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Methods for the Isolation and Characterization of Constituents of Natural Products

XI. Preparation of the 2,4-Dinitrophenylhydrazone Derivatives of Fatty Acid Esters of Glycolaldehyde and Separation of an Homologous Series by Thin-Layer Partition Chromatography

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Periodic acid oxidation of a 1-monoglyceride yields the glycolaldehyde ester of the fatty acid as one of the products. When this reaction is carried out in solution, formaldehyde is also present and methods for determining 1-monoglyceride content of a lipid are usually based on formaldehyde assay following periodate oxidation (2, 5). Earlier methods, in this regard, determine the excess of periodic acid remaining after the oxidation (1, 4). A more recent method employs the spectrophotometric estimation of the 2,4-dinitrophenylhydrazones of the glycolaldehyde esters liberated in the periodic acid reaction (10). None of these methods, however, are capable of indicating the nature of the fatty acid esterified in the monoglyceride and the actual quantitative isolation and identification of 1-monoglycerides from lipids involves rather tedious chromatographic procedures in which the danger of artifact formation is usually present.

In a recent paper (8), a method for carrying out the periodic acid reaction in a two-phase system was described. The oxidation of 1-monoglycerides by this technique gave nearly quantitative yields of glycolaldehyde esters determined spectrophotometrically as 2,4-dinitrophenylhydrazones. These results prompted us to attempt to develop a simple method for the quantitative and qualitative determination of 1-monoglycerides in lipids, and, as a necessary prerequisite, we have prepared and separated a homologous series of the 2,4-dinitrophenylhydrazone derivatives of glycolaldehyde esters of fatty acids. Van Duin (11) had prepared 7 members of this series, but these data are generally inaccessible, and only the melting points were available to us.

*Reagents and Apparatus*¹

Glycolaldehyde was obtained from the Aldrich Chemical Co., Milwaukee, Wisconsin; even-carbon acid chlorides and unsaturated acid chlorides were products of the Hormel Institute, Austin, Minnesota; the remaining acid chlorides were the highest purity products available from the Eastman Kodak Co., Rochester, New York; triethylenediamine [diazabicyclo (2.2.2.) octane] was obtained from the Matheson, Coleman and Bell Co., East Rutherford, N.J.; acidic alumina for chromatography, Brockmann activity grade I was obtained from the J. T. Baker Chemical Co., Phillipsburg, N.J., and was deactivated partially by the addition of 8% distilled water; calcium hydride was purchased from the Fisher Scientific Co., Silver Spring, Md.; the thin-layer equipment was the same as previously described (6). Sheets of silica gel coated on plastic (Baker Flex Silica Gel-1B) were obtained from the Baker Co.

EXPERIMENTAL METHODS AND RESULTS

Glycolaldehyde 2,4-dinitrophenylhydrazone was prepared according to the procedure of Machell and Richards (3), mp 164–165°C, lit. 162–163°C (3).

Fatty acid esters of glycolaldehyde 2,4-dinitrophenylhydrazone were prepared as follows: 11 mg (46 μ moles) of glycolaldehyde 2,4-dinitrophenylhydrazone were dissolved in 10 ml of benzene in a glass-stoppered 10-ml volumetric flask and a few small pellets of CaH₂ were added. When the evolution of gas ceased, 1 or 2 drops of acid chloride were added, the solution mixed by inversion and 5 mg (44 μ moles) of solid triethylenediamine were added. The solution became turbid immediately and was let stand for about 30 minutes at room temperature. The contents were transferred to an alumina column (10 g poured dry into a chromatography tube approximately 2 × 11 cm). The effluent was collected immediately and the column was washed with benzene until all color was removed below a slow-moving band which remained at or near the top of the column. Solvent was removed from the effluent under a stream of N₂ on the steam bath and the residue was recrystallized from absolute ethanol. Derivatives of unsaturated acids were recrystallized from *n*-hexane. Yields of the derivatives varied between 75 and 95%.

Several of the derivatives were also prepared by periodic acid oxidation of the 1-monoglyceride followed by derivatization with 2,4-dinitrophenylhydrazine. One hundred μ moles of the 1-monoglyceride dissolved in 10 ml of methylene chloride was passed over a 1-g column of calcium

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.

sulfate impregnated with aqueous periodic acid as described by Schwartz *et al.* (8). The solvent was removed from the effluent under a stream of N_2 on the steam bath, the residue was dissolved in absolute ethanol and added to a solution of 2,4-dinitrophenylhydrazine prepared according to Shriner and Fuson (9). The precipitate was collected the following day and recrystallized from absolute ethanol. Yields of the derivatives were in the vicinity of 85%. The melting point and chromatographic mobility of the derivatives made in this way agreed with those made via the fatty acid chlorides. However, 1-monoacetic and 1-monopropionic, which were oxidized with periodic acid in aqueous solution instead of on the column, did not give the expected products, but gave bis (2,4-dinitrophenylhydrazones) with melting points over $300^\circ C$ which were not further characterized.

Thin-layer neutral partition chromatography was carried out on 8×10 -inch plates according to the procedure of Schwartz and Brewington (6) but using hexane:benzene (65:35) saturated with polyethylene glycol 400 as mobile phase and sieved (through 200 mesh) Microcel T-38 as support.

Thin-layer partition chromatography on a weakly alkaline stationary phase. Schwartz *et al.* (7) described the thin-layer partition chromatography of 2,4-dinitrophenylhydrazones using an alkaline stationary phase. When the system described in that report was tried in the present study, little or no movement of the glycolaldehyde ester derivatives was observed. Since the polarity of the mobile phase could not be increased significantly, the KOH concentration in the stationary phase was accordingly decreased by a sixth which resulted in very fine separation of the series.

Two ml of 1 *N* methanolic KOH and 60 ml of methanol are added to 12.5 ml of polyethylene glycol 400 in a suitable vessel and 15 g of sieved Microcel T-38 are added. The slurry is shaken vigorously and spread over 8×8 -inch plates. The methanol is permitted to evaporate and the plates are stored in a desiccator over Ascarite. Benzene solutions of the derivatives are spotted and the plates are developed with hexane:benzene (65:35) saturated with polyethylene glycol 400. Unlike the stronger alkaline system, the weaker alkali does not cause the hydrazones to color violet but it does deepen the yellow color. Heating the finished plate at $100^\circ C$ for 15 minutes causes the glycolaldehyde ester derivatives to saponify, the liberated glycolaldehyde 2,4-dinitrophenylhydrazone turning deep violet. Other 2,4-dinitrophenylhydrazones including methyl esters of keto acids do not turn violet under these conditions and thus the glycolaldehyde ester hydrazones are easily distinguished by the color and also by the fact that the glycolalde-

TABLE 1
 SOME PROPERTIES OF THE 2, 4-DINITROPHENYLDRAZONE DERIVATIVES OF GLYCOLALDEHYDE ESTERS OF FATTY ACIDS

Carbons in parent acid	mp (°C)	Lit. mp (l) (°C)	$E \times 10^{-3a}$	Carbon (%)		Hydrogen (%)	
				Theory	Found ^b	Theory	Found ^b
2	152-154		20.5	42.6	42.7	3.5	3.6
3	112-113		19.5	44.6	44.9	4.0	3.8
4	89		20.9	46.4	46.9	4.5	4.1
5	72.5-73.5		20.8	48.4	49.5	4.9	4.7
6	76-77	77	20.5	49.7	50.0	5.3	5.8
7	76-77		19.9	51.1	50.9	5.7	5.9
8	79-80	76.5-77.5	19.4	52.4	52.3	6.0	6.4
9	78-80		19.3	53.7	53.7	6.3	6.8
10	84-86	81.5-83	19.9	54.8	54.5	6.6	7.1
11	85-87		22.0	55.8	55.3	6.9	7.1
12	87.5-89	87-88	20.3	56.8	57.5	7.1	7.2
13	90-91		20.5	57.7	57.7	7.3	7.9
14	93-94	91.5-92.5	20.2	58.6	58.0	7.6	7.8
16	97-98.5	81-84	21.9	60.2	59.9	8.0	8.3
18:0	98-100	95-98	20.5	61.6	61.8	8.3	8.8
18:1	56-58		21.0	61.9	61.8	7.9	8.5
18:2	31-32		20.8	—	—	—	—
18:3	37.5-38.5		20.3	62.0	62.1	7.5	7.4
Av			20.4				

^aAt 350 $m\mu$ in CHCl_3 .

^bC and H analyses by Schwartzkopf Laboratories, Long Island, N. Y.

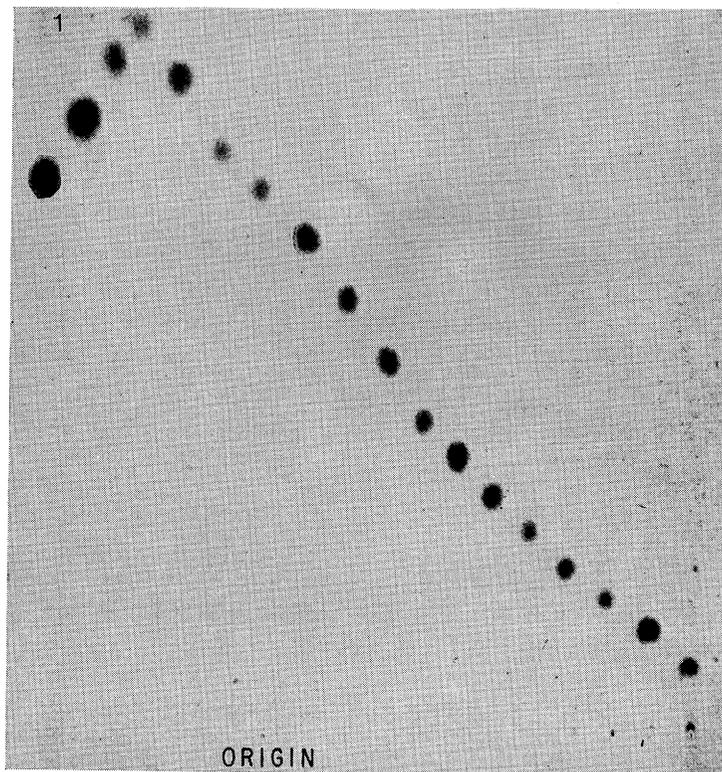


FIG. 1. Separation of a homologous series of the 2,4-dinitrophenylhydrazone derivatives of glycolaldehyde esters of fatty acids by thin-layer partition chromatography: stationary phase, polyethylene glycol 400; mobile phase, hexane:benzene (65:35) saturated with polyethylene glycol 400; support, Microcel T-38; (bottom right to top spot, C_2 - C_{13} , C_{15} , C_{17} , $C_{18}:0$; (bottom left to next to top spot), $C_{18}:3$, $C_{18}:2$, $C_{18}:1$).

hyde 2,4-dinitrophenylhydrazone will not move if the heated plate is redeveloped in the solvent.

Thin-layer, silver-ion chromatography. Flexible strips or sheets of silica gel 1B are impregnated with silver nitrate by dipping in a 5% solution of silver nitrate in acetonitrile. Impregnation may also be accomplished by allowing the acetonitrile solution to ascend the strip. The strips are air-dried until the odor of acetonitrile is dissipated, the derivatives are spotted and the chromatogram is developed with benzene.

Table 1 lists the derivatives prepared, their melting points, molar absorptivities and elemental analyses. All derivatives were yellowish or yellow-orange, and in chloroform solution showed an absorption maximum

at 350 $m\mu$. The lower derivatives are highly crystalline but as the chain length of the parent fatty acid increases, the ability to form a highly crystalline derivative from ethanol diminishes. No trouble was encountered in the preparation of any of the derivatives and only one recrystallization was necessary to obtain an analytically and chromatographically pure derivative.²

Separation of the homologous series of derivatives by thin-layer partition chromatography is depicted in Fig. 1. Resolution is very good, the whole series being separated in this one system. Oleic, linoleic, and linolenic acid esters are not well separated from other long-chain derivatives but can be resolved by silver-ion chromatography as is shown in Fig. 3. In practice, in this situation, the spot or band would be scraped from the partition plate and the scrapings placed directly onto a short (about 500 mg) column of the hydrated alumina contained in a medicine dropper. The color is then eluted with benzene, the stationary phase remaining

² The derivative of linolenic acid shows a small impurity on the Ag ion-silica gel chromatogram.

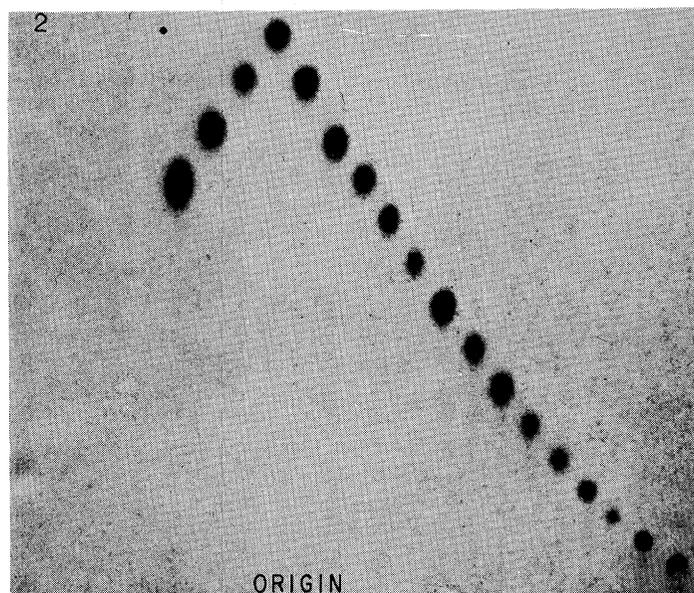


FIG. 2. Separation of a homologous series of the 2,4-dinitrophenylhydrazone derivatives of glycolaldehyde esters of fatty acids by thin-layer partition chromatography on an alkaline stationary phase; stationary phase, polyethylene glycol-KOH; mobile phase, hexane:benzene (65:35) saturated with polyethylene glycol 400; support, Microcel T-38; (bottom right to top spot), C_2 - C_{13} , C_{15} , C_{17} , C_{18} :0; (bottom left to next to top spot), C_{18} :3, C_{18} :2, C_{18} :1.

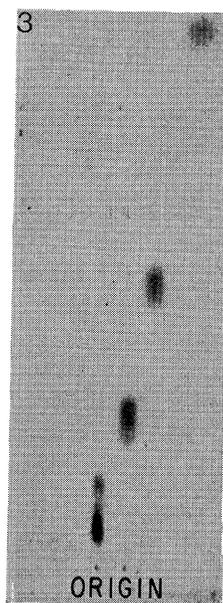


FIG. 3. Silver ion-silica gel chromatogram showing separation of unsaturated from saturated 2,4-dinitrophenylhydrazone derivatives of glycolaldehyde esters of fatty acids; (bottom to top), $C_{18}:3$, $C_{18}:2$, $C_{18}:1$, $C_{18}:0$; solvent, benzene.

adsorbed on the column. The eluted compound is then respotted onto the silver-ion-silica gel strip with the appropriate standards.

The ability of the alkaline partition system to resolve the series of derivatives is shown in Fig. 2. Resolution of the series is better than in the neutral partition system, and the series can be resolved on a smaller plate, and thus, in less time. No saponification of the ester linkage appears to take place as long as the plate is not heated.

SUMMARY

An homologous series of the 2,4-dinitrophenylhydrazone derivatives of the glycolaldehyde esters of fatty acids have been prepared. The derivatives are all solids having an absorption maximum at $350\text{ m}\mu$ (CHCl_3) and a molar extinction coefficient near 20,000. Neutral and alkaline thin-layer partition chromatographic procedures and a thin-layer, silver-ion adsorption system are described for resolving the derivatives.

REFERENCES

1. Handschumaker, E. and Linternis, L., A modified procedure for the determination of monoglycerides in fats and oils by oxidation with periodic acid. *J. Am. Oil Chemists' Soc.* **24**, 143-145 (1957).
2. Jensen, R. G. and Morgan, M. E., Estimation of the monoglyceride content of milk. *J. Dairy Sci.* **42**, 232-239 (1959).

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3. Machell, G. and Richards, G. N., Mechanism of saccharinic acid formation. II. The $\alpha\beta$ -dicarbonyl intermediate in formation of D-glucosaccharinic acid. *J. Chem. Soc.* **1960**, 1932-1938.
4. Martin, J. B., The equilibrium between symmetrical and unsymmetrical monoglycerides and determination of total monoglycerides. *J. Am. Chem. Soc.* **75**, 5483-5484 (1953).
5. Pohle, W. D. and Mehlenbacher, V. C., A modification of the periodic acid method for the determination of monoglycerides and free glycerol in fats and oils. *J. Am. Oil Chemists' Soc.* **27**, 54-57 (1950).
6. Schwartz, D. P. and Brewington, C. R., Methods for the isolation and characterization of constituents of natural products. II. Separation of homologous series of esters of pyruvic acid 2,6-dinitrophenylhydrazone by thin-layer chromatography. *Microchem. J.* **12**, 1-6 (1967).
7. Schwartz, D. P., Shamey, J., Brewington, C. R., and Parks, O. W., Methods for the isolation and characterization of constituents of natural products. X. New and improved methods for the analysis of carbonyl 2,4-dinitrophenylhydrazones and 2,4-dinitrophenylosazones. *Microchem. J.* **13**, 407-417 (1968).
8. Schwartz, D. P., Weihsrauch, J. L., and Burgwald, L. H., Methods for the isolation and characterization of constituents of natural products. A periodic acid column procedure for the oxidation of vic-glycols, epoxides and α -hydroxy acids at the micromole level. *Anal. Chem.*, in press.
9. Shriner, R. L. and Fuson, R. C., "Identification of Organic Compounds," 3rd ed., Wiley, New York, 1948.
10. Szonyi, C. and Sparrow, K., Spectrophotometric determination of small amounts of l-monoglycerides in fats. *J. Am. Oil Chemists' Soc.* **41**, 535-537 (1964).
11. Van Duin, H., *Verslag. Het Ned. Inst. voor Zuivelonderzoek, Wr.* **54**, Ede 46 (1961).