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Paper Chromatography of 2,4-Dinitrophenylhydrazones of Saturated Aliphatic Aldehydes

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► A rapid paper chromatographic procedure for separating saturated aliphatic aldehyde 2,4-dinitrophenylhydrazones is described. Two complementary systems were used. One employed filter paper impregnated with propylene glycol and ascending development by Skellysolve C fraction-methanol; the other used filter paper impregnated with vaseline and ascending irrigation with aqueous methanol. Separations were completed in $1\frac{3}{4}$ hours in the former and 6 hours in the latter. Derivatives C_1 to C_6 were separated by ascending development on propylene glycol impregnated paper with Skellysolve C fraction-methanol. Ascending development on vaseline-impregnated paper with aqueous methanol separated C_7 to C_{14} derivatives. Solvent path in both systems was $8\frac{1}{4}$ inches. Equipment required was simple, consisting of $11\frac{11}{16} \times 1\frac{1}{2}$ inch tubes and mer-

cury seals to prevent vapor pressure build-up. Equilibration was not necessary for successful separations.

VOLATILE aldehydes, ketones, and dicarbonyls influence the flavor and quality of a large number of food and natural products. Considerable activity has been directed in recent years to the isolation, separation, and identification of these compounds. Carbonyl compounds related to flavor have been separated by Burnett *et al.* (2) from vacuum packed dehydrated pork, Buss and MacKinney (4) from rancid corn oil, Dacre (7) from cheese, Forss *et al.* (10) and Tamsma (32) from milk and milk fat, Underwood *et al.* (33) from maple sirup, Spencer and Stanley (31) from tomatoes, Huelin (14) and others (13, 22) from apples, and David and Joslyn (8) from frozen green peas.

In most cases, carbonyls are converted into their 2,4-dinitrophenylhydrazone

derivatives for purposes of isolation and identification. As the carbonyls are present in small amounts, paper chromatography is particularly adapted to their separation and identification. A number of paper chromatographic systems have been proposed for the separation of 2,4-dinitrophenylhydrazones (3, 5, 14, 20, 23, 26, 28). Some of these (23, 26) have been reported (5) to be unsatisfactory because of streaking or poor resolution of mixtures. Other methods (4, 19, 20) require laborious and difficult pretreatment of the filter paper. Some can separate only a limited number of a homologous series. Methods which are suitable for the carbonyl derivatives from one natural product may not be adequate for the separation of a different assortment of derivatives from another product. Nearly all require extensive preparation and long periods of time for separation.

As the result of the authors' work on

the volatile carbonyl 2,4-dinitrophenylhydrazones (12) of oxidizing pork tissue, a rapid paper chromatographic procedure for separating the carbonyls into classes was developed. This method gives an apparent separation of the carbonyl 2,4-dinitrophenylhydrazones into saturated aliphatic aldehydes, ketones, 2-enals, 2,4-dienals, and dicarbonyl derivatives. The nature of these fractions was being studied, and a rapid, reproducible method was needed for separating and identifying the aliphatic aldehyde 2,4-dinitrophenylhydrazones without having to resort to alkali color changes for detection on the paper.

Silberstein (30) separated aliphatic carbonyl 2,4-dinitrophenylhydrazones from C₁ to C₆ on filter paper impregnated with 20% propylene glycol (1,2-propanediol) with descending irrigation with heptane-propylene glycol. Winteringham (34) used filter paper impregnated with vaseline in the separation of pyrethrins. Indovina *et al.* (16) separated several aliphatic aldehydes on paper impregnated with vaseline and Schiff's reagent or 2,4-dinitrophenylhydrazine. Seligman and Edmonds (29) recently reported separation of some saturated aldehydes, ketones, and unsaturated aldehydes and ketones on paper impregnated with olive oil. Both ascending and descending methods were used with aqueous methyl acetate and aqueous isopropyl alcohol. Their separations required 16 hours.

The method finally developed is rapid and requires only simple equipment. Ascending solvent development is used, and the technique employed is basically the test tube procedure of Rockland and Dunn (27) and Rice *et al.* (26). Two systems are used. The first employs filter paper impregnated with propylene glycol and Skellysolve (boiling point 91° to 95° C.)-methanol, and the second, filter paper impregnated with vaseline and aqueous methanol. The first separates *n*-aliphatic saturated aldehyde 2,4-dinitrophenylhydrazones from C₁ to C₆, and the second system, those from C₇ to C₁₄. Since the present paper was written, this method has been applied to the identification of saturated aliphatic aldehydes in rancid pork fat (11).

The work of Jones, Holmes, and Seligman (18) on the spectra of some 2,4-dinitrophenylhydrazones in basic solution shows the value of such measurements in the identification of classes. Their data on C₁ to C₆ saturated aliphatic aldehyde dinitrophenylhydrazones indicate the possibility of the existence of differences in color fading rates that might be useful in the identification of individual saturated aliphatic aldehydes. A study has therefore been made in this report of the

fading rates of the 2,4-dinitrophenylhydrazones of C₁ to C₁₄ saturated aliphatic aldehydes in alcoholic alkali.

SOLVENTS AND REAGENTS

Carbon tetrachloride (ACS specifications) was refluxed with 5 grams of 2,4-dinitrophenylhydrazine and 1 gram of trichloroacetic acid per liter and distilled. Some lots contained carbonyls, while others were relatively free.

Benzene (ACS specifications) was refluxed and distilled from 2,4-dinitrophenylhydrazine and trichloroacetic acid.

Methanol (ACS specifications) and commercial absolute ethyl alcohol were refluxed and distilled from sodium hydroxide.

Skellysolve C was refluxed over 2,4-dinitrophenylhydrazine and trichloroacetic acid and distilled. The fraction boiling from 91° to 95° C. was collected.

Alcoholic alkali (0.25*N*). Potassium hydroxide (1.4 grams) was dissolved in 15 ml. of distilled water and diluted to 100 ml. with ethyl alcohol. The solution was allowed to stand overnight and filtered before using. This solution was prepared fresh for each day's determination.

Uniformity of purified solvents was regularly checked by measuring the absorbance against water at 350 m μ .

Propylene glycol, U.S.P.

Vaseline (white petroleum jelly), Blue seal, Cheesbrough Manufacturing Co.

MATERIALS AND EQUIPMENT

Whatman No. 3 filter paper sheets, 18 $\frac{1}{4}$ × 22 $\frac{1}{2}$ inches. Sheets were cut in the machine direction into tapering strips 10 $\frac{1}{2}$ × 1 $\frac{3}{8}$ × 1 inch.

Test tubes approximately 11 $\frac{13}{16}$ × 1 $\frac{1}{2}$ inches (culture tubes, 300 × 38 mm.).

Mercury traps were used to regulate vapor pressure in the chromatographic chamber. They consisted of 25-ml. side-arm Erlenmeyer flasks containing mercury, with a glass tube leading from the chromatographic test tube immersed $\frac{1}{16}$ inch in the mercury.

Spectrophotometric measurements were made with a Beckman Model DU spectrophotometer using a tungsten lamp as light source.

EXPERIMENTAL

Normal saturated aliphatic aldehydes, C₁, C₂, C₃, C₄, C₅, and C₇, were obtained from commercial sources. Saturated aliphatic aldehydes, C₈, C₁₀, C₁₁, C₁₂, and C₁₄, were prepared by oxidation of the corresponding alcohol by the method of Delaby and Guillot-Allegre (9). Aldehydes, C₆, C₉, and C₁₃, were prepared by reduction of the methyl ester of the corresponding acid to the alcohol with lithium aluminum hydride (24) followed by oxidation in the usual man-

ner. 2,4-Dinitrophenylhydrazones of the 14 saturated aliphatic aldehydes were prepared by Iddles' (15) method. The hydrazones were purified by repeated recrystallization until a constant melting point was obtained.

Stock solutions of the hydrazones in carbon tetrachloride were prepared containing 25 mg. per liter (25 γ per ml.). Suitable amounts were taken from each stock solution to make up 100-ml. solutions of 30 μ moles per liter concentration. Aliquots of these solutions were used for measurement of maxima in carbon tetrachloride, ethyl alcohol, and alcoholic potassium hydroxide, and for preparation of mixtures for spotting chromatograms.

Two chromatographic systems were used to separate the saturated aliphatic aldehyde hydrazones. In the first system, paper strips were impregnated with propylene glycol by dipping once in 20% propylene glycol-80% methanol (by volume) and air-drying 1 hour. The strip was spotted with a mixture of equivalent amounts of hydrazones 1 $\frac{1}{4}$ inches from the bottom. Five milliliters (29) of 96% Skellysolve (boiling at 91° to 95° C.)-4% methanol (by volume—care should be taken that no methanol-rich phase is present) was placed in the large test tube and the paper strip inserted. The system was closed with a mercury trap. The developing solvent was allowed to ascend to a premarked point 8 $\frac{1}{4}$ inches above the starting point. This method separated C₁ to C₆ aldehydes in 1 $\frac{3}{4}$ hours. In the second system the paper strips were impregnated by dipping in a 7% solution of vaseline in Skellysolve (boiling at 91° to 95° C.). The strips were air-dried for 15 minutes, spotted, and developed in 6.00 ml. of 89% methanol-11% water in the large test tube closed by a mercury trap. This procedure separated saturated aliphatic aldehydes from C₇ to C₁₄ in 6 hours. All chromatograms were run at room temperature (27° C.).

Whatman No. 3 paper has a relatively large capacity; amounts spotted were calculated by the following formula:

$$x \text{ ml. of } 30 \mu\text{mole solution} = \frac{1.5 \text{ to } 3.0}{\text{absorbance of mixture of hydrazones}}$$

Each separation was run in groups of three strips, or more, for checking R_F 's and to supply enough material for further chromatography. Mixtures of C₁ to C₆, C₇ to C₁₄, and C₁ to C₁₄ aldehyde 2,4-dinitrophenylhydrazones were run. Individual dinitrophenylhydrazones were run simultaneously to determine variation in R_F values. Separated spots from the mixtures were extracted and run singly, and mixed with the appropriate hydrazones to determine completeness of resolution. In the separations, mixtures were prepared by combining equal volumes of solutions of 30 μ moles per liter concentration. The absorbance of this

mixture was determined, substituted in the above formula, and the volume of solution to be spotted determined.

The effect on the R_F values of variation in the amount of impregnating agent was examined. Propylene glycol was impregnated in 15, 20, and 25% quantities, and vaseline applied in 5, 7, and 9% amounts. Methanal, butanal, and hexanal in System 1, and tetradecanal, decanal, and heptanal in System 2 were chromatographed.

Fading studies of the hydrazones in alcoholic alkali were made by removing the solvent from a 3.00-ml. aliquot of 30 μ moles per liter solution (evaporation on steam bath with a jet of nitrogen gas), adding 0.3 ml. of benzene and 2.70 ml. of alcoholic potassium hydroxide, shaking, and reading at the predetermined major maximum at 2, 15, 30, 45, and 60 minutes. For purpose of calculation 2 minutes was taken as zero time.

RESULTS AND DISCUSSION

Table I shows the melting points of 2,4-dinitrophenylhydrazone derivatives prepared from C_1 to C_{14} n -aliphatic saturated aldehydes, and their wave lengths of maximum absorption in carbon tetrachloride, ethyl alcohol, and alcoholic alkali. These physical constants agree well with those reported in the literature (1, 6, 17, 18, 21). As far as the authors are aware, the absorption maxima of these compounds in carbon tetrachloride have not been previously reported. Determinations of absorbances of 30 μ moles per liter concentrations of the dinitrophenylhydrazones showed no appreciable differences between compounds, and between carbon tetrachloride, ethyl alcohol, and alcoholic potassium hydroxide solvents. This is borne out by molecular extinction coefficients reported in the literature (1, 17, 18, 21).

Fading studies of the aliphatic aldehyde dinitrophenylhydrazones in alcoholic potassium hydroxide indicate only limited value in differentiation of members of the homologous series. Methanal, as noted by Jones *et al.* (18), is in a class by itself. As shown in Figure 1, ethanal and propanal can be distinguished from the remaining members of the series by their more rapid decrease in absorbance with time. There appeared to be no significant differences in the fading of the rest of the dinitrophenylhydrazones (C_4 to C_{14}). This behavior correlates with the similarity of melting points and the differences in maxima in alcoholic alkali. A secondary maximum, which disappeared after 30 minutes (18), was observed at 520 $m\mu$ in the spectra of all the derivatives, except that of methanal, which faded much more rapidly.

The R_F 's of C_1 to C_6 saturated ali-

Table I. Physical Constants of Derivatives

2,4-Dinitrophenylhydrazone	Melting Point Range, °C.	Wave Length of Maximum Absorption, M_μ		
		CCl_4	EtOH	Alc. KOH
Methanal	167.5-168.2	330	346-9	420-6
Ethanal	159.5-160	343	355-8	430
Propanal	153-154	343	355-8	430
Butanal	121.8-122.2	343-6	355-8	430-4
Pentanal	107.5-108	343-6	355-8	430-4
Hexanal	105.9-106.3	343-6	355-8	430-4
Heptanal	106-106.7	343-6	355-8	430-4
Octanal	106.5-107	343-6	355-8	430-4
Nonanal	106-106.5	343-6	355-8	430-4
Decanal	104.2-104.8	346	355-8	430-4
Undecanal	104.4-105	346	355-8	430-4
Dodecanal	105-105.6	346	355-8	430-4
Tridecanal	108.2-108.7	346	355-8	430-4
Tetradecanal	108.5-109	346	355-8	430-4

* All melting points corrected.

Table II. Separation Data for Propylene Glycol (20%) and Skellysolve-Methanol (System 1)

Aliphatic Aldehyde 2,4-Dinitrophenylhydrazones	R_F				
	I, Amount, γ	II, Mixture	III, Individual	IV, Single, extd. from II	V, Mixture II, extd. and authentic
Methanal	2.54	0.26	0.27	0.27	0.27
Ethanal	2.71	0.39	0.39	0.40	0.38
Propanal	2.88	0.59	0.64	0.62	0.62
Butanal	3.05	0.72	0.74	0.75	0.74
Pentanal	3.22	0.82	0.88	0.87	0.87
Hexanal	3.50	0.89	0.94	0.94	0.91

* Based on averages of 3 or more paper strips.

phatic aldehyde 2,4-dinitrophenylhydrazones separated by System 1 (20% propylene glycol) are shown in Table II. Column I shows the approximate amount of material present in each spot. The R_F 's obtained when the entire mixture was separated are shown in column II. Column III indicates the R_F 's obtained when individual dinitrophenylhydrazones were run singly. The R_F 's of the extracted spots from column II when run singly are shown in column IV. There was no indication of further separation in this rechromatography. The R_F 's of individual spots from column II, mixed with corresponding dinitrophenylhydrazones and run individually, are listed in column V. There was no separation, and small dense spots were formed.

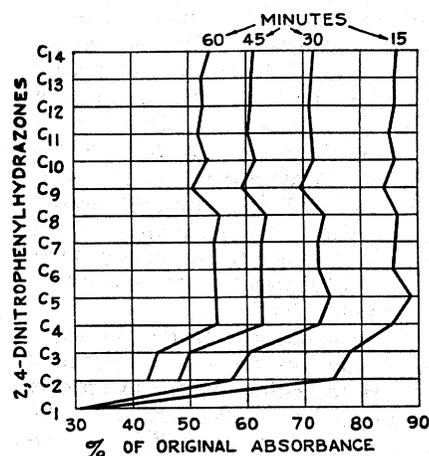


Figure 1. Fading of saturated aliphatic 2,4-dinitrophenylhydrazones in alcoholic alkali

Table III. Separation Data for Vaseline (7%) and Aqueous Methanol (System 2)

Aliphatic Aldehyde 2,4-Dinitrophenylhydrazones	R_F				
	I, Amount, γ	II, Mixture	III, Individual	IV, Single, extd. from II	V, Mixture II extd. and authentic
Heptanal	2.67	0.69	0.72	0.71	0.71
Octanal	2.79	0.62	0.65	0.65	0.66
Nonanal	2.92	0.55	0.60	0.60	0.60
Decanal	3.05	0.49	0.51	0.51	0.52
Undecanal	3.18	0.41	0.43	0.44	0.43
Dodecanal	3.30	0.34	0.38	0.38	0.37
Tridecanal	3.43	0.28	0.33	0.33	0.33
Tetradecanal	3.56	0.22	0.24	0.24	0.24

* Based on averages of 3 or more paper strips.

The R_F values of the separated mixture (column II) are lower than those of the singly-run compounds. The R_F values of columns IV and V are closely similar to the singly-run compounds in column III, indicating quantitative separation. Spots were well spaced in the propylene glycol system, but the interval between spots tended to decrease with height.

Table III shows the separations of C_7 to C_{14} aliphatic aldehydes obtained with System 2 (7% vaseline impregnated). The data are presented in the same manner as in Table II for System 1. Here, also, the mixture caused a depression in the R_F values (columns II and III). The data show complete separation of the mixture of the eight 2,4-dinitrophenylhydrazones. The spots were well spaced, with the intervals remarkably constant throughout. The second system has proved very reproducible in R_F values. However, the first system varies somewhat from day to day in R_F values. In the separation and identification of unknown derivatives, known compounds should always be chromatographed at the same time. It is extremely important that the chromatographic chamber be sealed with a mercury trap. If stoppers are used, the solvent ascends much more slowly and may not reach the required height. Often no separation is obtained, because after a partial separation the bands roll up on each other. This appears to be due to a build-up of pressure by the volatile solvents. Winteringham *et al.* (35) and Quayle (25) have designed apparatus for paper chromatography by ascending development at reduced pressure. This shortens the time necessary for equilibration.

In the separations on the two paper chromatography systems, a mixture of all fourteen 2,4-dinitrophenylhydrazones can be applied to either system. After completion of a separation on one system, unseparated material which followed the solvent front can then be extracted and applied to the other system. In extracting spots for spectrophotometric measurements or unresolved material for application to the next system, it was necessary to eliminate extracted propylene glycol or vaseline. This was done by extracting material from the first system with a Skellysolve fraction (boiling 91° to 95° C.), and the second system with cold methanol. The extracted material was spotted on untreated Whatman No. 3 paper and the hydrazones moved up and away from the impregnating agent with an appropriate solvent. Recoveries of extracted material were usually 85% or better.

Table IV shows the effect on R_F values of variation in the amount of impregnating agent in the paper. In-

crease in the amount of impregnating lowers the R_F values. The effect of fewer hydrazones in the mixture on the depression in the R_F values can be noted. Lowering of R_F values is less in the 20% propylene glycol and

Table IV. Effect of Variation in Amount of Impregnating Agent on R_F Values^a

Mixture of 2,4-Dinitrophenylhydrazones	Propylene Glycol, %		
	15	20	25
Methanal	0.35	0.26	0.20
Butanal	0.81	0.73	0.60
Hexanal	0.99	0.88	0.83
	Vaseline, %		
	5	7	9
Tetradecanal	0.28	0.23	0.19
Decanal	0.56	0.50	0.46
Heptanal	0.75	0.70	0.67

^a Based on average of 3 paper strips.

7% vaseline systems than shown in Tables II and III with a more complex mixture. Variation in amount of impregnating materials should prove useful in the separation of other homologous series. For instance, 2-enals and 2,4-dienals could be expected to run just behind hydrazones of aliphatic aldehydes of the same number of carbons in System 1 and just ahead in System 2 (22, 29).

The sensitivity of the propylene glycol system was shown by the separation of a low-melting preparation (melting at 149° C.) of ethanal 2,4-dinitrophenylhydrazones into two polymorphic forms. The low-melting form had an R_F value of 0.49 as compared with 0.39 for the high-melting form (melting at 159.5° to 160° C.) referred to in Tables I and II. The low-melting form was present in minor amounts and had the same absorption maximum as the high-melting form. Buss and Mackinney (4) have considered the possibility of polymorphism and the existence of meta-stable forms in the separation and identification of hydrazones.

SUMMARY

A rapid paper chromatographic method of separating aliphatic aldehyde 2,4-dinitrophenylhydrazones up to 14 carbon atoms has been described. A meticulous separation of a mixture of aliphatic aldehyde derivatives from C_1 to C_6 and C_7 to C_{14} was obtained, by using two complementary systems. The first one employed filter paper impregnated with propylene glycol and ascending development of Skellysolve C fraction-methanol, with complete

separation in $1\frac{3}{4}$ hours. The second system used filter paper impregnated with vaseline and ascending irrigation with aqueous methanol; separation was completed in 6 hours. With the simple apparatus used, equilibration was not necessary for successful separation. Regulation of vapor pressure, however, was an important factor in this type of chromatography. Pressure in the chromatographic chamber should not be allowed to exceed atmospheric pressure greatly.

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Mention of specific commercial materials or equipment does not constitute recommendation for their use above similar materials and equipment of equal value.

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