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Note

Detection of acetodiacylglycerols in milkfat lipids by thin-layer chromatography

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Numerous thin-layer chromatographic (TLC) procedures have been reported for separating mixtures of phospholipids, monoacylglycerols, 1,2- and 1,3-diacylglycerols, cholesterol, non-esterified fatty acids, triacylglycerols and cholesterol esters. While a variety of solvent systems have been used to achieve these separations, most contain up to 2% of either formic or acetic acid. The acid is included in order to prevent streaking and tailing especially when non-esterified fatty acids are present[1]. The presence and the amount of acid can alter the nature of the separation[2].

Reported thin-layer chromatograms for the separation of milkfat lipids[3,4] show that when acid is present in the developing solvent system, non-esterified fatty acids migrate ahead of cholesterol plus 1,3-diacylglycerols and behind the triacylglycerols. When milkfat lipids were separated in this laboratory using solvent systems without acid, non-esterified fatty acids remained near the origin. A spot, however, was observed between cholesterol plus 1,3-diacylglycerols and the triacylglycerols. This communication reports the characterization of the components of this spot as acetodiacylglycerols.

EXPERIMENTAL

Materials

Milkfat was obtained from freshly churned butter by melting in a water-bath at 45°, centrifuging and filtering the fat through a filter-paper to dry the sample.

Thin-layer chromatography

Samples dissolved in chloroform were applied to 20 × 20 cm glass TLC plates coated to a thickness of 0.3 mm with Kieselgel G (E. Merck, Darmstadt, G.F.R.). Plates were developed using the solvent system hexane–ethyl acetate (88:12). Spots were located by spraying with 10% phosphomolybdic acid in ethanol, followed by heating at 180° for 5 min. For photography the yellow background was removed by exposing the plate to ammonia vapour.

Preparative TLC was carried out using the same plate thickness and solvent system. Bands were visualised under ultraviolet light after spraying the plates with a methanolic solution containing 0.025% 2′,7′-dichlorofluorescein and 0.005% rhodamine B (ref. 5). The band representing the unknown component was scraped from

the plates into a chromatography column containing a 3-cm plug of Florisil and eluted with diethyl ether. When sufficient material was collected it was re-chromatographed on plates previously washed overnight in methanol. Impurities were removed from Florisil by washing with methanol and diethyl ether.

Infrared spectra

The infrared spectra of the samples as a 3% solution in carbon disulphide were recorded between 4000 cm^{-1} and 650 cm^{-1} using a Perkin-Elmer Model 237 infrared spectrophotometer.

Gas–liquid chromatography of triacylglycerols

The gas–liquid chromatographic (GLC) triacylglycerol profile of the sample was obtained on a 5% solution in chloroform[6] using 0.61 m × 2 mm I.D. stainless steel columns packed with 3% JXR on 100–120-mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). The columns were temperature-programmed from 150 to 300° at 4°/min. Temperatures of the injector block and detector base were 330 and 360°, respectively. The carrier gas flow-rate was 40 ml/min. A Varian Aerograph Model 1840 gas chromatograph equipped with flame ionization detectors was used for all analyses.

Detection of acetic acid

Approximately 10 mg of sample was saponified by refluxing with 1 ml of ethanol and 30 μl of 60% aqueous KOH. After 30 min the mixture was reduced to dryness. Three drops of concentrated HCl and subsequently 0.3 ml of distilled water were added. The liberated water-soluble acids were detected by GLC on a 1.52 m × 2 mm I.D. glass column, packed with 10% free fatty acid phase (FFAP) on 80–100 mesh Gas-Chrom P. Column, injector and detector temperatures were 115, 200 and 225°, respectively. Carrier gas flow-rate was 40 ml/min.

RESULTS AND DISCUSSION

The thin-layer chromatogram of milkfat lipids developed in the solvent system hexane–ethyl acetate (88:12) without acid is shown in Fig. 1. Under these conditions non-esterified fatty acid remains near the origin. A spot of reasonable intensity is seen between the spot representing cholesterol plus 1,3-diacylglycerols and the diffuse spot representing triacylglycerols. Milkfat triacylglycerols produce a diffuse spot as they are partially separated into molecular species that contain long- and short-chain components. GLC triacylglycerol profiles of milkfat show that it contains triacylglycerols of carbon number (total number of acyl carbon atoms in the triacylglycerol) ranging from 26 to 56 (refs. 6 and 7).

The infrared spectrum of the isolated component was similar to that of milkfat triacylglycerols except that strong absorption bands were observed at 1366 cm^{-1}, 1224 cm^{-1} and 1050 cm^{-1}. The band at 1366 cm^{-1} is considered to be due to C–H stretching in terminal methyl groups, that at 1224 cm^{-1} has been attributed to acetate groups while the band at 1050 cm^{-1} is found in the spectra of α-acetodiacylglycerols[8,9]. The spectrum was similar to that of an α-acetodiacylglycerol isolated from *Euonymus verrucosus* seed oil[8].

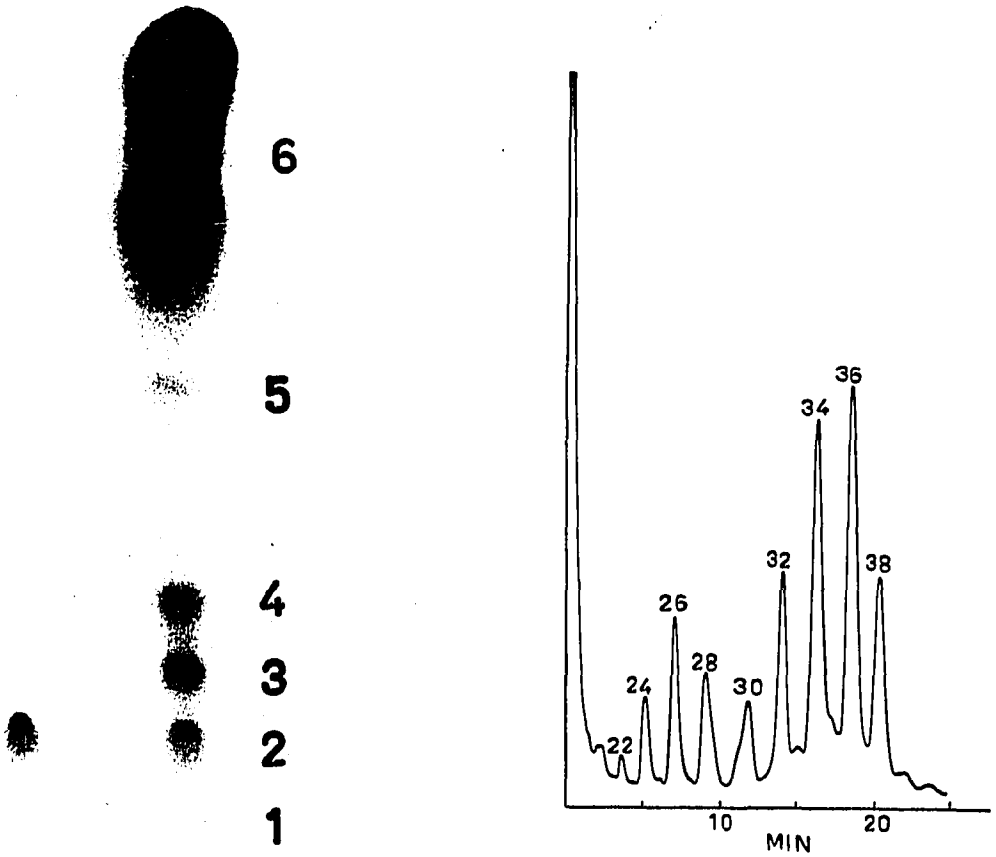

Fig. 1. Thin-layer chromatogram showing the position of acetodiacylglycerols in milkfat lipids. Left-hand spot, oleic acid. Right-hand spots, 75 μg of milkfat lipids. (1) Monoacylglycerols; (2) non-esterified fatty acids; (3) 1,2-diacylglycerols; (4) cholesterol + 1,3-diacylglycerols; (5) acetodiacylglycerols; (6) triacylglycerols.

Fig. 2. Gas chromatogram of the triacylglycerol distribution pattern of the acetodiacylglycerols of milkfat lipids. For operating conditions, see text. The peaks are identified by the total number of acyl carbon atoms in the triacylglycerols.

The GLC triacylglycerol profile of the acetodiacylglycerols is shown in Fig. 2. It shows the presence of triacylglycerols of carbon number 22 to 38. This is consistent with the sample being a monoacetodiacylglycerol. Milkfat triacylglycerols, which have a carbon number range from 26 to 56 are thus not separated from acetodiacylglycerol on the basis of molecular weight. The separation is no doubt due to the polarity of the acetate group. It has been shown that α-acetodiacylglycerols but not β-acetodiacylglycerols have an absorption band at 1050 cm^{-1}. The intensity of the band at 1050 cm^{-1} in this study does not indicate that the acetodiacylglycerol is present solely in the α-position. However, the presence of acetate in the terminal positions of milkfat acetodiacylglycerides is consistent with the pattern found for other short-chain fatty acids of milkfat which are found predominately in the terminal positions[10].

GLC analysis of the water-soluble fatty acids liberated from the sample after saponification showed that acetic acid was the major component. Butyric acid and a small amount of caproic acid were also detected. Other short-chain fatty acids, such as butyric acid, are possibly present in the acetodiacylglycerols, as the GLC triacylglycerol profile showed the existence of triacylglycerols of carbon numbers 22 and 24 which would account for the presence of acetobutylpalmitylglycerol and acetobutylstearylglycerol.

Although not normally reported as a constituent of milkfat, acetic acid in trace amounts has been found in butter and milkfat[11–15]. Whether acetic acid was esterified as triacylglycerol, or resulted from oxidation of unsaturated acids or was of microbial origin has not hitherto been certain.

Thin-layer chromatograms of milkfat from sheep and goats and the depot fat of oxen and sheep showed a spot in the same position as that occupied by bovine milkfat acetodiacylglycerols. Trace amounts of acetic acid have been reported in sheep milkfat[16] and the depot fat of oxen[17] and sheep[18].

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