A Simple Method of Preparation of Methyl *trans*-10,*cis*-12- and *cis*-9,*trans*-11-Octadecadienoates from Methyl Linoleate

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ABSTRACT: Pure conjugated isomers of linoleic acid were prepared on a large scale by alkali-isomerization of purified methyl linoleate. The methyl esters of alkali-isomerized linoleic acid contained mainly the methyl *cis*-9,*trans*-11- and *trans*-10,*cis*-12-octadecadienoates (44 and 47%, respectively). These two isomers were then separated and purified by a series of low-temperature crystallizations from acetone. The isomeric purity obtained for the *cis*-9,*trans*-11-octadecadienoate isomer was >90% and that of the *trans*-10,*cis*-12-octadecadienoate isomer was 89 to 97%. The isolated yield of the two isomers corresponded to 18 and 25.7%, respectively, of the starting material. The structure of the two isomers was confirmed using partial hydrazine reduction, silver nitrate–thin-layer chromatography of the resulting monoenes and gas chromatography coupled with mass spectrometry of the 4,4-dimethyloxazoline derivatives. Fourier transform infrared spectroscopy of the monoenes gave the confirmation of the geometry of each double bond.
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KEY WORDS: Conjugated linoleic acid (CLA), 4,4-dimethyloxazoline derivative, GC–FTIR, GC–MS, hydrazine reduction, isomerization, linoleic acid, low-temperature crystallization, NMR, silver nitrate–thin layer chromatography, UV.

Conjugated linoleic acid (CLA), a mixture of positional and geometrical isomers of linoleic acid with conjugated double bonds, has gained considerable attention because of its anticarcinogenic (1–5) and antiatherogenic (6–8) properties. CLA is found naturally in milk (8,9), dairy (9–13), and meat products from ruminants (1,12,14) because of its formation as an intermediate of hydrogenation of dietary polyunsaturated fatty acids by anaerobic bacteria in the rumen. In addition, isomers of CLA are produced by free radical-induced isomerization of linoleic acid (15–17) during commercial hydrogenation of vegetable oils (18).

The most abundant CLA isomer is 9*c*,11*t*-18:2, which is thought to be the most biologically active isomer (2,4). However, the mechanism by which CLA isomers may act is still unknown. One possibility is that they may have an effect on eicosanoid synthesis. Few data have been published so far on the metabolism of 18:2 isomers having conjugated double bonds (9,19,20). It was recently shown in rats that 9,11-18:2 and 10,12-18:2 may be converted into conjugated $C_{20:3}$ and $C_{20:4}$ isomers (21), as demonstrated for mono-*trans* isomers of linoleic acid (22,23), but it is not known yet which of the geometrical isomers *ct*, *tc*, or *tt* are the precursors.

To further study the metabolic pathways and the physiological effects of 9*c*,11*t*-18:2 and of 10*t*,12*c*-18:2, it is necessary to obtain substantial amounts of these compounds in a pure form. Here we describe a simple method of preparation and purification to generate both CLA isomers.

MATERIALS AND METHODS

Chemical reagents and solvents were purchased from Sigma-Aldrich (St Quentin Fallavier, France) and SDS (Solvants, Documentation, Syntheses; Peypin, France), respectively. Analytical thin-layer chromatography (TLC) was performed on 0.25 mm precoated silica gel plates containing a fluorescent indicator (Merck, Darmstadt, Germany).

Gas chromatography (GC). A Hewlett-Packard HP 5890 series II (Hewlett-Packard, Les Ulis, France) equipped with a splitless/split injector and a flame-ionization detector was used. The temperature of both the injector and detector was 250°C. Helium was the carrier gas. The analyses were performed using two columns of different polarities. The first one was a BPX-70 fused-silica capillary column (SGE, Melbourne, Australia, 0.25 µm film thickness, 50 m length, 0.33 mm i.d.). The second one was a DB Wax fused-silica capillary column (J&W Scientific, Rancho Cordo, CA), 30 m length, 0.25 mm i.d.. The oven temperature was programmed from 60 to 180°C at 20°C/min for the BPX column and from 60 to 200°C at 20°C/min for the DB Wax column; the flow rate of the nitrogen was 1 mL/min and 1.8 mL/min, respectively, for each column. Quantitative data were obtained using an SP 4400 Chromjet (Thermo Separation Products, Les Ulis, France) integrator.

Gas chromatography–mass spectrometry (GC–MS). A Hewlett-Packard 5890 gas chromatograph coupled to an HP model 5989 MS instrument was used for the GC–MS analyses. The latter was operated in the electron impact mode at 70 eV with a source temperature of 250°C. The GC separation

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was performed on a BPX-70 capillary column as described above, and helium was used as carrier gas. The oven temperature was programmed from 60 to 190°C at 20°C/min. Splitless injection was used with the injection port maintained at 250°C.

Gas–liquid chromatography–Fourier transform infrared spectroscopy (GC–FTIR). The gas-phase infrared spectra were obtained with a Bruker IFS 85 Fourier transform infrared spectrometer connected to a Carlo Erba (Massy, France) 5160 gas chromatograph equipped with an on-column injector and a flame-ionization detector both at 300°C. A BPX-70 fused-silica capillary column was used. The interface consisted of a gold-coated lightpipe (20 cm × 0.8 mm i.d.) maintained at 250°C. Helium was the carrier gas. The oven temperature was programmed from 60 to 250°C at 10°C/min, and then held isothermally for completion of the analyses. The spectra resolution was fixed at 8 cm^{-1} and 12 interferograms were collected per second.

Nuclear magnetic resonance spectrometry (NMR). NMR spectra were recorded on a Bruker AC-300-P Fourier transform NMR spectrometer (Bruker, Fallanden, Switzerland) with proton observation at 300 MHz and carbon observation at 75.5 MHz. Unless otherwise stated, spectra were recorded in deuteriochloroform ($CDCl_3$) and chemical shifts are given in δ-values in ppm downfield from tetramethylsilane (TMS).

Ultraviolet spectrometry (UV). The samples were redissolved in cyclohexane, and UV spectra were measured on a Beckman DU 640 spectrometer (Beckman Instruments Inc., Palo Alto, CA).

Purification of linoleic acid from safflower oil. Batches of 260 g of safflower oil (Lesieur Alimentaire, Neuilly, France) were saponified, and unsaponified matter was removed according to AOCS procedure Ca-6a-40. Fatty acids were esterified by refluxing for 5 h in a solution of 1% H_2SO_4 in methanol. The fatty acid methyl esters (FAME) were then fractionated by urea adduction. For that purpose, 750 g of the resulting methyl esters were dissolved in a hot solution of urea in methanol (750 g urea/3 L methanol). After cooling under nitrogen, the flask was set overnight at 4°C. The urea adduct and nonadduct fractions were separated by filtration. The crystals were washed with cold hexane. The filtrate was transferred into a separatory funnel with 5 L water and 37.5 mL concentrated HCl added, and the methyl esters were extracted with hexane. The nonadduct fraction was submitted to a second urea fractionation (urea/FAME, 0.5:1, vol/vol) and the urea adduct fraction was extracted as described.

Isomerization of linoleic acid. Methyl linoleate (110 g) was added to 216 mL of dry ethylene glycol containing 62.4 g of potassium hydroxide (24). The mixture was heated at 180°C for 13 h while a slow stream of nitrogen was passed through the reaction mixture. After cooling the mixture to room temperature, 480 mL of distilled water and 68 mL of concentrated HCl were added and the isomerized fatty acids were extracted with hexane (3 × 400 mL). The combined hexane layers were washed with distilled water, dried over anhydrous sodium sulfate, and hexane-evaporated under N_2. The isomerized fatty acids (108 g) were esterified in 330 mL of a solution of 4% HCl in methanol at 60°C for 30 min. FAME were extracted with hexane (3 × 400 mL) and the hexane layer was washed with distilled water, dried over anhydrous sodium sulfate, and hexane-evaporated.

Low-temperature crystallization. A solution of 110 g of isomerized FAME in 1200 mL of acetone was placed in a large acetone bath, the temperature of which was gradually reduced to −58°C over 2 h with solid carbon dioxide and kept for 1 h. The resulting crystals were then filtered through a Buchner funnel kept at −60°C in a deepfreeze. The precipitate was pressed down hard with a glass stopper and washed with cold solvent before being transferred to a flask by washing with solvent at room temperature. The methyl esters were recovered from the crystals and from the mother liquor by evaporating the solvent on a rotary film evaporator.

Preparation of 4,4-dimethyloxazoline (DMOX) derivatives. FAME (500 mg) were converted to their DMOX derivatives by treatment with 2-amino-2-methylpropanol (0.25 mL) in a sealed ampoule at 170°C for 8 h (25–27). The reaction mixture was cooled, dissolved in 3 mL of dichloromethane, and washed twice with 1 mL of water. After drying the organic phase, the solvent was removed under a stream of nitrogen and the sample was dissolved in hexane. The sample was applied to a short column of Florisil, which was subsequently washed with hexane prior to elution of the DMOX derivatives with a mixture of hexane/acetone (96:4, vol/vol) (28).

Hydrazine reduction. Polyunsaturated fatty acids (PUFA) were submitted to hydrazine reduction according to Ratnayake (29). Briefly, 30 mL of 96% ethanol and 1 mL of hydrazine (Pierce, Rockford, IL) were added to 3.0–3.5 mg of free fatty acid in a round-bottomed flask (125 mL). The reaction was performed at 40 ± 1°C under a light stream of oxygen with gentle magnetic stirring. At the end of the reaction, 120 mL of distilled water was added and the fatty acids were extracted three times with 40 mL of hexane. The hexane layer was washed with distilled water, dried over anhydrous sodium sulfate, and evaporated to dryness.

Each reduced fraction, containing a mixture of 18:0, monoenes, and unreacted conjugated methyl linoleate, was fractionated by silver nitrate-thin layer chromatography ($AgNO_3$–TLC). Silica gel plates (Merck, 0.25 mm thickness, 20 × 20 cm) were impregnated by dipping them for 30 min in a 10% silver nitrate solution in acetonitrile. The developing solvent was toluene (30). After spraying with a 0.2% solution of 2′,7′-dichlorofluorescein in ethanol, bands were detected under UV light at 254 nm, scraped off the plate, and placed into glass centrifuge tubes. A 1% solution of sodium chloride in 90% ethanol was added until the red color of the silver-dichlorofluorescein complex disappeared (31). The methyl esters were extracted with hexane after addition of water.

RESULTS AND DISCUSSION

Purification of methyl linoleate from safflower oil. GC analysis of the transesterified safflower oil revealed a mixture of

TABLE 1
Fatty Acid Composition (wt% of the total) of Aliquots of the Reaction Product Collected During Alkali-Isomerization of 10 g of Methyl Linoleate

	1 h	2 h	3 h	4 h	6 h	8 h	10 h
18:1n-9	2.15	2.24	2.19	2.21	2.19	2.19	2.26
18:2n-6	67.69	2.28	1.41	0.52	0.49	0.41	0.44
9*c*,12*t*- + 9*t*,12*c*-18:2			Trace	0.33	0.40	0.39	0.41
9*c*,11*t*-18:2	13.89	41.19	44.44	43.92	41.03	38.70	36.16
10*t*,12*c*-18:2	15.00	44.14	47.17	46.74	43.83	41.30	38.25
9*c*,11*c*- + 10*c*,12*c*-18:2	0.66	2.11	2.35	2.51	4.25	4.50	4.94
9*t*,11*t*- + 10*t*,12*t*-18:2	0.63	2.03	2.42	3.40	6.90	9.36	11.67
Other isomers					Trace	0.96	1.69

75.8% methyl linoleate, 13.2% methyl oleate, and 7.8% saturated FAME. Two successive urea crystallizations were necessary to isolate pure methyl linoleate. Hence, when a ratio of urea to FAME equal to 1:1 (w/w) was used, saturated and monounsaturated FAME formed crystalline adducts, whereas methyl linoleate remained primarily in the mother liquor. Thus urea complexation of 765 g of safflower FAME with 765 g of urea gave 508.7 g of a nonadduct fraction and 255.1 g of an adduct fraction. GC analysis of the nonadduct fraction showed an increase in the amount of methyl linoleate to 92% with 5.1% methyl oleate and 2.4% saturated FAME. A second urea complexation of 508.7 g of FAME with 254.3 g urea (ratio of urea/FAME, 0.5:1) gave 396.5 g of nonadduct and 109.8 g of adduct FAME fractions. GC analysis of the nonadduct fraction showed that the mixture contained 97.3% 18:2n-6 and 2.1% methyl oleate.

Alkaline isomerization. Heating methyl linoleate in the presence of alkali gave 95% of conjugated isomers, 9*c*,11*t*- and 10*t*,12*c*-18:2 mainly being produced in equal amounts as demonstrated by Nichols *et al.* (24). In order to study the reaction parameters, 10 g of methyl linoleate was heated at 180°C in a solution of KOH in ethylene glycol and the reaction was followed hourly by GC analysis of an aliquot of the alkali-isomerized mixture (Table 1). Isomerized free fatty acids in each aliquot were methylated by boron trifluoride (BF_3) in methanol under mild conditions (room temperature for 30 min) as described by Werner *et al*. (32). Under these conditions, the BF_3 did not modify the profile of the isomers and did not increase the proportion of di-*trans* isomers. GC analysis of the aliquots showed that linoleic acid was completely isomerized after 3 h and the mixture of 9*c*,11*t*- and of 10*t*,12*c*-18:2 isomers was formed, 44.5 and 47.17%, respectively. The amounts of di-*trans* isomers (9*t*,11*t*- and 10*t*,12*t*-18:2) and all *cis* isomers (9*c*,11*c*- and 10*c*,12*c*-18:2) were minor (Table 1). Traces of other positional conjugated isomers were not detected up to 4 h of reaction. However, the amounts of 9*c*,11*t*- and of 10*t*,12*c*-18:2 isomers decreased and those of 9*t*,11*c*-18:2, 10*c*,12*t*-18:2, and the di-*trans* isomers increased when the reaction was maintained for >3 h. For example, the di-*trans* isomers represented 11.7% of total fatty acids after 10 h of reaction. Additionally, peaks corresponding to other conjugated positional isomers appeared after 6 h of reaction. Accordingly, the reaction time was very important to obtaining the 9*c*,11*t*- and 10*t*,12*c*-18:2 isomers with a minimum of impurities. However, it was necessary to increase the reaction time when increasing the quantity of linoleic acid. Consequently, we had to heat the reaction mixture for 13 h to completely isomerize 110 g of linoleic acid. GC analysis showed that the composition of the 13-h isomerized fatty acid mixture was similar to that of an aliquot taken after 3 h in the first experiment (Table 2).

The length of the reaction was reduced using a higher temperature (190 or 200°C), but the amount of the di-*trans* isomers in the mixture increased and other positional conjugated isomers appeared.

The isomerization was carried out twice and the composition of the isomerized linoleic acids obtained in each reaction was similar. Two major conjugated isomers, 10*t*,12*c*- and

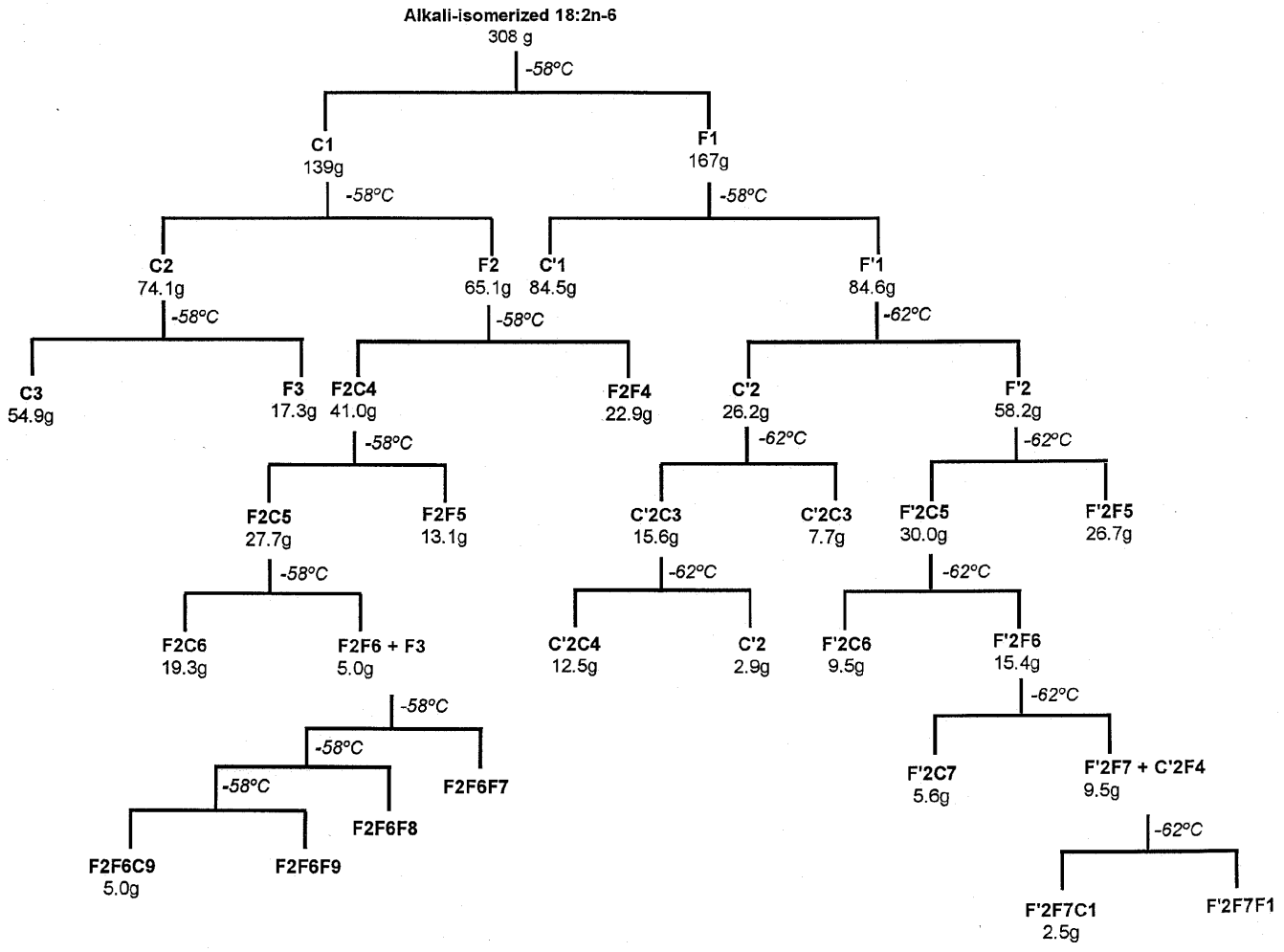
TABLE 2
Fatty Acid Composition (wt% of the total) of Aliquots of the Reaction Product Collected During Alkali-Isomerization of 110 g of Methyl Linoleate

	3 h	5 h	7 h	9 h	11 h	13 h
18:1n-9	2.19	2.20	2.18	2.16	2.35	2.18
18:2n-6	74.46	49.56	27.76	7.35	2.26	0.64
9*c*,12*t*- + 9*t*,12*c*-18:2	Trace	Trace	Trace	0.22	0.20	0.21
9*c*,11*t*-18:2	10.86	22.44	32.46	41.43	43.98	43.99
10*t*,12*c*-18:2	11.74	24.52	35.21	45.01	47.04	48.47
9*c*,11*c*-18:2	0.20	0.41	0.68	1.12	1.02	1.03
10*c*,12*c*-18:2	0.21	0.51	0.75	1.11	1.09	1.14
9*t*,11*t*- + 10*t*,12*t*-18:2	0.34	0.615	0.95	1.32	2.24	2.54

9*c*,11*t*-18:2, were separated and purified from isomerized linoleic acid by successive low-temperature crystallizations.

*Purification of 10*t*,12*c*-18:2 by crystallization.* Scheme 1 shows the procedure used for purification and isolation of 10*t*,12*c*-18:2 from isomerized methyl linoleate by successive low-temperature crystallization. Methyl esters of isomerized linoleic acid (308 g) were dissolved in acetone (12 mL/g) and the solution was gradually cooled to −58°C. The crystallization gave 139 g of precipitate (C1) and 167 g of mother liquor (F1). At −58°C, the 10*t*,12*c*-18:2 isomer crystallized from acetone while 9*c*,11*t*-18:2, oleic acid, di-*trans,* and all *cis* conjugated isomers remained primarily in the mother liquor. GC analysis of fraction C1 showed an increase in the amount of 10*t*,12*c*-18:2 to 72.6% with other components as listed in Table 3. Low-temperature crystallization of fraction C1 twice at −58°C in acetone, using the precipitate C2 in the second crystallization, yielded a precipitate C3 (54.9 g). GC composition of fraction C3 was as follows: 91.2% 10*t*,12*c*-18:2, 5.8% 9*c*,11*t*-18:2 (Table 3). Further, fraction F2, containing 63% of 10*t*,12*c*-18:2, was dissolved in acetone and submitted to two successive crystallizations at −58°C. In the final precipitate (19.3 g) the purity of 10*t*,12*c*-18:2 was 93.7% (Table 3). Finally, in order to recover most of the 10*t*,12*c*-18:2 isomer, fractions F3 and F2F6, which presented about the same FAME composition (52% 10*t*,12*c*-18.2 and 36% 9*c*,11*t*-18:2), were pooled and crystallized three times from acetone (12 mL/g) using the insoluble fraction in each instance. The final precipitate F2C9 (5.0 g) showed a pure 10*t*,12*c*-18:2 (97.8% the total FAME) (Table 3). The yield of the product corresponded to 25.7% of the starting material.

Structure of the 10*t*,12*c*-18:2 isomer was confirmed by GC–MS and GC–FTIR spectrometry. An aliquot of the fraction F2C9 containing 97.8% of 10*t*,12*c*-18:2 was converted to its DMOX derivative. GC–MS analysis of these gave a spectrum with an intense molecular ion (*m/z* 333) and the characteristic fragments shown in Table 4. For example, a mass interval of 12 units (instead of 14) occurred between *m/z* 210 (C_9) and 222 (C_{10}) and between *m/z* 236 (C_{11}) and 248 (C_{12}), indicating the presence of a conjugated double bond in the 10 and 12 positions. Intense ions at *m/z* 196 (C_8), 262 (C_{14}), and 290 (C_{15}) were also characteristic (33). The UV spectrum showed a UV_{max} = 232 nm characteristic of a conjugated diene system, and the GC–FTIR spectrum revealed the presence of an absorption band characteristic of



SCHEME 1

TABLE 3
Fatty Acid Composition (wt% of the total) of the Precipitates (C) and the Mother Liquors (F) During Purification of 10*t*,12*c*-18:2 by Low-Temperature Crystallization[a]

Crystallization	1		2		3		4		6		9
	C1	F1	C2	F2	C3	F3	F2C4	F2F4	F2C6	F2F6	F2C9
18:1n-9	1.2	3.35	Trace	Trace	Trace	2.15	1.0	3.1	Trace	2.45	Trace
18:2n-6	Trace	1.02	Trace	Trace	Trace	0.9	0.5	1.4	Trace	1.2	Trace
9*c*,11*t*-18:2	22.9	60.0	12.6	30.7	5.8	36.9	20.8	58.9	5.7	35.0	1.4
10*t*,12*c*-18:2	72.6	28.7	80.3	63.2	91.2	50.8	69.8	25.8	93.7	52.7	97.95
9*c*,11*c*- + 10*c*,12*c*-18:2	0.6	3.35	1.0	1.7	1.4	1.5	2.4	3.5	0.2	3.0	0.8
9*t*,11*t*- + 10*t*,12*t*-18:2	1.8	2.75	2.05	4.4	1.45	4.65	4.15	4.7	0.35	4.55	Trace

[a]See Scheme 1.

cis-trans or *trans-cis* conjugated double bonds (985 and 950 cm^{-1}) (34).

To confirm the position of the *trans* ethylenic bond, the conjugated fatty acid was submitted to hydrazine reduction. With hydrazine, reduction takes place without modification of the geometry or the position of the double bonds, so that the bonds in the monoenes obtained by reduction of the polyenes are representative of the original positions in the parent molecule (29). Hydrazine reduction resulted in the formation of two major monoenes, A and B. The mixture was then fractionated by $AgNO_3$–TLC and monoenes A (R_f = 0.60) and B (R_f = 0.79) were isolated and further analyzed by GC. To determine the position of the ethylenic bond in the monoenes, each monoene (A and B) was converted to its DMOX derivative and submitted to GC–MS analysis. The major fragments of A and B are reported in Table 4. As an example, for compound B, a mass interval of 12 units instead of the usual 14 units for a saturated chain occurred between *m/z* 210 (C_9) and *m/z* 222 (C_{10}), indicating a double bond in the Δ10 position in the parent molecule. Similarly, compound A is the 12-18:1 isomer. GC–FTIR studies revealed that only 10-18:1 had a *trans* double bond (IR band at 972 cm^{-1}).

The ^{1}H NMR spectrum of the 10*t*,12*c*-18:2 isomer showed: (δ_H) 0.89 (*t, J* = 7 Hz, 3H, CH_3); 1.27–1.42 (*m*, 18H, CH_2), 2.05–2.19 (*m*, 4H, 9-H2 and 14-H2); 2.30 (*t, J* = 7.7 Hz, 2H, 2-H2); 3.66 (*s*, 3H, COOCH_3); 5.26–5.34 (*dt*, $J_{12\text{-}13}$ = 11.0, $J_{13\text{-}14}$ = 7.6, 1H, 13-H); 5.60–5.69 (*dt,* $J_{10\text{-}11}$ = 15.0, $J_{9\text{-}10}$ = 7.0, 1H, 10-H); 5.90–5.97 (*dd,* $J_{12\text{-}13}$ = 11.0, $J_{12\text{-}11}$ = 11.0, 1H, 12-H); 6.24–6.34 (*dt,* $J_{11\text{-}10}$ = 15.0, $J_{11\text{-}12}$ = 11.0, 1H, 11-H). The ^{13}C NMR spectrum showed: (δ_C) 174.27 (*C*=O); 134.73 (C_{10}); 129.88 (C_{13}); 128.73 (C_{12}); 125.60 (C_{11}); 51.42 (*C*–O–C=O); 34.10 (C_2); 32.94; 31.78, 29.68; 29.42; 29.16; 29.13; 29.07; 28.96; 27.66; 24.96; 22.66; 14.13 (C_1).

The ^{1}H NMR spectrum of the 10*t*,12*c*-18:2 isomer showed four distinct signals for the four olefinic protons, two doublet-triplet (δ_H = 5.26 and 5.60), which correspond to the shifts of the protons of the outer *trans-cis* diene system (13-H and 10-H), and two doublet-doublet (δ_H = 5.90 and 6.24), which correspond to the shifts of the protons of the inner *trans-cis* diene system (12-H, 11-H). From the ^{13}C NMR spectrum, according to Lie Ken Jie *et al.* (35), the carbon signals of the methylene groups adjacent to the *trans-cis* conjugated double bond were readily recognized. The signals of the C-14 carbon atom adjacent to the *cis* double bond appeared at δ_C 27.66, while that of the C-13 adjacent to the *trans* double bond appeared at δ_C 32.94.

*Purification of 9*c*,11*t*-18:2 by crystallization.* The 9*c*,11*t*-18:2 isomer also was purified by low-temperature crystallization (Scheme 1). However, the starting material was fraction F1, corresponding to the mother liquor (F1) of the first crystallization, which contained mainly the 9*c*,11*t*-18:2 isomer (60%) accompanied by 28.7% of the 10*t*,12*c*-18:2 isomer (Table 1). To remove the 10*t*,12*c*-18:2 isomer, the fraction F1 was submitted to a crystallization in acetone (10 mL/g) at −58°C. In the mother liquor F′1, the amount of 10*t*,12*c*-18:2 decreased from 28.7 to 12.2%, while the amount of 9*c*,11*t*-18:2 increased from 60 to 71.5% (Table 5). A second crystallization was carried out from the fraction F′1 in acetone (10 mL/g); however, no precipitate was formed. An aliquot of the

TABLE 4
Characteristic Ions in Mass Spectra of 4,4-Dimethyloxazoline (DMOX) Derivatives of 9,11-18:2, 10,12-18:2, and Principal Fragments of the Monoenes Obtained by Hydrazine Reduction of 9,11-18:2 (C and D) and 10,12-18:2 (A and B)

Components	M^+ *m/z* (intensity %)	Fragments *m/z* (intensity %)
9*c*,11*t*-18:2	333 (28.6)	182 (16.3), 196 (3.2), 208 (2.8), 222 (5.5), 234 (3.0), 248 (5.7), 262 (18.5), 276 (15.8)
10*t*,12*c*-18:2	333 (25.7)	196 (6.2), 210 (2.2), 222 (6), 236 (3.5), 248 (1.3), 262 (3.5), 276 (17.6), 290 (15)
A	335 (14.3)	224 (9.2), 238 (1.9), 250 (4.4), 278 (16.8)
B	335 (19.1)	196 (13.8), 210 (3.9), 222 (3.9), 250 (28.1)
C	335 (16.7)	182 (21.9), 196 (5.4), 208 (4.5), 236 (19.6)
D	335 (18.8)	210 (8.2), 224 (3), 236 (4.5), 264 (23.3)

TABLE 5
Fatty Acid Composition (wt% of the total) of the Precipitates (C) and the Mother Liquors (F) During Purification of 9*c*,11*t*-18:2 by Low Temperature Crystallization[a]

Crystallization	1		2		3		4		5		6		7		8
	C′1	F′1	C′2	F′2	C′2C3	C′2F3	C′2C4	C′2F4	F′2C5	F′2F5	C′2C6	C′2F6	F′2C7	F′2F7	F′2C8
18:1n-9	3.3	4.3	4.6	4.1	4.5	4.7	2.3	6.6	4.65	3.7	3.85	4.5	3.55	4.7	4.0
18:2n-6	1.2	3.2	1.95	5.7	1.1	5.45	0.25	1.4	3.6	7.7	0.65	2.4	0.75	2.0	0.3
9*c*,11*t*-18:2	68.4	71.4	78.5	62.5	82.2	64.5	89.0	73.8	73.3	63.45	89.8	72.65	90.1	71.3	92.1
10*t*,12-18:2	24.25	12.15	10.9	13.4	8.5	11.9	7.25	13.5	10.6	14.1	3.5	12.1	3.4	13.1	2.8
9*c*,11*c*- + 10*c*,12*c*-18:2	2.05	4.2	2.85	6.8	1.5	9.6	0.5	1.85	4.8	7.6	1.2	4.8	1.3	4.5	0.4
9*t*,11*t*- + 10*t*,12*t*-18:2	0.85	1.2	1.5	2.6	1.2	2.25	0.6	1.5	1.6	3.0	0.85	1.65	0.55	1.8	0.4

[a]See Scheme 1.

fraction F′1 was converted to DMOX derivatives for GC–MS analysis. Analyses of fraction F′1 showed that the 10*t*,12*c*-18:2 isomer was accompanied by the 11,13 isomer, which did not crystallize from acetone at –58°C. These two isomers are not separable as methyl esters and coelute as a single component.

At –62°C, the 9*c*,11*t*- and 10*t*,12*c*-18:2 isomers crystallize from acetone, whereas the 11,13-18:2 isomer remains in the mother liquor. Therefore, the fraction F′1 was subjected to a low-temperature crystallization from acetone (10 mL/g) at –62°C. 26.2 g of precipitate (C′2) was isolated. The precipitate contained 78.5% 9*c*,11*t*-18.2 and 10.1% 11,13- and 9*t*,12*c*-18:2, while filtrate F′2 (58.2 g) contained 62.5% of 9*c*,11*t*-18:2. Fraction C′2 was redissolved in acetone (10 mL/g) and submitted to two successive crystallizations. The final precipitate C′2C4 (12.44 g) contained the 9*c*,11*t*-18:2 isomer as 89.1% of the total FAME. Filtrate F′2 was redissolved in acetone (10 mL/g) and submitted to two successive crystallizations. The final precipitate F′2C6 (9.5 g) contained nearly pure 9*c*,11*t*-18:2 (89.8%). Filtrate F′2F6, which contained 72.6% of 9*c*,11*t*-18:2, was crystallized to give 5.6 g of crystalline fraction F′2C7. The 9*c*,11*t*-18:2 in this fraction had a purity of 90.1%. Finally, filtrate fractions C′2F4 and F′2F7, which had similar FAME compositions, were pooled and crystallized two times from acetone (10 mL/g) to give 2.5 g of final precipitate. GC analysis of the crystalline fraction F′2C8 showed that 9*c*,11*t*-18:2 had a purity of 92.1% (Table 5). The yield of the product corresponded to 18% of the starting material.

As for 10*t*,12*c*-18:2, the structure of 9*c*,11*t*-18:2 was confirmed by GC–MS and GC–FTIR. An aliquot of the fraction containing 90.1% of 9*c*,11*t*-18:2 was converted to the DMOX derivative. GC–MS analysis (Table 4) confirmed the structure of 9,11-18:2 (36), the UV spectrum showed the UV_{max} = 232 nm as for the 10*t*,12*c*-18:2 isomer, and the GC–FTIR spectrum revealed the presence of an absorption band characteristic of *cis-trans* or *trans-cis* conjugated double bonds (985 and 950 cm^{-1}). The fraction containing the conjugated fatty acid was submitted to hydrazine reduction. This resulted in the formation of two major monoenes, C (R_f = 0.65) and D (R_f = 0.78). GC–MS studies of the DMOX derivatives (Table 4) showed that C and D were 9-18:1 and 11-18:1, respectively, and GC–FTIR studies revealed that only 11-18:1 had a *trans* double bond.

The ^{1}H NMR spectrum of the 9*c*,11*t*-18:2 isomer showed: (δ_H) 0.88 *(t, J* = 7 Hz, 3H, CH_3); 1.26–1.45 (*m*, 18H, CH_2), 2.05–2.18 (*m*, 4H, 8-H2, and 13-H2); 2.30 (*t, J* = 7.4 Hz, 2H, 2-H2); 3.66 (*s*, 3H, $COOCH_3$); 5.24–5.32 (*dt,* $J_{9\text{-}10}$ = 10.8, $J_{9\text{-}8}$ = 7.0, 1H, 9-H); 5.60–5.69 (*dt,* $J_{12\text{-}11}$ = 15.0, $J_{12\text{-}13}$ = 7.0, 1H, 12-H); 5.90–5.97 (*dd,* $J_{10\text{-}11}$ = 11.0, $J_{10\text{-}9}$ = 10.8, 1H, 10-H); 6.23–6.33 (*dt,* $J_{11\text{-}12}$ = 15.0, $J_{11\text{-}10}$ = 11.0, 1H, 11-H). And according to Lie Ken Jie *et al.* (35), the ^{13}C NMR spectrum showed: (δ_C) 174.26 (*C*=O); 134.73 (C_{12}); 129.88 (C_9); 128.73 (C_{10}); 125.60 (C_{11}); 51.42 (*C*–O–C=O); 34.09 (C_2); 32.94 (C_{13}); 31.78 (C_{16}); 29.68; 29.42 (C_{14}); 29.16; 29.13; 29.07 (C_4); 28.96 (C_{15}); 27.66 (C_8); 24.95 (C_3); 22.66 (C_{17}); 14.13 (C_1).

As for the 10*t*,12*c*-18:2 isomer, the ^{1}H NMR spectrum of the 9*c*,11*t*-18:2 isomer showed four distinct signals for the four olefinic protons, two doublet-triplet (δ_H = 5.24 and 5.60) which correspond to the shifts of the protons of the outer (9-H and 12-H) *cis-trans* diene system, and two doublet-doublet (δ_H = 5.60 and 5.90) which correspond to the shifts of the protons of the inner *cis-trans* diene system (10-H, 11-H). From the ^{13}C NMR spectrum, the carbon signals of the methylene groups adjacent to the *cis-trans* conjugated double bond were readily recognized. The signals of the C-8 carbon atom adjacent to the *cis* double bond appeared at δ_C 27.66, while that of the C-13 adjacent to the *trans* double bond appeared at δ_C 32.92. These results agreed with values reported by Lie Ken Jie *et al.* (35).

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