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Identification of 9(*E*),11(*E*)-18:2 Fatty Acid Methyl Ester at Trace Level in Thermal Stressed Olive Oils by GC Coupled to Acetonitrile CI-MS and CI-MS/MS, a Possible Marker for Adulteration by Addition of Deodorized Olive Oil

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The olive oil market is suffering from sophisticated illegal treatments. One common adulteration process consists of the addition to virgin olive oil of lower quality oils, such as "lampante" oil, an inexpensive oil and with some organoleptic defects, which is then submitted to thermal deodorization under vacuum processes for removal of the undesired flavor components. Such a blending may not have a huge influence on the chemical composition and may not significantly affect the parameters usually checked as quality indicators, although the organoleptic properties may change. As a consequence, a major effort is being devoted to find reliable markers able to unmask such adulterations. We report here the complete characterization of a compound, detected at trace levels exclusively in thermal stressed oils, which could be a candidate marker for adulteration. The investigation, carried out by GC-MS and GC-MS/MS, provided its complete structure, including the stereochemistry, shown to be a 9(*E*),-11(*E*)-18:2 fatty acid methyl ester. Experimental data also confirmed the influence of both temperature and heating time on formation and concentration of this compound.

KEYWORDS: Olive oil; adulteration; FAMEs; mass spectrometry

INTRODUCTION

In the world market, there is a growing interest in extra virgin olive oils because of their high nutritional properties and sensory qualities. Extra virgin olive oil can be considered a "natural fruit juice", since it is obtained from olive fruit just by physical operations (milling, pressing, centrifugation, and filtration). The composition of extra virgin olive oils is the result of complex interactions among olive variety, environmental conditions, fruit ripening, and extraction technology (*1*−*4*). Triacylglycerols are largely the main component (ca. 98%) of olive oil (*5*), whose nonglyceridic moiety (ca. 2%) consists of several components (volatile organic compounds, phenols, tocopherols, pigments, sterols, squalene, etc.) that are responsible for olive oil flavor, stability, and nutritional properties (*6*−*8*).

In recent years, analytical criteria have been established by international regulations to define olive oil quality grade (extra virgin, virgin, "lampante", "refined", etc.) and genuineness, through the detection of adulterations with seed oils or other solvent-extracted oils (*9*, *10*). Nevertheless, at present, no absolute analytical parameters have been identified to detect one of the latest most common modifications of extra virgin olive oil, the addition of deodorized-only lampante oil. In practice, this adulteration process consists of the mild thermal deodorization (80−120 °C) of such an oil, which is inexpensive and has an unpleasant taste, for the removal of the undesired flavor, followed by its dilution into extra virgin olive oil in variable amounts depending on the quality of the lampante oil itself. In general, such a blending does not produce an easily detectable modification of the chemical composition because of the mild conditions used in the deodorization. Indeed, the parameters usually checked as quality indicators, such as triacylglycerol composition, sterols, and newly formed steroid hydrocarbons, are not appreciably altered by the blending, although the organoleptic properties might change. Different approaches have been used trying to solve this problem, on the basis both of common analytical techniques, such as GC and HPLC, and of more advanced ones, such as GC-MS, HPLC-

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MS, LC-MS, NMR, and even circular dicroism (*11*, *12*), but none of them have been conclusive.

We now report for the first time, to the best of our knowledge, the presence at trace level of methyl 9(*E*),11(*E*)-octadecadienoate in the fatty acid methyl esters fraction (FAMEs) of some extra virgin and deodorized olive oils. Its structure was identified by mass spectrometric analysis and its stereochemistry by the comparison with pure standards. From preliminary data, this compound was heating dependent, thus representing a possible marker for the detection of deodorized oil addition to extra virgin olive oil.

MATERIALS AND METHODS

Chemicals. The mixture of conjugated linoleic acid methyl esters (CLAMEs) was purchased from Sigma-Aldrich (St. Louis, MO). The following FAMEs were obtained from Matreya, Inc. (Pleasant Gap, PA): methyl 9(*Z*),11(*E*)-octadecadienoate, methyl 9(*E*),11(*E*)-octadecadienoate, and methyl 9(*Z*),11(*Z*)-octadecadienoate. HPLC grade acetonitrile was purchased from Carlo Erba Reagenti (Rodano, MI, Italy) and isobutane 3.5 from El-chimie (Cinisello Balsamo, MI, Italy). Carapelli Firenze S.p.A. (Tavarnelle Val di Pesa, FI, Italy) supplied olive oil and refined seed oil samples.

Sample Preparation. Fatty acid methyl esters were prepared by trans-esterification with sodium methylate from acylglycerols to minimize changes in CLA isomers distribution (*13*). In a 10-mL screw top conical reaction vial, 6 mL of 0.2 N sodium methylate in methanol was added to 0.5 g of oil sample, and the resulting mixture was heated at 100 °C for 20 min. Additional heating could be necessary if the fatty substance is not completely liquefied and clear at room temperature. The following extraction of the FAMEs was carried out by adding 2 mL of hexane and 2 mL of water. The organic solution was totally evaporated under nitrogen, reconstituted by 1.5 mL hexane, and diluted as necessary for the analyses.

The heated olive oil samples have been prepared as follows: a glass vial open to air containing 100 mL of olive oil has been put into a mineral oil bath at 160 °C or 190 °C. Four samples were collected every 2 h and submitted to methylation process as described above.

The heated olive oil samples were prepared as follows: 100 mL of olive oil was heated from 90 °C up to 190 °C in a mineral oil bath. Samples were collected every 2 h and submitted to methylation as described above.

Instrumentation and Acquisition Methods. GC-FID analysis were performed on a Varian Star 3400CX GC (Varian, Inc., Walnut Creek, CA) equipped with a flame ionization detector and a model 1078 split/splitless injector. A 60 m × 0.25 mm i.d., 0.2 μm, CP-Sil 88 for FAME capillary column (Varian) was used, and hydrogen with 18 psi head pressure was the carrier gas. The injector and detector were held at 280 °C. The oven temperature program was the following: start 140 °C for 7 min, ramp to 195 °C at 1 °C/min, and hold for 10 min.

The GC-MS equipment consisted of a Varian Saturn 2000 MS/MS ion trap mass spectrometer coupled to a Varian 3800 gas chromatograph equipped with a model 1079 split/splitless injector and a CTC Analytics Combi PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). The separation was achieved by a 100 m × 0.25 mm i.d, 0.2 μm, CP-Sil 88 for FAME capillary column (Varian), supplied with helium carrier gas at 1 mL/min constant flow. The injector temperature was 280 °C and the oven temperature program was the following: start 100 °C, ramp to 160 °C at 15 °C/min, ramp to 225 °C at 1 °C/min, and hold for 5 min. The MS acquisitions were performed both by electron ionization (EI) and by chemical ionization (CI), in full scan, tandem mass spectrometry (MS-MS), and multiple reaction monitoring (MRM) modes, using the following methods:

Method 1: EI, full scan mode. Scan time, 1 s/scan; emission current, 10 μA. Temperatures: trap, 210 °C; transfer line, 170 °C; manifold, 100 °C. Mass range, *m*/*z* 25−450.

Method 2: Isobutane CI, full scan mode. Scan time, 1 s/scan; emission current, 10 μA; CI ionization and reaction storage level, *m*/*z* 25.0; reagent ion ejection amplitude, 7.4 V; CI background mass, *m*/*z* 65; target TIC, 10 000 counts; CI maximum ionization time, 2000 μs; CI maximum reaction time, 40 ms; automatic reaction control (ARC) prescan ionization time, 200 μs. Temperatures: trap, 210 °C; transfer line, 170 °C; manifold, 100 °C. Mass range, *m*/*z* 60−450.

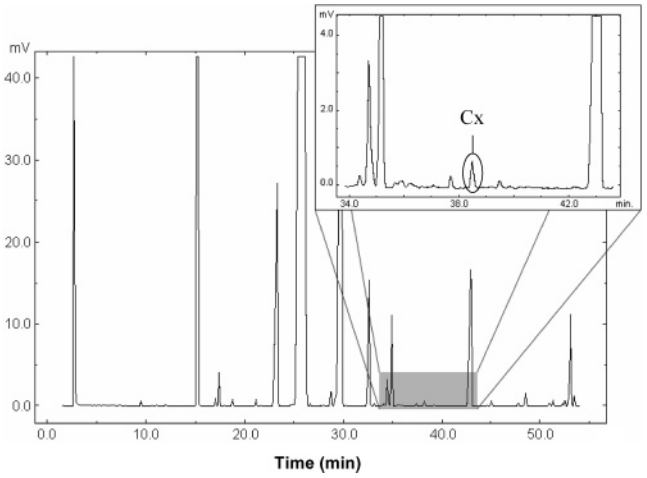

Figure 1. GC-FID chromatogram of a refined peanut oil and its expansion in the range of retention times 34–43 min. Cx is the unknown peak detected in oils submitted to thermal stress.

Method 3: Acetonitrile CI, full scan mode. Scan time, 1 s/scan; emission current, 10 μA; CI ionization and reaction storage level, *m*/*z* 25.0; reagent ion ejection amplitude, 9.0 V; CI background mass, *m*/*z* 65; target TIC, 8000 counts; CI maximum ionization time, 2000 μs; CI maximum reaction time, 120 ms; ARC prescan ionization time, 100 μs. Temperatures: trap, 210 °C; transfer line, 220 °C; manifold, 100 °C. Mass range, *m*/*z* 60−450.

Method 4: Isobutane CI, MRM mode. Scan time, 0.45 s/scan; emission current, 10 μA; CI ionization and reaction storage level, *m*/*z* 25.0; reagent ion ejection amplitude, 7.4 V; CI background mass, *m*/*z* 65; target TIC, 5000 counts; CI maximum ionization time, 2000 μs; CI maximum reaction time, 40 ms; ARC prescan ionization time, 200 μs. Precursor ions: *m*/*z* 245, 263, 295; excitation amplitude, 0 V. Temperatures: trap, 210 °C; transfer line, 220 °C; manifold, 100 °C. Mass range, *m*/*z* 244−296.

Method 5: Acetonitrile CI, full scan mode. Differs from Method 3 as follows: Scan time, 0.7 s/scan; CI background mass, *m*/*z* 150. Temperatures: trap, 175 °C; transfer line, 200 °C; manifold, 80 °C.

Method 6: Acetonitrile CI, MS-MS mode. Scan time, 0.7 s/scan; emission current, 10 μA; CI ionization and reaction storage level, *m*/*z* 25.0; reagent ion ejection amplitude, 9.0 V; CI background mass, *m*/*z* 150; target TIC, 8000 counts; CI maximum ionization time, 2000 μs; CI maximum reaction time, 120 ms; ARC prescan ionization time, 100 μs. Precursor ion, *m*/*z* 348; mass defect, 83 mu/100u; resonant waveform type; excitation amplitude, 1.2 V. Temperatures: trap, 175 °C; transfer line, 200 °C; manifold, 80 °C. Mass range, *m*/*z* 150−355.

With isobutane as the CI reagent, the gas pressure was adjusted to get the ratio of the peak heights at *m*/*z* 57, $[(CH_3)_3C]^+$, to *m*/*z* 43, $[(CH_3)_2CH]^+$, in the isobutane spectrum of approximately 1:1. When acetonitrile was used as a reagent, its pressure was adjusted so that the valley between *m*/*z* 41, $[CH_3CN]^{+\bullet}$, and *m*/*z* 42, $[CH_3CNH]^+$, in the spectrum was about 50% of the *m*/*z* 41 peak height.

RESULTS AND DISCUSSION

Involvement of Thermal Stress in the Formation of Cx (GC-FID Experiments). During our studies on the glyceridic fraction of seeds oil, we noticed a small unknown peak (Cx) in the GC-FID chromatograms of sunflower, peanut, and soybean oil FAME samples, more intense in the refined ones (**Figure 1**). The same compound was also present to a lower extent in refined olive oils and at trace level in some extra virgin and simply deodorized olive oils. Hence, experiments were carried

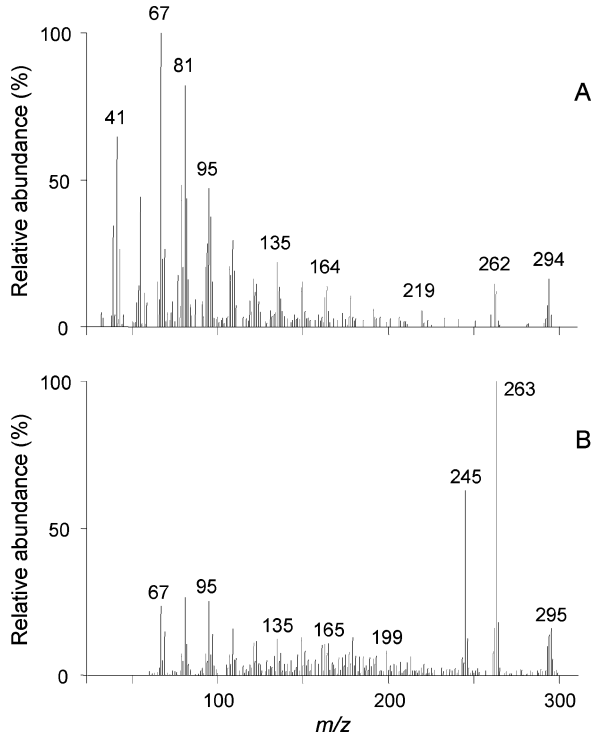

Figure 2. EI (**A**) and isobutane CI (**B**) mass spectra of Cx.

out to investigate the heating dependence of the formation of Cx. Lampante and extra virgin olive oils where heated at different temperatures ranging between 90 and 190 °C for 4−8 h. An increase of the compound under study was observed starting from 100 °C, becoming more intense with the raising of the temperature and the protraction of the heating time. The structure of Cx was then investigated by GC-MS.

Gas Chromatography in GC-MS Experiments. To achieve a chromatographic separation analogous to that of GC-FID experiments, a longer CP-Sil 88 column and a suitable oven temperature program were used. Furthermore, the relationship between GC-FID and GC-MS retention times was investigated. The refined peanut oil whose GC-FID chromatogram is shown in **Figure 1** was used to set up the GC-MS method because of its relatively strong peak of Cx. The retention times of some FAME known peaks were plotted with GC-FID times (Rt_{GC-FID}) on the horizontal axis and GC-MS times (Rt_{GC-MS}) on the vertical axis, and the resulting data points were then fitted by linear regression to a curve, having the following equation:

$$Rt_{GC-MS} = 0.91Rt_{GC-FID} + 11.39$$

The useful regression statistics are coefficient of determination (r^2), 0.998, and confidence interval for the prediction, $Rt_{GC-MS} \pm 0.35$. According to the equation above, as the experimental Rt_{GC-FID} of Cx is 36.60 min, the resulting predicted Rt_{GC-MS} is 46.52 ± 0.35 min, within the TIC chromatogram range, exhibiting one peak at 46.77 min. The correspondence of Cx with such a peak was further confirmed by its proximity with two main peaks, exactly as for Cx in the GC-FID chromatogram.

EI-MS and Isobutane CI-MS. Figure 2 shows both the EI and the isobutane CI mass spectra of Cx, acquired by methods 1 and 2, respectively. The molecular ion $[M]^{+\bullet}$ is at *m*/*z* 294, as confirmed by the $[M + H]^+$ ion at *m*/*z* 295 in the CI spectrum. Both ions lose 32 amu, typical neutral methanol loss of FAMEs, forming the ions at *m*/*z* 262 and *m*/*z* 263, respectively. In the CI spectrum, also a consecutive loss of water from $[M + H - 32]^+$ produces the ion at *m*/*z* 245 ($[M + H - 32 - 18]^+$). This leads us to suppose that the compound under investigation is an octadecadienoic acid methyl ester (18:2) (*14*). Moreover, its retention time, which is significantly higher than the one of linoleic acid methyl ester (38.78 min) (*15*), suggests a possible conjugation of the double bonds.

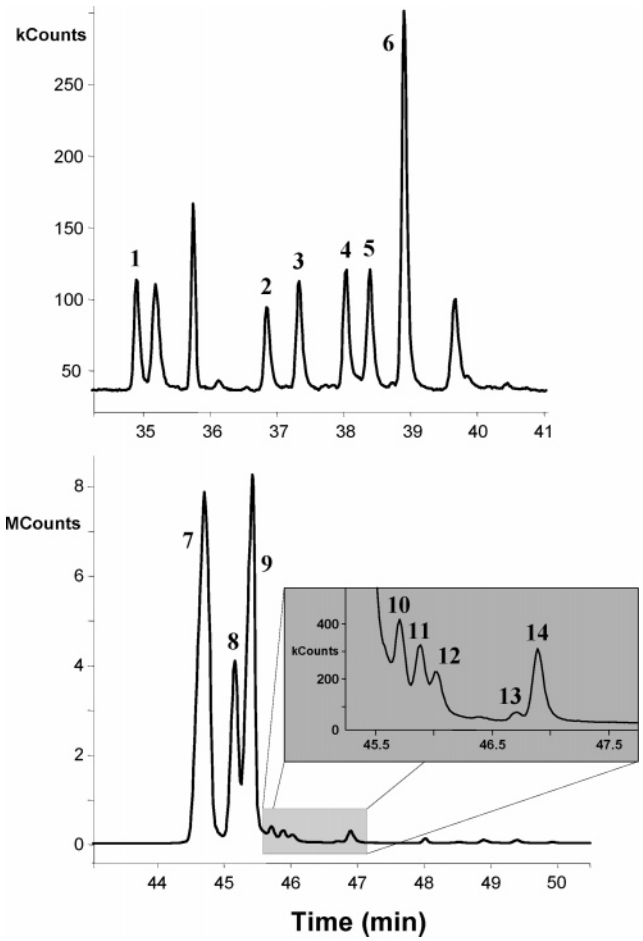

Figure 3. EI TIC chromatogram, in the time intervals 34–41 and 43–50 min, of a commercial mixture of conjugated 18:2 FAMEs, diluted 1:1000 in hexane. The acetonitrile CI-MS spectra revealed that it also contains, besides a large number of impurities, oleic acid methyl ester (**1**), homoallylic 18:2 isomers (**2**–**5**), linoleic acid methyl ester (**6**), and conjugated 18:2 isomers (**7**–**14**).

Acetonitrile CI-MS and -MS/MS. The investigation of both the conjugation and the position of the double bonds was carried out by acetonitrile CI-MS and acetonitrile CI-MS/MS, using the method first proposed by Brenna et al. (*16*−*20*). According to theory (*21*−*26*), the $[C_3H_4N]^+$ ion (*m*/*z* 54), generated by self-reaction of acetonitrile under CI conditions, adds to the C−C double bonds of unsaturated fatty acids to yield a $[M + 54]^+$ ion, which decomposes by loss of neutral methanol to yield a $[M + 54 - 32]^+$ ion. The ratio $[M + 54]^+/[M + 54 - 32]^+$ in the CI-MS spectrum of CLAMEs is 1 order of magnitude lower with respect to that of the equivalent homoallylic isomers (*20*). Collision-induced dissociation (CID) of the $[M + 54]^+$ ion generates two highly diagnostic ions, representative of the double bond positions.

Because of the low concentration of Cx in the matrixes under study, the acetonitrile CI-MS method was refined using a mixture of commercial "conjugated" 18:2 FAMEs, with a linoleic acid methyl ester content less than 1%. These were revealed as a mixture of homoallylic and conjugated 18:2 FAMEs. As shown in **Figure 3**, the total ion current chromato-

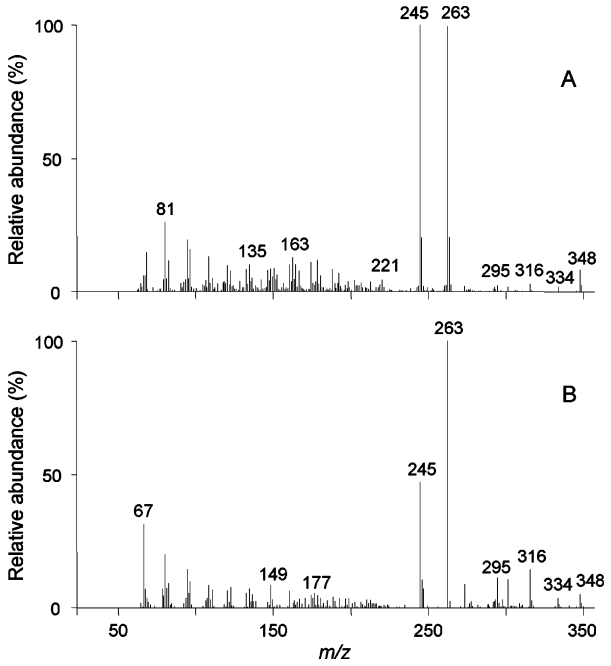

Figure 4. Acetonitrile CI-MS spectra of homoallylic (**A**) and conjugated 18:2 FAMEs (**B**).

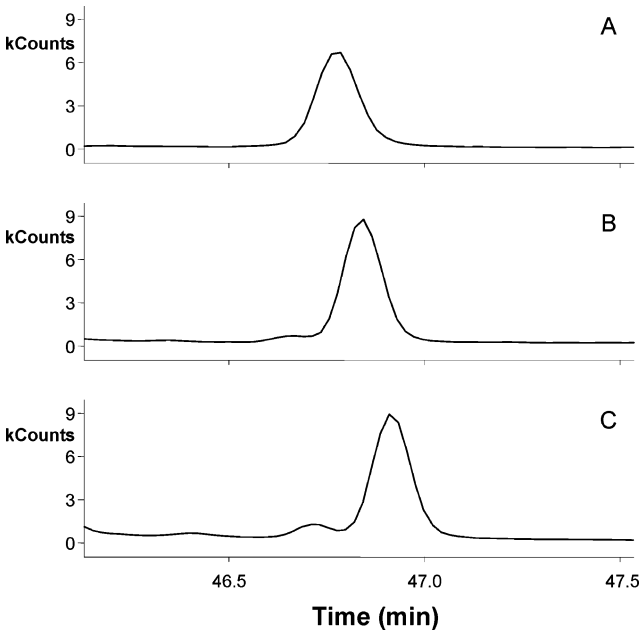

Figure 5. Comparison among the total ion current chromatograms, in the time interval 46.0–47.5 min ca., of commercial mixture of conjugated 18:2 FAMEs (**A**), sample obtained by adding a refined peanut oil to the commercial mixture (**B**), and refined peanut oil (**C**).

gram contains two separate groups of peaks (intervals 34−41 and 43−50 min), which were attributed to homoallylic isomers (peaks 2−6) and conjugated isomers (peaks 7−14) by the acetonitrile CI-MS spectra. **Figure 4** shows two spectra, relative to peaks 2 (spectrum A) and 10 (spectrum B) in **Figure 3**, which are representative of the compounds belonging to one of the two groups each, acquired by method 3. In spectrum A, the ion at *m*/*z* 348 has a relative abundance significantly higher than *m*/*z* 316, so that the ratio $[M + 54]^+/[M + 54 - 32]^+$, which is about 2.8, is representative of a homoallylic isomers. In contrast, in spectrum B, the ratio $[M + 54]^+/[M + 54 - 32]^+$ is about 0.3. Such a value is the evidence of the existence of conjugated double bonds.

Interestingly, the spectra of **Figure 4** contain many diagnostic ions, produced by several competitive reactions, characteristic of this ionization technique. We have already mentioned the following ions: $[M + H]^+$ (*m*/*z* 295), $[M + H - 32]^+ = [M + H - MeOH]^+$ (*m*/*z* 263), $[M + H - 32 - 18]^+ = [M + H - MeOH - H_2O]^+$ (*m*/*z* 245), $[M + 54]^+ = [M + C_3H_4N]^+$ (*m*/*z* 348), and $[M + 54 - 32]^+ = [M + C_3H_4N - MeOH]^+$ (*m*/*z* 316). Besides these, a $[M + 40]^+$ ion (*m*/*z* 334) has also been detected. It is probably a $[M + C_2H_2N]^+$ adduct, that was originally observed in acetonitrile CI-MS spectra of long-chain unsaturated hydrocarbons (*22*−*24*).

A comparison was carried out between the retention time of Cx and of the peaks obtained in the analysis of the commercial mixture to find a possible correspondence with one of the compounds in the mixture. Both peaks 13 and 14 in **Figure 3** have to be considered as possible candidates, though their retention times are slightly different from that of Cx in real samples, probably because of an influence of the matrix composition on retention times. The addition of measured amounts of five different oil samples containing Cx to five identical solutions of the commercial mixture, and the following analysis by method 4 of the resulting mixtures, confirmed the correspondence with peak 14. In fact, when the real sample is added, the ratio area of peak 14/area of peak 13 increases with respect to its original value in the pure mixture of FAMEs. Moreover, as shown in **Figure 5**, this addition leads to a

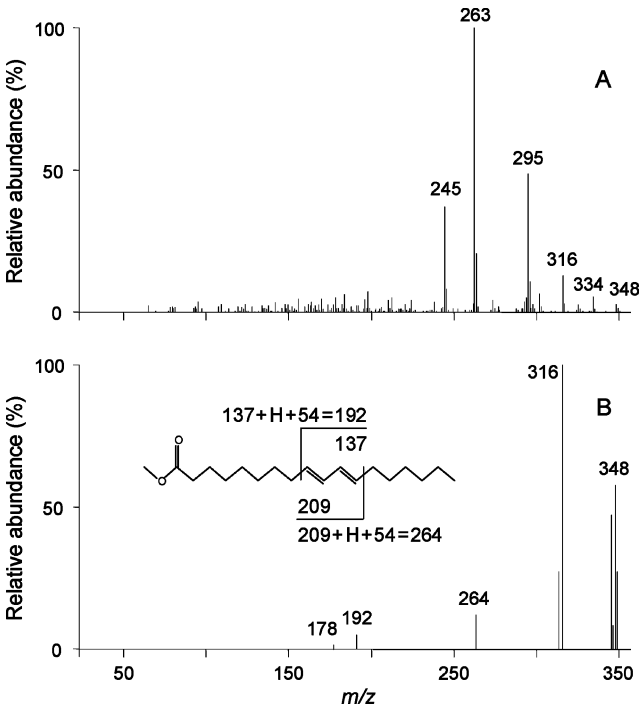

Figure 6. Acetonitrile CI-MS (**A**) and -MS/MS of *m*/*z* 348 (**B**) spectra of Cx.

retention time which is intermediate between those obtained for olive oil samples and commercial mixture. Although present at a low concentration level, compound 14 was suitable for refining the CI-MS method and setup of the CI-MS/MS method. As a consequence, the optimization of method 3 developed method 5, which was used as a starting point for the development of a tandem mass spectrometry based method (method 6). The acetonitrile CI-MS and -MS/MS spectra of compound 14 in the mixture of FAMEs and of Cx in the refined peanut oil provided the information needed. **Figure 6** shows the MS and MS/MS spectra of Cx. The ratio $[M + 54]^+/[M + 54 - 32]^+$ in the

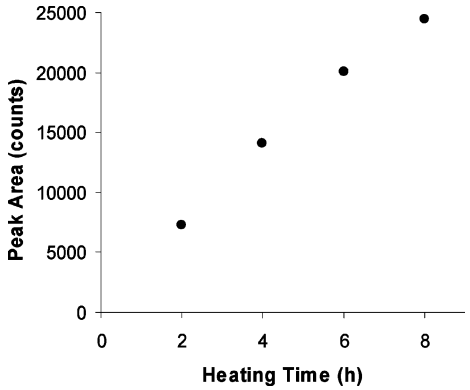

Figure 7. Effect of the heating time on the amount of Cx.

single mass spectrum, which is about 0.2, confirms the conjugation of the double bonds. In addition, the ions at *m*/*z* 264 and 192 in the CID spectrum of *m*/*z* 348 revealed their location, according to theory, at positions 9 and 11 of the main chain. One more product ion at *m*/*z* 178 in spectrum B of **Figure 6** suggests a possible coelution of trace of a 10,12−18:2 FAME, not confirmed by any ion at *m*/*z* 278 (*20*), even when a fine-tuning of the excitation amplitude was carried out. Hence, Cx is a methyl 9,11-octadecadienoate. The experiments showed how critical the method setup had to be on the real sample, as a sample with a relative high concentration of Cx exhibits a very poor signal in the MS/MS run (lower than 200 counts).

Stereochemistry. Mass spectrometry does not provide any definite information about the configuration of Cx. However, the four possible geometric isomers are easily distinguishable on the basis of their retention times. Thus, hexane solutions of three commercial 9,11−18:2 standards (9(*Z*),11(*E*), 9(*Z*),11(*Z*), and 9(*E*),11(*E*)), with a purity grade >96%, containing also traces of the fourth (9(*E*),11(*Z*)), not found on the market, were injected into the GC-MS system to register their retention times and compare them to that of Cx. The aim was achieved using the GC-MS oven program mentioned above, which offered a good separation and provided the elution order 9(*Z*),11(*E*) < 9(*E*),11(*Z*) < 9(*Z*),11(*Z*) < 9(*E*),11(*E*), which is in accordance with data available in the literature (*27*). As a final result, the clear correspondence of Cx with the 9(*E*),11(*E*) isomer was found, so that the compound under investigation had to be methyl 9(*E*),11(*E*)-octadecadienoate.

Involvement of the Heating Time in the Formation of Cx. The variation of the average peak areas of Cx in a commercial extra virgin olive oil submitted to heating process at 190 °C was investigated. For this purpose, five samples from the same oil were collected, one before the thermal treatment and four more after 2, 4, 6, and 8 h each of heating. All, after transesterification, were analyzed in triplicate by method 4. **Figure 7** shows the experimental results that clearly demonstrated that the heating time significantly affects the concentration of Cx. In practice, it caused a progressive increment of the amount of Cx, which was still in progress after 8 h.

In conclusion, for the first time, the presence at trace level of a conjugated linoleic acid methyl ester (Cx) has been reported in the FAMEs fraction of extra virgin and deodorized olive oils. A thorough GC-MS/MS study was carried out, leading to its certain identification as methyl 9(*E*),11(*E*)-octadecadienoate. From our data, Cx formation is related to the heating temperature and time in olive oil samples; therefore, it may represent a possible marker for the detection of deodorized oil addition to extra virgin olive oil. For this purpose, the GC-MS/MS method reported here is quite promising as it allows the selective detection of Cx at a very low level.

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