

## Occurrence of Diesters of 3-Chloro-1,2-propanediol in the Neutral Lipid Fraction of Goats' Milk

Janis Cerbulis,\* Owen W. Parks, Ray H. Liu, Edwin G. Piotrowski, and Harold M. Farrell, Jr.

The neutral lipid fraction of goats' milk has been found to contain, as a minor component, fatty acid diesters of 3-chloro-1,2-propanediol. This class of compounds migrates on thin-layer chromatography (TLC) plates in advance of the triglyceride fraction and was isolated by extraction of lyophilized milk with petroleum ether followed by silicic acid chromatography and preparative TLC, all in the absence of chlorinated solvents. Structures of the parent compounds and the product of the transmethylation reaction, 3-chloro-1,2-propanediol, were identified by desorption chemical ionization and high-resolution mass spectrometry. The transmethylated esters of fatty acids were identified by gas-liquid chromatography. Control experiments with triglyceride showed no generation of chlorolipid during the chemical characterization procedures. These results demonstrate the occurrence of this class of halolipids in a natural unprocessed food. Initially, the 3-chloro-1,2-propanediol esters were identified in one bulk sample, which was a composite of five commercial samples. They were subsequently identified in a hand-milked sample from a private herd in Pennsylvania and also in a bulk milk sample from California. Similar TLC spots were only barely detectable in the neutral fats of pooled cows' milks and ewes' milks.

Halolipid analogues have been prepared synthetically as probes for membrane structure (Adison, 1982; Esfahani et al., 1981; Sturtevant et al., 1979) or as potential anti-metabolites (Brachwitz et al., 1982). Velisek and co-workers have identified esters and diesters of 3-chloro-1,2-propanediol as components of protein hydrolysates that apparently arise from triglycerides present in vegetable meals or flours (Velisek et al., 1978, 1979, 1980; Davidek et al., 1980).

During the course of a detailed investigation of the lipids of goats' milk (Cerbulis et al., 1982, 1983), a neutral lipid fraction that migrated on thin-layer chromatography (TLC) plates slightly ahead of the major triglycerides was observed. Isolation and characterization of this fraction showed it to contain, as major components, diacyl fatty acid esters of 3-chloro-1,2-propanediol. This may represent the first report of the occurrence of this compound in unprocessed food, although it has recently been described in contaminated Spanish olive oil (Roach et al., 1983).

### MATERIALS AND METHODS

**Materials.** Raw goats' milk samples were obtained with the cooperation of a large commercial goat dairy company and were maintained at 5 °C in transit to the laboratory. Upon receipt, the samples were lyophilized and stored at -20 °C. Before lipid extraction, equal weights of five samples obtained from several geographical areas in Pennsylvania and New Jersey during the months of April through June were mixed together to minimize nutritional, environmental, and breed differences. A sample of pooled frozen raw goats' milk was obtained through J. Jeter of the California Goat Dairywomen's Association of Turlock, Ca. A sample was also obtained directly from the hand milking of four goats maintained on a private farm in southeastern Pennsylvania and not intended for commercial consumption.

**Reagents.** All solvents were of nanograde quality. Unisil silicic acid (100-200 mesh), silica gel G Uniplates, and precoated SIL G-25 TLC plates were obtained from Clarkson Chemical Co. (Williamsport, Pa), Analtech, Inc. (Newark, DE), and Brinkmann Instruments, Inc. (Westbury, NY), respectively. 3-Chloro-1,2-propanediol and 1,3-propanediol were from Aldrich Chemical Co. Inc.

(Milwaukee, WI). Synthetic 3-chloro-1,2-propanediol dipalmitate was obtained from Peter Yurawecz of the Food and Drug Administration, Division of Chemical Technology, Washington, DC 20204.

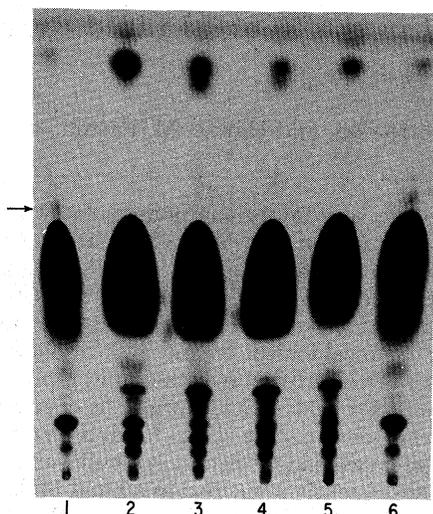
**Lipid Extraction.** The neutral or "free" lipids were obtained as previously described by Cerbulis et al. (1982), extracting the freeze-dried samples 4 times with nanograde petroleum ether. Lipid extracts and column fractions were analyzed by TLC; the developing solvent was petroleum ether-diethyl ether-acetic acid (90:10:1).

**Silicic Acid Column Separations.** Silicic acid (100-200 mesh), which was activated by treatment at 110 °C for 12 h, was used for all column separations. Free or neutral lipids (70 g) were dissolved in hexane and applied to a 3.5 × 35 cm column equilibrated with hexane. Stepwise elution was carried out with hexane, 6% benzene in hexane, 18% benzene in hexane, 50% benzene-hexane, and finally benzene; each step represented 10 column volumes at a flow of 150 mL/h.

**Transmethylation.** The isolated fraction was dissolved in 1.2 mL of 1 N methanolic HCl and allowed to stand at room temperature for 16 h (overnight). The mixture was evaporated to dryness under nitrogen at room temperature. One portion of residue was dissolved in methylene chloride and the methyl esters were subjected to gas-liquid chromatographic analysis (GLC) according to the procedure previously described by Cerbulis et al. (1982). The residue was also dissolved in methanol and spotted across a 12.5 × 10.0 cm four-scored TLC Uniplate with a 250- $\mu$ m layer of silica gel G. The TLC plate had been previously washed with methanol and dried in an oven for 1 h at 100 °C. The plate was developed 6 cm in 10% CH<sub>3</sub>OH-90% CHCl<sub>3</sub>, and the end plates were removed and dried. The alcohol portion of the isolated fraction was visualized under UV light by using a modification of the method of Schwartz (1958) in which ethyl acetoacetate replaces the acetyl acetone. The area corresponding to the alcohol was scraped from the inner plates, packed in a small glass column plugged with glass wool, and eluted with methanol. Trilaurin tested as described above served as a control for the transmethylation procedure. Alternatively, transmethylation was carried out with H<sub>2</sub>SO<sub>4</sub> as the catalyst.

**Mass Spectrometry.** A Finnigan MAT 311A (San Jose, CA) mass spectrometer was used for the following analyses. (a) Molecular weights of compounds in the isolated fraction were characterized by the desorption chemical ionization technique using ammonia as the

\*Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Philadelphia, Pennsylvania 19118.



**Figure 1.** Thin-layer chromatograms of the neutral lipid fractions of (1) goats' milk, (2) corn oil, (3) sunflower oil, (4) safflower oil, (5) peanut oil, and (6) goats' milk. The plates were silica gel G, and the solvent was freshly prepared petroleum ether–diethyl ether–acetic acid (90:10:1). The arrow indicates the position of the fraction studied in this paper. Each track represents approximately 0.50 mg of lipid applied to the plate.

reagent gas ( $\text{NH}_3/\text{DCI}$ ). (b) Authentic 3-chloro-1,2-propanediol and the alcohol portion of the transmethylation were analyzed in the methane chemical ionization ( $\text{CH}_4/\text{CI}$ ) mode with a direct inlet probe. (c) The isolated alcohol was further analyzed in the 70-eV electron impact (EI) mode for the accurate masses of major fragments with nominal masses  $m/e$  79, 81, and 61. The peak matching procedure was used for these analyses with benzene as the reference for the first two fragments and 1,3-propanediol as the reference for the third fragment.

## RESULTS AND DISCUSSION

The lipid composition of the milk samples in this study has been described by Cerbulis et al. (1982, 1983). The petroleum ether (free lipid) extract of freeze-dried milks contains the majority of the neutral lipids including tri-, di-, and monoglycerides and cholesterol. Closer examination of the thin-layer chromatogram revealed a small fraction that migrates in advance of the triglycerides (Figure 1). This fraction was present in all goats' milk samples. The fraction was not found in peanut, corn, sunflower, or safflower oils (Figure 1). The consistent occurrence of the unknown fraction in goats' milk samples prompted us to investigate further its nature.

The unknown, along with some of the triglycerides, was partially purified by silicic acid column chromatography as described under Materials and Methods. The fractions of interest were eluted with the second solvent used (6% benzene in hexane). The unknown was further purified by preparative TLC using petroleum ether–diethyl ether–acetic acid (90:10:1) and hexane–ether (85:15) as the developing solvents.

Schmid and Mangold (1966) reported the occurrence of neutral plasmalogens and alkoxy diglycerides in human depot fat; these classes of lipids migrate in advance of the triglyceride fraction on TLC. When the isolated fraction was passed over a Celite–phosphoric acid column as described by Parks et al. (1961), its mobility on TLC remained unchanged and no free aldehydes were liberated. Initial analysis of the transmethylation products of the isolated unknown fraction showed no compounds with TLC mobilities of alkyl glyceryl ethers. These tests ruled out the possibility that the unknown fraction represents

either neutral plasmalogen or an alkoxy diglyceride.

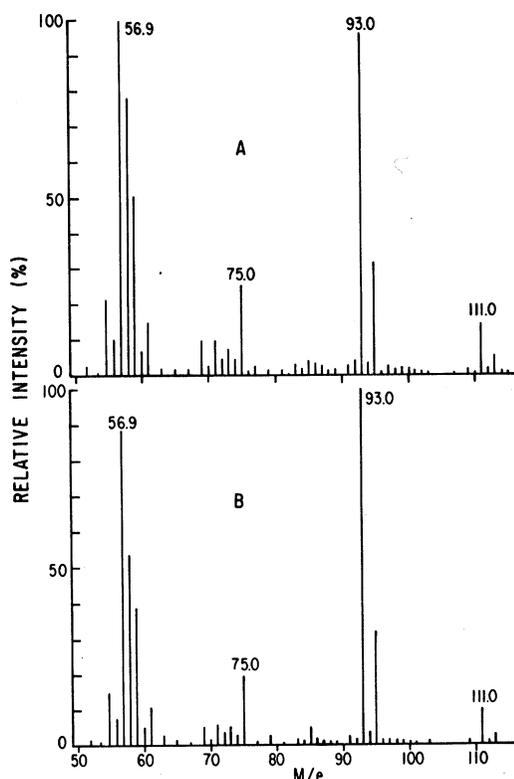
The  $\text{NH}_3/\text{DCI}$  analyses of the isolated fraction indicated the presence of nine major components with molecular weights of 502, 528, 530, 556, 558, 584, 586, 612, and 614. The quasi-molecular ions of these compounds showed characteristic isotopic patterns of one chlorine atom; specifically, the ratios, (quasi-molecular ion + 2)/(quasi-molecular ion), of these compounds are within  $\pm 2.2\%$  of the expected values. A sample calculation is as follows: the two compounds with molecular weights of 556 and 558 apparently partition into three major ions at  $m/e$  574, 576, and 578 (ammonium adduct ions) with intensities of 11 184, 37 823, and 13 520, respectively. The structure for  $m/e$  574 ion is proposed as  $\text{C}_{33}\text{H}_{65}\text{N}_1\text{O}_4^{35}\text{Cl}_1$ . Theoretical calculation indicated that the intensities of the companion  $m/e$  576 and 578 ions should be 39.94% and 2.57% of that of the  $m/e$  574 ion and correspond to 4467 and 287, respectively. Subtracting these values from the observed intensities of  $m/e$  576 and 578 ions resulted in net intensities of 33 357 and 13 233, respectively. Thus, the intensity of the  $m/e$  578 ion ( $\text{C}_{33}\text{H}_{67}\text{N}_1\text{O}_4^{37}\text{Cl}_1$ ) is  $13\,233/33\,357 = 39.67\%$  of that of the  $m/e$  576 ion ( $\text{C}_{33}\text{H}_{67}\text{N}_1\text{O}_4^{35}\text{Cl}_1$ ). The expected value is 39.95%.

Compounds with molecular weights of 638, 640, and 642 are also observed in the  $\text{NH}_3/\text{DCI}$  analyses. Under the experimental conditions used, the intensities of these ions are rather low. However, their characteristic isotopic pattern also showed, qualitatively, the presence of one chlorine atom in each compound.

GLC analyses of the methyl esters obtained from the transmethylation process showed that the major fatty acids present in these compounds are  $\text{C}_{10:0}$ ,  $\text{C}_{12:0}$ ,  $\text{C}_{14:0}$ ,  $\text{C}_{16:0}$ ,  $\text{C}_{18:0}$ , and  $\text{C}_{18:1}$ . On the basis of the fatty acid composition and the molecular weights observed, it was concluded that the alcohol of the isolated fraction had a molecular weight of 110—consistent with 3-chloro-1,2-propanediol [e.g., molecular weight of 502 =  $\text{C}_{10:0} + \text{C}_{16:0}$  (or  $\text{C}_{12:0} + \text{C}_{14:0}$ ) + 110 – 2( $\text{H}_2\text{O}$ ), molecular weight of 640 =  $\text{C}_{18:0} + \text{C}_{18:1} + 110 - 2(\text{H}_2\text{O})$ , etc.].

The  $\text{CH}_4/\text{CI}$  analyses of the authentic 3-chloro-1,2-propanediol compound and the alcohol portion purified by TLC from the transmethylation process are compared in Figure 2. The ions at  $m/e$  111 and 113 apparently represent the protonated molecular ions that have lost a molecule of  $\text{H}_2\text{O}$  and a molecule of  $\text{HCl}$  to give the  $m/e$  93 and 95 and the  $m/e$  75 fragments, respectively. To further confirm the structure of this isolated alcohol, the accurate masses of three major fragments obtained in EI analyses were measured and found to be  $78.9951 \pm 0.0003$ ,  $80.9921 \pm 0.0003$ , and  $61.0290 \pm 0.0002$ . These masses are in excellent agreements with the fragments,  $\text{C}_2\text{H}_4\text{O}_1^{39}\text{Cl}_1$  (78.995065),  $\text{C}_2\text{H}_4\text{O}_1^{37}\text{Cl}_1$  (80.992115), and  $\text{C}_2\text{H}_5\text{O}_2$  (61.028951) expected from 3-chloro-1,2-propanediol. TLC of the transmethylated trilaurin sample revealed glycerol as the only alcohol present, indicating that 3-chloro-1,2-propanediol was not generated during transmethylation of the isolated fraction. In addition, when  $\text{H}_2\text{SO}_4$  was used as the catalyst, the 3-chloro-1,2-propanediol was also observed. Schmid et al. (1967) reported only methyl esters, dimethyl acetals, and glycerol as the reaction products of transmethylation for neutral plasmalogens under similar conditions in their initial structural identifications. In addition, the compounds reported here comigrate and have identical chemical reactions with synthetic diesters of 3-chloro-1,2-propanediol.

From the results presented above, it would appear that the neutral lipid fraction of the large cross-sectional sample of commercial goats' milk contains a small but significant



**Figure 2.** Methane chemical ionization mass spectra of (A) authentic 3-chloro-1,2-propanediol and (B) the alcohol portion of the transmethylation product from the isolated fraction.

quantity of the fatty acid diesters of 3-chloro-1,2-propanediol (<1% of total neutral lipid). A sample of goats' milk obtained by hand milking directly from four goats from a private herd yielded a similar TLC spot. This spot was purified by preparative TLC and found to contain similar diesters of 3-chloro-1,2-propanediol. In this case these esters accounted for about 50% of the original TLC spot. A sample of raw goats' milk obtained from a commercial herd in California also exhibited these compounds. The initial stages of purification involve only lyophilization, extraction with petroleum ether, and liquid chromatography in hexane. Thus, the milk has not been exposed to chlorinated solvents in the laboratory. In the transmethylation experiments, neither substitution of  $H_2SO_4$  nor a companion experiment with trilaurin produced any 3-chloro-1,2-propanediol. Velisek et al. (1978, 1979, 1980) appear to have generated these compounds only under the condition of protein hydrolysis (6 M HCl at 110 °C).

As discussed by Addison (1982), it is rarely clear in the case of halolipids whether these compounds are dietary substances merely passed through an organism, are synthesized from homologous man-made (anthropogenic) compounds, or are de novo synthesized from halide ions. In the case of the fatty acid diesters found in goats' milk, the

former two alternatives appear more likely; the extensive lipolysis and biohydrogenation that occur in the rumen (Noble, 1981) seem to mitigate against a simple pass-through mechanism and argue for conversion of the diol to diester. Since chlorine-based sanitizers are widely used in dairy operations, the chlorine may be inadvertently introduced into the milks. However, reactions of these compounds with lipids (Ghenbari et al., 1982), under simulated food handling conditions, favor substitution across double bonds in polyunsaturated fatty acids. Regardless of the mechanism, the results presented here demonstrate the occurrence of this class of halolipids in goats' milk. Similar TLC spots were only barely detectable, in the neutral fats from pooled cows' milks and in ewes' milks.

#### ACKNOWLEDGMENT

We thank Dr. B. Ribadeau-Dumas of Laboratoire de Biochimie et Technologie Laitières, Institut National de la Recherche Agronomique, CNRZ 78350, Jouy-en-Josas, France, for the freeze-dried ewes' milks.

#### LITERATURE CITED

- Addison, R. F. *Prog. Lipid Res.* 1982, 21, 47-71.  
 Brachwitz, H.; Langen, R.; Hintsche, R.; Schildt, J. *Chem. Phys. Lipids* 1982, 31, 33-52.  
 Cerbulis, J.; Parks, O. W.; Farrell, H. M., Jr. *J. Dairy Sci.* 1982, 65, 2301-2307.  
 Cerbulis, J.; Parks, O. W.; Farrell, H. M., Jr. *Lipids* 1983, 18, 55-58.  
 Davídek, J.; Velisek, J.; Kubelka, V.; Janíček, G.; Simicová, Z. *Z. Lebensm.-Unters. -Forsch.* 1980, 171, 14-17.  
 Esfahani, M.; Cavanaugh, J. R.; Pfeffer, P. E.; Luken, D. W.; Devlin, T. *Biochem. Biophys. Res. Commun.* 1981, 101, 306-311.  
 Ghenbari, H. A.; Wheeler, W. B.; Kirk, J. R. *J. Food Sci.* 1982, 47, 482-485.  
 Noble, R. C. "Lipid Metabolism in Ruminant Animals"; Christie, W. W., Ed.; Pergamon Press: New York, 1981.  
 Parks, O. W.; Keeney, M.; Schwartz, D. P. *J. Dairy Sci.* 1961, 44, 1940-1943.  
 Roach, J. A. G.; Brumley, W. C.; Joshi, A.; Yurawecz, P.; Gardner, A. M.; Sphon, J. A., presented at the 31st Annual Conference on Mass Spectrometry and Allied Topics, Boston, MA, May 1983.  
 Schmid, H. H. O.; Baumann, W. J.; Mangold, H. K. *Biochim. Biophys. Acta* 1967, 144, 344-354.  
 Schmid, H. H. O.; Mangold, H. K. *Biochem. Z.* 1966, 346, 13-25.  
 Schwartz, D. P. *Anal. Chem.* 1958, 30, 1855-1856.  
 Sturtevant, J. M.; Ho, C.; Reimann, A. *Proc. Natl. Acad. Sci. U.S.A.* 1979, 76, 2239-2243.  
 Velisek, J.; Davídek, J.; Hájšlová, J.; Kubelka, V.; Janíček, G.; Mánková, B. *Z. Lebensm.-Unters. -Forsch.* 1978, 167, 241-244.  
 Velisek, J.; Davídek, J.; Kubelka, V.; Bartosová, J.; Tucková, A.; Hájšlová, J.; Janicek, G. *Lebensm.-Wiss. Technol.* 1979, 12, 234-236.  
 Velisek, J.; Davídek, J.; Kubelka, V.; Janíček, G.; Svobodová, Z.; Simicová, Z. *J. Agric. Food Chem.* 1980, 28, 1142-1144.

Received for review January 3, 1984. Accepted February 17, 1984. Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.