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SEPARATION OF THE 2,4-DINITROPHENYLHYDRAZONES OF SATURATED ALDEHYDES, METHYL KETONES, 2-ENALS AND 2,4-DIENALS

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Investigators attempting to characterize the normal and abnormal flavors in food products are directing more and more attention to the carbonyl compounds associated with such flavors. After a preliminary isolation, these carbonyl compounds are usually converted into 2,4-dinitrophenylhydrazone derivatives. Separation, identification and quantitative estimation of these colored derivatives are then made. Separation of the 2,4-dinitrophenylhydrazones is accomplished by chromatography, either on paper or on a partition column. There is a definite need for a chromatographic method which can separate a variety of 2,4-dinitrophenylhydrazones in a rapid, consistent, and quantitative manner. The partition column method was selected for study since it is more adaptable to quantitative measurement.

The partition chromatographic method of KRAMER AND VAN DUIN[1] as modified by MONTY[2] and BASSETTE[3] uses nitromethane as a stationary phase, supported on celite, and purified hexane as the mobile phase. This method, fine as it is, was found to have two main disadvantages. The first involves the use of nitromethane. Since nitromethane absorbs light strongly in the same region as the 2,4-dinitrophenylhydrazones a quantitative spectrophotometric measurement of the color in the eluate fractions can be made only after evaporating away the nitromethane and dissolving the residue in another solvent. The second disadvantage is that the detection and fractionation of the colored derivatives is done visually which may allow some small amounts of derivatives to pass by unseen.

In the present method these two disadvantages, we feel, have been satisfactorily alleviated. To replace the nitromethane, acetonitrile or 2-chloroethanol was found to be just as efficient and since neither absorbs light in the same region as the 2,4-dinitrophenylhydrazones, the eluate is ready for spectrophotometric measurement without any alteration. To detect the chromatographic bands, especially those invisible to the eye, the eluate is collected in 10 ml fractions and an absorbance measurement is made of each fraction as it is collected. Very small quantities of 2,4-dinitrophenylhydrazones can be detected in this way and a quantitative measure of the amount present is obtained at the same time.

The partition chromatographic method to be described is equally successful in separating the 2,4-dinitrophenylhydrazones of saturated aldehydes, saturated methyl ketones, 2-enals, or 2,4-dienals.

EXPERIMENTAL

Apparatus and reagents

Beckman Model B spectrophotometer with test tube holder and a minimum of three optically matched test tubes (15 × 95 mm or larger).

Measuring siphon to deliver 10 ml ± 0.2 ml.

Chromatographic tube approximately 25 × 350 mm with constricted tip and removable solvent reservoir. A permanent sintered-glass plate or pyrex wool plug can be used at the bottom of the tube.

Tamping rod. A stainless steel disc (1 × 20 mm) is perforated by drilling 20 to $24^1/_{16}$ in. holes in it. This disk is soldered onto the end of a stainless steel rod (5 × 450 mm) to form a T-shaped tamping rod.

Purified hexane. Reflux 3 l of high-purity *n*-hexane (Phillips Petroleum Company) with 50 ml concentrated sulfuric acid for 3 hours. Decant hexane from acid and wash with two 500 ml portions of distilled water. Decant hexane into distilling flask, add 3 teaspoons of sodium hydroxide pellets and mix. Distil, collecting the 69–70° fraction.

Acetonitrile. Distil and collect the 80–81.5° fraction.

2-Chloroethanol. Distil and collect the 127–129° fraction.

Dry celite. A sufficient quantity of analytical grade celite* is dried in a 160° oven for at least 24 hours before use.

Equilibrated hexane for chromatography. Purified hexane is shaken with an excess of acetonitrile or 2-chloroethanol and the hexane layer used.

Column preparation

Acetonitrile column. Twenty five grams of dried celite are placed in a blendor jar with 250 ml of hexane equilibrated with acetonitrile and blended for one or two minutes. Five-tenths ml of distilled water and 33 ml of acetonitrile are added slowly to the swirling suspension. When completely homogenous the celite slurry is poured through a funnel into the chromatographic tube, the tip of which has been closed with a piece of tubing and pinchcock. The tamping rod is moved through the slurry to remove all air bubbles. With the pinchcock opened, air pressure (6–8 lb./in.^{2}) is applied to the top of the column until the celite is compact, being careful that the celite is always covered with equilibrated hexane. More slurry is added, stirred, and compressed as before, until all the celite has been added. The tamping rod is used to firm and level the top of the column material. Flow-rate at this point should be 120–150 drops/min. A prepared column can be kept a week or more if necessary by closing the pinchcock

at the bottom and stoppering the top to prevent evaporation of the hexane covering the column material.

2-Chloroethanol column. This column is prepared in exactly the same manner as the acetonitrile column except that purified hexane saturated with 2-chloroethanol is used and 25 ml of 2-chloroethanol replace the 33 ml of acetonitrile. The flow-rate of a chloroethanol column is less than that of an acetonitrile column. By using slightly less air pressure to compress the column material a satisfactory flow-rate will result.

Column operation

Both columns are set up and operated in the same manner. The following method of operation has been found to give fast and reproducible results. The prepared column is supported near the spectrophotometer so both may be attended simultaneously. The 2,4-dinitrophenylhydrazones should be dissolved in 10 ml or less of equilibrated hexane. If necessary purified hexane can be substituted. When the volume of solvent covering the column is one ml or less the 2,4-dinitrophenylhydrazone solution is carefully added. Collection of the eluate is started immediately. When the sample solution has drained into the column, 2 ml portions of equilibrated hexane are added until no color can be seen in the hexane. The solvent reservoir is attached and filled with equilibrated hexane. The siphon is situated to receive the column eluate and to empty each 10 ml fraction directly into one of the matched spectrophotometer test tubes. The optical density of each 10 ml fraction is then measured and recorded while the next fraction is being collected. Equilibrated hexane is used as the reference blank. Since the optical density measurement is less time-consuming than the collection of the next fraction, a method of increasing the flow-rate of the column, when needed, was introduced. An air line adjusted to 1 or 1 ½ lb./in.^{2} pressure is connected to the solvent reservoir. At some convenient point in this air line a "T" tubing connection is inserted. When the open end of the "T" tube is closed, by placing a finger over it, air pressure is directed against the solvent in the reservoir increasing the flow-rate of the column. Pressure is released just as the fraction starts to siphon. About fifty 10 ml fractions can be collected and measured in one hour using this procedure.

DISCUSSION

Figs. 1 and 2 show the separations that can be expected from the two columns when they separate the 2,4-dinitrophenylhydrazones of the four homologous series studied. The subscripts (C_3, C_{10}, etc.) indicate the number of carbon atoms in the parent aldehyde or ketone. No attempt was made to chromatograph equal molar amounts of each derivative. To conserve space the formaldehyde (C_1) peak is not shown in Figs. 1(A) and 2(A). The formaldehyde curve would reach a maximum within fractions 210–215 in Fig. 1(A) and within fractions 255–260 in Fig. 2(A). The wavelength at which each fraction of a given series is measured in the spectrophotometer is also shown. These wavelengths of maximum absorption were determined on a mixture of appropriate 2,4-dinitrophenylhydrazones. Individual 2,4-dinitrophenylhydrazones

may give maxima which differ slightly from these average values. When dealing with unknown compounds the wavelength at which the readings should be made will also be unknown. In this case initial readings are made at 335 mμ; then one fraction from each "peak" is set aside to be measured at the other wavelengths (340, 355, and 370 mμ). The wavelength which gives the greatest absorbance places the unknown tentatively in one of the four classes, aldehyde, methyl ketone, enal, or dienal.

"Peak volume" is defined as that volume of mobile phase required to elute the

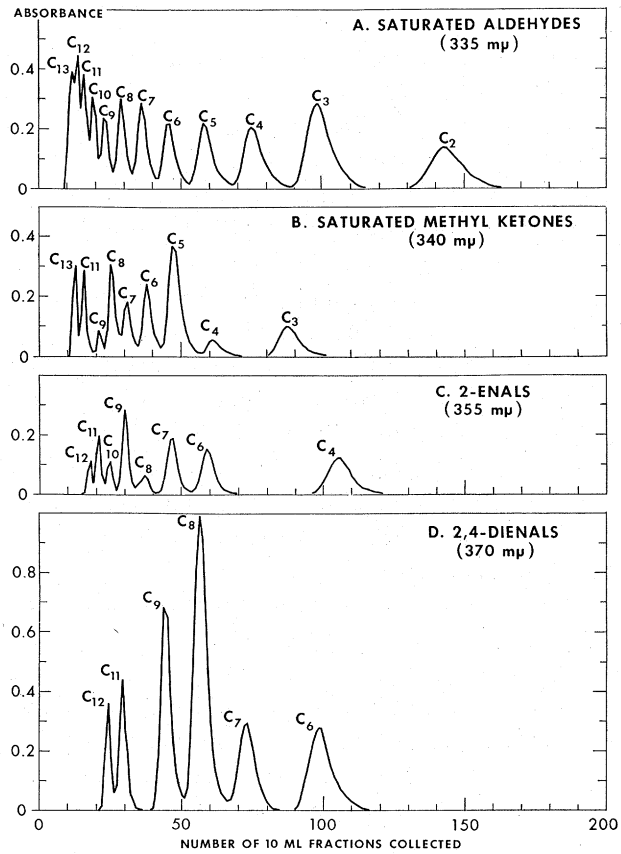

Fig. 1. Typical chromatograms using the acetonitrile column.

fraction of deepest color for each 2,4-dinitrophenylhydrazone. Peak volume is unaffected by the amount of material being chromatographed. Corrected peak volume is the peak volume minus the hold up volume, hold up volume being the amount of mobile phase held on the column. The hold up volume for a 25 g column (acetonitrile or chloroethanol) is 60 ml. This 60 ml value was obtained by weighing the chromatographic tube, the celite, and stationary phase needed to make a column and subtracting these weights from the total weight of the completed column. The completed column was weighed with no excess solvent present. The difference in weights was

converted into ml of mobile phase. This corrected peak volume is very similar to, but not identical with, the true retention volume described by KRAMER AND VAN DUIN[1]. These authors plotted the logarithms of their true retention volumes against the number of carbon atoms in the parent compound of two homologous series and obtained practically straight lines. A truly straight line would be taken to indicate an ideal liquid–liquid partition chromatographic system. Similar plots of our data, using corrected peak volumes, are shown in Figs. 3 and 4. All the lines are curved indicating

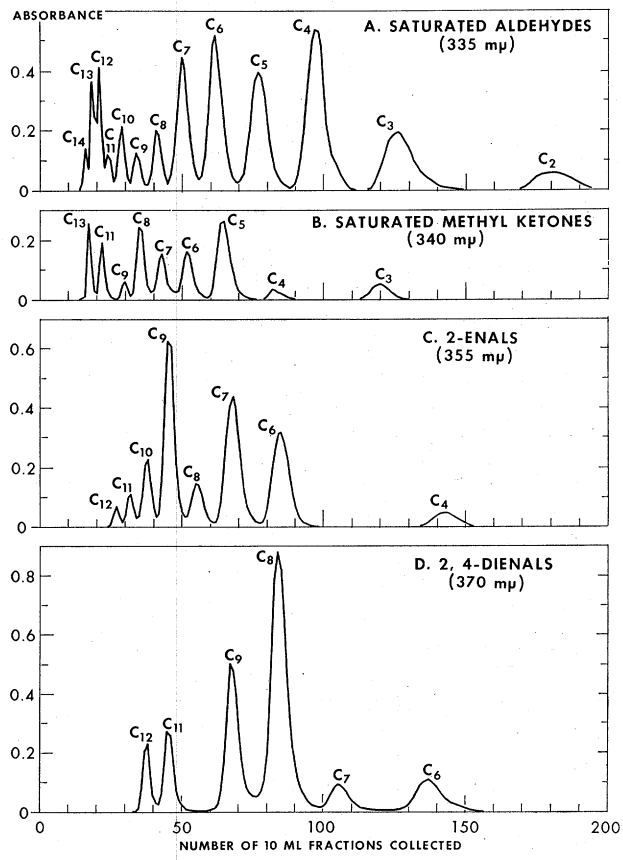

Fig. 2. Typical chromatograms using the 2-chloroethanol column.

non-ideality in the present partition systems. This deviation from ideal is attributed to the high moisture content of the columns, since some columns prepared without water gave plots which were not so curved.

A high moisture content is desirable in the acetonitrile column and a necessity in the chloroethanol column. In both columns the moisture present improves the resolving power, prevents cracking, channeling, or distortion of the top surface during preparation and manipulation of the column, and decreases the eluate flow to a more desirable rate. The eluate from a chloroethanol column prepared without water is

cloudy and impossible to measure in the spectrophotometer. The water (0.5 ml) added during column preparation usually alleviates this cloudiness. However, if a cloudy eluate is noticed, or suspected, a drop of absolute ethanol mixed into each fraction will quickly remove any cloudiness present.

Recovery data were obtained by measuring the optical density of a 2,4-dinitrophenylhydrazone solution, chromatographing 10 ml of this solution on a column, and measuring all color removable from the column. This was done with individual

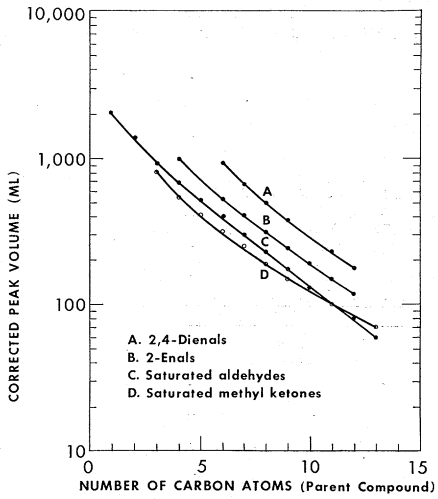

Fig. 3. Relationship of carbonyl chain length to peak volume, acetonitrile column.

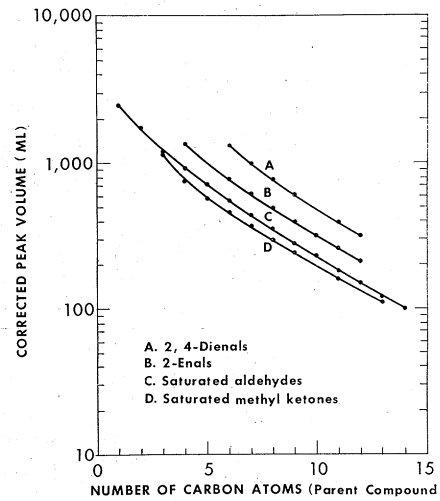

Fig. 4. Relationship of carbonyl chain length to peak volume, 2-chloroethanol column.

2,4-dinitrophenylhydrazones and with mixtures. The majority of recoveries ranged from 92 % to 102 %. In general, low recoveries (92–95 %) were obtained from the slower moving bands and higher recoveries (95–100 %) from the faster moving bands. The formaldehyde, acetaldehyde, and acetone derivatives gave the lowest recoveries (85–90 %).

The method of detecting the 2,4-dinitrophenylhydrazone bands as they move off the column is inherently sensitive. The equilibrated hexane used as the reference blank and the clear column eluate should have the same optical density; any increase in eluate optical density is taken to indicate the presence of 2,4-dinitrophenylhydrazone. The method can be defined as being sensitive to 1 μg of 2,4-dinitrophenylhydrazone per 10 ml fraction since this amount will increase the optical density 0.005 to 0.010 units. Adjacent homologs, up to the C_{10} derivative, can be qualitatively separated from each other when their concentrations differ by as much as 10 to 1. Adjacent C_{11} to C_{14} derivatives separate only when their relative concentrations approach equality. The columns are capable of handling milligram quantities of the 2,4-dinitrophenylhydrazones but best column performance is obtained by chromatographing 20 to 50 μg of the higher molecular weight derivatives (C_{14} to C_9) and from 50 to 100 μg of the lower molecular weight compounds (C_8 to C_1).

Some investigators have reported the decomposition of 2,4-dinitrophenylhydrazones on some chromatographic columns. FORSS AND DUNSTONE[4] found that appreciable decomposition of a number of 2,4-dinitrophenylhydrazones took place on silica gel columns when chromatographed for 24 hours or longer, and that the acetone derivative was affected on an alumina column. BASSETTE[3] has reported complete destruction of the methional (3-methylthiopropanal) derivative on a silica gel–nitromethane column. No decomposition of any compound has been noticed or suspected while using the acetonitrile or chloroethanol columns but it was deemed advisable to study the effect of the columns on methional 2,4-dinitrophenylhydrazone since it appears to be of an unstable nature. The methional derivative was chromatographed on each of the columns; visually the bands appeared unaffected; recovery from the acetonitrile column was 92 % and from the chloroethanol column 93 % The melting point of the recovered material was the same as that of the original material (123°). From these data it was concluded that no decomposition occurs on either column.

The use of air pressure to increase the eluate flow is needed to conserve time only when the spectrophotometer measurement takes less time than the collection of the next fraction. This intermittent and moderate use of pressure against the column does not affect the peak volume or the symmetry of the curves. Excessive or prolonged pressure, however, disrupts the equilibrium of the column causing some stationary phase to be washed off. If small globules of stationary phase are observed in the eluate it indicates excessive flow-rate and in this case peak volume values will be smaller and spectrophotometer readings will be erratic.

When dealing with unknown compounds or where quantitative data are desired each prepared column should be used for only one determination. Using a chloroethanol column more than once under any circumstance is not recommended. The acetonitrile column holds up well under repeated use but should be used more than once only when it is known to be clean of all previous compounds and when the identity of the 2,4-dinitrophenylhydrazones is known.

Of the two columns studied the acetonitrile column has been found to be the more reliable and the least liable to present difficulties of operation. It has slightly less resolving power than the chloroethanol column, and as a result it takes less time and less solvent to remove a particular compound. The chloroethanol column is the reverse, more resolving power but more time-consuming in operation.

Although the two columns are satisfactory for separating homologs of one series, it can be seen from Figs. 1 and 2 that the separation of a mixture of compounds from all four series, aldehydes, ketones, enals, and dienals, would probably not be possible.

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SUMMARY

Two similar partition chromatographic systems are described. Acetonitrile or 2-chloroethanol is used as the stationary phase on a celite column and hexane saturated with acetonitrile or 2-chloroethanol is the mobile phase. Both are suitable for the separation of the 2,4-dinitrophenylhydrazones of saturated aldehydes, saturated methyl ketones, 2-enals, and 2,4-dienals. Details of column preparation and operation are given. Graphs indicating the separations to be expected from each of the four homologous series are included.

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