Newly Recognized Anticarcinogenic Fatty Acids: Identification and Quantification in Natural and Processed Cheeses

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We previously isolated and identified a mixture of isomeric derivatives of *c*-9,*c*-12-octadecadienoic acid (linoleic acid) containing a conjugated double-bond system (designated CLA) in extracts of grilled ground beef. Synthetically prepared CLA was effective in partially inhibiting the initiation of mouse epidermal carcinogenesis by 7,12-dimethylbenz[*a*]anthracene. We now report that CLA is present in various natural and processed cheeses. A capillary GC/reversed-phase HPLC method was developed that separated nine CLA isomers from samples. Among the dairy products tested, the CLA content ranged from 28.3 ppm (raw whole milk) to 1815 ppm (Cheese Whiz), whereas grilled ground beef contained 994 ppm. Of the isomers, *c*-9,*t*-11-, *t*-10,*c*-12-, *t*-9,*t*-11-, and *t*-10,*t*-12-octadecadienoic acids accounted for more than 89% of total CLA, while the *c*-9,*c*-11-, *t*-9,*c*-11-, *c*-10,*c*-12-, *c*-10,*t*-12-, and *c*-11,*c*-13-octadecadienoic acids were minor contributors. Possible sources and mechanisms of formation of CLA are discussed.

The relationship between diet and cancer risk is exceedingly complex (Doll and Peto, 1981). Factors that appear to enhance carcinogenesis under one set of experimental conditions may have no effect or even inhibit carcinogenesis under different conditions (Pariza, 1988). With specific regard to meat and dietary fat, there are now several epidemiologic reports linking increased consumption with decreased risk of cancer of esophagus (Tuyns et al., 1987; Ziegler et al., 1981) and stomach (Geboers et al., 1985; Hill, 1987). Moreover, in a prospective epidemiologic study, Hirayama (1985) reported that colon cancer mortality was 3–4 times lower among Japanese subjects consuming diets containing both green and yellow vegetables *and* meat on a daily basis as opposed to those consuming diets containing either green and yellow vegetables *or* meat on a daily basis. This provocative finding suggests that interactions among cancer inhibitors from plant and animal food sources may be important in determining cancer risk in man.

We recently isolated from grilled ground beef isomeric derivatives of *c*-9,*c*-12-octadecadienoic acid (linoleic acid) containing a conjugated double-bond system (designated CLA) (Ha et al., 1987). Synthetically prepared CLA inhibited the initiation of mouse skin carcinogenesis by 7,12-dimethylbenz[*a*]anthracene (Ha et al., 1987) and forestomach tumorigenesis induced by benzo[*a*]pyrene (unpublished results). The mechanism of inhibition is not yet known. Studies in this area are of particular importance given that CLA has been isolated from human serum, bile, and duodenal juices (Cawood et al., 1983). The origin of CLA in human fluids is not known, but heat-processed meats and other fatty foods should be investigated as possible contributing sources.

The mechanism of CLA formation in foods is not understood very well, but heat treatment (Ha et al., 1987), free-radical-type oxidation of linoleic acid (Cawood et al., 1983), and microbial enzymatic reactions involving linoleic or linolenic acids in the rumen (Gurr, 1987; Viviani, 1970) are thought to be major contributors. Conjugated dienoic acids were determined in butterfat (Bartlet and Chapman, 1961; Scott et al., 1959) and in milkfat (Riel, 1963; Parodi, 1977) by spectrophotometric or GLC methods. A capillary GC method (Lanza and Slover, 1981) and a combination of UV spectrophotometry with HPLC (Brown and Snyder, 1982) were used to determine CLA in shortenings and soy oil, respectively. These methods lack specificity. Attempts were made to separate individual CLA isomers prepared from alkali-isomerized linoleic acid using a polar or nonpolar capillary column (Scholfield, 1981; Scholfield and Dutton, 1971). Because of the unknown biological function of CLA in vivo and the separation difficulties, little data on CLA in food are available. This paper describes the identification and quantification of CLA isomers in cheese and ground beef.

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Table I. Products Studied in This Investigation

	sample	descriptive characteristics
1.	Parmesan cheese (grated)	part-skim milk, cheese culture, enzymes, aged over 10 months
2.	cheddar cheese (American)	whole milk, cheese culture, enzymes, aged over 6 months
3.	Romano cheese (grated)	part-skim cow's milk, cheese culture, enzymes, aged over 5 months
4.	blue cheese	whole milk, cheese culture, enzymes, aged over 100 days
5.	pasteurized proc cheese (American)	American cheese, milkfat
6.	cream cheese	pasteurized milk, cream, cheese culture
7.	cheese spread (Roka blue)	pasteurized milk, milkfat, cream cheese
8.	Cheese Whiz	cheddar cheese, low-moisture part-skim mozzarella cheese, whey concentrate
9.	milk	
	pasteurized whole	cow's milk
	nonpasteurized whole	cow's milk
10.	ground beef	uncooked or grilled

MATERIALS AND METHODS

Materials. Organic solvents (HPLC grade; Burdick and Jackson Chemical Co., Muskegon, MI); 12-hydroxy-*c*-9-octadecenoic acid (ricinoleic acid, 99%), 12-hydroxy-*t*-9-octadecenoic acid (ricinelaidic acid, 99%), *c*-9,*c*-12-octadecadienoic acid (linoleic acid, 99%), and other fatty acid standards (Sigma Chemical Company, St. Louis, MO); and trifluoroacetic anhydride, (*R*)-(−)-2-phenylbutyric acid (PBA) and 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) (Aldrich Chemical Co., Milwaukee, WI) were used. A 1.0 mg of PBA/0.1 mL concentration was prepared with a 2:1 chloroform–methanol (v/v) mixture. Alkali-isomerized linoleic acid was prepared according to the method as

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previously described (Ha et al., 1987). Cheese and ground beef samples were purchased from a commercial grocery, and milk samples were obtained from the Department of Food Science, University of Wisconsin–Madison (Table I).

Preparation of CLA Isomer Standards. The methyl ester of *t*-10,*c*-12-octadecadienoate was prepared by crystallization from methyl esters of alkali-isomerized linoleic acid (Scholfield and Koritala, 1970). Methyl *t*-10,*t*-12- and *c*-10,*c*-12-octadecadienoate were prepared from the *t*-10,*c*-12 isomer by iodine and light isomerization (Tolberg and Wheeler, 1958). The prepared 10,12 isomers were purified by normal-phase semipreparatory HPLC as described below in HPLC Separation. A typical semipreparatory normal-phase HPLC profile of the methyl *t*-10,*c*-12-octadecadienoate prepared exhibited three components (peaks: 1, 40.1 min; 2, 47.5 min; 3, 65.1 min), which were present in relative proportions of 89, 2, and 9%, respectively. Subsequent capillary GC analyses, using conditions described in the GC section, of these peaks revealed that peak 1 is a methyl t-10,c-12 isomer of greater than 95% purity, while peaks 2 and 3 are unknown impurities. The remaining 10,12 geometrical CLA standards were similarly purified by this HPLC procedure.

The 9,11-octadecadienoic acid isomers (c,c; c,t; t,t) were prepared from ricinoleic acid or ricinelaidic acid (Schneider et al., 1964), and the individual isomers were separated by the argentation HPLC method (Scholfield, 1980).

Preparation of Free CLA. This procedure includes extraction and saponification of CLA. Sample material (1 g) containing 1.0 mg of PBA (internal standard) was homogenized with 20 mL of 2:1 chloroform–methanol (v/v) for 60 s in a Polytron homogenizer (Brinkman instruments, Westbury, NY) at medium speed. Another 10 mL of the chloroform–methanol mixture was used to rinse the Polytron probe and combined with the homogenate, followed by addition of 10 mL of double-distilled water. For milk, 5 g of sample, 5.0 mg of PBA, and 150 mL of the chloroform–methanol mixture were used. The homogenate was centrifuged at 2000 rpm for 30 min (4 °C). The organic layer was separated, dried over anhydrous Na_2SO_4, and rotoevaporated. Total fat content was determined from the residue. Free fatty acids were prepared by heating the fat extracts in 2 mL of 1.0 N sodium hydroxide in methanol (v/v) in a screw-capped test tube (15 × 1.5 cm). After being heated in a boiling water bath for 15 min, the solution was acidified to pH 1 with 5.5 N sulfuric acid in water (v/v). The free fatty acids were extracted with 3 × 10 mL portions of heptane. The organic extract was washed with water and dried over anhydrous Na_2SO_4, and the filtered solvent was removed under vacuum with a rotary evaporator.

The effect of this procedure on CLA formation was investigated. When linoleic acid (2.0 mg) was subjected to the procedure, no CLA was detected as determined by UV absorbance at 235 nm using a Beckman DU-50 spectrophotometer (Berkley, CA) and by the semipreparatory reversed-phase HPLC described below. This finding indicates that CLA was not formed as a result of extraction/saponification by our methods.

HPLC Separation. Separation and purification of CLA by HPLC were performed at room temperature on a Beckman Model 421A microcontroller system (Fullerton, CA) fitted with two solvent delivery modules (Beckman Model 110A) and a dual-channel UV detector (Micromeritics 788 Model; Norcross, GA). Eluent was monitored at 235 or 245 nm. Peak areas were recorded with a Spectra Physics 4270 integrator (Arlington, IL). CLA in the sample was separated on a semipreparatory reversed-phase column (Ultrasphere-ODS, 5 μm, 250 × 10 mm (i.d.), Beckman) with a gradient mobile phase (acetonitrile and water) as previously reported (Ha et al., 1987). The purification of individual isomers or alkali-isomerized linoleic acid was performed on a normal-phase semipreparatory column (Ultrasil-NH_2, 5 μm, 250 mm × 10 mm (i.d.), Beckman) with a gradient system. The starting mobile phase (99:1 hexane–ethanol, v/v) and flow rate (1.0 mL/min) were maintained for 20 min, and then both hexane proportion and flow rate were linearly increased to 100% and 4.0 mL/min, respectively, over 20 min. These conditions were held for an additional 40 min and then returned to the starting conditions for 10 min. The system was reequilibrated at least 10 min prior to the next injection.

Preparation of CLA Derivatives. CLA methyl esters were prepared from the free-acid form with boron trifluoride–methanol according to AOCS Method Ce2-66 (1973). The PTAD derivative of CLA methyl ester was prepared according to the method of Young et al. (1987) after methylation of CLA.

GC Analysis. GC analysis of CLA methyl ester or CLA methyl ester derivatized with PTAD was carried out with a Varian 3700 gas chromatograph fitted with a flame ionization detector (FID) and a Spectra Physics 4270 integrator. The column used was a Supelcowax-10 fused silica capillary column (Supelco Inc.) (60 m × 0.32 mm (i.d.), 0.25-μm film thickness). GC conditions consisted of an on-column injection system with helium as the carrier gas at 2 mL/min linear gas flow rate. Temperatures were programmed as follows: oven, 50–200 °C at 20 °C/min and held for 60 min; injector, 50–200 °C at 100 °C/min after injection. Detector temperature was 250 °C. The volume injected ranged from 1.0 to 2.0 μL, containing 0.5–5.0 μg of CLA/μL.

GC–MS Analysis. GC–MS analysis was conducted with a Finnigan 4510 GC-EI/CI automated mass spectrometer system (Sunvalle, CA) using a splitless injector and a Supelcowax-10 capillary column (60 m × 0.32 mm (i.d.), 2.5-μm film thickness). The column temperature was programmed as specified in GC Analysis. Electronic impact (EI) ionization and chemical ionization (CI) were carried out at 70 eV and 100 °C as a source temperature. The CI spectrum was obtained with isobutane as a reagent gas. For the analysis of PTAD derivatives of CLA methyl esters, a DB-5 glass capillary column (30 m × 0.3 mm (i.d.), 1.0-μm film thickness) was used with a temperature program: 60–250 °C at 10 °C/min after 1-min holding at 60 °C. The data were analyzed by a Data General NOVA/4 system (Data General Corp., Santhboro, MT) equipped with a CDC-CMD disk driver (Magnetic Peripherals Inc., Oklahoma City).

GC–FT/IR Analysis. GC–FT/IR analysis was performed with a Nicolet Model 60S FT/IR (Schaumburg, IL) using a Supelcowax-10 capillary column (60 m × 0.32 mm (i.d.), 0.25-μm film thickness). GC conditions were the same as those for GC analysis.

CLA Quantification. Quantification of individual CLA isomers in a sample was based on the internal standard method. To obtain correction factors (CF) for individual CLA isomers, a reference mixture consisting of known amounts of the isomers plus PBA was subjected to the extraction procedure and reversed-phase HPLC analysis. Pooled CLA and PBA peaks from the HPLC were chromatographed on a capillary GC column (Supelcowax-10) after methylation. The CF for the individual isomers was calculated as follows: $CF_x = (area_{IS}/weight_{IS}) \times (weight_x/area_x)$, where the subscript IS refers to internal

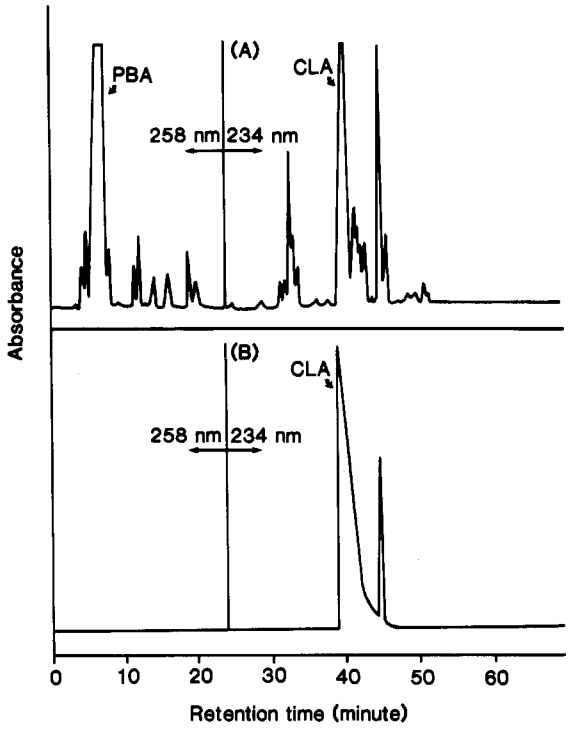

Figure 1. Semipreparatory reversed-phase HPLC elution profiles of Cheese Whiz sample containing PBA (A) and alkali-isomerized linoleic acid (B).

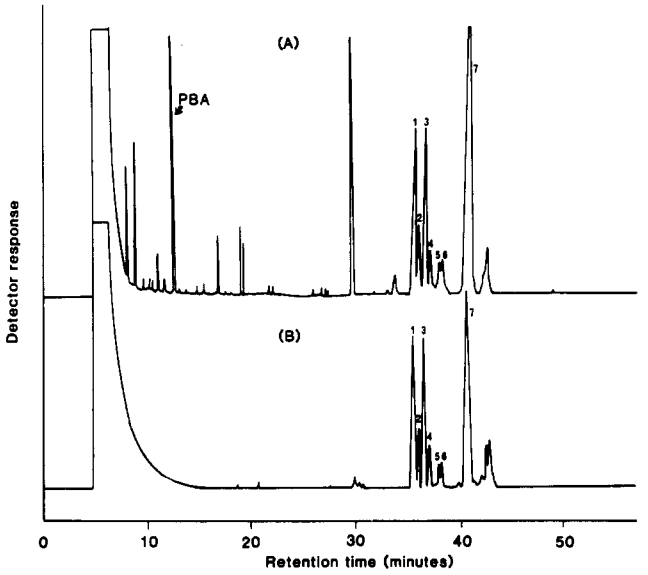

Figure 2. Capillary (Supelcowax-10 column) GC elution profiles of methylated CLA obtained form Figure 1A (A) and methylated alkali-isomerized linoleic acid (B) obtained from Figure 1B. Peak identification: 1–7, CLA methyl esters.

standard and the subscript x refers to a given CLA isomer. Using CF_x, the amount of each CLA isomer in the sample was calculated by $ppm_x = [(area_x/area_{IS}) \times weight_{IS}\ (mg)]/sample\ (g) \times CF_x \times 1000$.

RESULTS

GC/HPLC Separation. A reversed-phase semipreparatory HPLC of the sample effected separation of CLA from the other saturated or unsaturated fatty acids (Figure 1). Subsequent GC analysis of the methylated CLA peak indicated that seven components (peaks 1–7) eluted after linolenic acid; these peaks exhibited retention times identical with those of the methylated alkali-isomerized linoleic acid components (Figure 2). Two approaches were employed to identify the isomers: (1) determination of ECL values of CLA isomers; (2) spectral analyses of the CLA sample or alkali-isomerized linoleic acid containing unidentified peaks for which standards are not available or are difficult to obtain.

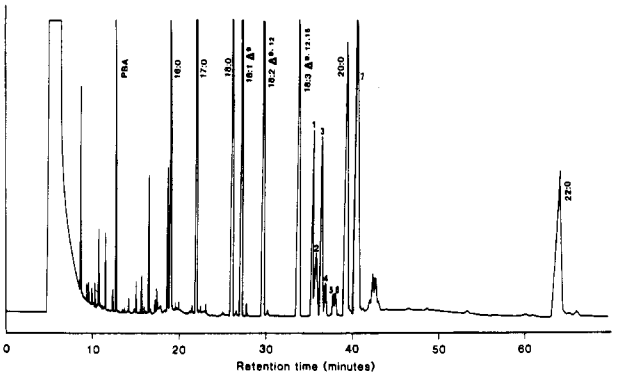

Figure 3. Capillary (Supelcowax-10 column) GC elution profile of fatty acid methyl esters. Various fatty acid methyl esters were cochromatographed with the methylated CLA sample obtained from Figure 1B.

Table II. Equivalent Chain Length (ECL) of CLA Isomers (Methyl Esters) on a Supelcowax-10 Fused Silica Capillary Column (60 m × 0.32 mm (i.d.), 0.25-μm Film Thickness)

peak no.[a]	ECL present study[b]	ECL Scholfield method[c]	ΔECL[d]	isomer identified by Scholfield[c]	isomer identified by present study[e]
1	19.49	20.72	1.23	c-9,t-11	c-9,t-11/t-9,c-11
2	19.53				c-10,t-12
3	19.62	20.86	1.24	t-10,c-12	t-10,c-12
4	19.67				c-11,c-13
5	19.80				c-9,c-11
6	19.82				c-10,c-12
7	20.01	21.22	1.21	t-9,t-11	t-9,t-11/t-10,t-12
		21.23	1.22	t-10,t-12	

[a] In Figure 2A. [b] ECL was calculated by the method of Scholfield (1981). [c] Data were obtained from Scholfield (1981) analyzed on a Silar-10C capillary column (50 m × 0.25 mm (i.d.)) at 200 °C. [d] ΔECL represents the absolute value obtained from the difference between two ECL values in same row. [e] Peaks were identified by cochromatography of standard and/or GC–MS and GC–FT/IR spectral analyses.

Determination of ECL. A chromatogram shown in Figure 3 presents the GC profile (Supelcowax-10) of the methyl esters of saturated fatty acid standards (C16:0, C17:0, C18:0, C20:0, C22:0) plus the methylated CLA sample obtained from Figure 1B. ECL values of CLA methyl ester isomers were determined by plotting carbon numbers vs retention times on semilog paper as described by Miwa et al. (1960) and Scholfield (1981). The ECL values of CLA methyl esters are shown in Table II, ranging from 19.49 for peak 1 to 20.01 for peak 7. Scholfield et al. (1981) and Scholfield and Dutton (1971) have reported that ECL values and elution orders for some of the geometrical/positional isomers of CLA methyl esters separated on a 100-m glass capillary Silar 10C column (Table II). The difference in ECL (ΔECL) between the two columns for the tested compounds is also shown (Table II). ΔECL remained constant within 0.01–0.03 unit for the isomers tested by both columns. The correlation coefficient (r value) was 0.9995 for the available standards on the two columns. The column (Supelcowax-10) that we used is only slightly less polar than a Silar 10C column. Therefore, the ECL data are comparable.

A CLA methyl ester standard was cochromatographed with a methylated CLA sample containing unidentified peaks. For those peaks that cochromatographed with a standard CLA methyl ester, identity is set as such. This relationship was then used to determine the identities of

the remaining unknown peaks.

According to the ECL relationship and cochromatographic results, peaks 1, 3, and 5–7 in Figure 2 were identified as methyl esters of *c*-9,*t*-11- and/or *t*-9,*c*-11-, *t*-10,*c*-12-, *c*-9,*c*-11-, and *c*-10,*c*-12-, and *t*-9,*t*-11- and/or *t*-10,*t*-12-octadecadienoate, respectively (Table II).

Identification of Peaks 2 and 4. The methyl esters of alkali-isomerized linoleic acid or sample CLA were subjected to GC–MS and GC–FT/IR analyses. EI-MS data of peaks 2 and 4 in Figure 2 are identical, yielding 67 (base peak), 294 (M^+), 74, 59, and 262; hence, these isomers could not be distinguished by this method.

Since under normal EI ionization conditions double bonds can migrate prior to fragmentation, making it difficult to determine their original positions, Doorlittle et al. (1985) used CI-MS to identify the double-bond position in hydrocarbon chains and fatty acids. CI-MS data of CLA methyl esters of peaks 2 and 4 exhibited a molecular weight of 294 ($M^+ + 1$, 295, 100%). Typical fragments (*m/e*) for peak 2 were 113 (3%), 213 (5%), 139 (1%), and 239 (12%) and for peak 4 were 99 (5%), 227 (8%), 125 (1%), and 253 (13%). Cleavage between carbons 10 and 11 and between 12 and 13 double bonds numbered from the carboxyl group yielded *m/e* 113 and 213, respectively. Cleavage between the 8–9 and 14–15 single-bond carbons produced *m/e* 139 and 239, respectively. Hence, peak 2 is identified as a 10,12 positional isomer of methyl octadecadienoate. Peak 4 had *m/e* 99 derived from cleavage of the double bond between carbons 11 and 12, *m/e* 227 from cleavage of the double bond between carbons 13 and 14, *m/e* 125 derived from single-bond cleavage between carbons 9 and 10, and *m/e* 253 from single-bond cleavage between carbons 15 and 16, indicating that this compound is an 11,13 positional isomer.

Additionally, peaks 1 and 5 contained *m/e* 127, 199, 153, and 225, indicating 9,11 isomers. Similarly, peaks 3 and 6 contained *m/e* 113, 139, 213, and 239 and were identified as 10,12 isomers. Peak 7 contaned *m/e* of both 9,11 and 10,12 isomers.

The CLA sample (methyl esters) derivatized with PTAD was chromatographed on a Supelcowax-10 column. All peaks of CLA methyl esters disappeared from the GC profile compared with those of underivatized CLA methyl esters (Figure 4). Since PTAD is electrophilic and therefore only reacts with a conjugated double-bond system in hydrocarbon chains or fatty acids via Diels–Alder reaction (Young et al., 1987), peaks 1–7 are identified as CLA positional isomers. The PTAD derivatives of CLA methyl esters (molecular weight 467) that had relatively high polarity were not eluted under these conditions. The Supelcowax-10 (polar) column was changed to a DB-5 (nonpolar) column to elute the derivatives. A different elution pattern was obtained from that observed with the former column. This method will not identify positional isomers, but it confirms the presence of the conjugated double bond in the sample and also indicates the location of CLA methyl esters in the GC chromatogram from the Supelcowax-10 column.

The major differences in GC–FT/IR spectra of peaks 2 and 4 were at the 1000–800-cm^{-1} range. Sharp absorption at 990 and 945 cm^{-1} (peak 2) and broad absorption at 990 cm^{-1} (peak 4) were observed, indicating that peak 2 is a cis,trans isomer and peak 4 a cis,cis isomer (Tolberg and Wheeler, 1958).

On the basis of results of spectral analyses, cochromatography, and ECL values, peaks 1–7 were identified as methyl esters of *c*-9,*t*-11- and/or *t*-9,*c*-11-, *c*-10,*t*-12-, *t*-10,*c*-12-, *c*-11,*c*-13-, *c*-9,*c*-11-, and *c*-10,*c*-12-, and *t*-9,*t*-11- and/or *t*-10,*t*-12-octadecadienoate, respectively.

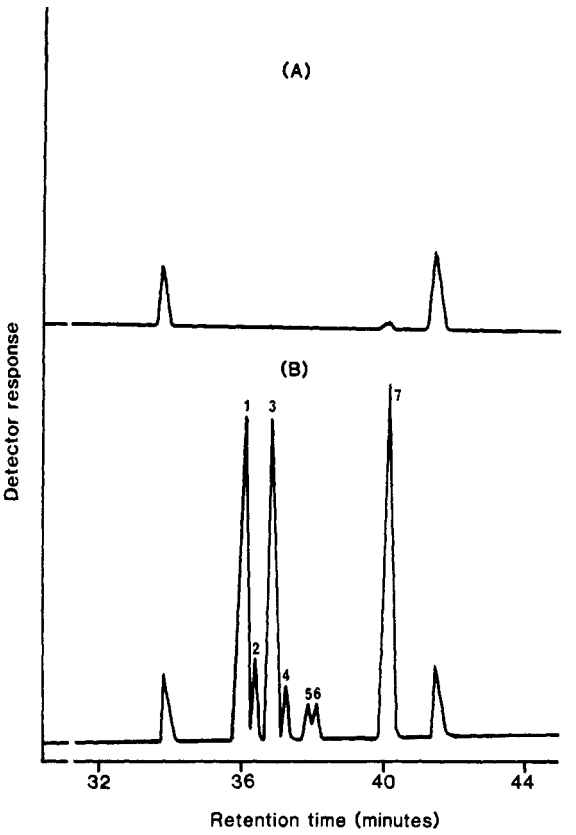

Figure 4. Capillary (Supelcowax-10 column) GC elution profiles of PTAD-derivatized (A) or nonderivatized (B) CLA (methylated) obtained from Figure 1A. Peak identification: 1–7, CLA methyl esters.

Application. The newly developed GC/HPLC method to analyze individual CLA isomers was applied to dairy products and beef. A CLA sample containing PBA was purified on the semipreparatory reversed-phase column (Figure 1A). PBA was eluted at 6.2 min and CLA at 40 min. The two pooled peaks were dried over anhydrous Na_2SO_4, and the organic solvent was evaporated under nitrogen. After methylation of the residue, it was analyzed by GC. A typical chromatogram shown in Figure 2A presents the profile of CLA isomers obtained from Cheese Whiz, and the identities are given in Table II. PBA was coeluted with some impurities on the HPLC column, but these impurities did not interfere with CLA isomer resolutions on the GC column.

Quantification of peaks 2 (c-10,t-12 isomer) and 4 (c-11,c-13 isomer) was based on an assumption that CF values of these isomers are equal to the average CF values of the remaining five CLA isomers: 0.17, c-9,t-11 isomer (peak 1); 0.16, t-10,c-12 isomer (peak 3); 0.17, c-9,c-11 isomer (peak 5); 0.16, c-10,c-12 isomer (peak 6); 0.17, t-9,t-11 or t-10,t-12 isomer (peak 7). The analytical data of CLA are shown in Table III. Total CLA content among cheeses ranged from 169.3 ppm (blue cheese) to 1815 ppm (Cheese Whiz). Of the aged natural cheeses (samples 1–4), Parmesan cheese aged more than 10 months contained the highest (622.3 ppm) and blue cheese aged over 100 days contained the lowest amounts of CLA (169.3 ppm), suggesting a positive relationship between the aging period and CLA content. In general, processed cheese contained more CLA than nautral cheese. It is interesting to note that raw and pasteurized whole milk both contained similar amounts of CLA. Grilled ground beef contained 994 ppm of total CLA, while uncooked ground beef contained 561.7 ppm. Fat content ranged from 4.0% (pasteurized

Table III. CLA Isomer Content in Samples[a]

	sample	CLA isomer in sample,[b] ppm 1	2	3	4	5	6	7	total	total fat,[c] %	total CLA in fat, ppm
1.	Parmesan cheese	147.3[d]	10.7	42.8	15.4	13.8	9.1	383.2	622.3 ± 15.0[e]	32.3 ± 0.9	1926.7
2.	cheddar cheese	79.5	10.0	60.4	16.5	3.2	1.9	269.1	440.6 ± 14.5	32.5 ± 1.7	1355.7
3.	Romano cheese	56.2	7.7	27.4	6.0	6.7	3.1	249.8	356.9 ± 6.3	32.1 ± 0.8	1111.9
4.	blue cheese	25.9	12.2	13.2	1.5	1.5	1.2	113.7	169.3 ± 8.9	30.8 ± 1.5	549.8
5.	processed cheese	102.8	16.3	49.3	14.5	7.6	5.3	378.3	574.1 ± 24.8	31.8 ± 1.1	1805.3
6	cream cheese	29.0	12.9	11.0	4.1	10.1	6.2	261.2	334.5 ± 13.3	35.5 ± 1.0	942.3
7.	Roka blue spread	32.4	3.4	24.2	8.9	2.2	2.9	128.6	202.6 ± 6.1	20.2 ± 0.8	1003.0
8.	Cheese Whiz	381.1	52.8	322.9	23.8	25.3	15.2	993.9	1815.0 ± 90.3	20.6 ± 1.1	8810.7
9.	milk										
	pasteurized whole	17.0	0.5	6.1	0.5	t[f]	t	4.2	28.3 ± 1.9	4.0 ± 0.3	707.5
	nonpasteurized whole	20.1	2.0	6.6	t	t	t	5.3	34.0 ± 1.0	4.1 ± 0.1	829.3
10.	ground beef										
	grilled	187.9	56.7	145.1	19.9	29.8	8.9	545.7	994.0 ± 30.9	10.7 ± 0.3	9289.7
	uncooked	116.8	23.6	109.5	10.7	18.5	2.8	279.8	561.7 ± 22.0	27.4 ± 0.2	2050.0

[a] CLA was analyzed by GC using a Supelcowax-10 capillary column (60 m × 0.32 mm (i.d.), 0.25-μm film thickness). [b] Identity of each isomer was shown in Table II. [c] Total fat was determined by the solvent extraction method (2:1 chloroform–methanol, v/v). [d] Reported values are the means of three measurements. [e] Mean ± SEM. [f] t = trace, less than 0.10 ppm. Statistics: Tukey's $w(0.05)$ = 71.6 for total CLA.

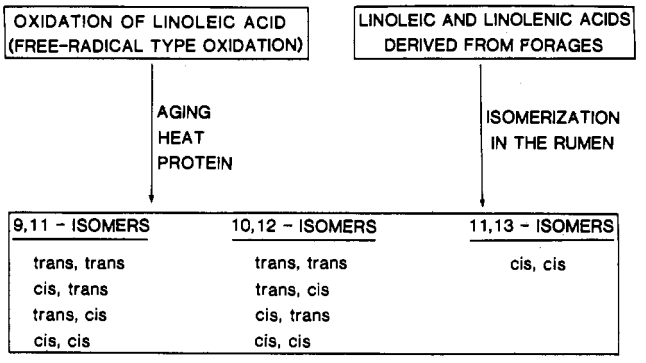

Figure 5. Factors that may contribute to CLA occurrence in dairy products and meat.

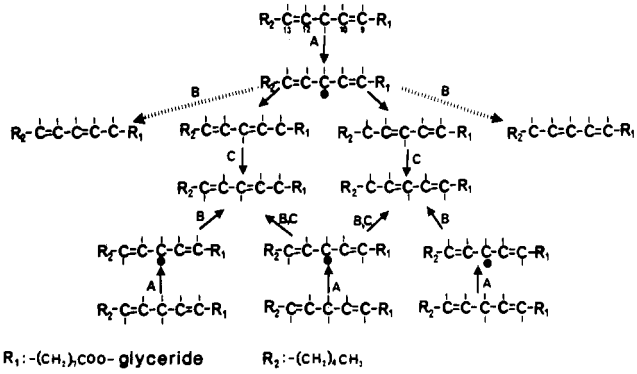

Figure 6. Model for CLA formation in dairy products and meat. Starting materials are *c*-9,*c*-12-octadecadienoic acid (top) or its geometric isomers (c-9,t-12; t-9,c-12; and t-9,t-12) in glycerides or phospholipids (bottom). Reaction: A, initiation; B, isomerization; C, stabilization. Solid line represents a major pathway and dotted line a minor pathway.

whole milk) to 35.5% (cream cheese). On the basis of total fat, the CLA content ranged from 549.8 ppm (blue cheese) to 9289.7 ppm (grilled ground beef).

Of the individual isomers, *t*-9,*t*-11-/*t*-10,*t*-12-, *c*-9,*t*-11-/*t*-9,*c*-11-, and *t*-10,*c*-12-octadecadienoic acid accounted for more than 89% of the total CLA in all measured samples. the t,t isomers ranged from 49.8% (uncooked ground beef) to 78.1% (cream cheese); however, in milk samples approximately 15% of the CLA was present as t,t isomers. The remaining CLA isomers (c-9,c-11; c-10,c-12; c-10,t-12; c-11,c-13) contributed less than 11% of the total CLA in the samples.

DISCUSSION

Origin of CLA. The origin of CLA in cheese and ground beef is not known. On the basis of our findings we propose the models shown in Figures 5 and 6. CLA formation may be attributed to two parameters as shown in Figure 5: (1) free-radical-type oxidation of linoleic acid effected by aging, heat treatment, and protein quality; (2) isomerization of linoleic and linolenic acids in the rumen.

Aging processes modify the physiochemical properties of cheese or beef to give typical characteristics. Examples include the oxidation of fats. Under anaerobic conditions this may occur during heating, where oxidation of linoleic acid in glycerides or phospholipids may be initiated to form an allyl radical. The radical would be stabilized through the formation of its resonance forms, which require hydrogens to form a conjugated double-bond system (steps A and B, Figure 6). The hydrogens are attributed to proteins that, in turn, form protein radicals. These radicals may be neutralized by α-tocopherol in the lipophilic regions (Pascoe et al., 1987). Iversen et al. (1984) found that when linoleic acid was oxidized by UV irradiation in the presence of albumin, 9,11-conjugated linoleic acid was formed rather than oxidation products, suggesting the importance of protein as a hydrogen source. The importance of protein in CLA formation was also supported by our finding that lactalbumin- and lactoglubulin-enriched cheese contained significantly higher amounts of CLA than cheeses not enriched (Table III). Cheese Whiz, which is enriched with whey concentrate (Table I), contained twice as much CLA as the other processed cheeses. Whey protein contains relatively high levels of lactalbumin and lactoglobulin (Harper, 1983; McDermott, 1987) that could provide a hydrogen source.

CLA isomers that were isomerized from linoleic and linolenic acids in the rumen may contribute directly to the CLA content in cheese or meat. A significant amount of CLA was present in raw whole milk and pasteurized whole milk (Table III). Strocchi et al. (1967) observed a positive correlation of conjugated dienoic C_{18} fatty acids of milk with trans isomers and linoleic acid in the diet. These conjugated dienoic fatty acids were also present in butter (1–4.5%) and were directly related to the linolenic acid content in the diet of cows (Bartlet and Chapman, 1961; Kuzdzal-Savoie, 1966; Viviani, 1970). During biohydrogenation of linoleic or linolenic acid by microorganisms in the rumen, cis double bonds undergo extensive isomerization (Gurr, 1987; Viviani, 1970). This may involve a shift in position along with the carbon chain (positional isom-

erization) or a change of geometrical configuration or both. Isomerization would be the rate-controlling step and would determine the final concentration of CLA. In milk or ruminant animal tissues, the isomer ratio is effected by the microbial population in the rumen which, in turn, is influenced by the amount of linoleic acid and/or linolenic acid fed (Viviani et al., 1970).

Formation of Positional/Geometrical Isomers. The t-9,t-11 and t-10,t-12 isomers and the c-9,t-11 and t-9,c-11 isomers could not be separately quantified in this study. However, if we assume that each of the t,t isomers contributes equally to the total amount of peak 7 and that the total concentration of the t-9,c-11 isomer coeluted with c-9,t-11 isomer in peak 1 is equal to the concentration of the c-10,t-12 isomer of peak 2, then the following conclusions may be drawn: (1) The molar concentration of the 9,11 positional isomer is identical with the molar concentration of the 10,12 positional isomer. (2) The concentrations of c-9,t-11 and t-10,c-12 isomers are equal. (3) There are four major isomers (t-9,t-11, c-9,t-11, t-10,t-12, t-10,c-12) and five minor isomers (c-9,c-11, t-9,c-11, c-10,c-12, c-10,t-12, c-11,c-13-). (4) There is a relatively higher concentration (61–78.1% of total CLA in cheese) of t,t isomers. These conclusions might be explained by the isomerization of linoleic acid and/or linoleic acid geometrical isomers (c-9,t-12, t-9,t-12, and t-9,c-12 isomers) as shown in Figure 6.

Linoleic acid radical containing an unpaired electron on the methylene-interrupted carbon (carbon number 11) would stabilize to form resonances via an electron shift. The shift occurs in either the carboxyl group or hydrocarbon terminal direction to make a conjugated double bond. The probability of the formation of 9,11 or 10,12 isomer is equal due to the distance of the carboxyl group from the double-bond system and/or to the esterified carboxyl group in the glycerides or phospholipids.

Theoretically, eight possible geometric isomers of 9,11- and 10,12-octadecadienoic acid (c-9,c-11; c-9,t-11; t-9,c-11; t-9,t-11; c-10,c-12; c-10,t-12; t-10,c-12; t-10,t-12) would form from the isomerization of *c*-9,*c*-12-octadecadienoic acid. As a result of the isomerization, only four isomers (c-9,c-11; c-9,t-11; t-10,c-12; c-10,c-12) would be expected (step B, Figure 6). However, of the four isomers, c-9,t-11 and t-10,c-12 isomers are predominantly produced during the autoxidation or alkali isomerization of *c*-9,*c*-12-linoleic acid due to the coplanar characteristics of five carbon atoms around a conjugated double bond and spacial conflict of the resonance radical or anion (Nichols et al., 1951). The remaining two c,c isomers are minor contributors.

The relatively higher distribution of the t,t isomers of 9,11- or 10,12-octadecadienoic acid in the sample apparently resulted from the further stabilization of c-9,t-11 or t-10,c-12 geometric isomers (step c, Figure 6), which is thermodynamicaly preferred, during an extended processing time or long aging period. Additionally the t,t isomer of 9,11- or 10,12-octadecadienoic acid that was predominantly formed during the isomerization of linoleic acid geometrical isomers (*t*-9,*t*-12-, *c*-9,*t*-12-, and *t*-9,*c*-12-octadecadienoic acid) (Nichols et al., 1951) may influence the final ratio of the isomers or the final CLA content in the samples. These linoleic acid geometrical isomers accounted for up to 11% of the milk fat (Deman and Deman, 1983) and for 13.6% of linoleic acid content in beef (Lanza and Slover, 1981). In the case of milk, we observed only 15% of the total CLA content was the t,t isomers, far lower than for the other samples. The reason for this may be that rumen microorganisms preferentially isomerized *c*-9,*c*-12-octadecadienoic acid to *c*-9,*t*-11-octadecadienoic acid (Hughes et al., 1982; Parodi, 1977). Subsequent pasteurization of the milk was not sufficient to effect the stabilization of c,t isomers to the t,t isomeric forms. It would be of interest to investigate the predominant CLA isomer peak (c-9,t-11 isomer) in raw milk as a nutrient.

Linoleic acid geometrical isomers also influence the distribution of minor contributors (c,c isomers of 9,11- and 10,12-, *t*-9,*c*-11-, and *c*-11,*t*-12-octadecadienoic acids) observed in this study. The 11,13 isomer might be produced as a minor product from *c*-9,*c*-12-octadecadienoic acid or from its isomeric forms during processing.

The newly developed methods reported herein combining reversed-phase HPLC with GC may be used to determine CLA and its isomeric forms in other food products. Such information should be of interest given the anticarcinogenic properties of CLA under certain conditions in animal experiments (Ha et al., 1987) and the fact that CLA has been isolated from human serum, bile, and duodenal juice (Cawood et al., 1983).

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Registry No. *c*-9,*t*-11-18:2, 2540-56-9; *t*-9,*c*-11-18:2, 872-23-1; *c*-10,*t*-12-18:2, 2420-44-2; *c*-9,*c*-11-18:2, 544-70-7; *c*-10,*c*-12-18:2, 7307-45-1; *t*-9,*t*-11-18:2, 544-71-8; *t*-10,*t*-12-18:2, 1072-36-2; *t*-10,*c*-12-18:2, 2420-56-6; *c*-11,*c*-13-18:2, 117624-52-9.

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Isolation and Characterization of Wheat Straw Lignin

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A method was developed for the isolation of lignin from wheat straw by ball milling and enzyme treatment. Grinding duration, cellulase hydrolysis time, and dioxane–water composition of the extraction solvent on lignin yield were examined. Ball-milling for 8 days followed by cellulose hydrolysis for 4 days were needed to maximize isolated lignin yield. Extraction of ball-milled, enzyme-treated straw with 50% dioxane–water resulted in twice as much lignin solubilization as seen for a 96% dioxane–water extraction. Nitrobenzene oxidation, solid-state and solution NMR, and infrared spectroscopy of isolated lignins indicated few differences between lignins in the various fractions. Lignin soluble in 96% dioxane had the least carbohydrate contamination and the highest concentration of cinnamic acids. Progressively more carbohydrate and less cinnamic acids were found in the 50% dioxane-soluble and water-soluble lignin fractions. Some acetyl groups and ethanol were found in the lignins. Lignin yields were high from this isolation procedure, and the data suggest that 50% dioxane lignins from herbaceous plants result in greater yields than 96% dioxane without major changes in lignin composition.

Although lignin has long been associated with poor forage fiber digestion by ruminant animals, the mechanism(s) of this inhibition has never been established (Jung and Fahey, 1983). Evidence is accumulating that chemical composition and structure of forage lignin plays a larger role in determining fiber digestibility than simply quantity of lignin. The relationship between forage digestibility and lignification is consistently different between legumes and grasses (Mowat et al., 1969). It was found that legume stems were much more digestible than grass stems of equal lignin content, and legumes contain lignin which has smaller amounts of sinapyl alcohol units (Jung et al., 1983b). As grasses mature, the inhibitory effects of additional lignin concentration become progressively smaller (Van Soest, 1967; Jung and Vogel, 1986) and a similar affect is seen for legumes, but the decline in lignin's effect on digestibility is sharper (Jung, H. G., 1986). It has been

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