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Detection of conjugated diene isomers of linoleic acid in liver lipids of rats fed a choline-devoid diet indicates that the diet does not cause lipoperoxidation

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Male and female F-344 rats were fed for 1 week choline-devoid or control choline-supplemented diets containing either 5% corn oil and 10% partially hydrogenated vegetable oils (PHO diets) or 15% corn oil (CO diets). HPLC/second derivative UV spectrophotometric analyses, combined with on-line atmospheric pressure ionization mass spectrometry, were used to determine whether conjugated diene isomers of linoleic acid (CLA), present in the diets, were assimilated into liver and adipose tissue lipids. The CLA content in the PHO diets was an order of magnitude greater than that in the CO diets. CLA were detected in the adipose tissue of all rats but in the liver of only rats fed the PHO diets. In adipose tissue, the CLA levels clearly reflected those present in the diets, and no sex differences, or differences between rats fed the choline-devoid or control diets were noted. In addition to CLA, conjugated linolenic and eicosatrienoic acids, arising probably from desaturation and elongation of CLA, were detected in the liver. The results provide evidence that the conjugated dienes detected in liver lipids of rats fed a PHO-containing choline-devoid diet are of dietary origin and do not reflect lipid peroxidation. (J. Nutr. Biochem. 6:281–289, 1995.)

Keywords: conjugated dienes; CLA; lipoperoxidation; HPLC; U.V. spectrophotometry; mass spectrometry

Introduction

Feeding a choline-devoid (CD) diet to male F-344 rats induces a series of morphologic, metabolic, and genomic alterations in the liver that culminate in the genesis of hepatocellular carcinomas after 12–16 months.[1–7] However, no liver tumors develop in female F-344 rats chronically fed the same diet.[8,9]

According to Ghoshal and Farber,[1] induction of a peroxidative process of liver cell membrane lipids, reaching a maximum within the first few days of feeding, is the key alteration underling the hepatocarcinogenicity of a CD diet in male F-334 rats. This view is held even though in previous studies,[10–12] which Ghoshal and Farber appear to be unaware of,[1] we obtained results that clearly contradict such a conclusion. Indeed, the conjugated dienes detected at early time points in liver lipids of rats fed the CD diet were found to be carried not by fatty acid hydroperoxides or hydroxides but by fatty acids of dietary origin. The fat in the

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CD diet used by both us and Ghoshal and Farber's group consists[13] of 5% corn oil (CO), and 10% partially hydrogenated vegetable oils (PHO). PHOs are known[14,15] to contain fatty acids with conjugated dienes (CFA), and the particular brand included in the CD diet was found to be no exception.[11,12] Moreover, when a diet containing only CO was used, neither we nor Ghoshal and Farber's group could detect any conjugated dienes in liver lipids.[12,16]

We recently showed[17] that the CFA present in the PHO included in the CD diet are conjugated diene isomers of linoleic acid (CLA). The identification was achieved by combining HPLC/second derivative UV spectrophotometric analyses with on-line atmospheric pressure ionization mass spectrometry (API-MS). We have applied this methodology to determine whether CLA can be detected in liver and adipose tissue lipids of F-344 rats fed CD or control choline-supplemented (CS) diets containing either 5% CO and 10% PHO (PHO diets), or 15% CO (CO diets). The results obtained are the object of this paper.

Methods and materials

Animals and diets

F-344 rats (Harlan Sprague–Dawley, Indianapolis, IN) were housed in suspended wire bottom cages, one per cage, in a constant temperature (22 ± 2°C) and humidity (50 ± 10%) environment with a 7 a.m. to 7 p.m. light period. Groups of 3 males and 3 females (100 ± 2.5 g body weight) were fed either CD (CD rats) and CS (CS rats) diets containing 10% PHO and 5% corn oil (CDPHO and CSPHO diets/rats), or CD and CS diets containing 15% CO (CDCO and CSCO diets/rats). The diets were all semipurified (DYETS, Inc., Bethlehem, PA USA), and feed and water were offered to the animals ad libitum. After 1 week, the body weight of the rats was recorded, the animals were decapitated, and samples of liver and perirenal adipose tissue were removed and immediately processed as indicated below. For reasons indicated elsewhere,[9] liver samples were taken from the left lobe in the case of males and the right lobe in the case of females.

Chemicals

All solvents used were of HPLC grade (Fisher Scientific, Pittsburgh, PA USA). The methyl esters of the following fatty acids were purchased from Sigma Chemical Co. (St. Louis, MO USA): arachidonic, linolenic, linoleic, oleic, and elaidic (18:1 trans). A mixture of CLA methyl esters was obtained from Nu Chek Prep, Inc. (Elysian, MN USA). Desferal (deferoxamine methanesulfonate), an iron chelator, was purchased from CIBA-Geigy (Basel, Switzerland). Samples of the CO and PHO included in the diets were obtained from DYETS, Inc. All other reagents and chemicals were of highest available purity.

Lipid extraction and preparation of fatty acid methyl esters

Total lipids were extracted from samples of the diets (~1 g) and rat tissues (~0.5 g) by the Folch procedure.[18] Aliquots (3 mg) of the lipids, and of CO and PHO, were dissolved in 5 mL of ethanol, and 100 μL of desferal (25 mg/mL of 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 hr. After addition of 10 mL of n-hexane acidified with 0.7 mL of 37% HCl to pH 3–4, the samples were shaken and centrifuged for 1 hr at 3000*g*. The hexane phase containing free fatty acids was collected, the solvent evaporated, and the residue dissolved in 0.5 mL of CH_3CN/0.14% CH_3CO_2H (vol/vol). Fatty acid methyl esters (FAME) were prepared as described.[19] 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 of CH_3CN/0.14% CH_3CO_2H (vol/vol) before HPLC analysis. All solvent evaporations were performed under vacuum, and lipids were quantified using Chiang's method.[20]

HPLC analyses of FAME

FAME separations were performed using a Hewlett-Packard 1090 Series II liquid chromatograph equipped with a Hewlett-Packard 1040 photodiode array detector, controlled by a Hewlett-Packard Chemstation (Hewlett-Packard, Palo Alto, CA USA). A 100 × 2.1 mm Hewlett-Packard C-18 ODS Hypersil column, 5 μm particle size, was used. The mobile phase was 64:26 CH_3CN-H_2O (vol/vol) containing 0.12% CH_3CO_2H and trace (1–2 mM) $NH_4O_2CCH_3$, 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 FAME with conjugated dienes) and a secondary monitoring wavelength of 200 nm (to detect nonconjugated diene FAME). Spectra (195–315 nm) of the eluate were obtained every 1.28 sec and were electronically stored. Second derivative spectra were generated using the Chemstation software. In second derivative UV spectra, the conjugated diene chromophore displays two minima, at ~233 and ~243 nm.[21]

HPLC/API-MS analyses of FAME

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, Canada). The interface was maintained at 5 kV and the orifice voltage was set at 70 V. 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. Analytes in the HPLC mobile phase, displaying second derivative UV spectra characteristic of the conjugated diene chromophore, were introduced directly into the nebulization ionization source with no splitting of the effluent. The mass spectrometer was set to scan over the range m/z 240 to 400 at a *m/z* 0.1 resolution, and a rate giving one scan/8.67 sec.

Quantitation of CFA methyl esters

CFA methyl esters were quantitated using CLA methyl ester reference standards, as previously described.[21] The same standard curve was used in the quantitation of 18:3 and 20:3 CFA methyl esters, since no reference standards are available for these fatty acids, and anticipating that their extinction coefficient would not greatly differ from that of CLA.

Statistics

Differences between means were evaluated by the Student' *t*-test and considered significant when $P < 0.05$.

Results

CLA in diets

As shown in *Table 1*, the content of CLA was an order of magnitude greater in PHO than in CO, while the contents of the diets reflected in general those of the fat(s) they were

prepared with. No significant differences were seen between CD and control CS diets.

CLA in rat liver total lipids

Table 2 shows the body weight, liver weight, and levels of liver total lipids in rats fed the four diets. As anticipated,[1–7] total lipids were significantly higher in CD than in CS rats. In male but not in female CD rats, the liver weight was also significantly greater than in CS rats. This result is most likely accounted for by the fact that in CD rats triacylglycerol (TG) deposition occurs uniformly throughout the liver of males, but predominantly or only in the right and caudate lobes of females.[9]

No CFA were detected in males[12,16] and females fed the CO diets, while essentially identical results were obtained in the case of male and female rats fed the PHO diets (data not shown), except as noted below (*Table 3*). *Figure 1* illustrates a typical FAME chromatogram monitored at both 200 and 234 nm. All assignments were validated by means of reference standards, except in the case of peaks 6 and 8. Only peaks 6, 7, and 8 displayed an absorbance maximum at 234 nm; their retention times were 22.7, 34.9, and 42.0 min, respectively. *Figures 2–4* show the UV and mass spectra of these three peaks. Peak 7 had a retention time (*Figure 1*), a second derivative UV spectrum (*Figure 3*, upper graph), and a mass spectrum (*Figure 3*, lower graph) identical to those obtained for the peak of the most abundant reference CLA-FAME isomer (see *Figures 5* and *6*). Second derivative UV spectra, typical of the conjugated diene chromophore, were displayed also by peaks 6 and 8 (*Figures 2* and *4*, respectively, upper graphs). The mass spectrum (*Figures 2–4*, lower graphs) of: peak 6 showed two major molecular ions ([M + H]+, 293.3 m/z, and [M + NH_4]+, 310.1 m/z), consistent with the analyte(s) therein being 18:3 FAME; peak 7, three major ions ([M + H]+, 295.2 m/z; [M + H − CH_3]+, 281.1 m/z; and [M + NH_4]+, 312.3 m/z), consistent with the analyte(s) being 18:2 FAME (see also *Figure 6*); and peak 8, two major molecular ions ([M + H]+, 321.2 m/z, and [M + NH_4]+, 338.1 m/z), consistent with the analyte(s) being 20:3 FAME. 18:3 and 20:3 isomers with conjugated dienes were not detected in the PHO and CO included in the diets nor in the fat extracted from the diets (data not shown). Their presence, therefore, suggests that desaturation and elongation of CLA might occur in rat liver. It should be noted, however, that a definitive assignment of the analytes in peaks 6 and 8 could not be made, due to unavailability of suitable reference compounds. Peaks having retention times typical of fatty acid hydroxides or hydroperoxides[12] were not observed in any of the HPLC analyses.

Table 3 shows the results of CLA quantitations. As already indicated, CFA were not detected in rats fed the CO diets. This result may be largely accounted for by the very low content of CLA in these diets (*Table 1*), and the short (1 week) feeding period. In CDPHO rats, the level of liver CLA was ~2 fold grater than in CSPHO rats. This finding may be accounted for by two factors: preferential incorporation of CLA into TG,[22] and TG accumulation in the liver of rats fed CD diets.[1–7] Notably, even in CSPHO rats the liver CLA level was greater (~2 fold) than that found in the rat's diet. This finding could indicate that CLA are not

Table 1 Conjugated diene isomers of linoleic acid (CLA) in samples of partially hydrogenated vegetable oils (PHO), in corn oil (CO), and in fat of semipurified choline-devoid (CD) and choline-supplemented (CS) diets

Sample	CLA (μg/mg of fat)
PHO	4.24 ± 0.07[b,c,d,e,f]
CO	0.33 ± 0.06[a,c,d]
CDPHO	2.76 ± 0.08[a,b,e,f]
CSPHO	2.62 ± 0.09[a,b,e,f]
CDCO	0.30 ± 0.07[a,c,d]
CSCO	0.29 ± 0.06[a,c,d]

Each value represents the mean ± SD of 5 samples.
Superscript letters after SD indicate a significant difference ($P < 0.01$) from [a]PHO, [b]CO, [c]CDPHO, [d]CSPHO, [e]CDCO, and [f]CSCO.

Table 2 Body and liver weights of the rats at sacrifice time, and total lipid content of the livers

Diet fed	Body wt, g	Liver wt, g	mg Lipids/g Liver
		Male Rats	
CDPHO*	128 ± 8	7.9 ± 1.2[b,d]	163 ± 26[b,d]
CSPHO	120 ± 7	4.9 ± 0.3[a,c]	28 ± 7[a,c]
CDCO	127 ± 5	7.6 ± 0.3[b,d]	154 ± 10[b,d]
CSCO	120 ± 14	5.6 ± 0.1[a,c]	28 ± 1[a,c]
		Female Rats	
CDPHO	113 ± 13	4.7 ± 1.3	107 ± 31[b,d]
CSPHO	116 ± 4	4.2 ± 0.1	26 ± 1[a,c]
CDCO	121 ± 9	4.8 ± 1.0	125 ± 15[b,d]
CSCO	110 ± 6	4.3 ± 0.3	31 ± 5[a,c]

Each value represents the mean ± SD of 3 rats.
*CDPHO and CSPHO, choline-devoid and choline-supplemented diets containing partially hydrogenated vegetable oils; CDCO and CSCO, choline-devoid and choline-supplemented diets containing only corn oil.
Superscript letters after SD indicate significant difference ($P < 0.01$) from [a]CDPHO, [b]CSPHO, [c]CDCO, and [d]CSCO.

Table 3 18:2, 18:3, and 20:3 fatty acid isomers with conjugated dienes in rat liver total lipids

Diet fed	18:2 (μg/mg)	18:3 (μg/mg)	20:3 (μg/mg)
Male rats			
CDPHO*	10.34 ± 0.97[b,c,d]	1.82 ± 0.15[b,c,d]	1.01 ± 0.31[b,c,d]
CSPHO	3.91 ± 0.72[a,c,d]	n.d.[a]	0.37 ± 0.09[a,c,d]
CDCO	n.d.[a,b]	n.d.[a]	n.d.[a,b]
CSCO	n.d.[a,b]	n.d.[a]	n.d.[a,b]
Female rats			
CDPHO	11.69 ± 1.21[b,c,d]	1.40 ± 0.34[b,c,d]	0.91 ± 0.31[b,c,d]
CSPHO	04.48 ± 0.84[a,c,d]	n.d.[a]	0.40 ± 0.22[a,c,d]
CDCO	n.d.[a,b]	n.d.[a]	n.d.[a,b]
CSCO	n.d.[a,b]	n.d.[a]	n.d.[a,b]

Each value represents the mean ± SD of fatty acid methyl ester analyses of the lipids from 3 rats.
*See legend to Table 2.
Supersript letters after SD as in Table 2; n.d., not detected.

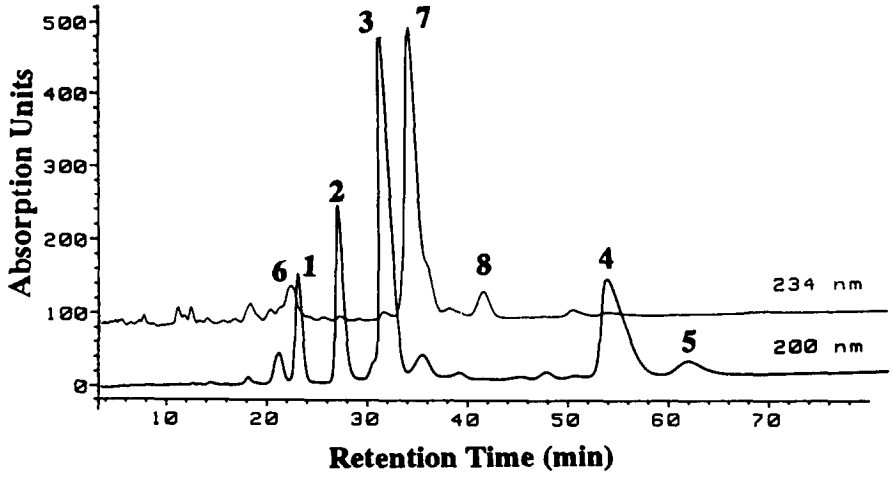
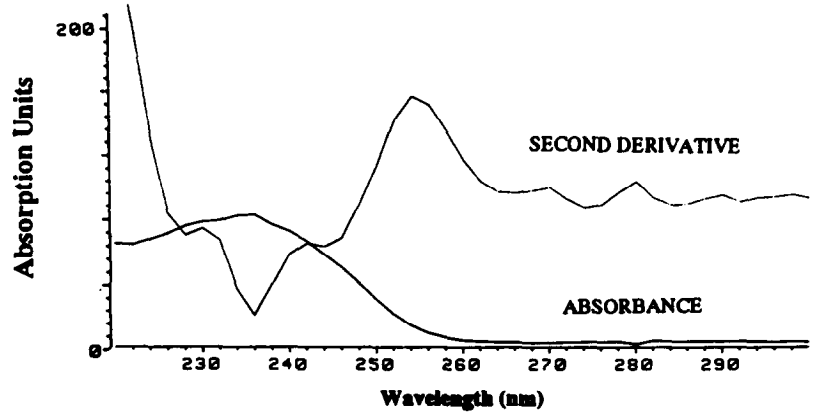


Figure 1 HPLC chromatogram of fatty acid methyl esters, prepared from liver total lipids of a female rat fed the CDPHO diet, recorded with the photodiode array detector at 200 and 234 nm. (1) methyl docosahesaenoate, (2) methyl arachidonate, (3) methyl linoleate, (4) methyl oleate, (5) methyl elaidate, (5) methyl ester(s) of 18:3 isomer(s) with conjugated dienes, (7) methyl esters of 18:2 isomers with conjugated dienes, and (8) methyl ester(s) of 20:3 isomer(s) with conjugated dienes. All assignments were validated with reference standards, except for 6 and 8.

metabolized in rat liver as readily as the parent compound, linoleic acid. *Table 3* also shows the levels of the presumed 18:3 and 20:3 conjugated diene isomers present in rats fed the PHO diets. No 18:3 isomers were detected in CSPHO rats, while the level of 20:3 isomers was again greater in CDPHO than in CSPHO rats. No significant sex differences were noted in the levels of CLA, or of the other CFA.

CLA in adipose tissue total lipids

Figure 7 illustrates a representative HPLC-chromatogram of FAME derived from adipose tissue. Only one peak with an absorbance maximum at 234 nm was noted, irrespective of the diet fed to the rats. Its retention time was 34.8 min, and its UV and mass spectrum (*Figure 8*) showed that it



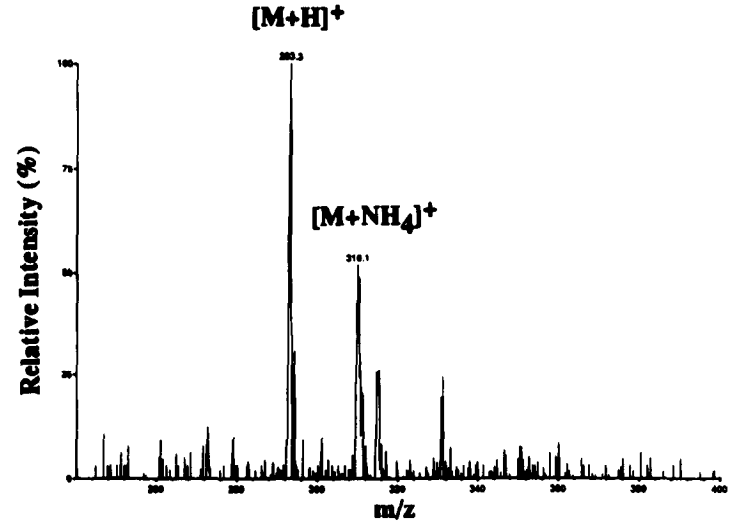


Figure 2 Conventional and second derivative UV spectra (upper graph) and mass spectrum (lower graph) of peak 6 in *Figure 1.*

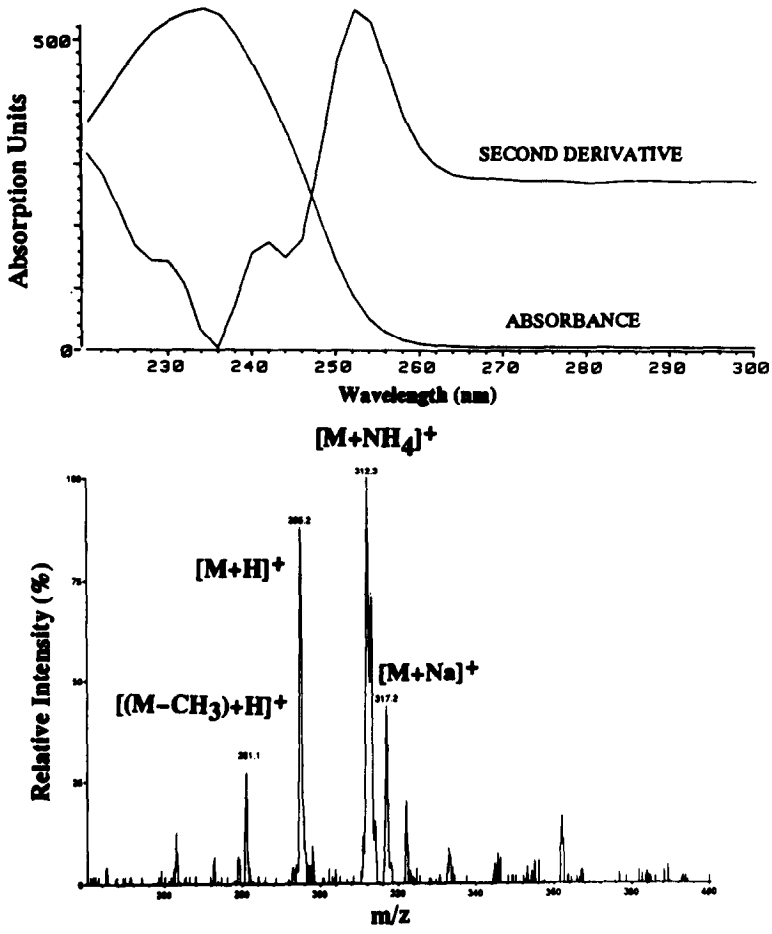


Figure 3 Conventional and second derivative UV spectra (upper graph) and mass spectrum (lower graph) of peak 7 in *Figure 1*.

consisted of CLA (see *Figure 6*). As shown in *Table 4*, the CLA concentration in adipose tissue lipids clearly reflected that in the diet fed to the rats. It was also ~5 fold greater than that in the fat of the diets (see *Table 1*), pointing again to a possible sluggishness in the metabolism of CLA. No sex differences, nor differences between rats fed the CD and control CS diets, were noted.

Discussion

The absorption and assimilation of dietary CLA by experimental animals and humans are well documented in the literature.[22–24] CLA have been detected in cholesteryl esters, phospholipids, and especially TG of body fluids and a variety of organs, including liver and, in particular, depot fat.[22–24] To our knowledge, though, this is the first report of the detection of 18:3 and 20:3 CFA in biological tissues. Since these two unusual fatty acids were not present in the diets fed to the rats, they appear to be the products of desaturation and elongation of CLA in the liver.

Evidence in the literature[24] suggests that the organ and lipid distributions of CLA, as well as their metabolism, depend on at least two variables: the amount present in the diet and the length of CLA intake. In the present study, diets containing disparate amounts of CLA were fed for 1 week, and while CLA were detected in both liver and adipose tissue of rats fed the PHO diets, they were detected only in adipose tissue of those fed the CO diets. A similar organ distribution of CFA was previously[12] observed in rats fed the four diets for up to 2 weeks; moreover, no CFA were detected in liver phospholipids, irrespective of the diet fed. Two additional factors may have contributed to the latter finding: (1) only one of the eight possible CLA isomers is incorporated into phospholipids[25,26]; and (2) the relative sensitivity of the methodologies used to analyze and quantitate CLA.[22]

PHOs have long been known to contain stable CFA,[14,15] and those present in the PHO we analyzed,[17] and in hydrogenated soybean oil and margarines,[27] have been shown to include CLA. Therefore, PHOs should be added to the list of foods known to contain CLA[23,24,28]; this communication may also be the first in which CLA, present in a PHO, have been found to be assimilated in tissue lipids. At the present time, CLA are the object of considerable interest, because they have been shown to exert anticarcinogenic activity in rodent models of skin, forestomach, and breast cancer,[25,26,29,30] and antiatherogenic activity in a rabbit model.[31] Whether CLA act as anticarcinogens in the CD diet model of hepatocarcinogenesis[1–7] has not been tested. For this purpose, experiments would have to be performed to determine the incidence of liver tumors in rats fed CD diets, containing increasing amounts of CLA.[30]

Differences in methodological approaches appear to be the primary reason for the conflicting interpretations given

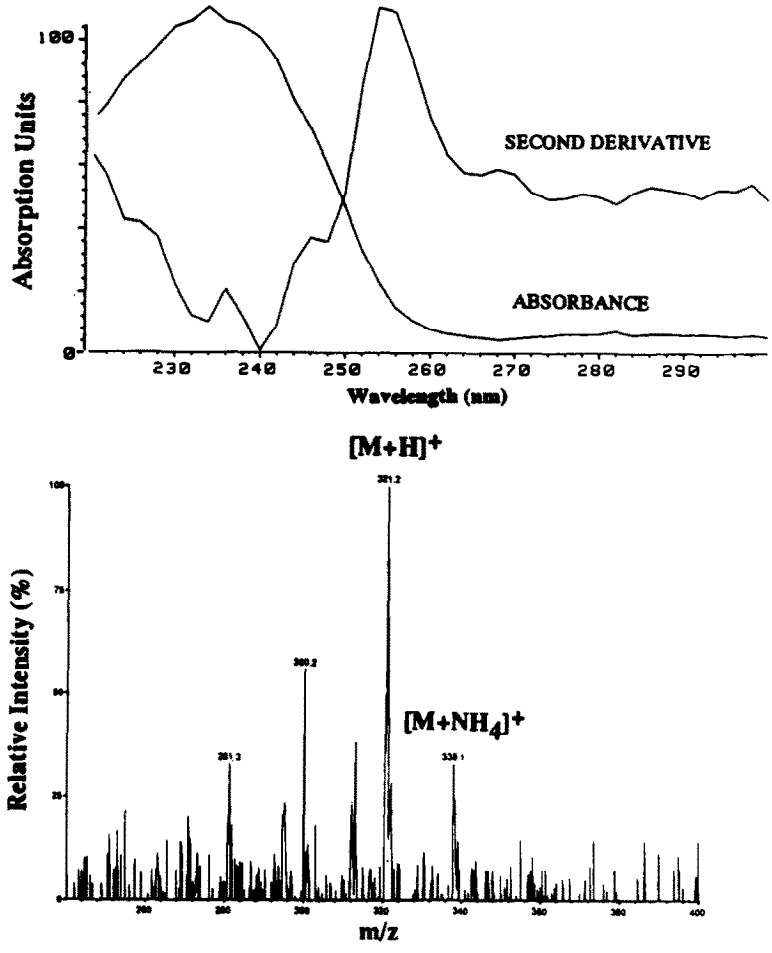


Figure 4 Conventional and second derivative UV spectra (upper graph) and mass spectrum (lower graph) of peak 8 in *Figure 1*.

by Ghoshal and Farber's group,[1] and by us,[6,12] to the detection of conjugated dienes in liver lipids of CD rats. Throughout their studies, for example, Ghoshal and Farber used only the difference spectrum method[32] to detect and quantitate conjugated dienes. We attribute to this fact, in particular, their failure to observe conjugated dienes in the CD diet,[33] given that this diet, and its control CS diet, contain the same amounts of PHO and CO and thus of conjugated dienes. There are then no reasons to anticipate that thiobarbituric acid reacting substances would be present in these diets when properly stored.[33]

Ghoshal and Farber also analyzed exclusively total lipids. It is apparent, however, that as long as a CD diet as presently formulated is used, a significant amount of CFA, specifically CLA, known to incorporate into a variety of acyl-esters, is also fed to the rats. Under these conditions, therefore, unequivocal evidence of an ongoing process of lipoperoxidation could come only from the detection of

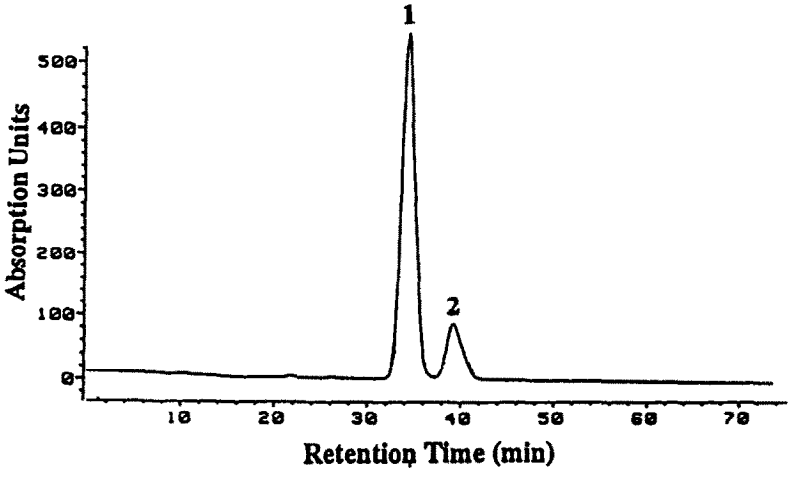


Figure 5 HPLC chromatogram of reference CLA fatty acid methyl esters, recorded with the photodiode array detector at 234 nm. Two peaks are evident, having retention times of 34.9 and 38.9 min.

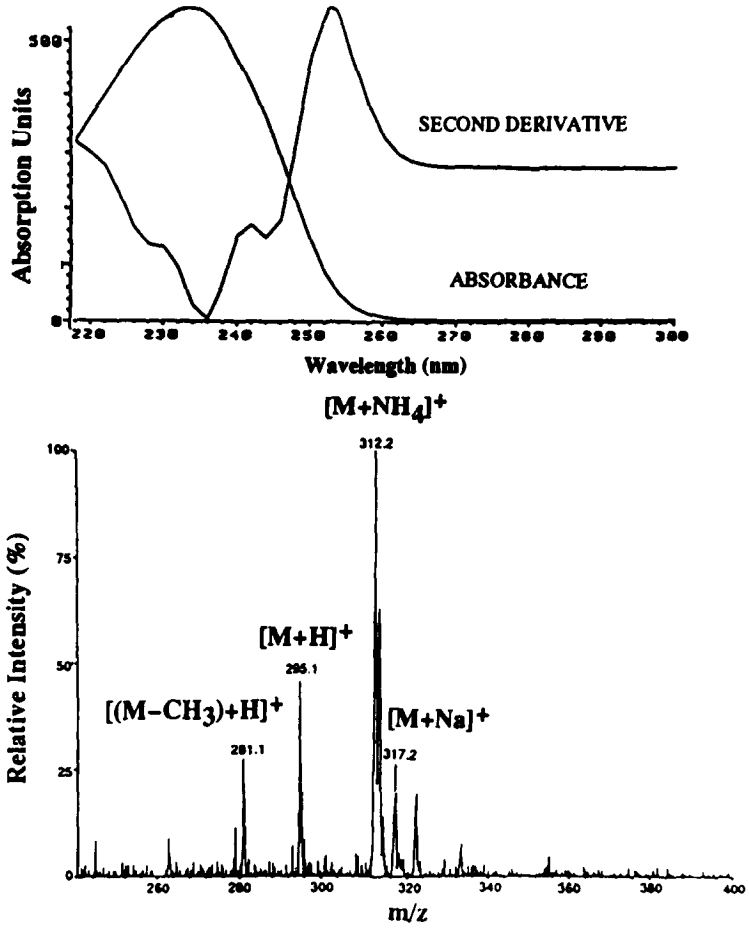


Figure 6 Conventional and second derivative UV spectra (upper graph), and mass spectrum (lower graph), of peak 1 in *Figure 5*.

fatty acid hydroperoxides or hydroxides in phospholipids. Previously,[12] we were able to readily detect, by means of HPLC analyses coupled to second derivative UV spectrophotometry, fatty acid hydroperoxides and hydroxides in liver phospholipids of rats treated with CCl_4, a well-proven pro-oxidant[34]; however, none were detected when the same methodologies were used to analyze liver phospholipids from CD rats. No fatty acid hydroperoxides or hydroxides were detected in the present study either. It is apparent, therefore, that the effects of a CD diet on rat liver, which have been attributed to lipoperoxidation,[1] must be open to alternate explanations. Production of CLA by free radical activity has been deemed to be extremely unlikely.[35,36]

One peculiarity of the CD diet model of hepatocarcinogenesis is that it results in tumor induction in male but not in female F-344 rats.[8,9] In the present study, no sex differences were observed in the level of CFA present in liver lipids of rats fed the PHO-diets. Thus, even assuming that

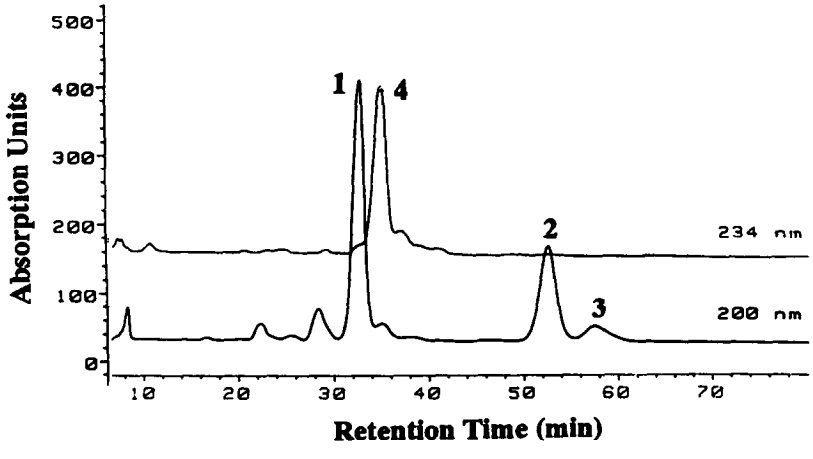


Figure 7 HPLC chromatogram of fatty acid methyl esters, prepared from adipose tissue lipids of a female rat fed the CDPHO diet, recorded with the photodiode array detector at 200 and 234 nm. (1) methyl linoleate, (2) methyl oleate, (3) methyl elaidate, and (4) methyl ester(s) of 18:2 isomers with conjugated dienes.

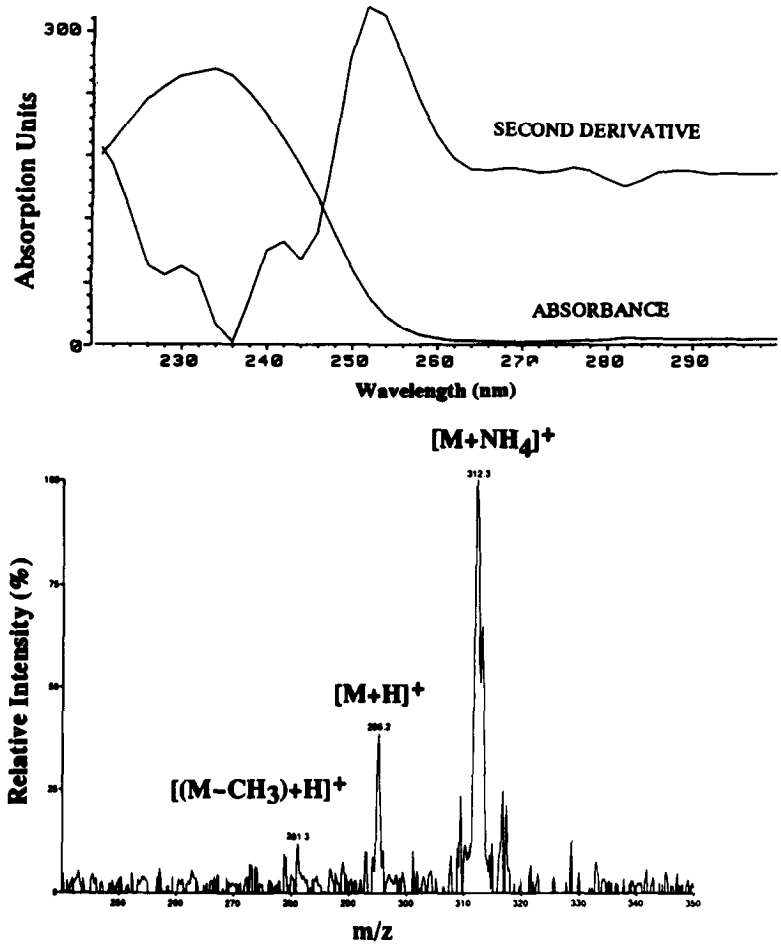


Figure 8 Conventional and second derivative UV spectra (upper graph) and mass spectrum (lower graph) of peak 4 in *Figure 7*.

the CFA reflected indeed a process of lipid peroxidation, it is apparent that the latter could not be the key alteration underlying the genesis of the tumors.[1] Moreover, liver tumors have never been reported to develop in rats fed control, choline-supplemented diets.[1–7]

Finally, our findings highlight the inadequacy of relying exclusively on detection of conjugated dienes, to evaluate lipid peroxidation in animals fed semipurified diets containing a PHO.

Table 4 Conjugated diene isomers of linoleic acid (CLA) in rat adipose tissue total lipids

	CLA (μg/mg of fat)	
Diet fed	Male rats	Female rats
CDPHO*	$13.30 \pm 1.02^{c,d}$	$12.90 \pm 1.21^{c,d}$
CSPHO	$13.11 \pm 0.75^{c,d}$	$12.60 \pm 0.87^{c,d}$
CDCO	$1.27 \pm 0.43^{a,b}$	$1.01 \pm 0.54^{a,b}$
CSCO	$1.41 \pm 0.50^{a,b}$	$1.30 \pm 0.45^{a,b}$

Each value represents the mean ± SD of fatty acid methyl ester analyses of the lipids from 3 rats.
*See legend to Table 2.
Superscript letters after SD as in Table 2.

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