

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

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*Received January 18, 1968*

A number of reports from this and other laboratories describing thin-layer methods for class separation (1, 2, 9, 10, 13) and also for separation of a more or less extensive homologous series of 2,4-dinitrophenylhydrazone (1, 6, 12) and dinitrophenylosazone (3) derivatives of carbonyl compounds have appeared. In a number of instances, problems have arisen in this laboratory that necessitated the simplification or modification of existing methods to facilitate the characterization of an unknown class of carbonyl compounds or for the identification of members of a given class. We would, therefore, like to present some of the modified and new methods which we have employed over the past few years for the analysis of carbonyl compounds isolated from natural products.

MATERIALS AND APPARATUS<sup>1</sup>

Magnesium oxide (cat. no. 2477) suitable for chromatographic use was obtained from the J. T. Baker Co., Phillipsburg, N. J. The powder had an adsorption index (Food and Drug Yellow no. 4) of 12-13 and was used without further treatment. The original container was subdivided into 6 or 7 lots and these were stored in tightly sealed containers and stored in the absence of moisture. One lot was used up before opening another in order to maintain the activity of the original

<sup>1</sup> Reference to certain products or companies does not imply an endorsement by the Department over others not mentioned.

MgO. Polyethylene glycol 400 (Baker) was used as received. Analytical grade Celite and Microcel T-38 were products of the Johns-Manville Co., Baltimore, Md. The Microcel T-38 was sieved through a 200 mesh screen. Methanol,  $\text{CHCl}_3$ , and benzene were ACS grade. Hexane was the high purity grade produced by the Phillips Petroleum Co., Bartlesville, Okla. The thin-layer chromatography equipment was the same as described earlier (8).

#### EXPERIMENTAL METHODS

All thin-layer separations were run in equilibrated tanks lined with filter paper. All finished plates were placed in a tank containing cotton wetted with diethylamine to intensify the spots unless otherwise noted.

(A) *Class separation of dicarbonyl bis(2,4-dinitrophenylhydrazones)* by thin-layer chromatography. Cobb (2) and Schwartz, *et al.* (9) described thin-layer chromatography (TLC) procedures for separating  $\alpha$ -ketoaldehydes,  $\alpha$ -diketones, and glyoxal on plates prepared from Seisorb 43 and Celite 545. In the procedure of Schwartz *et al.* (9), it was necessary to partially deactivate the adsorbent at high temperatures before preparing the plates. In the procedure presented below, deactivation of the adsorbent is unnecessary thereby simplifying the procedure and eliminating the need for a muffle furnace.

Three g of MgO and 7 g of Analytical Grade Celite are vigorously shaken with 50 ml of distilled water in a stoppered 200-ml Erlenmeyer flask until a lump-free suspension is obtained. The slurry is spread immediately in the usual manner over five  $8 \times 8$ -inch plates. The plates are air-dried for 30 minutes and then heated at  $100^\circ\text{C}$  for 1 hour. The osazones are spotted from ethyl acetate solution and the plate is developed with  $\text{CHCl}_3$ :MeOH (95:5) for about 90 minutes.

(B) *Separation of a homologous series of  $\alpha$ -keto aldehyde bis(2,4-dinitrophenylhydrazones) by thin-layer partition chromatography.* Approximately 12.5 ml of polyethylene glycol 400 is dissolved in 70 ml of absolute ethanol in a 200-ml Erlenmeyer flask and 15 g of sieved Microcel T-38 is added. The flask is stoppered and shaken vigorously for a few minutes and the plates ( $8 \times 10$ -inch) are prepared in the usual manner. The plates are air-dried for 10 minutes and then heated at  $100^\circ\text{C}$  for 5 minutes. The  $\alpha$ -keto aldehyde derivatives are spotted

from ethyl acetate solution and the plate is developed with benzene:hexane (3:2) saturated with stationary phase.

(C) *Separation of a short series of  $\alpha$ -diketone bis (2,4-dinitrophenylhydrazones) by thin-layer partition chromatography.* The thin-layer partition plates are prepared as described in (B). The diketone derivatives are spotted from ethyl acetate solution and the plate developed using hexane:benzene (3:1) saturated with stationary phase.

(D) *Class separation of aliphatic monocarbonyl 2,4-dinitrophenylhydrazones by thin-layer adsorption chromatography.* A thin-layer adsorption system employing Seasorb 43 and Celite 545 as the plate coating was described from this Laboratory for separating methyl ketones, saturated aldehydes, 2-enals and 2,4-dienals (10). In that procedure it was essential to sieve the Seasorb 43 in order to obtain satisfactory plates. Also in that procedure, the shorter member(s) of a faster moving class moved into the class immediately following it. Thus, acetone moved with the saturated aldehydes, acetaldehyde and formaldehyde with the 2-enals, and crotonaldehyde and acrolein with the 2,4-dienals. In the procedure described below, all of the lower members move with their respective class resulting in clean-cut separation of the 4 classes. Also the sieving step is eliminated.

The plates are prepared in the same manner as outlined for preparation of the dicarbonyl class plates (procedure A), except that the plates are held at room temperature open to the air for 48 hours and are not heated. The hydrazones are spotted from benzene solution and the plate is developed for about 90 minutes with hexane:CHCl<sub>3</sub> (95:25).

(E) *Separation of homologous series of methyl ketones, saturated aldehydes, 2-enals and 2,4-dienals by normal thin-layer partition chromatography.* The system described below is very similar to that published by Badings and Wassink (1), but is considered to be more economical and simpler to use while being just as effective. Moreover, the plates can be used in about 15 minutes after preparation.

The plates are prepared as described under (B). The derivatives are spotted from benzene solution and the plate developed for about an hour with hexane saturated with stationary phase.

(F) *Separation of homologous series of longer-chain 2,4-dinitrophenylhydrazones by thin-layer partition chromatography using an*

*alkaline stationary phase.* Normal partition systems described in the literature for separating a series of 2,4-dinitrophenylhydrazones usually only separate about the first 12 members effectively. Higher members in the series are ordinarily not separated or separate inadequately. One must then resort to reverse-phase partition chromatography in order to resolve the longer-chain members. Reverse-phase chromatography is usually not as smoothly executed as normal partition chromatography mainly because of solubility problems of the longer-chain members in the mobile phase. Thus, one must resort to applying smaller amounts of these to eliminate streaking and this may result in difficulty in visualizing the spot or band on the finished chromatogram. The system described below has been used extensively in this Laboratory, and has virtually eliminated the need for reverse-phase partition chromatography of the 2,4-dinitrophenylhydrazones of saturated aldehydes, 2-enals, and 2,4-dienals. The system is unique in that longer-chain derivatives move fastest and the shorter members slowest as is usual in normal partition chromatography, but, in contrast to normal partition chromatography, the longer chain derivatives are separated better from each other than are the shorter members from each other. It is thus possible to separate easily, a series of about C<sub>5</sub> through C<sub>18</sub> on a single 8-inch plate in about 45 minutes. Furthermore, the spots are intensified due to the alkaline conditions and one can more readily follow the separation during development of the plate.

The plates are prepared by slurring 12.5 ml of polyethylene glycol 400 dissolved in 65 ml of 1% methanolic KOH with 15 g of the sieved Microcel T-38. The plates are spread in the usual manner and permitted to air-dry for 15 minutes. They are then stored in a desiccator over Ascarite and used as needed.

The hydrazones are spotted from benzene solution and color different shades (depending on the class) as the benzene evaporates from the plate. The 2,4-dienals color rose-red, 2-enals pinkish-red, saturated aldehydes tan and methyl ketones yellowish-grey. The plate is developed with hexane saturated with stationary phase for about 40 minutes.

(G) *Separation of dicarbonyl bis(2,4-dinitrophenylhydrazones) into classes by column adsorption chromatography.* One g of MgO and 9 g of Celite 545 (previously dried for 24 hours at 100°C) are shaken

with 50 ml of benzene at high speed on a mechanical shaker (Eberbach Corp., Ann Arbor, Mich.) for 15 minutes. The slurry is quickly poured into a chromatography tube (approximately 2.2 cm i.d.  $\times$  23 cm). The slurry is packed immediately using moderate gas pressure leaving about 2 ml of benzene above the bed. Any solids adhering to the sides of the tube are washed down carefully with benzene and the mixture of osazones dissolved in benzene are quantitatively transferred to the column, and, after washing the sides of the tube down development of the chromatogram is begun. The diketones are eluted with 350 ml of  $\text{CHCl}_3$ :benzene (3:1); the  $\alpha$ -keto aldehydes are then removed with 300 ml of  $\text{CHCl}_3$  and glyoxal is eluted last with 4% methanol in  $\text{CHCl}_3$ .

#### RESULTS AND DISCUSSION

*Class separation of dicarbonyl bis(2,4-dinitrophenylhydrazones) by thin-layer adsorption chromatography* (see procedure A). Figure 1 is a reproduction of the thin-layer plate showing separation of the  $\alpha$ -diketones,  $\alpha$ -ketoaldehydes, and glyoxal into classes. As in the procedure reported by Cobb *et al.* (2) and by Schwartz *et al.* (9), separation of the classes is cleanly effected. Also as reported by

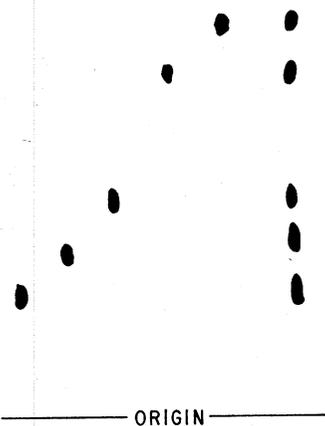


FIG. 1. Separation of dicarbonyl bis(2,4-dinitrophenylhydrazones) into classes. Support:  $\text{MgO}$ -celite (3:7); solvent:  $\text{CHCl}_3$ : $\text{MeOH}$  (95:5). Diagonally from top to bottom: 2,3-octanedione, 2,3-butanedione,  $\alpha$ -ketononanal,  $\alpha$ -ketopropanal, and glyoxal. Column on right is a mixture of all compounds.

Schwartz *et al.* (9), the  $\alpha$ -diketone class is blue, and the  $\alpha$ -ketoaldehydes and glyoxal are violet. The present procedure can detect  $9 \times 10^{-5}$   $\mu$ moles of an osazone. Schwartz *et al.* (9) reported  $4 \times 10^{-5}$   $\mu$ moles as the lower limit. Only the extremes in each class which were on hand were chromatographed. The intermediate members would move between the extremes.

*Separation of a homologous series of  $\alpha$ -ketoaldehyde bis(2,4-dinitrophenylhydrazones) by thin-layer partition chromatography* (see procedures B and C). Separation of a series of  $\alpha$ -ketoaldehyde derivatives from  $C_3$  through  $C_9$  by thin-layer partition chromatography is shown in Fig. 2. Exposure of the finished plate to diethylamine vapor gives violet spots and permits the detection of about  $4 \times 10^{-5}$   $\mu$ moles of an osazone. Cobb *et al.* (3) used reverse phase chromatography to separate this series, but it was necessary to develop the plate for about 7 hours. Schwartz (7) and Corbin (4) separated this series by column partition chromatography.

*Separation of  $\alpha$ -diketone bis(2,4-dinitrophenylhydrazones) by thin-layer partition chromatography.* Separation of the short series of  $\alpha$ -diketone derivatives which we had on hand is shown in Fig. 3. Exposure of the finished plate to diethylamine vapor gives blue spots and permits the detection of about  $9 \times 10^{-5}$   $\mu$ moles.

*Class separation of aliphatic monocarbonyl 2,4-dinitrophenylhydrazones by thin-layer adsorption chromatography* (see procedure D).

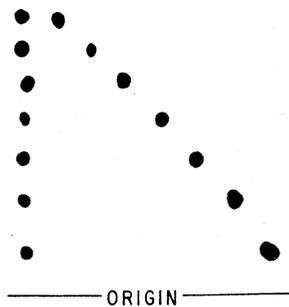


FIG. 2. Separation of a series of  $\alpha$ -ketoaldehyde bis(2,4-dinitrophenylhydrazones) by thin-layer partition chromatography. Support: Microcel T-38; stationary phase: polyethylene glycol 400; mobile phase: benzene:hexane (3:2) saturated with stationary phase. Diagonally from top to bottom  $C_9$  through  $C_3$ . Column on left is a mixture of the 7 members.

Fig. 4 depicts the separation of methyl ketones, saturated aldehydes, 2-enals, and 2,4-dienals from each other; the colors on the plate are similar to those on the Seasorb plate (10) but do not contrast as well. However, class differentiation is still aided by differences in color. A long-chain member and one or more short-chain members of each class were selected for study as before (10) to maximize the difficulty of separation. The following lower limits of detection were observed:

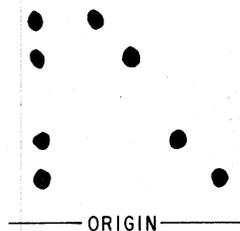


FIG. 3. Separation of some  $\alpha$ -diketone bis(2,4-dinitrophenylhydrazones) by thin-layer partition chromatography. Support: Microcel T-38; stationary phase: polyethylene glycol 400; mobile phase: hexane:benzene (3:1) saturated with stationary phase. Diagonally from top to bottom: 2,3-octanedione, 2,3-heptanedione, 2,3-pentanedione, 2,3-butanedione. Column on left is a mixture of the 4 members.

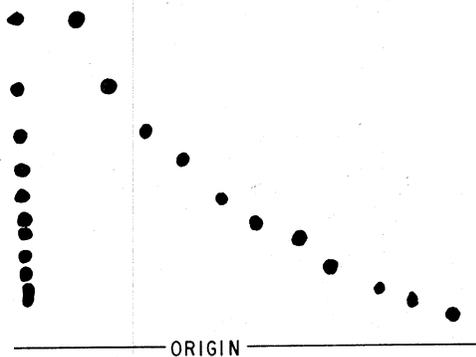


FIG. 4. Class separation of aliphatic monocarbonyl 2,4-dinitrophenylhydrazones by thin-layer adsorption chromatography. Support: MgO-Celite 545 (3:7); solvent: hexane:  $\text{CHCl}_3$  (95:25). Diagonally from top to bottom: 2-nonadecanone, acetone, *n*-octadecanal, propanal, ethanal, methanal, octadec-2-enal, crotonal, acrolein, octadec-2,4-dienal, and pent-2,4-dienal. Column on left is a mixture of all of the compounds.

methyl ketones,  $3 \times 10^{-4}$ ; saturated aldehydes,  $2 \times 10^{-4}$ ; 2-enals,  $1.4 \times 10^{-4}$ ; and 2,4-dienals,  $1.4 \times 10^{-4}$   $\mu$ moles.

Separation of the classes is best achieved when low and equimolar concentrations (up to 5 times the lower limit of detection) of the members are spotted. It is, therefore, recommended [as in a previous report (10)] that the TLC class separation be used as a check on the column procedure (12) or on an unknown spot or band cut from a thin-layer partition plate.

*Separation of homologous series of methyl ketones, saturated aldehydes, 2-enals and 2,4-dienals by normal thin-layer partition chromatography* (see procedure E). Resolution of homologous series of these 4 classes are similar to those shown by Badings and Wassink (1). The first 10 members of the methyl ketone series and first 13 members of the saturated aldehyde series separate satisfactorily. Detection limits after exposure to diethylamine vapor is approximately  $6 \times 10^{-4}$   $\mu$ moles. For the 2-enals and 2,4-dienals, the first 16 members are resolved although the longer-chain members are somewhat crowded. About  $5 \times 10^{-4}$   $\mu$ moles can be detected after exposure of the plate to diethylamine vapor.

*Separation of a series of longer-chain 2,4-dinitrophenylhydrazones by thin-layer partition chromatography using an alkaline stationary phase* (see procedure F). Separation of a series of longer-chain saturated aldehydes is shown in Fig. 5. Note the good separation of the long-chain derivatives. The normal partition system separated only the first 13 members. Essentially similar separations of the 2-enals and 2,4-dienal series were observed. The use of an alkaline stationary phase for separating the longer-chain methyl ketones proved to be ineffective, separation similar to that obtained in the normal partition system being observed. Increasing the strength of the methanolic KOH used in preparing the plates did not improve separation of the longer members.

We have found the alkaline partition plate useful also for separating pairs of hydrazones which migrate the same in the normal partition system. Thus, acrolein and acetaldehyde are readily separated in the alkaline system, but do not separate in the normal system.

There are also color differences in the alkaline system between the four classes. These colors are similar to the colors displayed by the classes on the MgO plates or columns.

No evidence of any breakdown or isomer formation has been observed on the alkaline partition plates. The stability of 2,4-dinitrophenylhydrazones and 2,4-dinitrophenylosazones under strongly alkaline conditions has been demonstrated (7, 11).

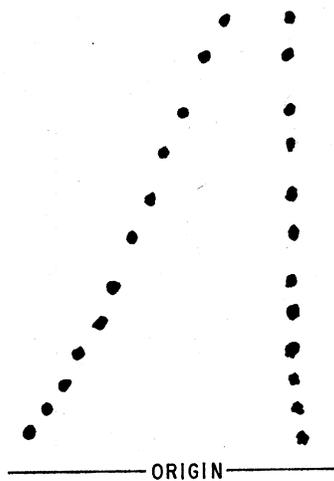


FIG. 5. Separation of a series of longer-chain saturated aldehyde 2,4-dinitrophenylhydrazones by thin-layer partition chromatography utilizing an alkaline stationary phase. Support: Microcel T-38; stationary phase: alkaline polyethylene glycol 400; mobile phase: hexane saturated with neutral polyethylene glycol 400. Diagonally from top to bottom  $C_{18}$ ,  $C_{16}$ ,  $C_{14}-C_5$ . Column on right is mixture.

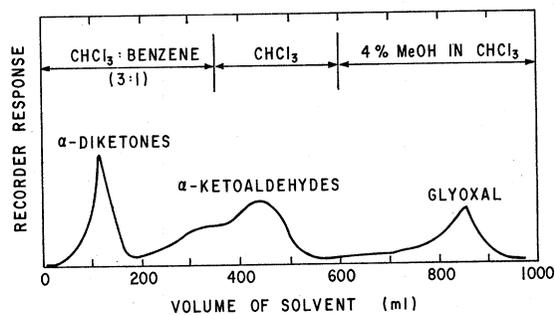


FIG. 6. Class separation of dicarbonyl bis(2,4-dinitrophenylhydrazones) on a MgO-Celite column.

*Separation of dicarbonyl bis(2,4-dinitrophenylhydrazones) into classes by column adsorption chromatography* (see procedure G). The separation of a mixture of  $\alpha$ -diketones,  $\alpha$ -ketoaldehydes, and glyoxal into classes on a column of MgO: Celite is shown in Fig. 6. Approximately 0.03  $\mu$ moles each of the derivatives of 2,3-octanedione, 2,3-butanedione, 2-keto nonanal, 2-keto butanal, 2-keto propanal, and glyoxal were chromatographed. The pooled fractions constituting a given peak were checked for proper classification on the MgO plates (procedure A) and the individual members identified in the thin-layer partition system for the dicarbonyls (procedures B and C). Recoveries were checked and found to be nearly quantitative.

As in the thin-layer procedure (procedure A) the color of the diketone class (blue) aids in distinguishing it from the  $\alpha$ -ketoaldehydes (violet) and glyoxal (violet).

Van Duin (5) first reported separation of the bis(2,4-dinitrophenylhydrazones) of carbonyls by column adsorption chromatography. He employed zinc carbonate as the adsorbent and a pyridine-containing solvent as eluant. He also described differences in color of the classes.

#### SUMMARY

New and improved thin-layer and column adsorption and partition chromatographic systems are described for classifying and separating series of 2,4-dinitrophenylhydrazone and bis(2,4-dinitrophenylhydrazones). Improved and simplified thin-layer adsorption chromatographic methods for separating classes of monocarbonyls (methyl ketones, saturated aldehydes, 2-enals, and 2,4-dienals) and dicarbonyls ( $\alpha$ -diketones,  $\alpha$ -ketoaldehydes, and glyoxal) are outlined. Partition systems employing a neutral and a basic stationary phase are presented.

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