Chromatographic Separation of Lactone Precursors and Tentative Identification of the γ-Lactones of 4-Hydroxy Octanoic and 4-Hydroxy Nonanoic Acids in Butterfat[1]

J. E. KINSELLA, S. PATTON and P. S. DIMICK, Lipids Laboratory, Division of Food Science and Industry, The Pennsylvania State University, University Park, Pennsylvania

Abstract

A combination of silicic acid column and thin layer chromatography which separates γ- and δ-hydroxy acid containing glycerides from butterfat is described. This procedure facilitates the isolation of the γ- and δ-hydroxy acids (lactone precursors) while avoiding lactonization. The method indicated that the hydroxy acids are entirely esterified to the glyceride in fresh butterfat. Hydrolysis, using pancreatic lipase, showed that these polar acids are located on the α-positions of the glycerides. The γ-lactones of 4-hydroxy octanoic and 4-hydroxy nonanoic acids were tentatively identified by gas liquid chromatography. These occurred in quantities of 0.25–0.5 ppm and approximately 0.2 ppm respectively in the butterfat samples investigated.

Introduction

THE ESSENTIAL ROLE of the major aliphatic lactones (δ-decalactone and δ-dodecalactone) in imparting a characteristic and desirable flavor to butter and foods containing butterfat and causing an undesirable nonoxidative off flavor in stored whole dry milk is well known (1–3). Currently these lactones are incorporated into margarine to improve its palatability (4). Many other aliphatic lactones have been identified in milk and butterfat (5); however, their overall contribution to the flavor of butter has not yet been established. Including both series, i.e., the saturated γ- and δ-aliphatic lactones, 17 have been identified and quantified from butterfat (5,6). The precursors of these lactones are the corresponding γ- and δ-hydroxy alkanoic acids which occur in butterfat esterified in glyceride form (2,3). These glyceride containing hydroxy acids are very unstable towards heat and acidity. The hydroxy acids easily hydrolyze off and spontaneously lactonize.

This communication describes chromatographic methods facilitating the isolation and separation of these lactone precursor glycerides in their natural state by avoiding lactonization. Using this method both the γ-lactones of the corresponding 4-hydroxy octanoic and nonanoic acids were isolated and tentatively identified.

Experimental

Reagent grade solvents were redistilled from glass prior to use.

Silicic Acid Column Chromatography (SACC)

One hundred grams of Mallinckrodt silicic acid (80–100 mesh) prepared according to Hirsch and Ahrens (7) was slurried into a large glass column (60 cm × 4.5 cm I.D.) in petroleum ether bp 35–42C. The silicic acid was thoroughly packed by gently tapping the tube and the gel bed washed with an additional 300 ml petroleum ether to give a gravity flow rate of 5–6 ml/min. The temperature of this column was kept slightly below that of the eluting solvents used. This precaution avoided bubble formation and consequent cracking of the column packing. Fifteen grams of butterfat, prepared from butteroil by centrifugation (1) was dissolved in 50 ml of 1% diethyl ether in petroleum ether and gently applied onto the column. Two 25-ml quantities of the same solvent mixture were successively applied to wash the sample completely into the column packing and a solvent head of ½ in. was maintained above the packing.

After some initial experimentation, the following four eluting solvents were found to give the most efficient separation of the polar glycerides: 1) 250 ml 1% diethyl ether in petroleum ether; 2) 300 ml of 5% diethyl ether in petroleum ether; 3) 250 ml 8% diethyl ether in petroleum ether; 4) 2 × 250 ml 1% methanol in diethyl ether. This sequential elution by solvent mixtures 1–3 removed the nonpolar lipids in a gradual manner. This minimized the mutual solubilizing effect of the normal glycerides on the hydroxy acid containing glycerides enabling the latter to be adsorbed to the silicic acid. Effluents from 1, 2, and 3 were pooled and their solvent evaporated. The final eluate (fraction 4) with the triglycerides containing hydroxy fatty acids (HTG), was very carefully concentrated under a stream of nitrogen.

Thin-Layer Chromatography

One-dimensional thin-layer chromatography (TLC) was used in all cases. Glass plates (20 × 20 cm) were coated with a slurry of 20g Silica Gel G, (250 μ thickness) in 40 ml of water. These plates were activated at 120C for 2 hr before use. The plates were developed in starch-sealed glass tanks lined with solvent-saturated filter paper in a solvent system consisting of petroleum ether:diethyl ether:acetic acid (63:37:0.5 v/v). The lipid spots were made visible using sulfuric acid-potassium dichromate spray reagent (10%) and heating (120C for 30 min). Recovery of specific spots from developed TLC chromatograms was achieved by revealing a side strip of the plate containing both standards and sample, with iodine spray (10% v/w in methanol) and scraping the corresponding area of the unsprayed portion. After scraping the authentic spots on the intact side strip were made visible by sulfuric acid-potassium dichromate charring. This served as a qualitative check on the scraped fractions. The lipids were extracted from TLC scrapings by eluting with diethyl ether and filtering through glass wool to remove the silica gel particles.

Cholesterol was a regular contaminant of the HTG isolated by silicic acid column chromatography

[1] Authorized for publication on June 22, 1966, as Paper No. 3156 in the journal series of the Pennsylvania Agricultural Experiment Station.

(SACC). Under the TLC conditions employed throughout this investigation, the chromatographic behavior of cholesterol waas predictable and since it was easy to locate on the TLC plate, (UV light), cholesterol was used as an internal standard in comparing the Rf values of the relatively polar HTG fractions. Thus, the migration of the various compounds under study were expressed relative to cholesterol, i.e. $R_{chol} = \frac{\text{distance of unknown from origin}}{\text{distance of cholesterol from origin}}$ (9). This characteristic was found to be constant on numerous chromatograms developed under similar conditions.

Gas-Liquid Chromatography

Lactones were routinely separated by gas-liquid chromatography (GLC) on diethylene glycol adipate (8). For detection of the trace lactones (less than 1 ppm) a Barber-Colman Model 5000 gas chromatograph with a hydrogen flame ionization detector was used. To confirm the identity of unknown peaks both polar (polyester) and nonpolar (Apiezon) coated column packings were used (Table I). Authentic lactone standards were used for comparison.

Samples analyzed included the glyceride containing hydroxy acids isolated by SACC. Since these compounds are highly unstable upon heating the temperature of the injection port (210C) caused immediate hydrolysis from the glyceride moiety with simultaneous lactonization of these γ- and δ-hydroxy acids. The resulting chromatograph scan indicated the quantity and type of lactones present (8). By saponifying (8) and/or hydrolyzing with lipase (10) aliquots of the HTG and separating the products by TLC distinct fractions were recovered which contained γ- and δ-lactones almost exclusively (Fig. 1). Methyl esters of the normal fatty acids from the HTG were prepared and analyzed as described elsewhere (11).

Infrared spectra were obtained in carbon tetrachloride solution (5% w/v) on a Perkin-Infracord spectrophotometer using sodium chloride microcells.

Results

The pooled fraction from SACC elutions 1, 2, and 3 contained the hydrocarbons, sterol esters, triglycerides, and most of the unesterified fatty acids (FFA). Gravimetrically, these accounted for 98%+ of the sample applied to the column. This pooled fraction when heated for 3 hr at 120C did not produce any lactone odor indicating the absence of HTG in this fraction. If present, these would yield lactones which are detectable, olfactorily, at a concentration of 1–5 ppm. The final eluate from the silicic acid column contained FFA, cholesterol, glycerides containing hydroxy acids (HTG), diglycerides and monoglycerides. This fraction accounted for 0.75% of the applied butterfat sample. When the solvent was very carefully evaporated from this fraction by a stream of N_2 in the cold, the characteristic coconut odor of lactones was absent, which indicated tthat the γ- and δ-hydroxy fatty acids present were esterified in the glycerides. The HTG moiety usually showed a range in polarity of R_{chol} 0.8–1.2 on TLC (Fig. 1).

The structure of a typical triglyceride containing hydroxy fatty acid is shown (Fig. 2). Upon heating (180C) in the presence of trace amounts of water (15), this hydroxy acid is hydrolyzed and simultaneously lactonized as indicated. Likewise, injection

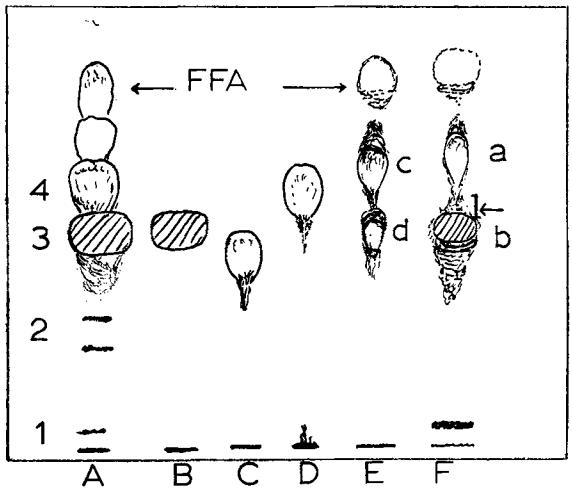

FIG. 1. Composite thin-layer chromatogram of the HTG fractions. Legend; A = polar eluate (fraction 4) from the silicic acid column consisting of monoglyceride, diglycerides, cholesterol, glyceride containing hydroxy acids and free fatty acids denoted A1, A2, A3, A4, and FFA, respectively; B = cholesterol; C and D = standard δ- and γ-lactone mixtures; E = saponifiable compounds derived from HTG fraction. Spot Ec contains mostly γ- and δ-lactones and spot Ed contains hydroxy fatty acids and δ-lactones. F = products obtained from A by hydrolyzing with lipase. Spot Fb, excluding the cholesterol, (hatch marked) consists of hydroxy acids and δ-lactones and spot Fa contains γ-lactones and short chain fatty acids. The maximum concentrations of γ-C8 and γ-C9 lactones are indicated by arrow.

of such glycerides into a GLC column (175C) releases the lactones (odorous) corresponding to the precursor hydroxy alkanoic acids (odorless).

The GLC chromatogram obtained following injection of the HTG showed that this fraction contained predominantly the C8 through C14 delta lactone and C10 and C12 gamma lactone precursors. The presence of smaller quantities of other lactone precursors

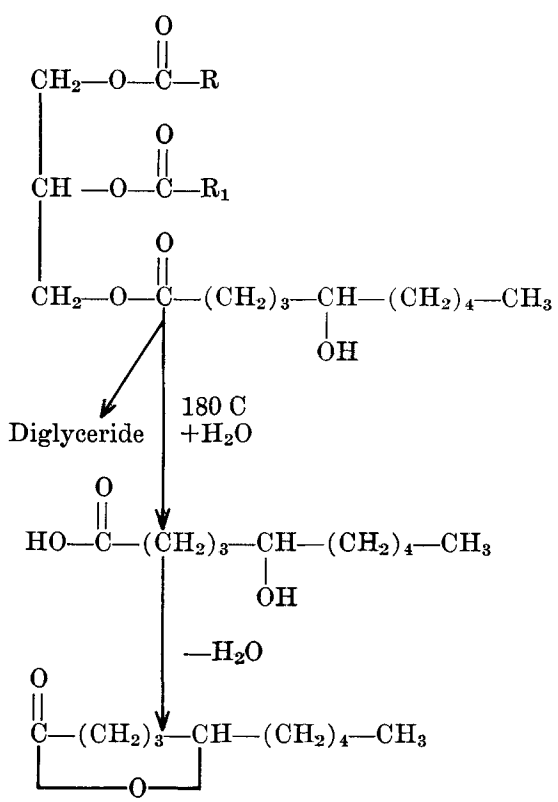

FIG. 2. Mechanism of δ-lactone formation.

TABLE I

Retention Time of γ-C8 Lactone Under Various Gas Chromatographic Conditions

GC[a] model	Length, ft	Column packing[b]	Temp, C	Inlet gas[c] pressure, lb	Detector		Retention time (min)	
					Type[d]	Voltage	Unknown	Standard γ-C8
5000	6	10% DEGA	180	20	HFI		2.3	2.2
			175	16			1.9	1.9
10	6	10% DEGA	187	30	R-226	750	3.5	3.6
			187	20		1000	5.9	5.9
10	6	5% Apiezon L	180	15	R-226	750	7.6	7.6
20	8	12% DEGS	180	12	S	1500	6.4	6.4

[a] All machines were Barber-Colman models.
[b] From Applied Science Laboratories, State College, Pa.
[c] Argon carrier gas used in all cases.
[d] HFI—Hydrogen flame ionization; R-226—Radium argon ionization; S—Strontium detector.

in this series was also apparent but the larger lactone peaks rendered it difficult to reliably determine their retention times. Nevertheless, peaks corresponding to the δ-lactone of 5-hydroxy hexanoic acid (δ-C6), the γ-lactone of 4-hydroxy octanoic acid (γ-C8), the γ-lactone of 4-hydroxy nonanoic acid (γ-C9), and the γ-lactone of 4-hydroxy tetradecanoic acid (γ-C14) were tentatively identified from a plot of the log of their retention time versus their carbon number. The saponifiable compounds from the HTG, i.e. the free fatty acids (FFA), hydroxy fatty acids and lactones, were separated on a TLC plate (Fig. 1). The spot having an R_{chol} 1.16–1.20 contained the γ-C8, γ-C9, γ-C10, γ-C12, γ-C14, γ-C16 lactones. These had retention times identical to the corresponding authentic lactones. Both the γ-C8 and γ-C9 lactones cochromatographed with authentic standard γ-C8 and γ-C9 lactones on the gas chromatographs used. The γ-C8 lactone peak was confirmed by GLC analysis on different columns (Table I).

The HTG fraction was hydrolyzed using pancreatic lipase and the products then separated by TLC. The spot, with R_{chol} 1.2, contained both γ-lactones and γ-hydroxy fatty acids. The GLC peaks of all the γ-lactones from this fraction exactly coincided with the retention times of standard γ-lactones. The γ-C8 and γ-C9 lactones occurred in highest concentration in an area corresponding to R_{chol} 1.05–1.13 on the TLC plate (Fig. 1). These lactones were quantitated by comparison with peak areas of standard γ-C8 and γ-C9 lactones according to the procedure of Dimick et al. (1). The quantity of γ-C8 lactone varied between 0.25–0.5 ppm and γ-C9 was approximately 0.2 ppm.

The pancreatic lipase hydrolyzed the lactone precursors (γ- and δ-hydroxy fatty acids) from the HTG molecule at the α-position since the resulting monoglycerides, when analyzed by GLC, did not contain any lactones. These data strongly suggest that the γ- and δ-hydroxy acids are esterified to the α-positions of their constituent glyceride molecules. It was not established whether there were one or two molecules of hydroxy fatty acids per glyceride molecule but chromatographic behavior (polarity) on TLC plates suggested the presence of only one hydroxyl function on these triglycerides.

The fatty acid composition of the monoglycerides are presented in Table II. The normal fatty acids recovered from the saponified HTG consisted predominantly of myristic (5%), palmitic (50%), stearic (20%), oleic (20%+) and traces of the other fatty acids usually found in butterfat.

An IR spectrum of the saponifiable compounds (hydroxy fatty acids and lactones) of the HTG from which the normal fatty acids had been removed by TLC, is shown (Fig. 3). The OH stretching peak at 3560 cm^{-1} is characteristic of hydroxy groups located in a hydrocarbon chain (12). Association of carboxyl groups (dimerization) by H bonding, especially in dilute nonpolar solution, accounts for the broad absorption at 2000–3000 cm^{-1}. The absorptions at 1770 cm^{-1} and 1720 cm^{-1} are evidence of the presence of γ- and δ-lactones, respectively (13,14). This spectrum demonstrates the presence of both the lactones and their precursor hydroxy acids in the saponifiable matter from the HTG sample. On heating an aliquot of this, the OH absorption peak disappeared. Evidently, the heating caused lactonization and loss of the free OH function, indicating that it was located in γ- and/or δ-position of the hydrocarbon chain of the fatty acid.

Discussion

Methods for isolating γ- and/or δ-hydroxy alkanoic acids (or their esters) as such are challenging because of their inherent instability. The relative intramolecular electronegativity of the hydroxyl group (inductive effect) renders it very susceptible to an electrophilic attack by either the adjacent carboxyl group of the acid (or carbonyl group of the ester) causing these to lactonize under most experimental (and practical) conditions, except in alkaline solution. The chromatographic conditions described above minimize the amount of preparative manipulation of samples and enable the immediate lactone precursors, i.e., the triglycerides containing hydroxy acids, to be isolated in their natural state. Such a method is desirable to facilitate the complete elucidation of the structure and quantification of these triglyceride containing hydroxy fatty acids. It should also greatly aid the investigation of the kinetics of lactone formation.

The above chromatographic procedures were indispensable in the isolation of the precursors and identification of the γ-C8 and γ-C9 aliphatic lactones. The odor of these two gamma lactones is sweeter and sharper than that of delta decalactone, in fact these are more characteristic of the odor from clean fresh coconuts, perhaps, because of their higher volatility. The individual contribution of these two gamma lac-

TABLE II

Fatty Acids in Position 2 of the Glycerides Containing Hydroxy Acid

Fatty acid carbon No.	Percentage composition range
10	t – 2.0
12	t – 5.0
14	18.0–19.0
15	t – 1.5
16	45.0–55.5
16:1	t – 3.0
17	t – 1.5
18	6.0–10.0
18:1	13.0–20.0
18:2	t – 1.6

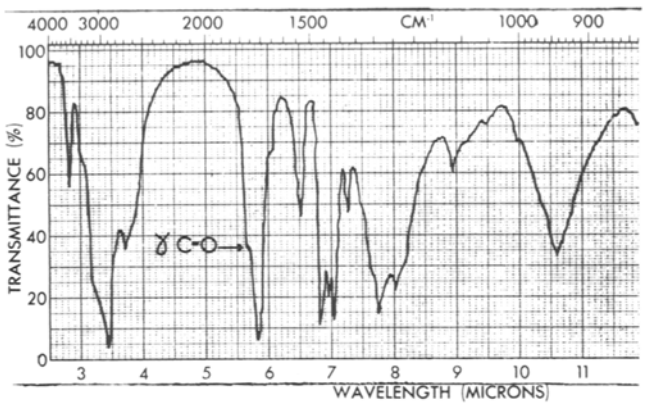

FIG. 3. An IR spectrum of the polar derivatives (hydroxy acids and lactones) obtained from the HTG by saponification.

tones to the flavor of milk and butter is negligible. In cooking materials, pastries and confectionaries which contain appreciable amounts of butterfat the contribution of these gamma lactones to the overall desirable flavor is very important.

With the tentative identification of these two lactones the existence of a homologous series of both odd and even carbon numbered γ- and δ-hydroxy alkanoic acids (δ-C6–δ-C16; δ-C7–δ-C15 and γ-C8–γ-C16; γ-C9–γ-C15) in butterfat is now virtually confirmed (5). Formation of the acetoxy methyl esters of the hydroxy fatty acids from the HTG fraction of butterfat and also, of authentic delta lactones, according to Tulloch's (14) method, is feasible and has been achieved in this laboratory but the maximum yield obtained rarely exceeded 30%. Analysis of these esters by GLC using a polyester succinate column produced a series of peaks which corresponded to the retention times of the even-numbered carbon hydroxy aliphatic acids C10 through C18 inclusive. The corresponding series of lactones has recently been isolated from butterfat (16).

The quantities of free lactones detectable in the HTG fraction of fresh butterfat samples used in this investigation were almost negligible as determined by olfaction which is far more perceptive than any of the GLC detectors used. This observation is in accordance with a previous report (3). Hence, the conclusion is that in very fresh butterfat the lactones are entirely in their precursor forms, i.e., γ- and δ-hydroxy acids esterified to the α-positions of the respective glycerides. The present work has indicated that most treatments (acidification, heating and prolonged storage), excluding alkali saponification, cause hydrolysis of these hydroxy acid acyl bonds with spontaneous lactonization. On prolonged storage of butterfat and dry whole milk powder these lactones are generated spontaneously and impart an off flavor to these products (2).

The specific biological origin of these lactone precursors is presently under investigation. The logical implication from the hydrolysis data, i.e., α-position of the hydroxy acids and fatty acid composition of the resultant monoglyceride, which is very similar to that derived from high molecular weight milk triglycerides (17), is that the mammary tissue is actively involved in the elaboration of the hydroxy acid containing glycerides. Whether the glandular tissue synthesizes these hydroxy acids de novo or obtains them from the blood is being studied.

ACKNOWLEDGMENT

Supported by Agricultural Research Service, USDA, Grant No. 12-14-100-7980 (73), administered by the E. Utiliz. Res. Dev. Div., Philadelphia, Pa.

REFERENCES

1. Dimick, P. S., N. J. Walker and J. E. Kinsella, Cereal Science *11*, 479–481 (1966).
2. Patton, S., Food Quality, Publication No. 77 of AAAS., p. 165 (1965).
3. Boldingh, J., and R. J. Taylor, Nature *194*, 909 (1962).
4. Boldingh, J., and R. J. Taylor (Unilever), U. S. Patent 2,819,169 (1958).
5. Jurriens, G., and J. M. Oele, JAOCS *42*, 857 (1965).
6. Parliment, T. H., W. W. Nawar and I. S. Fagerson, J. Dairy Sci. *48*, 615 (1965).
7. Hirsch, J., and E. E. Ahrens, J. Biol. Chem. *233*, 311 (1958).
8. Dimick, P. S., S. Patton, J. E. Kinsella and N. J. Walker, Lipids *1*, 387 (1966).
9. Stahl, E., Thin Layer Chromatography, Academic Press, New York (1965).
10. Luddy, F. E., R. A. Barford, JAOCS *41*, 693 (1964).
11. Kinsella, J. E., Can. J. Biochem. *44*, 247 (1966).
12. Showell, J. S., Progress in Chemistry of Fats and Other Lipids, Pergamon Press, New York, 1965.
13. Silverstein, R. M., and G. C. Bassler, Spectrometric Identification of Organic Compounds, John Wiley, New York, 1964.
14. Tulloch, A. P., JAOCS *41*, 833 (1964).
15. Wyatt, C. J., E. A. Day and R. L. Pereira, J. Dairy Sci. *49*, 701 (1966).
16. Kinsella, J. E., S. Patton and P. S. Dimick, JAOCS, submitted, (1967).
17. Dimick, P. S., R. D. McCarthy and S. Patton, J. Dairy Sci. *48*, 735 (1965).

[Received July 5, 1966]