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ORIGINAL ARTICLE

Identification of C18 Intermediates Formed During Stearidonic Acid Biohydrogenation by Rumen Microorganisms In Vitro

S. P. Alves · M. R. G. Maia · R. J. B. Bessa · A. J. M. Fonseca · A. R. J. Cabrita

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Abstract In vitro batch incubations were used to study the rumen biohydrogenation of unsaturated fatty acids. An earlier study using increasing supplementation levels of stearidonic acid (18:4n-3), revealed that the rumen microbial population extensively biohydrogenates 18:4n-3 after 72 h of in vitro incubation, though several intermediates formed were not completely characterized. Therefore, in the present study, samples were reanalyzed in order to identify the 18:2, 18:3 and 18:4 biohydrogenation intermediates of 18:4n-3. Gas–liquid chromatography coupled to mass spectrometry was used to characterize these intermediates. The acetonitrile chemical ionization mass spectrometry of the fatty acid methyl esters derivatives enabled the discrimination of fatty acids as non-conjugated or conjugated biohydrogenation intermediates. In addition, the acetonitrile covalent adduct chemical ionization tandem mass spectrometry yielded prominent ions indicative of the double bond position of the major 18:3 isomers, i.e. Δ5,11,15 18:3. Furthermore, the 4,4-dimethyloxazoline derivatives prepared from the fatty acid methyl esters enabled the structure of novel 18:2, 18:3 and 18:4 biohydrogenation intermediates to be elucidated. The intermediates accumulated in the fermentation media after 72 h of incubation of 18:4n-3 suggest that similar to the biohydrogenation pathways of linoleic (18:2n-6) and α-linolenic (18:3n-3) acids, the pathway of the 18:4n-3 also proceeds with the formation of conjugated fatty acids followed by hydrogenation, although no conjugated dienes were found. The formation of the novel biohydrogenation intermediates of 18:4n-3 seems to follow an uncommon isomerization pattern with distinct double bond migrations.

Keywords Biohydrogenation intermediates · Stearidonic acid · Gas–liquid chromatography · Mass spectrometry · 4,4-Dimethyloxazoline · Covalent adduct chemical ionization

Abbreviations

CACI	Covalent adduct chemical ionization
CACIMS/MS	Covalent adduct chemical ionization tandem mass spectrometry
CI	Chemical ionization
DHA	Docosahexaenoic acid
DM	Dry matter
DMOX	4,4-Dimethyloxazoline
EI	Electron impact
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl ester(s)
GLC	Gas–liquid chromatography
MS	Mass spectrometry
PFI	Polyenoic fatty acid isomerase

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PUFA	Polyunsaturated fatty acid methyl ester(s)
SDA	Stearidonic acid
Ag^{+}-SPE	Silver ion solid-phase extraction

Introduction

Stearidonic acid (SDA; 18:4n-3) is a polyunsaturated fatty acid (PUFA) that constitutes the first metabolite of α-linolenic acid (18:3n-3) in the metabolic pathway leading to the endogenous production of eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) [1]. An increased interest has emerged in the last few years on these long chain n-3 PUFA due to their benefits to human health [2, 3]. Recently it was suggested that 18:4n-3 itself may be a surrogate for 20:5n-3 for health promotion and disease prevention [4]. In dairy products, the potential of 18:4n-3 enriched soybean oil to increase the n-3 fatty acid content was demonstrated by Bernal-Santos et al. in rumen fistulated cows [5].

In ruminants, PUFA are biohydrogenated in the rumen, often resulting in the formation of *cis* and *trans* isomers, including conjugated isomers, *trans* monoenes or others intermediates. The biohydrogenation of PUFA by the rumen microbial population has been widely studied in vitro, mostly by using linoleic acid (18:2n-6), 18:3n-3, 20:5n-3, and 22:6n-3 as fatty acids or oil sources [6–11]. Recently, in vitro batch incubations were conducted to evaluate the biohydrogenation of 18:4n-3 by the rumen microbial population with increasing supplementation levels [12]. These in vitro incubations showed an extensive biohydrogenation of 18:4n-3. However, at high 18:4n-3 supplementation levels several 18:2, 18:3 and 18:4 biohydrogenation intermediates accumulated after 72 h of incubation. As far as we know, there are no reports on the characterization of the intermediates formed during the biohydrogenation of 18:4n-3. A more complete characterization of these intermediates is important to understand the overall biohydrogenation process and underlying mechanisms for their formation. The importance of disclosure all the detailed information on isomeric profile is linked to the current knowledge that slight structural differences can profoundly modify the biological action of these fatty acids [13].

Gas–liquid chromatography coupled to mass spectrometry (GLC–MS) has been widely used for the characterization of fatty acids, despite the location of the double bonds being particularly difficult. For this, several methods have been used, including covalent adduct chemical ionization tandem mass spectrometry (CACIMS/MS) of fatty acid methyl esters (FAME) derivatives [14, 15], and the mass spectrometry analysis of others derivatives, such as 4,4-dimethyloxazoline (DMOX), picolinyl, and pyrrolidides [16–20]. The objective of this study was to determine the structure and composition of the novel biohydrogenation intermediates formed during in vitro incubations with 18:4n-3, and the effect of increasing supplementation levels on the pattern of biohydrogenation intermediates formed.

Materials and Methods

Chemicals

All reagents and solvents were analytical and chromatographic grade, and were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ag^{+}-ion SPE cartridges (750 mg/6 mL) and a mixture of FAME standards were purchased from Supelco Inc. (Bellefonte, PA, USA). A bacterial acid methyl esters mix was purchased from Matreya LLC (Pleasant Gap, PA, USA).

In Vitro Incubations

Samples from batch incubations were obtained from an experiment described elsewhere [12]. Briefly, Hungate tubes containing 4.5 mL buffered ruminal fluid, obtained from 3 adult cows, were supplemented with increasing levels of 18:4n-3 (pure free fatty acid from Sigma-Aldrich, St. Louis, MO, USA) and incubated at 39 °C for 72 h. Stearidonic acid was supplemented at 0 (control), 0.25, 0.50, 0.75, 1.00, 1.25, and 1.50 mg per 33 mg total mixed ration (TMR, dry matter (DM) basis). Each treatment was replicated in triplicate within the incubation. Reactions were stopped after 72 h by cooling in an ice-slurry, and tubes stored at −20 °C until analysis.

Preparation of Fatty Acid Methyl Esters

Lipids from in vitro incubations were extracted from freeze-dried whole fermentation media using a mixture of dichloromethane and methanol (2:1, by vol.) and FAME were prepared by a combined transesterification method, as described by Maia et al. [12]. In order to detect FAME coelutions during GLC analysis, selected samples were fractioned into saturated, *trans*-18:1, *cis*-18:1, 18:2, 18:3 and 18:4 isomers by Ag^{+}-SPE using hexane with increasing amounts of acetone, according to Kramer et al. [21].

Preparation of 4,4-Dimethyloxazoline Derivatives

4,4-Dimethyloxazoline derivatives were prepared from FAME by adding 0.5 μL of 2-amino-2-methyl-1-propanol,

and left at 170 °C for 16 h under nitrogen. Once cooled, DMOX derivatives were extracted twice with a mixture of diethyl ether and hexane (1:1, by vol.) and rinsed with water. After drying over anhydrous sodium sulphate the solvent was evaporated under nitrogen at 34 °C and the residue dissolved in 1 mL of hexane (GC-grade).

Instrumentation

Quantification of FAME was determined using a gas chromatograph HP6890A (Hewlett-Packard, Avondale, PA, USA), equipped with a flame-ionization detector (GLC-FID) and a CP-Sil 88 capillary column (100 m; 0.25 mm i.d.; 0.20 μm film thickness; Chrompack, Varian Inc., Walnut Creek, CA, USA). The injector and detector temperatures were 250 and 280 °C, respectively. Initial oven temperature of 100 °C was held for 1 min, increased at 50 °C/min to 150 °C and held for 20 min, increased at 1 °C/min to 190 °C and held for 5 min, and then increased at 1 °C/min to 200 °C and held for 35 min. Helium was used as carrier gas at a flow rate of 1 mL/min, the split ratio was 1:10 and 1 μL of sample was injected.

Identification of C18 biohydrogenation intermediates of FAME and DMOX derivatives was achieved by GLC–MS analysis using an ion trap Varian Saturn 2200 system (Varian Inc., Walnut Creek, CA, USA) and the same capillary column and oven temperatures used in GLC-FID analysis. The ion trap parameters used in the present analyses are similar to those described in Alves and Bessa [22]. Identification of fatty acids was achieved by electron impact (EI) and chemical ionization (CI) mass spectrometry, using acetonitrile as the reagent of CI. Additionally, the acetonitrile CACIMS/MS technique was used for location of the double bond position in some biohydrogenation intermediates.

Statistical Analysis

The effect of increasing 18:4n-3 supplementation levels on the concentration of the novel biohydrogenation intermediates after 72 h of incubation was analyzed by a single effect model using the GLM procedure of SAS (SAS Inst. Inc., 2002, Cary, NC). When a significant effect was observed the means were compared using the least square difference method.

Results

Identification of C18 FAME Derivatives by EI and CI Mass Spectrometry

Two partial GLC chromatograms of the C18 FAME region from ruminal in vitro incubations supplemented with 0 and 1.5 mg of 18:4n-3 per 33 mg of TMR DM are presented in Figs. 1 and 2. Both figures show that several 18:2, 18:3 and 18:4 fatty acids were produced during the incubation of 1.5 mg of 18:4n-3. The mass spectrometry analysis under EI or CI, of the C18 FAME intermediates allowed the recognition of the carbon chain length and number of double bonds in incubation samples. Electron impact mass spectra of homoallylic (methylene-interrupted) 18:3n-3,

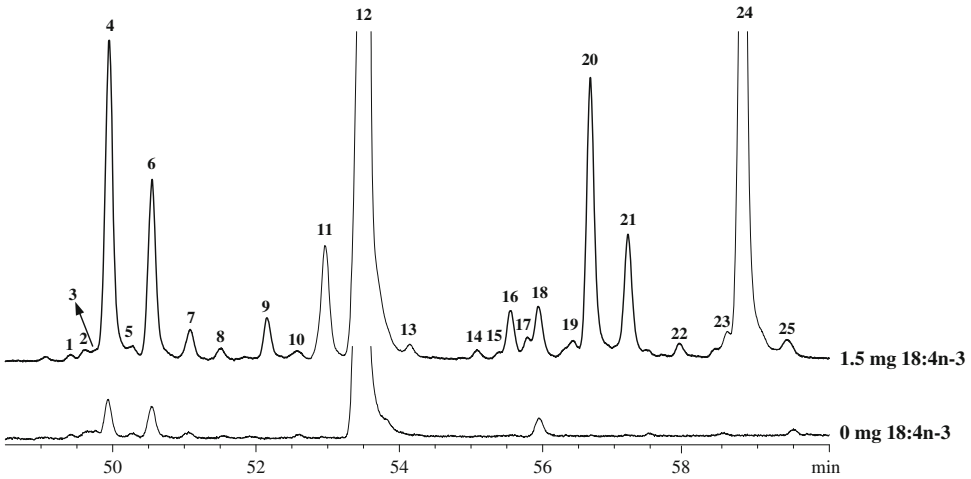


Fig. 1 Partial GLC-FID chromatograms using a CP-Sil 88 capillary column, showing the 18:1, 18:2 and 18:3 FAME region from ruminal in vitro incubation samples supplemented with 0 (control) and 1.5 mg of 18:4n-3 per 33 mg total mixed ration (DM basis). Peak identification: *1* unresolved *trans*-6 to *trans*-8 18:1; *2* *trans*-9 18:1; *3* *trans*-10 18:1; *4* *trans*-11 18:1; *5* *trans*-12 18:1; *6* *cis*-9 + *trans*-13 + *trans*-14 18:1; *7* *cis*-11 + *trans*-15 18:1; *8* *cis*-12 18:1; *9* *trans*-5,*trans*-10 18:2; *10* unresolved *trans*-16 and *cis*-14 18:1; *11* unresolved *cis*-15 18:1 and *trans*-5,*trans*-11 18:2; *12* internal standard-19:0; *13* 18:2 unidentified; *14* *cis*-16 18:1; *15* *trans*-9,*cis*-12 18:2; *16* *trans*-11,*cis*-15 18:2; *17* unidentified peak; *18* 18:2n-6; *19* unidentified peak; *20* Δ5,11,15 18:3; *21* Δ5,11,14 18:3; *22* Δ5,10,15 18:3; *23* unidentified peak; *24* Δ5,11,15 18:3; *25* 20:0

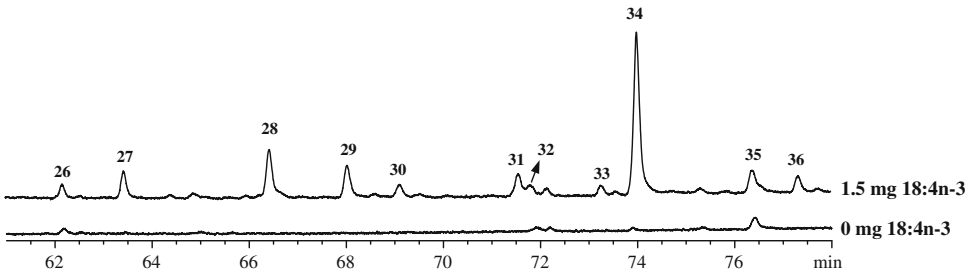
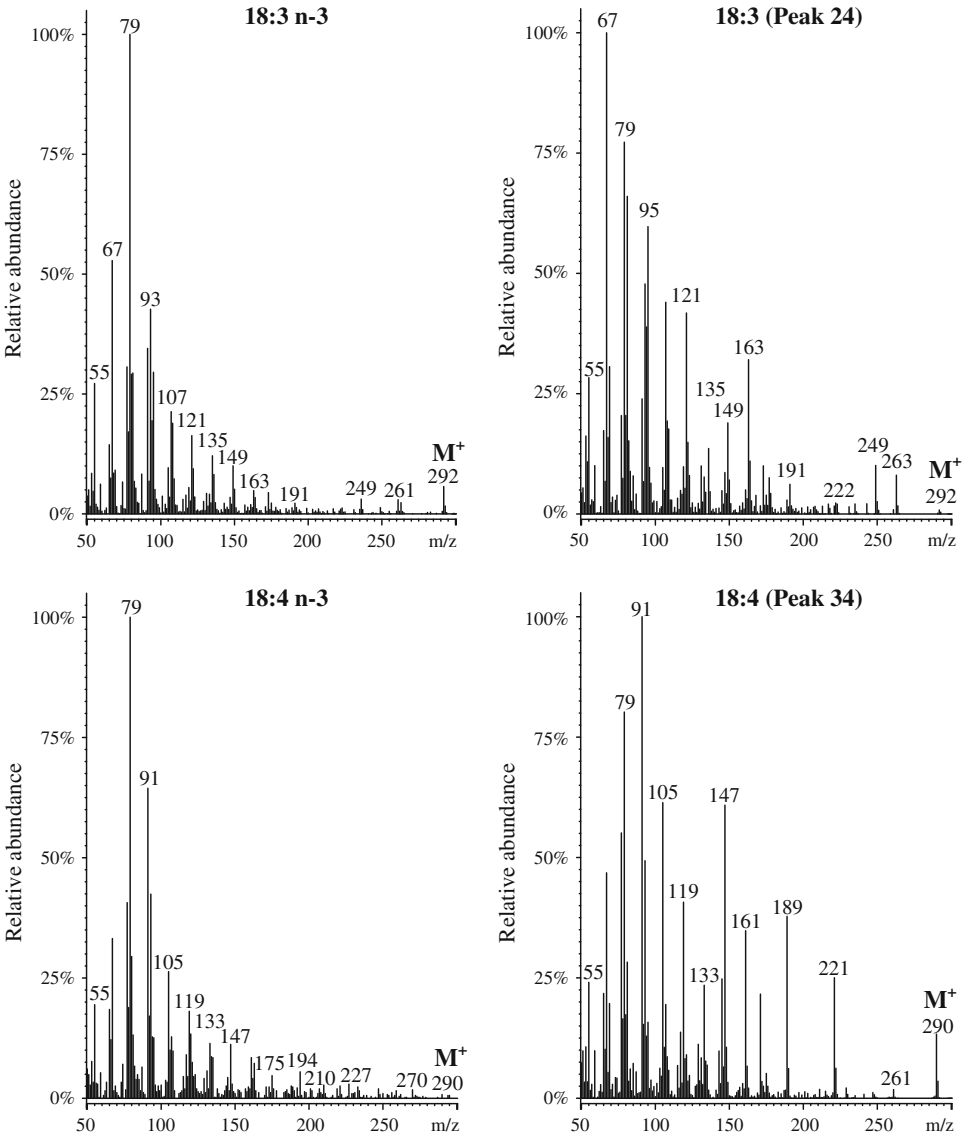


Fig. 2 Partial GLC-FID chromatograms using a CP-Sil 88 capillary column, showing the 18:4 FAME region from ruminal in vitro incubation samples supplemented with 0 (control) and 1.5 mg of 18:4n-3 per 33 mg total mixed ration (DM basis). Peak identification: *26* 18:3n-3; *27* CLA *cis*-9,*trans*-11; *28* 18:4n-3; *29* 18:3 unidentified; *30* 18:3 unidentified; *31* Δ5,7,11,15 18:4; *32* 22:0; *33* Δ5,8,10,15 18:4; *34* Δ5,8,10,15 18:4; *35* unidentified peak; *36* Δ5,8,10,15 18:4

Fig. 3 EI mass spectra of 18:3 and 18:4 isomers. Peak numbers correspond to peaks in Figs. 1 and 2



18:4n-3 and of two novel biohydrogenation intermediates formed are presented in Fig. 3. There are small differences between 18:3 mass spectra, however no diagnostic ions were produced to clarify the position of the double bonds. Similarly, there are no diagnostic ions in the EI mass spectrum of the novel 18:4 isomer (peak 34), although its mass spectrum is quite different from the mass spectrum of 18:4n-3. The ion at *m*/*z* 91 is presumably formed from the

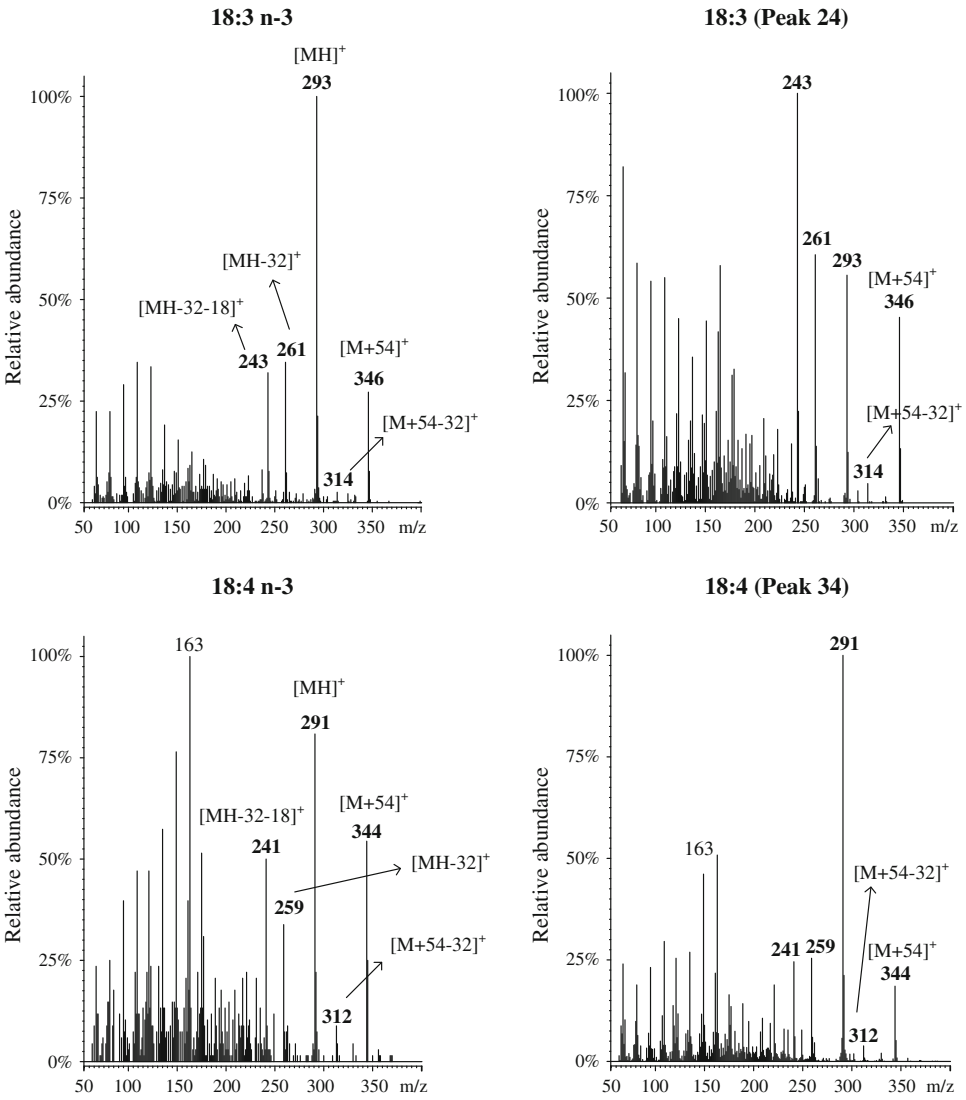


Fig. 4 CACIMS spectrum of 18:3 and 18:4 isomers showing the base peak $[M + 54]^+$ and the common ions $[MH]^+$, $[MH - 32]^+$ and $[M + 54 - 32]^+$. Peak numbers correspond to peaks in Figs. 1 and 2

tropylium ion. The homologous series *m/z* 105, 119, 133, 147 are also probably tropylium ions with alkyl substituent [23].

The acetonitrile CI mass spectra also allowed the identification of the number of double bonds in C18 fatty acid intermediates. The product of acetonitrile self-reaction, generated under CACIMS conditions, reacts with the fatty acid double bond to yield molecular ions with 54 atomic mass units above the parent analyte. The $[M + 54]^+$ ions at *m/z* 346 and 344 for the 18:3 and 18:4 isomers, respectively, are shown in Fig. 4. The CACIMS also produce other ions, corresponding to the protonated molecule ($[MH]^+$), and losses of methanol from the protonated molecule ($[MH-32]^+$) and from adduct ($[M + 54 - 32]^+$), at *m/z* 293, 261 and 314 for the 18:3 isomers and at *m/z* 291, 259 and 312 for the 18:4 isomers. The intensity ratios of $[M + 54]^+/[M + 54 - 32]^+$ have previously been shown to be related to double bond geometry [14, 24, 25]. Table 1 shows the CACIMS $[M + 54]^+/[M + 54 - 32]^+$ ratios for the 18:2, 18:3 and 18:4 isomers. The non-conjugated 18:2 isomers showed ratios around 4.33 whereas the conjugated linoleic acid isomer (CLA) *cis*-9,*trans*-11 18:2 showed a ratio of 0.35. Similarly, the 18:3n-3 and others four isomers eluting before the 18:3n-3 showed ratios between 22.1 and 61.3, and other two 18:3 isomers eluting after 18:3n-3 showed ratios lower than 3. The 18:4n-3 showed a ratio of 61, whereas its isomers showed ratios between 3.25 and 4.79.

Identification of C18 DMOX Derivatives by EI Mass Spectrometry

The DMOX derivatives were prepared from FAME incubation samples in order to characterize the novel C18 biohydrogenation intermediates formed. Table 2 presents the characteristic ion fragments of DMOX derivatives of C18 biohydrogenation intermediates. The base peak in all

Table 1 CACIMS $[M + 54]^+/[M + 54 - 32]^+$ ratios for non-conjugated, partially conjugated and conjugated 18:2, 18:3 and 18:4 isomers identified in samples from ruminal in vitro incubation of 1.5 mg of 18:4n-3 per 33 mg total mixed ration (DM basis)

Peak[a]	FAME	$[M + 54]^+/[M + 54 - 32]^+$
	18:2 isomers	
	Non-conjugated	
9	*trans*-5,*trans*-10	4.76
11	*trans*-5,*trans*-11	5.47
16	*trans*-11,*cis*-15	3.85
18	18:2n-6	3.86
	Conjugated	
27	*cis*-9,*trans*-11	0.35
	18:3 isomers	
	Non-conjugated	
20	Δ5,11,15	55.2
21	Δ5,11,14	61.3
22	Δ5,10,15	41.0
24	Δ5,11,15	22.1
26	18:3n-3	30.3
	Partially conjugated	
29	18:3*	2.54
30	18:3*	1.82
	18:4 isomers	
	Non-conjugated	
28	18:4n-3	61.0
	Partially conjugated	
31	Δ5,7,11,15	3.33
33	Δ5,8,10,15	3.67
34	Δ5,8,10,15	4.79
36	Δ5,8,10,15	3.25

[a] Peak numbers correspond to peaks in Figs. 1 and 2

* Unidentified 18:3 isomers

fatty acids was observed at *m/z* 126 formed by cyclization-displacement reaction. The abundant ion at *m/z* 113 is also a characteristic ion in DMOX derivatives and is produced by a McLafferty rearrangement.

Two of the five 18:2 biohydrogenation intermediates identified in incubation samples at high supplementation levels are novel intermediates and their EI mass spectra of DMOX derivatives (Fig. 5) showed the molecular ion at *m/z* 333. The even mass homologous series at *m/z* 126 + 14 amu is interrupted in the region of the double bond, with a gap of 12 amu. Although the mass spectra of both 18:2 peaks, showed a gap of 13 amu at *m/z* 140/153 and a gap of 12 amu at *m/z* 208/220 and 222/234, for peaks 9 and 11, respectively. The presence of odd-numbered ion at *m/z* 153 accompanied by the *m/z* 152, are diagnostic ions in the mass spectra of DMOX derivatives of fatty acids with their first double bond at carbon-5 [26, 27]. The published rules for unsaturated fatty acids reveal the presence of intense peaks containing n − 2 and n + 2 carbon atoms, therefore the presence of the intense ion at *m/z* 208 in spectrum of peak 9 presumed a double bond at carbon-11. However, the 12 amu gap was observed between *m/z* 208 and 220, locating the double bond at carbon-10. The order of elution of either peaks 9 and 11 in GLC-FID presumed a *trans,-trans* configuration system.

Four novel 18:3 non-conjugated fatty acids were putatively identified by their DMOX mass spectra, showing all the molecular ion at *m/z* 331 (Fig. 6). Peaks 20 and 24 showed identical mass spectra, both having a prominent ion at *m/z* 262 (Fig. 6), which is characteristic of a *bis*-methylene interrupted double bond system, resulting in the formation of stabilized allylic radical fragments, and confirming the location of double bonds at carbons-11, 15 in either peaks 20 and 24. The third double bond was putatively identified at carbon-5 because a gap of 12 amu was observed between *m/z* 140 and 152. However, this does not match with the published rules for the presence of a double bond at carbon-5 and what was observed for the Δ5,10 and Δ5,11 dienes. The published rules for the DMOX derivatives with double bonds at C-4, C-5 and C-6, presume that the ions (i.e., *m/z* 138, 152 and 166, respectively) derived from cleavage at the double bond are accompanied by intense odd-numbered ions (i.e., *m/z* 139, 153 and 167, respectively). Nevertheless, as none of these intense odd ions were observed, peaks 20 and 24 were putatively characterized as Δ5,11,15 18:3 having different *cis/trans* configurations. Seeing that peak 20 elutes before peak 24 in the CP-Sil88 capillary column, and taking in account the order of elution of geometric 18:3 isomers [28, 29], we assume that peak 20 might contain three *trans* double bonds and peak 24 might contain at least one *trans* double bond.

The mass spectrum of the DMOX derivatives of peaks 21 and 22 (Fig. 6) showed a gap of 12 amu between ions at *m/z* 140 and 152, similarly to what was observed for the Δ5,11,15 18:3 isomers, suggesting that both peaks might have a double bond at carbon-5. Moreover, peak 21 contained ion fragments with mass interval of 12 amu at *m/z* 222/234 and 262/274 that were separated by gaps of 14 amu, which enabled the characterization of peak 21 as Δ5,11,14 18:3. Additionally, the mass spectrum of peak 22 showed ion fragments with gaps of 12 amu at *m/z* 220/234 and 276/288, allowing the Δ5,10,15 18:3 structure to be elucidated. On the other hand, the geometry of the double bonds of both Δ5,11,14 and Δ5,10,15 18:3 isomers could not be determined.

Four partially conjugated 18:4 isomers were identified in incubation samples at high supplementation levels of 18:4n-3. The mass spectra of DMOX derivatives of 18:4n-3 and peaks 28, 31 and 34 are presented in Fig. 6. The mass

Table 2 Characteristic ion fragments recorded during GLC–MS analysis of DMOX derivatives of novel C18 biohydrogenation intermediates in samples from ruminal in vitro incubation of 1.5 mg of 18:4n-3 per 33 mg total mixed ration (DM basis)

Peak[a]	Fatty acid	Characteristic ion fragments (m/z, relative intensity)
9	*trans*-5,*trans*-10 18:2	113(90), 126(100), **140**(7), **152**(14), 153(16), 166(52), 180(13), 194(20), **208**(28), **220**(2), 234(7), 248(7), 262(19), 276(5), 290(8), 304(3), 318(5), 333(M^+, 7)
11	*trans*-5,*trans*-11 18:2	113(96), 126(100), **140**(6), **152**(15), 153(17), 166(58), 180(13), 194(17), 208(29), **222**(3), **234**(5), 248(10), 262(15), 276(5), 290(6), 304(2), 318(2), 333(M^+, 7)
20	Δ5,11,15 18:3	113(35); 126(100), **140**(8), **152**(10), 166(40), 180(9), 194(21), 208(28), **222**(4), **234**(4), 248(4), 262(**77**), **276**(3), **288**(5), 302(4), 316(5), 331(M^+, 4)
21	Δ5,11,14 18:3	113(41), 126(100), **140**(7), **152**(10), 166(37), 180(8), 194(16), 208(21), **222**(2), **234**(5), 248(11), **262**(16), **274**(1), 288(4), 302(5), 316(2), 331(M^+, 1)
22	Δ5,10,15 18:3	112(18), 126(100), **140**(8), **152**(10), 166(38), 180(13), 194(21), **208**(21), **220**(4), 234(6), 248(16), 262(16), **276**(3), **288**(5), 302(8), 316(2), 331(M^+, 1)
24	Δ5,11,15 18:3	113(53), 126(100), **140**(8), **152**(10), 166(41), 180(8), 194(23), 208(28), **222**(2), **234**(5), 248(4), 262(**81**), **276**(3), **288**(4), 302(4), 316(5), 331(M^+, 4)
31	Δ5,7,11,15 18:3	113(28), 126(100), **140**(3), **152**(5), **166**(44), **178**(4), 192(11), 206(28), **220**(3), **232**(5), 246(3), 260(**94**), **274**(2), **286**(2), 300(1), 314(5), 329(M^+, 22)
33	Δ5,8,10,15 18:4	113(48), 126(100), **140**(5), **152**(12), 166(22), 167(19), **180**(16), **192**(14), **206**(4), **218**(22), 232(12), 246(41), 260(9), **274**(1), **286**(2), 300(8), 314(7), 329(M^+, 16)
34	Δ5,8,10,15 18:4	113(48), 126(100), **140**(7), **152**(10), 166(22), 167(15), **180**(13), **192**(16), **206**(7), **218**(18), 232(13), 246(38), 260(21), **274**(1), **286**(4), 300(8), 314(6), 329(M^+, 17)
36	Δ5,8,10,15 18:4	113(45), 126(100), **140**(6), **152**(10), 153(4), 166(19), 167(13), **180**(13), **192**(8), **206**(5), **218**(17), 232(11), 246(38), 260(19), **274**(2), **286**(2), 300(7), 314(5), 329(M^+, 14)

Bold numbers indicate ions fragments used for the location of the double bonds

[a] Peak numbers correspond to peaks in Figs. 1 and 2

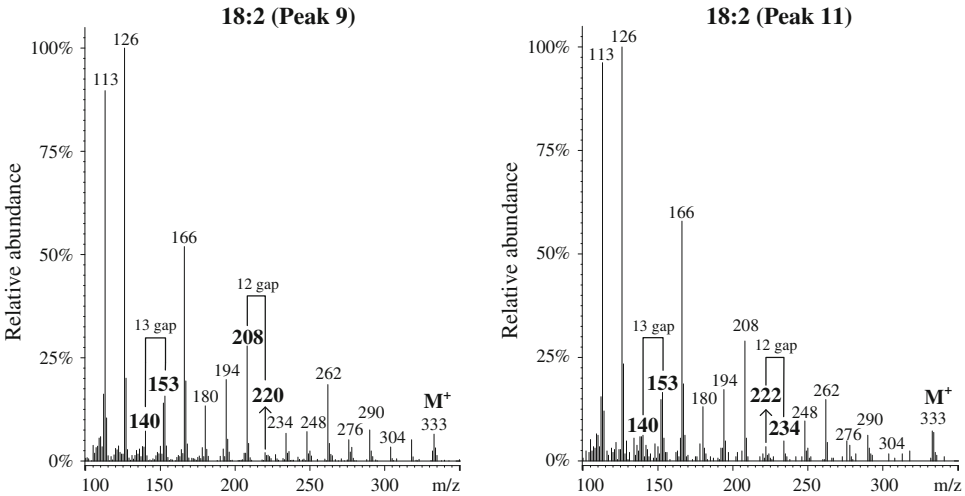


Fig. 5 GLC–MS mass spectra of DMOX derivatives of 18:2 isomers

spectra of 18:4n-3 (peak 28) clearly shows the 12 amu gaps at *m/z* 194/206, 234/246 and 274/286 enabling the three end double bonds to be located at carbons-9, 12, 15. The first double bond was identified at carbon-6 by the presence of an intense odd ion at *m/z* 167 and the even ion at *m/z* 166, confirming the presence of the Δ6,9,12,15 18:4 (18:4n-3).

In the mass spectrum of peak 31, an intense peak at *m/z* 260 characteristic of an ethylene interrupted double bond system together with gaps of 12 amu at *m/z* 220/232 and 274/286 enabled the location of two double bonds at carbons-11, 15. The conjugated double bond was putatively identified at carbons-5, 7 by the gap of 12 amu at *m/z* 140/152 and 166/178. Thus, peak 31 was tentatively identified as Δ5,7,11,15 18:4.

Three 18:4 peaks (33, 34 and 36) showing identical DMOX mass spectra were identified in the incubation samples, their mass spectra containing ion fragments with a mass interval of 12 amu at *m/z* 180/192, 206/218 and 274/286, which enabled the conjugated double bond to be located at carbons-8, 10 and a terminal double bond at carbon-15. The first double bond was putatively identified

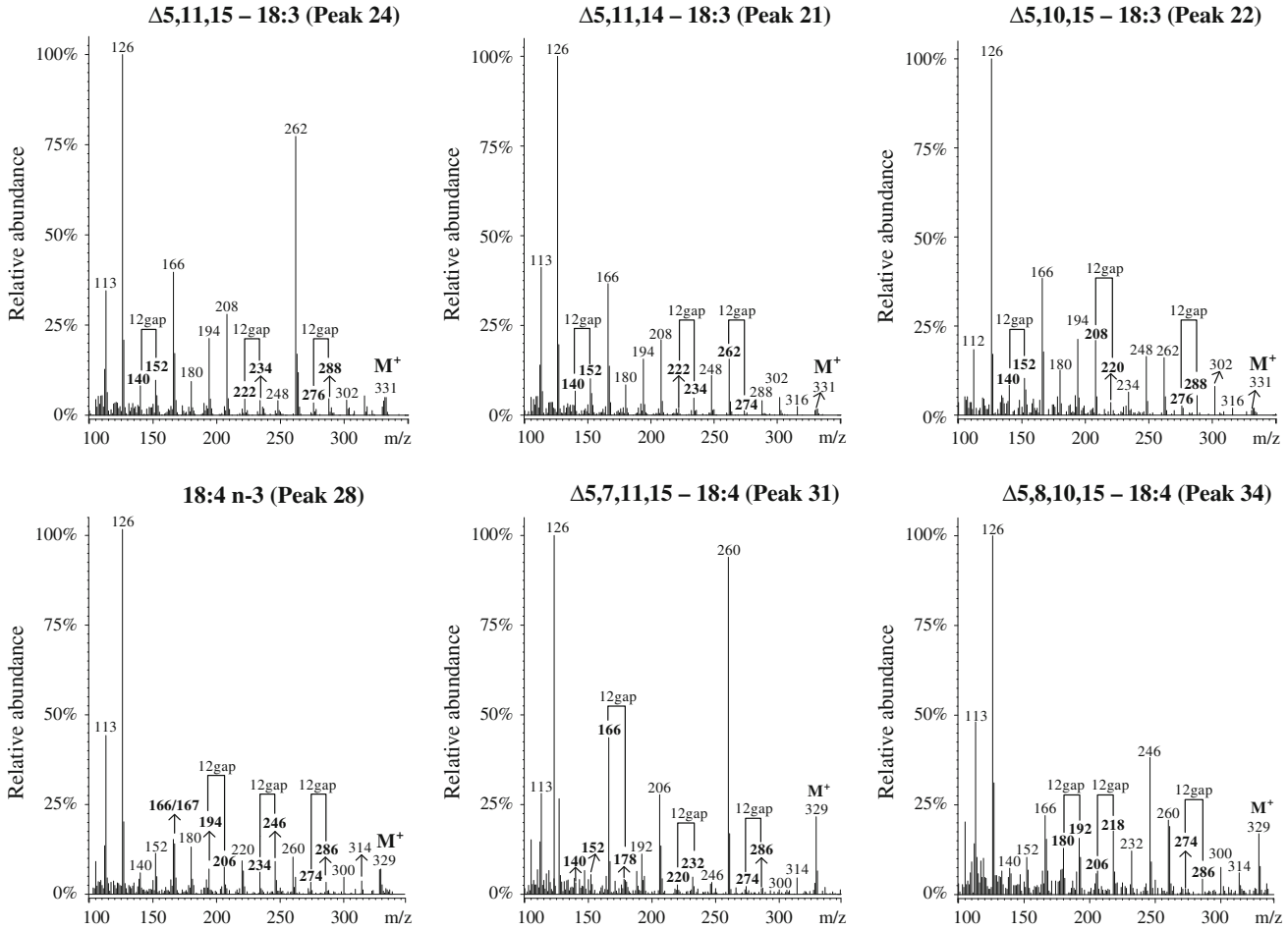


Fig. 6 GLC–MS mass spectra of DMOX derivatives of 18:3 and 18:4 isomers

at carbon-5 (Fig. 6) by the gap of 12 amu between ion fragments at *m/z* 140 and 152. Once more, no intense odd ions were observed accompanying the even ion at *m/z* 152 and 166.

Identification C18 FAME Derivatives by CACIMS/MS Mass Spectrometry

The CACIMS/MS technique was applied for the identification of the major 18:3 biohydrogenation intermediates. Peaks 20 and 24 showed identical CACIMS/MS mass spectra, peak 24 being obtained by collisionally activated dissociation of the $[M + 54]^+$ ion at *m/z* 346 (Fig. 7). The ion at *m/z* 318 corresponds to a cleavage between C16 and C17, indicating a double bond at carbon-15. The series of ions containing the carboxyl group at *m/z* 264, 276 and 290, and the end of the molecule at *m/z* 162, 176 and 190, indicates a double bond at carbon-11. Finally, the fragment at *m/z* 246 corresponds to a cleavage vinylic to the double bond between C4 and C5. These fragmentations allowed to establish the structures as Δ5,11,15 18:3, however the geometry could not be determined. The identification of the others minor 18:3 and 18:4 intermediates by CACIMS/MS was not successfully achieved, due to the low amount of these intermediates in samples or because mass spectra did not show prominent ions useful to establish the double bond.

Effect of 18:4n-3 Supplementation on C18 Biohydrogenation Intermediates

Table 3 shows the effect of increasing 18:4n-3 supplementation levels on 18:2, 18:3 and 18:4 biohydrogenation intermediates accumulated after 72 h of ruminal incubations. Supplementations at 0 and 0.25 mg of 18:4n-3 per 33 mg of TMR DM were not included because almost no novel biohydrogenation intermediates were detected at these levels. Increasing the 18:4n-3 supplementation level, increased the number of novel biohydrogenation intermediates formed. Indeed, the *trans*-5,*trans*-10 18:2, Δ5,11,15 18:3, Δ5,11,14 18:3, Δ5,12,15 18:3 and Δ5,7,11,15 18:4 accumulate from supplementation level of 1.0 mg onwards.

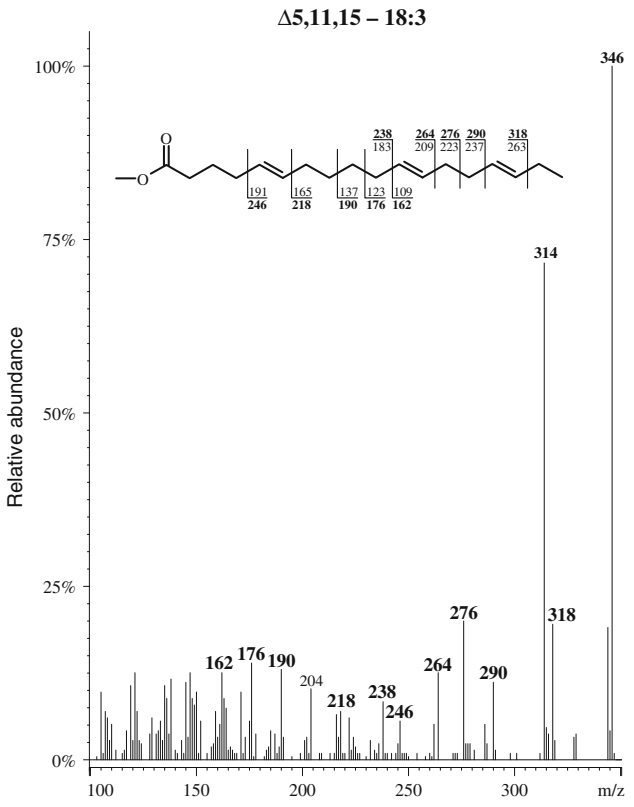


Fig. 7 CACIMS/MS spectra obtained upon collisionally deactivated dissociation of $[M + 54]^+$ ion for Δ5,11,15 18:3 isomer

The 18:3 and 18:4 biohydrogenation intermediates that occurred at greater percentages were the Δ5,11,15 18:3 (peak 24) and the Δ5,8,10,15 18:4 (peak 34), reaching 64.4 and 66.9% of the total 18:3 and 18:4 fatty acids identified in 1.50 mg supplementation level, respectively.

Discussion

Recently, we reported that the supplementation of 18:4n-3 by the rumen microbial population in vitro promoted an accumulation of several unknown octadecapolyenoic biohydrogenation intermediates, though not completely characterized [12]. The present work reports the characterization of those C18 biohydrogenation intermediates.

The octadecapolyenoic intermediates comprised five 18:2, four 18:3, and four 18:4 fatty acids, which were almost undetected at supplementation level of 0.25 mg of 18:4n-3 per 33 mg of TMR DM. The electron impact and chemical ionization GLC–MS analysis of FAME were used for the identification of carbon chain length and number of double bonds. According to the literature, the location and geometry of double bonds in EI mass spectra of FAME cannot be accurately determined because positional and geometrical isomers show almost identical mass spectra. Indeed, the double bond becomes mobile and moves up and down the chain under EI ionization, hence its original position cannot be reliably determined. This is mainly due to the high sensitivity of the carboxyl group to fragmentation and to double bond migration [30, 31]. Isomerization of double bonds during DMOX preparation was reported by some authors [32], therefore the identification of double bond position using DMOX derivatives should be used carefully. Thus, complementary methods that do not use chemical treatment of the sample such as CACIMS are preferred.

The acetonitrile CI mass spectra of FAME derivatives, apart from the recognition of carbon chain length and number of double bonds, allows a rapid recognition of the non-conjugated, partially conjugated and fully conjugated structure. The CACIMS $[M + 54]^+/[M + 54 - 32]^+$ ratios of non-conjugated fatty acids are substantially higher than the partially conjugated and fully conjugated ratios [14, 24, 25]. Lawrence and Brenna [24] reported that partially conjugated 18:3 isomers showed ratios around 2 whereas fully conjugated 18:2 and 18:3 isomers showed ratios lower than 1. Based on these rules, we could discriminate the novel 18:3 and 18:4 fatty acids as non-conjugated or partially conjugated structures. The GLC retention time in the cyanopropylpolysiloxane stationary phase can also be used to predict the conjugated structure seeing that FAME with conjugate double bonds have higher retention times compared with homologous non-conjugated FAME [33]. Indeed, the partially conjugated 18:4 isomers showed higher retention times than the homoallylic 18:4n-3. Conversely, the novel non-conjugated 18:3 isomers identified in incubated samples showed much lower retention times compared with the 18:3n-3.

The single stage MS analysis of FAME does not give information about the location of the double bond position. Although the MS/MS using acetonitrile, i.e., CACIMS/MS, has been successfully used for the identification of double bond position in a variety of fatty acids including methylene-interrupted [15, 34], conjugated and partially conjugated [14, 24], and long chain non-methylene interrupted highly unsaturated FAME [25]. Therefore, the CACIMS/MS technique was used to clarify the position of the double bonds of 18:3 and 18:4 isomers. However, only the major 18:3 biohydrogenation intermediates were successfully identified by CACIMS/MS. Nitrogen containing derivatives, like DMOX derivatives, have also been used for the location of double bond in a variety of fatty acids [16, 20, 35], these derivatives being only slightly less volatile than FAME and with comparable resolution. Indeed, during EI mass spectrometry the amide moiety carries the charge when the molecule is ionized preventing the double bond to become mobile and move up and down through the chain [23]. In the mass spectra of DMOX derivatives, a saturated

Table 3 Effect of increasing 18:4n-3 supplementation levels on the 18:2 (% total 18:2), 18:3 (% total 18:3) and 18:4 (% total 18:4) biohydrogenation intermediates, total fatty acid concentration (mg/g DM) and total 18:2, 18:3 and 18:4 (g/100 g total fatty acids)

Peak[B]	Fatty acid	Supplementation level[A]					SEM	*P*
		0.50	0.75	1.00	1.25	1.50		
	Total fatty acid (mg/g DM)	14.1c	15.6c	16.3bc	20.1ab	20.8a	1.28	0.016
	18:2 isomers (% of total 18:2)							
18	18:2n-6	45.3a	39.0a	27.1b	16.6b	20.7b	3.76	0.001
9	*trans*-5,*trans*10	n.d.[4]	n.d.	9.16b	15.4a	11.6b	0.83	0.012
11	*trans*-5,*trans*-11[C]	9.75b	14.7b	18.2b	43.1a	35.7a	2.73	<0.001
13	18:2 unidentified	13.6a	16.2a	14.2a	6.40b	6.87b	1.465	0.002
15	*trans*-9,*cis*-12	7.46b	8.99ab	9.35a	1.84c	1.48c	0.564	<0.001
16	*trans*-11,*cis*-15	17.9ab	16.1bc	19.3a	14.7c	17.7ab	0.85	0.026
27	*cis*-9,*trans*-11	6.04	5.09	5.82	2.01	5.97	1.412	0.286
	Total 18:2 (g/100 g total FA)	3.89c	3.81c	7.32b	11.5a	8.16b	0.941	<0.001
	18:3 isomers (% of total 18:3)							
26	18:3n-3	100a	100a	26.2b	1.15c	0.76c	6.04	<0.001
20	Δ5,11,15	n.d.	n.d.	35.1a	29.9b	21.4c	0.95	0.006
21	Δ5,11,14	n.d.	n.d.	11.2b	14.7a	9.79b	0.642	0.008
22	Δ5,10,15	n.d.	n.d.	15.0a	1.31b	1.37b	3.782	0.067
24	Δ5,11,15	n.d.	n.d.	27.9c	50.4b	64.4a	3.17	<0.001
	Total 18:3 (g/100 g total FA)	0.45c	0.40c	2.61c	30.5b	39.1a	1.22	<0.001
	18:4 isomers (% of total 18:4)							
28	18:4n-3	100a	66.2b	66.8b	18.7c	11.3c	8.97	<0.001
31	Δ5,7,11,15	n.d.	n.d.	8.24	12.7	8.61	2.425	0.411
33	Δ5,8,10,15	n.d.	n.d.	n.d.	5.04	4.60	0.365	0.439
34	Δ5,8,10,15	n.d.	33.8c	37.5bc	54.4ab	66.9a	5.61	0.016
36	Δ5,8,10,15	n.d.	n.d.	n.d.	9.21	8.56	1.172	0.714
	Total 18:4 (g/100 g total FA)	0.26d	0.81cd	1.03c	2.60b	5.43a	0.203	<0.001

Within a row, means with different letters are significantly different, $P < 0.05$

n.d. Not detected

[A] Supplementation level: supplementation with 0.50, 0.75, 1.00, 1.25, and 1.50 mg of 18:4n-3 per 33 mg of a commercial total mixed ration per dry matter

[B] Peak numbers correspond to peaks in Figs. 1 and 2

[C] This peak co-elutes with *cis*-15 18:1

chain is indicated by regular gaps of 14 amu between ions, in unsaturated derivatives an interval of 12 amu between ions, corresponding to fragments containing n − 1 and n, identifies a double bond positioned between carbons n and n + 1 [27]. In addition, some exceptions can be found for double bonds closer to the carbonyl group. Several authors reported that odd numbered fragments at *m*/*z* 139, 153 and 167 accompanied by even mass ions at *m*/*z* 138, 152, and 166 are used to locate double bonds at C-4, C-5 and C-6, respectively [26, 27]. This rule was used for the identification of peaks 9 and 11 (Δ5,10 and Δ5,11, showing intense ions at *m*/*z* 152 and 153) and 18:4n-3 peak (*cis*-6,9,12,15, showing intense ions at *m*/*z* 166 and 167). However, it could not be applied for the identification of the others 18:3 and 18:4 biohydrogenation intermediates. The intense ion at *m*/*z* 180 in the DMOX mass spectra of peaks 20, 21, 22, 24, 31, 33, 34 and 36, suggests that a double bond exists at carbon-4, -5 or -6. However, a double bond at carbon-4 implies a reduction in the intensity of the ion at *m*/*z* 126, which was not observed. Therefore, as no odd ions at *m*/*z* 153 and 167 were observed, the gap of 12 amu rule was used to infer the double bond position at carbon-5 for the 18:3 isomers (Δ5,11,15, Δ5,11,14 and Δ5,10,15) and 18:4 isomers (Δ5,7,11,15 and Δ5,8,10,15). Although geometry of the double bonds could not be determined, at least one *trans* double bond should be present in the novel biohydrogenation intermediates, as two Δ5,11,15-18:3 isomers and three Δ5,8,10,15-18:4 isomers were identified from 1.00 mg 18:4n-3 level onwards. Considering the order of elution in the cyanopropylpolysiloxane stationary phase, we can expect that the two Δ5,11,15-18:3 isomers might be the *trans*-5,*trans*-11,*trans*-15 and the *trans*-5,*trans*-11,*cis*-15. The tentative allocation of *cis* double bound position at carbon-15 was achieved by biological plausibility given that this isomer was the highest biohydrogenation intermediate accumulated in samples and because the *trans*-11,*cis*-15 double bond system is commonly found in ruminal C18 intermediates.

The biohydrogenation intermediates formed in 1.00, 1.25, and 1.50 mg supplementation levels, suggest that different patterns of isomerizations and hydrogenations had occurred. The 18:4n-3 biohydrogenation pathway in the rumen is still unknown, however, taking into account the recognized biohydrogenation pathways of 18:2n-6 and 18:3n-3 [36, 37], the results herein obtained suggest that the 18:4n-3 pathway also involves the formation of conjugated fatty acids and hydrogenation of double bonds. The

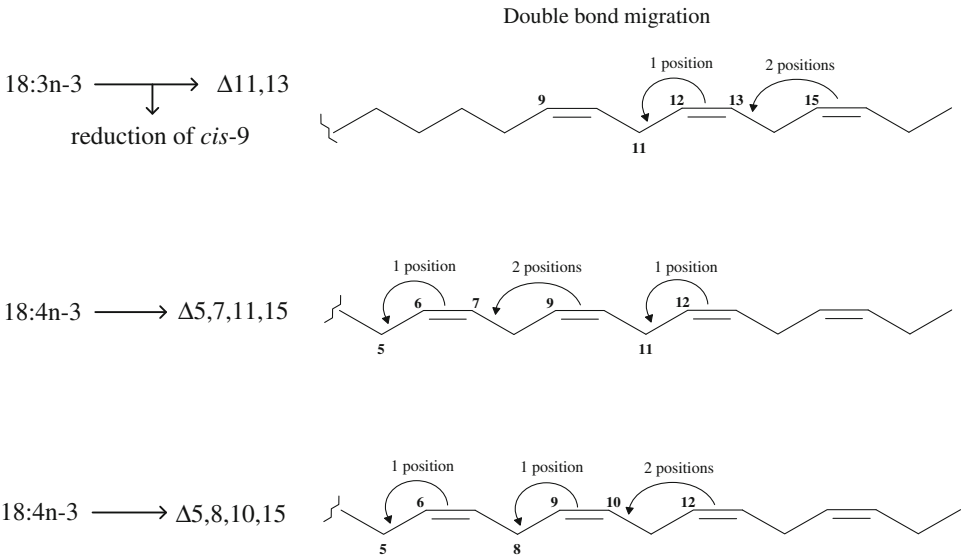


Fig. 8 Schematic representation of the double bonds migration from 18:3n-3 and 18:4n-3 into conjugated dienoic derivatives. The *arrows* do not imply proven biochemical conversions

main biohydrogenation pathway of 18:2n-6 in the rumen involves the formation of the conjugated diene *cis*-9,*trans*-11, followed by reductions to *trans*-11 18:1 and to 18:0. However, others pathways have been hypothesized because new intermediates have been reported to be formed during the 18:2n-6 metabolism in the rumen, like the CLA isomers Δ10,12, Δ11,13 and others Δ9,12 isomers with different *cis/trans* configurations [38, 39]. The main biohydrogenation pathway of 18:3n-3 in the rumen involves the formation of the partially conjugated *cis*-9,*trans*-11,*cis*-15 18:3, which is subsequently hydrogenated to the non-conjugated *trans*-11,*cis*-15 18:2 and further hydrogenated to 18:1 isomers and 18:0 [36]. However, several new 18:3 biohydrogenation intermediates have been identified [37, 40–43]. Very recently, rumen incubation of ^{13}C labelled 18:3n-3 was described to produce fourteen 18:3 isomers, 5 non-conjugated 18:2 isomers and 8 CLA isomers, indicating that 18:3n-3 biohydrogenation pathways are more complex than those previously reported [42]. Similarly, rumen biohydrogenation of 18:4n-3 might also produce 18:4 conjugated isomers, which is supported by the tentatively identification of several partially conjugated 18:4 isomers after 72 h of rumen incubations.

The formation of the partially conjugated 18:4 isomers may involve multiple *cis/trans* and carbon–carbon isomerizations, including shifts of the double bonds two-carbons away, and isomerizations close to the carboxyl group. Indeed, three double bonds in the novel 18:4 isomers moved position, despite not being clear if these multiple migrations occurred sequentially or simultaneously. A sequential migration of the double bonds implies the formation of intermediate isomers between 18:4n-3 and the conjugated 18:4 isomers identified. We could not detect these intermediates, but it is not clear if they were not formed, which would exclude the sequential double bond migration, or if they were transient and were not present after the 72 h of incubation.

The 18:4 biohydrogenation intermediates identified showed isomerizations of double bonds to two-carbons away from the initial position, i.e., the double bonds at carbons-9 and -12 in 18:4n-3 were converted into double bonds at carbons -7 and -10 in the conjugated Δ5,7,11,15 and Δ5,8,10,15, respectively (Fig. 8). A migration of double bonds two-carbons away might also occur during the biohydrogenation of 18:3n-3 because *trans*-11,*cis*-13 and *trans*-11,*trans*-13 have been proposed as intermediates of 18:3n-3 [42, 44–46], suggesting a shift of double bonds at carbons-12, 15. A polyenoic fatty acid isomerase (PFI) found in *Ptilota filicina* marine algae was described to perform a single enzymatic isomerization of two double bonds, one allylically and one homoallylically [47, 48]. These authors reported the conversion of *cis*-6,9,12 18:3 (γ-linolenic) into a conjugated triene by the PFI, in which double isomerization in that the double bond at carbon-9 is transferred to the carbon-8 and the double bond at carbon-12 migrates two-carbons away to form the double bond at carbon-10, producing the conjugated triene *cis*-6,*trans*-8,*trans*-10. Although not yet described in the rumen ecosystem, a similar unusual enzymatic mechanism might occur in microbial isomerases. However, further work is necessary to evaluate the occurrence of this feature in the rumen microbial ecosystem. Hypothetical migrations of the double bonds from both 18:3n-3 and 18:4n-3 into conjugated derivatives are presented in Fig. 8.

Although isomerization from carbon-6 to carbon-5 have not been reported, Kairenius et al. [16] recently suggested that hydrogenation of long chain polyenoic fatty acids in the rumen may proceed via two distinct mechanisms that involve sequential reduction and/or isomerization of *cis* double bonds closest to the carboxyl group.

The three 18:3 non-conjugated isomers putatively identified are probably formed by hydrogenation of the second double bond (closest to the carbonyl group) in the partially conjugated 18:4 isomers. The double bond at carbon-15 may be further hydrogenated to produce the novel 18:2 biohydrogenation intermediates identified in samples. However, the double bond at carbon-15 is considered less likely to be hydrogenated in the first steps of biohydrogenation as demonstrated through the known pathways of 18:3n-3, in which its biohydrogenation leads to the formation of the triene *cis*-9,*trans*-11,*cis*-15 and the dienes *trans*-11,*cis*-15 [36]. Nevertheless, recent results showed that the biohydrogenation of 18:3n-3 can produce the conjugated diene *cis*-9,*trans*-11. Lee and Jenkins [42] found an enrichment of about 40% of *cis*-9,*trans*-11 with labeled ^{13}C from 18:3n-3, implying a reduction of the double bond at carbon-15. The same authors also detected enrichments in others CLA isomers, including *trans*-9,*cis*-11, *cis*-9,*cis*-11, *trans*-11,*trans*-13, *trans*-8,*trans*-10 and *cis*-10,*cis*-12 at 3–48 h of incubation. In the present study, no increase of conjugated dienes was found during the biohydrogenation of 18:4n-3. This suggests that either the 18:4n-3 biohydrogenation pathway does not involve the formation of CLA isomers or that after 72 h of incubation they could have been reduced to monoenoic acids.

A putative metabolic pathway for the formation of the novel 18:2, 18:3 and 18:4 biohydrogenation intermediates identified in incubation samples at the 1.00 mg supplementation level and greater is presented in Fig. 9. The intermediates accumulated after 72 h of incubation are probably the main intermediates formed in the 18:4n-3 biohydrogenation pathway and are not end products because in the incubations at lower supplementation levels of 18:4n-3, none of these novel intermediates were found.

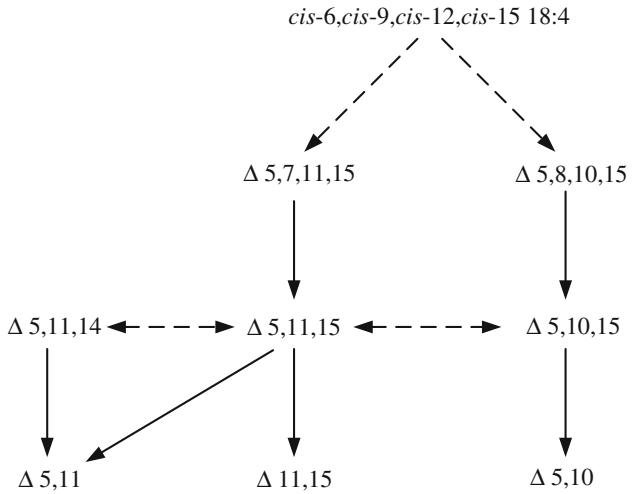


Fig. 9 Putative metabolic pathway for the formation of the novel 18:2, 18:3 and 18:4 biohydrogenation intermediates from 18:4n-3 by rumen microbes. *Dotted arrows* represent suggested carbon–carbon isomerization and *solid arrows* represent proposed hydrogenations, based on the identified biohydrogenation intermediates

In vitro incubation of rumen fluid with increasing supplementation levels of 18:4n-3 produced several novel biohydrogenation intermediates comprising non-conjugated 18:2 and 18:3, and partially conjugated 18:4 isomers, particularly at greater levels (1.0–1.5 mg 18:4n-3 per 33 mg TMR DM). The identification of these intermediates was achieved by gas–liquid chromatography coupled to mass spectrometry analysis of fatty acid methyl esters and DMOX derivatives. However, the *cis* and *trans* geometry of the novel intermediates was not determined.

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References

1. Yamazaki K, Fujikawa M, Hamazaki T, Yano S, Shono T (1992) Comparison of the conversion rates of [alpha]-linolenic acid (18:3n-3) and stearidonic acid (18:4n-3) to longer polyunsaturated fatty acids in rats. Biochim Biophys Acta 1123:18–26
2. Guil-Guerrero JL (2007) Stearidonic acid (18:4n-3): metabolism, nutritional importance, medical uses and natural sources. Eur J Lipid Sci Technol 109:1226–1236
3. Horrocks LA, Yeo YK (1999) Health benefits of docosahexaenoic acid (DHA). Pharmacol Res 40:211–225
4. Whelan J (2009) Dietary stearidonic acid is a long chain (n-3) polyunsaturated fatty acid with potential health benefits. J Nutr 139:5–10
5. Bernal-Santos G, O'Donnell AM, Vicini JL, Hartnell GF, Bauman DE (2010) Hot topic: enhancing omega-3 fatty acids in milk fat of dairy cows by using stearidonic acid-enriched soybean oil from genetically modified soybeans. J Dairy Sci 93:32–37
6. Fowad A, Marie-Claude N, Pierre W, Francis E (2006) Effects of preconditioning and extrusion of linseed on the ruminal biohydrogenation of fatty acids. 2. In vitro and in situ studies. Anim Res 55:261–271
7. Troegeler-Meynadier A, Nicot MC, Bayourthe C, Moncoulon R, Enjalbert F (2003) Effects of pH and concentrations of linoleic and linolenic acids on extent and intermediates of ruminal biohydrogenation in vitro. J Dairy Sci 86:4054–4063
8. Boeckaert C, Vlaeminck B, Mestdagh J, Fievez V (2007) In vitro examination of DHA-edible micro algae: 1. effect on rumen lipolysis and biohydrogenation of linoleic and linolenic acids. Anim Feed Sci Technol 136:63–79
9. AbuGhazaleh AA, Jenkins TC (2004) Disappearance of docosahexaenoic and eicosapentaenoic acids from cultures of mixed ruminal microorganisms. J Dairy Sci 87:645–651
10. AbuGhazaleh AA, Holmes LD, Jacobson BN, Kalscheur KF (2006) Short communication: eicosatrienoic acid and docosatrienoic acid do not promote vaccenic acid accumulation in mixed ruminal cultures. J Dairy Sci 89:4336–4339
11. AbuGhazaleh AA, Jenkins TC (2004) Short communication: docosahexaenoic acid promotes vaccenic acid accumulation in mixed ruminal cultures when incubated with linoleic acid. J Dairy Sci 87:1047–1050

12. Maia MRG, Correia CAS, Alves SP, Fonseca AJM, Cabrita ARJ (2011) Technical note: stearidonic acid metabolism by mixed ruminal microorganisms. J Anim Sci. doi:10.2527/jas.2011-4118
13. Pariza MW (2004) Perspective on the safety and effectiveness of conjugated linoleic acid. Am J Clin Nutr 79:1132S–1136S
14. Michaud AL, Yurawecz MP, Delmonte P, Corl BA, Bauman DE, Brenna JT (2003) Identification and characterization of conjugated fatty acid methyl esters of mixed double bond geometry by acetonitrile chemical ionization tandem mass spectrometry. Anal Chem 75:4925–4930
15. Michaud AL, Diau GY, Abril R, Brenna JT (2002) Double bond localization in minor homoallylic fatty acid methyl esters using acetonitrile chemical ionization tandem mass spectrometry. Anal Biochem 307:348–360
16. Kairenius P, Toivonen V, Shingfield K (2011) Identification and ruminal outflow of long-chain fatty acid biohydrogenation intermediates in cows fed diets containing fish oil. Lipids 46:587–606
17. Christie W, Brechany E, Holman R (1987) Mass spectra of the picolinyl esters of isomeric mono- and dienoic fatty acids. Lipids 22:224–228
18. Christie WW, Robertson GW, McRoberts WC, Hamilton JTG (2000) Mass spectrometry of the 4,4-dimethyloxazoline derivatives of isomeric octadecenoates (monoenes). Eur J Lipid Sci Technol 102:23–29
19. Christie WW, Brechany EY, Johnson SB, Holman RT (1986) A Comparison of pyrrolidide and picolinyl ester derivatives for the identification of fatty-acids in natural samples by gas chromatography–mass spectrometry. Lipids 21:657–661
20. Destaillats F, Sebedio JL, Berdeaux O, Juaneda P, Angers P (2005) Gas chromatography-mass spectrometry determination of metabolites of conjugated cis-9,trans-11,cis-15 18: 3 fatty acid. J Chromatogr B: Biomed Sci Appl 820:15–22
21. Kramer JKG, Hernandez M, Cruz-Hernandez C, Kraft J, Dugan MER (2008) Combining results of two GC separations partly achieves determination of all *cis* and *trans* 16: 1, 18: 1, 18: 2 and 18: 3 except CLA isomers of milk fat as demonstrated using Ag-ion SPE fractionation. Lipids 43:259–273
22. Alves SP, Bessa RJB (2007) Identification of *cis*-12,*cis*-15 octadecadienoic acid and other minor polyenoic fatty acids in ruminant fat. Eur J Lipid Sci Technol 109:879–883
23. Dobson G, Christie WW (2002) Mass spectrometry of fatty acid derivatives. Eur J Lipid Sci Technol 104:36–43
24. Lawrence P, Brenna JT (2006) Acetonitrile covalent adduct chemical ionization mass spectrometry for double bond localization in non-methylene-interrupted polyene fatty acid methyl esters. Anal Chem 78:1312–1317
25. Alves SP, Tyburczy C, Lawrence P, Bessa RJB, Thomas Brenna J (2011) Acetonitrile covalent adduct chemical ionization tandem mass spectrometry of non-methylene-interrupted pentaene fatty acid methyl esters. Rapid Commun Mass Spectrom 25:1933–1941
26. Luthria DL, Sprecher H (1993) 2-Alkenyl-4,4-dimethyloxazolines as derivatives for the structural elucidation of isomeric unsaturated fatty-acids. Lipids 28:561–564
27. Spitzer V (1996) Structure analysis of fatty acids by gas chromatography low resolution electron impact mass spectrometry of their 4,4-dimethyloxazoline derivatives: a review. Prog Lipid Res 35:387–408
28. Kramer JKG, Blackadar CB, Zhou J (2002) Evaluation of two GC columns (60-m SUPELCOWAX 10 and 100-m CP Sil 88) for analysis of milkfat with emphasis on CLA, 18:1, 18:2 and 18:3 isomers, and short- and long-chain FA. Lipids 37:823–835
29. Alves SP, Bessa RJB (2009) Comparison of two gas-liquid chromatograph columns for the analysis of fatty acids in ruminant meat. J Chromatogr A 1216:5130–5139
30. Christie WW (1998) Gas chromatography–mass spectrometry methods for the structural analysis of fatty acids. Lipids 33:343–353
31. Murphy RC (1993) Mass spectrometry of lipids. Plenum Press, New York
32. Lamberto M, Ackman RG (1995) Positional isomerization of *trans*-3-hexadecenoic acid employing 2-amino-2-methyl-propanol as a derivatizing agent for ethylenic bond location by gas-chromatography/mass spectrometry. Anal Biochem 230:224–228
33. Kramer JKG, Cruz-Hernandez C, Zhou JQ (2001) Conjugated linoleic acids and octadecenoic acids: analysis by GC. Eur J Lipid Sci Technol 103:600–609
34. Van Pelt CK, Brenna JT (1999) Acetonitrile chemical ionization tandem mass spectrometry to locate double bonds in polyunsaturated fatty acid methyl esters. Anal Chem 71:1981–1989
35. Wasowska I, Maia MRG, Niedzwiedzka KM, Czauderna M, Ribeiro JMCR, Devillard E, Shingfield KJ, Wallace RJ (2006) Influence of fish oil on ruminal biohydrogenation of C18 unsaturated fatty acids. Br J Nutr 95:1199–1211
36. Harfoot CG, Hazlewood GP (1997) Lipid metabolism in the rumen. In: Hobson PN (ed) The rumen microbial ecosystem. Elsevier, London, pp 382–426
37. Jenkins TC, Wallace RJ, Moate PJ, Mosley EE (2008) Board-invited review: recent advances in biohydrogenation of unsaturated fatty acids within the rumen microbial ecosystem. J Anim Sci 86:397–412
38. Shingfield K, Bernard L, Leroux C, Chilliard Y (2010) Role of *trans* fatty acids in the nutritional regulation of mammary lipogenesis in ruminants. Animal 4:1140–1166
39. Or-Rashid MM, AlZahal O, McBride BW (2011) Comparative studies on the metabolism of linoleic acid by rumen bacteria, protozoa, and their mixture in vitro. Appl Microbiol Biotechnol 89:387–395
40. Bessa RJB, Alves SP, Jeronimo E, Alfaia CM, Prates JAM, Santos-Silva J (2007) Effect of lipid supplements on ruminal biohydrogenation intermediates and muscle fatty acids in lambs. Eur J Lipid Sci Technol 109:868–878
41. Gomez-Cortes P, Tyburczy C, Brenna JT, Juarez M, de la Fuente MA (2009) Characterization of *cis*-9 *trans*-11 *trans*-15 C18:3 in milk fat by GC and covalent adduct chemical ionization tandem MS. J Lipid Res 50:2412–2420
42. Lee YJ, Jenkins TC (2011) Biohydrogenation of linolenic acid to stearic acid by the rumen microbial population yields multiple intermediate conjugated diene isomers. J Nutr 141:1445–1450
43. Destaillats F, Trottier JP, Galvez JMG, Angers P (2005) Analysis of alpha-linolenic acid biohydrogenation intermediates in milk fat with emphasis on conjugated linolenic acids. J Dairy Sci 88:3231–3239
44. Jouany JP, Lassalas B, Doreau M, Glasser F (2007) Dynamic features of the rumen metabolism of linoleic acid, linolenic acid and linseed oil measured in vitro. Lipids 42:351–360
45. Dannenberger D, Nuernberg K, Nuernberg G (2009) Diet-dependent occurrence of CLA isomers in rumen and duodenal digesta of slaughtered bulls. Eur J Lipid Sci Technol 111: 553–562
46. Fukuda S, Nakanishi Y, Chikayama E, Ohno H, Hino T, Kikuchi J (2009) Evaluation and characterization of bacterial metabolic dynamics with a novel profiling technique, real-time metabolotyping. PLoS One 4:e4893
47. Wise ML, Hamberg M, Gerwick WH (1994) Biosynthesis of conjugated triene-containing fatty acids by a novel isomerase from the red marine alga *Ptilota filicina*. Biochem 33:15223–15232
48. Wise ML, Rossi J, Gerwick WH (1997) Characterization of the substrate binding site of polyenoic fatty acid isomerase, a novel enzyme from the marine alga *Ptilota filicina*. Biochem 36: 2985–2992