SEPARATION OF ORGANIC ACIDS BY THIN-LAYER CHROMATOGRAPHY OF THEIR 2,4-DINITROPHENYLHYDRAZIDE DERIVATIVES AND THEIR ANALYTICAL DETERMINATION*

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(Received June 28th, 1965)

INTRODUCTION

Along with qualitative and quantitative determination of plant fatty acids underway at this laboratory, we are investigating the behavior of a series of 2,4-dinitrophenylhydrazides (2,4-DNP hydrazides) of organic acids by thin-layer chromatography (TLC). At the present time the best and most rapid method is by gas-liquid chromatography (GLC) of the acids or esters.

General column, paper, and thin-layer chromatographic procedures for separating the acids and their derivatives have been described[1-7]. The procedures outlined for these methods are deficient in terms of quantitation, degradation, as well as difficulty in achieving the desired separation and recovery of materials for further structural studies. Results of work reported here represent our effort to quantitate the separation of fatty acid 2,4-DNP hydrazides.

EXPERIMENTAL

Preparation of 2,4-dinitrophenylhydrazides

According to the general method outlined by INOUYE AND NODA[8], 1-g samples of the organic acids were refluxed with 2 ml of thionyl chloride for 1 h. Excess thionyl chloride was removed by gentle heat under vacuum. Two grams of 2,4-DNP hydrazine in 200 ml of benzene was added to the acid chloride and the mixture rapidly refluxed for 30 min. While hot the solution was filtered and allowed to cool. On cooling, the hydrazides of lower molecular weight precipitated and were filtered. When precipitation of the hydrazide did not occur on cooling, the benzene was removed at 60° under vacuum. Crude hydrazides were purified by recrystallization from either benzene or 95 % ethanol. Their purity was monitored by infrared, melting point, thin-layer chromatography until a single spot was obtained, and elemental analysis.

Preparation of mercury adducts

Mercury adducts of oleic ($C_{18:1}$) and linoleic ($C_{18:2}$) acid 2,4 DNP hydrazides were prepared by reacting the derivatives with mercuric acetate in methanol at room

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temperature according to the procedure of JANTZEN AND ANDREAS[9] for fatty acid methyl esters.

Preparation of chromatographic layers

Thin-layer plates (20 × 20 cm) were prepared for R_B determination (ratio of sample to Butter Yellow migration) by the following procedure: To 35 g of Silica Gel G according to STAHL was added 75 ml of distilled water and mixed thoroughly. The mixture was then applied to the glass plate with a 250 μ Brinkmann-Desaga spreader. After air drying, the plates were baked in an oven for 15 min at 120°. Aluminum Oxide G according to STAHL (30 g) was stirred into 60 ml of distilled water in a blender and then applied to the plates. The plates were activated by heating in an air convection oven for 15 min at 120°. Polyamide powder according to STAHL (15 g) was mixed thoroughly with 60 ml of 95 % ethanol and then applied to the plates and allowed to dry for 2 h before use.

In order to determine R_B values all chromatograms were prepared by the ascending method at room temperature in a solvent-saturated atmosphere with Butter Yellow as the marker. The compounds (1 μg in benzene solution) were spotted 2 cm from the bottom of the thin-layer plate. The plate was submerged in the solvent to a depth of 5 to 10 mm and the solvent allowed to run a distance of 15 cm.

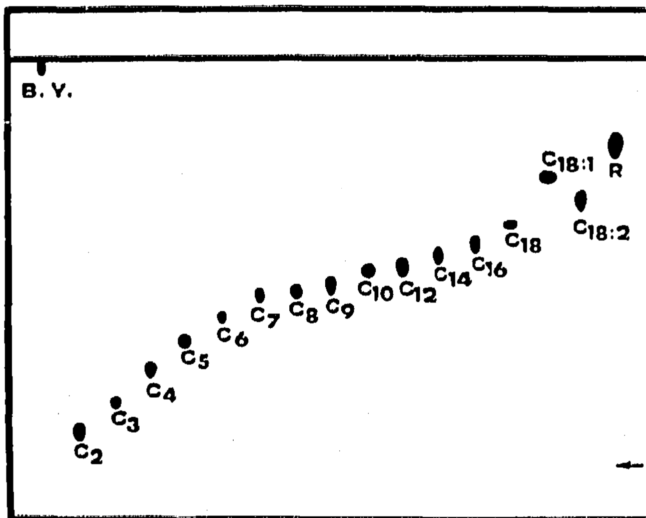
The size of the polyamide plates used for the quantitative separation of 2,4-DNP hydrazides of fatty acids was 20 × 60 cm. A mixture of known concentration of fatty acid 2,4-DNP hydrazides in benzene was streaked across the plate 2 cm from the bottom. The plate was developed in a 12 × 24 in. tank for the entire length of the plate. The visible bands were scraped off with a microscope slide into a petri dish and each component was eluted with 95 % ethanol. With polyamide, wetting of the slide with ethanol prior to scraping was required to counteract the electrostatic charge present on the plate so that the powder would not scatter during removal. The fatty acid 2,4-DNP hydrazide extracts were made to volume and the ultraviolet and visible spectra determined. The molar absorptivity was determined from the ultraviolet absorption maxima at 330 mμ.

RESULTS AND DISCUSSION

In order to fully evaluate the TLC characteristics of the hydrazides, 15 fatty acids were chosen to represent the saturated and unsaturated alkyl acids. Table I summarizes the physical constants for the group of 2,4-DNP hydrazides. Butter Yellow as a marker was satisfactory in the polyamide system (Fig. 3), but was of no consequence in the alumina and Silica Gel systems as it migrated with the front.

TLC separation was achieved with three supports and solvent systems. Fig. 1 is a representative chromatogram of the 2,4-DNP hydrazides on alumina developed in a chloroform–methanol–diethylamine (99:1:0.5, v/v/v) solvent system. The separations are sufficient for quantitation of the lower membered derivatives but not for the higher molecular weight derivatives. With this support and solvent system, the R_B was equal to the R_F since the Butter Yellow migrated with the solvent front.

Chromatography of the same 2,4-DNP hydrazides on Silica Gel G in the same solvent system with a lower concentration or chloroform and an increase in methanol (chloroform–methanol–diethylamine, 98:2:0.5, v/v/v) is shown in Fig. 2. Here, as



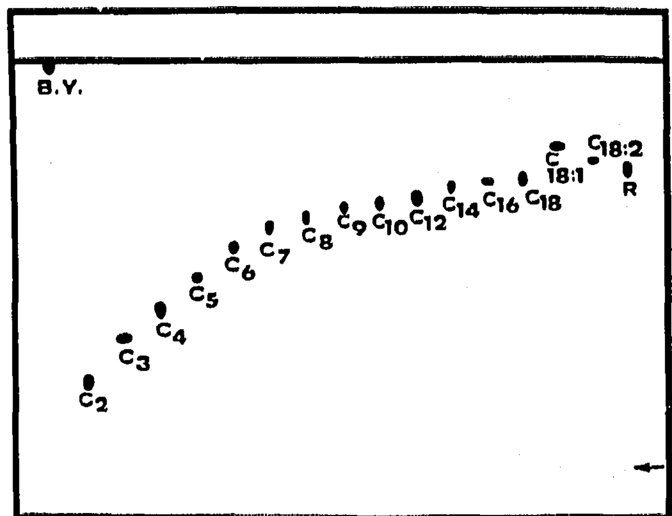
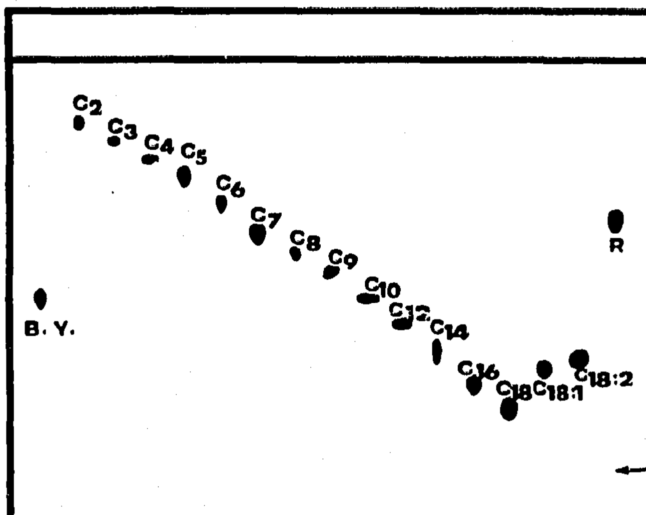


Fig. 1. Chromatogram of 2,4-DNP hydrazides on Aluminum Oxide G developed in a chloroform–methanol–diethylamine (99:1:0.5, v/v/v) solvent system. B.Y. is Butter Yellow and R is 2,4-dinitrophenylhydrazine.

Fig. 2. Chromatogram of 2,4-DNP hydrazides on Silica Gel G developed in chloroform–methanol–diethylamine (98:2:0.5, v/v/v) solvent system.



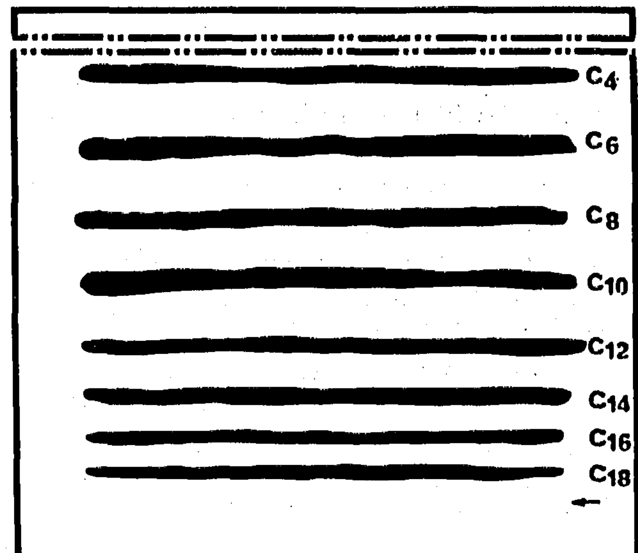


Fig. 3. Chromatogram of 2,4-DNP hydrazides on polyamide powder developed in methanol–water (9:1, v/v).

Fig. 4. Chromatogram of the quantitative separation of a representative mixture of 2,4-DNP hydrazides on polyamide plate (20 × 60 cm) developed in methanol–water (9:1, v/v).

TABLE I

CHARACTERIZATION OF 2,4-DINITROPHENYLHYDRAZIDES

Acids	Melting points (°C)		Elemental analysis; difference in calcd. and found			$R_B \times 100$		
	Lit.	Found[a]	C	H	N	b	c	d
C_2	197	196–198				210	8	5
C_3		186–188	0.04	0.05	0.00	200	15	14
C_4	168	162–164				190	20	22
C_5		127–128	0.27	0.03	0.05	170	23	29
C_6	112	107–108				150	27	35
C_7		89– 91	0.18	0.02	0.06	140	29	40
C_8	102	96– 98				130	31	42
C_9		104–106	0.04	0.01	0.01	110	34	45
C_{10}	113	110–112				100	35	47
C_{12}	116	114–116				84	37	48
C_{14}	118	115–117				66	39	51
C_{16}	120	119–120				46	42	52
C_{18}	122	120–121				35	45	55
$C_{18:1}$	Oil	80– 82	0.09	0.02	0.23	54	64	59
$C_{18:2}$	Oil	68– 69	0.05	0.09	0.11	62	60	57
Butter yellow						100	100	100

[a] Melting points taken on a Fisher-Johns apparatus are uncorrected.
[b] Polyamide plates developed in methanol–water (9:1, v/v).
[c] Aluminum Oxide G plates developed in methanol–chloroform–diethylamine (1:99:0.5. v/v/v).
[d] Silica Gel G plates developed in methanol–chloroform–diethylamine (2:98:0.5, v/v/v).

in the alumina system, the separations were not sufficient to allow quantitation of the higher fatty acid derivatives. Alkyl acids C_2 through C_7, however, could be readily separated from the higher fatty acid derivatives.

The polyamide system (Fig. 3) was superior to the other two systems for separation of saturated acid derivatives. Irrigation in methanol–water (9:1, v/v) gave a straight line separation within the homologous series C_2 through C_{18}. Efforts to increase the separation ratio of each component by increasing the amount of water in the system resulted in a greater separation of the derivatives C_2 through C_7, but a spreading and overlapping of the slower running derivatives (higher mol. wt.). Multiple development of the polyamide plate resulted in the same phenomenon.

The presence of unsaturation in the chain increases the R_B value in the polyamide system resulting in the inadequate separation of the saturated and unsaturated derivatives (Fig. 3). The unsaturated 2,4-DNP hydrazides can be removed from the saturates by TLC of their mercury adducts on Silica Gel G. Table II gives the results of chromatography of the mercury adducts of oleic and linoleic 2,4-DNP hydrazides along with four 2,4-DNP hydrazides. In the chloroform–ethyl ether (8:2, v/v) system (solvent a) the adducts remain at the origin while the free 2,4-DNP hydrazides move. Separation of the individual addition products may be accomplished either by rechromatography on Silica Gel G in isopropanol–acetic acid (100:1, v/v) solvent system (solvent b) or by fission of the adducts with hydrochloric acid and chromatography of the derivatives in the polyamide system.

Quantitative separation of a representative mixture of 2,4-DPN hydrazides is shown in Fig. 4. For maximum separations the solvent mixture should be placed in the chromatograph jar 6 to 8 h prior to plate development to insure complete

TABLE II

CHROMATOGRAPHY OF A MIXTURE OF 2,4-DINITROPHENYLHYDRAZIDES AND MERCURY ADDUCTS ON ACTIVATED SILICA GEL G

Acids	$R_F \times 100$	
	Solvent a	*Solvent b*
C_4	26	85
C_{18}	52	94
$C_{18:1}$	53	94
$C_{18:2}$	52	95
$C_{18:1}$–Hg	00	78
$C_{18:2}$–Hg	00	46

Solvent a = chloroform–ethyl ether (8:2, v/v).
Solvent b = isopropanol–acetic acid (100:1, v/v).

tank saturation. The increased separation observed in Fig. 4 as compared with that in Fig. 3 was probably due to the longer development path.

Table III summarizes the results of efforts to quantitate the derivatives listed in Fig. 4. The total recovery (average of two determinations) as measured by ultraviolet absorption was 100.0 %. Routinely, recoveries within 1 % of theoretical were obtained. The quantitative nature of the TLC separation is indicated by the maximum error of 2.6 % for C_{16} fatty acid derivative and a minimum error of 0.6 % for the C_{10} derivative. Efforts to increase the separation ratio of the components on the long plate by increasing the thickness of the polyamide support resulted in increased streaking and overlapping of the slower moving bands.

TABLE III

QUANTITATIVE ESTIMATION OF FATTY ACID 2,4-DINITROPHENYLHYDRAZIDES

Acid	*Standard 2,4-DNP hydrazides (moles/l × 10^{-5})*	*Mixture 2,4-DNP hydrazides after TLC (moles/l × 10^{-5})*	*Percent error*
C_4	3.73	3.80	1.9
C_6	3.38	3.36	0.7
C_8	3.09	3.11	0.6
C_{10}	2.84	2.84	0.0
C_{12}	2.63	2.67	1.5
C_{14}	2.45	2.40	2.0
C_{16}	2.29	2.23	2.6
C_{18}	2.15	2.17	1.1

The ultraviolet and visible spectra of derivatives listed in Table I show absorption maxima at 330 mμ in neutral and acid solution and 425 mμ in basic solution. The molar absorptivity of each derivative was $1.46 \cdot 10^4 \pm 0.02$ from the absorption maxima at 330 mμ.

SUMMARY

The thin-layer chromatographic characteristics of a number of organic acid 2,4-dinitrophenylhydrazides on three types of supports are described. The solvent system for the supports Silica Gel G and Aluminum Oxide G was chloroform–methanol–diethylamine in concentrations of 98:2:0.5, v/v/v and 99:1:0.5, v/v/v, respectively. The acids C_2 through C_7 can be individually separated from higher molecular weight derivatives in these two systems.

Straight line separations of C_2 through C_{18} 2,4-dinitrophenylhydrazides are given for polyamide support irrigated in methanol–water (9:1, v/v). Preliminary removal of unsaturated derivatives can be accomplished by converting them to the mercury addition compounds and chromatographing on Silica Gel G in chloroform–ethyl ether (8:2, v/v).

Details are described for the separation and quantitation of eight fatty acid 2,4-dinitrophenylhydrazides by thin-layer chromatography and ultraviolet spectrometry, respectively.

REFERENCES

1 D. C. Malins, *J. Am. Oil Chemists' Soc.*, 37 (1960) 576.
2 L. J. Morris, *Chem. Ind. (London)*, (1962) 1238.
3 L. J. Morris, R. T. Holman and K. Fontell, *J. Am. Oil Chemists' Soc.*, 38 (1961) 316.
4 S. Ruggieri, *Nature*, 193 (1962) 1282.
5 E. Vioque and R. T. Holman, *J. Am. Oil Chemists' Soc.*, 39 (1962) 63.
6 J. W. H. Lugg and B. T. Overell, *Nature*, 160 (1947) 87.
7 R. I. Cheftel, R. Munier and M. Macheboeuf, *Bull. Soc. Chim. Biol.*, 34 (1952) 1095.
8 Y. Inouye and M. Noda, *Bull. Agr. Chem. Soc. Japan*, 19 (1955) 86.
9 E. Jantzen and H. Andreas, *Chem. Ber.*, 92 (1959) 1427.