Evaluating Acid and Base Catalysts in the Methylation of Milk and Rumen Fatty Acids with Special Emphasis on Conjugated Dienes and Total *trans* Fatty Acids

John K.G. Kramer[a,]*, Vivek Fellner[a], Michael E.R. Dugan[a], Frank D. Sauer[a], Magdi M. Mossoba[b], and Martin P. Yurawecz[b]

[a]Center for Food and Animal Research, Research Branch, Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada, K1A 0C6, and [b]U.S. FDA, Center for Food Safety and Applied Nutrition, Washington, DC 20204

ABSTRACT: Milk analysis is receiving increased attention. Milk contains conjugated octadecadienoic acids (18:2) purported to be anticarcinogenic, low levels of essential fatty acids, and *trans* fatty acids that increase when essential fatty acids are increased in dairy rations. Milk and rumen fatty acid methyl esters (FAME) were prepared using several acid- (HCl, BF_3, acetyl chloride, H_2SO_4) or base-catalysts ($NaOCH_3$, tetramethylguanidine, diazomethane), or combinations thereof. All acid-catalyzed procedures resulted in decreased *cis/trans* (Δ9*c*,11*t*-18:2) and increased *trans/trans* (Δ9*t*,11*t*-18:2) conjugated dienes and the production of allylic methoxy artifacts. The methoxy artifacts were identified by gas–liquid chromatography (GLC)-mass spectroscopy. The base-catalyzed procedures gave no isomerization of conjugated dienes and no methoxy artifacts, but they did not transesterify *N*-acyl lipids such as sphingomyelin, and $NaOCH_3$ did not methylate free fatty acids. In addition, reaction with tetramethylguanidine coextracted material with hexane that interfered with the determination of the short-chain FAME by GLC. Acid-catalyzed methylation resulted in the loss of about 12% total conjugated dienes, 42% recovery of the Δ9*c*,11*t*-18:2 isomer, a fourfold increase in Δ9*t*,11*t*-18:2, and the formation of methoxy artifacts, compared with the base-catalyzed reactions. Total milk FAME showed significant infrared (IR) absorption due to conjugated dienes at 985 and 948 cm^{-1}. The IR determination of total *trans* content of milk FAME was not fully satisfactory because the 966 cm^{-1} *trans* band overlapped with the conjugated diene bands. IR accuracy was limited by the fact that the absorptivity of methyl elaidate, used as calibration standard, was different from those of the other minor *trans* fatty acids (e.g., dienes) found in milk. In addition, acid-catalyzed reactions produced interfering material that absorbed extensively in the *trans* IR region. No single method or combination of methods could adequately prepare FAME from all lipid classes in milk or rumen lipids, and not affect the conjugated dienes. The best compromise for milk fatty acids was obtained with $NaOCH_3$ followed by HCl or BF_3, or diazomethane followed by $NaOCH_3$, being aware that sphingomyelins are ignored. For rumen samples, the best method was diazomethane followed by $NaOCH_3$.

Lipids 32, 1219–1228 (1997).

Interest in milk fatty acid composition was renewed because of ongoing studies to increase the essential fatty acid content (1,2), to determine accurately the *trans* content (3–8), and lately, to quantitate the conjugated octadecadienes (9–15) which have been associated with inhibition of carcinogenesis and tumorigenesis (16–18). Milk and rumen fatty acid compositions are complex (1,19–21). Not only is there a large range in chain length from C_4 to C_{26}, including branch-chain fatty acids, but milk also contains many positional and geometric isomers of mono-, di-, and tri-unsaturated fatty acids, and many of these fatty acids are present in very low concentrations. In 1991, Jensen *et al.* (19) estimated 400 fatty acids to be present in bovine milk.

No single method is presently able to resolve all 400 fatty acids. The availability of long polar capillary columns (50 to 100 m) for gas–liquid chromatography (GLC) has improved the resolution of many positional and geometric fatty acid isomers (3–8,10–12,15,22). Prior separations with argentation chromatography still show several regions with overlapping peaks particularly in the mono- and diunsaturated fatty acid region (3–8,22–25). The development of a routine method for the analysis of total milk and rumen fatty acids, without extensive secondary fractionations, would be desirable.

Difficulty may occur in quantitatively preparing esters from such a great variety of fatty acids and lipid classes that are present in milk and rumen lipids. Sodium methoxide ($NaOCH_3$)-catalyzed methylations have been used (3,9), but free fatty acids and *N*-acyl lipids (i.e., sphingolipids and glycosphingolipids) are not methylated under these conditions (26). Acid-catalyzed methylations convert all known lipid classes; however, reports have indicated that the conjugated dienes are isomerized (12,27–29). The very short-chain fatty acid methyl esters (FAME) are difficult to quantitate because the methyl esters are volatile, water-soluble, and require correction factors (30,31). Some of the difficulties with the very

*To whom correspondence should be addressed at 4046 Neatby Bldg., Center for Food and Animal Research, Agriculture and Agri-Food Canada, Ottawa, Ontario, Canada, K1A 0C6.

Abbreviations: ATR, attenuated total reflection; FTIR, Fourier transform infrared; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; IR, infrared; MS, mass spectrometry; TLC, thin-layer chromatography.

short-chain fatty acids were avoided by preparing H_2SO_4-catalyzed isopropyl esters for milk analyses (4,6,8,24,32).

In the present study, several acid- and base-catalyzed methylation methods were evaluated for the analyses of milk and rumen lipids, including a combination of methods. The methods included the following catalysts in anhydrous condition: HCl/methanol (33), BF_3/methanol (34), acetyl chloride/methanol (35), $NaOCH_3$/methanol (36), tetramethylguanidine/methanol (13,27,37), H_2SO_4/isopropanol (4,6,24,32), and diazomethane in diethyl ether/methanol (26). Several criteria were considered in evaluating methods for the analysis of milk and rumen lipids: (i) completion of methylation of free fatty acids and *O*- and *N*-esters as judged by thin-layer chromatography (TLC); (ii) coextraction of artifacts with hexane that may interfere with the direct analysis of the hexane extract by GLC; (iii) extent of isomerization of conjugated dienes and methoxy artifact formation as judged by GLC; and (iv) comparison of total *trans* content determined by GLC and infrared (IR) spectroscopy. A chromatographic separation is presented using a 100-m capillary column that resulted in the separation of about 180 peaks from milk lipids.

MATERIALS AND METHODS

Materials. Milk and rumen samples were obtained from cows used in two separate experiments to test the effect of ionophores on milk and rumen fatty acid composition. Other reports have detailed the effect of ionophores on rumen and milk lipids (38–40). Methyl ester (Nu-Chek-Prep, Inc., Elysian, MN) and phospholipid (Matreya Inc., Pleasant Gap, PA) standards were obtained commercially.

Extraction of lipids. Total rumen lipids were extracted with chloroform/methanol (2:1, vol/vol) as described previously (38). Total milk lipids were extracted with a modified Folch procedure described by Jensen (41). The total lipids were taken up in 10 mL of chloroform and stored at −70°C until analyzed.

Preparation of fatty acid esters. The total rumen and milk lipids were methylated in 15-mL culture tubes equipped with Teflon-lined screw caps, under N_2, and in anhydrous conditions. HCl/methanol (33), acetyl chloride/methanol (35), and H_2SO_4/isopropanol (6,32) were heated for 1 h at 80°C. $NaOCH_3$/methanol (36) and 14% BF_3/methanol (34) were heated for 10 min at 50°C. Tetramethylguanidine/methanol was heated for 2 min at 100°C (27,37). Diazomethane in diethyl ether/methanol was left for 10 min at room temperature (26). The excess diazomethane and solvents were then removed by a stream of nitrogen and the residue dissolved in 2 mL hexane and analyzed directly by GLC. To all other reactions, 5% water was added to the alcohol solution, and the esters were extracted with 2 mL of hexane and analyzed directly by GLC. To avoid any loss of short-chain esters, the hexane layer was not reduced in volume. Base-catalyzed reactions were neutralized with aqueous HCl prior to hexane extraction. To ensure completion of reaction, the total lipids were extracted using the Bligh and Dyer mixture (42). The extent of neutral lipid esterification was checked by TLC using the developing solvent hexane/diethyl ether/acetic acid (85:15:1, by vol). The polar lipids were checked by TLC using the developing solvent chloroform/methanol/water (65:25:4, by vol). The FAME were extracted with hexane and analyzed directly by GLC to determine the extent of isomerization of conjugated dienes.

Several combinations of methylations were also tested with standard neutral- and phospholipids mixtures: $NaOCH_3$/methanol for 10 min at 50°C followed by an excess of HCl/methanol for 10 min at 80°C; $NaOCH_3$/methanol for 10 min at 50°C followed by an excess of BF_3/methanol (43) for 10 min at 50°C; and diazomethane for 10 min at room temperature followed by evaporation to dryness in a stream of N_2, and reaction with $NaOCH_3$/methanol for 10 min at 50°C. The extent of methylation of the total lipid extract was checked by TLC, while the extent of isomerization of the conjugated dienes was checked by GLC.

Analyses of esters by GLC. FAME were analyzed by GLC (model 5890; Hewlett-Packard, Palo Alto, CA) using a SP-2560 fused silica capillary column (100 m × 0.25 mm i.d. × 0.2 μm film thickness; Supelco Inc., Bellefonte, PA), H_2 as the carrier gas, a 1:15 split mode, and a flame-ionization detector. For comparison, FAME were also analyzed using a CP-Sil 88 WCOT fused silica column (100 m × 0.25 mm i.d. × 0.2 μm film thickness; Chrompack, Middleburg, The Netherlands). Both columns were operated at 70°C for 4 min, then temperature-programmed at 13°C/min to 175°C, held there for 27 min, programmed at 4°C/min to 215°C, and finally held there for 31 min; total run was 80 min. Starting at 70°C permitted the resolution of the short-chain FAME. Maintaining the temperature at 215°C for a longer period of time allowed the emergence of the very long-chain FAME. The temperature program for the isopropyl esters was similar to FAME except that it was started at 90°C, taken to 195°C, and held at 215°C for 36 min.

Fractionation of FAME by high-performance liquid chromatography (HPLC). Total milk and rumen FAME were separated on a reverse-phase Supelcosil™ LC-8DB column (25 cm × 4.6 mm i.d.; 5-μm particle size; Supelco Inc.) using an HPLC (model 1090; Hewlett-Packard) equipped with an autosampler. The system was operated at room temperature with a flow rate of 1.5 mL/min. A portion of the eluate was diverted (three-way valve #02-0124; Alltech Associates Inc., Deerfield, IL) to an evaporative light-scattering detector (model MKIII; Varex Alltech Associates Inc.) operated at 75°C and 2.5 L/min of N_2, and the remainder was collected using a fraction collector (Gilson model 203; Mandel Scientific Co. Ltd., Guelph, Ontario, Canada). Approximately 1 mg of total lipids in 25 μL hexane was applied onto the column. Fractions were eluted isocratically with 87.5% methanol and 12.5% water and collected at 1-min intervals from 3 to 35 min. After each run, the column was regenerated with dichloroethane.

Analyses of FAME by GLC–FTIR (Fourier transform infrared) spectroscopy and GLC–MS (mass spectrometry). The same GLC conditions as described above were used to ana-

lyze the FAME by GLC–FTIR using a Hewlett-Packard system (models 5890 and 5965B) as described previously (38). In addition, the FAME were analyzed by a GLC–MS system (model 4500; Finnigan, San Jose, CA) using the same GLC conditions. Mass spectra were acquired in 1-s increments ranging in mass from 30 to 500.

Measurement of trans *absorption of FAME by attenuated total reflection (ATR)–FTIR spectroscopy.* An FTIR spectrometer (model FTS-60A; Bio-Rad, Digilab Division, Cambridge, MA) was used equipped with a Graseby Specac (Fairfield, CT) Golden Gate Single Reflection Diamond ATR cell that required about 1 µL test sample. This accessory contained a 2×2 mm diamond with an active sampling area of 0.6 mm in diameter. It was prealigned in the factory, and preattached to its baseplate that attached to the floor of the FTIR spectrometer sample compartment. Mounting holes and pegs in the baseplate ensured its proper positioning and alignment.

Neat (without solvent) FAME test aliquots (1 µL) were placed without weighing on the horizontal (face-up) diamond sampling surface of the cell. For quantitative work, the test aliquots had to cover all of the horizontal surface of the diamond. Two hundred and sixty-four scans were collected at 4 cm^{-1} resolution. After a measurement was made, the surface of the diamond was cleaned by wiping it with low-lint paper.

ATR quantitation was based on the measurement of the integrated area between the limits of 990 and 945 cm^{-1}, which included all C-H out-of-plane deformation absorptions of isolated *trans* double bonds, and *trans* contained in methylene and nonmethylene-interrupted 18:2 isomers at 966 cm^{-1}. The contribution of overlapping conjugated *cis,trans* and *trans,trans* diene bands at 985 and 948 cm^{-1} (44) was subtracted digitally. A calibration plot of "area" vs. "percentage" *trans* FAME was generated for reference mixtures in the range of 0.81–14.4% methyl elaidate in methyl oleate which was used as the infrared (IR) reference material. The regression line parameters for the *trans* standards were 0.001194 (intercept) and 0.01777 (slope), with a correlation coefficient of 0.9996. A commercial FAME reference mixture was used which consisted of 16:0 (6%), 18:0 (3%), 18:1n-9 (35%), 18:2n-6 (50%), 18:3n-3 (3%), and 20:4n-6 (3%) (#RM-1; Matreya Inc., Pleasant Gap, PA); all double bonds were *cis.* Absorption spectra were obtained by "ratioing" the test sample single-beam spectra against that of the reference mixture. For test samples, the percentage of total *trans* was calculated from the absorption area between the limits of 990 and 945 cm^{-1}, and the linear regression equation that described the calibration plot. The lower limit of quantitation was about 1%. Using cells with 1.5 mL and 50 µL capacity, the ATR method was successfully used to determine the *trans* levels in hydrogenated oils and commercial food products (45,46), and this method was recently adopted as Recommended Practice (47).

Statistics. Milk and rumen FAME were analyzed by the analysis of variance.

RESULTS AND DISCUSSION

Analysis of milk FAME by GLC. A typical GLC chromatogram of total milk FAME using a 100-m polar fused silica capillary column (SP-2560; Supelco Inc.) is shown in Fig-

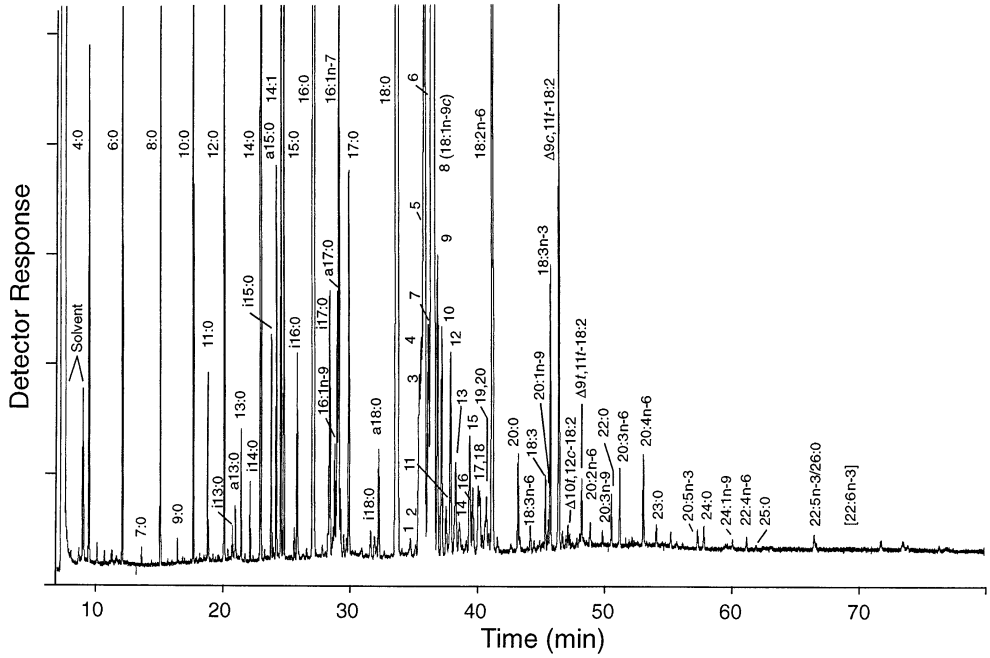

FIG. 1. A typical gas–liquid chromatogram of total bovine milk fatty acid methyl ester prepared by $NaOCH_3$/methanol (10 min at 50°C) followed by HCl/methanol (10 min at 80°C), and separated on a 100-m fused silica capillary column (SP-2560; Supelco Inc., Bellefonte, PA). i, iso; a, anteiso; numbers 1–20 are an arbitrary consecutive numbering of all the peaks in the region between 18:0 and 18:2n-6 using this column. [22:6n-3] is not present in these milk samples but the position at which it should emerge in the chromatogram is indicated.

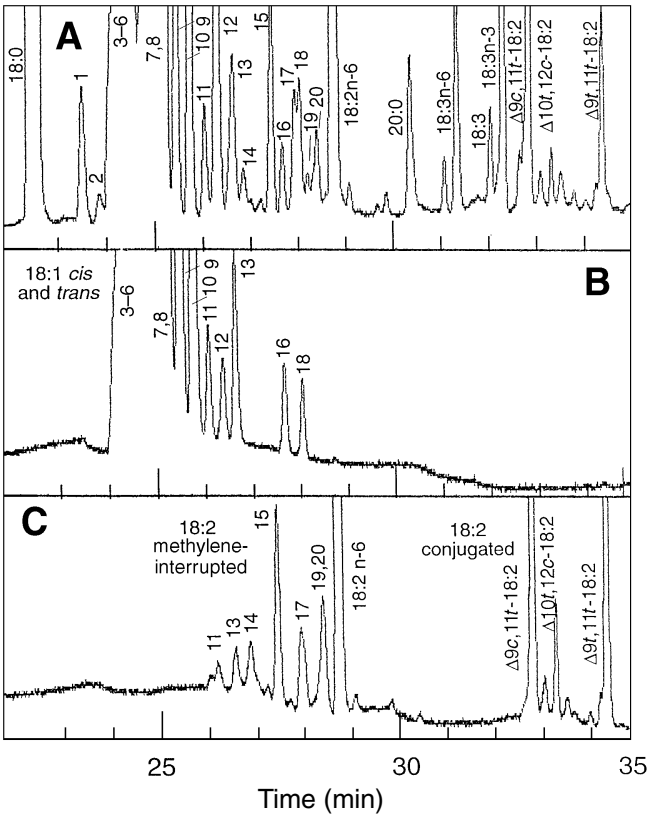

FIG. 2. The methyl stearate (18:0) to conjugated octadecadiene region of the gas–liquid chromatogram of total bovine milk fatty acid methyl esters (A), and methyl octadecenoates [B; emerged at 8 min from high-performance liquid chromatography (HPLC) column] and methyl octadecadienoates (C; emerged at 11 min from HPLC column) isolated from total milk fatty acid methyl esters by HPLC. Gas–liquid chromatography (GLC) analysis was on the same type of column as Figure 1. Numbers 1–20 refer to the same numbers as in Figure 1 between 18:0 and 18:2n-6.

ure 1. Using a temperature program from 70 to 215°C permitted a separation from the short-chain (4:0) to the very long-chain saturated (26:0) and polyunsaturated (20:2n-6, 20:3n-6, 20:4n-6, 20:5n-3, 22:4n-6, 22:5n-3) FAME found in milk. A total of about 180 peaks were observed. Many of the peaks were identified by comparison to known FAME standards and confirmed by GLC–MS and GLC–FTIR. The 100-m CP-Sil 88 WCOT fused silica column gave similar results to the SP-2560 column with a few minor exceptions. There appeared to be a better resolution of *trans*-16:1 and iso-17:0, anteiso-17:0 and 16:1n-7, and 20:1n-9 and 18:3n-3 with the CP-Sil 88 column, while the resolution of 22:1n-9 and 20:3n-3, and 20:5n-3 and 24:0 was better with the SP-2560 column. Resolution of critical pairs was sometimes improved by changing the column temperature, as shown previously (48). However, that process was limited, because a compromise was needed to deal with the wide range of fatty acids present in milk and rumen lipids.

The major unresolved peaks with either column occurred between 18:0 and 18:2n-6 and included several positional isomers of *cis*- and *trans*-18:1, and the methylene-interrupted and noninterrupted 18:2 isomers, many of them overlapping. The 18:1–18:2 region was resolved by HPLC into mono- and diunsaturated FAME (Fig. 2). With the use of GLC–FTIR, the identity of the major *cis* and *trans* absorption peaks was determined, but not the minor peaks (i.e., peaks 1,2,11,13–20). Based on the HPLC separation and GLC–FTIR results, the peaks in 18:1–18:2 region were identified as *trans*-18:1 (peaks 3–7, and part of peak 10), *cis*-18:1 (peaks 8–13,16,18), and 18:2-isomers (peaks 11,13–15,17,19,20). No attempt was made to further separate *cis*- and *trans*-18:1 or identify each of the isomers, since this was adequately described by others in recent publications on milk and milk fat lipids (4–8,22–25).

Analysis of conjugated dienes using either base- or acid-catalyzed methylations. The composition of the conjugated dienes was greatly affected by the catalyst used in the methylation procedure. Base-catalyzed methylation of milk using $NaOCH_3$ showed Δ9*c*,11*t*-18:2 as the major conjugated diene, with smaller amounts of eight other isomers including Δ9*t*,11*c*-18:2, Δ10*t*,12*c*-18:2, Δ9*c*,11*c*-18:2, and Δ9*t*,11*t*-18:2 (Fig. 3). On the other hand, all acid-catalyzed methylation procedures resulted in decreased Δ9*c*,11*t*-18:2 and increased Δ9*t*,11*t*-18:2, and the formation of additional peaks between 20:2n-6 and 20:3n-9 (Fig. 3). The peaks formed during acid-catalyzed methylation were identified as positional allylic methoxy isomers of 18:1 by GLC–MS. All the methoxy artifacts had molecular ion at *m/z* 326. These allylic methoxy compounds could have been formed from hydroxy 18:1 present in milk (19). We also confirmed that these allylic methoxy derivatives were formed from the conjugated dienes by heating the standard methyl esters of Δ9*c*,11*t*-18:2 and Δ9t,11t-18:2 with HCl/methanol for 1 h at 80°C. The GLC and GLC–MS of the resultant FAME showed the formation of the corresponding methoxy derivatives as major product (data not shown). The formation of methoxy artifacts during acid-catalyzed methylations of conjugated dienes was first pointed out in a letter to the editor of *Lipids* as early as 1972 (29), but there was no follow-up manuscript, and its findings appeared to be largely ignored by many investigators. Similar allylic methoxy derivatives were reported recently following acid-catalyzed methylation of allylic hydroxy oleates (49). The GLC–MS results, isomer identification, and possible mechanism of formation of these allylic methoxy derivatives formed during acid-catalyzed methylation of milk lipids will be presented in a separate publication.

Acid-catalyzed methylation has been used extensively for the analysis of conjugated dienes in milk and dairy products. The catalysts used by several groups included HCl (16,17,28), acetyl chloride (20,27,35,50), BF_3 (5,10–12,15,20,22,23,27, 32,43,48,50), and H_2SO_4 (4,6,8,24,32). Some authors recognized the isomerization of the conjugated dienes during acid-catalyzed methanolysis and recommended that the reaction of BF_3/methanol (12,15) or HCl/methanol (28) be carried out at room temperature to reduce this isomerization. Our TLC results showed that methylation was not complete with either BF_3 or HCl as a catalyst under these mild conditions (data not shown). GLC analyses showed evidence of isomerization and artifact (methoxy) for-

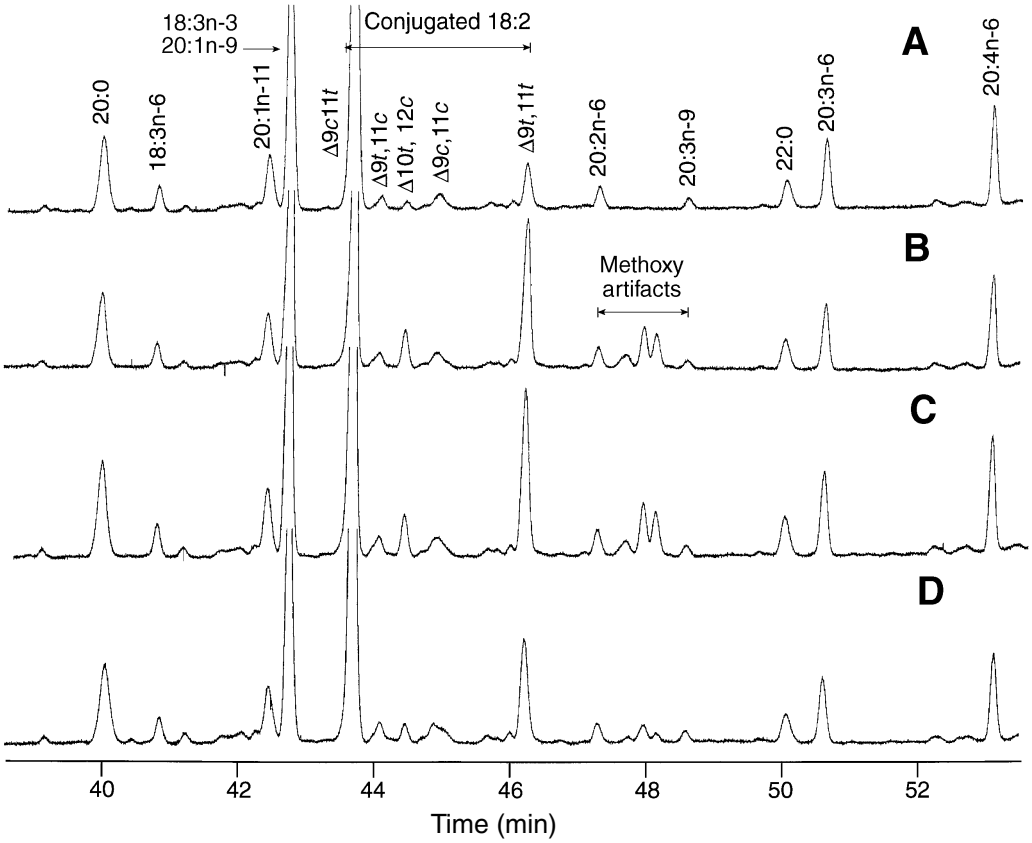

FIG. 3. The methyl eicosanoate (20:0) to methyl arachidonate (20:4n-6) region of the GLC of total bovine fatty acid methyl esters prepared by using the following catalysts in anhydrous methanol: (A) $NaOCH_3$, (B) HCl, (C) acetyl chloride, or (D) BF_3. Analysis was on the same type of GLC column as Figure 1. See Figure 2 for abbreviation.

mation. In one of the milk samples, the ratio of Δ9*c*,11*t*-18:2 to Δ9*t*,11*t*-18:2 was 3.6 with $NaOCH_3$ and 2.7 with either HCl or BF_3 at room temperature. Therefore, not even mild acid-catalyzed methylation at room temperature is appropriate for the analysis of conjugated dienes. Furthermore, it should be pointed out that in two publications additional peaks were observed in the GLC chromatogram of conjugated dienes after acid-catalyzed methylation (12,27), but the peaks were not identified.

In several recent publications, Wolff's group recommended the preparation of isopropyl esters using H_2SO_4 as catalyst (4,6,8,24,32). This method was proposed to overcome the need for the use of correction factors for the short-chain FAME (24,30,32) and to improve the resolution of 4:0 from the solvent front (4). However, this method did not address the issue of isomerization of conjugated dienes. Reevaluation of H_2SO_4-isopropanol methylation reaction showed extensive isomerization of conjugated dienes and artifact formation, just as for HCl/methanol. In one publication (32) isopropyl esters were prepared by acid (H_2SO_4) and base (Na) catalysts, but no comparison was made of the distribution of 18:2 *cis,trans* conjugated dienes. However, their results showed a higher content of total conjugated dienes (18:2 *c,t*) with base- than with acid-catalyzed methylation (Table V, Ref. 32), which was similar to our finding (Table 1).

Recently, Shantha *et al.* (27) proposed the use of tetramethylguanidine, a base-catalyzed reagent developed by Schuchardt and Lopes (37), for the analysis of conjugated dienes. It was reported that tetramethylguanidine caused little isomerization of the *cis/trans* to the *trans/trans* conjugated dienes (27) and that free fatty acids were methylated (27,37). At first glance, this method appeared to be an ideal methylation reagent for total milk lipids. However, we found that tetramethylguanidine gave incomplete methylation of phosphatidylcholine, and did not methylate sphingomyelin as judged by TLC. Furthermore, hexane coextracted unknown material after the reaction which interfered with the determination of 4:0, and 8:0 to 10:0, present in milk. Introducing a water wash was unsuccessful in removing the interfering broad peaks, but a significant reduction was observed in the content of 4:0 and 6:0 FAME after the extra wash. For these reasons, tetramethylguanidine alone was not considered suitable for the methylation of total milk lipids.

Evaluating a combination of catalysts for the methylation of total milk lipids. Total milk lipids contain about 98% triacylglycerols, 1% phospholipids (phosphatidylcholine, phosphatidylethanolamine and sphingomyelin in about equal proportions), 0.3% cholesterol, 0.3% diacylglycerols, and trace amounts of cholesteryl esters and free fatty acids (20). Methylation using only $NaOCH_3$/methanol gave no isomerization of conjugated dienes and artifact formation, but did not methylate free fatty acids and sphingomyelin. The AOCS

TABLE 1
Fatty Acid Composition (area %) of Milk Lipids from Cows Fed Control or Ionophore-Treated Diets (for 2 wk). Comparison of Transesterification Procedures

Fatty	Control		Ionophore-treated		
acids (FA)	$NaOCH_3$	HCl	$NaOCH_3$	HCl	SE[e]
4:0–13:0	10.9[f]	10.2	10.2	9.8	0.7
14:0–18:0	48.0	46.9	47.1	47.4	0.7
20:0–26:0	0.29[b]	0.38[a]	0.28[b]	0.37[a]	0.01
iso FA[g]	1.02	1.00	1.03	1.00	0.02
anteiso FA[h]	0.71	0.70	0.71	0.71	0.01
18:1 *trans*(*t*)	3.6[b]	3.6[b]	6.3[a]	6.0[a]	0.1
18:1 *cis*(*c*)	26.5[a]	26.8[a]	23.5[b]	23.0[b]	0.4
18:2 *c,t*[i]	0.25[d]	0.49[c]	0.65[a]	0.61[b]	0.01
18:2n-6	3.6[b]	3.6[b]	3.8[a]	3.8[a]	0.05
18:2 conjugated	0.74[c]	0.67[d]	1.38[a]	1.21[b]	0.02
Δ9*c*,11*t*-18:2	0.52[b]	0.23[d]	1.08[a]	0.44[c]	0.03
Δ9*t*,11*t*-18:2	0.07[d]	0.25[b]	0.12[c]	0.48[a]	0.01
Δ9*c*,11*t*/9*t*,11*t*[j]	7.2[b]	0.9[c]	9.1[a]	0.9[c]	0.2
Methoxy artifacts[k]	0[c]	0.21[b]	0[c]	0.41[a]	0.01
n-6 PUFA[l]	0.29[b]	0.32[a]	0.29[b]	0.33[a]	0.01
n-3 PUFA[m]	0.53[b]	0.59[a]	0.54[b]	0.63[a]	0.01

[a-d]Means within a row with different superscript letters are different ($P < 0.05$).
[e]SE, pooled standard error.
[f]Each value represents the mean of the milk from 10 cows/diet. The same milk fat extract was transesterified with either $NaOCH_3$/methanol or HCl/methanol.
[g]Sum of all iso fatty acids: 13:0, 14:0, 15:0, 16:0, 17:0, and 18:0.
[h]Sum of all anteiso fatty acids: 13:0, 15:0, 17:0, and 18:0.
[i]Sum of all 18:2 methylene-interrupted *c,c, c,t, t,c,* and *t,t* isomers.
[j]Ratio of Δ9*c*,11*t*-18:2 to Δ9*t*,11*t*-18:2.
[k]Sum of all allylic methoxy artifacts.
[l]Sum of all n-6 PUFA (polyunsaturated fatty acids): 20:2n-6, 20:3n-6, 20:4n-6, and 22:4n-6.
[m]Sum of all n-3 PUFA: 18:3n-3, 20:5n-3, and 22:5n-3.

TABLE 2
Comparison of *trans* Content (% of total fatty acid methyl esters) of Milk from Two Separate Studies in Which Cows Were Treated with Ionophores (for 2 or 3 wk[a]).

		Control[b]		2 Wk		3 Wk	
Expt.	Method	$NaOCH_3$	HCl	$NaOCH_3$	HCl	$NaOCH_3$	HCl
1	GLC[c]	4.9[d]	5	8.3	8.2		
	GLC – CD[e]	4.1	4.3	6.9	6.9		
	IR[f]	5.8[g]	4.6	8.9	6.2[h]		
2	GLC	5.2	5.3	13.8	14.8	13.5	14.2
	GLC – CD	4.4	4.4	12.8	13.3	12.4	12.9
	IR	5.1	3.9[h]	12.6	6.7[h]	12.7	7.0[h]

[a]As measured by gas–liquid chromatography (GLC) and attenuated total reflection infrared (IR) spectroscopy. The experimental conditions of these studies are published elsewhere (Ref. 40).
[b]The total fat extract of each milk sample was analyzed by both methods.
[c]The GLC method involved summing all *trans* monounsaturates (peaks 3–7, half of 10; Fig. 1), *cis/trans* 18:2 (peaks 14,15,17,19,20; Fig.1) and total conjugated dienes.
[d]Each GLC value represented the mean of the milk from 10 cows.
[e]Total *trans* determined by GLC ([c]) minus total conjugated dienes (CD).
[f]The IR values represented the total *trans* absorption from monoenes, and methylene- and nonmethylene-interrupted 18:2. Conjugated dienes were excluded, see text.
[g]Each IR value represented the mean of two determinations. Milk samples 1 to 5 and 6 to 10 were combined because of insufficient material.
[h]Acid-catalyzed methanolysis showed interferences in the *trans* IR region, see text.

method recommends a combination of $NaOCH_3$ and BF_3/methanol (43). We tried the combination of $NaOCH_3$ (10 min at 50°C) followed by either BF_3/methanol (10 min at 50°C), or HCl/methanol (10 min at 80°C). The results showed that both these methods methylated free fatty acids, and most of the sphingomyelin as judged by TLC (data not shown). The GLC results showed that the ratio of Δ9*c*,11*t*-18:2 to Δ9*t*,11*t*-18:2 was only slightly reduced, with the formation of trace amounts of methoxy derivatives. This combination of methods appears to be the best compromise for the methylation of total milk lipids. A saponification followed by diazomethane has been used successfully for the analysis of conjugated dienes (51), but this method also ignores the sphingomyelins which are not saponified.

Quantitative analysis of conjugated dienes and trans *FAME of milk.* A summary of the milk fatty acid composition determined by using either $NaOCH_3$/methanol or HCl/methanol is compared in Table 1. Milk samples were obtained from ongoing studies at Agriculture and Agri-Food Canada to test the effect of ionophores on milk fatty acid composition (39,40). The most noted difference between methods was on the complex mixture of conjugated diene isomers (Fig. 3). $NaOCH_3$/methanol gave ~12% higher values of total conjugated dienes compared with HCl/methanol (Table 1). Of the total conjugated dienes, ~74% consisted of the Δ9*c*,11*t* isomer using $NaOCH_3$/methanol, compared with only ~35% when HCl/methanol was used, while the Δ9*t*,11*t* isomer increased from ~9% with $NaOCH_3$ to ~39% with HCl. The ratio of Δ9*c*,11*t* to Δ9*t*,11*t* for the two methods decreased from ~8 to 0.9 (Table 1). Some of the conjugated dienes were converted to allylic methoxy derivatives during acid-catalyzed methylation. However, the sum of the conjugated dienes plus the methoxy derivatives in acid-catalyzed methanolysis gave ~18% higher values than those observed for the $NaOCH_3$/methanol method (Table 1). This suggests that some of the methoxy derivatives may have been derived from preexisting hydroxy fatty acids (49). The methoxy artifacts did not appear to be derived from either 18:1 (*cis* or *trans*) or 18:2n-6 in milk during these acid methylation conditions, since the levels of these fatty acids were not significantly different between the two methods; see Table 1. In addition to the changes in conjugated dienes, HCl/methanol also gave slightly higher values for the long-chain saturated (20:0–26:0) and polyunsaturated fatty acids (n-6 and n-3) compared with $NaOCH_3$/methanol (Table 1). This was not surprising, since the acid catalyst methylated all the lipid components, including sphingomyelin rich in long-chain saturates, and free fatty acids plus minor acidic lipids which also contain polyunsaturated fatty acids.

Based on the GLC analyses, the total *trans* content of milk samples was similar using either $NaOCH_3$ or HCl as catalyst (Table 2). The total *trans* content was obtained by adding the relative percentage of all *trans* monounsaturated fatty acids (peaks 3–7, and half of peak 10; Fig. 1), *cis/trans* 18:2 (peaks 14,15,17,19,20; Fig. 1), and total conjugated dienes in the milk chromatograms (Table 2). In the milk samples investigated, the *trans* content increased from about 5 to 8% (Experiment 1, Table 2), and from about 5 to 14% (Experiment 2,

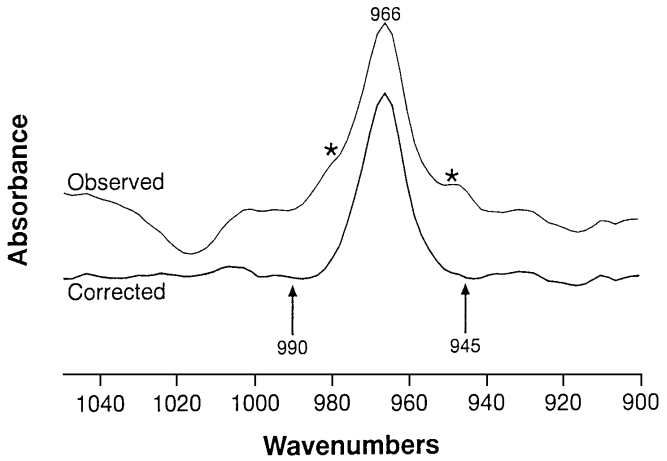

FIG. 4. Expanded infrared spectral range showing the out-of-plane deformation bands of isolated *trans* (966 cm^{-1}), and "*trans*-containing" conjugated dienes (985 and 948 cm^{-1}, denoted by asterisks) present in total bovine milk fatty acid methyl esters prepared by $NaOCH_3$/methanol (observed). The total *trans* area was integrated between the limits of 990 and 945 cm^{-1}, after subtracting the contribution of the conjugated dienes (corrected).

Table 2). The total FAME from the two experiments and the two methylation methods were subsequently analyzed by ATR–IR spectroscopy, which has been applied to rapid quantitation of total *trans* content in food products (45,46). The IR spectra (Fig. 4) showed absorbances due to the *trans* monounsaturated fatty acids and methylene-interrupted and noninterrupted 18:2 at 966 cm^{-1} and absorbances due to "*trans*-containing" conjugated dienes at 985 and 948 cm^{-1} (44). The IR calibration was based only on the absorptivity of methyl elaidate, which appeared to be inappropriate for the quantitation of conjugated dienes. Therefore, the absorbances at 948 and 985 cm^{-1} were subtracted before quantitation. However, even after removing the conjugated diene contribution, the remaining IR *trans* values were sometimes higher than those determined by GLC minus the conjugated dienes (Table 2). This may be due to additional unknown or overlapping *trans* isomers in the GLC chromatogram with different absorptivities than those of methyl elaidate. Until an appropriate *trans*-free reference material is found that is close to that of milk fat composition both in isolated and conjugated *trans* fatty acids, the accurate determination of total *trans* content in milk by IR will be difficult. The results in Table 2 showed that HCl methylation leads to lower *trans* values as determined by IR, due to the production of interfering material with absorbances in the *trans* region, which made these IR determinations of total trans content unreliable. This pointed to a further disadvantage of preparing total milk FAME using only HCl/methanol.

Preparation and analysis of total rumen FAME. Rumen lipids contain a complex mixture of phospho-, sphingo-, neutral-, and sterol lipids including high levels of free fatty acids, mainly from plant origin (21). The present study was undertaken to develop a method for the preparation of FAME from total rumen lipids. $NaOCH_3$/methanol was ineffective because of the high content of free fatty acid, while diazomethane methylated the free fatty acids, but it did not react with the ester lipids. Diazomethane did not isomerize the conjugated dienes or produce methoxy artifacts (Fig. 5A). On the other hand, HCl/methanol caused isomerization of the conjugated dienes, but did not form allylic methoxy derivatives (Fig. 5B). There is no explanation for this finding, but it may suggest the absence of hydroxy fatty acids in rumen lipids or an effect of free fatty acids. A combination of $NaOCH_3$ (10

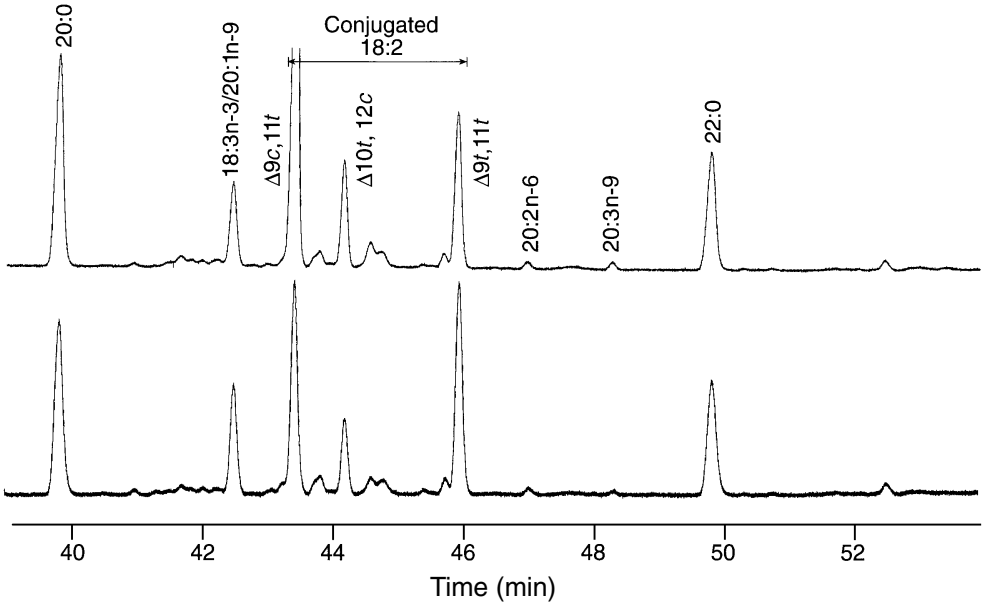

FIG. 5. The methyl eicosanoate (20:0) to methyl arachidonate (20:4n-6) region of the gas–liquid chromatogram of total rumen fatty acid methyl esters prepared by using (A) diazomethane and (B) HCl as catalysts in anhydrous methanol. Analysis was on the same type of GLC column as Figure 1. See Figure 2 for abbreviation.

min at 50°C) and HCl (10 min at 80°C) gave a Δ9*c*,11*t*-18:2 to Δ9*t*,11*t*-18:2 ratio of 4:3, while a combination of diazomethane and $NaOCH_3$ gave a ratio of 4:1. Both methods would appear to be suitable for total rumen FAME preparation. In our previous study (38), HCl/methanol was used to prepare the FAME of total rumen lipids which isomerized the conjugated dienes. However, in that study (38), only the total conjugated dienes were presented, which should be only slightly lower than if a base catalyst had been used (~12% based on milk results; Table 1).

Rumen FAME showed a similar pattern to that observed in milk FAME except for greatly reduced levels of very short-chain saturated (4:0–10:0) and long-chain C_{20} and C_{22} PUFA, and a higher content of *trans*-18:1 isomers and long-chain (22:0 to 26:0) saturated fatty acids (compare with Refs. 38 and 39). A comparison of the content of conjugated dienes suggests that it was twice as high in milk (present study, Table 1) than in rumen samples (39), both in control (0.7 vs. 0.3%) and ionophore treatments (1.4 vs. 0.7%), respectively. The content of Δ9*c*,11*t*-18:2 found in control milk (0.7%) agrees well with the value obtained by others (3) when a base-catalyzed method was used (0.85%).

Summary. The analysis of the conjugated dienes in milk and dairy products needs to be accurate, in view of their beneficial claims, and the assumption that only one of the isomers (Δ9*c*,11*t*-18:2) may be active in carcinogenesis and tumorigenesis (18). The evidence suggests that the formation of the conjugated dienes is enzymatic both by rumen bacteria (52–54) and in biological material as products of free radical *in vivo* lipid peroxidation (55–57). Careful analysis of the conjugated dienes in biological material, using only base-catalyzed methods that do not isomerize the conjugated diene system, has shown that Δ9*c*,11*t*-18:2 was the only isomer present (51). The presence of larger amounts of other isomers suggests subsequent isomerization during processing or improper (acid-catalyzed) methodology. There are many references in the literature reporting the content of conjugated dienes in milk and dairy products (3,4,6,8–15,24,27). Many of these studies show a high content of isomers other than Δ9*c*,11*t*-18:2 (10–13,15,27). Reinvestigation using proper techniques is needed.

Given the data presented here, a single procedure cannot simultaneously methylate all milk or rumen lipid components and avoid isomerization of conjugated dienes and the formation of allylic methoxy artifacts. Base-catalyzed methods caused no isomerization and produced no methoxy artifacts. However, $NaOCH_3$ did not methylate free fatty acids and *N*-acyl lipids, and diazomethane did not methylate esters. Tetramethylguanidine did not methylate sphingomyelin or phospholipids completely, which makes this method unsuitable for tissue lipids high in these lipids, as was reported recently (58). In addition, tetramethylguanidine gave undesirable products that extracted with hexane and interfered with the analyses of short-chain FAME, such as found in milk. Acid-catalyzed methods (HCl, BF_3, acetyl chloride, and H_2SO_4) methylated all lipid classes. However, these catalysts caused extensive isomerization of conjugated dienes, formed allylic methoxy artifacts, and are therefore not recommended by themselves for the analysis of milk and rumen lipids. A combination of methods appears to be most suitable, but that increases the loss of short-chain FAME during solvent removal or excessive handling. For milk, $NaOCH_3$-HCl (or BF_3) resulted in small losses of unconverted sphingomyelin, and minor isomerization of conjugated dienes and allylic methoxy formation, while diazomethane-$NaOCH_3$ ignored the sphingomyelin lipids. For rumen, either diazomethane-$NaOCH_3$, or $NaOCH_3$-HCl (or BF_3) gave good results. Analysis of isopropyl, instead of methyl esters, has the added advantage of not requiring the use of correction factors for FAME (24,30–32), but the isopropyl esters need to be prepared using Na isopropionate as catalyst instead of H_2SO_4/isopropanol (6,32). The determination of total isolated *trans* content in milk by IR methods was complicated by interferences from significant amounts of conjugated dienes.

ACKNOWLEDGMENTS

The authors thank R.C. Fouchard (CFAR) and Arran Bibby (Graseby Specac) for their technical support, and Graceby Specac for the generous loaning of a diamond ATR cell for the duration of the study. Contribution number 2425 from the Center of Food and Animal Research. Conjugated dienes were a gift from R.O. Adlof, USDA, Northern Regional Laboratory, Peoria, IL.

REFERENCES

1. Christie, W.W. (1981) The Effects of Diet and Other Factors on the Lipid Composition of Ruminant Tissues and Milk, in *Lipid Metabolism in Ruminant Animals* (Christie, W.W., ed.) pp. 193–226, Pergamon Press, Oxford.
2. Sutton, J.D., and Morant, S.V. (1989) A Review of the Potential of Nutrition to Modify Milk Fat and Protein, *Livest. Prod. Sci. 23,* 219–237.
3. Henninger, M., and Ulberth, F. (1994) *Trans* Fatty Acid Content of Bovine Milk Fat, *Milkwissenschaft 49,* 555–558.
4. Wolff, R.L. (1994) Contribution of *trans*-18:1 Acids from Dairy Fat to European Diets, *J. Am. Oil Chem. Soc. 71,* 277–283.
5. Chen, Z.-Y, Pelletier, G., Hollywood, R., and Ratnayake, W.M.N. (1995) *Trans* Fatty Acid Isomers in Canadian Human Milk, *Lipids 30,* 15–21.
6. Wolff, R.L. (1995) Content and Distribution of *trans*-18:1 Acids in Ruminant Milk and Meat Fats. Their Importance in European Diets and Their Effect on Human Milk, *J. Am. Oil Chem. Soc. 72,* 259–272.
7. Precht, D. (1995) Variation of *trans* Fatty Acids in Milk Fats, *Z. Ernährungswiss. 34,* 27–29.
8. Chardigny, J.-M., Wolff, R.L., Mager, E., Bayard, C.C., Sébédio, J.-L., Martine, L., and Ratnayake, W.M.N. (1996) Fatty Acid Composition of French Infant Formulas with Emphasis on the Content and Detailed Profile of *trans* Fatty Acids, *J. Am. Oil Chem. Soc. 73,* 1595–1601.
9. Fogerty, A.C., Ford, G.L., and Svoronos, D. (1988) Octadeca-9,11-dienoic Acid in Foodstuffs and in the Lipids of Human Blood and Breast Milk, *Nutr. Rep. Internat. 38,* 937–944.
10. Ha, Y.L., Grimm, N.K., and Pariza, M.W. (1989) Newly Recognized Anticarcinogenic Fatty Acids: Identification and Quantitation in Natural and Processed Cheeses, *J. Agr. Food Chem. 37,* 75–81.

11. Shantha, N.C., Decker, E.A., and Ustunol, Z. (1992) Conjugated Linoleic Acid Concentration in Processed Cheese, *J. Am. Oil Chem. Soc. 69,* 425–428.
12. Werner, S.A., Luedecke, L.O., and Shultz, T.D. (1992) Determination of Conjugated Linoleic Acid and Isomer Distribution in Three Cheddar-Type Cheeses: Effects of Cheese Cultures, Processing, and Aging, *J. Agric. Food Chem. 40,* 1817–1821.
13. Shantha, N.C., Ram, L.N., O'Leary, J., Hicks, C.L., and Decker, E.A. (1995) Conjugated Linoleic Acid Concentrations of Dairy Products as Affected by Processing and Storage, *J. Food Sci. 60,* 695–697, 720.
14. Banni, S., Gianfranca, C., Contini, M.S., Angioni, E., Deiana, M., Dessì, M.A., Melis, M.P., and Corongiu, F.P. (1996) Characterization of Conjugated Diene Fatty Acids in Milk, Dairy Products, and Lamb Tissues, *Nutr. Biochem. 7,* 150–155.
15. Jiang, J., Bjoerck, L., Fondén, R., and Emanuelson, M. (1996) Occurrence of Conjugated *cis*-9, *trans*-11-Octadecadienoic Acid in Bovine Milk: Effects of Feed and Dietary Regimen, *J. Dairy Sci. 79,* 438–445.
16. Ha, Y.L., Storkson, J., and Pariza, M.W. (1990) Inhibition of Benzo(a)pyrene-induced Mouse Forestomach Neoplasia by Conjugated Dienoic Derivatives of Linoleic Acid, *Cancer Res. 50,* 1097–1101.
17. Ip, C., Chin, S.F., Scimeca, J.A., and Pariza, M.W. (1991) Mammary Cancer Prevention by Conjugated Dienoic Derivative of Linoleic Acid, *Cancer Res. 51,* 6118–6124.
18. Belury, M.A. (1995) Conjugated Dienoic Linoleate: A Polyunsaturated Fatty Acid with Unique Chemoprotective Properties, *Nutr. Rev. 53,* 83–89.
19. Jensen, R.G., Ferris, A.M., and Lammi-Keefe, C.J. (1991) The Composition of Milk Fat, *J. Dairy Sci. 74,* 3228–3243.
20. Jensen, R.G. (1996) The Lipids in Human Milk, *Prog. Lipid Res. 35,* 53–92.
21. Harfoot, C.G. (1981) Lipid Metabolism in the Rumen, in *Lipid Metabolism in Ruminant Animals* (Christie, W.W., ed.) pp. 21–55, Pergamon Press, Oxford.
22. Ratnayake, W.M.N., and Beare-Rogers, J.L. (1990) Problems of Analyzing C_{18} *cis*- and *trans*-Fatty Acids of Margarine on the SP-2340 Capillary Column, *J. Chromatogr. Sci. 28,* 633–639.
23. Ratnayake, W.M.N., and Pelletier, G. (1992) Positional and Geometric Isomers of Linoleic Acid in Partially Hydrogenated Oils, *J. Am. Oil Chem. Soc. 69,* 95–105.
24. Wolff, R.L., Bayard, C.C., and Fabien, R.J. (1995) Evaluation of Sequential Methods for the Determination of Butterfat Fatty Acid Composition with Emphasis on *trans*-18:1 Acids. Application of the Study of Seasonal Variations in French Butters, *J. Am. Oil Chem. Soc. 72,* 1471–1483.
25. Molkentin, J., and Precht, D. (1995) Optimized Analysis of *trans*-Octadecenoic Acids in Edible Oils, *Chromatographia 41,* 267–272.
26. Christie, W.W. (1982) *Lipid Analysis,* 2nd edn., Pergamon Press, Oxford.
27. Shantha, N.C., Decker, E.A., and Hennig, B. (1993) Comparison of Methylation Methods for the Quantitation of Conjugated Linoleic Acid Isomers, *J. AOAC Internat. 76,* 644–649.
28. van den Berg, J.J.M., Cook, N.E., and Tribble, D.L. (1995) Reinvestigation of the Antioxidant Properties of Conjugated Linoleic Acid, *Lipids 30,* 599–605.
29. Koritala, S., and Rohwedder, W.K. (1972) Formation of an Artifact During Methylation of Conjugated Fatty Acids, *Lipids 7,* 274.
30. Bannon, C.D., Craske, J.D., and Hilliker, A.E. (1985) Analysis of Fatty Acid Methyl Esters with High Accuracy and Reliability. IV. Fats with Fatty Acids Containing Four or More Atoms, *J. Am. Oil Chem. Soc. 62,* 1501–1507.
31. Craske, J.D., Bannon, C.D., and Norman, L.M. (1988) Limitations of Ambient Temperature Methods for the Methanolysis of Triacylglycerols in the Analysis of Fatty Acid Methyl Esters with High Accuracy and Reliability, *J. Am. Oil Chem. Soc. 65,* 262–266.
32. Wolff, R.L., and Fabien, R.J. (1989) Utilisation de l'isopropanol pour l'extraction de la matière grasse de produits laiters et pour l'estérification subséquente des acides gras, *Le Lait 69,* 33–46.
33. Stoffel, W., Chu, F., and Ahrens, E.H. (1959) Analysis of Long-Chain Fatty Acids by Gas–Liquid Chromatography. Micromethod for Preparation of Methyl Esters, *Anal. Chem. 31,* 307–308.
34. Morrison, W.R., and Smith, L.M. (1964) Preparation of Fatty Acid Methyl Esters and Dimethylacetals from Lipids with Boron Fluoride-Methanol, *J. Lip. Res. 5,* 600–608.
35. Lepage, G., and Roy, C.C. (1986) Direct Transesterification of all Classes of Lipid in a One-Step Reaction, *J. Lip. Res. 27,* 114–120.
36. Christopherson, S.W., and Glass, R.L. (1970) Preparation of Milk Fat Methyl Esters by Alcoholysis in an Essentially Nonalcoholic Solution, *J. Dairy Sci. 52,* 1289–1290.
37. Schuchardt, U., and Lopes, O.C. (1988) Tetramethylguanidine-Catalyzed Transesterification of Fats and Oils: A New Method for Rapid Determination of Their Composition, *J. Am. Oil Chem. Soc. 65,* 1940–1941.
38. Fellner, V., Sauer, F.D., and Kramer, J.K.G. (1995) Steady-State Rates of Linoleic Acid Biohydrogenation by Ruminal Bacteria in Continuous Culture, *J. Dairy Sci. 78,* 1815–1823.
39. Fellner, V., Sauer, F.D., and Kramer, J.K.G. (1997) Effect of Ionophores with Different Binding Selectivity for Cations on Fermentation and Lipid Metabolism by a Continuous Culture of Ruminal Bacteria, *J. Dairy Sci. 80,* 921–928.
40. Sauer, F.D., Fellner, V., Kinsman, R., Kramer, J.K.G., Jackson, H.A., Lee, A.J., and Chen, S. (1997) Methane Output and Lactation Response in Holstein Cattle with Monensin or Unsaturated Fat Added to the Diet, *J. Anim. Sci.*, in press.
41. Jensen, R.G. (1989) The Lipids of Human Milk, pp. 30, CRC Press Inc., Boca Raton.
42. Bligh, E.G., and Dyer, W.J. (1959) A Rapid Method of Total Lipid Extraction and Purification, *Can. J. Biochem. Physiol. 37,* 911–917.
43. *Official Methods and Recommended Practices of the American Oil Chemists' Society,* (1989) Preparation of Methyl Esters of Long-Chain Fatty Acids (Ce 2-66), 4th edn., American Oil Chemists' Society, Champaign.
44. Mossoba, M.M., McDonald, R.E., Armstrong, D.J., and Page, S.W. (1991) Identification of Minor C_{18} Triene and Conjugated Diene Isomers in Hydrogenated Soybean Oil and Margarine by GC-MI-FT-IR Spectroscopy, *J. Chromatogr. Sci., 29,* 324–330.
45. Mossoba, M.M., Yurawecz, P.M., and McDonald, R.E. (1996) Rapid Determination of the Total *trans* Content of Neat Hydrogenated Oils by Attenuated Total Reflection Spectroscopy, *J. Am. Oil Chem. Soc. 73,* 1003–1009.
46. Ali, L.H., Angyal, G., Weaver, C.M., Rader, J.I., and Mossoba, M.M. (1996) Determination of Total *trans* Fatty Acids in Foods: Comparison of Capillary-Column Gas Chromatography and Single-Bounce Horizontal Attenuated Total Reflection Infrared Spectroscopy, *J. Am. Oil Chem. Soc. 73,* 1699–1705.
47. *Official Methods and Recommended Practices of the American Oil Chemists' Society,* (1996–1997) Isolated *trans* Isomers—Infrared Spectroscopic Methods (Cd 14d-96), 4th edn. with additions and revisions, American Oil Chemists' Society, Champaign.
48. Wolff, R.L. (1994) Analysis of Alpha-Linolenic Acid Geometric Isomers in Deodorized Oils by Capillary Gas–Liquid Chromatography on Cyanoalkyl Polysiloxane Stationary Phases: A Note of Caution, *J. Am. Oil Chem. Soc. 71,* 907–909.
49. Yurawecz, M.P., Hood, J.K., Roach, J.A.G., Mossoba, M.M., Daniels, D.H., Ku, Y., Pariza, M.W., and Chin, S.F. (1994) Conversion of Allylic Hydroxy Oleate to Conjugated Linoleic Acid and Methoxy Oleate by Acid-Catalyzed Methylation Procedures, *J. Am. Oil Chem. Soc. 71,* 1149–1155.

50. Bitman, J., and Wood, D.L. (1987) Comparison of Direct Transesterification of Fatty Acids with Procedures Applied to Extracts of Human and Cow Milk Fat, *J. Am. Oil Chem. Soc. 64,* 637.
51. Smith, G.N., Taj, M., and Braganza, J.M. (1991) On the Identification of a Conjugated Diene Component of Duodenal Bile as 9*Z*,11*E*-Octadecadienoic Acid, *Free Rad. Biol. Med. 10,* 13–21.
52. Kepler, C.R., Hirons, K.P., McNeill, J.J., and Tove, S.B. (1966) Intermediates and Products of the Biohydrogenation of Linoleic Acid by *Butyrivibrio fibrisolvens, J. Biol. Chem. 241,* 1350–1354.
53. Hughes, P.E., Hunter, W.J., and Tove, S.B. (1982) Biohydrogenation of Unsaturated Fatty Acids, *J. Biol. Chem. 257,* 3643–3649.
54. Fujimoto, K., Kimoto, H., Shishikura, M., Endo, Y., and Ogimoto, K. (1993) Biohydrogenation of Linoleic Acid by Anaerobic Bacteria Isolated from Rumen, *Biosci. Biotech. Biochem. 57,* 1026–1027.
55. Cawood, P., Wickens, D.G., Iversen, S.A., Braganza, J.M., and Dormandy, T.L. (1983) The Nature of Diene Conjugation in Human Serum, Bile and Duodenal Juice, *FEBS Lett. 162,* 239–243.
56. Iversen, S.A., Cawood, P., Madigan, M.J., Lawson, A.M., and Dormandy, T.L. (1984) Identification of a Diene Conjugated Component of Human Lipid as Octadeca-9,11-dienoic Acid, *FEBS Lett. 171,* 320–324.
57. Dormandy, T.L., and Wickens, D.G. (1987) The Experimental and Clinical Pathology of Diene Conjugation, *Chem. Phys. Lipids 45,* 353–364.
58. Belury, M.A., and Kempa-Steczko, A. (1997) Conjugated Linoleic Acid Modulates Hepatic Lipid Composition in Mice, *Lipids 32,* 199–204.