Characterization of two steroidal ketones and two isoprenoid alcohols in dairy products

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Abstract Two steroidal ketones, Δ^4-cholesten-3-one and $\Delta^{3,5}$-cholestadiene-7-one, were isolated and identified for the first time in anhydrous milk fat and in nonfat dry milk. Together with these, two isoprenoid alcohols, phytol and dihydrophytol, were identified in anhydrous milk fat. Their identities were established on the basis of chromatographic and mass spectral data and confirmed by comparison with authentic materials.

Supplementary key words Δ^4-cholesten-3-one · $\Delta^{3,5}$-cholestadiene-7-one · phytol · dihydrophytol · gas–liquid chromatography · mass spectrometry

Thus far, investigations on the oxidation of milk lipids have primarily been aimed at the characterization of the off-flavor constituents (1) and concerned themselves with heat-, light-, and metal-catalyzed oxidations of glycerides (2–4). To date, only a few steroids have been isolated and identified in milk lipids. Probably, most of them are of plant origin (5–7) and were introduced via feed. Little work has been directed toward the study of their oxidation in milk fat, including the oxidation of cholesterol, which is by far the most abundant sterol. The only ketosteroid previously found in a dairy product is Δ^7-cholesten-3-one (8). Its origin and/or significance have not been elucidated. In the present investigation, a commercial sample of nonfat dry milk (NFDM) was examined for the presence of oxidation products of cholesterol. An analogous examination was conducted on anhydrous milk fat (AMF).

EXPERIMENTAL PROCEDURES[5]

Isolation of neutral lipids from NFDM

2.8 kg of NFDM, purchased at a local supermarket and stored at room temperature for 2 yr, was hydrated, extracted with *n*-hexane (J. T. Baker Chemical Co., Phillipsburg, N.J.),[6] and chromatographed over hydrated alumina as previously described (7). This brought about the removal of the hydrocarbons. The alumina column was then eluted with about 300 ml of a 1:6 (v/v) mixture of ether–hexane, and the eluate was dried over Na_2SO_4 and concentrated on a steam bath under a gentle stream of air. The residue was subjected to gas–liquid chromatographic–mass spectrometric (GLC–MS) analysis.

Fractionation of the unsaponifiable matter of AMF

200 g of AMF, prepared from mixed-herd milk, Beltsville, Md., and stored for 18 months at 5°C, was saponified without a solvent as reported earlier (9). The saponified material was ground in a mortar with an equal volume of Celite 545 (Fisher Scientific Co., Fair Lawn, N.J.) then transferred to a Soxhlet thimble and continuously extracted for 24 hr with *n*-hexane. (Both Celite and extraction thimble were previously washed with CH_2Cl_2 and dried.) The hexane extract was dried with Na_2SO_4 and evaporated to near dryness, and the residue was dissolved in the minimum amount of CH_2Cl_2. This solution was chromatographed on a column of 150 g of alumina (2 cm ID) previously hydrated to a 6% water content, and irrigation was started with 200 ml of *n*-hexane, which brought about elution of the hydrocarbons. The eluent was then changed to a 1:6 (v/v) mixture of ether–hexane, and two 200-ml fractions of eluate were collected. A fourth 400-ml fraction was collected by eluting with pure ether. Each one of these four fractions, designated as AMF-I to AMF-IV, was evaporated to near dryness and analyzed by GLC. The chromatographic profile of the first three fractions indicated that they did not contain oxygenated ste-

Abbreviations: AMF, anhydrous milk fat; NFDM, nonfat dry milk; GLC, gas–liquid chromatography; MS, mass spectrometry.

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[5] All solvents were distilled in all-glass systems prior to use.

[6] Reference to brand or firm names does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

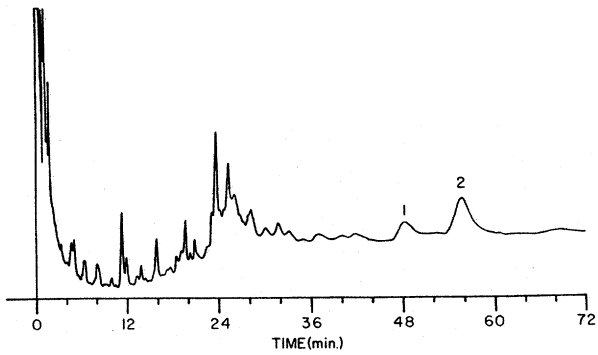

Fig. 1. Gas–liquid chromatogram (flame ionization detector) of the *n*-hexane extract of NFDM after removal of the hydrocarbons by chromatography over hydrated acid alumina. Programming: 4°C/min, from 90 to 220°C.

roids, whereas fraction AMF-IV seemed to consist of cholesterol alone. The latter was dissolved in just enough hot *n*-hexane to achieve solution, then it was cooled to −5°C in an ice–salt bath and quickly centrifuged. The supernate was concentrated to half volume, cooled to −5°C, and centrifuged; the final supernate was worked up as above. This procedure was repeated four times in order to get rid of most of the cholesterol. The last supernate thus obtained, designated as AMF-IV-S, was evaporated to near dryness, and the residue was dissolved in a small volume of *n*-hexane. This solution was placed on top of a 0.7 × 9.0 cm column of dry-packed acid alumina, Brockmann activity grade I (Baker). Eight 10-ml fractions were collected using eluents progressively richer in ether as follows: 1, ether–hexane 15:85; 2, ether–hexane 30:70; 3, ether–hexane 45:55; 4, ether–hexane 60:40; 5 and 6, ether; 7 and 8, methanol. Each fraction was evaporated to near dryness and analyzed by GLC. The chromatogram of the first four fractions, AMF-IV-S-1 to AMF-IV-S-4, showed little more than the solvent peak. AMF-IV-S-5 was fractionated further using another 0.7 × 9.0 cm column of acid alumina, with ether as the irrigant. Five 10-ml subfractions were eluted, which will be referred to as fractions AMF-IV-S-5A to AMF-IV-S-5E.

GLC and GLC–MS

The chromatograph was a Hewlett-Packard 5750B instrument equipped with a flame ionization detector and a 10:1 splitter. The column was a 4 ft × 0.125 inch (OD) stainless steel column treated with dimethylchlorosilane and packed with 3% OV-225 on Gas-Chrom Q (Applied Science Laboratories, State College, Pa.). The carrier gas was helium, supplied at a head pressure of 40 psi, and the column was programmed from 90 to 220°C at 4 or 6°C/min.

The low-resolution mass spectra were determined with an LKB 9000 GLC–MS system operating at an ionizing energy of 70 eV, an accelerating voltage of 3.5 kV, a separator temperature of 240°C, and an ion source temperature of 290°C. The samples were introduced into the mass spectrometer via the GLC inlet. Column and conditions were the same as those described above.

The high-resolution mass spectra were determined with a DuPont 21-110B double-focusing mass spectrometer at 70 eV. The spectra were recorded on a photoplate, and accurate mass measurements were made by means of a comparator, with perfluorokerosene as internal standard. The compounds for which high-resolution mass spectra were determined were trapped off the OV-225 column.

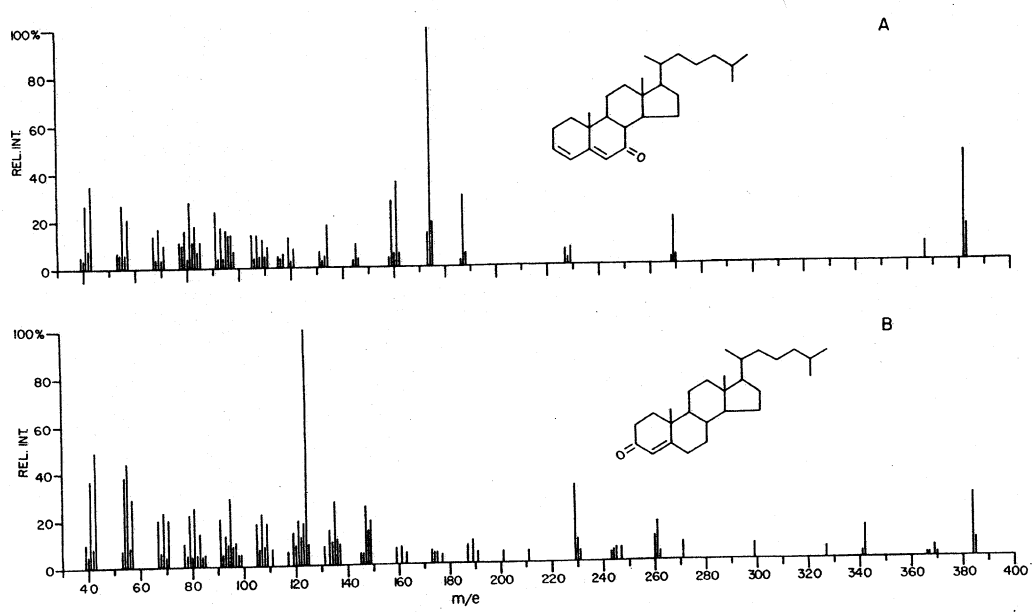

Fig. 2. Mass spectra of $\Delta^{3,5}$-cholestadiene-7-one (*A*) and Δ^{4}-cholesten-3-one (*B*) isolated from NFDM and AMF.

Reference compounds

Phytol was purchased from the Aldrich Chemical Co., Milwaukee, Wis. Δ^4-Cholesten-3-one and $\Delta^{3,5}$-cholestadiene-7-one were obtained from Steraloids, Inc., Pawling, N.Y. Dihydrophytol was from Analabs, Inc., North Haven, Conn. Cholesterol (purified via the dibromide) was purchased from Eastman Organic Chemicals, Rochester, N.Y. The oxidation of dihydrophytol and the methylation of the resulting phytanic acid were conducted on a microscale using the methods of Schwartz and Weihrauch (10) and Schwartz and Bright (11).

RESULTS AND DISCUSSION

Analysis of the neutral lipid fraction from NFDM

Fig. 1 shows the chromatogram of the neutral lipid fraction from NFDM. This same fraction was also analyzed by GLC–MS. Attention was focused on the peaks marked *1* and *2* (Fig. 1) because the mass spectra of the compounds associated with them showed several ions characteristic of steroid molecules. The low-resolution mass spectra of the compounds associated with peak 1 and peak 2 (Fig. 1) are shown in **Fig. 2,** *A* and *B,* respectively. The high-resolution mass spectral measurements of compound 1 indicated that the exact molecular weight was 382.3238, consistent with the elemental composition $C_{27}H_{42}O$, for which the calculated molecular weight is 382.3235. The absence of $[M - H_2O]^{+\cdot}$ and $[M - CH_3 - H_2O]^+$ species rules out the presence of a hydroxyl group. Therefore, the oxygen is most likely present in a carbonyl moiety. Assuming a tentative tetracyclic steroidal structure, there must be present two double bonds to account for the remaining two sites of unsaturation. The ion at *m/e* 269 ($M - C_8H_{17}$) resulting from the loss of the side chain at C_{17} and that at *m/e* 227 ($M - C_8H_{17} - 42$) supply conclusive evidence that the carbonyl and the two double bonds are present in the tetracyclic moiety. The rearrangement ion at *m/e* 174 (base peak) and those at *m/e* 187 and *m/e* 161 give prominent peaks in the spectrum of compound 1 and in that of $\Delta^{3,5}$-sitostadiene-7-one (12). Actually, the two spectra are virtually identical except for differences due to the ethyl substituent at C_{24} in $\Delta^{3,5}$-sitostadiene-7-one. We concluded that compound 1 was quite likely $\Delta^{3,5}$-cholestadiene-7-one. The spectrum of the authentic material was identical with that of the unknown. The gas–liquid chromatographic retention times also were in excellent agreement.

The high-resolution mass spectrum of the compound associated with peak 2 (Fig. 1) provided 384.3379 as the exact molecular weight, consistent with the elemental composition $C_{27}H_{44}O$, for which the calculated molecular weight is 384.3392. Assuming here too a tentative steroidal structure, the presence of the *m/e* 271 ($M - C_8H_{17}$) and *m/e* 229 ($M - C_8H_{17} - 42$) peaks suggests that the double bond and the oxygenated function are located in the polycyclic moiety. The intense ion at *m/e* 124 (base peak), together with that at *m/e* 342 ($M - 42$, loss of ketene), is strong indication of a Δ^4-3-keto moiety (13). Therefore, compound 2 was identified as Δ^4-cholesten-3-one. The mass spectrum and chromatographic retention time of the authentic material agree with those of the unknown.

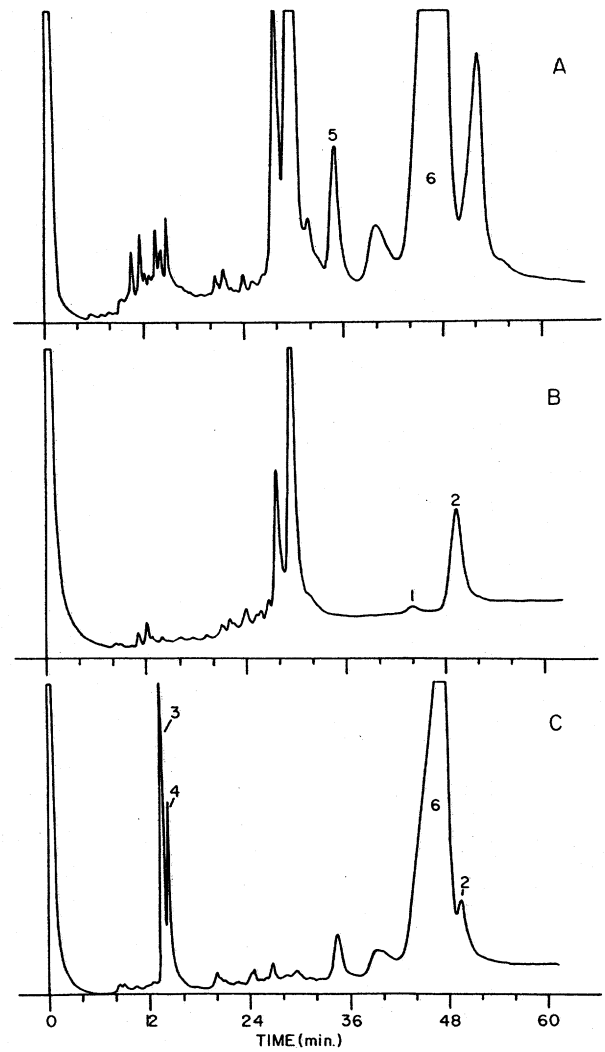

Fig. 3. Gas–liquid chromatogram of various fractions of the unsaponifiable matter of AMF. *A,* fraction AMF-IV-S-5; *B,* fraction AMF-IV-S-5C; *C,* fraction AMF-IV-S-6. Programming: 6°C/min, from 90 to 220°C.

Analysis of the unsaponifiable fraction of AMF

The presence of $\Delta^{3,5}$-cholestadiene-7-one and Δ^4-cholesten-3-one in the unsaponifiable fraction of AMF could be established only after several purification steps as described in Experimental Procedures. These were required by the presence of large amounts of cholesterol and lanosterol. The results of this purification procedure are shown

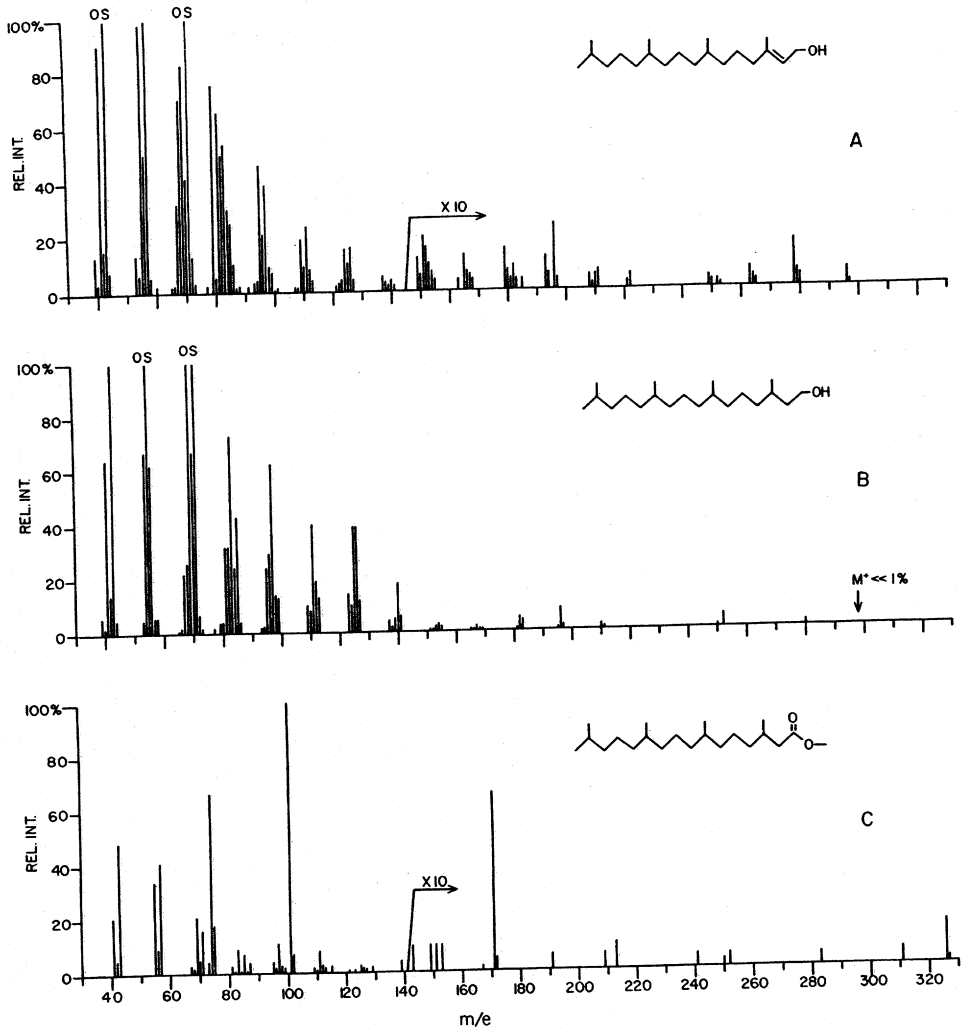

Fig. 4. Mass spectra of phytol (*A*) and dihydrophytol (*B*) from fraction AMF-IV-S-6, and of methyl phytanate (*C*). OS, off scale.

by the chromatograms of **Fig. 3.** Fraction AMF-IV-S-5 (Fig. 3*A*), although low in cholesterol (peak 5) content, still contains a substantial amount of lanosterol (peak 6). The peak of the latter sterol hides those of the two steroidal ketones, which can be seen in the chromatogram of fraction AMF-IV-S-5C (Fig. 3*B*), obtained after removal of the hydroxy-containing constituents by a second chromatography over acid alumina. The chromatogram of Fig. 3*B* also shows that the quantitative ratio of Δ^4-cholesten-3-one and $\Delta^{3,5}$-cholestadiene-7-one is different from that observed in NFDM (see Fig. 1). The mass spectra of both compounds agreed with those of authentic samples.

The chromatogram of fraction AMF-IV-S-6 (Fig. 3*C*) showed that it contained, in addition to lanosterol, Δ^4-cholesten-3-one, cholesterol, and several other minor unidentified compounds, at least two more products that gave relatively large and well-resolved peaks (peaks 3 and 4). The molecular ion of the unknown associated with peak 4 (Fig. 3*C*) was readily identified in its low-resolution mass spectrum at *m/e* 296. The spectrum (**Fig. 4,** *A*) also shows loss of one molecule of water (*m/e* 278) and a fragmentation pattern very similar to that of *trans*-phytene (9). The unknown was then tentatively identified as phytol, and its identity was confirmed by comparison of its GLC retention time and mass spectrum with those of an authentic sample.

The molecular ion (*m/e* 298) of the unknown associated with peak 3 (Fig. 3*C*) is much less than 1% of the base peak (Fig. 4*B*) and could be seen only through introduction of a relatively large sample into the LKB 9000 spectrometer. The high-resolution mass spectrum did not show the molecular ion. However, its elemental composition could be inferred by exact mass measurement of the only ions of reasonable abundance beyond *m/e* 210. They were the ion of *m/e* 280 (elemental composition $C_{20}H_{40}$, $[M - H_2O]^{+\cdot}$) and that of *m/e* 252 (elemental composition $C_{18}H_{36}$, $[M - H_2O - C_2H_4]^{+\cdot}$). The elimination of water coupled with the expulsion of ethylene suggests

that the unknown is a primary alcohol unbranched at the α and β positions. In this case, the ion of m/e 252 can be arrived at, from the molecular ion, through a six-membered transition state (14). The unknown was tentatively assumed to be dihydrophytol. It was trapped off the OV-225 GLC column and oxidized on a microcolumn of chromic acid on Celite (10), and the reaction product was methylated with diazomethane (11). The mass spectrum of the final product (Fig. 4*C*) was identical with that of phytanic acid methyl ester (15). The identify of the unknown compound associated with peak 3 (Fig. 3*C*) was finally confirmed by comparison with authentic dehydrophytol.

The present investigation is the first to report the presence of $\Delta^{3,5}$-cholestadiene-7-one and of Δ^{4}-cholesten-3-one in dairy products. $\Delta^{3,5}$-Cholestadiene-7-one is a major product of autoxidation of cholesterol (16), and its formation via 7-ketocholesterol has been studied (16). The origin of Δ^{4}-cholesten-3-one is less certain. It has been recognized as an intermediate metabolite of cholesterol in animals and plants (17, 18); however, it has also been identified as a photo-derived product (19) and as an autoxidation product of cholesterol under drastic conditions (20). As for the presence of both steroid ketones in NFDM and AMF, it is uncertain whether or not they can be regarded as normal constituents of fresh milk.

The following experiment was conducted to verify whether or not $\Delta^{3,5}$-cholestadiene-7-one and Δ^{4}-cholestene-3-one might have formed from cholesterol during the separation process. Cholesterol (2.55 g) was dissolved in hexane (500 ml) and subjected to the same chemical and physical conditions as the extract of NFDM and the unsaponifiable fraction of AMF. These included concentration on a steam bath under a stream of air, and chromatography on alumina. No trace of either ketone could be detected.

Phytol and dihydrophytol are the first two isoprenoid alcohols isolated from milk. Other isoprenoid compounds previously found in milk fat are pristane, phytane, phytanic acid, pristanic acid, 4,8,12-trimethyltridecanoic acid (21), and phytene (9). A likely source of phytol found in milk fat is ingested chlorophyll, but the metabolic intermediates in its conversion to dihydrophytol in the cow's body have not yet been elucidated. In this connection, it is worth mentioning that Patton and Benson (22) have found not only phytol but also dihydrophytol in the rumen contents of lactating cows.

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