, ketone, hydroxy, etc). In order to recover the internal standards in the fraction of interest, it was necessary to use their corresponding acid forms, tridecanoic and pentadecanoic acids. After the separation of nonpolar FAME, the polar fraction was subjected to methylation with diazomethane, transforming the internal standards into FAME, before the analysis by GC. Fig. 2A shows the expanded zone of monoepoxides after separation of the nonpolar FAME fraction corresponding to thermoxidized SO sample. As expected from the results obtained in model systems of triolein and trilinolein (3), six peaks were identified by GC–MS and assigned as *trans*-9,10- (**1**) and *cis*-9,10-epoxystearate (**3**) coming from oleate and *trans*-12,13- (**2**), *trans*-9,10- (**4**), *cis*-12,13- (**5**) and *cis*-9,10-epoxyoleate (**6**) coming from linoleate acyl groups.

Confirmation of the correct peak assignation was alternatively obtained by hydrogenation. Samples were hydrogenated before transmethylation and then, the polar FAME fraction was separated. As can be observed in Fig. 2B, corresponding to the epoxide zone of hydrogenated samples, the six peaks in Fig. 2A were converted into four peaks corresponding to the saturated monoepoxy compounds. *Trans*-12,13- (**2**) and *cis*-12,13-epoxyoleate (**5**) were reduced to *trans*-12,13- (**2′**) and *cis*-12,13-epoxiestearate (**5′**), respectively. In the case of monoepoxides coming from oleate, their areas were increased as a consequence of the reduction of *trans*-9,10- and *cis*-9,10-epoxyoleate.

It is interesting to note that nonpolar FAME accounted for more than 98% of the initial samples and more than 85% of the heated samples. After

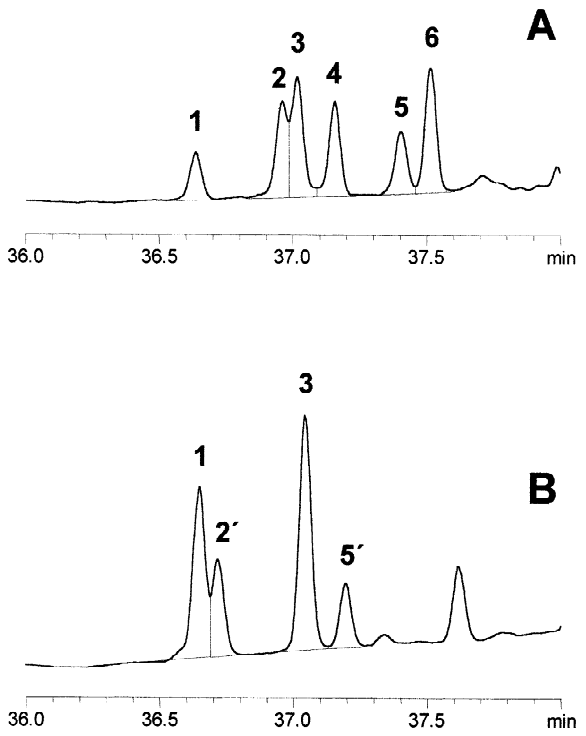


Fig. 2. Significant part of the gas chromatograms corresponding to the epoxide zone of fatty acid methyl esters of sunflower oil heated at 180 °C for 15 h before (A) and after hydrogenation (B). Peak assignments: 1=*trans*-9,10-epoxystearate; 2=*trans*-12,13-epoxyoleate; 3=*cis*-9,10-epoxystearate; 4=*trans*-9,10-epoxyoleate; 5=*cis*-12,13-epoxyoleate; 6=*cis*-9,10-epoxyoleate; 2′=*trans*-12,13-epoxiestearate; 5′=*cis*-12,13-epoxiestearate.

nonpolar FAME elimination, the concentration of the compounds of interest is enormous, thus facilitating their quantitation at minimum levels. Fig. 3 shows the zone of monoepoxides corresponding to initial SO sample before (A) and after (B) separation of the nonpolar FAME fraction. Although practically negligible in the total sample, the elimination of the non-polar FAME fraction allowed the quantitation of total monoepoxy compounds at levels as low as 0.303 mg/g oil found for initial SO and as 0.328 mg/g oil for initial OO sample. As can be observed, there is still a small peak corresponding to C22:0, probably due to diazomethane methylation of free fatty acids, but it does not interfere with the quantitative analysis of epoxides (Fig. 3A).

Tables 1 and 2 show quantitative data obtained for OO and SO samples, respectively. Monoepoxy

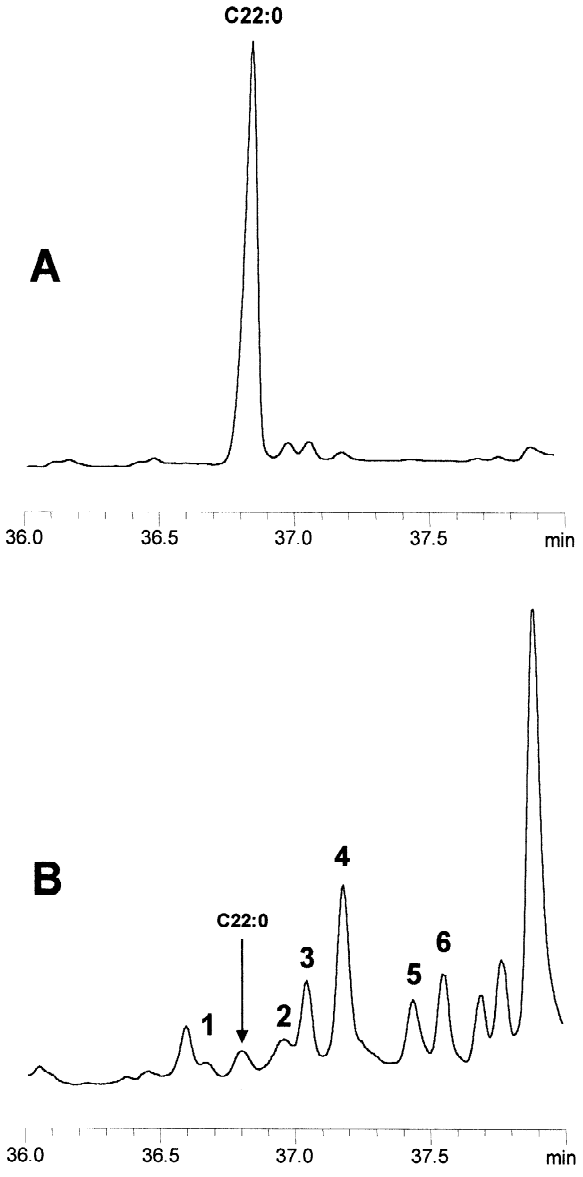


Fig. 3. Significant part of the gas chromatograms corresponding to the epoxide zone of fatty acid methyl esters of initial sunflower oil (A) and its polar fraction (B). Peak assignments: C22:0, behenic acid methyl ester; 1=*trans*-9,10-epoxystearate; 2=*trans*-12,13-epoxyoleate; 3=*cis*-9,10-epoxystearate; 4=*trans*-9,10-epoxyoleate; 5=*cis*-12,13-epoxyoleate; 6=*cis*-9,10-epoxyoleate.

FAME were quantitated in triplicate samples of polar FAME fractions. Response factors (RF) relative to both internal standards were calculated for methyl *cis*-9,10-epoxystearate in the range 0–0.5 mg/ml for split–injection analyses and 0–0.025 mg/ml for on-column injection analyses. The FID response linearity was satisfactory, with coefficients of correlation ranging from 0.998 to 0.999. Linearity was checked up to 3 mg/ml for split and up to 0.15 mg/ml for on-column injections. Only a minor difference was found in RF referred to methyl tridecanoate between split–injection ($1.06\pm0.011$) and on-column injection ($0.94\pm0.035$). However, when RF was referred to methyl pentadecanoate no significant difference was found between split ($0.97\pm0.011$) and on-column injection ($0.94\pm0.023$). Quantitative results for the epoxides presented in Tables 1 and 2 were obtained using methyl pentadecanoate as internal standard and the calculated RF for methyl *cis*-9,10-epoxystearate.

Recovery of monoepoxy FAME after separation of polar FAME by adsorption chromatography was checked by comparing the results with those obtained in the analysis of the total sample. Except for methyl *trans*-9,10-epoxystearate, overlapping with C22:0, neither the sum of the rest of monoepoxides nor the amounts of individual compounds were significantly different when the amounts were statistically compared in both samples. Recovery of these compounds ranged between 100 and 102%, which indicates not only that the recovery of monoepoxy FAME and I.S. was complete but also that there was a complete methylation of I.S. with diazomethane after separation of nonpolar FAME.

Repeatability was assessed by means of the relative standard deviation (RSD) for triplicate samples (Tables 1 and 2). The results obtained for each compound showed an excellent repeatability with RSD ranging between 2.2 and 5.1% for on-column injection and 0.1 and 2.0% for split injection. Although on-column injection is claimed to improve precision of the chromatographic analysis, the small differences found could be attributed to the higher precision of automatic injection against manual injection. However, as can be observed in Tables 1 and 2, the precision was significantly improved when considering the total amount of monoepoxy FAME. On the other hand, as observed in Tables 1 and 2, significant differences between the two injection systems were found for *trans*-12,13-EO in both oils. However, the slightly higher amounts found in this

Table 1
Quantitation of monoepoxy fatty acid methyl esters in olive oil heated at 180 °C for 15 h

| | On column | | Split | |
|---|---|---|---|---|
| | Mean (mg/g oil) | RSD (%) | Mean (mg/g oil) | RSD (%) |
| *trans*-9,10-ES | 7.29 | 3.8 | 7.57 | 0.3 |
| *trans*-12,13-EO | 0.82* | 2.6 | 0.93* | 1.3 |
| *cis*-9,10-ES | 4.07 | 5.1 | 3.85 | 0.3 |
| *trans*-9,10-EO | 0.80 | 4.8 | 0.79 | 1.5 |
| *cis*-12,13-EO | 0.25 | 4.5 | 0.25 | 0.6 |
| *cis*-9,10-EO | 0.32 | 2.2 | 0.35 | 0.9 |
| Total | 13.52 | 1.3 | 13.72 | 0.1 |

ES, epoxystearate; EO, epoxyoleate. Results are mean of three samples of polar FAME.
* $P<0.05$.

Table 2
Quantitation of monoepoxy compounds in sunflower oil heated at 180 °C for 15 h

| | On column | | Split | |
|---|---|---|---|---|
| | Mean (mg/g oil) | RSD (%) | Mean (mg/g oil) | RSD (%) |
| *trans*-9,10-ES | 0.87 | 2.3 | 0.84 | 2.0 |
| *trans*-12,13-EO | 2.94* | 2.7 | 3.16* | 1.4 |
| *cis*-9,10-ES | 1.55 | 4.2 | 1.45 | 1.5 |
| *trans*-9,10-EO | 1.85 | 4.5 | 1.81 | 0.4 |
| *cis*-12,13-EO | 1.17 | 4.7 | 1.14 | 0.1 |
| *cis*-9,10-EO | 2.51 | 4.0 | 2.46 | 0.7 |
| Total | 10.88 | 2.4 | 10.87 | 0.7 |

ES, epoxystearate; EO, epoxyoleate. Results are mean of three samples of polar FAME.
* $P<0.05$.

compound for split injection were parallel to the lower mean values found for *cis*-9,10-ES, which have to be attributed to small differences in the separation of both peaks which were not totally resolved (Fig. 2A).

To sum up, the methodology proposed was very useful for quantitation of monoepoxy fatty acids in thermoxidized oils and even in refined oils where they are present at ppm levels.

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