Improved Separation of Conjugated Fatty Acid Methyl Esters by Silver Ion–High-Performance Liquid Chromatography

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ABSTRACT: Operating from one to six silver ion–high-performance liquid chromatography (Ag^+–HPLC) columns in series progressively improved the resolution of the methyl esters of conjugated linoleic acid (CLA) isomeric mixtures from natural and commercial products. In natural products, the 8 *trans*, 10 *cis*-octadecadienoic (18:2) acid was resolved from the more abundant 7 *trans*, 9 *cis*-18:2, and the 10 *trans*, 12 *cis*-18:2 was separated from the major 9 *cis*, 11 *trans*-18:2 peak. In addition, both 11 *trans*, 13 *cis*-18:2 and 11 *cis*, 13 *trans*-18:2 isomers were found in natural products and were separated; the presence of the latter, 11 *cis*, 13 *trans*-18:2, was established in commercial CLA preparations. Three Ag^+–HPLC columns in series appeared to be the best compromise to obtain satisfactory resolution of most CLA isomers found in natural products. A single Ag^+–HPLC column in series with one of several normal-phase columns did not improve the resolution of CLA isomers as compared to that of the former alone. The 20:2 conjugated fatty acid isomers 11 *cis*, 13 *trans*-20:2 and 12 *trans*, 14 *cis*-20:2, which were synthesized by alkali isomerization from 11 *cis*, 14 *cis*-20:2, eluted in the same region of the Ag^+–HPLC chromatogram just before the corresponding geometric CLA isomers. Therefore, CLA isomers will require isolation based on chain length prior to Ag^+–HPLC separation. The positions of conjugated double bonds in 20:2 and 18:2 isomers were established by gas chromatography–electron ionization mass spectrometry as their 4,4-dimethyloxazoline derivatives. The double-bond geometry was determined by gas chromatography–direct deposition–Fourier transform infrared spectroscopy and by the Ag^+–HPLC relative elution order.

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Recently we reported for the first time the separation of a complex mixture of conjugated linoleic acid (CLA) isomers by silver ion–high-performance liquid chromatography (Ag^+–HPLC) as their methyl esters using 0.1% acetonitrile in hexane as the mobile phase (1). With this method, a commercial CLA mixture was separated into three groups of *trans,trans*, *cis/trans*, and *cis,cis* octadecadienoic acid (18:2) isomers. Each of the three groups of geometric isomers was shown to contain four major positional CLA isomers 8,10-18:2, 9,11-18:2, 10,12-18:2, and 11,13-18:2. Application of Ag^+–HPLC to the separation of natural products revealed additional CLA peaks, including several that were not resolved. For instance, 7 *trans*, 9 *cis*-18:2 was reported for the first time to be present in cow and human milk, cheese, beef and human adipose tissue, but it coeluted with 8 *trans*, 10 *cis*-18:2 (2). Furthermore, it was reported recently that mouse liver microsomes elongated 9 *cis*, 11 *trans*-18:2 to 11 *cis*, 13 *trans*-20:2 (3). The longer-chain 20:2 conjugated fatty acids (CFA) were synthesized by alkali isomerization of 11 *cis*, 14 *cis*-20:2, but no supporting evidence was provided (3). Therefore, it was not known where longer-chain CFA such as 20:2 eluted relative to CLA on Ag^+–HPLC columns. Based on the elution order of saturated fatty acid methyl esters (FAME) or their triacylglycerols by Ag^+–HPLC using a similar acetonitrile in hexane mobile phase, an inverse relationship of retention volume and chain length was expected (4).

In the present communication, we report the markedly improved resolution of the CLA isomers, and the separation of the 18:2 from the 20:2 CFA isomers, by using two to six Ag^+–HPLC columns in series. This resulted in the separation of several previously coeluting pairs of CLA isomers found in foods and biological systems.

MATERIALS AND METHODS

The polyunsaturated fatty acids (PUFA) linoleic acid (9 *cis*, 12 *cis*-18:2) and 11 *cis*, 14 *cis*-eicosadienoic acid (11 *cis*, 14 *cis*-20:2) were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). CLA standards (9 *cis*, 11 *trans*-18:2 and 10 *trans*, 12 *cis*-18:2) were obtained from Matreya, Inc. (Pleasant Gap, PA). A 10% solution of trimethylsilyldiazomethane in hexane was obtained from TCI America (Portland, OR). 2-Amino-2-methyl-1-propanol (95%) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). All solvents and chemicals were reagent grade.

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Abbreviations: CFA, conjugated fatty acids; *cis/trans*, refers to all the CLA isomers having either a *cis,trans* or a *trans,cis* configuration; CLA, conjugated linoleic acid; DMOX, 4,4-dimethyloxazoline; FAME, fatty acid methyl esters; GC–DD–FTIR, gas chromatography–direct deposition–Fourier transform infrared; GC–EIMS, gas chromatography–electron ionization mass spectrometry; Ag^+–HPLC, silver ion–high-performance liquid chromatography; PUFA, polyunsaturated fatty acids.

The two PUFA 9 *cis*, 12 *cis*-18:2 and 11 *cis*, 14 *cis*-20:2 were isomerized according to published procedures (5,6). Briefly, 20 mL of ethylene glycol was placed in a three-necked round-bottom flask (100 mL) equipped with a magnetic stirrer, a condenser, and a thermostatically controlled heater, and heated to 110°C. Argon was passed through the reaction flask throughout the heating process. KOH (5 g) was carefully added to the flask, and the temperature was raised to 160°C after the KOH had dissolved. At this point, a PUFA was added and heated for 40 min at 160 ± 5°C. The flask was allowed to cool to room temperature, and the liquid was transferred into a 500-mL separatory funnel containing 100 mL of 6 N HCl; final pH 1–2. The liquid was extracted with two portions of 40 mL petroleum ether/diethyl ether (1:1). The combined organic phase was washed with 80 mL distilled water and dried over Na_2SO_4.

The 4,4-dimethyloxazoline (DMOX) derivatives of the CFA were prepared directly from the free fatty acids by reaction with a threefold excess of 2-amino-2-methyl-1-propanol at 170°C for 0.5 h in a 1-mL vial (7). The DMOX derivatives were analyzed by gas chromatography–electron ionization mass spectrometry (GC–EIMS) (1). The CFA were methylated with trimethylsilyldiazomethane according to Hashimoto *et al.* (8). The FAME were then analyzed by GC using a 100-m CP-Sil 88 column (9). FAME were also measured by GC–direct deposition–Fourier transform infrared (GC–DD–FTIR) (10). A Bio-Rad (Cambridge, MA) Tracer™ GC–FTIR 60A spectrometer system was used. This system was used with a 50-m CP-Sil 88 capillary column as described earlier (11).

Ag^+–HPLC separation of the CFA methyl esters was carried out using an HPLC (Waters 510 solvent delivery system; Waters Associates, Milford, MA), equipped with a 100-µL injection loop (Waters 600E, System Controller), a photodiode array detector (Waters 996) operated at 233 nm, and an operating system (Waters Millennium™ version 2.15). Two to six ChromSpher 5 Lipids analytical silver-impregnated columns (each 4.6 mm i.d. × 250 mm stainless steel; 5 µm particle size; Chrompack, Bridgewater, NJ) were used in series. The mobile phase was 0.1% acetonitrile in hexane and operated isocratically at a flow rate of 1.0 mL/min; a flow rate of 2.0 mL/min sharpened all the peaks but led to less resolution in the *trans,trans* and *cis/trans* regions. The flow was initiated 0.5 h prior to sample injection. Typical injection volumes were 5–15 µL. The column head pressure increased from about 350 psi to about 750, 1050, 1300, and 2000 psi as the number of columns in series increased from one to two, three, four, and six, respectively. The silica LC-Si, phenyl LC-DP, and diol LC-Diol columns, each 4.6 mm i.d. × 250 mm stainless steel, 5 µm particle size, were obtained from Supelco Inc. (Bellefonte, PA).

RESULTS AND DISCUSSION

The separation of CLA isomers progressively improved by increasing the number of Ag^+–HPLC columns connected in series (up to six) as demonstrated with a beef lipid mixture in Figure 1. This is the first chromatographic separation of peaks attributed to the minor CLA isomers 8 *trans*, 10 *cis*-18:2 and 10 *trans*, 12 *cis*-18:2 in natural products, which were previously unresolved (2). The use of three columns in series was sufficient to begin resolving 8 *trans*, 10 *cis*-18:2 from 7 *trans*, 9 *cis*-18:2 and 10 *trans*, 12 *cis*-18:2 from 9 *cis*, 11 *trans*-18:2. The presence of these minor CLA isomers was previously confirmed in cheese lipids by GC–EIMS even though they had not been resolved with a single Ag^+–HPLC column (2,12). Further examples of three-column separation of total CLA methyl esters are presented for cheese (Fig. 2A) and cow milk (Fig. 2B). In a recent publication, the presence of the 10 *trans*, 12 *cis*-18:2 isomer in cheese was indirectly deduced by using partial hydrazine reduction and GC techniques (13).

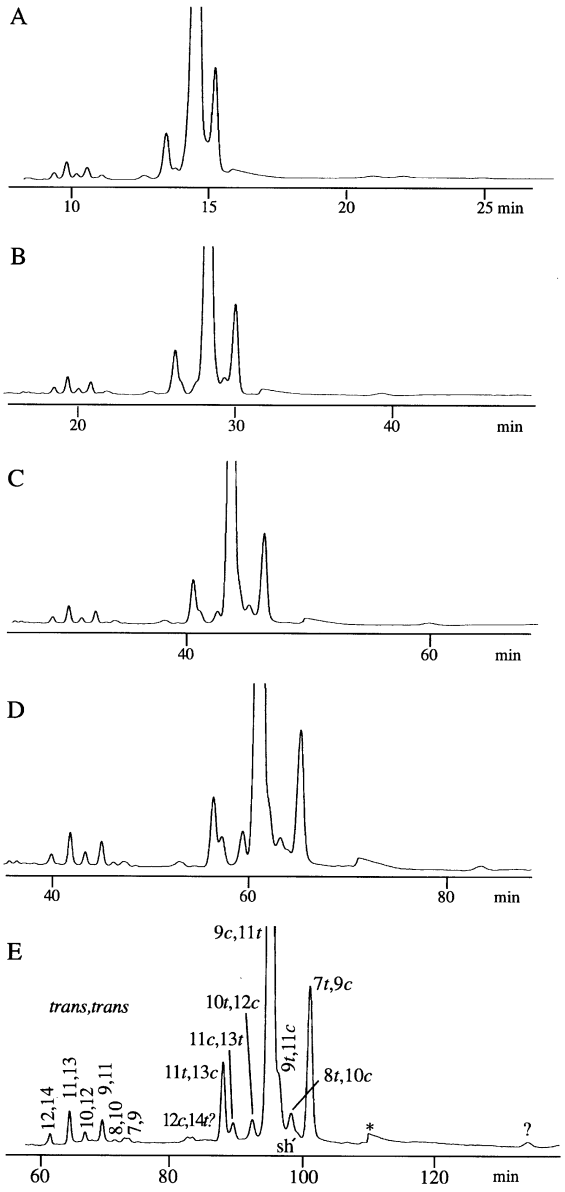

FIG. 1. Separation of total beef conjugated fatty acids using one (A), two (B), three (C), four (D), and six (E) silver ion–high-performance liquid chromatography (Ag^+–HPLC) columns in series. The asterisk (*) denotes an ultraviolet absorption at 233 nm due to methyl oleate. A shoulder after the 8*t*,10*c* peak was abbreviated "sh."

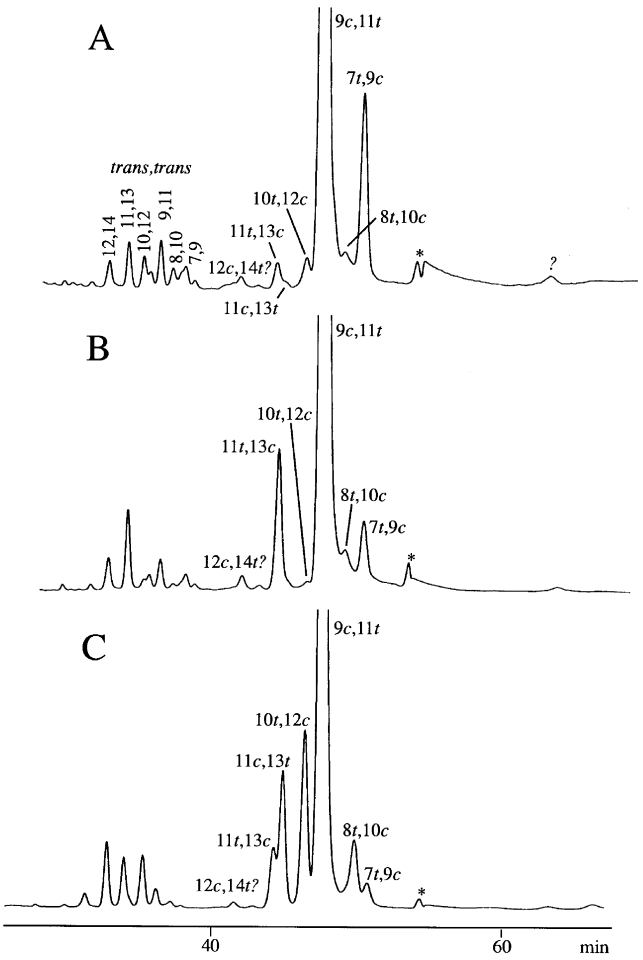

FIG. 2. Ag^+–HPLC profiles of cheese (A) and cow milk (B) using three silver-ion columns in series. Profile for coinjected mixtures of cow milk (C) with a commercial preparation from Nu-Chek-Prep (Elysian, MN). The extra peaks in the *trans,trans* region have not been identified. For asterisks and abbreviation see Figure 1.

Furthermore, the use of two to six Ag^+–HPLC columns in series clearly resolved the two 11,13-18:2 geometric isomers (Fig. 1B–E). The identification of these two isomers was achieved by coinjection of each of the natural product mixtures investigated with a known Nu-Chek-Prep commercial preparation of CLA; the resulting chromatogram for the mixture of cow milk plus Nu-Chek-Prep is shown in Figure 2C. The geometric configuration of the 11,13-18:2 isomer in the commercial CLA preparation was previously established as 11 *cis*, 13 *trans*-18:2 based on its chromatographic elution order by GC and Ag^+–HPLC (2,12,14). Therefore, the first-eluting major 11,13-18:2 isomer in cow milk (Fig. 2B) was tentatively assigned to be 11 *trans*, 13 *cis*- 18:2. The 11 *trans*, 13 *cis*-18:2 isomer was also found to be the most abundant 11,13-18:2 isomer in beef (Fig. 1), cheese (Fig. 2A), and human adipose tissue (data not shown), unlike the human milk that we examined which showed about equal amounts of the 11,13-18:2 pair of isomers.

A shoulder on the tail of the 9 *cis*, 11 *trans*-18:2 peak, tentatively attributed to the 9 *trans*, 11 *cis*-18:2 isomer, was observed only when the separation was carried out with six columns connected in series (Fig. 1E). A similar partial resolution of the *cis/trans* 9,11-18:2 pair of isomers was recently reported by using two Ag^+–HPLC columns in series under different experimental conditions, namely, 0.5% acetonitrile in hexane and detection at 206 nm (4); 9 *trans*, 11 *cis*-18:2 eluted as a shoulder on the leading edge of the 9 *cis*, 11 *trans*-18:2 peak in that case (15). The presumed reversal of the elution sequence of this pair of *cis/trans* geometric isomers may be an effect of mobile phase composition and is being investigated further.

The relative proportion of the different minor CLA isomers varied slightly in the different biological matrices investigated. For example, the 7 *trans*, 9 *cis*-18:2 was more abundant than 11 *trans*, 13 *cis*-18:2, except for cow milk, whereas 11 *trans*, 13 *cis*-18:2 was more abundant than 11 *cis*, 13 *trans*-18:2, except for human milk. These findings probably are not general characteristics but may be attributed to the limited number of test samples investigated. Also unclear is the dependence on diet of these variations in CLA distribution in human tissue lipids.

In using the previously identified Nu-Chek-Prep commercial mixture as reference (1), the separation of CLA mixtures was further investigated by joining several normal-phase HPLC columns, such as silica, phenyl, and diol columns, before or after a silver-ion column in series. In general, a degradation in resolution was obtained. The best combination of all of these columns was achieved by using a silica column followed by a silver-ion column (Fig. 3A). However, this result did not match the excellent separation obtained by using even two Ag^+–HPLC columns in series (Fig. 3B).

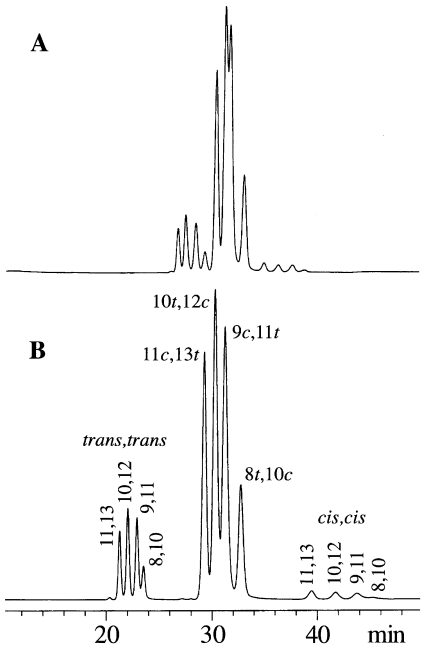

FIG. 3. Separation of a commercial conjugated linoleic acid preparation (Nu-Chek-Prep, Elysian, MN), using a combination of a silica column followed by a silver-ion column (A), and two silver-ion columns in series (B).

The resolution of synthetic C_{18} and C_{20} CFA mixtures was also enhanced with multiple Ag^+–HPLC columns. The isomerized FAME products of either 9 *cis*, 12 *cis*-18:2 (Fig. 4A) or 11 *cis*, 14 *cis*-20:2 (Fig. 4B) gave rise to two peaks in each of the *trans,trans*, *cis/trans*, and *cis,cis* regions of the Ag^+–HPLC chromatogram. The major CFA peaks were due to the *cis/trans* isomers. In using two Ag^+–HPLC columns in series, the retention times (volumes) for these two *cis/trans* pairs of CFA were close, as seen from the analysis of a mixture of these CFA mixtures (Fig. 4C). In each case, the three pairs of C_{20} CFA eluted before the corresponding C_{18} CFA in the *trans,trans*, *cis/trans*, and *cis,cis* regions, respectively. By comparison, using a single Ag^+–HPLC column was inadequate (Fig. 4D). A similar partial resolution was previously reported (16) for some positional C_{18} and C_{20} monounsaturated fatty acid isomers, using a single Ag^+–HPLC column. Furthermore, the inverse relationship between elution order and chain length was similar to that reported for saturated FAME and their triacylglycerols (4). These conjugated C_{18} and C_{20} products were also analyzed by GC. The two *cis/trans* C_{18} CFA isomers eluted at the same GC retention times as those of the standards 9 *cis*, 11 *trans*-18:2 and 10 *trans*, 12 *cis*-18:2 on the 100-m CP-Sil 88 column. The two *cis/trans* C_{20} CFA isomers eluted shortly after arachidonic acid. Their equivalent chain lengths were 22.48 and 22.57 for 11,13-20:2 and 12,14-20:2, respectively; the equivalent chain length of arachidonic acid on this GC column was 22.40.

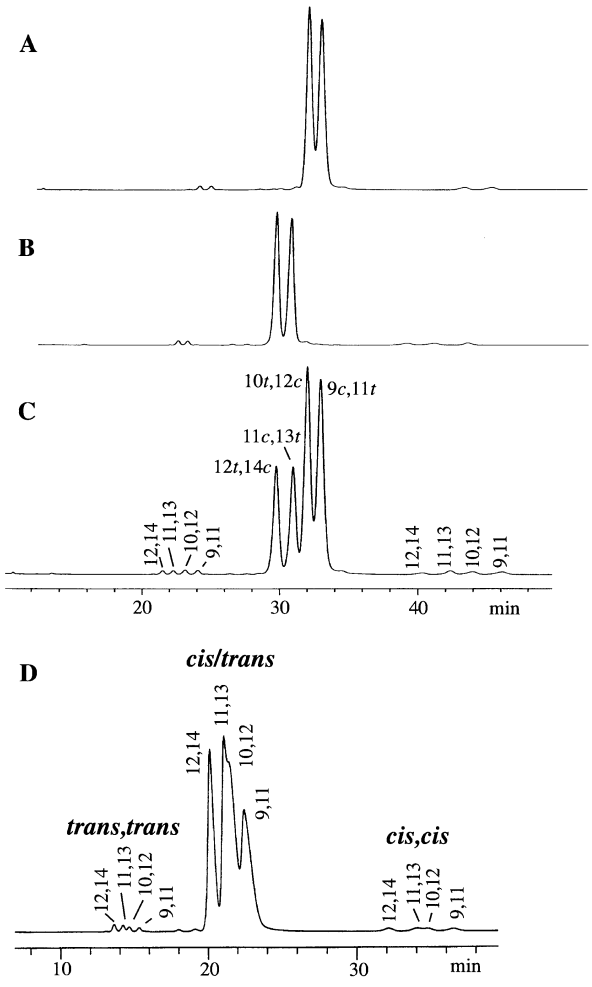

FIG. 4. Ag^+–HPLC separation using two silver-ion columns in series for (A) a CLA mixture obtained by alkali isomerization of linoleic acid (9 *cis*, 12 *cis*-18:2), (B) a conjugated fatty acid mixture obtained by alkali isomerization of 11 *cis*, 14 *cis*-20:2, and (C) coinjection of mixtures (A) and (B). Chromatogram (D) shows the separation obtained by analyzing a mixture of (A) and (B) on a single Ag^+–HPLC column. Different amounts of conjugated 18:2 and 20:2 mixtures were analyzed in (C) and (D). For abbreviations see Figures 1–3.

The isomerization products of 9 *cis*, 12 *cis*-18:2 were identified as 9,11-18:2 and 10,12-18:2 (data not shown), and those of 11 *cis*, 14 *cis*-20:2 were found to be 11,13-20:2 and 12,14-20:2 by GC-EIMS as the DMOX derivatives (Fig. 5). The characteristic fragment ions due to the double bond occurred at *m/z* 224, 236, 250, and 262 for 11,13-20:2 (Fig. 5A), and at *m/z* 238, 250, 264, and 276 for 12,14-20:2 (Fig. 5B). Favorable allylic cleavages occurred at *m/z* 210 and 290 for 11,13-20:2 and *m/z* 224 and 304 for 12,14-20:2. In addition, the ion that was 14 mass units greater than the higher- mass allylic ion was also abundant, i.e., *m/z* 304 and 318, for 11,13-20:2 and 12,14-20:2, respectively.

The *cis/trans* geometries of the C_{18} and C_{20} CFA methyl ester isomers were established by GC–DD–FTIR (Fig. 6). These compounds exhibited characteristic =C-H stretching vibrations at 3020 and 3002 cm^{-1} and deformation bands at 985 and 946 cm^{-1}, as previously described for *cis/trans* conjugated dienes (10). The relative intensities of the C-H stretching vibrations for *cis/trans* C_{18} and C_{20} CFA methyl esters were compared in Figure 7 after normalization relative to the ester carbonyl band. The results showed that, whereas the asymmetric C-H stretch (2952 cm^{-1}) for the CH_3 group in these two CFA isomers was similar, the asymmetric (2924 cm^{-1}) and symmetric (2853 cm^{-1}) CH_2 stretching vibration bands were more intense in C_{20} than in C_{18} CFA. These data were consistent with the formation of 11 *cis*, 13 *trans*-20:2 and 12 *trans*, 14 *cis*-20:2, respectively.

The results of the present study demonstrated the improved resolution of CLA isomers observed by using two or more Ag^+–HPLC columns in series. It would appear that the use of three Ag^+–HPLC columns was the best compromise to achieve, in a timely manner, resolution of most CLA isomers in biological matrices. More than three columns should only be used to resolve specific critical pairs of isomers. Furthermore, this method resolved the 11,13-18:2 pair of geometric CLA isomers. No improvements or advantages were gained by combining a silver-ion column with any of the normal-phase columns, such as silica, phenol, or diol.

In addition, the results show that the CFA with different chain lengths eluted in the same region of the Ag^+–HPLC chromatogram. Therefore, for future investigations of CLA metabolites a prior isolation based on chain length, such as reversed-phase HPLC (17,18), may be required.

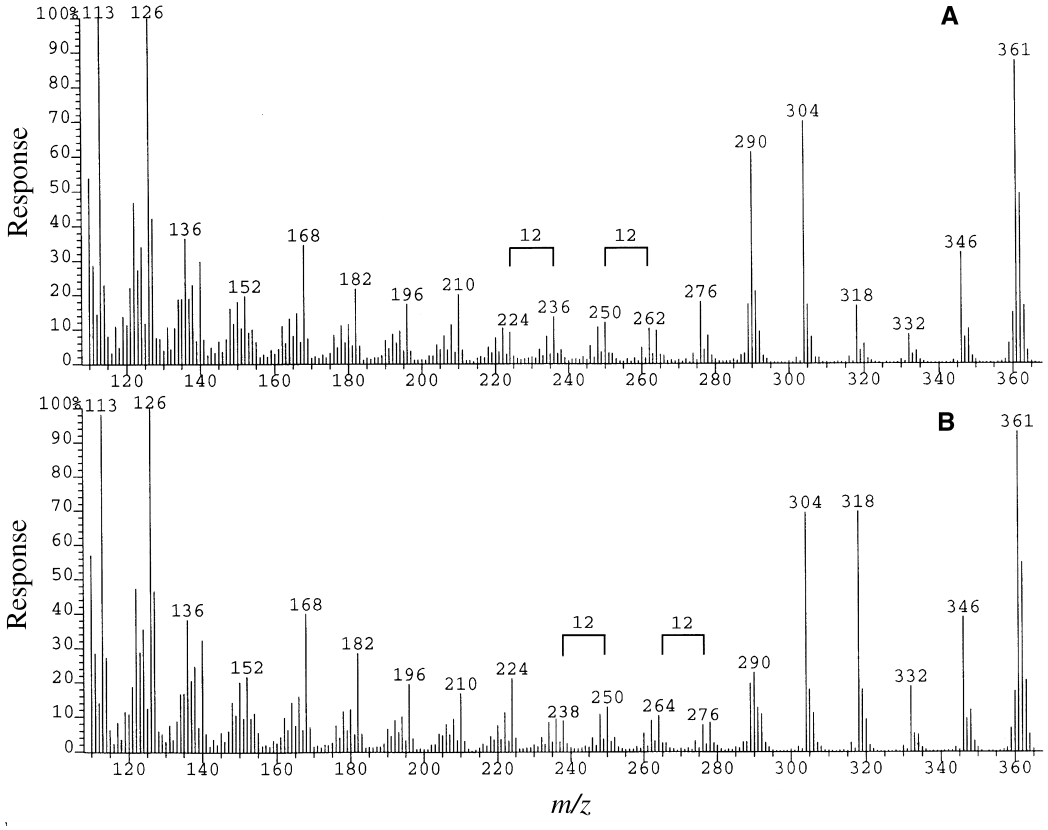

FIG. 5. Gas chromatography–electron ionization mass spectrometry of the 4,4-dimethyloxazoline derivative of isomerized 11 *cis*, 14 *cis*-20:2. The two major *cis/trans* isomers were 11,13-20:2 (A) and 12,14-20:2 (B).

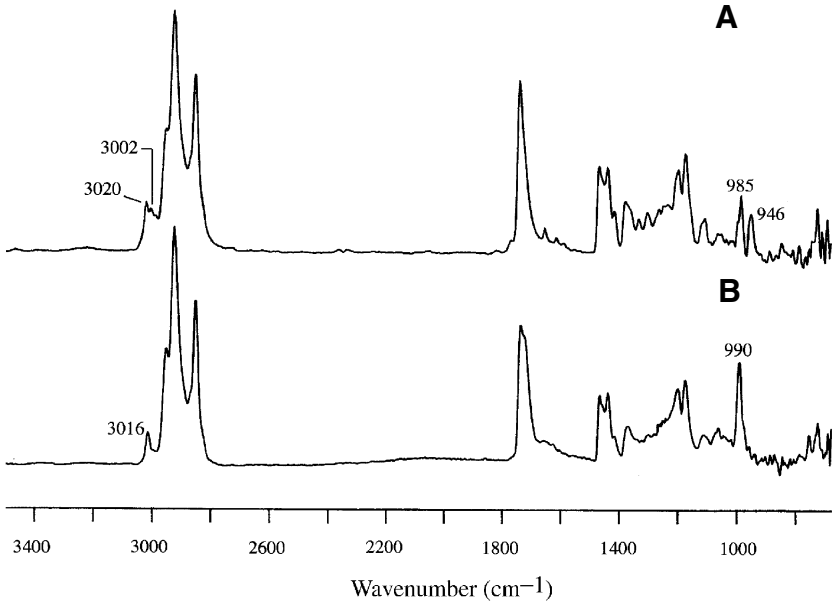

FIG. 6. Characteristic gas chromatography–direct deposition–Fourier transform infrared spectral regions, indicating *cis/trans* (A) and *trans,trans* (B) conjugated double bonds in 20:2 fatty acid methyl esters.

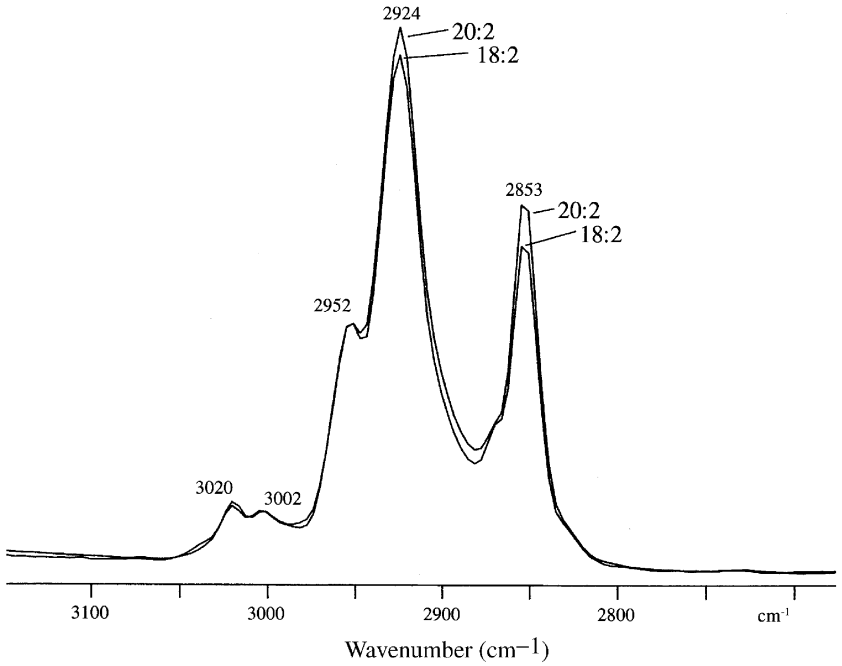

FIG. 7. Comparison of the relative intensity of C-H stretching vibrations for *cis/trans* C_{18} and C_{20} conjugated fatty acid methyl esters after normalization relative to the ester carbonyl band: asymmetric C-H stretch for the CH_3 group at 2952 cm^{-1}, asymmetric (2924 cm^{-1}) and symmetric (2853 cm^{-1}) C-H stretch for the CH_2 groups.

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