LIQUID–LIQUID PARTITION CHROMATOGRAPHY WITH THE SYSTEM CHLOROFORM–CYCLOHEXANE–NITROMETHANE

WENDELL F. SMITH, Jr. AND KENNETH L. EDDY
Research Laboratories, Eastman Kodak Company, Rochester, N.Y. 14650 (U.S.A.)
(Received October 22nd, 1965)

INTRODUCTION

Liquid–liquid partition chromatography (LLC) can be divided into two major categories: (1) column partition chromatography, and (2) paper chromatography. Both techniques were introduced in the early 1940's by MARTIN and his co-workers[1,2]. Since that time paper chromatography has seen a phenomenal development, whereas column partition chromatography has progressed at a much slower rate. This is particularly true with regard to instrumentation which, when compared to the elegant tools available for gas chromatography, is still in a stage of infancy. At least two laboratories are currently working in this field[3–5]. The popularity of paper chromatography has undoubtedly been due in large part to the convenience with which small amounts of materials may be separated.

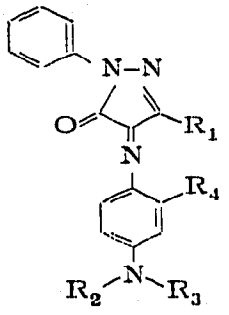
A similar situation exists in the separation of synthetic dyestuffs. The vast majority of dye separations by partition chromatography* have been carried out with paper as the support[6,7]. Furthermore, since most synthetic dyestuffs have acidic or basic groups, practically all of these separations have employed water or hydroxylated solvents as the stationary phase. DEREPENTIGNY AND JAMES[10] have reported one of the few separations of dyes by column partition chromatography. These workers separated two isomeric aminofluoresceins using 0.2 *M* sodium phosphate buffer supported on kieselguhr as the stationary phase, and *n*-butanol–cyclohexane mixtures for the mobile phase. In this paper, we describe a completely organic system which has been found highly efficient for the separation of many hydrophobic dyes.

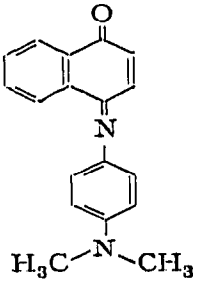
It was of interest to separate quantitatively, as well as qualitatively, mixtures of azomethine and indoaniline dyes, such as those with structures I–XIII: I–X are magenta dyes, XI is a cyan dye, and XII and XIII are yellow dyes. These dyes are, in general, sensitive towards acids and bases and consequently often deteriorate when examined by adsorption chromatography on the common adsorbents such as alumina and silica gel. Although we have been able to resolve some mixtures on polyamide columns with no loss of dye, more often we have found that alumina or silica gel is required.

To solve our problem we turned to liquid–liquid partition chromatography. We have investigated several liquid–liquid systems for separating azomethine and

* We are not here concerned with the incorporation of indicator dyes in the stationary phase to render zones of colorless acidic materials, such as fatty acids, visible on a column[1,8,9].

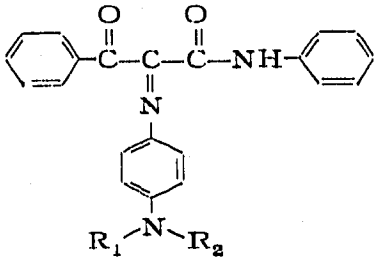
indoaniline dyes and have found the chloroform–cyclohexane–nitromethane system to be the most satisfactory. With this system, we have also effected separations within other classes of materials, including chloroplast pigments and azo dyes such as those (XIV–XIX) used by BROCKMANN AND SCHODDER[11] for determining the activity of alumina.



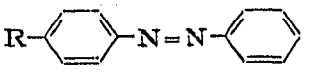


(XI)

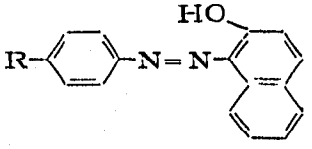
(I) $R_1 = CH_3$; $R_2 = R_3 = C_2H_5$; $R_4 = H$
(II) $R_1 = R_2 = CH_3$; $R_3 = C_2H_5$; $R_4 = H$
(III) $R_1 = R_2 = R_3 = CH_3$; $R_4 = H$
(IV) $R_1 = CH_3$; $R_2 = C_2H_5$; $R_3 = R_4 = H$
(V) $R_1 = R_2 = CH_3$; $R_3 = R_4 = H$
(VI) $R_1 = CH_3$; $R_2 = R_3 = R_4 = H$
(VII) $R_1 = C_6H_5$; $R_2 = R_3 = CH_3$; $R_4 = H$
(VIII) $R_1 = R_2 = R_3 = R_4 = CH_3$
(IX) $R_1 = NHCOC_6H_5$; $R_2 = R_3 = R_4 = CH_3$
(X) $R_1 = C_6H_5$; $R_2 = CH_3$; $R_3 = R_4 = H$



(XII) $R_1 = R_2 = CH_3$
(XIII) $R_1 = CH_3$; $R_2 = H$



(XIV) R = H
(XV) R = OCH_3
(XVI) R = NH_2
(XVII) R = OH



(XVIII) R = H (Sudan yellow)
(XIX) R = C_6H_5N = N (Sudan red).

RESULTS AND DISCUSSION

The system chloroform–cyclohexane–nitromethane

The binoidal curve (Fig. 1) for the ternary system was determined at laboratory temperature (24°) by titration, with the same solvents which were to be used for chromatographic purposes. The solvents were Eastman Kodak Spectro Grade nitromethane, Practical Grade chloroform, and Eastman Grade cyclohexane. The addition of chloroform to cyclohexane–nitromethane mixtures is endothermic. Almost all of the

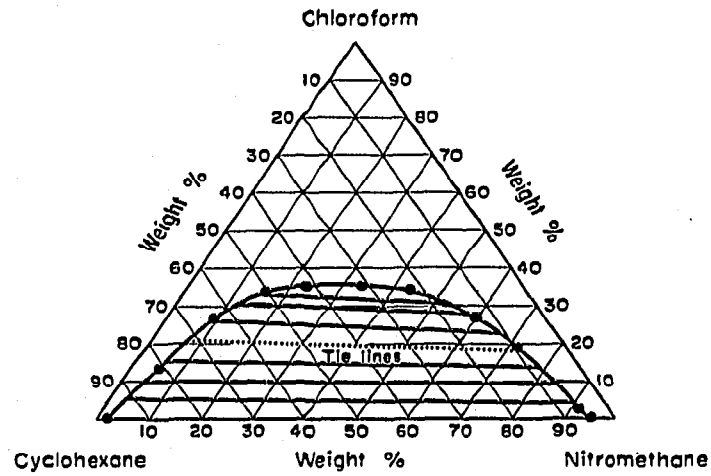


Fig. 1. Solubility curve at 24° for the system chloroform–cyclohexane–nitromethane.

chloroform necessary to give one phase was therefore added to the cyclohexane–nitromethane mixtures, and these solutions were then allowed to stand at room temperature for about two hours before the additional chloroform necessary to give one phase was added. The results are given in Table I and Fig. 1.

To establish the ends of the tie lines, refractive index measurements were used. The refractive index of the upper (non-polar) phase changes slowly with changing composition, but it changes more rapidly with changing composition of the lower

TABLE I

SOLUBILITY CURVE AT 24° FOR CHLOROFORM–CYCLOHEXANE–NITROMETHANE

Chloroform (*wt.* %)	*Cyclohexane* (*wt.* %)	*Nitromethane* (*wt.* %)
0.0	97.8*	2.2*
13.7	77.6	5.7
26.8	63.9	9.3
33.9	50.1	16.0
35.4	41.3	23.3
35.7	30.7	33.6
34.3	22.3	43.4
27.2	13.5	59.3
18.5	9.8	71.7
2.3	6.3	91.4
0.0	5.1*	94.9*

* Ref. 12 (value at 25°).

phase. A point of each tie line was determined by the composition of a two-phase mixture. A second point was established by measuring the refractive index of the lower phase. The data are presented in Table II and Fig. 1. The dotted tie line in Fig. 1 indicates the phase pair which we have found satisfactory for our separations. The composition of the phases are (by weight): lower (stationary) phase: 18.1 % $CHCl_3$, 9.7 % C_6H_{12}, 72.2 % CH_3NO_2; upper (mobile) phase: 21.1 % $CHCl_3$, 72.1 % C_6H_{12}, 6.8 % CH_3NO_2. Other phase pairs may prove better for other classes of compounds.

TABLE II

THE LINE DATA FOR CHLOROFORM–CYCLOHEXANE–NITROMETHANE

	Chloroform (wt. %)	*Cyclohexane (wt. %)*	*Nitromethane (wt. %)*
Upper phase	5.5	91.8	2.7
Lower phase	4.8	5.7	89.5
Upper phase	10.3	86.1	3.6
Lower phase	9.3	7.0	83.7
Upper phase	15.8	79.1	5.1
Lower phase	13.7	8.1	78.2
Upper phase	21.1	72.1	6.8
Lower phase	18.1	9.7	72.2
Upper phase	26.2	64.5	9.3
Lower phase	21.9	11.3	66.8
Upper phase	31.0	56.5	12.5
Lower phase	28.0	14.0	58.0
Upper phase	33.5	51.0	15.5
Lower phase	30.8	17.0	52.2

Preparation of phases and columns

To prepare the mobile and stationary phases, any mixture on the selected tie line, properly equilibrated, may be used. From the practical standpoint, one usually desires a larger quantity of mobile than of stationary phase (for elution), although occasionally the reverse is true (for coating the support). For the former case, we use (*cf.* dotted tie line, Fig. 1) 796 ml of $CHCl_3$, 496 ml of CH_3NO_2 and 5000 ml of C_6H_{12}, stirred for at least three hours. The mobile (upper) phase is then stored in bottles over a few milliliters of stationary phase.

As a support, powdered cellulose (Whatman CF 11 Chromedia) is excellent. Although silica gel is capable of supporting more stationary phase than cellulose and therefore gives columns of higher capacity, separations are much slower on such columns because some adsorption still occurs. Consequently, dyes are more apt to decompose on LLC columns in which silica gel is the support. Cellulose has also proved more satisfactory than several diatomaceous-earth preparations.

The column design is important. It is essential that the neck of the column (consisting of a standard-taper outer joint to accommodate a solvent reservoir) be

slightly larger in diameter than the rest of the column. This permits a machined cylindrical Teflon packing plug, approximately 1 in. long, to fit snugly in the column. The plug has a hole drilled in the center of one end. The hole is threaded to fit the threaded end of a stainless-steel rod of a length convenient for packing purposes. If the Teflon plug does not fit snugly, the column may be packed unevenly, *i.e.*, one side tighter than the other, and uneven bands and streaking may result during a separation.

A typical procedure for preparing a 1-in. diameter by 30-in. long column follows:

(1) *Coating the cellulose.* A 2-l, three-necked Morton-flask[13], equipped with a dropping funnel and an air-driven, propeller-type stirrer, is charged with 150 g of cellulose powder (Whatman CF 11 Chromedia) and enough mobile phase to cover the cellulose completely. To this is slowly added, with rapid stirring, 45 g (30 % by weight of the cellulose) of nitromethane *or* 45 g of lower phase* (Table II). The coated cellulose is kept covered with mobile phase at all times.

(2) *Packing the column.* The column is packed by the conventional wet-packing technique. Enough cellulose is added at a time to give about 2 in. of packed material. The material is compressed using the tool described above, about 65 lb./sq.in. pressure being applied. Pressure rings do not adversely affect the performance of these columns. When the cellulose is packed, its upper surface is covered with a little sand, and approximately 1 in. of mobile phase is left above the sand for column storage. It is advisable not to leave a large amount of mobile phase over a column (*e.g.*, in a solvent reservoir) since temperature fluctuations in the laboratory may cause some phase separation. If this happens droplets of (mainly) nitromethane form on top of the sand; the droplets are readily removed with a syringe, however, and this must be done before the column is put into use. Thermostatting the column and reservoir would probably circumvent this problem and also prolong the life of the column. We have found that our columns give satisfactory separations for about twenty chromatograms, the efficiency slowly decreasing with use.

Solutes are readily recovered from the eluents by rotary evaporation with a Rinco evaporator at room temperature. Traces of residual nitromethane may be removed by adding benzene or methanol and re-evaporating the solution.

Column efficiency

The height equivalent to a theoretical plate (H.E.T.P.) was measured for a freshly packed, 1-in. diameter column, by Method 2 of JAMES AND MARTIN[14]. With dye III as solute and an elution rate of 3.0 ml/min, the H.E.T.P. was 0.5 mm.

R_F values

The R_F values were calculated from the formula:

$$R_F = R\left(\frac{A_m}{A}\right)$$

* Columns prepared with cellulose coated with nitromethane give initially better separations than those prepared with cellulose coated with lower phase. However, with use the two become equivalent.

in which[1]:

R = (movement of position of maximum concentration of solute)/(simultaneous movement of surface of developing fluid in empty part of tube above chromatogram),

A = area of cross section of the column,

A_m = area of cross section of the mobile phase.

The values were determined with 1-in. diameter columns, by using an elution rate of approximately 3 ml/min. The results for the dyes are given in Table III, in which the R_F values by LLC are also compared with those by TLC (Al_2O_3 plates with benzene as eluent). The least-squares relationship (Fig. 2) is R_F (LLC) $\cong$ 0.30 + 0.81 R_F (TLC) $\pm$ 0.10. This allows at least a rough estimate to be made of the separation to be expected by LLC on the basis of a quick TLC experiment.

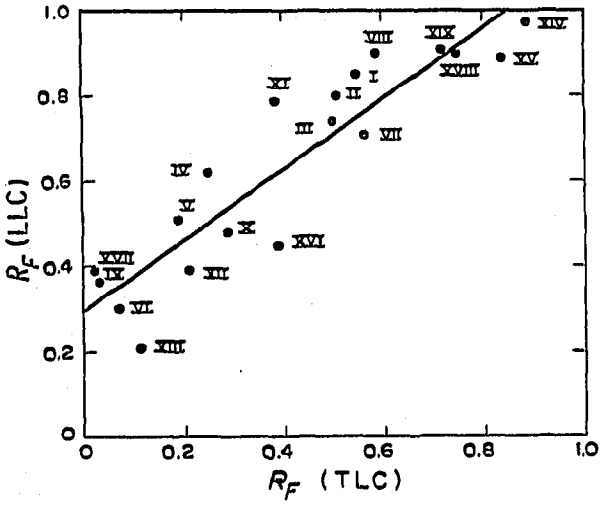


Fig. 2. R_F values by TLC (alumina plates, benzene eluent) *versus* R_F values by LLC.

The R_F values for the plant pigments of a grass-leaf extract are given in Table IV. Identification is based upon the TLC data of ANWAR[15] and ROLLINS[16]. On a 36-in.

TABLE III

R_F VALUES OF DYES I–XIX

Dye	*TLC**	*LLC*	*Dye*	*TLC**	*LLC*
I	0.55	0.85	XI	0.39	0.79
II	0.51	0.80	XII	0.21	0.39
III	0.50	0.74	XIII	0.11	0.21
IV	0.25	0.62	XIV	0.89	0.97
V	0.19	0.51	XV	0.84	0.89
VI	0.07	0.30	XVI	0.39	0.45
VII	0.56	0.71	XVII	0.02	0.39
VIII	0.59	0.90	XVIII	0.75	0.90
IX	0.03	0.36	XIX	0.72	0.91
X	0.29	0.48			

* Elution with benzene on alumina plates.

column, complete resolution of four of the xanthophylls is obtained. Chlorophylls *a* and *b* are resolved, but each is contaminated with a carotene, pheophytin, or xanthophyll.

TABLE IV

R_F VALUES OF PLANT-LEAF PIGMENTS

Band	*Identification*	R_F *value*
Yellow	Carotene	0.54
Gray	Pheophytin	0.53
Blue-green	Chlorophyll *a*	0.50
Yellow	Xanthophyll	0.47 +
Green	Chlorophyll *b*	0.47 —
Yellow	Xanthophyll	0.41
Yellow	Xanthophyll	0.36
Yellow	Xanthophyll	0.32
Yellow	Xanthophyll	0.21

SUMMARY

The system chloroform–cyclohexane–nitromethane has been investigated at 24°, and the application of one phase pair to liquid–liquid partition chromatography described. The use of powdered cellulose as the support for the stationary (polar) phase has given excellent separations of some plant-leaf pigments and azomethine, indoaniline, and azo dyes.

REFERENCES

1 A. J. P. MARTIN AND R. L. M. SYNGE, *Biochem. J.*, 35 (1941) 1358.
2 R. CONSDEN, A. H. GORDEN AND A. J. P. MARTIN, *Biochem. J.*, 38 (1944) 224.
3 S. M. LAMBERT AND P. E. PORTER, *Anal. Chem.*, 36 (1964) 99.
4 S. M. LAMBERT, *Anal. Chem.*, 37 (1965) 959.
5 K. J. BOMBAUGH AND J. N. LITTLE, *J. Chromatog.*, 16 (1964) 47.
6 E. LEDERER AND M. LEDERER, *Chromatography*, Elsevier, New York, 1937, Chap. 15.
7 K. VENKATARAMAN, *The Chemistry of Synthetic Dyes*, Academic Press, New York, 1952, pp. 1326–1331.
8 A. H. GORDON, A. J. P. MARTIN AND R. L. M. SYNGE, *Biochem. J.*, 37 (1943) 79.
9 H. F. LIDDELL AND H. N. RYDON, *Biochem. J.*, 38 (1944) 68.
10 J. DEREPENTIGNY AND A. T. JAMES, *Nature*, 174 (1954) 927.
11 H. BROCKMANN AND H. SCHODDER, *Ber.*, 74B (1941) 73.
12 H. J. WECK AND H. HUNT, *Ind. Eng. Chem.*, 46 (1954) 2521.
13 A. A. MORTON AND D. M. KNOTT, *Ind. Eng. Chem. (Anal. Ed.)*, 13 (1941) 649.
14 A. J. JAMES AND A. J. P. MARTIN, *Biochem. J.*, 50 (1952) 681.
15 M. H. ANWAR, *J. Chem. Educ.*, 40 (1963) 29.
16 C. ROLLINS, *J. Chem. Educ.*, 40 (1963) 32.