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Gas chromatography–mass spectrometry determination of metabolites of conjugated *cis*-9,*trans*-11, *cis*-15 18:3 fatty acid

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Abstract

Structural determination of polyunsaturated fatty acids by gas chromatography–mass spectrometry (GC–MS) requires currently the use of nitrogen containing derivatives such as picolinyl esters, 4,4-dimethyloxazoline or pyrrolidides derivatives. The derivatization is required in most cases to obtain low energy fragmentation that allows accurate location of the double bonds. In the present work, the following metabolites of rumelenic (*cis*-9,*trans*-11,*cis*-15 18:3) acid, from rat livers, were identified: *cis*-8,*cis*-11,*trans*-13,*cis*-17 20:4, *cis*-5,*cis*-8,*cis*-11,*trans*-13,*cis*-17 20:5, *cis*-7,*cis*-10,*cis*-13,*trans*-15,*cis*-19 22:5, and *cis*-4,*cis*-7,*cis*-10,*cis*-13,*trans*-15,*cis*-19 22:6 acids by GC–MS as their 4,4-dimethyloxazoline and methyl esters derivatives. Specific fragmentation of the methyl ester derivatives revealed some similarity with their corresponding DMOX derivatives. Indeed, intense ion fragments at $m/z = M^+ - 69$, corresponding to a cleavage at the center of a bis-methylene interrupted double bond system were observed for all identified metabolites. Moreover, intense ion fragments at $m/z = M^+ - 136$, corresponding to allylic cleavage of the *n*-12 double bonds were observed for the C20:5, C22:5, C22:6 acid metabolites. For the long chain polyunsaturated fatty acids from the rumelenic metabolism, we showed that single methyl esters derivatives might be used for both usual quantification by GC–FID and identification by GC–MS.

Keywords: Conjugated fatty acid metabolites; Gas chromatography–mass spectrometry; Long chain polyunsaturated fatty acid; Rat liver

1. Introduction

Gas chromatography–mass spectrometry (GC–MS) is classically used for structural determination of unsaturated fatty acids [1]. Methyl esters prepared at room temperature by alkali-catalyzed transesterification are currently used for fatty acid profile determination by GC–FID [2]. These derivatives rarely provide characteristic ion fragments that allow accurate location of the ethylenic double bonds [1]. In order to obtain low ionization potential, the carboxyl group must be derivatized with a reagent containing a nitrogen atom [1]. In this case, the nitrogen group carries the charge and double bond migration and ionization are subsequently minimized [1]. Picolinyl esters [3,4] and 4,4-dimethyloxazolines (DMOX) [5] are extensively used, and their GC–MS properties have been reviewed in details by Christie [1]. DMOX derivatives exhibit good chromatographic properties [1,3]; indeed, only slight differences in the temperature programming are necessary to obtain similar retention times as for their corresponding fatty acid methyl esters (FAME). However, drastic thermal conditions (150–190 °C) and long reaction

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time (6–12 h) are necessary to obtain sufficient derivatization yield, resulting in partial degradation of sensitive samples such as long chain polyunsaturated fatty acids (PUFA) [1]. Picolinyl esters can be prepared by transesterification such as methyl esters at moderate temperature [4], but their analysis requires column temperatures about 50 °C higher than for methyl esters [1]. Using appropriate stationary phases, the resolution problem is greatly lessened but resolution of PUFA tends to be difficult particularly in complex mixtures.

In the present study, structural identification of some metabolites in the rat of rumelenic (*cis*-9,*trans*-11,*cis*-15 18:3) acid, a minor component of milk fat, was achieved using both DMOX and FAME. Mass spectra of both derivatives were compared and the results showed that FAME can efficiently be used for GC–MS characterization of these partially conjugated long chain PUFA in complex samples.

2. Materials and methods

2.1. Samples

Liver lipid samples from rats fed a mixture of conjugated *cis*-9,*trans*-11,*cis*-15 18:3 and *cis*-9,*trans*-13,*cis*-15 18:3 isomers of α-linolenic acid (CLNA) kindly donated by Naturia Inc. (Sherbrooke, PQ, Canada) were obtained from a previous study [5].

2.2. Preparation of fatty acid derivatives (FAME)

Methylation of *O*-acetylated fatty acids was carried out with sodium methoxide in methanol [2]. DMOX derivatives of FA were prepared using modified literature procedures [3,6] by heating total liver lipids (10 mg) directly with 2-amino-2-methyl-1-propanol (500 μL) under nitrogen atmosphere at 150 °C overnight.

2.3. GC–MS analysis of FAME and DMOX derivatives

FAME and DMOX derivatives were analyzed by GC–MS (Hewlett-Packard model 6890 Series II gas chromatograph attached to an Agilent model 5973N selective quadrupole mass detector; Palo Alto, CA) under an ionization voltage of 70 eV at 230 °C, and connected to a computer with a Hewlett-Packard ChemStation. The injector (splitless mode) and the interface temperatures were maintained at 250 °C, while He was used as carrier gas under constant flow (2.4 mL min^{-1}). GC separation was performed on a BPX-70 capillary column (SGE, Melbourne, Australia; 50 m, 0.32 mm i.d., 0.25 μm film thickness). Temperature programming mode consisted of 60 °C isothermal for 1 min, increased to 170 °C at 20 °C min^{-1}, and held isothermal for 40 min at 170 °C.

3. Results and discussion

Rumelenic (*cis*-9,*trans*-11,*cis*-15 18:3) acid, a minor component of ruminant fats [7–9], is an intermediate of the "biohydrogenation" process of α-linolenic (*cis*-9,*cis*-12,*cis*-15 18:3) acid in the rumen. Metabolism of conjugated dienoic FA has been extensively studied over the past years [10–13]. Indeed, by analogy, we have studied the metabolism of the rumelenic acid [5] in the rat resulting in the proposed biochemical pathway (Fig. 1).

The octadecatetraenoic intermediate has not been detected but the elongation product, the *cis*-8,*cis*-11,*trans*-13,*cis*-17 20:4 acid, has been identified from total liver lipid. The spectrum of its DMOX derivative is shown in Fig. 2A. The molecular ion at *m*/*z* = 357 and the loss of the terminal methyl group (*m*/*z* = 342) confirmed the eicosatetraenoic acid structure. Ion fragments at *m*/*z* = 328 and 302 allow the location of an ethylenic double bond in position Δ17, which is corroborated by the intense ion fragment at *m*/*z* = 288. Such characteristic fragment is typical for bis-methylene interrupted double bond systems [1,14,15].

Ions fragments at *m*/*z* = 274, 260, 248, 234, and 222 allow the location of the 11,13-conjugated system, while the double bond at position 8 is confirmed either by ion fragments at *m*/*z* = 182 and 194 or allylic cleavage fragments at *m*/*z* = 222 and 168. The key diagnostic ion for this FA is the intense ion fragment at *m*/*z* = 288 representing the loss of a radical fragment of 69 atomic mass unit (amu). The spectrum

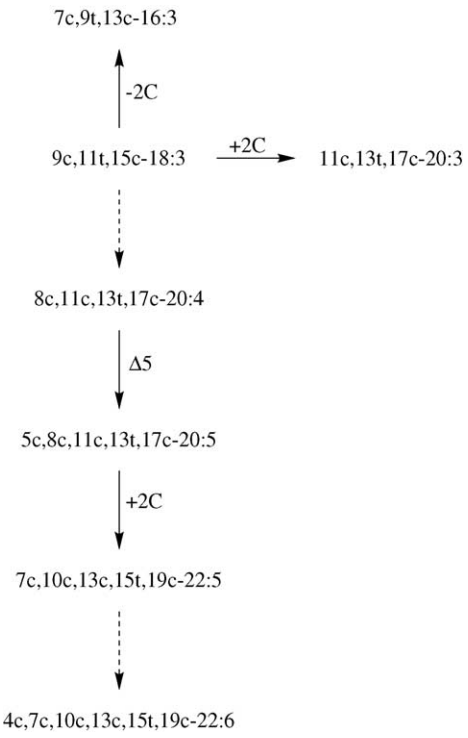

Fig. 1. Proposed biochemical pathway for the metabolism of rumelenic (*cis*-9,*trans*-11,*cis*-15 18:3) acid (adapted from [5]).

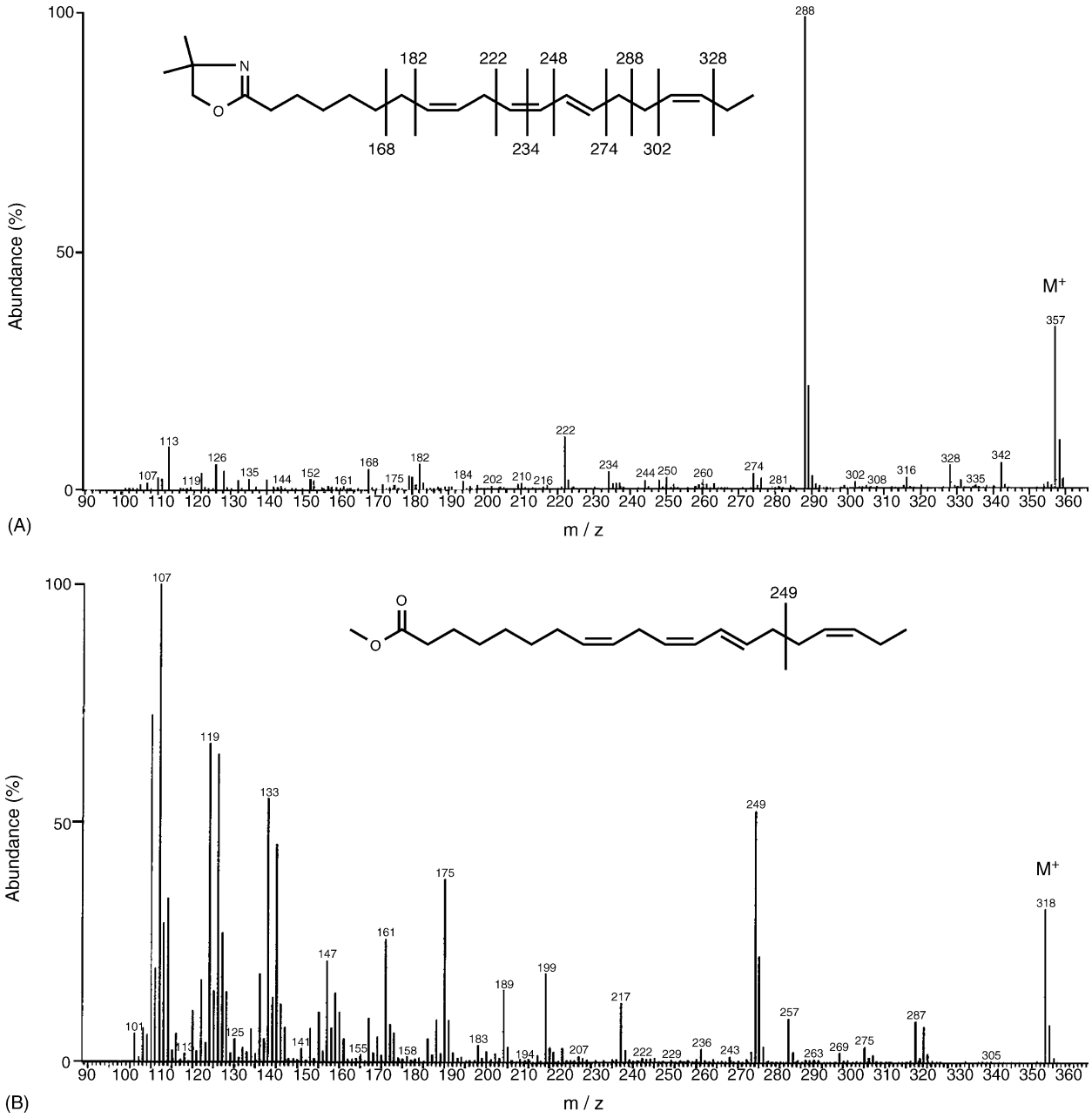

Fig. 2. Mass spectra of DMOX derivatives of *cis*-8,*cis*-11,*trans*-13,*cis*-17 20:4 (A), *cis*-5,*cis*-8,*cis*-11,*trans*-13,*cis*-17 20:5 (C), *cis*-7,*cis*-10,*cis*-13,*trans*-15,*cis*-19 22:5 (E), *cis*-4,*cis*-7,*cis*-10,*cis*-13,*trans*-15,*cis*-19 22:6 (G) acids, and methyl esters derivatives of *cis*-8,*cis*-11,*trans*-13,*cis*-17 20:4 (B), *cis*-5,*cis*-8,*cis*-11,*trans*-13,*cis*-17 20:5 (D), *cis*-7,*cis*-10,*cis*-13,*trans*-15,*cis*-19 22:5 (F), *cis*-4,*cis*-7,*cis*-10,*cis*-13,*trans*-15,*cis*-19 22:6 (H) acids. Fragmentations occurred towards the carboxyl group.

of its methyl ester derivative, shown in Fig. 2B, exhibits an intense fragment at $m/z = 249$ which corresponds to a loss of a 69 amu (C_5H_9) fragment from the parental molecular ion at $m/z = 318$.

The DMOX derivative spectrum of the *cis*-5,*cis*-8,*cis*-11,*trans*-13,*cis*-17 20:5 acid is shown in Fig. 2C. The molecular ion at $m/z = 355$ confirms the eicosapentaenoic acid structure. The intense ion fragment at $m/z = 286$ confirmed a bis-methylene interrupted structure, and allow the location of the Δ13 and Δ17 double bonds. Ion fragments at $m/z = 180$, 192, 220 and 232 allow the location of the double bonds in positions Δ8 and Δ11, respectively. The odd numbered ion fragment at $m/z = 153$ is characteristic for the Δ5 ethylenic double bond and resulted from the ionization of the nitrogen atom followed by cyclization and cleavage of the rest of the aliphatic chain [3]. The corresponding methyl ester derivative spectrum is presented in Fig. 2D. Similarly to the DMOX derivative of 20:4 acid, the methyl ester deriva-

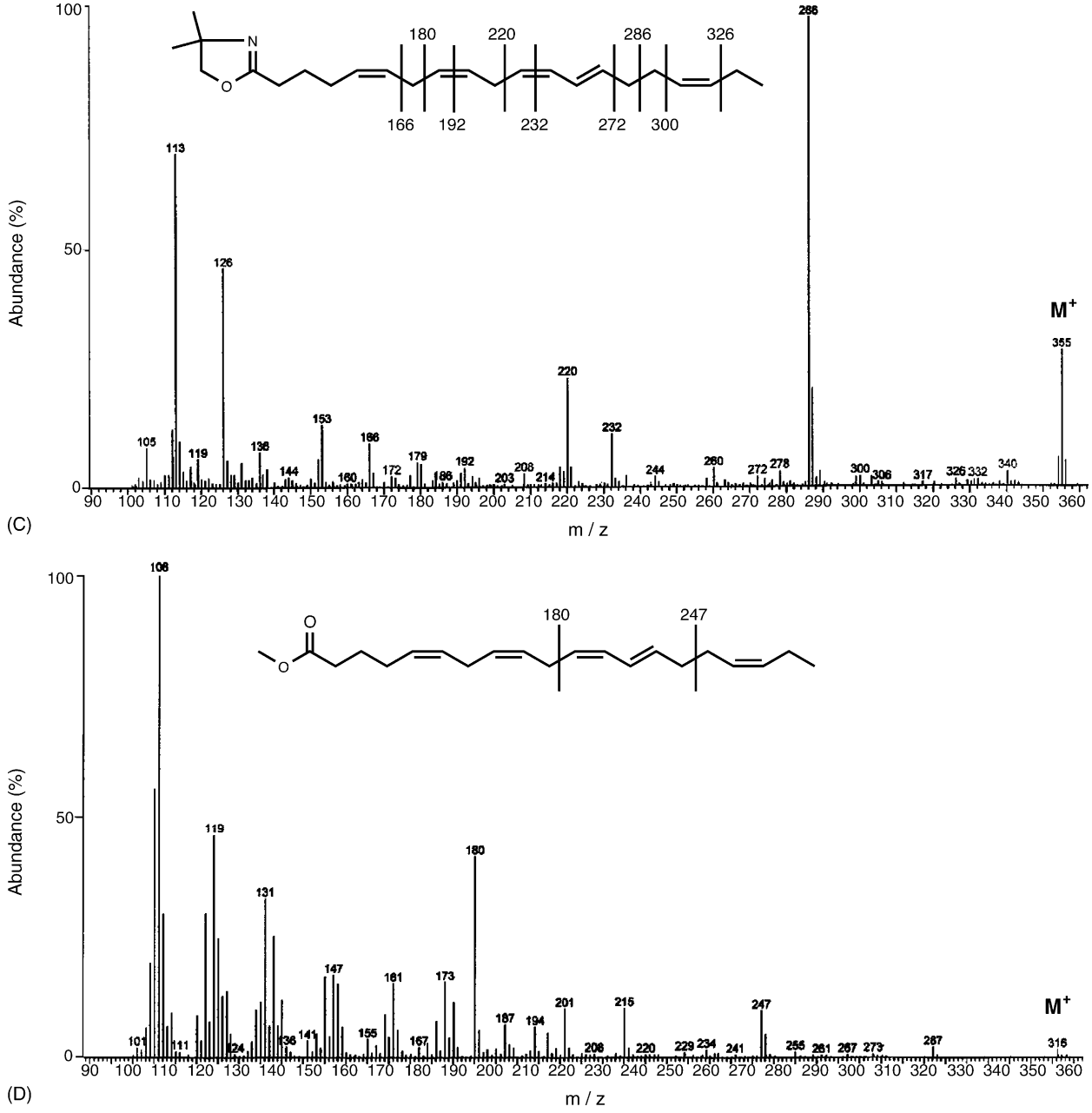

Fig. 2. (*Continued*)

tive spectrum presents a characteristic ion fragment at 247 corresponding to $M^+ - 69$ amu. Moreover, the spectrum exhibits an intense ion fragment at $m/z = 180$ corresponding to a cleavage between carbons 10 and 11. Indeed, this diagnostic ion is also present in the DMOX spectrum (Fig. 2C) at $m/z = 220$.

Charge remote fragmentation mechanism similar to that proposed by Dobson et al. [16], leads to the formation of ion fragment at $M^+ - 69$ amu for both DMOX and methyl ester metabolites of 9,11,15 18:3 acid is given in Fig. 3 [17]. The abstraction of a bis-allylic hydrogen radical consecutively to the formation of a positive radical molecular ion, results in a series of delocalization that gives, after disruption of the C_{15}–C_{16} sigma bond, a stable penta-unsaturated ion fragment with four conjugated ethylenic double bonds. Formation of intense ion fragments at $M^+ - 135$ and $M^+ - 136$ amu for DMOX and methyl ester derivatives, respectively, could be the result of allylic cleavage of the *n*-12 ethylenic double bond.

The DMOX derivative spectrum of *cis*-7,*cis*-10,*cis*-13,*trans*-15,*cis*-19 22:5 acid is given in Fig. 2E. The in-

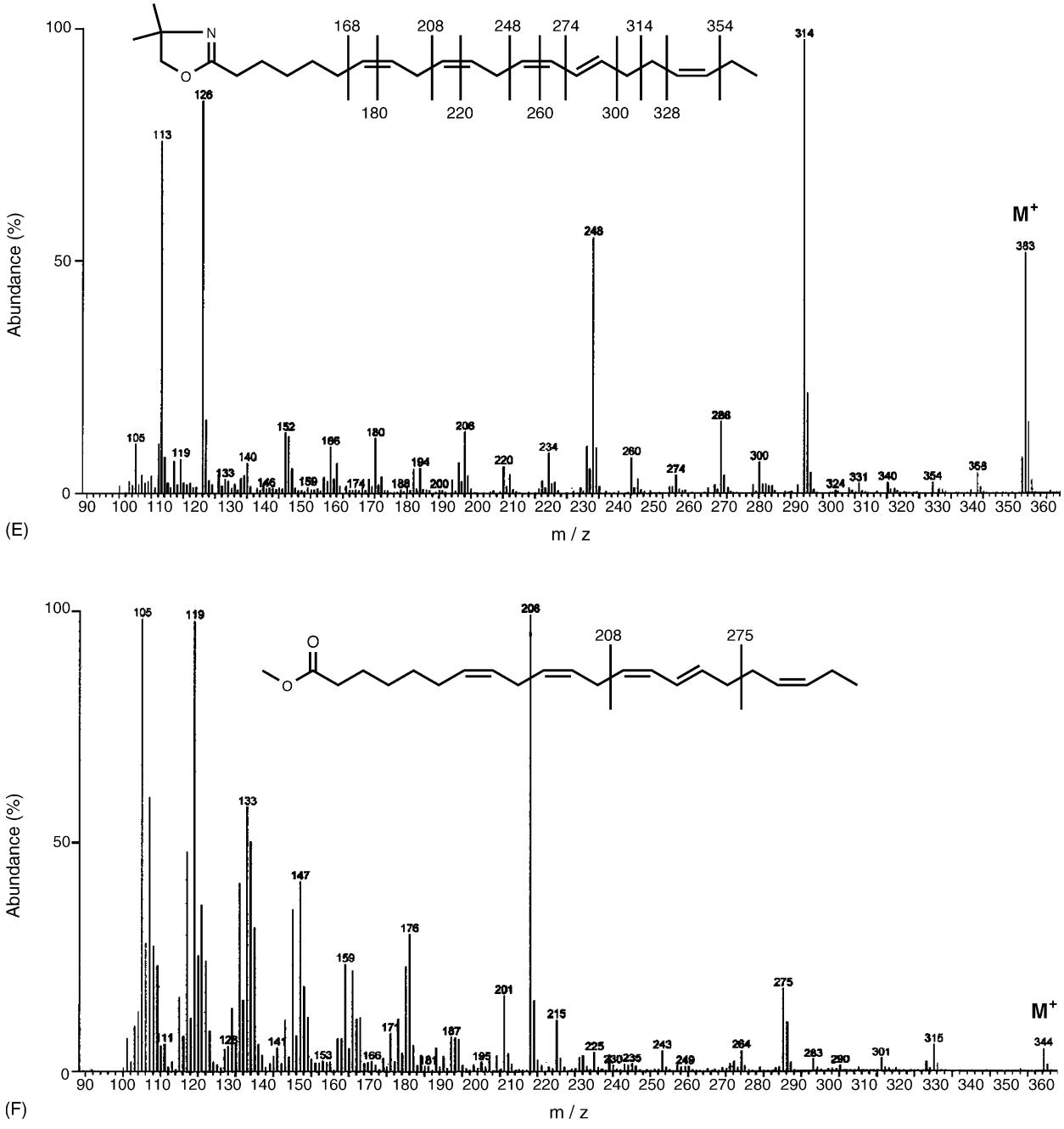

Fig. 2. (*Continued*)

tense ion fragment at $m/z = 383$ and the ion fragment at $m/z = 368$, corresponding to a loss of the terminal methyl group, confirmed the docosapentaenoic acid structure. The intense ion fragment at $m/z = 314$ corresponding to the cleavage at the center of the bis-methylene interrupted double bond allow the location of both Δ19 and Δ15 double bonds. Additional intense allylic fragment at $m/z = 248$ corroborated the location of Δ13,15 conjugated system. Ion fragments at $m/z = 220$, 208 and 180, 168 allow the location of double bonds Δ10 and Δ7, respectively. The key diagnostic ion for the DMOX derivative of 7,10,13,15,19 22:5 are the intense ion fragments at $m/z = 314$ and 248 representing the loss of 69 and 135 amu, respectively. The methyl ester spectrum of this FA (Fig. 2F) exhibits similar ion fragmentation and presents two intense ion fragments at $m/z = 275$ and 208.

The DMOX derivative spectrum of the 4,7,10,13,15,19 22:5 acid which is an intermediate (Fig. 1) in the formation of the partially conjugated docosahexaenoic acid, is il-

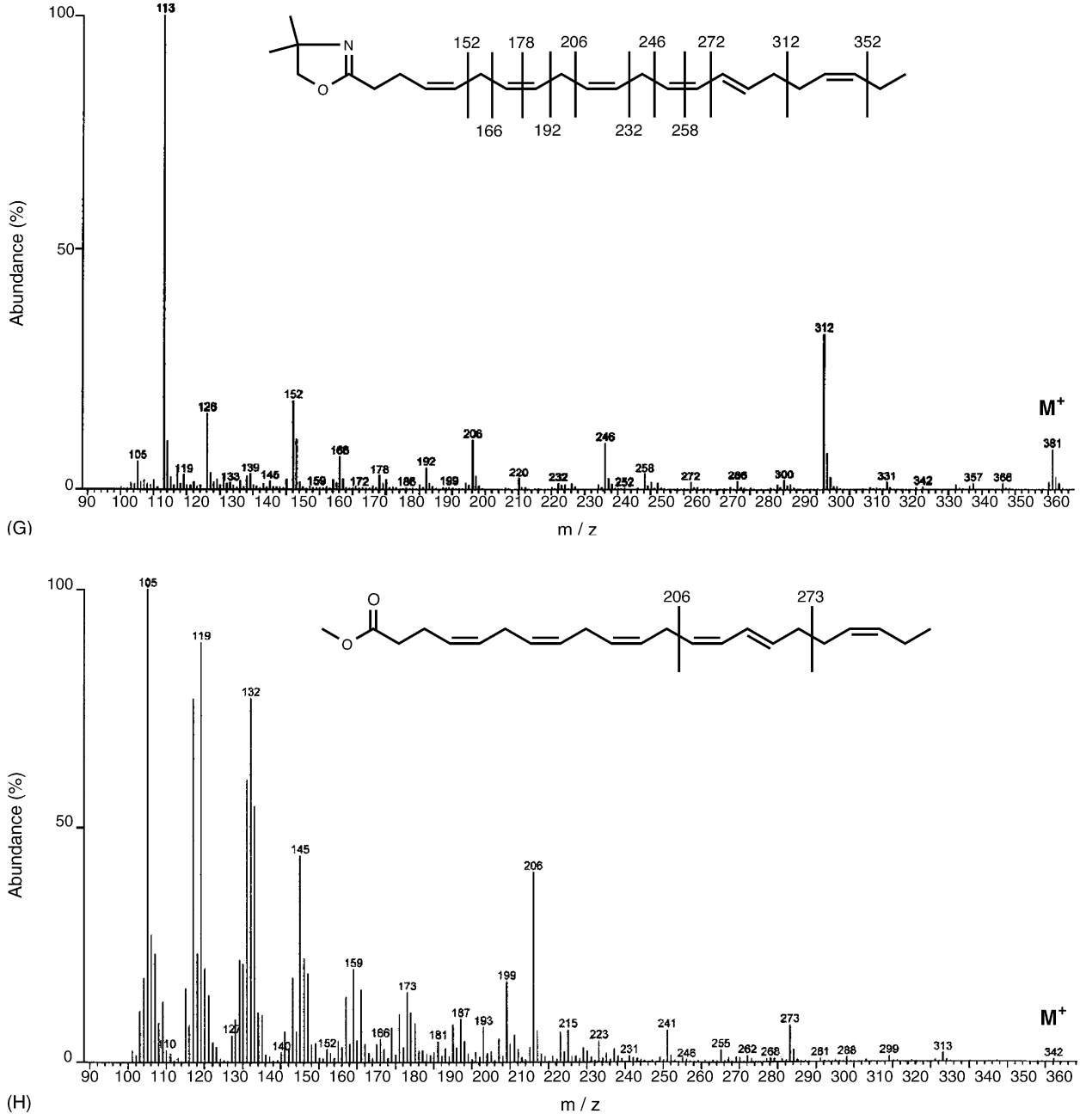

Fig. 2. (*Continued*).

lustrated in Fig. 2G. The molecular ion at $m/z = 381$ and the ion fragment at $m/z = 366$ corresponding to the loss of the terminal methyl group confirmed the docosahexanoic acid structure. The intense ion fragment at $m/z = 312$ confirmed the bis-methylene interrupted structure and corroborates both location of Δ19 and Δ15 ethylenic double bonds. The ion fragment at $m/z = 246$ confirmed the Δ13,15 conjugated system. Methylene interrupted Δ7 and Δ10 double bonds can be assignated by ion fragments at $m/z = 166$, 178 and $m/z = 206$ and 232, respectively. Moreover, ion fragment at $m/z = 220$ is indicative of the loss of vinylic carbon at position 11 (loss of 12 amu from $m/z = 232$). Similarly, the loss of vinylic carbon at position 16 (loss of 12 amu) resulted in ion fragment at $m/z = 286$, from 298. The odd numbered ion fragment at $m/z = 139$ is an unambiguous evidence for the location of a Δ4 double bond [3]. The methyl ester derivative spectrum of this PUFA is given in Fig. 2H, and the observed characteristic ion fragments at $M^+ - 69$ ($m/z = 273$) and $M^+ - 136$ (206), are also present in this spectrum.

The methyl ester derivatives of the metabolites of *cis*-9,*trans*-11,*cis*-15 18:3 (rumelenic) acid exhibit spe-

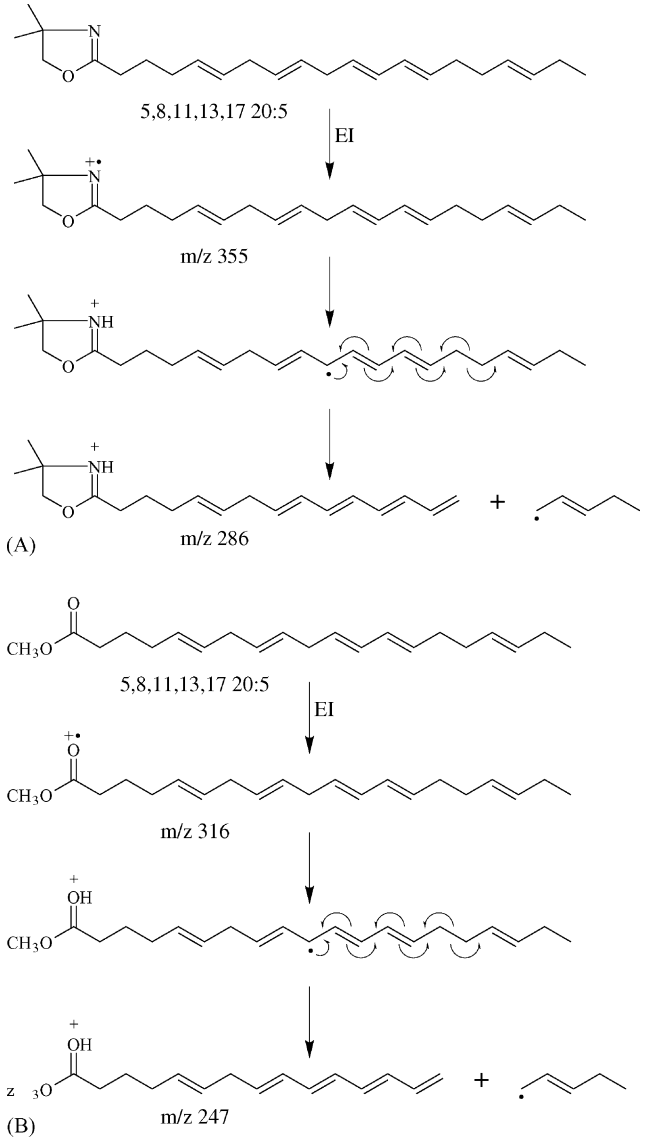

Fig. 3. Proposed chemical mechanism for the formation of ion fragment at $m/z = M^+ - 69$ by charge remote fragmentation in electronic impact ionization mass spectrometry of *cis*-5,*cis*-8,*cis*-11,*trans*-13,*cis*-17 20:5 acid as its DMOX and methyl ester derivatives.

cific mass spectral properties: intense ion fragments at $m/z = M^+ - 69$ corresponding to the cleavage at the center of the bis-methylene interrupted double bond system are observed in all cases. Moreover, mass spectra of the C20:5, C22:5, C22:6 methyl ester derivatives of this partially conjugated PUFA group, exhibit an intense ion fragment at $m/z = M^+ - 136$, specific for the allylic cleavage of the *n*-12 ethylenic double bond. Indeed, these ion fragments, not usually observed for other PUFA methyl ester derivatives, permit their accurate detection in GC–MS (Fig. 4). Our mass spectral observations are correlated with the analysis of their respective DMOX derivatives spectra.

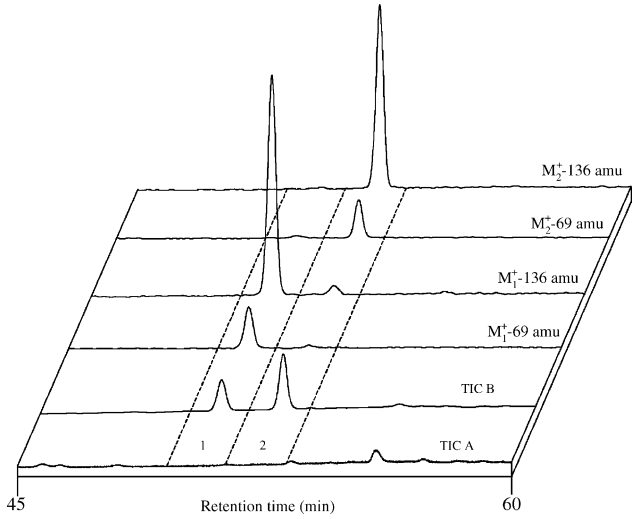

Fig. 4. Reconstructed ion chromatograms for methyl esters derivatives of *cis*-7,*cis*-10,*cis*-13,*trans*-15,*cis*-19 22:5 (1) and *cis*-4,*cis*-7,*cis*-10,*cis*-13,*trans*-15,*cis*-19 22:6 (2) acids isolated from rat liver lipid (total ion current B, TIC B). TIC A corresponds to a liver lipid sample from rat non-fed with rumelenic acid.

4. Conclusion

The DMOX derivatization, widely used for lipid analysis, requires high reaction temperature (150–190 °C) and long reaction time (6–12 h) that may result in partial degradation of PUFA [1]. We have shown that methyl esters derivatives, used for routine fatty acid profile quantification by GC–FID, may be used for structural authentification of metabolites of rumelenic acid by GC–MS.

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