SEPARATION OF HOMOLOGOUS SERIES OF 2,4-DINITROPHENYLOSAZONES BY COLUMN PARTITION CHROMATOGRAPHY

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This manuscript describes chromatographic systems for separating homologous series of the 2,4-dinitrophenylosazone* derivatives of α-keto-aldehydes and 2,3-diketones. To the author's knowledge, only CORBIN[1] has successfully resolved the members of these two classes.

In the work reported below, column partition systems are described in which the stationary phase is alkaline. It is well known that both 2,4-dinitrophenylhydrazones and 2,4-dinitrophenylosazones change from a yellow or orange hue in neutral solvents to various shades of red, purple or blue under the proper alkaline conditions. This phenomenon, which is also shown by other *meta*-dinitro compounds[2], has been studied extensively and has been discussed by JONES AND HANCOCK[3], JONES *et al.*[4], BRAUDE AND JONES[5], TIMMONS[6], BOHLMANN[7], NEUBERG AND STRAUSS[8], and in references cited by these investigators. Since the wavelength of maximum absorption of the 2,4-dinitrophenylhydrazones or osazones going from neutral to alkaline conditions is shifted toward the visible region of the spectrum, visual detection of a spot or zone is enhanced. Paper chromatograms of 2,4-dinitrophenylhydrazones are usually sprayed with alcoholic base for this reason.

TURBA AND SCHRADER-BEIELSTEIN[9], VAN DUIN[10,11] and SCHWARTZ *et al.*[12,13] have employed alkaline adsorbents for fractionation of 2,4-dinitrophenylhydrazones. However, no partition system employing a strongly alkaline phase has been described for separating an homologous series of 2,4-dinitrophenylhydrazones. The stability of 2,4-dinitrophenylhydrazones and osazones adsorbed on a strong anion-exchange resin has been demonstrated by SCHWARTZ *et al.*[14]. It, therefore, seemed feasible to attempt to introduce partition systems employing a strong base.

EXPERIMENTAL

*Reagents and apparatus***

Ethanolamine. Fisher's highest purity grade is redistilled under reduced pressure. The fraction boiling at 56–58° at 4 mm pressure is collected and stored at 4° in a tightly

* The term 2,4-dinitrophenylosazone or merely osazone in this manuscript is used synonymously with bis-(2,4-dinitrophenylhydrazone). It should also be understood that only derivatives were investigated even though the derivative term has sometimes been omitted in the text.

** 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.

stoppered, brown glass bottle. Ethanolamine is hygroscopic and should not be unduly exposed to moist air.

Benzene. ACS grade benzene is redistilled and then dried over anhydrous sodium sulfate for 24 h before use.

Benzene saturated with ethanolamine. Redistilled, dried benzene is saturated by shaking, or more conveniently by stirring (preferably magnetically with a Teflon covered bar) 50 ml of redistilled ethanolamine per gallon of benzene for at least 2 h. Complete phase separation takes at least 16 h. However, a faint turbidity in the benzene phase is not detrimental and in the prescribed method will develop anyhow.

Celite. Johns-Manville Analytical Grade Celite is dried at 140–150° for 96 h. The dried product should be protected against moisture.

Chromatographic column. A borosilicate glass column 2.6 cm × 29 cm with a coarse sintered glass disc is suitable.

Siphon. A siphon to deliver approximately 5 ml is used. If necessary fractions smaller than 5 ml may be collected in order to obtain purer fractions.

Cation-exchange resin. Amberlite IR 120 (H^+), 16–50 mesh, 8–10 % cross linked, is washed with distilled water until a colorless effluent is obtained. It is then washed with 6 column volumes of ACS grade methanol and then with 4 column volumes of redistilled benzene.

Column preparation

The same mechanical procedure is used in the preparation of all columns. Equipment should be clean and dry.

α-Keto-aldehydes. Seven ml ethanolamine and 0.5 ml distilled water are mixed in a 6-in. mortar and 15 g celite is added. The contents of the mortar are ground with a pestle until a homogeneous preparation is obtained. This is best accomplished by grinding, scraping the sides of the mortar completely with a stainless steel spoon and regrinding. The scraping and regrinding procedure is repeated 4 or 5 times. The impregnated celite is transferred to a 500 ml Erlenmeyer flask and 200 ml of mobile phase (benzene saturated with ethanolamine) is added. A 1.5-in. Teflon-coated stirring bar is dropped in, the mouth of the flask covered with aluminum foil and the contents stirred for 15 min maintaining a speed sufficient to produce a visible vortex in the slurry. A layer of glass wool should be placed between the flask and magnetic stirrer before the onset of stirring. This will prevent the temperature of the solution from rising significantly. At the end of this period the bar is removed and the slurry poured through a wide stemmed (so-called powder) funnel to within 2 in. from the top of a chromatographic tube clamped at the outlet. Air bubbles are removed by rotating a stainless steel rod through the slurry in the tube. The column is then packed under nitrogen pressure (2–3 p.s.i.) until the height of mobile phase above the packing is about 1 in. The remainder of the slurry is then added, the flask rinsed clean with mobile phase and the rinsings added to the column. Air bubbles are removed from the remainder of the slurry by stirring above the packed portion of the column. Nitrogen pressure is applied again until the last of the mobile phase has entered the column. The top of the column is then carefully leveled by applying light pressure from a stainless steel tamping rod similar to that described by CORBIN *et al.*[15].

It is important for best results to obtain an even, level column surface. A small amount of celite may resist firm packing and may obscure observation of the true

column surface. If this occurs, it is best to rinse the sides of the tube down carefully with small (about 10 ml) portions of mobile phase, pour out the suspended celite and then repeat this until the surface is free of unpacked celite. The column surface may then be retamped until smooth and perpendicular at all points to the walls of the tube.

Applications of sample. Collection of effluent is begun upon application of the sample. The sample of α-keto-aldehydes in a 10 ml beaker is dissolved in 5 ml of mobile phase. It is important that complete solution be obtained. With the amounts of dicarbonyls chromatographed in this study, 5 ml is sufficient to effect solution. Solution may be hastened by covering the beaker securely with aluminum foil and heating gently for a few minutes on the steam bath and then allowing the solution to cool to room temperature. The solution is applied to the column by allowing it to run from a pipette rotated rapidly around the upper inner wall of the tube. In quantitative work the beaker should be rinsed with the minimum volume of mobile phase until all color is removed and the rinsings applied to the column in the manner described above. After the last of the colored solution has just entered the column, the sides of the tube are rinsed down with a few milliliters of mobile phase. The latter is allowed to percolate into the column and 10 ml of mobile phase is added. A ball of glass wool (about 2 g) previously saturated with ethanolamine by soaking and squeezing out the excess, is then pushed into the column to within approximately 0.5 in. of the column surface and the tube filled with mobile phase. The glass wool impregnated with stationary phase is necessary to compensate for changes in the composition of mobile phase if a constant temperature room is not used. If the chromatogram is not developed at constant temperature, solution of some stationary phase will occur if the temperature rises. The use of glass wool impregnated with ethanolamine insures that the mobile phase remains saturated with stationary phase throughout the development.

Removal of ethanolamine from effluent. The effluent from the column contains a fine suspension of ethanolamine which renders the fractions unsuitable for spectrophotometric evaluation. It is, of course, also necessary to remove the ethanolamine from the osazones at some point if further analysis of the fractions is desired. Both of these problems are overcome by adsorbing the base onto a cation-exchange resin. The use of a cation-exchange resin in the microanalysis of 2,4-dinitrophenylhydrazones and osazones has been described by SCHWARTZ *et al.*[14]. Their results showed that all types of commonly occurring 2,4-dinitrophenylhydrazones and osazones pass through the resin quantitatively and without change.

For the purpose of removing ethanolamine the exchanger is packed into a coarse sintered glass funnel (approximately 2.2 cm i.d. × 5 cm from top to sintered disc). Five grams of resin (dry basis) will quantitatively take up the ethanolamine from about 250 ml of mobile phase. The conditioned exchanger is placed just below the siphon. The exchanger is changed at a suitable time, that is, when there is a minimum of 3 blank fractions emerging from the column (see Figs. 3 and 4). Regeneration of the exchanger is accomplished by washing with 5 column volumes of *N* HCl, followed by distilled water until a neutral effluent is obtained and then with methanol and benzene as described under reagents and apparatus.

2,3-Diketones. The procedure for preparation of the column for separating 2,3-diketones is exactly the same as that for the α-keto-aldehydes except that 8 ml ethanolamine is used and the water is omitted.

Glyoxal. Glyoxal, the only vicinal dialdehyde, requires a special column for chromatography. It is much too slow moving in the systems described above to be eluted in a reasonable length of time. It can be separated from all other saturated dicarbonyls on a column composed of 7.5 g celite, 1.5 ml water, and 3.5 ml ethanolamine. The column is constructed as described for the other classes.

RESULTS AND DISCUSSION

Figs. 1 and 2 are photographs, and Figs. 3 and 4 plots of absorbancy *vs.* fraction number of the α-keto-aldehyde and diketone chromatograms, respectively.

Coloring

There is a sufficient contrast in hue (see Table I) between the 2 classes so that visual differentiation can be made with confidence*. This color difference has always been manifested provided that coloring occurs. Coloring (and separation) will not occur if (a) insufficient stationary phase is used, (b) too much water is present, or (c) excessively high flow rates are employed.

TABLE I

COLOR, R-VALUES AND RECOVERY OF 2,4-DINITROPHENYLOSAZONES

2,4-Dinitrophenyl-osazone of	*Color*	R_{Bu}*	R_{Mg}*	R_{Khex}*	*Recovery %*
α-Ketononanal	blue	—	0.09	0.42	98
α-Ketooctanal	blue	—	0.11	0.54	95
α-Ketoheptanal	blue	—	0.15	0.70	102
α-Ketohexanal	blue	—	0.22	1.00	98
α-Ketopentanal	blue	—	0.31	1.44	97
α-Ketobutanal	blue	—	0.57	2.65	97
α-Ketopropanal (methylglyoxal)	blue	—	1.00	4.58	96
2,3-Octanedione	grey	0.24	—	—	102
2,3-Heptanedione	grey-violet	0.30	—	—	100
2,3-Hexanedione	—	0.43**	—	—	—
2,3-Pentanedione	violet	0.62	—	—	102
2,3-Butanedione (diacetyl)	violet	1.00	—	—	101
Glyoxal	blue	—	3.90	—	99

* Abbreviations: Bu = 2,3-butanedione; Mg = methylglyoxal; Khex = α-ketohexanal.

$$R = \frac{\text{volume of effluent to peak of compound}}{\text{volume of effluent to peak of standard}}.$$

** Value calculated from graph of $\log_{10}$ of effluent volume *vs.* number of carbons in parent compound.

With approximately equimolar concentrations of members of a given class, the intensity of the color increases on the chromatogram as the chain length of the member decreases. 0.05 μm of methylglyoxal or diacetyl show up as intensely colored bands under the prescribed conditions, even after having moved the full length of the column.

* Separation of the various classes of 2,4-dinitrophenylosazones (*e.g.*, non-vicinal diketones and dialdehydes, vicinal diketones, α-keto-aldehydes and vicinal dialdehyde) has been accomplished in this laboratory. The method greatly simplifies the analysis of complex mixtures of dicarbonyls and makes color differences between classes less critical, but still desirable from a confirmatory standpoint.

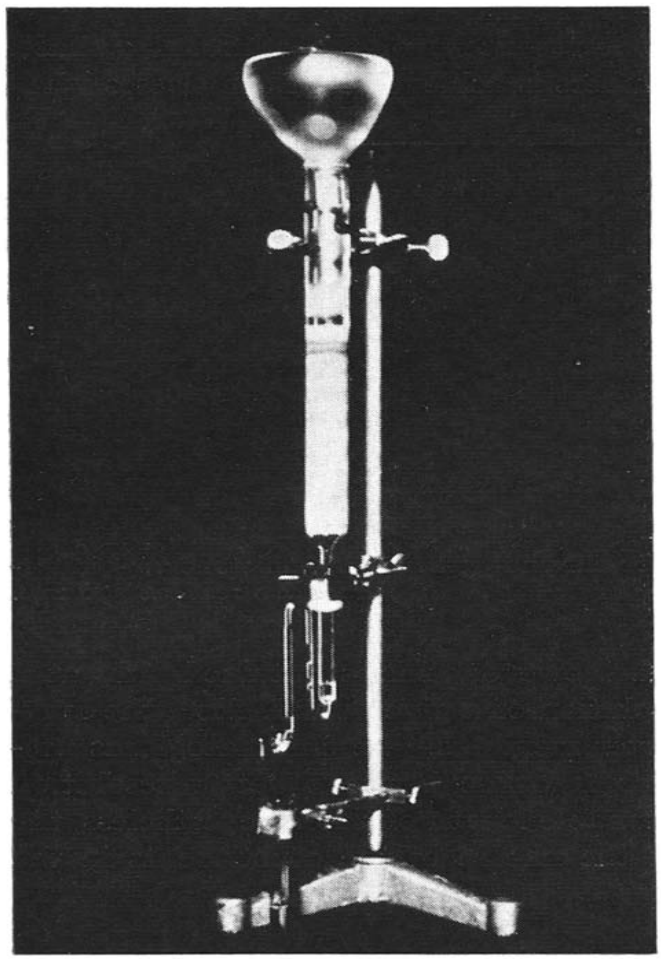

Fig. 1. Chromatogram of α-keto-aldehyde 2,4-dinitrophenylosazones. 1 h development. Approximately 0.05 μm each.

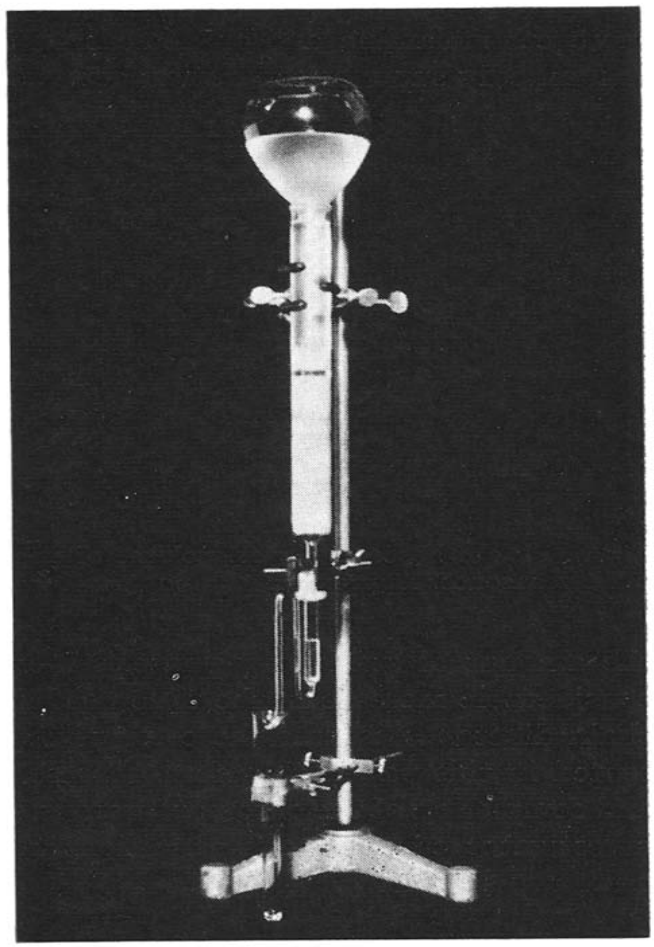

Fig. 2. Chromatogram of 2,3-diketone 2,4-dinitrophenylosazones. 45 min development. Approximately 0.05 μm each.

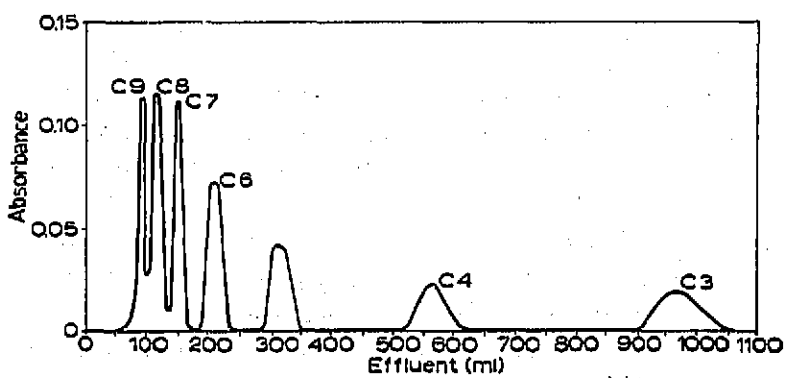

Fig. 3. Plot of absorptivity *vs.* volume of effluent for chromatogram of α-keto-aldehyde 2,4-dinitrophenylosazones. Approximately 0.05 μm each; flow rate = 133 ml/h; fractions read at 390 mμ.

The colors of the bands can be intensified by using more ethanolamine in the case of the diketones. This effect can be produced with the α-keto-aldehydes by using more ethanolamine and/or decreasing the water content of the stationary phase. A markedly superior separation between members is achieved for both classes when

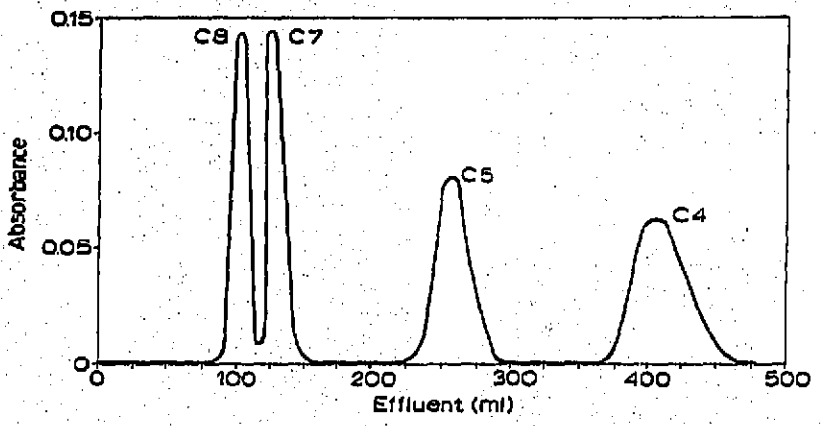

Fig. 4. Plot of absorptivity *vs.* volume of effluent for chromatogram of 2,3-diketone 2,4-dinitrophenylosazones. Approximately 0.05 μm each; flow rate = 92 ml/h; fractions read at 390 mμ.

the amount of stationary phase is increased. However, this is obtained only at a considerable increase in the time needed for elution of a given band. Also, the long-chain diketones assume the color of the shorter members. The chromatograms presented in this paper, therefore, represent a compromise between better resolution and completion of the chromatogram in a reasonable length of time. Nevertheless, the investigator can vary his conditions to suit the nature of the constituents in the unknown sample.

Reproducibility

The volume of mobile phase needed to elute a given zone was found to vary considerably from one chromatogram to the next. This was due largely to variations in flow rate. However, the volume of mobile phase needed to elute a given compound relative to an internal standard was found to be quite constant. It is felt that the *R* values (Table I) can be used with greater confidence than trying to control all of the variables involved, especially in light of the critical role played by moisture.

Stability and recovery studies

Although ample evidence attesting to the stability of 2,4-dinitrophenylhydrazones and osazones, under alkaline conditions is accumulating, it was felt that the long exposure of some of the osazones to ethanolamine warranted investigation of additional stability and recovery data. For this purpose, pairs of diketones and α-keto-aldehydes, which are well separated from each other (*e.g.*, C_8 and C_5 diketones, C_9 and C_6 α-keto-aldehydes, etc.) were chromatographed in the usual manner. The bands were collected and recoveries determined spectrophotometrically in chloroform. Each recovered compound was then cochromatographed with the same concentration of authentic unchromatographed derivative in the proper system recommended by CORBIN[1]. A continuous flow analyzer (Canal Industrial Corp., Bethesda, Md.) with a 390 mμ filter was used to monitor the chromatogram.

Recoveries are given in Table I. All compounds chromatographed as a single peak with no other bands being detectable either visually or by the instrument. These data indicate that dinitrophenylosazones are quite stable in alkali, a fact which might find considerable application in view of the good solubility of these compounds in non-aqueous bases.

Miscellaneous

The chromatogram of glyoxal is shown in Fig. 5. All other dicarbonyls investigated in this solvent system move with the front and do not change color in the system. It is not known whether other classes of aliphatic dicarbonyls (*i.e.*, unsaturated) would form osazones which would be blue on the chromatogram under the conditions for preparing the glyoxal column.

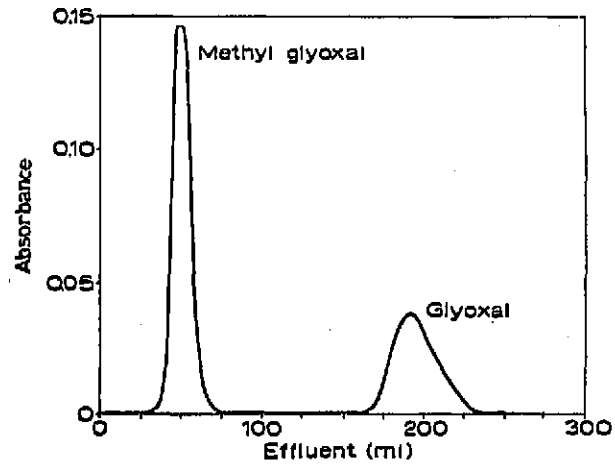

Fig. 5. Plot of absorptivity *vs.* volume of effluent for chromatogram of glyoxal and methylglyoxal 2,4-dinitrophenylosazones. Approximately 0.05 μm each; flow rate = 150 ml/h; fractions read at 390 mμ.

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SUMMARY

Ethanolamine–benzene systems are described for the partition chromatography of the 2,4-dinitrophenylosazone derivatives of homologous series of α-keto-aldehydes and 2,3-diketones. Each class produces a different vivid color on the chromatogram facilitating differentiation and permitting very small amounts of derivative to be followed visually down the column.

A special system is also described for the chromatography of the 2,4-dinitrophenylosazone of glyoxal, the only saturated vicinal dialdehyde.

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