Isolation and characterization of the methyl ketone precursor in butter fat

OWEN W. PARKS, MARK KEENEY, IRA KATZ, and DANIEL P. SCHWARTZ

Dairy Products Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Washington, D.C., and Department of Dairy Science University of Maryland, College Park, Maryland

SUMMARY The direct isolation of the methyl ketone precursor in butter fat is described. Evidence is presented which demonstrates that the precursors of the C_5 through C_{15} odd-numbered methyl ketones are triglycerides containing one β-keto acid and two fatty acid moieties. These glycerides accounted for 0.045% of the butter fat.

RECENTLY, Van der Ven et al. (1) demonstrated the presence of β-keto acids in milk fat by isolating pyrazolones formed on the reaction of butter fat with Girard's T reagent. These same workers presented evidence supporting the suggestion of Wong et al. (2), that β-keto esters are the precursors of the methyl ketones liberated in butter fat by saponification or heating in the presence of moisture. This paper substantiates the role of β-keto acids as the methyl ketone precursor in butter fat and presents evidence that these acids are esterified in the triglycerides.

EXPERIMENTAL METHODS

Isolation of Crude Methyl Ketone Precursor

Butter churned from 40% cream at 10° was melted at 45° and washed with warm water and the clear butter oil obtained by centrifugation. In a typical experiment 100.6 g of butter oil made up to 500 ml with hexane (3) was passed through a chromatographic column containing 30 g of a 2:1 mixture of Celite 545[1] (Johns-Manville Company, New York) and Magnesia 2665 (Fisher Scientific Company, Silver Spring, Md.).

[1] The use of trade names is for the purpose of identification only, and does not imply endorsement of the product or its manufacturer by the U. S. Department of Agriculture.

Following passage of the solution, the column was washed with approximately 500 ml of hexane until an aliquot of the effluent was free of residue on evaporation. It is essential that all nonadsorbable lipid be eluted from the column, since the methyl ketone precursor (which remains on the column) cannot be separated from triglycerides in the subsequent purification step. The total hexane effluent yielded 98.2 g of lipid residue upon evaporation of the solvent. The nonsaponifiable fraction of the fat passing through the magnesia–Celite column contained no methyl ketones (4), indicating complete extraction of the precursors by the basic adsorbent. The magnesia–extractable material was eluted from the adsorbent by passing 75 ml of a benzene–methanol (3:2) solution containing 2% concd HCl through the column followed by 250 ml of hexane. The combined benzene–methanol and hexane effluent, containing 1.4 g of lipid, was washed with 50 ml of distilled water, followed by three 50-ml washings with 90% methanol. The hexane–benzene layer was dried with sodium sulfate, filtered, and evaporated to dryness on a steam bath with the aid of a stream of nitrogen. The resulting residue (62.5 mg) was taken up in 10 ml of hexane.

Purification of the Methyl Ketone Precursor by TLC

The fraction recovered from the magnesia was further fractionated by TLC on neutral Silica Gel G (see Fig. 1). The plates were prepared by making a slurry of 30 g of Silica Gel G with 60 ml of distilled water and applying it to 20 x 20 cm plates with a Research Specialties Applicator (Research Specialties Company, Richmond, Cal.). The plates were allowed to dry at room temperature for 30 min and activated for 16 hr at 100°. Approximately 0.5 mg of crude fraction was applied in

hexane solution to each of nine spots per plate. The chromatograms were developed in 30 min with a hexane–ethyl ether solvent system (1:1). The guide strips were sprayed with 10% phosphomolybdic acid in 95% ethanol and heated to develop blue spots on a yellow background. The desired fraction of the remaining strips was scraped from the plates and the lipids were recovered by slurrying the Silica Gel G in one volume of redistilled methanol. Three volumes of redistilled hexane were added to the methanol slurry and the mixture was stirred thoroughly and filtered. To the filtrate in a separatory funnel was added one volume of distilled water. The mixture was shaken, the hexane layer was recovered, dried with sodium sulfate, and filtered, and the hexane removed under vacuum. An aliquot of the residue (17.6 mg) on heating or saponification in 4% KOH liberated the C_5 through C_{15} odd-numbered methyl ketones, as determined by column chromatography retention volumes and ultraviolet spectral studies of the 2,4-dinitrophenylhydrazones[2] (5).

Quantitative Methyl Ketone Data

A sample of butter oil or the isolated methyl ketone precursor, sealed in a glass ampoule in the presence of moisture and nitrogen, was heated for 40 hr at 100° (glycerol bath). Following the heating period, the ampoule was washed thoroughly, dried, and crushed under carbonyl-free hexane (6). In the case of the methyl ketone precursor, the sample was cooled to −12° prior to crushing under hexane. The DNP–hydrazones were prepared and the methyl ketone DNP–hydrazones obtained by methods previously reported (7, 8). The DNP–hydrazones were separated by column chromatography by the method of Corbin et al. (5). The C_{11}, C_{13}, and C_{15} methyl ketone DNP–hydrazones, collected from the Corbin column as one fraction, were separated by reverse phase chromatography on silanized Hyflo Super-Cel (Johns-Manville Company, New York) employing dodecane as the immobile phase and 85% aqueous acetonitrile as the mobile phase.[3] Optical density determinations of the DNP–hydrazones were obtained in chloroform at 365 mμ and concentrations calculated on the basis of a molar extinction coefficient of 22,500.

Infrared Study

An infrared spectral analysis of the methyl ketone precursor was made with a Perkin-Elmer Model 21 (Perkin-Elmer Corp., Norwalk, Connecticut) instrument. The lipid material was examined in either Baker spectrophotometric grade carbon tetrachloride or
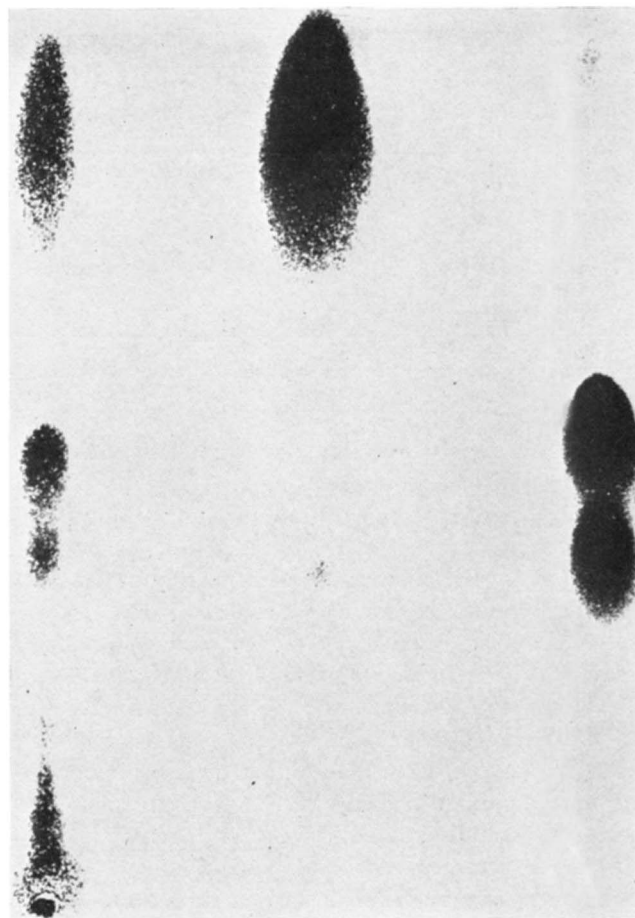

FIG. 1 Thin-layer chromatogram (excluding the spots that moved with the solvent front) of crude methyl ketone precursor fraction (left), methyl ketone precursor isolated from TLC plates (middle), and the heated methyl ketone precursor (right).

chloroform (J. T. Baker Chemical Co., Muirkirk, Md.) at a concentration of approximately 8 mg/ml in a 0.5 mm sodium chloride cell using a matched reference cell. A sample of the methyl ketone precursor was also sealed under nitrogen in a 10 ml glass ampoule, to which had been added a few drops of distilled water, and heated for 40 hr at 100°. An infrared spectrum of the heated precursor was obtained and the fraction was recovered from the cell and subjected to TLC (Fig. 1). The resulting two spots were recovered individually from the plate and an infrared spectrum was obtained on each.

Gas–Liquid Chromatography (GLC)

Analyses were obtained with a Research Specialties Model 600 instrument equipped with an argon ionization detector.

The fatty acid compositions of the original butter fat and the diglycerides recovered by TLC of the heated precursor were determined (using the methyl esters)

TABLE 1 METHYL KETONES OBTAINED BY HEATING BUTTER FAT AND THE ISOLATED METHYL KETONE PRECURSOR

DNP Hydrazone	8.3 g Butter Fat	4.0 mg Methyl Ketone Precursor
	μmoles	*μmoles*
Pentanone-2	0.58	0.62
Heptanone-2	1.19	1.38
Nonanone-2	0.56	0.74
Undecanone-2	0.59	0.61
Tridecanone-2	0.59	0.71
Pentadecanone-2	1.34	1.31
Total	4.85	5.37

on a 6 ft stainless steel column containing 10% Apiezon L on 60–80 mesh, acid-washed Celite.

Ester to glycerol molar ratios of the diglycerides were determined by hydrogenolysis and acetylation according to the method of Horrocks and Cornwell (9). The chromatographic separations of the acetates were conducted on a 6 ft stainless steel column containing 18% diethylene glycol succinate polyester on 80–90 mesh Anakrom AB (Analytical Engineering Laboratories, Hamden, Conn.). The response factors of reference samples were determined by chromatographing known quantities of glyceryl triacetate, oleyl acetate, and palmityl acetate. Glyceryl triacetate was purchased commercially and also obtained by the hydrogenolysis and acetylation of dipalmitin. Oleyl acetate and palmityl acetate were prepared from methyl oleate and dipalmitin (Hormel Institute, Austin, Minn.).
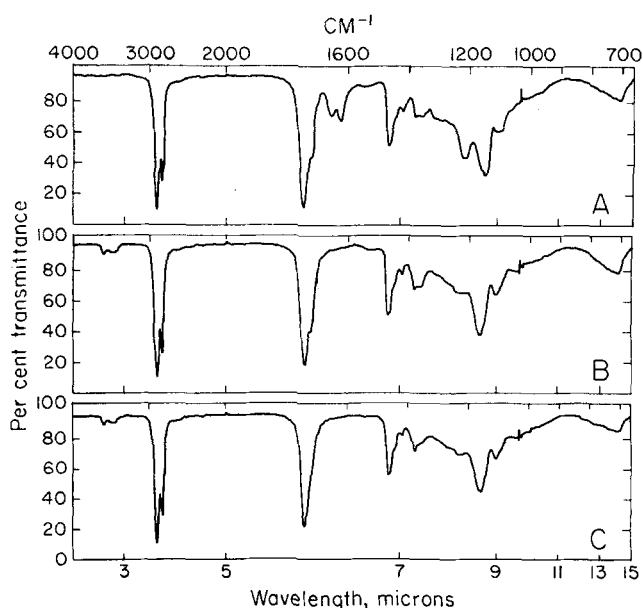
The acetates were also chromatographed on an Apiezon column to confirm the presence of glyceryl triacetate.

RESULTS AND DISCUSSION

Table 1 presents the number of micromoles of methyl ketones formed from heating 8.3 g of butter fat and 4.0 mg of the isolated methyl ketone precursor. It can be deduced from these data and the recovery of 17.6 mg of precursor from 100.6 g of the butter oil employed in this study that 42% of the precursor was recovered. Most of the loss can be accounted for in the three aqueous methanol extractions of the magnesia-absorbable material, the guide strips sprayed with phosphomolybdic acid, and the procedures employed for recovering the precursor from the TLC plates. The molar ratios of the methyl ketones liberated from an aliquot of the precursor are approximately the same as observed in the original fat as well as in other butter fats examined.[3] It can be concluded that the lipid material isolated is representative of the total methyl ketone precursor in butter fat.

Figure 2 contains the infrared spectra of (A) the methyl ketone precursor, (B) the heated methyl ketone precursor, and (C) the faster moving portion (TLC) of the heated precursor (slower moving portion gave the same spectrum). An over-all interpretation of these infrared spectra leads to the conclusion that the methyl ketone precursor is a triglyceride in which one of the acids esterified to the glycerol molecule is a β-keto acid.

Esters of β-keto acids have been reported (10, 11) to exhibit characteristic infrared absorption bands. Spectrum A shows, in addition to normal absorptions at 5.72 and 5.82 μ due to the presence of ester and ketone carbonyl groups, two absorption bands at 6.05 and 6.15 μ. These bands are the result of partial enolization of the β-keto esters and have been ascribed to the ester carbonyl group after chelation to the enolic hydroxyl group and the C=C, respectively. Heating the precursor (spectrum B) results in the complete loss of bands at 6.05 and 6.15 μ, with a corresponding increase in absorption of the ketone carbonyl group at 5.82 μ. Further influence of the β-keto ester enolization is reflected in the 8.0–9.0 μ region. Typical triglycerides do not usually show absorption at 8.2 μ and the strongest C—O stretching band usually absorbs nearer to 8.55 μ (12), whereas that in spectrum A is at 8.7 μ. Heating the precursor, however, results in the loss of the peak at 8.2 μ and a shift in the major C=O stretching band to 8.6 μ, so that the entire 8.0–10.0 μ region exhibits absorption patterns typical of diglycerides (12). The appearance of two O—H stretching vibration peaks with maxima at 2.8 and 2.9 μ further

FIG. 2 Infrared spectra in CCl$_4$ solution of (A) the methyl ketone precursor, (B) the heated methyl ketone precursor, and (C) the faster moving fraction (TLC) from the heated precursor.

substantiates the presence of a diglyceride in spectrum B. Spectrum C, in which the methyl ketones have been removed by TLC, is typical of the infrared spectra of diglycerides.

The ester to glycerol molar ratios of the two diglyceride fractions recovered from the TLC plates, as determined by GLC following hydrogenolysis and acetylation, were found to average 2.17 and 2.02. The identification of glycerol was based on comparisons of retention times of the triacetate on polyester and Apiezon stationary phases with those of standards.

The fatty acid composition of the diglycerides recovered from the TLC plates, as revealed by GLC of the methyl esters and fatty alcohol acetates, was typical of butter fat (13, 14) although the palmitic acid content was slightly higher than that of the original butter fat.

Inasmuch as 4 mg of pure methyl ketone precursor yielded 5.37 μmoles of methyl ketone, and 4.85 μmoles of methyl ketone resulted from 8.3 g of the original butter fat, it is estimated that the methyl ketone precursor represents 0.045% of the butter fat used in this study.

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