Liquid Chromatographic-Mass Spectrometric Analysis of Conjugated Diene Fatty Acids in a Partially Hydrogenated Fat

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A commercially available partially hydrogenated fat was analyzed for fatty acids containing conjugated dienes. The fatty acids were isolated by high-performance liquid chromatography (HPLC), and analyzed with a photodiode array detector and an atmospheric-pressure ionization mass spectrometer. Conventional and second-derivative ultraviolet (UV) spectra of the peaks eluting from the HPLC were recorded with the photodiode array detector, and peaks displaying second-derivative UV spectra characteristic of the conjugated diene chromophore were analyzed by mass spectrometry. The UV and mass spectra of the fatty acids with conjugated dienes, present in the partially hydrogenated fat, were identical to those of reference preparations of linoleic acid isomers with conjugated dienes. The results obtained emphasize that care must be exercised in the interpretation of clinical and experimental data concerning the detection of conjugated dienes in tissues or body fluids of humans and experimental animals. The conjugated dienes may not reflect an ongoing process of lipid peroxidation, but may be of dietary origin.

KEY WORDS: Conjugated dienes, fatty acids, HPLC, lipoperoxidation, mass spectrometry, partially hydrogenated fats, spectrophotometry.

It is well known that partially hydrogenated vegetable oils (PHO) contain a wide range of unusual positional and geometric fatty acid isomers (1), the preponderance of which carry *trans* double bonds (*trans* fatty acids). *Trans* fatty acids have been the object of numerous studies, which have shown, in particular, that their absorption, metabolism and body distribution occur with no significant deviations from those of normal *cis* fatty acids (2–4). In contrast, little attention has been given to the fact that PHO also contain small but not negligible amounts of stable fatty acid isomers with conjugated diene configurations (1), which originate from carbon-centered fatty acid radicals formed during the process of partial hydrogenation (5). In previous studies (6–8), evidence was obtained that these isomers are also absorbed and assimilated by rat tissues. Groups of rats were fed semipurified diets that contained either 10% PHO and 5% corn oil, or 15% corn oil. Fatty acids with conjugated dienes were detected in lipids of the intestinal mucosa, liver and adipose tissue of the former rats, but not of the latter. Moreover, high-performance liquid chromatography (HPLC) analyses of the lipids revealed the presence not of fatty acid hydroperoxides or hydroxides, but of fatty acids with conjugated dienes that eluted with the same retention times as those present in the dietary PHO (7,8). Identification of the fatty acids, however, was not attempted, partly because of the unavailability of suitable reference compounds. Commercial preparations of conjugated diene isomers of linoleic acid (CLA) are now available, and it was therefore decided to investigate whether the fatty acids with conjugated dienes, present in the PHO, are CLA. In this communication, we present evidence that this is indeed the case. The evidence was obtained by combining HPLC/second-derivative ultraviolet (UV) analyses with on-line atmospheric-pressure ionization mass spectrometry (MS).

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MATERIALS AND METHODS

Chemicals. All solvents were of HPLC-grade (Fisher Scientific, Pittsburgh, PA). The following fatty acids and their methyl esters were purchased from Sigma Chemical Co. (St. Louis, MO): arachidonic, linolenic, linoleic, oleic and elaidic acids (18:1 *trans*). Desferal (deferoxamine methanesulfonate), an iron chelator, was purchased from Ciba-Geigy (Basel, Switzerland). Preparations consisting of a mixture of CLA, or of their methyl esters, were obtained from Nu-Chek-Prep, Inc. (Elysian, MN). According to the supplier, the preparations had the following composition: 41.2% *c*9,*t*11- and *t*9,*c*11-CLA; 44.1% *t*10,*c*12-CLA; 9.4% *c*10,*c*12-CLA; 1.3% *t*9,*t*11- and *t*10*t*12-CLA; 1.1% *c*9,*c*11-CLA; and <0.7% linoleic acid (unchanged parent compound). Samples of the PHO (a mixture of partially hydrogenated soybean and palm oils), included in the diets in the previous studies (6–8), were obtained from DYETS, Inc. (Bethlehem, PA). All other chemicals and reagents were of highest available purity.

Preparation of fatty acids and fatty acid methyl esters (FAME) from PHO. Aliquots (3 mg) of the PHO were dissolved in 5 mL ethanol, and 100 μL Desferal (25 mg/mL H_2O), 1 mL of a 25% water solution of ascorbic acid, and 1 mL of 10 N KOH were added. The solutions were left in the dark at room temperature for 14 h. After addition of 10 mL *n*-hexane, acidified with 0.7 mL of 37% HCl to pH 3–4, the samples were shaken and centrifuged for 1 h at 3000 × *g*. The hexane phase containing free fatty acids was collected, the solvent was evaporated, and the residue was dissolved in 0.5 mL of CH_3CN/0.14% CH_3CO_2H (vol/vol). Aliquots (8 μL) of the latter were injected into the HPLC system.

FAME were prepared as described elsewhere (9). Briefly, free fatty acids (≈3 mg) were dissolved in 14% BF_3/CH_3OH and left at room temperature for 10 min. The methyl esters were extracted with *n*-hexane, an aliquot of the extract was taken to dryness and the residue was dissolved in 0.5 mL CH_3CN/0.14% CH_3CO_2H (vol/vol) before HPLC analysis.

All solvent evaporations were performed under vacuum, and lipids were quantitated by using Chiang *et al.'s* method (10).

HPLC analyses. Free fatty acid and FAME separations were performed with a Hewlett-Packard 1090 Series II liquid chromatograph, equipped with a Hewlett-Packard 1040 photodiode array detector and controlled by a Hewlett-Packard Chemstation (Hewlett-Packard, Palo Alto, CA). A 100 × 2.1 mm Hewlett-Packard C-18 ODS Hypersil column, 5 μm particle size, was used for all

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separations. The mobile phase was 74:26 CH_3CN/H_2O (vol/vol) containing 0.12% CH_3CO_2H and a trace (1–2 mM) of $NH_4O_2CCH_3$, flowing at a rate of 0.2 mL/min. The photodiode array detector was set at a primary monitoring wavelength of 234 nm (to detect fatty acids and FAME containing conjugated dienes) and a secondary monitoring wavelength of 200 nm (to detect nonconjugated diene fatty acids and FAME). Spectra (195–315 nm) of the eluate were obtained every 1.28 s and were electronically stored. Second-derivative UV spectra were generated by the Chemstation software. In second-derivative spectra the conjugated diene chromophore displays two minima, at ≈233 and ≈243 nm (11,12).

HPLC-MS analyses. Pneumatically assisted electrospray mass spectra were obtained on a Perkin Elmer/Sciex API I mass spectrometer, equipped with an atmospheric-pressure ionization source and an ionspray interface (Sciex, Toronto, Ontario, Canada). The interface was maintained at 5 kV, and the orifice voltage was set at 70V. High-purity air was used as the nebulizing gas at an operating pressure of 40 psi, and the curtain gas was high-purity N_2 flowing at 0.6 L/min. The analytes in the HPLC mobile phase, after photodiode array analysis, were introduced directly into the nebulization ionization source with no splitting of the effluent. The mass spectrometer was set to scan over the range of *m/z* 240 to 400 at an *m/z* resolution of 0.1, and a rate of one scan per 8.67 s.

RESULTS

A series of analyses was performed, first on mixtures of reference free fatty acids (4 μg each) and CLA, or FAME and CLA FAME, to validate the methodological approach, and then on the PHO samples. Because the acquisition time of each scan by the MS detector was relatively long, fatty acids eluting with similar retention times, such as linoleic acid and CLA, were found to overlap. For this reason, HPLC-MS analyses were performed only on FAME, which were resolved under our conditions.

Analysis of reference free fatty acids and FAME. Figures 1 and 2 illustrate typical HPLC separations of the fatty acids and FAME, respectively. It is evident that CLA were readily detected when the primary monitoring wavelength of the photodiode array detector was set at 234 nm. The CLA eluted as two peaks, with retention times of 35.5 and 40.7 min, respectively. Each peak displayed an absorbance maximum at 234 nm, and two minima in second-derivative UV spectra (Fig. 2, upper graphs). The minima of the first peak were at 236 and 245 nm, while those of the second were at 232 and 242 nm. These results, and the shoulder seen on peak 6 (Fig. 1), attest that the reference CLA preparations used were mixtures of isomers.

A typical HPLC-single quadrupole pneumatically assisted electrospray MS analysis of the reference FAME is shown in Figure 3. Fatty acids, both with and without conjugated dienes, were detected by the mass spectrometer. The mass spectra of the seven individual peaks in Figure 3 are shown in Figures 4, 5 and 6. Protonated molecular species $[M + H]^+$ and ammoniated molecular species $[M + NH_4]^+$ were detected for all species. All but methyl arachidonate and methyl docosahexaenoate also yielded demethylated fragments $[M + H - CH_3]^+$. Whereas the ammoniated molecular species obviously

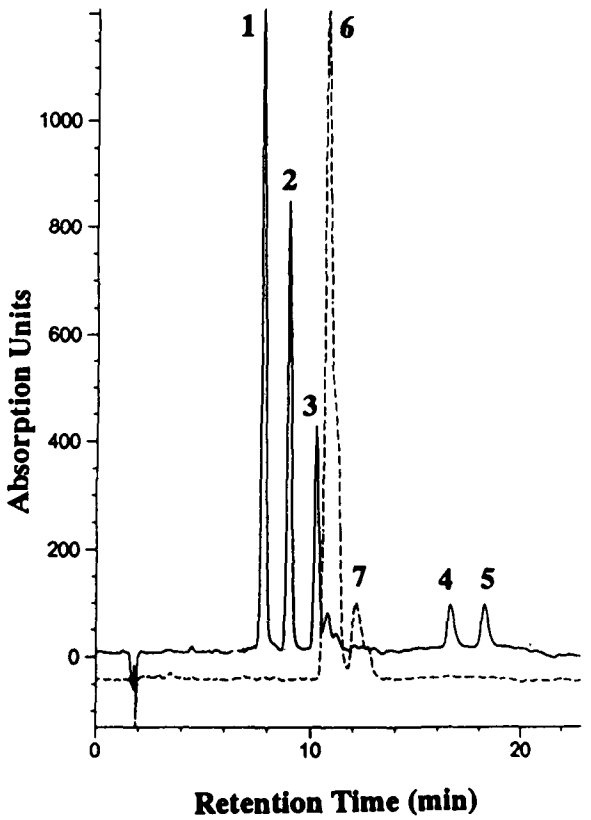

FIG. 1. Reversed-phase C-18 high-performance liquid chromatography chromatogram of free fatty acid standards detected at 200 nm (—) and at 234 nm (- - -): 1, linolenic acid; 2, arachidonic acid; 3, linoleic acid; 4, oleic acid; 5, elaidic acid; 6 and 7, linoleic acid isomers with conjugated dienes.

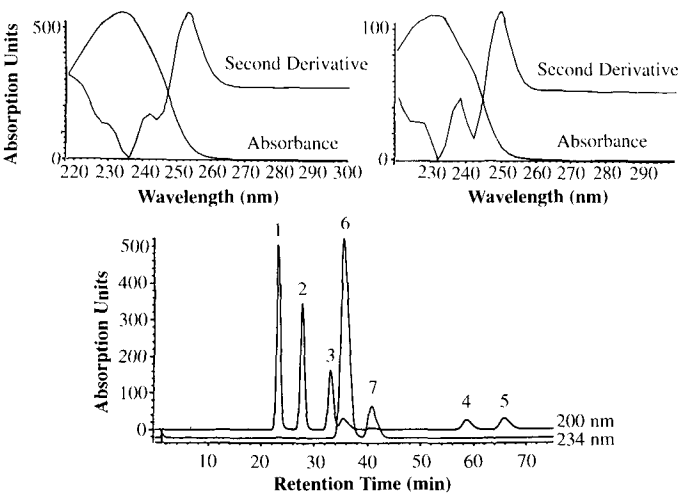

FIG. 2. Lower graph—reversed-phase C-18 high-performance liquid chromatography chromatogram of methyl esters of fatty acid standards monitored at 200 and 234 nm: 1, methyl linolenate; 2, methyl arachidonate; 3, methyl linoleate; 4, methyl oleate; 5, methyl elaidate; 6 and 7, methyl linoleate isomers with conjugated dienes. Upper graphs—conventional and second-derivative ultraviolet spectra of peaks 6 (left) and 7 (right).

arose from cationization by the $NH_4O_2CCH_3$ in the mobile phase, it is unknown whether the demethylated fragments $[M + H - CH_3]^+$ arose from hydrolysis or

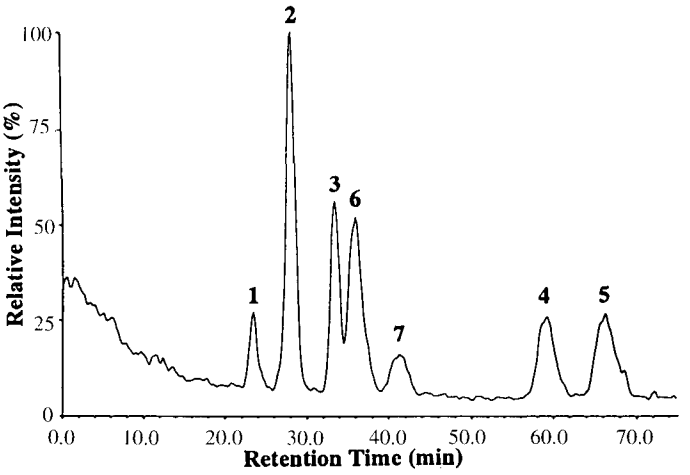

FIG. 3. Atmospheric-pressure ionization mass spectrometric total ion (*m/z* 240 to 400) chromatogram of the high-performance liquid chromatography eluate in Figure 2.

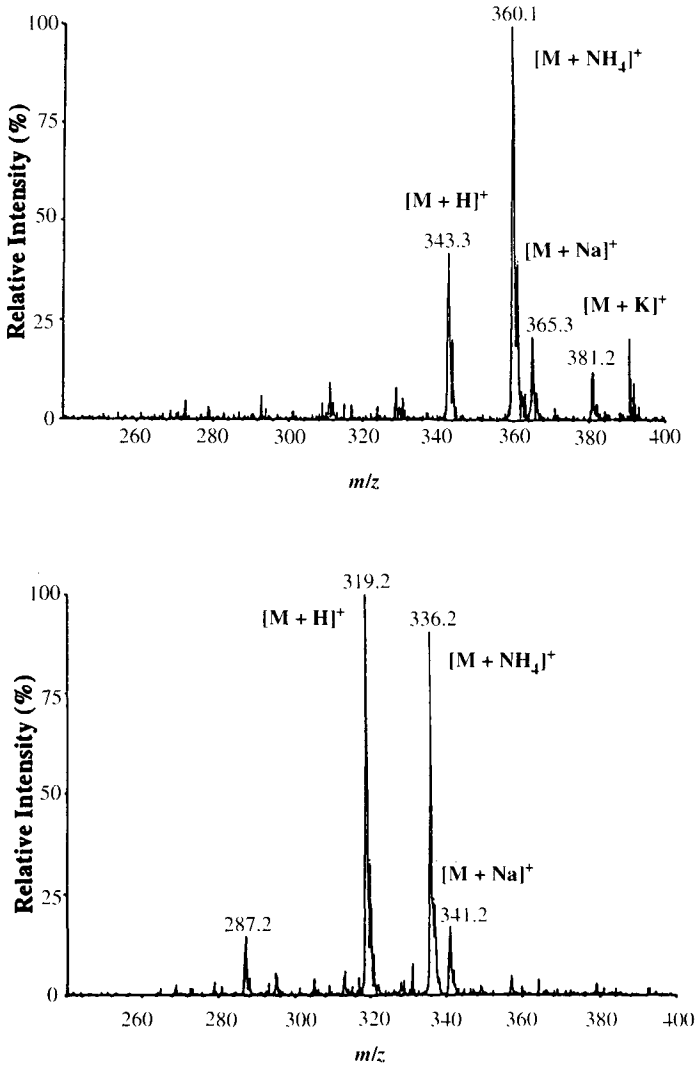

FIG. 4. Mass spectra of peaks 1 (methyl linolenate, upper graph) and 2 (methyl arachidonate, lower graph) shown in Figure 3.

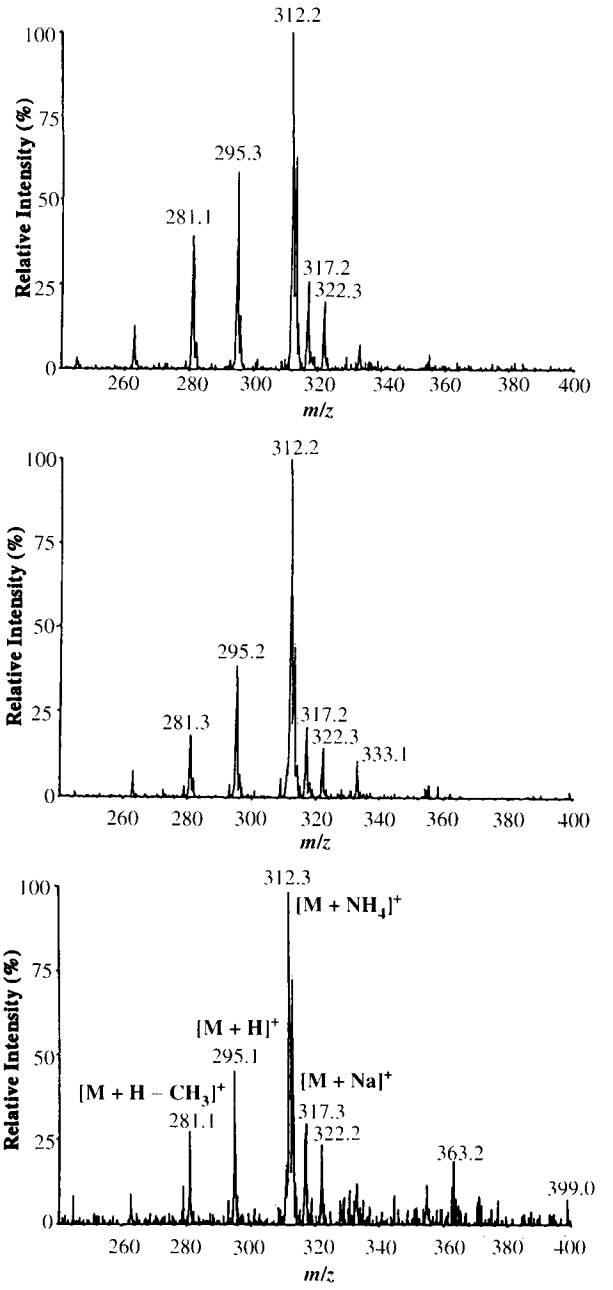

FIG. 5. Mass spectra of peak 3 (methyl linoleate, upper graph) and peaks 6 and 7 (methyl esters of linoleic acid isomers with conjugated dienes, middle and lower graphs) shown in Figure 3.

electronic fragmentation in the source; it is apparent, however, that they originated from parent methyl esters, and not from contaminating fatty acids, as these had quite different retention times in the HPLC system used (compare Fig. 1 with Fig. 2). The molecular mass of each FAME could be determined, but the use of a single quadrupole instrument with a soft ionization source precluded determination of the geometry and position of the double bonds in the analytes. Indeed, a molecular mass of 294 was obtained for the methyl esters of linoleic acid as well as those of the CLA (Fig. 5). Similarly, a molecular mass of 296 was obtained for the methyl esters of both oleic and elaidic acid (Fig. 6).

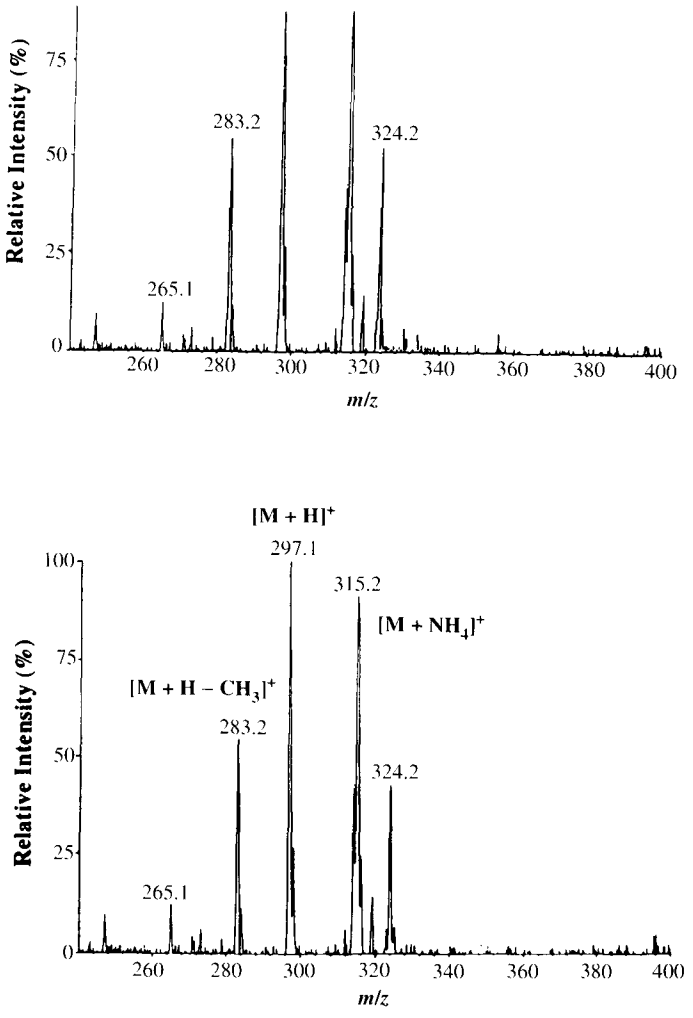

FIG. 6. Mass spectra of peaks 4 (methyl oleate, upper graph) and 5 (methyl elaidate, lower graph) shown in Figure 3.

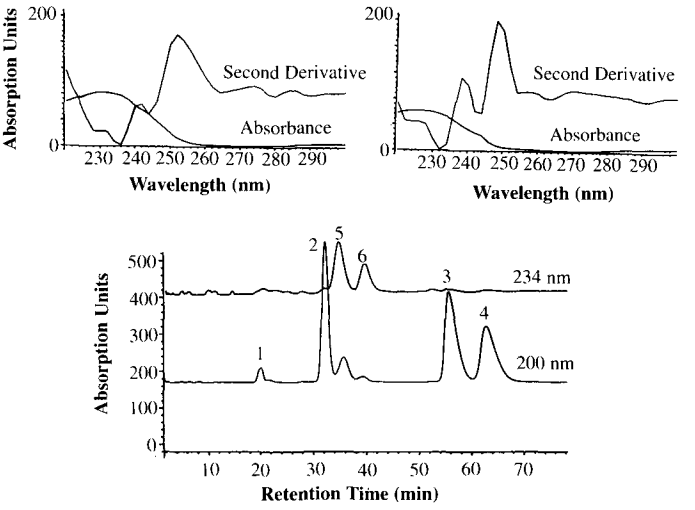

FIG. 7. Lower graph—reversed-phase C-18 high-performance liquid chromatography chromatogram of the methyl esters of fatty acids isolated from samples of a partially hydrogenated fat, detected at 200 and 234 nm: 1, methyl linolenate; 2, methyl linoleate; 3, methyl oleate; 4, methyl elaidate; 5 and 6, fatty acid methyl esters eluting with the same retention times as those of the methyl esters of reference linoleic acid isomers with conjugated dienes. Upper graphs—conventional and second-derivative ultraviolet spectra of peaks 5 (left) and 6 (right).

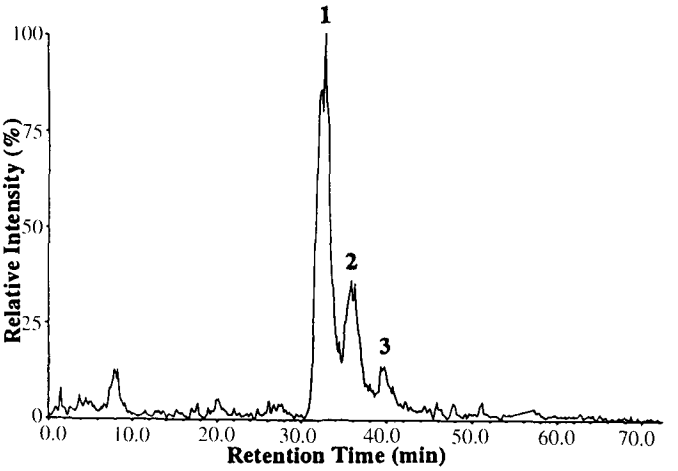

FIG. 8. Single ion chromatogram (*m/z* 294.5 to 295.5) of the atmospheric-pressure ionization mass spectrometric monitoring of the high-performance liquid chromatography eluate from Figure 7. The ion range selected is that of the $[M + H]^+$ species of linoleic acid methyl esters. 1, methyl linoleate; 2 and 3, methyl linoleate isomers with conjugated dienes.

Analysis of PHO FAME. A typical HPLC chromatogram obtained with the photodiode array detector while monitoring at both 200 and 234 nm is shown in Figure 7. The nonconjugated fatty acids detected at 200 nm included *trans* 18:1. At 234 nm, two major peaks were noted. As shown by their second-derivative UV spectra (Fig. 7, upper graphs), both peaks contained conjugated dienes. The retention times of the two peaks were 34.8 and 40.2 min, respectively, essentially the same as those of the two peaks observed with the methyl esters of the reference CLA (Fig. 2). Moreover, as in the latter case, the second-derivative spectrum of each peak showed a different set of minima (Fig. 7, upper graphs), indicating that they, too, consisted of mixtures of isomers. The content of fatty acids with conjugated dienes, in the brand of PHO analyzed, was previously (7) determined to be 4.01 ± 0.08 mg/g (mean ± SD, n = 6).

The PHO-derived 18:2 FAME yielded mass spectra similar to those obtained for the reference standards (data not shown). A typical selected ion chromatogram (*m/z* 294.5 to 295.5, corresponding to the $[M + H]^+$ ion of 18:2 FAME) is shown in Figure 8. Three components were evident, the first having the same retention time as methyl linoleate, and the other two retention times corresponding to those of the methyl esters of fatty acids containing a conjugated diene chromophore (compare Fig. 7 with Fig. 8).

DISCUSSION

A series of HPLC/UV/MS analyses were performed to characterize the fatty acids with conjugated dienes that are present in an edible brand of PHO. This PHO is also used in the formulation of experimental diets (6–8,13).

Fatty acids with conjugated dienes were efficiently analyzed by coupling HPLC with monitoring of conventional and second-derivative UV spectra. By diverting the emerging effluent directly into a pneumatically assisted electrospray mass spectrometer, it was possible to characterize the mass of the analytes. The results obtained clearly show that the PHO fatty acids with conjugated dienes are isomers of linoleic acid.

Conjugated *cis-trans* and *trans-trans* isomers of linoleic acid were recently found to be present in hydrogenated soybean oil and margarine by means of capillary gas chromatography–matrix isolation–Fourier transform-infrared spectroscopy (14). It appears, therefore, that partially hydrogenated fats should be included in the growing list of foods known to contain CLA (Ref. 15, and references therein). At the present time, there is much interest in CLA, because they have been shown to possess a strong anticarcinogenic activity in experimental animal models (16).

Our present and previous studies (6–8,17) highlight also another significant issue of practical importance, already emphasized by other investigators (18). PHO are widely consumed by human populations, and are used with some frequency in the formulation of experimental diets. Detection of conjugated dienes in tissues or body fluids of humans and experimental animals is almost invariably taken as evidence of an ongoing process of lipid peroxidation (13). Such a conclusion, though, is unwarranted, unless proven that the conjugated dienes are present in fatty acid hydroperoxides or hydroxides, rather than in fatty acid isomers of dietary origin.

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