Isolation and identification of new constituents in milk fat

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ABSTRACT After removal of cholesterol on a digitonin column, the unsaponifiable matter of milk fat was examined for alcoholic substituents. Derivatization with pyruvic acid chloride 2,6-dinitrophenylhydrazone and fractionation of the derivatives gave four main fractions. The second, the hexane–benzene fraction, was shown by thin-layer chromatography to have a mobility similar to many common sterols. The hexane–benzene fraction was saponified and gave rise to free alcohols, which were then analyzed on a combination gas–liquid chromatograph–mass spectrometer. Dihydrolanosterol, previously unreported in milk fat, and lanosterol, previously identified but never confirmed, were characterized. The sterols which were precipitated on the digitonin column, were removed, and by the use of the combination gas–liquid chromatograph–mass spectrometer β-sitosterol was identified. In addition, lanosterol and dihydrolanosterol were isolated from the unsaponifiable matter by chromatography on Florisil.

SUPPLEMENTARY KEY WORDS unsaponifiable matter · digitonin column · gas–liquid chromatography · mass spectrometry · lanosterol · dihydrolanosterol · β-sitosterol · Florisil

PART OF THE interest in the composition of milk fat has been directed toward the components in the unsaponifiable matter, which represent 0.30–0.45% (by weight) of the milk fat. Cholesterol is the constituent in highest concentration in the unsaponifiable matter, and a recent review (1) summarizes most of the compounds found to date.

Abbreviations: GLC, gas–liquid chromatography; TLC, thin-layer chromatography; GLC–MS, combination gas–liquid chromatograph–mass spectrometer.

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In 1951, Morice (2) separated the unsaponifiable matter from 4 kg of milk fat into fractions according to their solubility in methanol and further divided each fraction by adsorption chromatography using alumina. She was able to isolate and identify a material that was eluted immediately before cholesterol as lanosterol, a triterpene which is an intermediate in the biosynthesis of cholesterol. In addition, she isolated four other compounds which were not characterized.

McCarthy, Kuksis, and Beveridge (3) in 1962 worked with molecular distillates of 777 lb of milk fat. Their attention was mostly directed to a fraction that contained the bulk of the unsaponifiable matter. The only compounds identified with any degree of certainty were cholesterol and squalene. Also isolated was a sterol, which they thought to be lanosterol. However, even with the use of GLC and infrared spectroscopy, they were not able to confirm its identity with lanosterol.

Eisner, Wong, Firestone, and Bond (4) reported the column chromatographic fractionation of the unsaponifiable matter of milk fat using Florisil as the adsorbent. They investigated a fraction that contained 90% of all the sterols and found, even with extreme overloading, only one large GLC peak, which was identified as cholesterol. Later, they turned their attention to another fraction that was believed to contain tocopherols, high molecular weight alcohols, and triterpenoid alcohols (5). GLC of this fraction indicated the presence of a series of aliphatic alcohols (C_{22}–C_{31}) as well as α-, β-, γ-, and δ-tocopherols, and β-amyrin. However, none of these were positively identified.

The purpose of this work was to investigate further the composition of milk fat. Since the glyceride portion of the fat has received much attention and since its composition is well known, it was thought that the unsaponi-

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fiable matter should be more closely investigated. Because the report by Morice (2) of the occurrence of lanosterol in milk fat has not been confirmed, there remains some doubt that lanosterol is present. One of the problems in dealing with the unsaponifiable matter of milk fat is the relatively large amount of cholesterol present, and this could possibly account for the inability of McCarthy et al. (3) and Eisner et al. (4) to detect and confirm the presence of lanosterol. This aspect of the subject was also pursued.

EXPERIMENTAL METHODS[1]

Preparation of Milk Fat

Freshly pasteurized cream (obtained from mixed-herd milk, Agricultural Research Center, Beltsville, Md.) was churned in an electric drink mixer. The butter was isolated, melted at 52°C, and stored overnight at 4°C. The butter was remelted at 63°C, and the buttermilk removed. The fat phase was centrifuged at 5000 rpm, and the clear oil was removed with a pipette.

Saponification of the Milk Fat

The procedure used for the saponification of the milk fat was, with slight modification, that described by Schwartz, Burgwald and Brewington (6). A total of 125 g of milk fat was saponified, and the unsaponifiable matter was recovered from the Celite–soap mixture by elution with 1 liter of benzene (redistilled throughout unless otherwise noted).

Removal of Cholesterol from the Milk Fat

The removal of cholesterol from milk fat was accomplished by using a slight modification of the procedure described by Schwartz, Brewington, and Burgwald (7). A solution of digitonin was made by dissolving 1 g of digitonin (Mann Research Labs. Inc., New York) in 17 ml of water, with warming. The solution was then thoroughly ground into 34 g of Celite 545 (Johns-Manville, Baltimore, Md.), and the homogeneous mixture was packed firmly in a glass chromatographic column (3.5 cm I.D. × 29 cm). One-third of the benzene eluate from the saponification step was reduced in volume to approximately 50 ml on a steam bath under a stream of nitrogen; this was then placed on the digitonin column. The volume of the benzene eluate and of the washings was such that it occupied the dry bed volume of the column and was allowed to remain in contact with the column for 10 min until the digitonides formed. The material not precipitated by the digitonin was then eluted with 100 ml of benzene.

Derivatization of the Hydroxy Compounds

The benzene solution from the digitonin column was evaporated to dryness on a steam bath under a stream of nitrogen, and the residue, which weighed approximately 40 mg, was derivatized with pyruvic acid chloride 2,6-dinitrophenylhydrazone (J. T. Baker Chemical Co., Phillipsburg, N.J.) as described by Schwartz and Brewington (8). The derivatives were eluted from an alumina column using chloroform instead of benzene. Reference derivatives were also made of individual alcohols in the manner described by Schwartz and Brewington (8).

Alcohol-free benzene, the solvent used in the derivatization procedure, was prepared by passing benzene through a column of chromic acid and Celite. The column was prepared by dissolving 2 g of chromic acid in 24 ml of water and grinding the solution with 36 g of Analytical Grade Celite (Johns-Manville) in a mortar. This mixture was packed dry in a chromatographic column (2.3 cm I.D. × 63), and the impure benzene was passed through it. The benzene was then passed through a column containing 50 g of basic alumina in order to remove acidic impurities present and and also to dry the benzene. The benzene was then redistilled.

Column Chromatography of the Alcohol Derivatives from the Unsaponifiable Matter

The chloroform solution of the alcohol derivatives was evaporated to dryness on a steam bath under a stream of nitrogen, and the residue was dissolved in 2 ml of benzene. The alcohol derivatives were then fractionated on a column of 8% (v/w) hydrated acidic alumina. The alumina was prepared by the addition of distilled water to the dry alumina (Brockmann Activity Grade 1; J. T. Baker Chemical Co.). The mixture was shaken until all the lumps were broken and then equilibrated overnight. 20 g of the acidic alumina was placed in a chromatography tube (2.2 cm I.D. × 27 cm) half-filled with hexane. Just as the last of the excess hexane drained off, the benzene solution of the derivatives was placed on the column. As the last of the benzene solution entered the column, a wad of glass wool was placed just above the alumina so that the column would remain undisturbed during the addition of solvents. The column was immediately washed with 50 ml of hexane (redistilled throughout unless otherwise noted), which removes some of the hydrocarbons and other nonderivatives in the unsaponifiable matter. Next, 150 ml of hexane–benzene 1:1 was added until a yellow band was eluted; this is referred to as the hexane–benzene fraction. Another yellow band moving behind the hexane–benzene

[1] Mention of brand or firm names does not constitute an endorsement by the Department of Agriculture over others of a similar nature not mentioned.

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fraction was then removed with 100 ml of benzene. The rest of the material was eluted with 100 ml of chloroform.

Thin-Layer Chromatography of Alcohol Derivatives

A few microliters of the hexane–benzene fraction was spotted on Silica Gel G plates along with the derivatives of the C_{16}–C_{26} series of saturated, continuous-chain aliphatic alcohols as well as some of the more common sterols including cholesterol, cholestanol, ergosterol, stigmasterol, testosterone, β-sitosterol, lathosterol, estrone, lanosterol, and dihydrolanosterol. The plate was developed with benzene–hexane 3:1 in an equilibrated chromatographic tank. To facilitate visual inspection of the plate, a wad of cotton saturated with diethylamine was placed in the tank. Under these basic conditions the derivatives turned violet, thus making the spots much easier to see.

Silica Gel G-silver nitrate TLC plates were also used. The plates were made by dissolving 1 g of silver nitrate in 65 ml of H_2O and slurrying the solution with 30 g of Silica Gel G. The slurry was spread on 20 × 20 cm plates to give layers 25 μ in thickness. The plates were dried at 100°C for at least 2 hr and kept in a desiccator until used. The derivatives of the hexane–benzene fraction were spotted and the plate was developed two times in benzene–hexane 5:3 in an equilibrated chromatographic tank.

Regeneration of the Parent Alcohols

The hexane–benzene fraction from the alumina column was evaporated to dryness on a steam bath under a stream of nitrogen, and the derivatives were dissolved in 1.0 ml of acetone. 2 ml of 1 N methanolic potassium hydroxide (Fisher Scientific Company, Silver Spring, Md.) was ground into 4 g of Celite 545 in a mortar, and the acetone solution was added. The mixture was then ground in the shortest feasible time to homogeneity with care taken that the derivatives were absorbed to the Celite 545. In the presence of the base, the derivatives turn a bright violet. The mortar was immediately placed in an oven at 100°C. When the mixture turned brownish-yellow (about 15 min) signifying the completion of the saponification, the mortar was removed, cooled, and the powder transferred to a chromatography tube (1.7 cm I.D. × 13 cm). The free alcohols were eluted with 25 ml of benzene, and the benzene solution was evaporated to 1 ml under nitrogen.

Removal of Sterols from the Digitonin Column

The removal of sterols precipitated by the digitonin column was accomplished using a modification of the method of Katz and Keeney (9). The sterols that had formed the digitonides were eluted using 500 ml of dimethyl sulfoxide. The eluate including the bed volume of benzene was collected using moderate air pressure. The eluate was then extracted in a separatory funnel first with 150 ml of hexane and then twice with a total of 400 ml of hexane–benzene 1:1. It was necessary that the two phases be thoroughly mixed; however, care was taken not to shake the mixture vigorously, in order to avoid emulsions and unclean extractions. The extracts were then combined and evaporated on a steam bath under a stream of nitrogen to a volume of approximately 15 ml. An equal amount of water was added and shaken thoroughly with the extract in a centrifuge tube. It was usually necessary to use centrifugation to separate the layers. The upper layer was then pipetted off and evaporated to dryness on a steam bath under a stream of nitrogen. The residue containing mostly cholesterol was dissolved in 2–3 ml of chloroform.

GLC Trapping

The gas chromatograph used for the trapping procedure was the F & M Model 5150 (Hewlett-Packard Co., Avondale, Pa.) equipped with a thermal-conductivity detector. The column was a 4 mm I.D. × 1 m coiled glass column packed with 2.5% (w/w) SE-30 on 80–100 mesh silanized Gas Chrom Z. The column was preconditioned for 24 hr at 280°C with a helium flow rate of 10 ml/min. The operating conditions of the instrument were: column 240°C; injection port 250°C; detector 250°C; flow rate of helium carrier gas 75 ml/min.

A few microliters of the chloroform solution of the free sterols was injected into the instrument. Just as the cholesterol peak began to return back to the baseline, a capillary tube (1.5 mm O.D. × 10 cm) was fitted on the horizontal teat of the exit port. To insure condensation of the desired material, the capillary tube rested in cut-out vertical slots of a paper cup surrounded by dry ice. The capillary tube was removed in approximately 8 min, which was sufficient time for the desired material to elute from the column. After approximately five of the trappings were performed, the capillary containing the trapped material was sealed at the opposite end from where the trapped material was located, and the sides were washed down with 10 μl of chloroform. The capillary was centrifuged in order to remove residual chloroform from the sides, thus concentrating the solution. This was reinjected in its entirety into the gas chromatograph, and the material after cholesterol, which now appeared as fairly large peaks, was trapped in the same manner. The bulk of cholesterol had now been removed. The capillary tube was again sealed at one end, the sides rinsed with 10 μl of chloroform, and centrifuged. The solution in its entirety was then analyzed on the GLC–MS.

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Fractionation of Unsaponifiable Matter on Florisil

The final third of the unsaponifiable matter from the 125 g of milk fat was fractionated on Florisil as described by Eisner et al. (4). All of the fractions were evaporated to dryness on a steam bath under a stream of nitrogen, and the residues were dissolved in 5 ml of benzene. Fractions 3 and 4 were then analyzed using the GLC–MS.

GLC–Mass Spectrometry

The instrument used was the LKB 9000 manufactured by LKB Instruments, Inc., Rockville, Md. The gas chromatographic column employed was a 3 m × 4 mm (I.D.) coiled glass column packed with 3% (w/w) SE-30 on 100–120 mesh silanized Gas Chrom Z (Applied Science Laboratories Inc., State College, Pa.). The column was preconditioned for 24 hr at 280°C with a helium carrier gas flow rate of 10 ml/min. The column was programmed between 200°C and 230°C (2°C/min) for analysis of the regenerated hexane–benzene fraction. For the trapped material and fractions 3 and 4 from the Florisil, the column was kept at 230°C. Other standard operating conditions were: helium flow rate 30 ml/min; flash heater 250°C; separator 250°C; ion source 290°C. The mass spectra were obtained at a constant accelerating voltage of 3500 v with an electron energy of 70 ev and a scanning time of 4.5 sec over a m/e range of 12–450.

Reference Sterols[2]

Lanosterol and dihydrolanosterol were both obtained from Steroloids, Inc., Pawling, N.Y. The dihydrolanosterol was found to be pure by TLC and GLC, but the lanosterol was found to contain approximately 25% dihydrolanosterol. β-Sitosterol was obtained from the Applied Science Laboratories Inc., and was found by TLC and GLC to contain about 5% campesterol.

RESULTS

The Silica Gel G chromatogram indicated that all of the authentic alcohol derivatives moved in an area similar to the derivatives in the hexane–benzene fraction except for testosterone and estrone, which moved very little from the origin. The most intensely colored region of the hexane–benzene fraction, which was very complex, moved at the same rate as lanosterol. The chromatogram of the Silica Gel G-silver nitrate TLC plates was not greatly different from that of the Silica Gel G-adsorbent. One obvious feature of the chromatogram was the separation of the most intensely colored portion of the hexane–benzene fraction into two distinct spots. The new spot of lesser intensity moved ahead of the main spot and had an R_f similar to dihydrolanosterol, which usually accompanies lanosterol in nature.

The GLC chromatogram of the regenerated hexane–benzene fraction is shown in Fig. 1. Peaks A and B were identified as squalene and cholesterol, respectively, by their mass spectra and retention times. Peak B disappeared if the regenerated hexane–benzene fraction was passed over a small column of digitonin indicating that a small amount of the cholesterol escaped the digitonin column initially. Peaks C and D had the same retention times and mass spectra as dihydrolanosterol and lanosterol, respectively. The mass spectra are shown in Figs. 2 and 3. When the fraction was cochromatographed with first authentic lanosterol and then authentic dihydrolanosterol, the intensity of the respective peaks increased proportionately. None of the other peaks were identified although the mass spectra of some of them were taken and studied.

A typical GLC chromatogram of the trapped material can be seen in Fig. 4. The retention time and mass spectrum of peak B were identical to that of authentic β-sitosterol. None of the other peaks were identified except A which proved to be cholesterol.

The GLC chromatogram of fraction 4 from the Florisil column indicated that it was essentially all cholesterol as found by Eisner et al. (4). Fraction 3, however, gave the GLC chromatogram shown in Fig. 5. Peak A was identified as cholesterol. The peak of greatest intensity (C), which did not appear in the chromatogram of Eisner et al. (5) was proved by retention time and mass

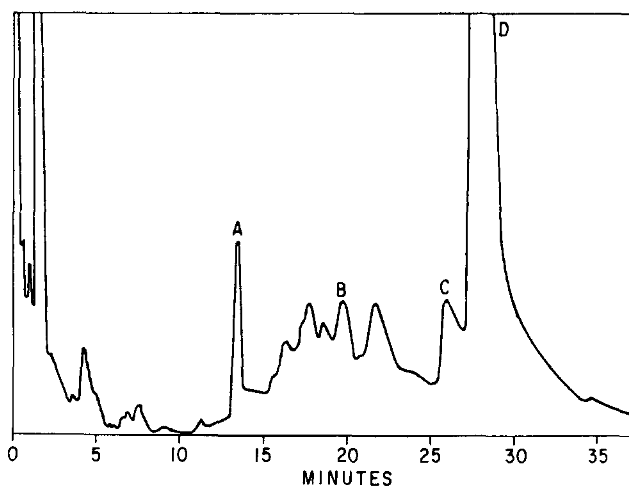

FIG. 1. GLC chromatogram of the lipid components in the regenerated hexane–benzene fraction. (*A*), squalene; *B*, cholesterol; *C*, dihydrolanosterol; *D*, lanosterol. Column: 3% SE-30. Temperatures: column 200–230°C programmed at 2°C/min; flash heater 250°C; separator 250°C; ion source 290°C. Carrier gas: helium, 30 ml/min.

[2] Names of sterols used in this paper are: lanosterol [8,24,(5α)-cholestadien-4,4,14α-trimethyl-3β-ol]; dihydrolanosterol [8(14),-(5α)-cholesten-4,4,14α-trimethyl-3β-ol]; β-sitosterol [5-cholesten-24β-ethyl-3β-ol].

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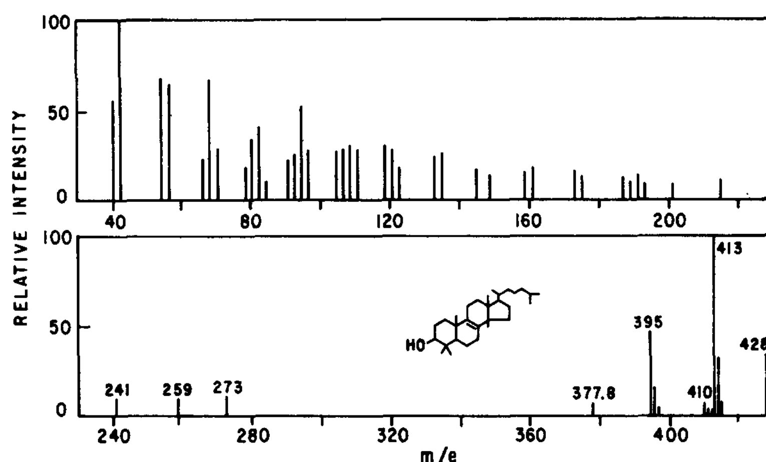

Fig. 2. Mass spectrum of dihydrolanosterol.

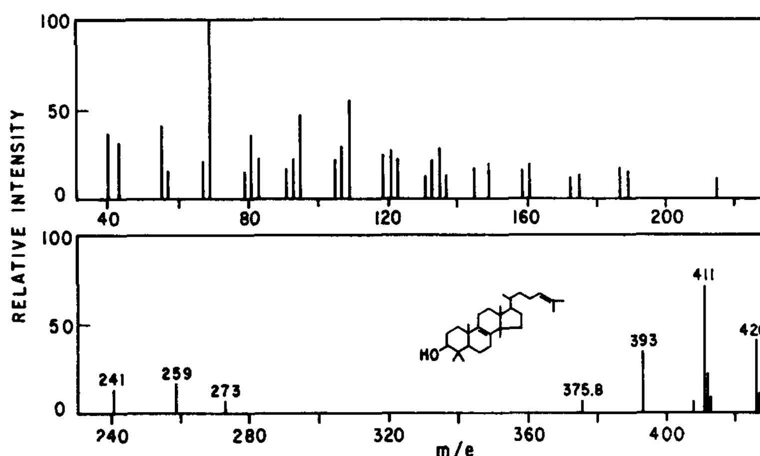

Fig. 3. Mass spectrum of lanosterol.

spectral analysis to be identical with lanosterol. Peak B was subsequently identified as dihydrolanosterol.

DISCUSSION

In previous studies the only sterols found in milk fat in sufficiently large quantities for positive identification were cholesterol and lanosterol. Lanosterol was identified by Morice (2) in 1951, but no one has since confirmed its presence. In a search for new constituents in milk fat, this study reports the isolation and identification of two sterols, dihydrolanosterol and β-sitosterol, as well as the confirmation of the presence of lanosterol. Neither dihydrolanosterol nor β-sitosterol occur in amounts comparable to that of lanosterol. The presence of dihydrolanosterol is important because it is a proposed intermediate in the biosynthesis of cholesterol, as is lanosterol. β-Sitosterol occurs largely in plants, so its presence in milk fat is not surprising.

The saponification of the milk fat was a critical step in this particular approach. Since most of the unidentified compounds in the unsaponifiable matter are thought to be hydroxy compounds, the isolation of them as derivatives was considered worthwhile. Consequently, it was necessary that the alcoholic solvents, used in other saponification procedures, be absent. The only procedure available that did not utilize alcoholic solvents in the actual saponification step was described by Schwartz et al. (6), and this procedure was used successfully.

The unsaponifiable matter of milk fat contains large quantities of cholesterol, and for an adequate analysis, it is helpful if the cholesterol is removed. The use of digitonin to precipitate cholesterol has long been a method for the isolation and determination of cholesterol (10). The compound is specific for 3-β-hydroxy steroids, but various degrees of precipitability have been found (11, 12) with steroids other than cholesterol having this configuration. It is then significant that dihydrolanosterol and lanos-

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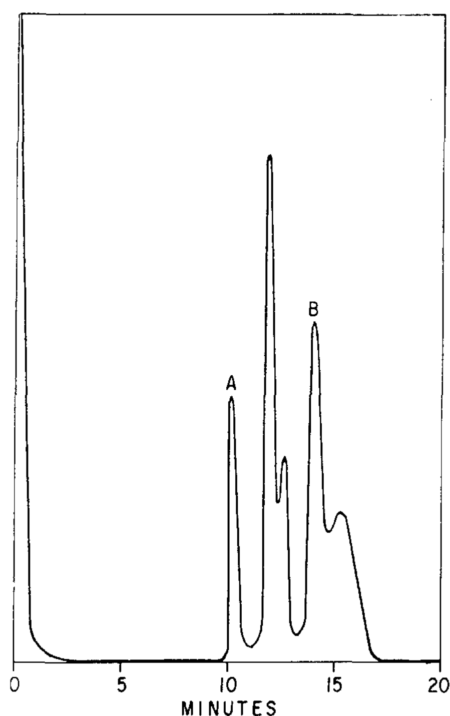

FIG. 4. A typical GLC chromatogram of GLC trapped components (other than cholesterol) eluted from the digitonin column. *A*, residual cholesterol; *B*, β-sitosterol. Column: 3% SE-30. Temperatures: column 230°C; flash heater 250°C; separator 250°C; ion source 290°C. Carrier gas: helium, 30 ml/min.

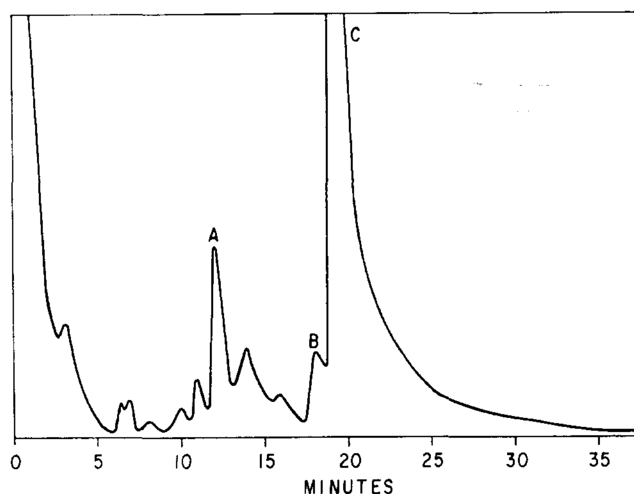

FIG. 5. GLC chromatogram of the unsaponifiable matter in fraction 3 from Florisil. *A*, cholesterol; *B*, dihydrolanosterol; *C*, lanosterol. Column: 3% SE-30. Temperatures: column 230°C; flash heater 250°C; separator 250°C; ion source 290°C. Carrier gas: helium, 30 ml/min.

terol were not precipitated by the digitonin column. Morice (2) reported that a precipitate with lanosterol formed within an hour in solution. Ruzicka, Denss, and Jeger (13) found that lanosterol was precipitated by digitonin in solution within 24 hr, while Homer and Virtanen (14) found that no precipitate was formed within 24 hr. It is, therefore, not surprising that lanosterol and dihydrolanosterol did not precipitate in the contact time allowed on the digitonin column, even though column reactions are generally more efficient. The digitonin column of Schwartz et al. (7) has particular value in that it offers an expedient and convenient means of removing not only cholesterol but also sitosterols, when they are constituents in lipids, from other lipid materials. More specifically, because of the steric requirement of digitonin, it offers an easy way to separate 3-β-OH sterols containing two methyl groups at the four position from other 3-β-OH sterols that do not contain two methyl groups at the four position.

A good mass spectrum was obtained for all three of the compounds identified. In Table 1 an explanation and list are given for the more important ions in the fragmentation pattern of lanosterol and dihydrolanosterol. The mass spectra of lanosterol and dihydrolanosterol are very similar as one would expect. Both exhibit rather intense molecular ions (426 and 428, respectively). The loss of a methyl group is, in both cases, the most intense peak in the high m/e region. A peak at M–33 indicating the loss of water and a methyl group, is fairly intense, but in contrast to many other sterols, a peak indicating the loss of water (M–18) is only weak.

Of particular importance in the identification of lanosterol and dihydrolanosterol is the presence of metastable peaks at 375.8 and 377.8, respectively. Both of the metastable peaks encountered were easily distinguishable and quite intense. It would seem that these peaks could be used to characterize compounds of this structure. They were formed from the M–15 to M–33 fragmentations.

The fragmentation giving rise to the peak at 273 for both lanosterol and dihydrolanosterol is characteristic of most sterols as pointed out by Biemann (15) and Budzikiewicz, Djerassi, and Williams (16). It represents the elimination of the side chain plus the loss of 42 mass units, which is believed to be the loss of carbon atoms 15, 16, and 17 of ring D. The peaks at 259 and 241 occurred in the spectra of both lanosterol and dihydrolanosterol.

TABLE 1 IMPORTANT FRAGMENTATION PROCESSES OF LANOSTEROL AND DIHYDROLANOSTEROL

Lanosterol Ion	Molecular Ion	Dihydrolanosterol Ion
426	M–15(CH_3)	428
411	M–18(H_2O)	413
408	M–18(H_2O)	410
393	M–33(CH_3 + H_2O)	395
375.8	Metastable	377.8
273	M–side chain + 42	273
259	M–side chain + 42 + CH_3	259
241	M–side chain + 42 + CH_3 + H_2O	241

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They cannot be easily explained, but it appears logical that they resulted from the loss of the side chain and 42 mass units, as above, plus the loss of a methyl group in the one case and the loss of a methyl group and water in the other.

The mass spectrum of β-sitosterol that was obtained but not shown here is very similar to the spectrum published and explained by Knights (17).

The fact that Eisner et al. (4, 5) did not find evidence for lanosterol in milk fat is puzzling. As shown in Fig. 4, lanosterol is present in relatively large amounts in fraction 3. It is important to note that lanosterol was eluted from the Florisil column before cholesterol. This finding is also in agreement with Morice (2) who found that lanosterol did move ahead of cholesterol on alumina. Even if the conditions were somewhat different because of differences in Florisil, etc., and the lanosterol did elute with fraction 4, the sterol fraction, cholesterol would not conceal its presence. Of course, the possibility always exists that the milk fats were grossly different in composition, but the authors feel that this is only a remote possibility.

The absence of saturated, continuous-chain aliphatic alcohols deserves mention. It was determined that they would appear in the hexane–benzene fraction if they were present. They have been found in the unsaponifiable matter of all vegetable oils as pointed out by Jacini, Fedeli, and Lanzani (18) and identified tentatively by Eisner et al. (5) in milk fat. From the present mass taken no evidence was found for their presence in milk fat in the present study.

Jacini et al. (18) gave the composition of the unsaponifiable matter of some of the more common vegetable oils. Two of the more frequently encountered constituents are cycloartenol and β-amyrin, both methyl sterols similar to lanosterol. They were also indicated from the work of Eisner et al. (5) to occur in milk fat. It was found that if they were present in the unsaponifiable matter of milk fat, they would occur in the hexane–benzene fraction; however, no evidence for their presence was found. In both cases, especially with β-amyrin, the retention times and R_f values are very close to lanosterol. Consequently, isolation and identification would be somewhat difficult due to the relatively large amounts of lanosterol present.

The fraction that was the most thoroughly analyzed was the hexane–benzene fraction. Mass spectra were obtained for all the GLC peaks, but identification of the peaks, other than the ones mentioned above, was not accomplished. Some work was also done on the benzene and chloroform fractions using the same approach, but at present none of the components has been identified.

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