Journal of Chromatography A, 1575 (2018) 72–79

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

# A gas chromatography full scan high resolution Orbitrap mass spectrometry method for separation and characterization of 3-hydroxymethyl pyridine ester of fatty acids at low levels

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ARTICLE INFO

*Article history:*
Received 22 May 2018
Received in revised form 30 August 2018
Accepted 6 September 2018
Available online 7 September 2018

*Keywords:*
FAME
3-hydroxymethyl pyridine ester
GC
EI
Orbitrap
Q-Exactive
NIST
Ionic liquid

ABSTRACT

Fatty acid methyl esters (FAMEs), which are commonly used to characterize lipids, have several limitations to conclude on many structures. 3-Pyridylcarbinol esters (3-PCE) are used to characterize fatty acid structures [1], in particular, to identify ring and double bond positions on the carbon chain. Chromatographic separation of these esters is complex due to their polarity and high boiling points. In this study, we used a column with high resolutive power based on ionic liquids to increase the separation quality in gas chromatography (GC). In addition, we used a high-resolution detector (Orbitrap) to limit non-specific signals and improve the detection limits. This detector could be used with a mass filter at 5 ppm for the rapid determination of 3-PCE from its characteristic ions ($m/z$ = 108.0441 and 92.0495). This filter allowed the identification of derivative fatty acids with good sensibility. Thus, it was possible to characterize 3-PCE by measuring the exact fragment masses to confirm structures such as C19:2n12cyclo$\Delta$9.



## 1. Introduction

Several plants and organisms (yeast, fungi, bacteria, algae, etc.) are known to produce common and uncommon fatty acids with beneficial properties for health and nutrition, as well as potential applications in the chemical and cosmetics industries. The carbon chains of such uncommon fatty acids can be saturated, polyunsaturated, straight, odd, branched, cycloalkylated, hydroxylated, etc. When bonded to lipids, their proportions and compositions can strongly affect the physicochemical properties of oils or biological membranes (fluidity) [1–4]. These properties are directly linked to the chemical structure of the fatty acids (position of double bonds, rings, *cis-trans* configurations, etc.) serving as a "signature" for each organism [1–8]. However, there are limited methods available for their identification, and the structural determination of fatty acids can be complicated and often imprecise.

Among the unusual fatty acids, branched-chain fatty acids with a cyclopropane ring in the acyl group are of great interest in various agri-foods and other industrial fields [1,9–11]. These fatty acids can be hydrogenated and then used to produce lubricants and plastics [1,11]. A broad variety of organisms (bacteria) and plants synthesize carboxylic fatty acids (CFAs) [12–15]. The most common CFAs are C17 and C19, synthesized from the monounsaturated fatty acids C16:1 and C18:1, respectively, by methylation of the double bonds in the phospholipids [16–20]. In all cases, CFAs are formed by a soluble enzyme, CFA synthase, which transfers a methylene group from *S*-adenosyl-L-methionine to the cis double bonds of the unsaturated fatty acids in membrane phospholipids. The enzyme acts on the double bonds at the $\Delta$9 or $\Delta$11 positions in the fatty acids [16–20] to generate different structures (C17, C19, a putative C19:2$\Delta$12cyclo$\Delta$9) [12,14,21,22].

Gas chromatography-mass spectrometry (GC–MS) is the most valuable technique for characterizing many fatty acid species in plants and bacteria after their derivatization [14,23,24]. Information on the position of the rings and double bonds can be obtained [25] on the basis of the 3-PC esters. Radical-induced cleavage at

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https://doi.org/10.1016/j.chroma.2018.09.010
0021-9673/

each carbon-carbon bond leads to the formation of a series of ions stabilized by the charge localized on the nitrogen atom of the pyridine ring. Structural features of the fatty acid molecule, in particular, the position of unsaturation or chain branching, can be easily deduced from the resulting fragmentation pattern. Characterization of esters is a challenge in gas-phase chromatographic separation [23]. Generally, the boiling temperatures of esters are 40 °C higher than those of the corresponding fatty acid methyl ester (FAME) homologs [26,27]. Because of the polarity of the 3-pyridylcarbinol ester (3-PCE), CARBOWAX™ or SUPELCOWAX™ [26] GC columns are commonly used for separation. However, separation is limited to chains with 22 carbons or fewer and is directly linked to the maximum temperature of the system. To bypass this, it is possible to separate 3-PCE by thin layer chromatography (TLC) [28] and then analyze the esters with high boiling points by pyrolysis [28,29] on 5MS [27] columns (supporting higher temperatures) or by direct injection. These columns can operate up to 350 °C, but under these extreme conditions, the resolution is not satisfactory.

In this work, we aimed to identify the different cyclic structures synthesized after the expression of the *E. coli cfa* gene in yeast. To preserve these structures, we used 3-PCE and analyzed the electronic impact mass spectrometry profiles at 30 eV [25]. For 3-PCE GC separation, we used a column based on an ionic liquid to separate FAMEs [30] successfully. This column was found to be resolutive with particle sizes of 0.2 μm and a maximum temperature of 300 °C, which is 20 °C higher than that of the SUPELCOWAX™ column. By slightly exceeding the manufacturer's recommendations, it is possible to observe 3-PCE up to C26:1 (unpublished data). To characterize ions, we coupled an Orbitrap-type detector that allows the measurement of exact mass with a precision less than 2 ppm (external calibration). The combination of this ultrasensitive detector and the specific fragmentation of 3-PCE permitted the screening of 3-PCE peaks below the column's nonspecific signals.

## 2. Materials and methods

### 2.1. Chemicals

3-Pyridylcarbinol, Potasium Tert-butoxyde, and n-heptane were purchased from Sigma-Aldrich (St-Quentin-Fallavier, France). Standard FAME37 (Supelco) and the bacterial standard C19:1cyclo$\Delta$9 (Matreya Inc., Pleasant Gap, PA) were obtained commercially. THF (Biosolve), heptane (VWR, Fontenay-sous-Bois, France), and sulfuric acid (Supelco) were purchased.

### 2.2. Biological material

The *Y. lipolytica* strain (JMY5578) transformed with the *E. coli* bacterial gene encoding a cyclopropane fatty acid synthase (*cfa*) was provided by INRA Grignon. This recombinant strain was derived from the wild-type strain P1d [31].

### 2.3. Preparation of derivative

#### 2.3.1. FAME preparation

The FAME was prepared from oil extracted by the Bligh and Dyer method [32] with an internal two-step procedure. Saponification of the lipids was first performed. First, 200 μL of KOH (5 M 10%)/methanol was added to 100 μL of oil, and the mixture was incubated for 1 h at 80 °C in closed tubes. Thereafter, 200 μL of $H_2SO_4$ (10 N) was added to neutralize the excess KOH. Next, 1.5 mL of methanol/chloroform (2:1 v/v) was added and vortexed for 30 s, then 500 μL of chloroform and 1 mL of $H_2O$ were added to facilitate phase separation. After centrifugation for 5 min at 2000 *g*, the chloroform phase was transferred to a new tube and evaporated under a stream of nitrogen to recover the FAs. After, the FAs were methylated by adding 750 μL of HCl (4%)/methanol and allowed to react at 80 °C for 1 h. Then, 1.5 mL of heptane was added and the extracted FAMEs were recovered in the top layer. The extraction was repeated two times. The pooled organic layers were evaporated under a stream of nitrogen. The FAMEs were mixed with 200 μL of heptane for GC analysis. The experiments were carried out in duplicate.

#### 2.3.2. Fatty acid 3-pyridylcarbinol ester (3-PCE) preparation

The protocol followed was based on the method reported by Destaillats et al. [33]. First, 100 μL of FAME in heptane were evaporated to dryness under a nitrogen stream, then 1 mL of dichloromethane was added. A solution of 1 M *tert*-butoxide (100 μL) in tetrahydrofuran was added to 200 μL of 3-Pyridylcarbinol. After homogenization of this reagent, 100 μL of FAME was added and the reaction occurred at 40 °C for 30 min. Next, 1 mL of MilliQ water and 2 mL of heptane were added and vortexed for 30 s. Once decanted, the organic phase was recovered, evaporated, and diluted to a total volume of 60 μL with heptane before transferring to GC vials.

### 2.4. Instrumentation and method development

#### 2.4.1. Gas chromatography-flame ionization detection (GC-FID) analysis

GC-FID analysis was performed on GC-FID 2010 Plus Shimadzu equipment (Shimadzu, Japan) using a BPX 70 capillary column (SGE, 30 m × 0.25 mm, 0.25 μm, SGE Analytical Science, UK). The column temperature was initially held at 120 °C for 2 min after injection and then was increased to 250 °C with a 5 °C/min heating ramp for 2 min. The carrier gas was $H_2$ with a flow of 1.34 L/min. The injected volume was 1 μL of FAME and the split ratio was 1/5. The injector temperature was 250 °C. FAMEs were detected by flam ionization using a detector at 250 °C with $H_2$ as the carrier gas. The targeted compounds were identified by a comparison of their retention times with a corresponding standard Supelco 37 Component FAME mix (Supelco, Bellefonte, USA) and by calculating the equivalent chain length (ECL). The compositions of the fatty acids were expressed as a relative percentage of their peak areas with respect to the total peak area of all the fatty acids.

#### 2.4.2. GC-HRMS qualitative analysis

FAMEs and 3-PCE were identified using a Q-Exactive™ GC Orbitrap GC–MS/MS (Thermo-Fisher, Villebon, France). Mass spectrometer control and spectral processing were carried out using the Xcalibur™ software (Version 4.0.27), the 2014 NIST mass spectral library, and an in-house high-resolution mass spectrometry (HRMS) database. The acquired data in the HRMS system was obtained in the range of 50–500 *m/z* in 60,000-resolution mode at 70 and 30 eV for electronic impact ionization (EI). For chemical ionization (CI), methane was used with a flow rate of 1.5 mL/min. The spectrometer was tuned at both energies and calibrated externally. The GC was equipped with a SUPELCO SLB™ IL60 capillary column (30 m × 0.25 mm i.d., 0.20 μm film thickness, Supelco, Bellefonte, USA). Helium was used as the carrier gas at a flow rate of 1.2 mL/min. The injector port and transfer line were heated to 280 °C. One microliter of diluted sample was injected in split mode (50:1) or splitless mode. The initial oven temperature was held at 150 °C for 2 min and then was ramped at 15 °C/min to 300 °C and held for 4 min. The MS source temperature was maintained at 280 °C.

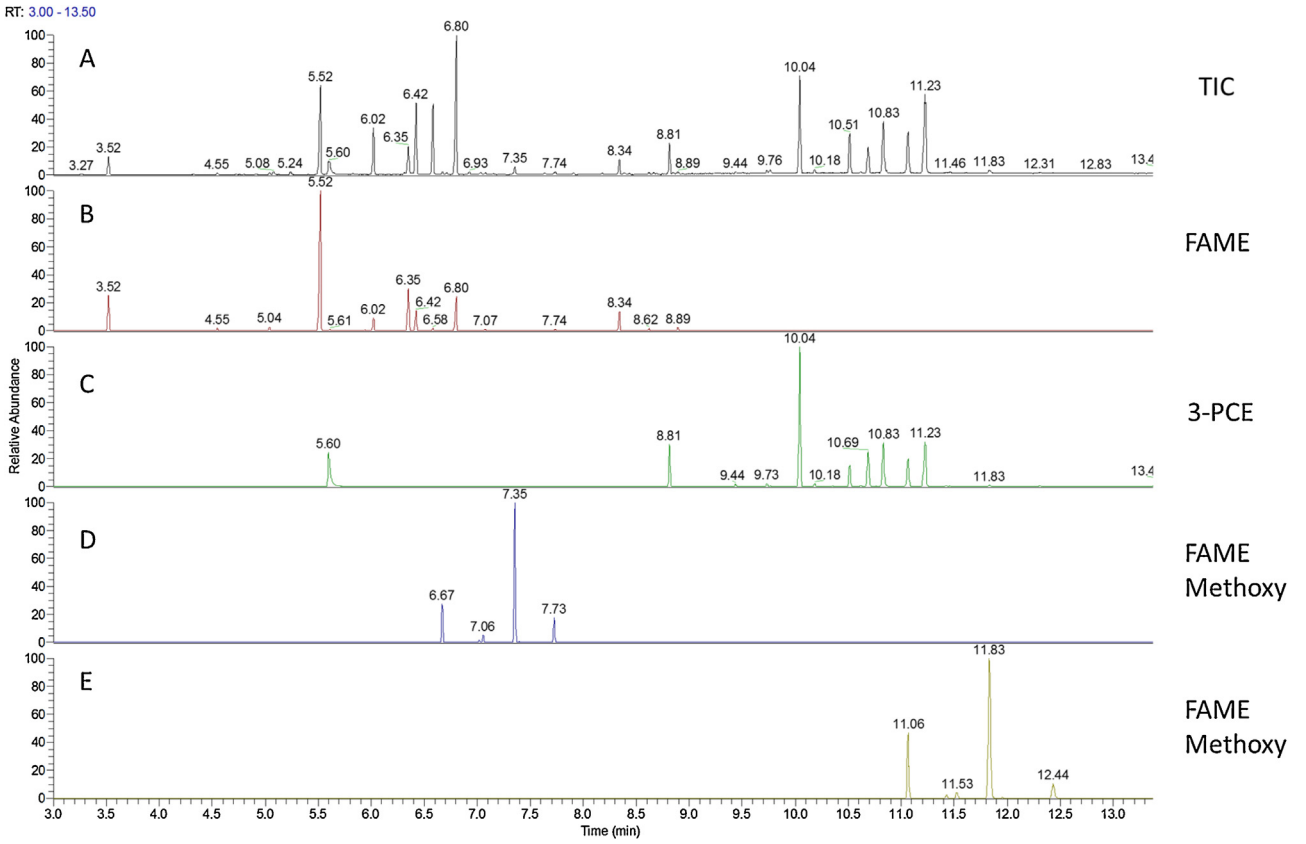


**Fig. 1.** Chromatogram after 3-PCE derivatization. (A) TIC, (B) EIC for 87.0443 (FAME), (C) EIC for 108.0441 (3-PCE), (D) EIC for 201.1488 (Methoxy FAME) and (E) EIC for 278.1751 (Methoxy 3-PCE). All EIC with 5 ppm extraction window.

**Table 1**
Semi-quantification of FA by FAME-GC-FID, confirmed by GC-EI-HRMS with FAME and 3-PCE.

| FA | GC-FID (FAME) | | GC-EI-HRMS (FAME) | GC-EI-HRMS (3-PCE) | |
|---|---|---|---|---|---|
| | % | Ecard type | % | % | ID (NIST 2014) |
| C14:0 | 0.35 | 0.19 | 0.60 | 0.72 | 3-Picolinyl stearate |
| C15:0 | 0.48 | 0.09 | 0.86 | 0.94 | 3-Picolinyl stearate |
| C16:0 | 22.10 | 0.24 | 30.52 | 37.86 | 3-Picolinyl stearate |
| C16:1n9 | 0.70 | 0.41 | 0.58 | 1.07 | cis-9-Hexadecenoic acid, picolinyl ester |
| C17:0 | ND | – | 0.20 | 0.21 | 3-Picolinyl stearate |
| C11:1cyclo$\Delta$9 | 9.21 | 1.05 | 5.76 | 6.23 | Picolinyl 9,10-methylene-hexadecanoate |
| C18:0 | 5.72 | 0.16 | 9.06 | 10.57 | 3-Picolinyl stearate |
| C18:1n9t | 16.49 | 3.81 | 0.66 | 15.01 | trans-9-Octadecenoic acid, picolinyl ester |
| C18:1n9c | ND | – | 9.20 | 0.20 | cis-9-Octadecenoic acid, picolinyl ester |
| C18:2n9c,12c | 15.03 | 1.34 | 20.14 | 9.31 | cis-9, cis-12-Octadecadienoic acid, picolinyl ester |
| C19:1cyclo$\Delta$9 | 24.48 | 1.50 | 16.83 | 14.35 | Picolinyl 9,10-methylene-octadecanoate |
| C19:2n12cyclo$\Delta$9 | 0.74 | 0.08 | 0.80 | 0.09 | n/d |
| C20:0 | 0.36 | 0.01 | 0.38 | 0.33 | Picolinyl 19-methylheneicosanoate |
| C22:0 | ND | – | 0.08 | 0.29 | Picolinyl 20-methylheneicosanoate |
| C24:0 | 2.81 | 0.26 | 4.00 | 2.52 | Picolinyl 19-methyleicosanoate |

**Table 2**
Characteristic (CI) and Base pic (BP) ions for functional groups in GC-HRMS orbitrap in range of 50–500 *m/z*.

| | Mass range : 50–500 m/z | | | |
|---|---|---|---|---|
| Function | BP | Raw formula | CI | Raw formula |
| FAME, Saturated | 87.0443 | C4H7O2 | | |
| FAME with 1 or 2 double bond | 81.0700 | C6H9 | | |
| FAME with 3 double bond | 79.0544 | C6H7 | | |
| TMS | 73.0469 | C3H9Si | | |
| FAME, Methoxy of CFA $\Delta$9 | 137.0964 | C9H13O | 201.1488 | C11H21O3 |
| 3-PCE | 92.0495 | C6H6N | 108.0441 | C6H6NO |
| 3-PCE, Methoxy of CFA $\Delta$9 | 278.1751 | C16H24O3N | 108.0441 | C6H6NO |

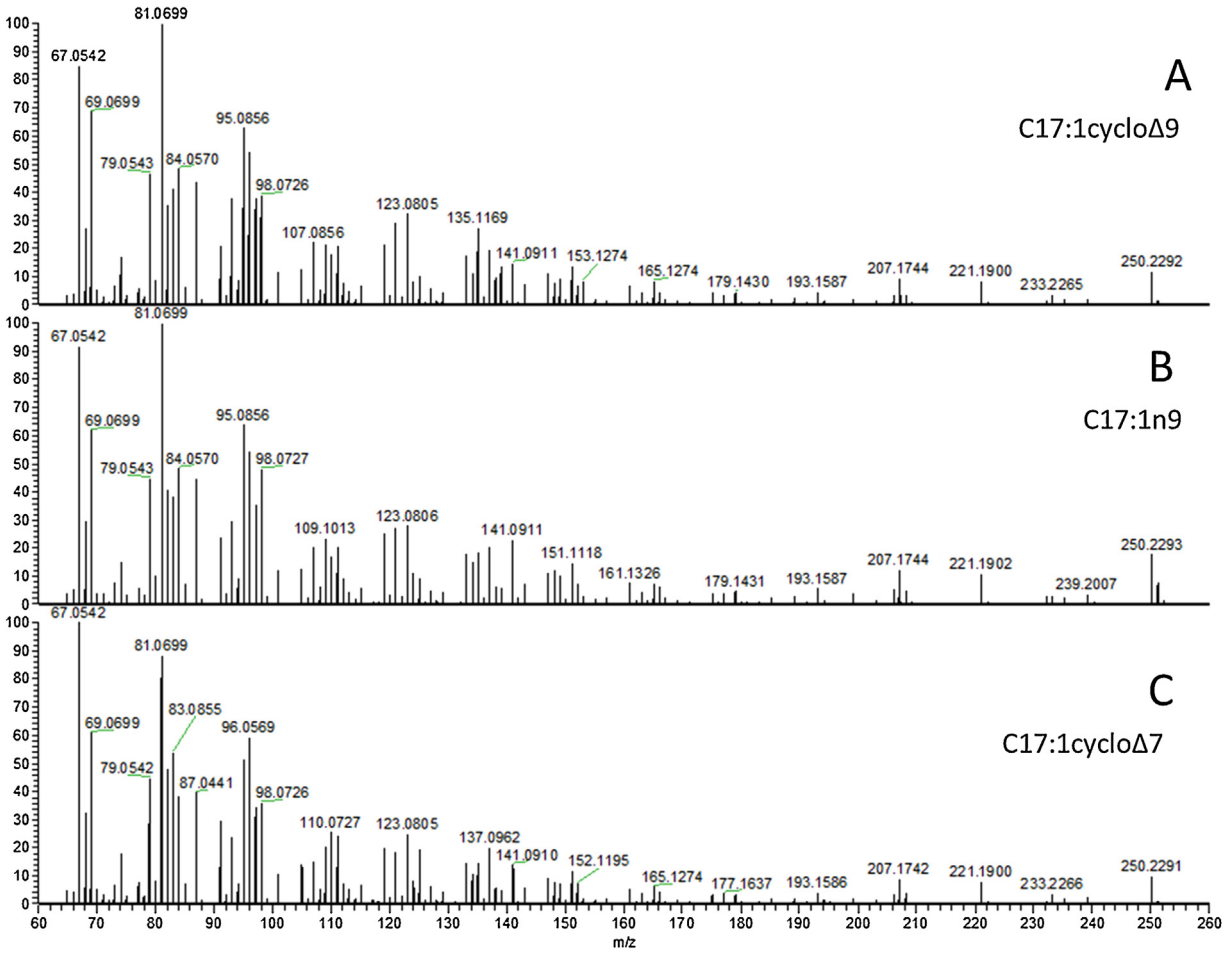


**Fig. 2.** GC-EI-HRMS at 70 eV for (A) C17:1cyclo9, (B) C17:1n9 and (C) C17:1C17:1cyclo7 FAME.

## 3. Results

### 3.1. FAME separation

Transformed *Yarrowia* with *cfa E. coli* gene was chosen for this study because the fatty acids generated had new complex structures to be identified. A wide range of unsaturated, cyclic (C17:1cyclo; C19:1cyclo; C19:2cyclo), and unknown fatty acids were detected in the sample (Fig. 1). After preparation, as described in the Materials and Methods section, the FAMEs were separated and analyzed by GC-HRMS with an ionic liquid column. Identification was made by comparing retention times (rt) with both the bacterial standards and FAME37 (Table 1). Mass spectra obtained by electronic impact of each entity were both compared with NIST databases and a specific base dedicated to FAMEs built with the GC-Orbitrap. To increase the reliability of our results, molecular ions were confirmed by CI. Based on these data, we confirmed the identity of unusual fatty acids like C17:1cycloΔ9 and C19:1cycloΔ9. However, these spectra (EI 70 eV) did not provide enough information to determine the position of the cyclopropanic ring on the carbon chain. Using this method, the retention time of the C17:1cycloΔ9 and C17:1cycloΔ7 FAMEs were similar [17]. Thus, this method could not be used to distinguish them. Moreover, the FAME EI spectra of these molecules were also similar (Fig. 2). In this study of fatty acid analysis in recombinant *Yarrowia*, the chromatograms also revealed a molecule that could be of the C19:2cycloΔ9 [22] type and a compound likely generated during preparation of FAME from CFA [5,18]. Different groups working on the extraction and transesterification methods [26,34] have identified several degradation compounds from biological extracts and from a C19:1cycloΔ9 commercial standard. These compounds contained an additional oxygen atom from methanol during acid methanolysis, resulting in typical ions [17] (Table 2) and the ion $C_{11}H_{21}O_3$ at $m/z = 201.1488$, which contains 3 oxygens (Fig. 1).

### 3.2. PCE separation

To confirm the results previously described concerning the molecules with cyclopropanic rings and other unknown compounds, 3-PCEs from FAME were tested on a SLB-IL60 column, yielding chromatograms in which the compounds could be distinguished. Application of a mass filter based on the specific ions from chemical groups ($m/z = 108.0441$) to the GC-HRMS results allowed for rapid sorting of the compounds (Fig. 1).

### 3.3. Confirmation of the structures of C17:1cycloΔ9 and C19:1cycloΔ9

The structures of the fatty acids C17:1cycloΔ9 and C19:1cycloΔ9 have been confirmed from 3-PCE mass spectra showing marker ions by Christies et al. [26] (Tables 1 and 2). In addition, the relative peak intensity was similar to those obtained by Christies et al. [26]. The $m/z = 164.0706$ signal, corresponding to a split in the α position of the n3 carbon in the carbon chain, was more intense than that found previously [26]. As previously described for the FAME analysis, this difference was due to the Orbitrap detector, which modified the signal intensity [35]. From the exact mass measurements, it was possible to distinguish three

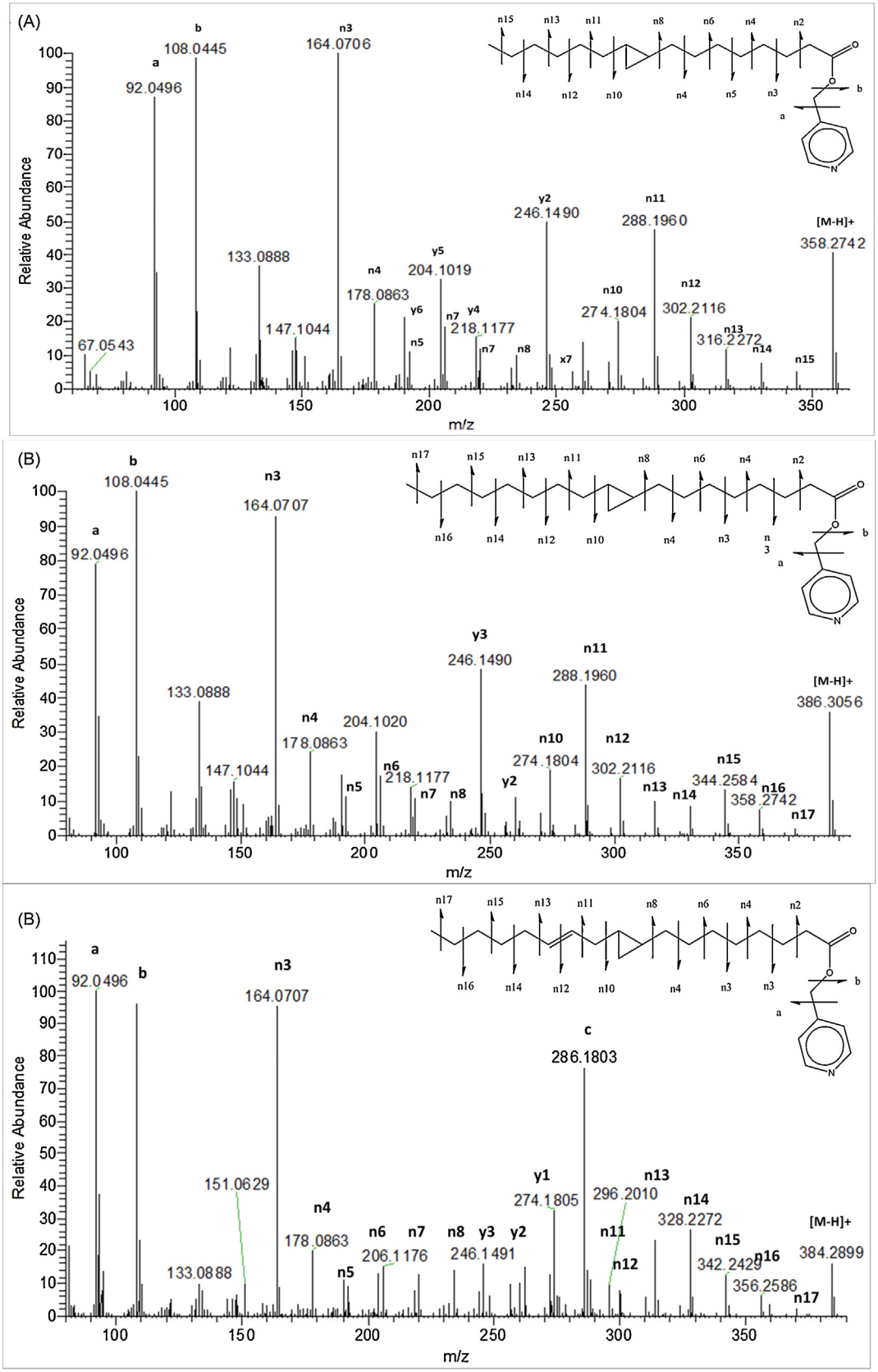


**Fig. 3.** EI 30ev HRMS of C17:1cyclo$\Delta$9 (A), C19:1cyclo$\Delta$9 (B), C19:2cyclo$\Delta$9, n12 (C).

ion families (Fig. 3). The "n" family corresponds to the breaking of the carbon chain after each methyl group. The "y" family was specifically observed with CFA, linked to the loss of a methyl group from the ion $m/z = 274.1804$. Ion $m/z = 246.1490$ was also more abundant with the Orbitrap detector than with common mass analyzers, such as a quadrupole. These differences were

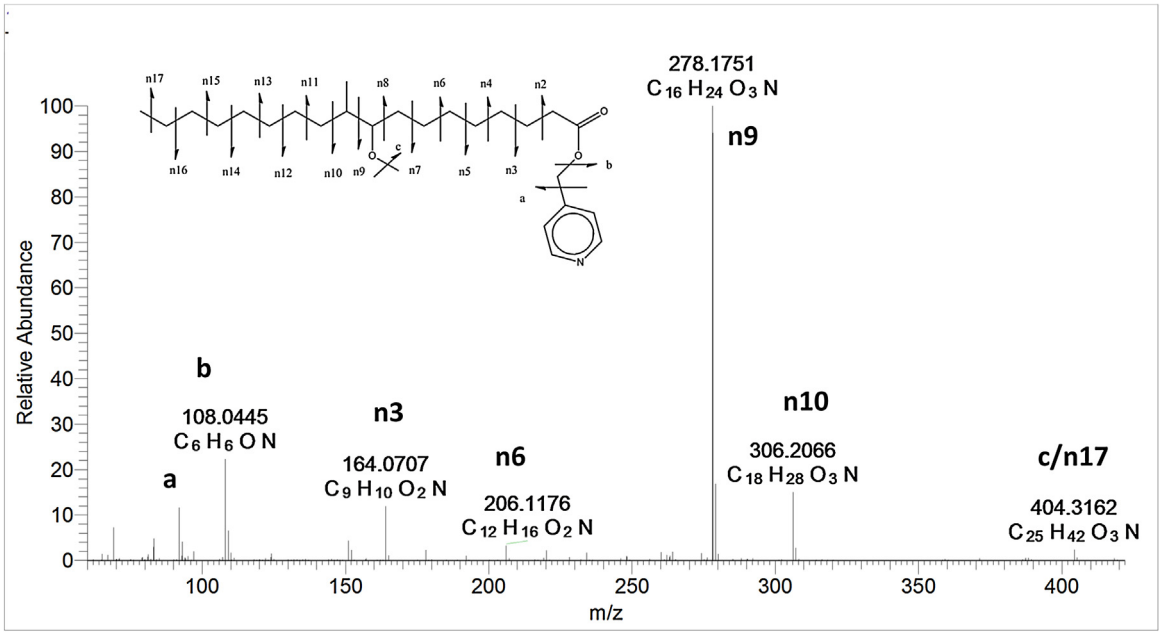


**Fig. 4.** EI 30ev HRMS for major methoxy compounds.

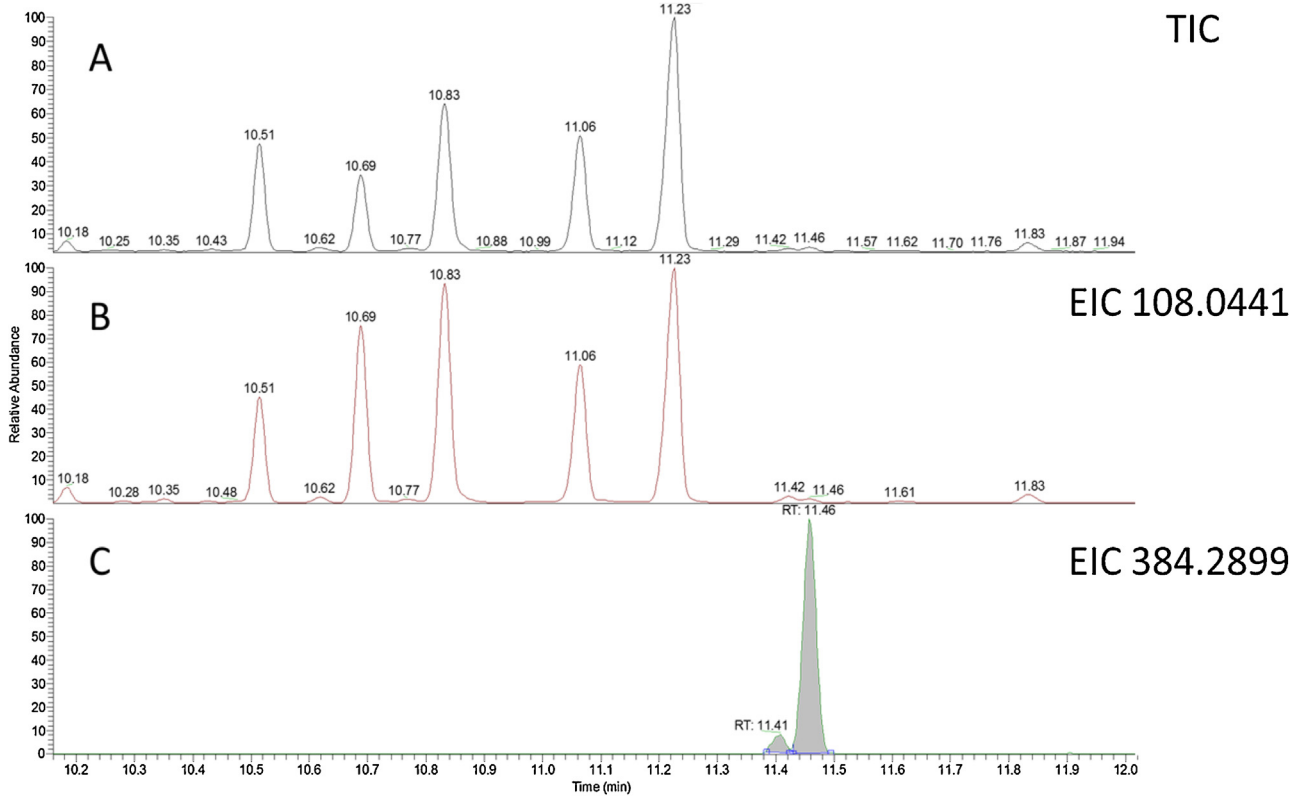


**Fig. 5.** GC-EI-HRMS spectrum at 30 eV of 3-PCE in IL60 column. (A) TIC, (B) EIC of $m/z = 108.0441 \pm 5$ ppm corresponding of characteristic ions of 3-PCE in GC-ORBITRAP, (C) EIC for C19:2n12cyclo$\Delta$9 $[M-H]^+$ ion.

observed both for commercial standards and produced molecules (data not shown). A third family, "x", was characterized by the loss of a $H_2O$ molecule linked to a molecular rearrangement at the carboxy functional group (supplementary data, Table S1&2, and Fig. S4 & S5). We have also noted an intensity inversion for peaks corresponding to the two marker ions of each 3-PCE (a,b). These modifications observed with the Orbitrap detector can be compared to those observed with FAME equivalents. However, determining the ion number for 3-PCE is much more important than the corresponding FAMEs and permitted the use of the NIST algorithm. According to these results, a database specific to 3-PCE can be developed, similar to the FAME database.

### 3.4. Characterization of C19:2n12cyclo∆9 with 3-PCE

Analysis of the GC-EI-HRMS spectrum at 30 eV (Fig. 3) of the chromatography peak of ion $m/z$ = 384.2899 at a retention time of 11.46 min (Fig. 5) confirmed the presence of C19:2n12cyclo∆9, which also confirmed the hypothesis of Maschida et al. [22]. The $[M-H]^+$ ion lost 2 H atoms compared to its saturated equivalent. The "n12″ ion was less abundant than others in the same region. A 26 uma mass difference between the n11 and n12 ions was observed and corresponded exactly to $C_2H_6$, indicating the presence of a double bond at this position. Unlike C19:1cyclo∆9, the $m/z$ = 246.1491 ion was less abundant. The chromatogram shows the presence of a small signal at a retention time of 11.41 min. This peak was too weak and partially eluted with C20:0 3-PCE, making it difficult to interpret the mass spectra. The spectra showed an abundant $m/z$ = 286.1803 signal corresponding to 2 uma less than C19:1cyclo∆9. This suggests that a molecular rearrangement occurred to stabilize the molecule into the Orbitrap detector.

### 3.5. Identification major methoxy derivative

Ours chromatograms also revealed the presence of a compound with an additional oxygen atom (Fig. 1). Previous researchers [17,26] suggested that such compounds are linked to the CFA degradation during methanolysis. In this case, degradation compounds such as methoxy can be generated through derivatization by $H_2SO_4$, boron trifluoride, boron trichloride, and HCl [35]. These reagents attack the cyclopropane ring and generate branched fatty acids and methoxy derivatives. The KOH method generated significantly less methoxy derivative, indicated by the detection of $C_{16}H_{24}O_3N$ (278.1751) and $C_9H_{13}O$ (137.0964) ions for 3-PCE. The signal intensities were similar for FAME and 3-PCE (Fig. 1). The mass spectra of the most abundant compound showed a methoxy structure, likely resulting from a ring opening followed by the addition of a methanol at this position. The main fragment results from a cut both in the alpha position of the n10 methyl group and in the beta position of the oxygen. The ion detected at 404.3162 corresponded to a cut at the alpha position of the lateral oxygen. The low signal intensity of the ions between n9 and n10, associated to a mass variation of 28 uma, confirmed the methyl position on carbon n10 (Fig. 4).

## 4. Discussion

The resolution of both the chromatography column and the mass detector, coupled with a high working temperature of the IL-60 column, allowed FAME and 3-PCE to be efficiently separated to C26:0. The incomplete derivatization procedure made it possible to detect FAME and 3-PCE in the same chromatogram. The sensitivity and mass accuracy of the GC Orbitrap allowed the isolation of 3-PCE by applying a mass filter. By applying a mass filter to the characteristic fragments of 3-PCE, in particular, fragment b̈with $m/z$ 108.0445 ($C_6H_6NO$), it was possible to isolate molecules with significantly different response, such as C19:2cyclo∆9.

The mass accuracy made it possible to rapidly obtain all the fragments of the molecule, thus facilitating the interpretation of the electron impact spectrum at 30 eV. By comparing with the mass spectra of C19:2∆12cyclo∆9, also present in the sample, it was possible to identify similarities and differences, validating the structure. This also confirmed the results obtained by Machida et al. [22], who observed a new unusual fatty acid and suspected this type of structure. Some differences in the fragmentation ion intensities were observed. They may have been due to the Orbitrap detector, as was reported by Mol [36]. We have also observed a specific alteration of the EI spectra for 3-PCE. Such changes were observed for some pesticides [36] and for FAME (Merlier, personal data). These modifications lowered the identification scores in the EI spectra of the databases (NIST, Wiley). This made it possible to quickly identify classes of compounds by applying an exact mass filter (Fig. 1).

In this study, analysis of the mass spectra revealed the presence of other compounds. The resolving capabilities of the IL60 liquid ion column allowed for efficient separation of 3-PCEs. Coupling with the Orbitrap detector yielded mass spectra, even for weakly concentrated compounds (Fig. 5). This allowed identification of the structure of the degradation compound C19:2cyclo∆9. Other compounds were detected for FAME and 3-PCE. However, their low levels and their preferential fragmentation made it difficult to identify their structures with certainty. It is possible that they were due to ions of a CFA derivative in position n9.

## 5. Conclusion

In this work, we separated FAME and the 3-PCE fatty acid using an IL60 column. It was thus possible to determine the position of the cyclopropane ring at the cis-9.10 position and the presence of a double bond at position 12, and to identify new compounds. These results were obtained even with relatively low concentrations due to the specificity and sensitivity of the GC-Orbitrap detector. We were able to confirm the hypotheses concerning the formation of methoxy groups from CFA during acid methanolysis.

## Acknowledgments

This work was performed in partnership with the SAS PIVERT, under the guidelines of the French Institute for the Energy Transition (Institut pour la Transition Energétique (ITE) P.I.V.E.R.T. (www.institut-pivert.com), selected as an Investment for the Future (Investissements d'Avenir). This work was supported by the Investments for the Future of the French Government under the reference ANR-001. The Regional Council of Picardie and European Union co-funded the equipment utilized within CPER 2007–2020. We thank Bertrand Beaujard for the preparation of the samples used for this study.

Reference to a journal publication

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2018.09.010.

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