A STUDY OF CHROMATOGRAPHIC METHODS

by

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INTRODUCTION

The experiments for this study were conducted in order to investigate the possibilities of satisfactorily separating mixtures of certain organic compounds by paper and thin-layer chromatographic methods.

Parts I, II and III were concerned with the separation of a mixture of amino acids. In Part I the mixture of amino acids was separated on narrow chromatographic paper strips, using the ascending method. Large sheets of chromatographic paper were used in Part II for the one-dimensional and the two-dimensional descending chromatographic methods. In the one-dimensional descending method the mixture was separated by the use of a single solvent system, whereas in the two-dimensional descending method two distinct solvent systems were employed, the second running at right angles to the first. The circular ascending chromatographic method used in Part III required the use of chromatographic paper rolled into cylinders.

Parts IV and V dealt with the separation of a mixture of derivatives of carbonyl compounds, the 2,4-dinitrophenylhydrazones. The ascending paper chromatographic method was used in Part IV, employing narrow chromatographic paper strips. In Part V the separation of the carbonyl derivatives was attempted by means of thin-layer chromatography, using a thin layer of a adsorbent material spread upon a glass plate.

PART I

SEPARATION OF AMINO ACIDS BY ASCENDING PAPER CHROMATOGRAPHY

Purpose

The purpose of this experiment was to determine the R_f values of several amino acids on chromatographic paper using various solvent systems at room temperature and to separate a mixture of these amino acids, thereby acquiring experience in the techniques of paper chromatography.

Principle

In paper chromatography, the mixture to be analyzed is distributed between two liquid phases: a stationary liquid phase, which is the water held by the cellulose fibers of the paper; and a mobile liquid phase, which is the solvent that is used.

The R_f value of the zone (the separated constituent of the mixture) is defined as the velocity of movement of the zone along the chromatographic paper divided by the velocity of movement of the mobile phase. In the separation of two or more substances by paper chromatography, the solutes distribute themselves between the two liquid phases according to their relative affinities for the stationary phase. The process is one of continuous adsorption and desorption, at different rates for the different solutes, and continues until the constituents of the sample become separated.

Review of Literature

Although a great amount of work has been done with the separation of amino acids by paper chromatography since the publication of the paper by Consden, Gordon and Martin (Biochem. J. 38, 224, 1944) which has become a classic in the field, improvements in techniques and apparatus are still being made, so that the volume of literature concerning this problem has continued to increase.

A complete review of the literature of this field is beyond the scope of this paper, but some references that may be cited are Block (Anal. Chem. 22, 1327, 1950), Crumpler and Dent (Nature 164, 441, 1949), Franklin and Quastel (Science 110, 446, 1949), Novellie (Nature 166, 1000, 1950) and Landua and Awapara (Science 109, 385, 1949). Recent reviews on chromatography include Strain (Anal. Chem. 33, 1733, 1961) and Wilkins (Anal. Chem. 33, 1844, 1961); a recent publication on the separation of amino acids is the work of Dunn and Murphy (Anal. Chem. 33, 997, 1961).

Apparatus

The chromatographic chamber was first set up using a $5\frac{1}{2}$ x $6\frac{1}{2}$ x $10\frac{1}{2}$ in. Corning glass jar. Two small evaporating dishes placed in the bottom of the jar held the solvent. The paper strips were hung from two glass rods which had cork stoppers fitted over the ends in order to hold the rods securely in place. Clothespins were used to clip the paper strips to the rods. Handi-Wrap (Dow Company) was fitted over the top of the jar in order to form an air-tight chamber. (Figure I, page 5)

This apparatus was found to be unsatisfactory because the Handi-Wrap did not keep the jar sufficiently air-tight. A glass frame for supporting the rods was constructed that would allow the rods to be held completely within the jar so that the jar could be covered with a glass plate. Glass rods were bent to such a shape that they would be held tightly in place against the walls of the jar. Indentations were made in the top of the frame so that the rods could rest in these notches and not roll freely. Paper clips were used instead of clothespins to clip the paper strips to the glass rods, since these were found to be more convenient. An 8 x 10 in. glass plate was placed over the top of the jar and sealed with Lubriseal so that the chamber would be air-tight. (Figure II, page 6)

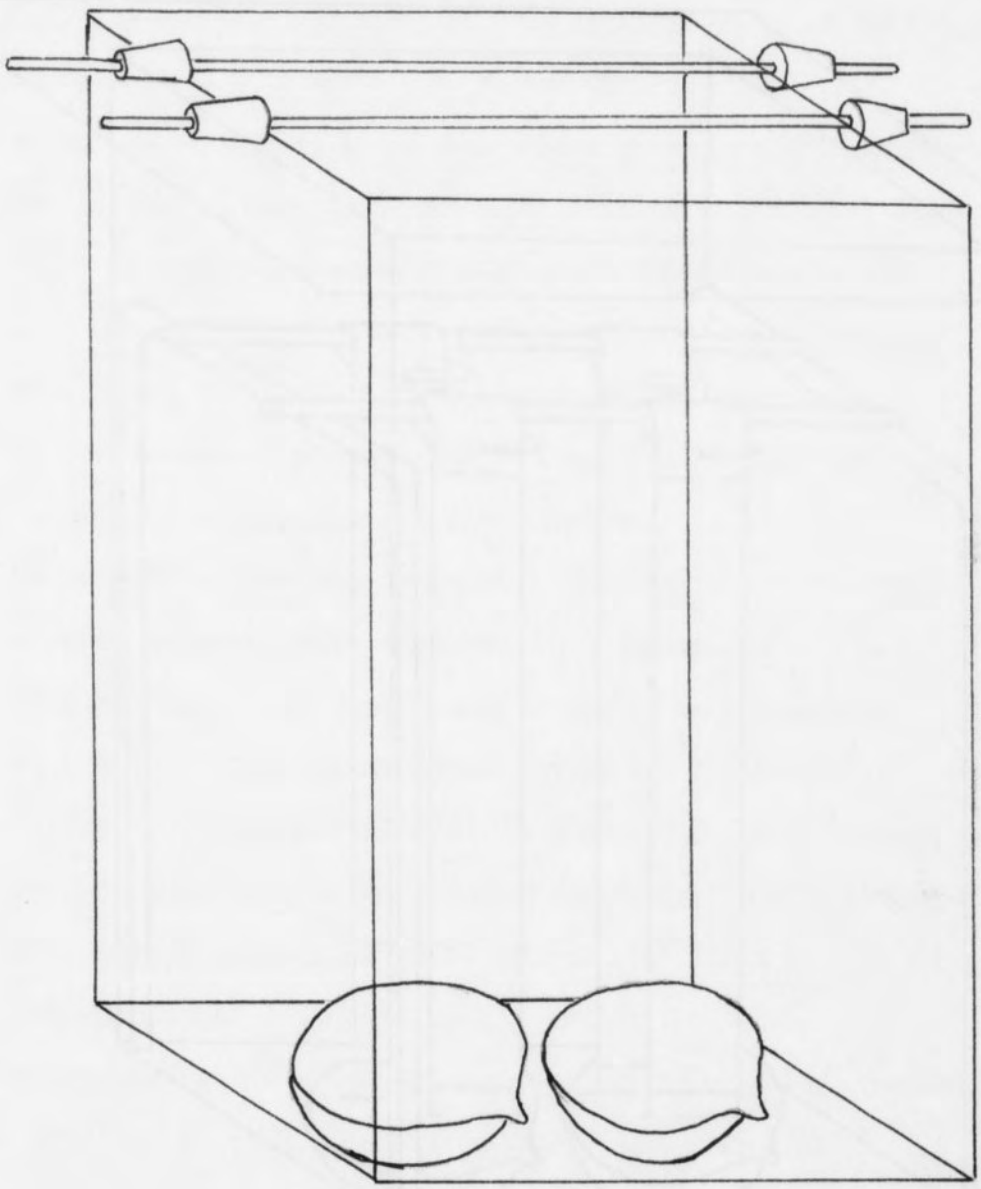

FIGURE I

Chromatographic Chamber

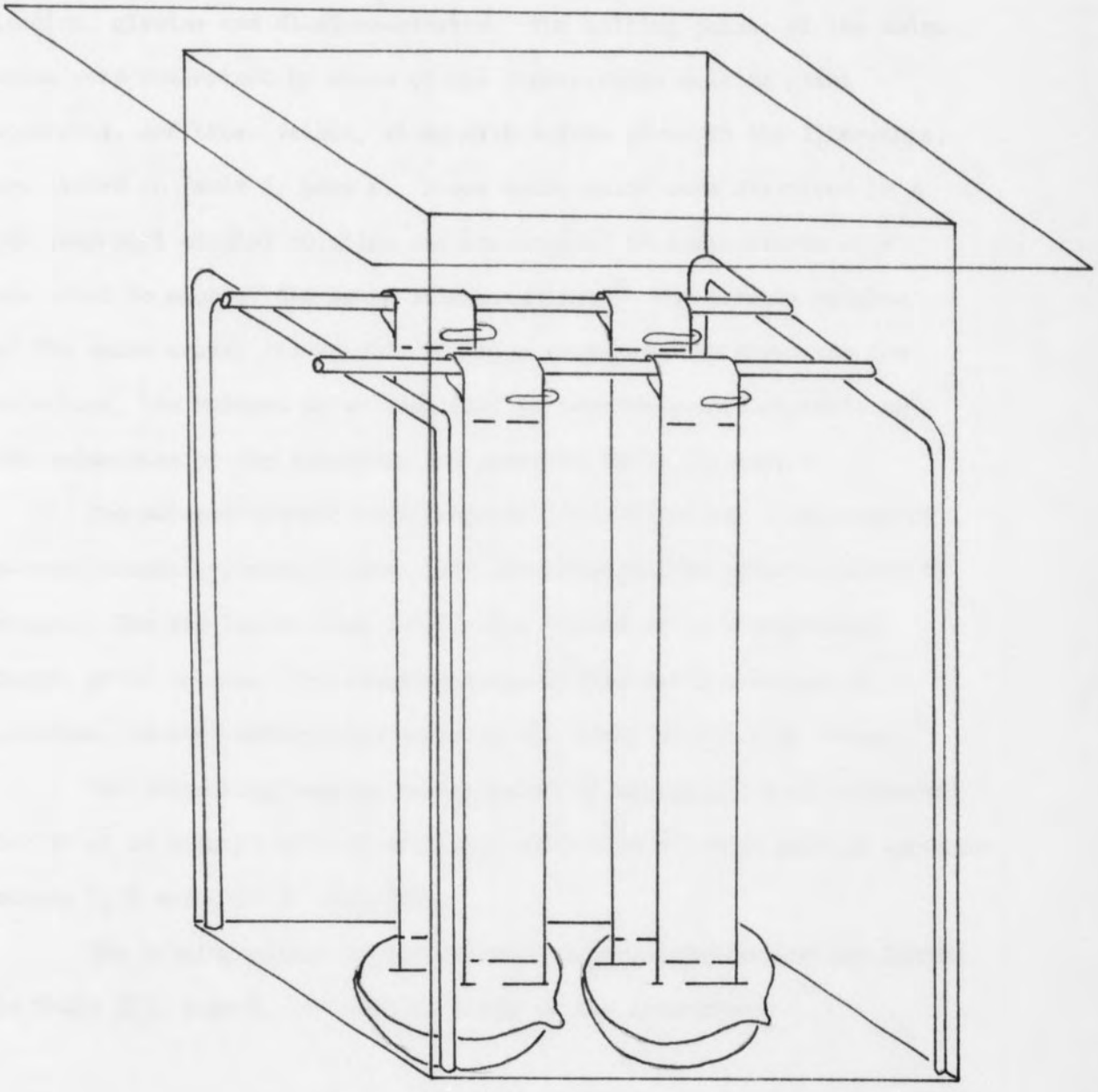

FIGURE II

Chromatographic Chamber

Preparation of Solutions

The amino acids used were tyrosine, phenylalanine, tryptophane, leucine, glycine and dl-alpha-alanine. The melting points of the amino acids were determined by means of the Fisher-Johns melting point apparatus, and these values, along with values given in the literature, are listed in Table I, page 8. These amino acids were dissolved in a 10% isopropyl alcohol solution and one drop of 6N hydrochloric acid was added to each of the amino acid solutions.[1] The formula weights of the amino acids, the weights of amino acids used in preparing the solutions, the volumes of solvent used in preparing the solutions and the molarities of the solutions are given in Table II, page 8.

Two solvent systems were prepared. The first was a solution of n-butyl alcohol, glacial acetic acid and water in the ratio of 4:1:5 by volume. The two layers that formed were separated in a separatory funnel prior to use. The second solvent system was a solution of pyridine, isoamyl alcohol and water in the ratio of 7:7:6 by volume.[2]

The indicating reagent was prepared by adding 1.5 g of ninhydrin to 120 ml of n-butyl alcohol saturated with water.[3] This gave an approximately 1.5% solution of ninhydrin.

The boiling points of the solvents were determined and are listed in Table III, page 9, with values given in the literature.

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[1] Block, LeStrange and Zweig, Paper Chromatography: A Laboratory Manual, Academic Press, Inc., New York: 1952, p. 45.

[2] Op. cit., p. 55.

[3] Op. cit., p. 59.

TABLE I

MELTING POINTS OF AMINO ACIDS

Amino Acid	Melting Point (Exp)(°C)	Melting Point (Acc)(°C)
Tyrosine	280	295 †
Phenylalanine	233-40	264 ‡
Tryptophane	261-2	275-82 ‡
Leucine	265	293-5 *
Glycine	233-5	232-6 *
dl-α-Alanine	250	295 †

* Lange's Handbook of Chemistry, Ninth Edition.

† Handbook of Chemistry and Physics, Thirty-eighth Edition.

‡ Cheronis and Entrikin, Semimicro Qualitative Organic Analysis, Second Edition, 1957, p. 546.

TABLE II

PREPARATION OF AMINO ACID SOLUTIONS

Amino Acid	Formula Weight*	Weight Used(g)	Vol. Solvent Used(ml)	Molarity
Tyrosine	181.2	0.2	10	0.1
Phenylalanine	165.2	0.4	20	0.1
Tryptophane	204.2	0.2	10	0.1
Leucine	131.2	0.3	20	0.1
Glycine	75.1	0.2	30	0.1
dl-α-Alanine	89.1	0.2	20	0.1

*Lange's Handbook of Chemistry, Ninth Edition.

TABLE III

BOILING POINTS OF SOLVENTS

Solvent	Boiling Point (Exp)(°C)	Boiling Point (Acc)(°C)*
Isopropyl alcohol	79-82	82.4
n-Butyl alcohol	111-115	118.0

*Lange's Handbook of Chemistry, Ninth Edition.

Procedure[4]

The n-butyl alcohol- acetic acid- water solution, after having been separated, was placed in the evaporating dishes and put in the chamber in order to allow the solvent to come to equilibrium with its vapor overnight.

Whatman's #4 chromatographic paper, 2.54 cm wide, was used. Each strip was cut 23 cm long. A light pencil line was marked 3 cm from either end of the strip, one line to be used as the line of origin and the other to be used as the boundary of the solvent front. The strips were allowed to become saturated with the solvent vapor in the chamber before use. One-tenth milliliter of the amino acid solution was placed on the line of origin, using a 1-ml pipet. The solution was applied a small amount at a time, allowing time for the spot to dry before each successive application, in order to prevent spreading. The final spots were no larger than 1 cm in diameter. The first letter of the name of

[4]Consden, Gordon and Martin, Biochem. J. 38, 224, 1944.

the amino acid was written in the upper margin for purposes of identification after developing and spraying. After the spots were dry the strips were clipped to the glass rods and placed in the chamber so that the bottom edge came into contact with the solvent in the evaporating dish. The chamber was then closed. The chromatograms were developed and then dried in the oven at 100°C. After drying they were sprayed with the ninhydrin reagent, replaced in the oven and dried again.

The positions of the zones were indicated by the colored spots that were formed when the ninhydrin reacted with the amino acids. The R_f values of the zones were calculated according to the formula

$$R_f = \frac{\text{distance moved by the zone}}{\text{distance moved by the solvent front}}.$$

The distance moved by the zone was measured from the line of origin to the center of the zone as indicated by the ninhydrin. The distance moved by the solvent front was the distance between the two marked lines.

Trials were run for each amino acid using both the n-butyl alcohol- acetic acid- water solution and the pyridine- isoamyl alcohol- water solution as the solvent systems. R_f values are given in Table IV, page 12.

A mixture of amino acids was prepared by adding 2 ml each of the solutions of tyrosine, phenylalanine, tryptophane, leucine, glycine and dl-alpha-alanine. The mixture was heated to 100°C in a beaker of boiling water. The procedure outlined above was followed, using 0.1 ml of the mixture. Since the solution of n-butyl alcohol, acetic acid and water had given the better separation of the two solvent systems, as evidenced by the range of the R_f values of the zones, it was used as the solvent system in the separation of the mixture.

After the chromatograms were developed, dried, sprayed with the ninhydrin reagent and dried again, the amino acids were identified by means of their relative R_f values, and further identification was made by means of the colors. From the line of origin to the top of the chromatogram the amino acids separated in the following order: glycine, dl-alpha-alanine, tyrosine, tryptophane, and phenylalanine and leucine. The last two were not appreciably separated and hence their R_f values could not be determined. Four trials were run. The determined R_f values are given in Table V, page 13.

TABLE IV

R_f VALUES OF AMINO ACIDS

Amino Acid	Solvent: n-Butyl alcohol- Acetic-acid- Water	Solvent: Pyridine- Isoamyl alcohol- Water
Tyrosine (pink-purple spot)	0.46 0.46	0.52 0.51 0.52
Phenylalanine (dark purple spot)	0.60 0.61	0.57 0.56
Tryptophane (purple-brown spot)	0.49 0.48	0.50 0.56
Leucine (dark purple spot)	0.61 0.62	0.52 0.54
Glycine (blue-violet spot)	0.19 0.20	0.20 0.18
dl-α-Alanine (dark red-violet spot)	0.26 0.26	0.26 0.26
Average Values		
Tyrosine	0.46	0.52
Phenylalanine	0.60	0.56
Tryptophane	0.48	0.53
Leucine	0.62	0.53
Glycine	0.20	0.19
dl-α-Alanine	0.26	0.26

TABLE V

R_f VALUES OF AMINO ACIDS IN THE SEPARATION OF A MIXTURE

Amino Acid	Trial 1	Trial 2	Trial 3	Trial 4
Glycine	0.19	0.20	0.19	0.19
dl-α-Alanine	0.29	0.30	0.29	0.28
Tyrosine	0.44	0.44	0.44	0.43
Tryptophane	0.52	0.53	0.52	0.51
Phenylalanine	---------- indeterminable ----------			
Leucine	---------- indeterminable ----------			

Discussion

In most of the trials the zones indicated by the ninhydrin reagent had not spread excessively from the original size; they were on the average 1-2 cm in diameter. This would indicate that the solvent system carried the solute up the paper uniformly. The color of the spots, vivid shades of purple, facilitated the determination of the final position of the zones and hence the determination of the R_f values.

Tailing was evident in several of the trials, especially in the case of the tyrosine solution. The cause for the tailing is unknown.

The length of time required for developing the chromatograms was different for the two solvent systems. For the n-butyl alcohol- acetic acid- water solution, the average length of time required was 3 hours; for the pyridine- isoamyl alcohol- water solution, the time required was 2 hours. The range of R_f values obtained by using the n-butyl alcohol-

acetic acid- water solution was greater than the range obtained with the pyridine- isoamyl alcohol- water solution; in the former case the range was 0.42; in the latter, 0.37. This indicated that the degree of separation increases as the length of time of developing increases.

Better separation of the mixture of amino acids might have been achieved if a longer strip of chromatographic paper had been used. This would have allowed the solvent to flow over a greater distance and would also have lengthened the time required for developing. However, limitations of the apparatus prevented the use of a longer strip.

Throughout the experiments efforts were made to keep the conditions as constant as possible. The chromatographic system was allowed to come to equilibrium before trials were run, including leaving the paper strips in the chamber overnight to permit them to become saturated with the solvent vapor before use.

Better agreement between the determined R_f values of each amino acid would probably have been obtained if the length of time that the paper strips were left in the chamber to come to equilibrium with the solvent vapor before use had been kept constant for each trial. This factor may also have been the cause of the slight variation in the length of time required for developing the chromatograms.

Room temperature varied less than 1° during the developing of the chromatograms, remaining relatively constant at $24^{\circ}C$.

Conclusion

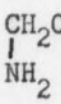
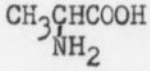
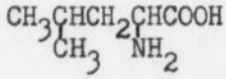
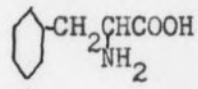
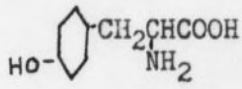
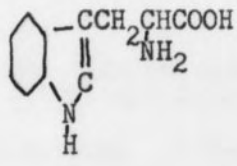
The R_f values of the amino acids, and hence the degree of separation of amino acids from a mixture, are dependent upon (1) the condition of equilibrium of the system, (2) the solvent system used and (3) the length

of time that the solvent flows, which is dependent upon the nature of the solvent.

An attempt was made to relate the R_f values with the molecular weights of the amino acids, to determine if they were proportional to each other. Evidence for this relationship was not found (Figure III, page 17). Nor does it appear from a study of structural formulas that the R_f value increases with increasing complexity of the structure of the molecule, as shown in Table VI, page 16. However, it may be noted from Table VI that in the case of the four amino acids that separated from the mixture, namely glycine, dl-alpha-alanine, tyrosine and tryptophane, the relationship between increasing complexity of molecular structure and increasing R_f value seems probable. The exceptions are phenylalanine and leucine, which did not separate. Further experimentation may provide more evidence for either establishing or rejecting this relationship.

TABLE VI

RELATIONSHIP BETWEEN R_f VALUES AND MOLECULAR STRUCTURE

Amino Acid	R_f Value	Structural Formula
Glycine	0.20	
dl-α-Alanine	0.26	
Leucine	0.62	
Phenylalanine	0.62	
Tyrosine	0.46	
Tryptophane	0.48	

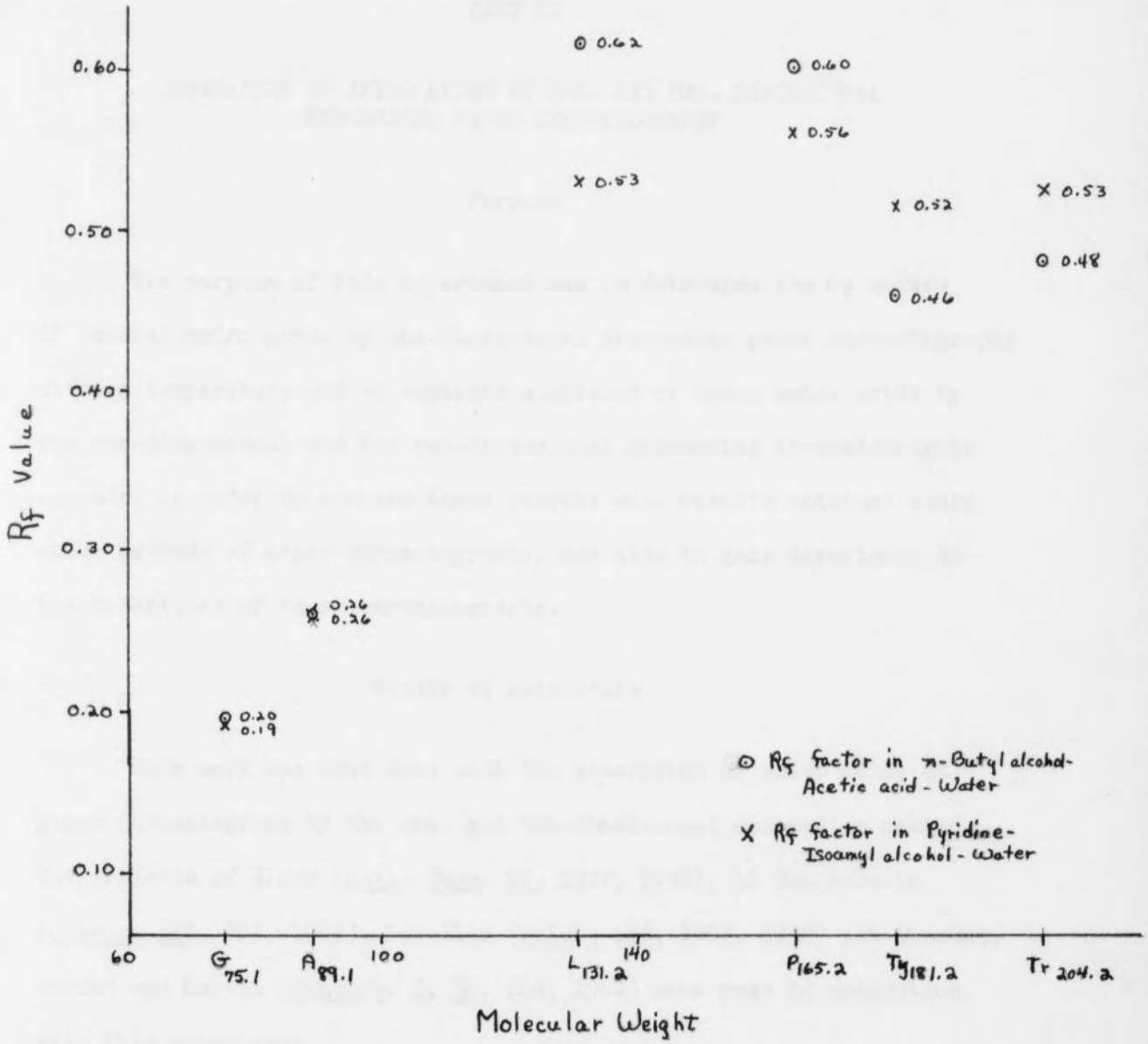


FIGURE III

R_f Values versus Molecular Weight

Key:

G - Glycine
A - dl-alpha-Alanine
L - Leucine
P - Phenylalanine
Ty - Tyrosine
Tr - Tryptophane

PART II

SEPARATION OF AMINO ACIDS BY ONE- AND TWO- DIMENSIONAL DESCENDING PAPER CHROMATOGRAPHY

Purpose

The purpose of this experiment was to determine the R_f values of several amino acids by one-dimensional descending paper chromatography at room temperature and to separate a mixture of these amino acids by the one-dimensional and the two-dimensional descending chromatographic methods, in order to compare these results with results obtained using other methods of paper chromatography, and also to gain experience in the techniques of paper chromatography.

Review of Literature

Much work has been done with the separation of amino acids on paper chromatograms by the one- and two-dimensional descending methods. The articles of Block (Anal. Chem. 22, 1327, 1950), Li and Roberts (Science 110, 425, 1949), Novellie (Nature 166, 1000, 1950) and Consden, Gordon and Martin (Biochem. J. 38, 224, 1944) were read in connection with this experiment.

Apparatus

The Chromatocab (Research Specialties Company) that was used consisted of a glass solvent trough cradled on glass supports near the top of the cabinet. (The cabinet itself could accommodate four such troughs.)

Glass antisiphon rods were placed above each edge of the glass trough to hold the paper away from the trough and thus prevent siphoning of the solvent. An anchor rod was used to hold the paper in place. One trough could accommodate two large sheets of chromatographic paper, one hanging from each side. (Figure IV-a, page 20)

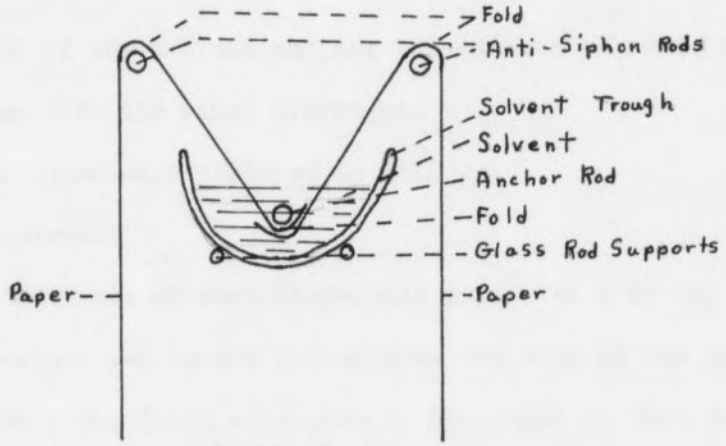


Figure IVa
Solvent Trough
(side view)

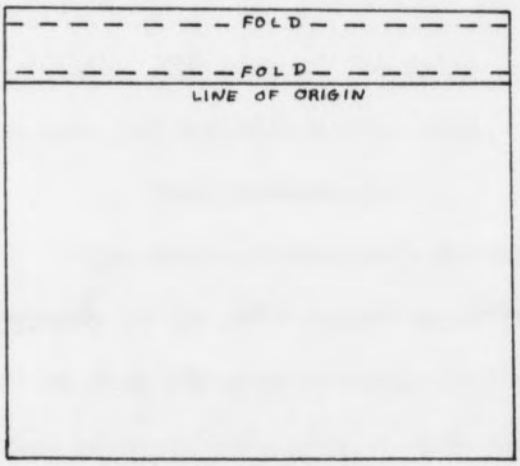


Figure IVb
One-Dimensional Layout

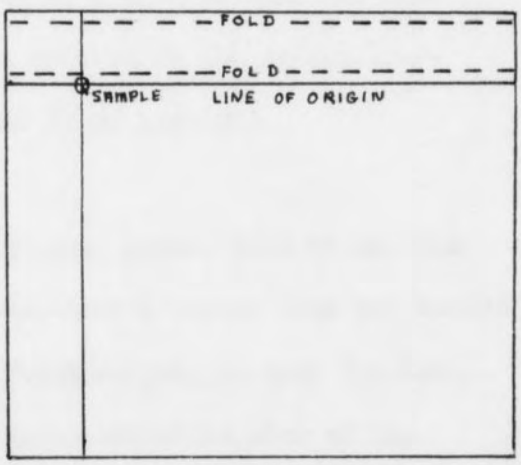


Figure IVc
Two-Dimensional Layout

FIGURE IV

Apparatus for Descending Paper Chromatography
(From Manufacturer's Manual)

Procedure

The amino acid solutions prepared in Part I were used.

The n-butyl alcohol- acetic acid- water solution was placed in a beaker on the floor of the cabinet and the solvent was allowed to establish equilibrium with its vapor overnight.

The sheets of paper were prepared as follows:

One-dimensional:

One sheet of Whatman's #1 chromatographic paper, 46 x 64 cm, was used. The line of origin was marked 7.6 cm from the edge of the paper along the longer side. Two folds were made in the paper so that it would hang from the trough vertically: the first fold was made 1.3 cm from the edge and folded back, and the other fold was made forwards 6.4 cm from the edge. Two 0.1 ml- samples of each of the amino acid solutions and of the mixture of amino acids were spotted long the line of origin. The name of the amino acid was written in the margin above the spot for identification later. (Figure IV-b, page 20)

Two-dimensional:

One sheet of Whatman's #1 chromatographic paper, 46 x 64 cm, was prepared in the same manner as above, except that a second line was marked 7.6 cm from the edge adjacent to the one first marked, so that the two lines were perpendicular to each other. One-tenth milliliter of the mixture of amino acids was placed at the point of intersection of the two lines. (Figure IV-c, page 20)

Both the one- and the two-dimensional sheets were then hung in the Chromatocab, one hanging from each side of the trough, and anchored with the heavy glass rod. The n-butyl alcohol- acetic acid- water solvent was poured into the trough, and the cabinet was closed again.

The chromatograms were allowed to develop for 14 hours. They were then removed from the cabinet, the solvent fronts were marked and the chromatograms were hung up to dry.

When the two-dimensional sheet had dried it was folded along the second side, in the same manner as before. The cabinet in the meantime had been allowed to come to equilibrium with respect to the pyridine-isoamyl alcohol- water solvent. The chromatogram was replaced in the cabinet, the second solvent was placed in the trough and the chromatogram was allowed to develop for 15 hours. The sheet was removed from the cabinet, the solvent front was marked and the chromatogram was dried.

Both the one- and the two-dimensional chromatograms were sprayed with the ninhydrin reagent and were placed in the oven to dry at 100°C. R_f values were determined; these are listed for the one-dimensional chromatogram in Table VII, page 23, and for the two-dimensional chromatogram in Table VIII, page 23.

TABLE VII

R_f VALUES OF AMINO ACIDS BY ONE-DIMENSIONAL DESCENDING METHOD

Amino Acid	R_f Value	
	Individual Trial	Mixture
Glycine	0.08	0.08
	0.09	0.09
dl-α-Alanine	0.19	0.18
	0.19	0.18
Tyrosine	0.25	0.24
	0.25	0.24
Tryptophane	0.35	0.35
	0.36	0.34
Phenylalanine	0.48	0.47
	0.49	0.47
Leucine	0.56	0.59
	0.57	0.59

TABLE VIII

R_f VALUES OF AMINO ACIDS BY TWO-DIMENSIONAL DESCENDING METHOD

Amino Acid	R_f Value	
	n-Butyl alcohol-Acetic acid-- Water	Pyridine- Isoamyl alcohol- Water
Glycine	0.12	0.02
dl-α-Alanine	0.22	0.03
Tyrosine	0.28	0.08
Tryptophane	0.35	0.13
Phenylalanine	0.50	0.14
Leucine	0.62	0.22

Discussion

In both the one-dimensional and the two-dimensional chromatograms it was noted that the zones had spread considerably. The cause for this is unknown.

Tailing was evident in the case of the tyrosine solution and also, but to a lesser degree, in the case of the tryptophane solution.

In the separation of the mixture of amino acids by both the one- and the two-dimensional methods, good separation was obtained. This was especially noted in the cases of phenylalanine and leucine, which had not separated on the shorter paper strips.

There was close agreement in the R_f values of the amino acids on the one-dimensional chromatogram among the individual trials and the separation of the mixture. This close agreement was probably due to the fact that all the trials were run simultaneously and under the same conditions, whereas a slight variance in the conditions would have caused the differences that were evident in the R_f values determined when the paper strips were used.

The average rate of advance of the solvent front in the descending method was 0.30 mm/min; for the ascending paper strips, it was 0.95 mm/min. The difference may have been due to a variance in the porosity and in the length of the paper. The slower rate of advance of the solvent front gave the better separation.

Conclusion

In Part I it was concluded that the R_f values and hence the degree of separation of amino acids from a mixture are dependent upon (1) the condition of equilibrium of the system, (2) the solvent system used and

(3) the length of time that the solvent flows, which is dependent upon the nature of the solvent. To these conclusions it may be added that the degree of separation of amino acids from a mixture is also dependent upon (4) the length of the paper used and hence the distance that the solvent front moves and (5) the rate at which the solvent front advances.

The one- and two- dimensional descending paper chromatographic methods using the large sheets are more satisfactory than the paper strip method of Part I because, although more time is required for developing the chromatogram, the greater distance through which the solvent front flows makes possible better separation of the amino acids.

PART III

SEPARATION OF AMINO ACIDS BY ASCENDING CIRCULAR PAPER CHROMATOGRAPHY

Purpose

The purpose of this experiment was to determine the R_f values of several amino acids by the method of ascending circular paper chromatography at room temperature and to separate a mixture of these amino acids by this method, in order to compare these results with those obtained by using other methods of paper chromatography, and to gain further experience in the techniques of paper chromatography.

Review of Literature

Although this method is not used as extensively as one- and two-dimensional descending paper chromatography for the separation of amino acids, it was successfully employed by Williams and Kirby (Science 107, 481, 1948).

Apparatus

The Chromatocab was used. Four 2-liter beakers were placed on the floor of the cabinet to hold the rolled sheets of paper. This experiment was run simultaneously with that of Part II.

Procedure

The solutions of amino acids (glycine, dl-alpha-alanine, tyrosine, tryptophane, phenylalanine and leucine) and the n-butyl alcohol- acetic

acid- water solvent prepared in Part I were used in this experiment.

The solvent was poured into each of the beakers to a height of about 2 cm and the system was allowed to establish equilibrium overnight.

Four sheets of Whatman's #1 chromatographic paper, 12.5 x 51 cm, were used. The line of origin was marked 4 cm from the bottom of the paper along the short side. Two 0.1 ml-samples of each of the amino acid solutions and three 0.1 ml-samples of the mixture of amino acids were spotted along the line of origin on the four papers. The sheets were then rolled into cylinders and clasped with paper clips. The cylinders were stood upright in the 2-liter beakers containing the solvent in the Chromatocab. (Figure V, page 28)

The chromatograms were allowed to develop for 14 hours. They were then removed from the cabinet, the solvent fronts were marked and the papers were hung up to dry. After drying, the chromatograms were sprayed with ninhydrin and dried in the oven at 100°C. R_f values were calculated and are listed in Table IX, page 29.

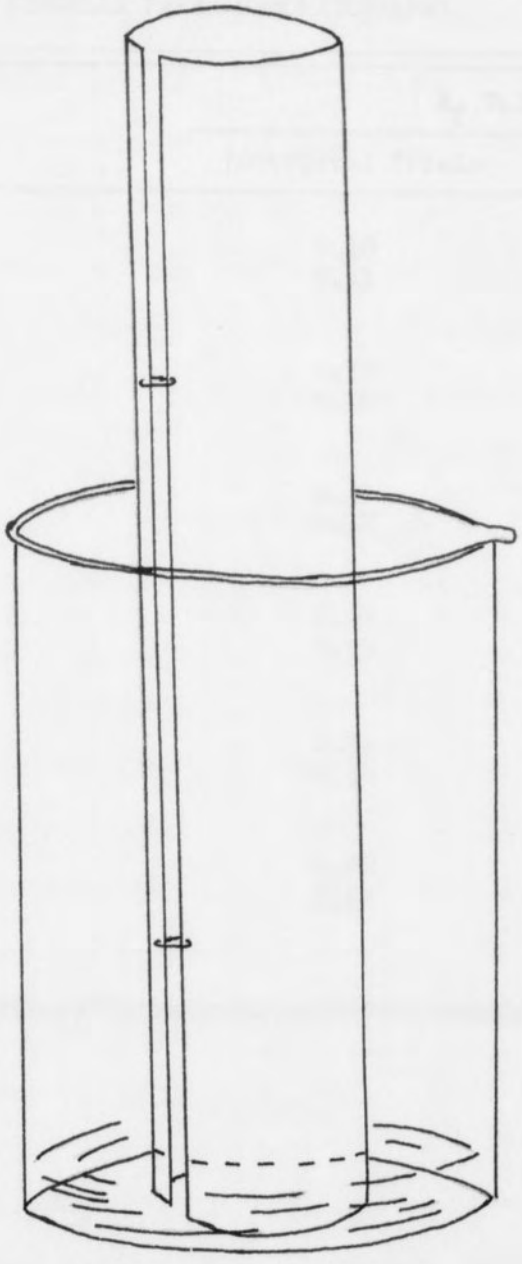


FIGURE V

Apparatus for Ascending Circular Paper Chromatography

TABLE IX

R_f VALUES OF AMINO ACIDS BY ASCENDING CIRCULAR PAPER CHROMATOGRAPHY

Amino Acid	R_f Values	
	Individual Trials	Mixture
Glycine	0.10	0.09
	0.11	0.10
		0.09
dl-α-Alanine	0.19	0.18
	0.19	0.19
		0.19
Tyrosine	0.18	0.22
	0.18	0.22
		0.22
Tryptophane	0.24	0.25
	0.23	0.27
		0.26
Phenylalanine	0.51	0.45
	0.53	0.45
		0.44
Leucine	0.62	0.56
	0.62	0.56
		0.56

Discussion

The R_f values obtained by the separation of the mixture of the amino acids agreed fairly closely with those obtained by the analysis of the individual solutions. This was due to the fact that all trials were run simultaneously and under the same conditions.

There was considerable spreading of the spots in the individual trials, but this effect was less evident in the separation of the mixture. Tailing was noted in the trials with the tyrosine solution, although this effect was not noted in the separation of the mixture.

The method of ascending circular chromatography gave better separation of the mixture than did the paper strips used in Part I, due to the greater distance through which the solvent front was permitted to flow.

The average rate of advance of the solvent front in ascending circular chromatography was 0.36 mm/min, as compared with 0.30 mm/min for the descending chromatography and 0.95 mm/min for the ascending paper strips. The slower rate of advance of the solvent front gave the better separation of the mixture.

Room temperature at the beginning of the experiment was 28°C.

Conclusion

Although the descending chromatograms on the large sheets and the ascending circular chromatograms on the long cylinders took longer to develop, these methods are preferable to the ascending paper strip method because the longer distance through which the solvent front moves affords better separation of the components of the mixture of amino acids.

PART IV

SEPARATION OF CARBONYL COMPOUNDS BY ASCENDING PAPER CHROMATOGRAPHY

Purpose

The purpose of this experiment was to determine the R_f values of several aldehydes and ketones on chromatographic paper at room temperature and to separate a mixture of these carbonyl compounds, thus gaining experience in the techniques of paper chromatography. The results obtained by this method were to be compared with the results obtained using the method of thin-layer chromatography.

Review of Literature

The separation of carbonyl compounds has not been treated extensively in the literature. Research done by Rice, Keller and Kirchner (Anal. Chem. 23, 194, 1951) in the separation of 2,4-dinitrophenylhydrazones of aldehydes and ketones was referred to in this experiment; other references include Blom and Caris (Nature 184, Suppl. No. 17, 1313, 1959) and Schepartz (J. Chromatog. 6, 185, 1961).

Apparatus

The chamber described in Part I was used for the separation of the carbonyl compounds. (Figure II, page 6)

Purification of Compounds

The purity of the carbonyl compounds used in this experiment was tested by determining the boiling points or the melting points of the compounds; these values and the values given in the literature are listed in Table X, page 33.

As the liquid carbonyl compounds were found to be impure, evidenced by the ranges of their boiling points, these compounds were purified by distillation. The boiling points of the purified compounds are given in Table XI, page 33.

Preparation of Solutions

Two solutions were prepared for use as solvent systems: (1) a 5% diethyl ether in petroleum ether solution (boiling point 40-50°C) and (2) a 30% aqueous acetone solution containing 1 ml of petroleum ether per 50 ml of solution (boiling point 37-100°C).[5]

A solution of 2,4-dinitrophenylhydrazine was prepared for use as an indicator by saturating 70 ml of 2N hydrochloric acid with 2,4-dinitrophenylhydrazine.[6] The solution was allowed to stand overnight and the excess solute was removed by filtration the next day.

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[5]Rice, Keller and Kirchner, Anal. Chem. 23, 194, 1951.

[6]Cheronis and Entrikin, Semimicro Qualitative Organic Analysis, Interscience Publishers, Inc., New York; Second Edition, 1957, p. 399.

TABLE X

BOILING POINTS OF CARBONYL COMPOUNDS

Compound	Boiling Point (°C) (Exp)	(Acc)*
n-Butyraldehyde	73-120	75.7
p-Tolualdehyde	198-203	204-5
Crotonaldehyde	97-102	102.2
p-Anisaldehyde	242-248	247-8
trans-Cinnamaldehyde	246-251	252
Cyclopentanone	125-131	130.7
Acetophenone	200-203	202.0
Benzophenone	m.p. 44	m.p. 45.8

*Lange's Handbook of Chemistry, Ninth Edition.

TABLE XI

BOILING POINTS OF PURIFIED CARBONYL COMPOUNDS

Compound	Boiling Point (°C)
n-Butyraldehyde	74-76
p-Tolualdehyde	201.5-203.0
Crotonaldehyde	98.0-100.0
p-Anisaldehyde	247.0-249.0
trans-Cinnamaldehyde	248.0-250.0
Cyclopentanone	129.0-131.0
Acetophenone	201.0-202.0

Procedure

The diethyl ether- petroleum ether solvent was placed in the evaporating dishes and put into the chromatographic chamber. The chamber was allowed to stand overnight to permit the solvent to establish equilibrium with its vapor.

The procedure was essentially the same as that outlined in Part I. Whatman's #1 chromatographic paper, 2.54 cm wide, was cut into strips 23 cm long and placed in the chamber to become saturated with the solvent vapor. A sample of 10 µl of the compound was placed on the line of origin 2 cm from the bottom edge of the paper, using a Lang-Levy Lambda-Pette capillary pipet (Research Specialties Company). Several trials were run using the liquid carbonyl compounds. Various reagents were tried as indicators, including Fehling's solution, potassium permanganate and Seliwanoff's reagent, but notone of them was successful. The solution of 2,4-dinitrophenylhydrazine indicated the positions of the zones, but it was noted that the zones had moved only slightly, if at all, away from the line of origin.

The failure of the zones to move was thought possibly due to the solvent, which was extremely volatile. The first solvent was replaced by the aqueous acetone- petroleum ether solution, and the chamber was allowed to come to equilibrium. Trials were run with the same carbonyl compounds, but again the indicator failed to show the positions of the zones.

Because of the difficulties involved in the chromatographic separation of the pure aldehydes and ketones, the 2,4-dinitrophenylhydrazones (DNP's) of these compounds were prepared in order to determine their R_f values. The melting points of the DNP's are given in Table XII, page 35, along with the values given in the literature.

TABLE XII

MELTING POINTS OF DNP's OF
CARBONYL COMPOUNDS

Compound	Melting Point (°C) (Exp)	(Acc)*
n-Butyraldehyde DNP	138	123
p-Tolualdehyde DNP	244-245	234
Crotonaldehyde DNP	190-191	190
p-Anisaldehyde DNP	254	253-254
trans-Cinnamaldehyde DNP	254-255	255
Cyclopentanone DNP	144-146	146
Acetphenone DNP	250	238-240 (250)†
Benzophenone DNP	193-194	238-239
Formaldehyde DNP	166-169	166
Acetaldehyde DNP	142-150	148 & 168‡
n-Propionaldehyde DNP	144-151	155

*Cheronis and Entrikin, Semimicro Qualitative Organic Analysis, Second Edition, 1957; pp. 582-584, 663-666.

†Sublimation of some crystals occurred at 240°; others melted at 250°.

‡Acetaldehyde DNP exists as a stable isomer that melts at 168.5° and as an unstable isomer that melts at 157°. A mixture of these two isomers melts at about 150°. (Cheronis, ibid., p. 586.)

Solutions were made by dissolving the DNP's in chloroform. The calculated formula weights of the DNP's, the weights of DNP's used in preparing the solutions, the volumes of solvent used and the molarities are given in Table XIII, page 37.

Samples of the prepared solutions, and dilutions of the ratios 1:1, 1:2, 1:3 and 1:4, were tested on the paper strips. After the chromatograms had developed they were placed in the oven to dry at 100°C. They were sprayed with potassium hydroxide solution, the positions of the zones were marked and the chromatograms were dried again. R_f values were calculated and are given in Table XIV, page 38.

One milliliter of each of the solutions of the DNP's of formaldehyde, acetaldehyde, n-propionaldehyde and n-butyraldehyde were placed in a test tube and 12 ml of chloroform were added. Four trials were run using 10 µl of the mixture in each trial. R_f values were calculated and are given in Table XV, page 39.

TABLE XIII

PREPARATION OF SOLUTIONS OF DNP'S

Compound	Formula Weight	Weight Used (mg)	Vol. Solvent Used (ml)	Molarity
n-Butyraldehyde DNP	252.2	83	20	0.016
p-Tolualdehyde DNP	316.3	75	20	0.012
Crotonaldehyde DNP	264.2	151	20	0.030
p-Anisaldehyde DNP	300.3	65	20	0.010
trans-Cinnamaldehyde DNP	300.3	75	20	0.013
Cyclopentanone DNP	250.2	168	20	0.032
Acetophenone DNP	362.3	82	20	0.014
Formaldehyde DNP	210.2	73	20	0.017
Acetaldehyde DNP	224.2	75	20	0.017
n-Propionaldehyde DNP	238.2	73	20	0.015

TABLE XIV

R_f VALUES OF DNP'S

Compound	Molarity	R_f Value
Formaldehyde DNP	0.017	0.59
	0.008	0.59
		0.72
		0.72
	0.006	0.58
		0.74
		0.73
	0.004	0.57
		0.72
		0.74
	0.003	0.74
		0.76
Acetaldehyde DNP	0.017	0.63
	0.008	0.61
		0.68
		0.69
	0.006	0.61
		0.70
		0.68
	0.004	0.56
		0.79
		0.76
	0.003	0.62
		0.75
		0.75
n-Propionaldehyde DNP	0.015	0.56
	0.008	0.70
		0.71
	0.005	0.49
		0.76
		0.71
	0.004	0.46
		0.70
		0.72
	0.003	0.71
		0.71
n-Butyraldehyde DNP	0.016	0.75
	0.008	0.86
		0.83
		0.82
	0.005	0.80
		0.84
		0.84
	0.004	0.84
		0.80
		0.79
	0.003	0.80
		0.78
		0.75

TABLE XV

R_f VALUES OF A MIXTURE OF THE DNP'S OF FORMALDEHYDE, ACETALDEHYDE, n-PROPIONALDEHYDE AND n-BUTYRALDEHYDE

Trial	R_f Values
Trial 1	0.60
Trial 2	0.57
Trial 3	0.56
Trial 4	0.56

Discussion

There was no indication in any of the trials of movement of the DNP's of p-tolualdehyde, crotonaldehyde, p-anisaldehyde, trans-cinnamaldehyde, cyclopentanone or acetophenone. On the other hand, there was movement of the DNP'S of formaldehyde, acetaldehyde, n-propionaldehyde and n-butyraldehyde. The cause of the failure of the former group to move is unknown, but it might be due to a too-highly concentrated solution.

The brown color that formed when the potassium hydroxide reacted with the DNP's made detection of the positions of the zones easy and hence facilitated the determination of the R_f values.

The spots showing the positions of the zones of the n-butyraldehyde DNP were well defined, indicating that the solvent carried the zone up the strip uniformly. However, there was some tailing more pronounced in the trials with the more concentrated solutions. In the trials with the DNP's of formaldehyde, acetaldehyde and n-propionaldehyde, there were no well-defined spots; instead there was considerable tailing in all these cases.

Several dilutions of each of the DNP solutions were tested to determine which concentration would give the best results. The 1:2 and 1:3 dilutions seemed to be the most satisfactory; this was most evident in the case of the n-butyraldehyde DNP. For this solution, there was more tailing in the 1:1 dilution, and the final zone in the trial with the 1:4 dilution was barely detectable.

There was no conclusive evidence for the dependence of the R_f value on the molarity of the solution. In some cases, namely those of formaldehyde DNP and acetaldehyde DNP, the R_f value increased with increased dilution; while in other cases, namely n-propionaldehyde DNP and n-butyraldehyde DNP, the R_f value decreased with increased dilution.

There was not much difference among the R_f values of the DNP's of formaldehyde, acetaldehyde and n-propionaldehyde, which were 0.67, 0.67 and 0.65, respectively. The n-butyraldehyde DNP had an R_f value of 0.80.

The average length of time required for developing the chromatograms was 1 hour 25 minutes.

The temperature remained relatively constant at 24°C for all the trials except those run on the last day of the experiment, when the temperature rose to 29°C.

Efforts were made during the experiment to keep the equilibrium conditions constant. The chromatographic system was allowed to establish equilibrium before each trial was run. The paper strips that had been prepared for use were kept in a second chamber containing the solvent in equilibrium with its vapor, so that the strips could become saturated with the solvent vapor. The strips were left in the chamber overnight before use.

The separation of the mixture of the DNP's of formaldehyde, acetaldehyde, n-propionaldehyde and n-butyraldehyde was not successful; only one zone was indicated when the strips were sprayed with potassium hydroxide. Other dilutions or other solvent systems would have to be tried in order to find a combination of factors that would separate the mixture successfully.

Conclusion.

The R_f values were not sufficiently different to permit the separation of the mixture under the conditions of this experiment.

PART V

SEPARATION OF CARBONYL DERIVATIVES BY THIN-LAYER CHROMATOGRAPHY

Purpose

The purpose of this experiment was to determine the R_f values of the 2,4-dinitrophenylhydrazones (DNP's) of several aldehydes and ketones by thin-layer chromatography at room temperature and to separate a mixture of these compounds, thereby acquiring experience in the techniques of thin-layer chromatography. Results obtained by this method were to be compared with results obtained by the method of paper chromatography.

Principle

In thin-layer chromatography the mixture to be analyzed is distributed between a solid adsorbent phase and a liquid mobile phase. The components of the mixture are distributed between the two phases according to their relative affinities for the adsorbent; the more strongly adsorbed solute proceeds less rapidly than those which are adsorbed to a lesser degree. The process is one of continuous adsorption and desorption, at different rates for the different solutes, and continues until the constituents of the mixture become separated.

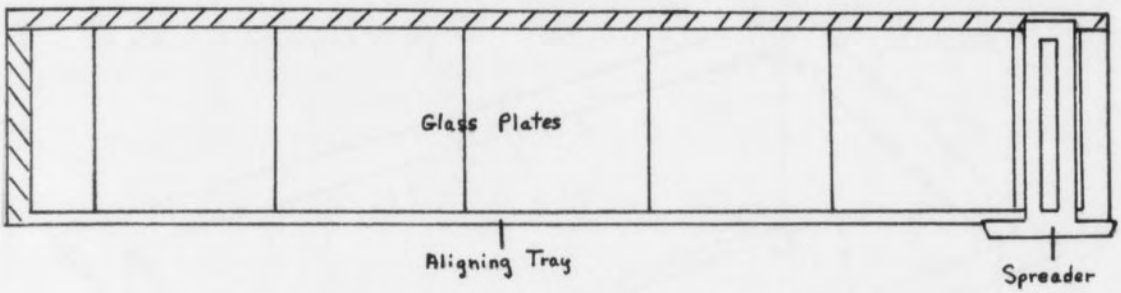
In this experiment the adsorbent phase was the thin layer of silica gel held on the glass plate; the mobile phase was the solvent, a solution of acetone, water and petroleum ether.

Review of Literature

Thin-layer chromatography has recently come into use, therefore, limited references are available concerning the separation of carbonyl DNP derivatives by this method. The publications consulted were Rosmus and Deyl (J. Chromatog 6, 187, 1961), DeMole (J. Chromatog 1, 24, 1958) and Wolfrom and Arsenault (Anal. Chem. 32, 693, 1960).

Apparatus

The apparatus used in this experiment was manufactured by the Research Specialties Company. The equipment included a nickel-plated spreader for application of a thin layer of adsorbent on a glass plate; an aligning tray, which held the glass plates and guided the spreader straight and smoothly over them; twenty 8 x 8 in. glass plates; silica gel and aluminum oxide for use as adsorbents; six Lang-Levy Lambda-Pette micropipets, three each of 10 µl and 20 µl; a plastic template for marking the ends of the line of origin and the boundary of the solvent front on the plates and for spacing the samples on the line of origin; a glass jar $8\frac{1}{4}$ x 3 x 9 in. for use as a developing chamber, provided with a glass lid and a stainless steel support rack that holds two glass plates upright and back-to-back with their lower edges immersed in the solvent in the bottom of the jar; a storage rack of stainless steel with a ten-plate capacity for minimum handling of the plates; and a set of spray reagents. (Figures VI and VII, pages 44 and 45)



Arrangement for Applying Adsorbent Layer

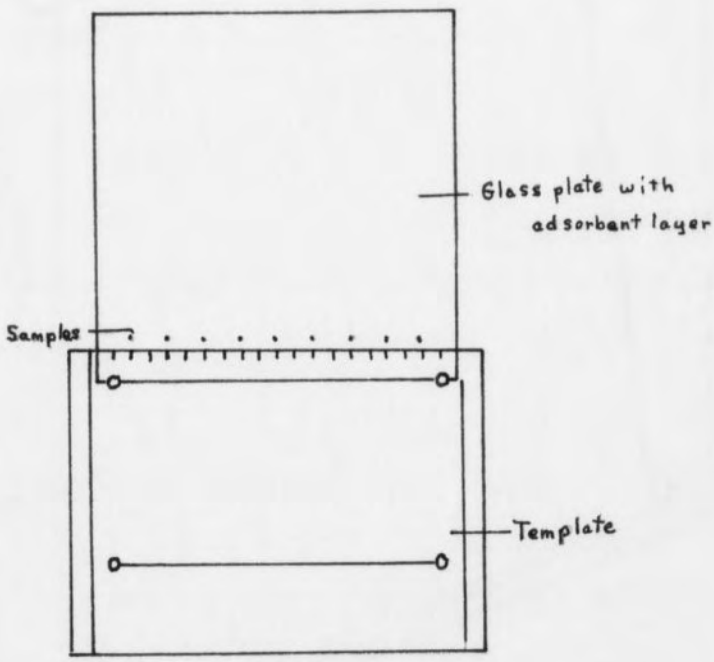


FIGURE VI

Apparatus for Thin-Layer Chromatography
(From Manufacturer's Manual)

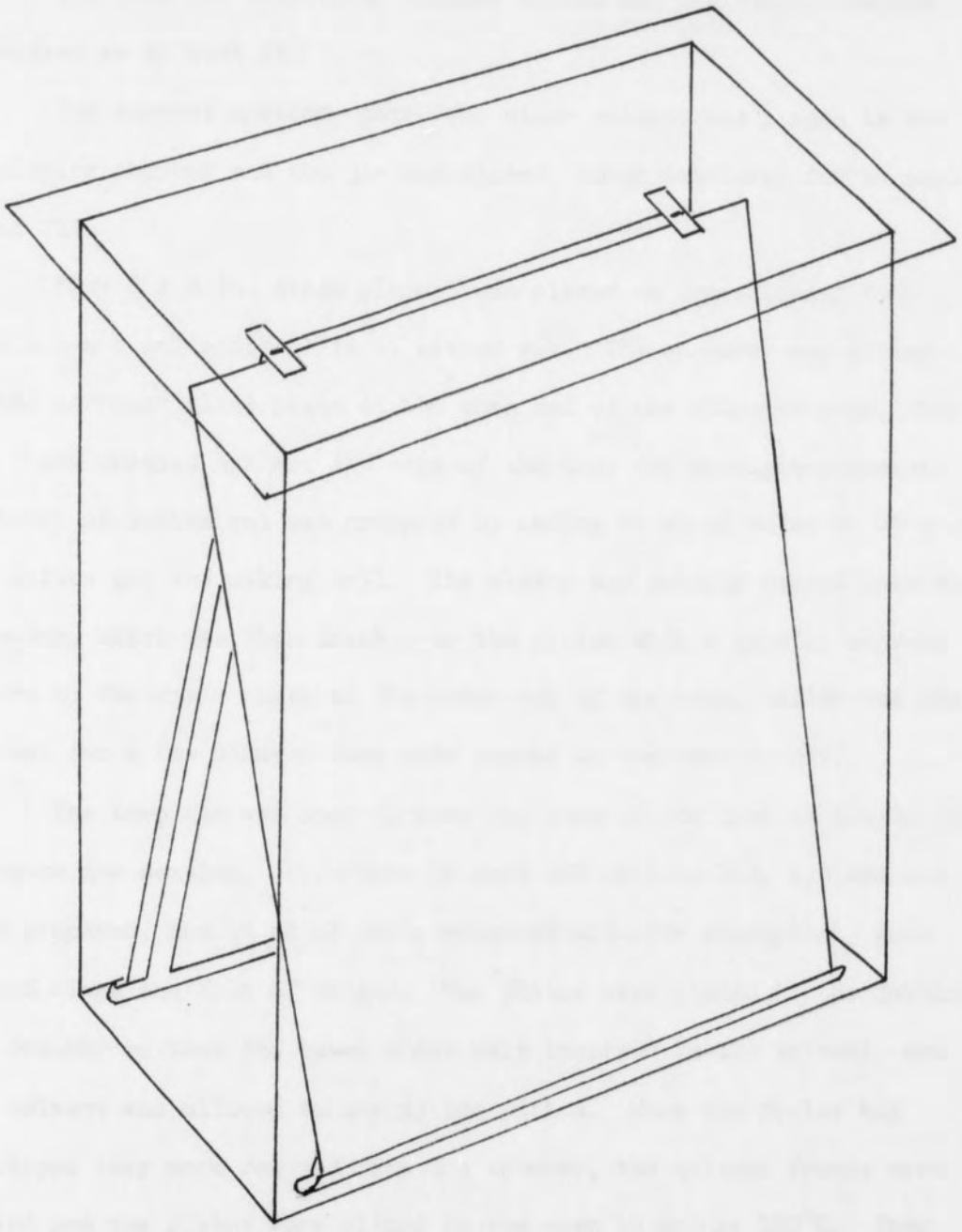


FIGURE VII

Apparatus for Thin-Layer Chromatography

Procedure

The same DNP solutions, solvent system and indicating reagent were used as in Part IV.

The aqueous acetone- petroleum ether solvent was placed in the developing chamber and the jar was closed, using Lubriseal for an air-tight fit.

Five 8 x 8 in. glass plates were placed on the aligning tray with a 3 x 8 in. glass plate at either end. The spreader was placed on the narrower glass plate at the open end of the aligning tray, with the T-arm pressed against the edge of the tray for straight movement. A slurry of silica gel was prepared by adding 40 ml of water to 20 g of the silica gel and mixing well. The slurry was quickly poured into the spreader, which was then drawn over the plates with a smooth, uniform motion to the small plate at the other end of the tray. After the plates had set for a few minutes they were placed in the oven to dry.

The template was used to mark the ends of the line of origin and to space the samples. Dilutions of each DNP of 1:1, 1:2, 1:3 and 1:4 were prepared, and 10 µl of each, measured with the micropipet, were placed along the line of origin. The plates were placed in the developing chamber so that the lower edges were immersed in the solvent, and the solvent was allowed to ascend the plates. When the plates had developed they were removed from the chamber, the solvent fronts were marked and the plates were placed in the oven to dry at 100°C. They were then sprayed with potassium hydroxide solution, the positions of the zones were marked and the plates were replaced in the oven to dry again. Several trials were run for each dilution. R_f values were calculated and are given in Table XVI, page 47.

TABLE XVI

R_f VALUES OF DNP'S

Compound	Molarity	R_f Value
Formaldehyde DNP	0.008	----
	0.006	----
	0.004	0.21
		0.23
		0.26
	0.003	0.27
		0.27
		0.26
Acetaldehyde DNP	0.008	0.19
		0.29
		0.27
	0.006	0.28
		0.33
		0.30
	0.004	0.86
		0.89
		0.89
	0.003	0.89
		0.92
		0.90
n-Propionaldehyde DNP	0.008	0.30
		0.29
		0.24
	0.005	0.20
		0.20
		0.21
	0.004	----
	0.003	----
n-Butyraldehyde DNP	0.008	0.95
		0.88
		0.89
	0.005	0.89
		0.91
		0.92
	0.004	0.89
		0.88
		0.86
	0.003	0.87
		0.87
		0.86

The mixture of DNP's prepared for separation by paper chromatography, including the DNP's of formaldehyde, acetaldehyde, n-propionaldehyde and n-butyraldehyde, was also used for separation by the thin-layer method. Five 10 µl-samples were spotted along the line of origin on the plate of silica and the plate was developed. After drying it was sprayed with potassium hydroxide solution. R_f values were calculated and are given in Table XVII below.

TABLE XVII

R_f VALUES OF A MIXTURE OF THE DNP'S OF FORMALDEHYDE, ACETALDEHYDE, n-PROPIONALDEHYDE AND n-BUTYRALDEHYDE

Trial	R_f Values
Trial 1	0.87
Trial 2	0.86
Trial 3	0.88
Trial 4	0.92
Trial 5	0.93

Discussion

The spots formed by the reactions of the DNP's with the potassium hydroxide solution were well defined in most cases, although there was considerable tailing of all the DNP solutions except that of n-butyraldehyde. On two of the plates the zones showed no sign of movement; the cause of this is unknown.

Generally, there was no particular dilution that gave more satisfactory results. However, in the case of the acetaldehyde DNP there was a marked difference in the R_f values of the 1:1 and 1:2 dilutions on one plate and the 1:3 and 1:4 dilutions run on another, although the two plates were developed simultaneously. The cause for this is unknown, but the difference may have been due to variances in the layer of adsorbent that was applied to each of the two plates. Difficulties caused by non-uniformity in the layer can be overcome only by practice in preparing the plates.

All the trials using the thin-layer chromatographic method were run on the same day except the separation of the mixture, so that the temperature was relatively constant at 24°C. The room temperature during the separation of the mixture was 29°C.

In the separation trial, only one zone was detected by means of the indicating reagent. The cause of the failure to get good separation is unknown; it may possibly be due to the nature of the adsorbent or the solvent. Further experimentation would clarify the problem.

Conclusion

Sufficient data were not obtained from which to draw conclusions for this experiment.

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