

Use of Ion-Exchange Resins in the Micro Analysis of 2,4-Dinitrophenylhydrazones

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This paper deals with the utilization of ion-exchange resins in the microanalysis of the 2,4-dinitrophenylhydrazine derivatives of carbonyl compounds. The procedures which will be described have been successfully used in this Laboratory for purification of micro amounts of carbonyl dinitrophenylhydrazones isolated from natural products.

Apparatus and Reagents

The chromatographic columns used are approximately 1 cm. i.d. by 15 cm. Satisfactory columns for all of the procedures to be described can be made by cutting a 10-ml. graduated pipet in half and placing a small wad of glass wool in the constricted portion of the tube.

One ion-exchange resin used is AG5OW-X4, 200-400 mesh (Bio-Rad Laboratories).^{*} This is a light-colored cation exchanger equivalent to Dowex-50. It is used in preference to the dark-colored resins since the adsorbed material is easily seen on it. Dowex 1-X4, 50-100 mesh (Dow Chemical Company) is also used.

All solvents used are ACS grade. Carbonyl-free or purified solvents are used where specified. Distilled water is used throughout.

Reagents used include 1*N* NaOH; 1*N* HCl; benzene, redistilled; benzene, carbonyl-free, prepared by the method of Schwartz and Parks¹; methanol; methanol, treated to reduce its carbonyl content (One l. of methanol is made 0.1*N* in HCl and refluxed for 72 hr. with

^{*} The use of trade names is for the purpose of identification only, and does not imply endorsement of the product or its manufacturer by the U. S. Department of Agriculture.

10 g. of 2,4-dinitrophenylhydrazine (hereafter DNPH or reagent). It is then distilled twice in glass equipment. It has not been found possible to prepare carbonyl-free methanol by this procedure. However, it is rendered sufficiently pure for its intended purpose.); alumina, grade F-20 (Aluminum Co. of America), activated by heating 24 hr. at 150°C., then partially deactivated by the addition of 6% (w/w) water. The wet alumina is shaken until all lumps are broken and then allowed to equilibrate for 16 hr. before use.

Experimental

Selective Removal of 2,4-Dinitrophenylhydrazine on AG 50W-X4

The presence of excess DNPH in a solution or precipitate containing 2,4-dinitrophenylhydrazones (hereafter hydrazones) presents a problem where the hydrazones possess polarities of the same order as the reagent. Separation of the reagent from the desired product(s) may be quite difficult, especially since the reagent may be present in several hundredfold excess. Chromatography on adsorbents has been used for this purpose,^{2,3} but, as has been pointed out,⁴ these methods are time-consuming and often incomplete. Oxidation of the excess DNPH with Benedict's reagent has been used successfully.^{4,5} Another method consumes the excess reagent with carbonyl compounds which yield derivatives that can be readily separated from the desired product(s).⁴ In quantitative analysis at the microgram level, however, these methods may be undesirable for obvious reasons.

In the procedure described below, advantage is taken of the fact that DNPH is sufficiently basic to be taken up by a strong cation exchange resin.

AG 50W-X4 cation exchanger is slurried in water and transferred to a chromatography tube. One gram of resin (moisture-free basis) will readily hold 100 mg. of DNPH. The resin in the tube is treated with the following sequence of reagents: (a) 2 column volumes of *N* NaOH; (b) water until effluent is neutral; (c) 2 column volumes of *N* HCl; (d) water until effluent is neutral; (e) 4 column volumes of methanol; and (f) 2 column volumes of purified methanol: carbonyl-free benzene (1:1).

The hydrazone-DNPH mixture is dissolved in the minimum of purified methanol:carbonyl-free benzene (1:1) and transferred to the column. The solution may be put through the column at a rate of 3

ml./min. using nitrogen pressure, if desired, with complete adsorption of DNPH still being maintained. The resin is washed with the purified solvent mixture until the effluent is colorless. The resin should be discarded after use. It has not been found possible to regenerate the resin for reuse.

Adsorption of 2,4-Dinitrophenylhydrazones by Dowex 1-X4

In this procedure advantage is taken of the weakly acidic properties of 2,4-dinitrophenylhydrazones. The acid strength of a number of hydrazones has been measured by Timmons.⁷ The mechanism of ionization in alkali is associated with the formation of a colored quinoidal compound and has been discussed by Bohlmann,⁶ Timmons,⁷ Braude and Jones,⁸ and Jones and Hancock⁹ and in references cited therein. The ionization of *m*-dinitro compounds in general has been dealt with by Porter.¹⁰

Dowex 1-X4 is slurried in water and transferred to a chromatographic tube. One gram of resin (moisture-free basis) will readily take up about 15 μ moles of hydrazones. The resin in the tube is treated with following sequence of reagents: (a) 1 column volume of *N* HCl; (b) water until effluent is neutral; (c) 1 column volume of *N* NaOH; (d) water until effluent is neutral; (e) 4 column volumes of methanol; and (f) 2 column volumes of methanol:benzene (8:2).

The sample containing the hydrazones (free of DNPH) is dissolved in the minimum volume of methanol:benzene (8:2) and applied to the column. If the sample should not completely dissolve in this solvent combination, such as might be the case when a relatively large amount of a fat or oil is present, the benzene concentration is increased until solution is attained. However, as the methanol concentration is reduced the capacity of the resin for hydrazones is also reduced. If this step should be found necessary, the resin should be washed in sequence step (f) with the solvent combination used to dissolve the sample.

After applying the sample to the resin, the column is washed free of neutral contaminants with four column volumes of the methanol:benzene combination. The adsorbed hydrazones, easily visible on the resin as a deep red-purple zone in the case of monocarbonyls and as a blue zone in the case of the bis(hydrazones) of vicinal dicarbonyls, are then eluted with 10% (v/v) glacial acetic acid in methanol:benzene (8:2) until the effluent is colorless.

Removal of Acids from Dinitrophenylhydrazones

Free organic acids can be expected to contaminate the hydrazones obtained from the anion exchange resin (Dowex-1). There is also a slight degree of saponification of glycerides as the latter pass through the resin bed. If the residue from the acetic acid effluent contains sufficient non-volatile organic acids to interfere with subsequent analysis of the hydrazones, they can be efficiently removed in the following manner: 2 g. of alumina is added a little at a time with shaking to a chromatography tube containing about 4 ml. of chloroform. The column is packed under light air pressure to remove air bubbles. The dry residue from the acetic acid effluent is dissolved in a minimum of chloroform and transferred to the column. Collection of the effluent is begun immediately. Chloroform is added until the effluent becomes colorless.

Results and Discussion

General

The hydrazones investigated and the concentrations employed are listed in Table I. Concentrations were determined spectrophotometrically in chloroform using the molar absorptivities and maxima listed in the table. All hydrazones studied were chromatographically pure in the concentrations given in Table I.

The possibility that decomposition, formation of artifacts, or other changes might take place during the various steps was investigated. Liquid-liquid partition chromatography on a Celite support was employed for all compounds. Each hydrazone obtained in the various steps was chromatographed with the same concentration of the untreated authentic sample. Aliphatic monocarbonyls were checked in the acetonitrile-hexane system described by Corbin et al.¹¹ Aromatic hydrazones were chromatographed in an acetonitrile-methyl cyclohexane system.¹² Bis(hydrazones) of vicinal dicarbonyls were run in an acetonitrile-methyl cyclohexane-ethyl acetate system.¹²

It should be noted in Table I that a large number of different classes of carbonyls are represented in the aliphatic series. Also, in this series, hydrazones of long, short, and intermediate chain length were selected. It is assumed that all hydrazones devoid of an additional functional group which fall within the limits of the extremes will behave analogously.

TABLE I
Recovery of 2,4-Dinitrophenylhydrazones from Dowex 1-X4

Hydrazone of	γ_{\max} , m μ	$E \times 10^{-3}$	Concentration investigated, μ mole	Recovery, %
Nonadecanone-2	365	22.5	1.87	97
Octanone-2			0.71	100
Acetone			1.76	95
Octadecanal	355	22.5	0.77	96
Octanal			1.52	92
Acetaldehyde			6.10	100
Formaldehyde	345	19.0	4.35	95
Octadeca-2-enal	373	27.5	0.07	104
Deca-2-enal			0.10	105
Crotonal			0.30	100
Acrolein	367	26.0	0.95	93
Octadeca-2,4-dienal	390	37.5	0.08	102
Deca-2,4-dienal			0.06	100
Penta-2,4-dienal			0.70	103
Phorone	385	24.3	0.43	100
5-hexen-2-one			1.05	90
2,3-Butanedione ^a	390	40.0	0.03	103
2,3-Octanedione ^a			0.15	98
Glyoxal ^a	390	40.0	0.01	101
Methyl glyoxal ^a			0.04	100
2-keto octanal ^a			0.67	104
Acetophenone	386	30.6	0.76	93
Phenylacetaldehyde	377	28.5	0.27	100
Cinnamaldehyde	390	28.8	0.08	100
Benzaldehyde	377	28.5	0.19	98

^a All dicarbonyls investigated as the bis(hydrazones).

Use of AG-50W-X4

All hydrazones passed through this resin quantitatively (98–101% recovery). One symmetrical peak was obtained for each hydrazone when subjected to chromatography.

DNPH is quantitatively taken up by the resin. If impure DNPH

is applied to the resin, colored impurities will pass through. The nature of these impurities has not been established, although it has been ascertained that there are three main colored components. Freshly recrystallized DNPH (from benzene) will give a colorless effluent. However, impurities form in the recrystallized solid even after standing a few hours. The formation of colored impurities is accelerated in solution in the presence of acid. Although this manuscript is not concerned with the formation of 2,4-dinitrophenylhydrazones, these observations are stated with the intention that they may aid the analyst in pertinent work.

Use of methanol or benzene contaminated with carbonyls will give rise to dinitrophenylhydrazones even at the high flow rates (3 ml./min.) used in this procedure. This is presumably due to the good contact made between the free carbonyls in the impure solvents and the zone of DNPH which builds up on the resin as the methanol-benzene solution passes through. AG-50W, being a strong acid, catalyzes the reaction. The use of solvents purified as described obviates this possibility.

Hydrazones containing a basic function may be expected to be adsorbed by the resin along with DNPH. Although no hydrazones of this type were investigated, there is a good possibility that they may be recovered from the resin free of DNPH. This statement is based on the observation that DNPH is not eluted with 5*N* H₂SO₄ or 5*N* HCl or with 1% trichloroacetic acid in carbonyl-free benzene. These suggest that DNPH is irreversibly held by the resin once exchange has occurred.

Use of Dowex 1-X4

Recoveries of hydrazones from Dowex-1 are given in Table I. All hydrazones eluted from the resin were chromatographically pure. No syn-anti isomerism which sometimes occurs with the hydrazones of aldehydes¹³ was evident.

Neutral and basic impurities in the hydrazone sample theoretically will pass through the resin. Model experiments were performed using butterfat, cholesterol, and cholesterol esters singly and in combinations. Each of these constituents passed through the resin quantitatively when applied to the column in amounts ranging from 5 to 45 mg. A slight white residue is always obtained from the column due

to decomposition of the resin. However, this residue is insoluble in chloroform, hexane, and benzene and thus will not interfere with subsequent analyses.

In practice, hydrazones isolated directly from butterfat¹⁴ which were contaminated with small amounts of lipid were obtained as crystalline solids free of lipid. Other applications of this procedure have involved purification of hydrazones cut from reversed-phase papergrams and contaminated with stationary phase, and also purification of fractions obtained from column partition chromatograms.

The data indicate that 2,4-dinitrophenylhydrazones are stable to strongly alkaline conditions at least for the contact periods used in this study, a maximum of approximately 7 min. Advantage can be taken of this where sparsity of material is critical and more analytical data are needed. Fading rate and maxima in alcoholic alkali are useful analytical tools for the classification of hydrazones.¹⁵ It may thus be feasible to recover the hydrazone from an alkaline solution for reuse. In this connection, the blue color given by vicinal dicarbonyl bis(hydrazones) on the anion exchanger as opposed to the red-purple color given by monocarbonyls may be helpful in characterizing an unknown without sacrificing any of the sample.

Experiments with Dowex 1-X4 in forms other than hydroxyl were performed. The resin in the borate, phosphate, citrate, carbonate, and bicarbonate forms will hold some hydrazones and allow others to pass through. The possibility that fractionation of hydrazones can be achieved by these procedures is being investigated.

Use of Alumina

The procedure outlined for adsorbing acids away from the hydrazones on alumina was investigated using the hydrazones in Table I in the recorded concentration. These hydrazones are not adsorbed by the alumina, passing through the column with the solvent front. All are recovered unchanged and quantitatively within a maximum of 20 ml. of solvent. Two model acids, stearic and oleic, were selected for study on the basis of their weakly acidic properties relative to shorter chain, more polar acids. Six milligrams of these acids were applied to individual columns and the columns washed with 20 ml. chloroform. The residues were treated with dry methanolic HCl in order to convert any acid to methyl esters. Gas chromatography of the entire residue failed to reveal any peaks.

References

1. Schwartz, D. P., and O. W. Parks, *Anal. Chem.*, **33**, 1396 (1961).
2. Veitch, F. P. Jr., and H. S. Milone, *J. Biol. Chem.*, **158**, 61 (1945).
3. Hilmer, P. E., and W. C. Hess, *Anal. Chem.*, **21**, 822 (1949).
4. Reich, H., K. F., Crane, and S. J. Sanfilippo, *J. Org. Chem.*, **18**, 822 (1953).
5. Reich, H., S. J. Sanfilippo, and K. R. Crane, *J. Biol. Chem.*, **198**, 713 (1952).
6. Bohlmann, F., *Chem. Ber.*, **84**, 490 (1951).
7. Timmons, C. J., *J. Chem. Soc.*, **1957**, 2613.
8. Braude, E. A., and E. R. H. Jones, *J. Chem. Soc.*, **1945**, 498.
9. Jones, L. A., and C. K. Hancock, *J. Am. Chem. Soc.*, **82**, 105 (1960).
10. Porter, C. C., *Anal. Chem.*, **27**, 805 (1955).
11. Corbin, E. A., D. P. Schwartz, and M. Keeney, *J. Chromatog.*, **3**, 322 (1960).
12. Corbin, E. A. Unpublished data.
13. Van Duin, H., *Nature*, **180**, 1473 (1957).
14. Schwartz, D. P., H. S. Haller, and M. Keeney, Abstracts of papers, 136th Meeting, ACS, Atlantic City, N. J., September, 1959, p. 15A.
15. Jones, L. A., J. C. Holmes, and R. B. Seligman, *Anal. Chem.*, **28**, 191 (1956).