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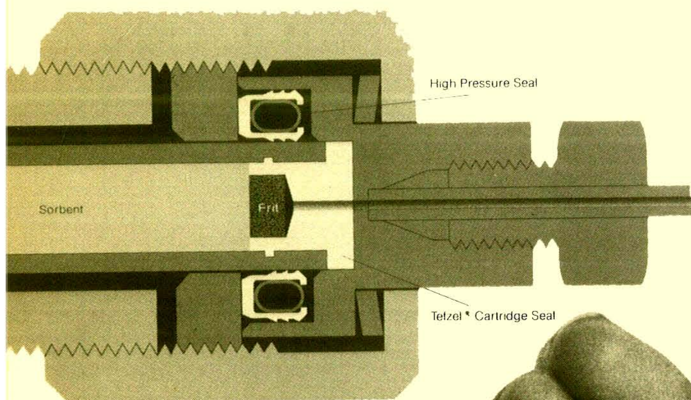

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THIN-LAYER CHROMATOGRAPHY

Edited by HALEEM J. ISSAQ
Frederick Cancer Research Center
Frederick, Maryland

and

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This is a special issue of *Journal of Liquid Chromatography*, Volume 4, Number 12, 1981.

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JOURNAL OF LIQUID CHROMATOGRAPHY

Volume 4, Number 12, 1981

Special Issue on Thin-Layer Chromatography

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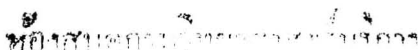

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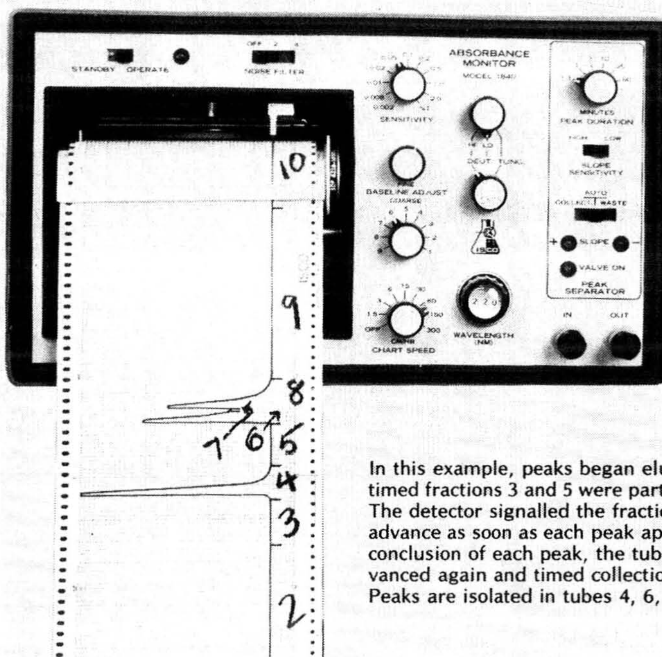

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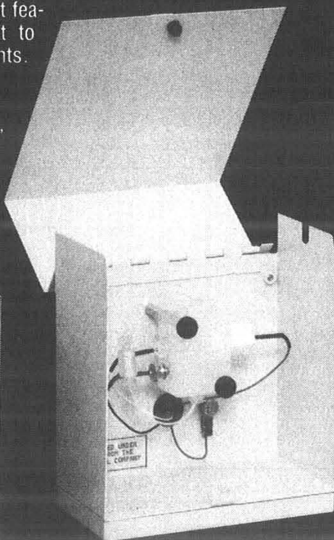

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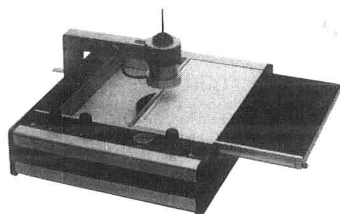

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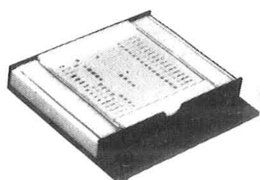

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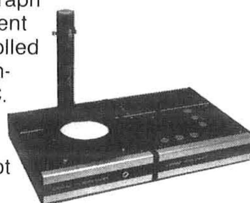

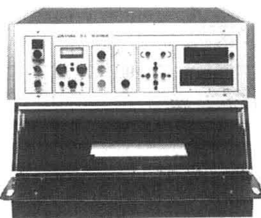

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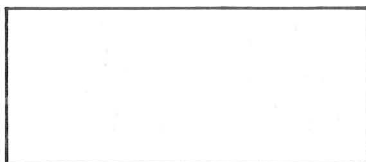

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JOURNAL OF LIQUID CHROMATOGRAPHY, 4(12), xiii-xiv (1981)

EDITORIAL

This is the fourth special issue of this Journal devoted to thin layer chromatography (TLC). Each one of these issues has included a review, new technologies, and advances of existing modes of TLC. In addition, research articles dealing with the separation of complex mixtures, their visualization and quantification were published. We will continue to present the analyst and reader these special issues which have found a favorable response and are widely accepted.

During the past two years we've witnessed increased activity in meetings and symposia dealing with TLC advances which we believe are totally justified, and we commend their organizers. These meetings include the Second Biennial TLC Symposium which was held in Philadelphia, PA in November 1980. The Third in this series is tentatively planned for the first week of November 1982, in Philadelphia. Two sessions devoted to TLC advances were held at the Nineteenth Eastern Analytical Symposium in New York City, in November 1980. This year, two sessions are planned for November 18-20 in the same place. The First International Symposium on Instrumental TLC (HPTLC) was held last year in Germany. The second symposium will be held in Interlaken, Switzerland, May 1982.

Another International Symposium, the First American-Hungarian Symposium on TLC and HPLC applications was held in Szegad, Hungary, May 1981. Scientists from Hungary, the Soviet Union and the United States participated in the discussion. At the close of the meeting the organizing committee decided to expand the symposium to include other Eastern Eurpoean Countries. The Second Symposium is planned for June 16-18, 1982 in Szeged, Hungary.

There is always half a day session devoted to TLC at the Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy. Other Chromatography meetings include research papers dealing with TLC.

We are pleased to see this increased activity and the forums which help to advance the theory and application of TLC. The Journal's editors and publishers are very pleased to be a part of this exciting re-birth. These special issues of the journal, devoted to TLC, will remain a forum where all chromatographers can publish their original research and innovative findings.

Keep up the good work!

Haleem J. Issaq
Jack Cazes

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(12), xv (1981)

NEW SYMPOSIUM SERIES

A symposium on advances in thin-layer chromatography and high-performance liquid chromatography was held in Szeged, Hungary, at the Biological Research Center, May 14-15, 1981, and was sponsored by the Hungarian Academy of Sciences. Four scientist from the U.S.A., ten from Hungary and three from the U.S.S.R. participated at the Symposium.

At the close of this meeting it was decided to expand the symposium to include scientists from other countries and the increase the number of participants. It was also decided to make the symposium a yearly event. The next symposium is scheduled for June 16-17, 1982 in Szeged, Hungary.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 4(12), 2091-2120 (1981)

A SYSTEMATIC STATISTICAL METHOD OF SOLVENT SELECTION FOR OPTIMAL SEPARATION IN LIQUID CHROMATOGRAPHY

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Jerome E. Haky and Gary M. Muschik
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Frederick, MD 21701.

ABSTRACT

A systematic method is described for selecting the optimum ternary mobile phase for both thin layer and high performance liquid chromatography. The statistical data analysis employs overlapping resolution mapping in which a contour plot is made by plotting resolution against solvent composition. The computer analysis predicts optimum mobile phase regions, from which the analyst can select the least viscous, and cheapest, mobile phase. Peak crossover is taken into consideration. Good agreement was observed between predicted and experimental data. The method is simple and easy to apply to liquid chromatography.

INTRODUCTION

The selection of a solvent system which will give optimum resolution in liquid chromatography (adsorption, partition or ion exchange) is not a simple matter. The most important considerations are the properties of the material being separated and the solid phase. The mobile phase can be selected only when these two factors have been defined. When the solvent is a binary, ternary...etc. mixture, solvent-solute and solvent-solvent interactions must be taken into consideration. A trial-and-error procedure is generally used to

*Author to whom correspondence should be addressed.

find a mobile phase which will satisfactorily resolve all the components of the mixture. When the mobile phase is composed of more than one solvent the task of selection becomes complex. In TLC, unlike HPLC, the process of solvent selection is less time consuming because the analyst can spot as many plates as he has developing tanks and develop them in different solvent mixtures or use a unit like the Selecta Sol or the Vario KS-Chamber in which up to 16 different solvents can be simultaneously tried on a 20 X 20 cm plate (1). Although it has been shown that TLC solvents can be used as mobile phases for HPLC (2,3), this simple approach is by no means a systematic one leading to the selection of an optimum mobile phase. We define an optimum mobile phase as that solvent mixture which would give base-line-separation of all the components of a sample mixture in the minimum amount of time.

Glajch et al (4), Belinky (5), and others (6-8) described a systematic solvent optimization procedure which employs statistical methods of data analysis. Our study describes a systematic approach to selecting a ternary solvent mixture based on a plot of pair resolution versus solvent composition, and overlapping resolution mapping (ORM) data analysis similar to that employed by Glajch et al (4). Peak crossover is taken into consideration. The method is simple and can be applied to both partition and adsorption liquid chromatography. In addition to solvent optimization, the answers obtained give the analyst the opportunity to select (a) the least viscous mobile phase (least back pressure), (b) the cheapest solvent mixture and (c) the shortest retention times.

EXPERIMENTAL

Materials: All solvents were glass distilled (Burdick and Jackson). The chemicals were analytical grade (Aldrich Chemical Co.) and used without further purification.

Apparatus: A modular HPLC system consisting of Laboratory Data Control (LDC) constametric I and II Pumps attached to an LDC Gradient Master, a Chromatronix

dual-channel uv absorbance detector, a Rheodyne injector, and a strip-chart recorder operated at 0.2 in/min was used.

The RP-8 and RP-18 reverse phases columns were all 250 mm X 4.6 mm prepacked with 10 μm particle size materials (Merck). 10 μl samples were injected. Experiments were run at room temperature using a mobile phase flow rate of 1.2 ml/min. Retention times, peak widths (W) and resolution (R_S) were determined by a 3352A Laboratory Data System (Hewlett-Packard) linked through a Hewlett-Packard 1865 A/D converter to the UV detector output of the liquid chromatograph. The output from the data system was recorded on a 9866A thermal line printer (Hewlett-Packard). Silica gel and reverse phase (RP-8 and RP-18) TLC plates were purchased from Whatman, Inc. Standard TLC tanks and equipment were used. Plates were spotted with 5 μl disposable micropipettes.

Procedure: A combination of the three initial solvents is devised according to Table 1. Other combinations may also be used. The initial solvents maybe pure

Table 1

Combination of solvents A, B and C used in this study to predict optimum mobile phase compositions.

% SOLVENT A	% SOLVENT B	% SOLVENT C
100	0	0
0	100	0
0	0	100
50	50	0
50	0	50
0	50	50
33	33	33
67	16	16
16	67	16
16	16	67

or a mixture of two organic solvents (normal phase) or a mixture of water/organic modifier (reverse phase). After selecting the solvents and proportions to be used (Table 1), 10 data points, one for each solvent combination are collected. These are used to calculate the resolutions of each pair of compounds in the mixture. If no peak crossover takes place the resolution between each pair (1-2, 2-3, 3-4.....etc) is used. If peak crossover does occur the resolution between all the peaks is calculated (1-2, 1-3, 1-4, 2-3, 2-4, 3-4..etc.), and used in determining the optimum mobile phase.

Two computer programs are used to predict optimum solvent composition. The first (Appendix 1) is a FORTRAN program (PEAKIN) which rearranges resolutions to correct for crossover, and if necessary, prints a table similar to Table 2 or Table 3, and produces a data file suitable for use in the next program. The second program (Appendix 2) is a SAS (Statistical Analysis System - version 79.5) route (9). A DATA paragraph converts the three-dimensional solvent compositions to a two-dimensional triangle representation as used by Snee (10). The data is fitted into a cubic model for a three dimensional system. The parameters of the cubic equation for each set of peak resolutions are computed using the general

Table 2

Mobile phase ratios and resolutions obtained for aflatoxins B_1, B_2, B_1 and G_2 using silica gel plates and acetone:chloroform (10:90), methanol:chloroform (5:95) and ethyl acetate:chloroform (30:70).

SOLVENT	MOBILE PHASES RATIOS									
10% ACETONE	1.00	0.0	0.0	0.0	0.5	0.50	0.33	0.16	0.16	0.67
5% MEOH	0.0	1.00	0.0	0.50	0.50	0.0	0.33	0.16	0.67	0.16
30% ETHYL-ACETATE	0.0	0.0	1.00	0.50	0.0	0.50	0.33	0.67	0.16	0.16
$R_s(B_1 - B_2)$	6.20	9.60	7.10	6.70	7.50	7.90	7.10	5.30	6.70	7.50
$R_s(B_2 - G_1)$	4.20	5.80	3.30	6.20	5.80	3.70	5.00	4.50	6.30	5.00
$R_s(G_1 - G_2)$	5.80	0.40	5.00	3.80	2.50	4.10	5.00	4.80	3.30	4.50

Table 3

Solvent compositions and resolutions obtained for the peak pairs N - M, M - E and E - D on reverse phase TLC plates using 95% $CH_3OH:H_2O$, 80% $CH_3CN:H_2O$ and 75% 2-ethoxyethanal:H_2O.

SOLVENT	MOBILE PHASE RATIOS									
95% CH_3OH	1.00	0.0	0.0	0.50	0.50	0.0	0.33	0.67	0.16	0.16
80% CH_3CN	0.0	1.00	0.0	0.50	0.0	0.50	0.33	0.16	0.67	0.16
75% 2ETHO	0.0	0.0	1.00	0.0	0.50	0.50	0.33	0.16	0.16	0.67
R_s(N - M)	6.3	7.4	7.1	7.5	7.9	7.9	7.5	7.5	7.0	8.3
R_s(M - E)	4.1	5.9	7.1	5.9	5.4	6.7	5.4	4.5	7.1	7.5
R_s(E - D)	2.9	1.5	3.3	2.5	3.4	2.9	2.1	3.8	2.5	2.9

linear model (GLM) procedure. The PRINT procedure lists predicted resolutions of each peak pair for all solvent combinations varying each solvent from zero to 100 percent by 2% increments (see for example Tabl e 4). Contour plots of the region where the predicted resolution above a desired level determined by the analyst are produced (see for example Figs. 1-3) using the PLOT procedure. The union of these plots showing the region where all resolutions are above this level, Fig. 4, and plots showing the area of maximum total resolution, Fig. 5, are also produced using PLOT. A flow chart of the procedure is shown in Fig. 6. The programs are run on an IBM model 370/168, and uses 210 K of core.

Ideally, where a combination of three modifiers and a base solvent is used the region of the optimum mobile phase mixture found from the ORM calculations will be in the center of the triangle. If one of the modifiers (A) is not ideal, the optimum mixture will be composed of the other two modifiers (B and C), with only a small amount of A. Therefore, the optimum region can indicate which of the three modifiers is a poor choice. Examples will be described later. The base solvents are water for reverse phase and hexane for normal phase (4). Other solvents for normal phase are also used.

Table 4

A sample of computer tabulation of mobile phase compositions and resolutions obtained using ORM calculations.

SOLVENT STUDY - A=CH3OH,B=CH3CN,C=THF

OBS	A	B	C	PEAK1	PEAK2	RESPRED
12741	0.8	0.10	0.10	1	2	1.6178
12742	0.8	0.10	0.10	1	3	6.4093
12743	0.8	0.10	0.10	1	4	8.9504
12744	0.8	0.10	0.10	1	5	10.5934
12745	0.8	0.10	0.10	2	3	4.7915
12746	0.8	0.10	0.10	2	4	7.3326
12747	0.8	0.10	0.10	2	5	8.9709
12748	0.8	0.10	0.10	3	4	2.5411
12749	0.8	0.10	0.10	3	5	4.1808
12750	0.8	0.10	0.10	4	5	1.6383
12751	0.8	0.12	0.08	1	2	1.3231
12752	0.8	0.12	0.08	1	3	6.5454
12753	0.8	0.12	0.08	1	4	8.8780
12754	0.8	0.12	0.08	1	5	10.7395
12755	0.8	0.12	0.08	2	3	5.2223
12756	0.8	0.12	0.08	2	4	7.5548
12757	0.8	0.12	0.08	2	5	9.4134
12758	0.8	0.12	0.08	3	4	2.3326
12759	0.8	0.12	0.08	3	5	4.1920
12760	0.8	0.12	0.08	4	5	1.8586
12761	0.8	0.14	0.06	1	2	1.0106
12762	0.8	0.14	0.06	1	3	6.6990
12763	0.8	0.14	0.06	1	4	8.8448
12764	0.8	0.14	0.06	1	5	10.9388
12765	0.8	0.14	0.06	2	3	5.6884
12766	0.8	0.14	0.06	2	4	7.8341
12767	0.8	0.14	0.06	2	5	9.9269
12768	0.8	0.14	0.06	3	4	2.1457
12769	0.8	0.14	0.06	3	5	4.2389
12770	0.8	0.14	0.06	4	5	2.0928
12771	0.8	0.16	0.04	1	2	0.6804
12772	0.8	0.16	0.04	1	3	6.8703
12773	0.8	0.16	0.04	1	4	8.8509
12774	0.8	0.16	0.04	1	5	11.1913
12775	0.8	0.16	0.04	2	3	6.1899
12776	0.8	0.16	0.04	2	4	8.1705
12777	0.8	0.16	0.04	2	5	10.5114
12778	0.8	0.16	0.04	3	4	1.9806
12779	0.8	0.16	0.04	3	5	4.3213
12780	0.8	0.16	0.04	4	5	2.3409
12781	0.8	0.18	0.02	1	2	0.3324
12782	0.8	0.18	0.02	1	3	7.0592
12783	0.8	0.18	0.02	1	4	8.8963
12784	0.8	0.18	0.02	1	5	11.4970
12785	0.8	0.18	0.02	2	3	6.7268
12786	0.8	0.18	0.02	2	4	8.5639
12787	0.8	0.18	0.02	2	5	11.1670
12788	0.8	0.18	0.02	3	4	1.8370
12789	0.8	0.18	0.02	3	5	4.4394

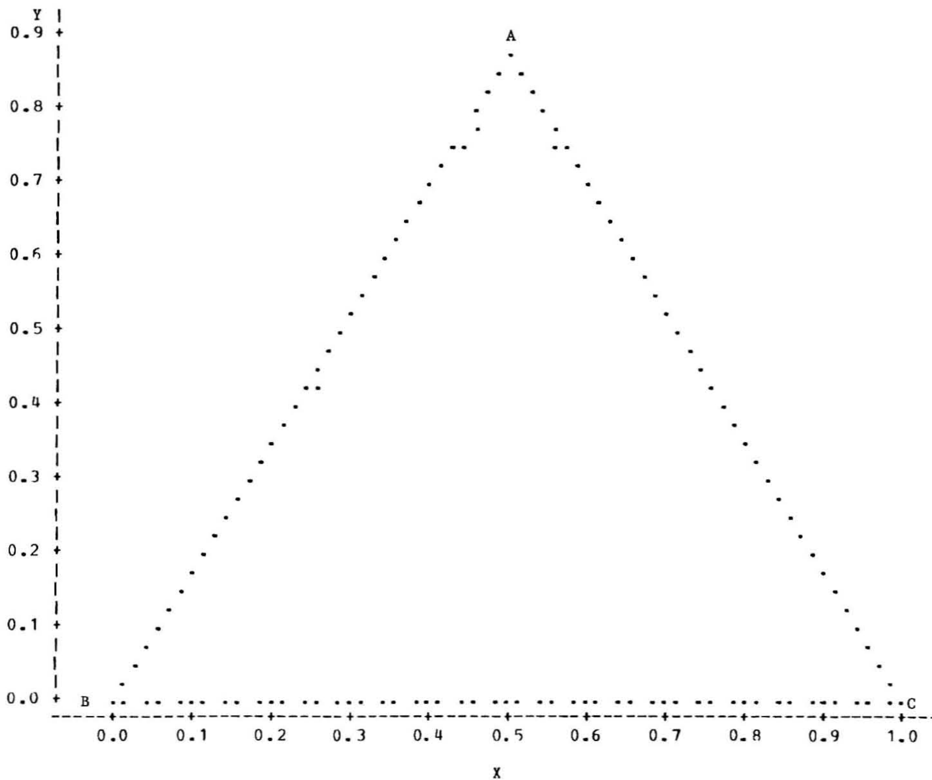

Fig. 1 Contour plot of aflatoxins B_1 and B_2, A = 10% acetone/chloroform, B = 5% methanol/chloroform, C = 30% ethylacetate/chloroform

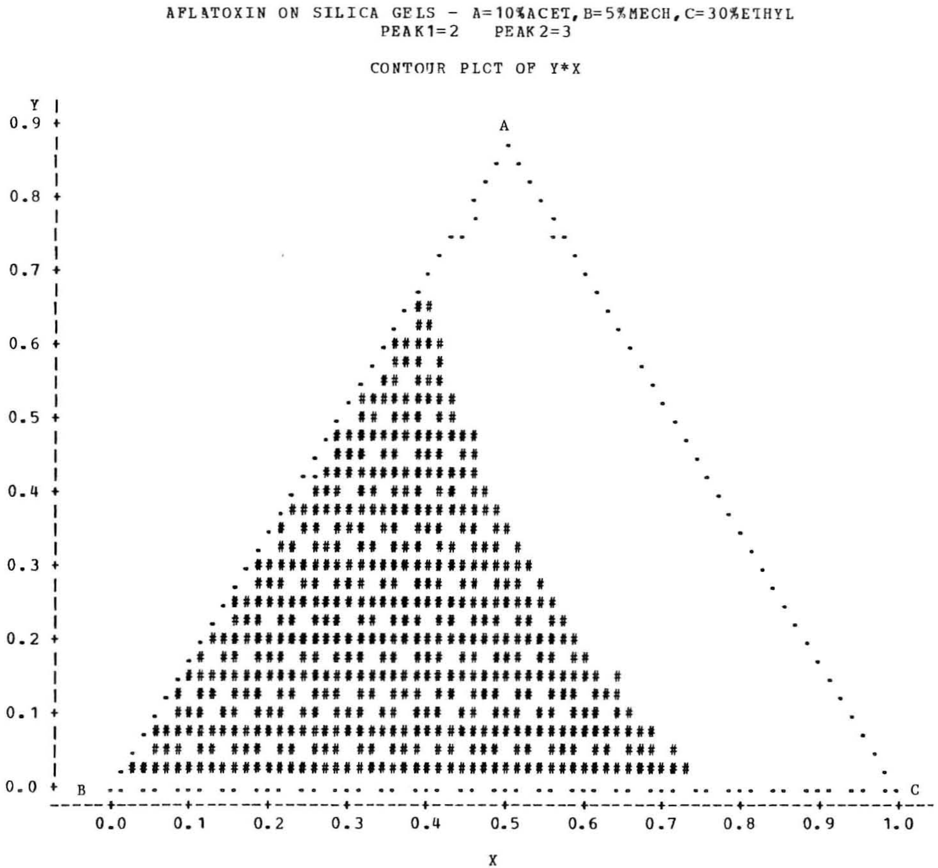

Fig. 2 Contour plot of aflatoxins B_2 and G_1, solvents as in Fig. 1. Shaded area designate mobile phase compositions that would give resolution greater than 5.15.

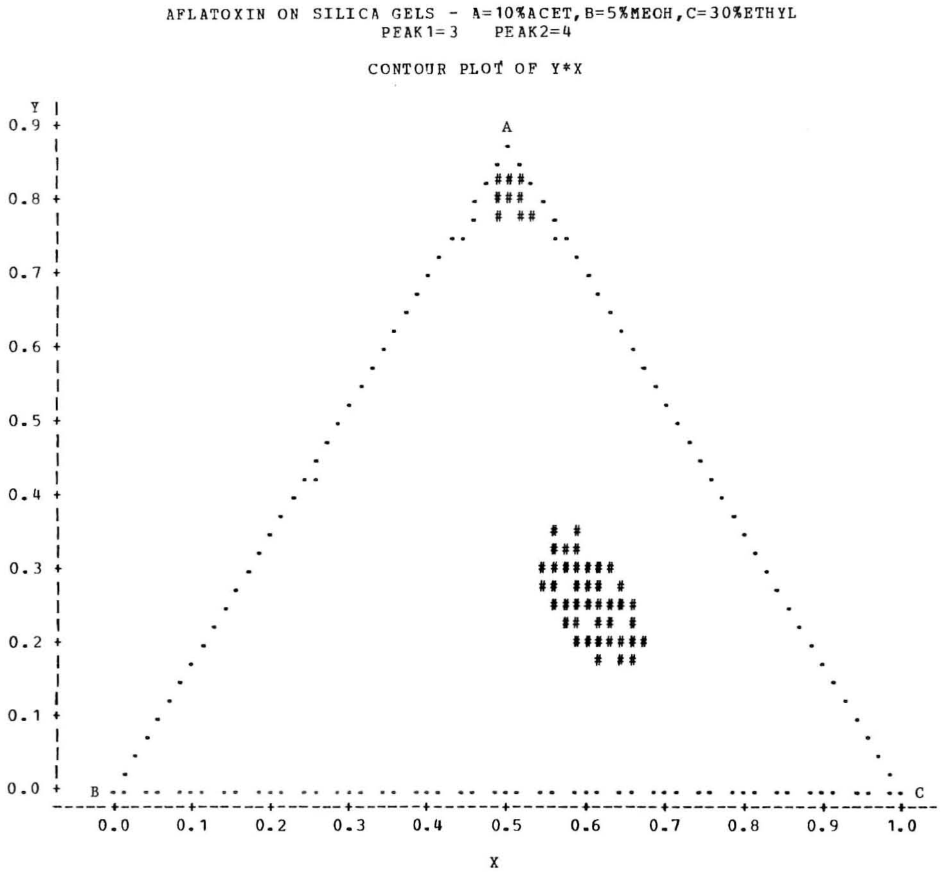

Fig. 3 Contour plot of aflatoxins G_1 and G_2, solvents as in Fig. 1. Shaded area designate mobile phase compositions that would give resolution greater than 5.15.

S T A T I S T I C A L   A N A L Y S I S   S Y S T E M

NUMBER OF PEAKS WHERE RESOLUTION>5.15

AFLATOXIN ON SILICA GELS - A=10%ACET, B=5%MECH, C=30%ETHYL

CONTOUR PLOT OF Y*X

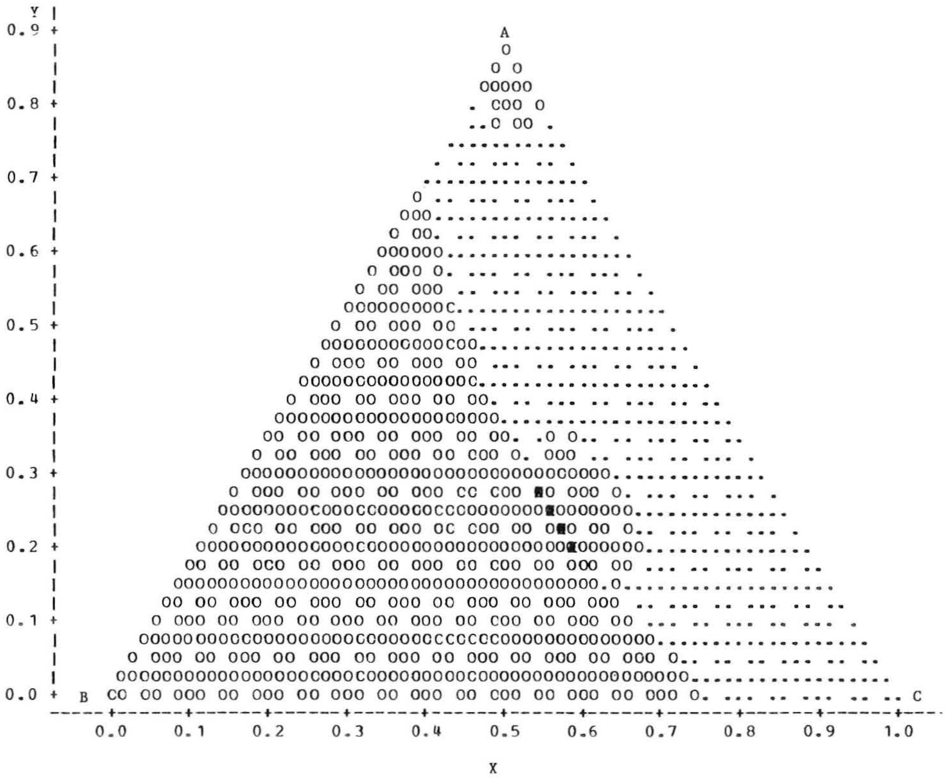

Fig. 4 Contour plot showing the area of optimum solvent composition where the resolution between each pair of the four aflatoxins is greater than 5.15 (■). Solvents as in Fig. 1.

S T A T I S T I C A L   A N A L Y S I S   S Y S T E M

SUM OF PEAK RESOLUTIONS FROM EACH MIX

AFLATOXIN ON SILICA GELS - A=10%ACET,B=5%MEOH,C=30%ETHYL

CONTOUR PLOT OF Y*X

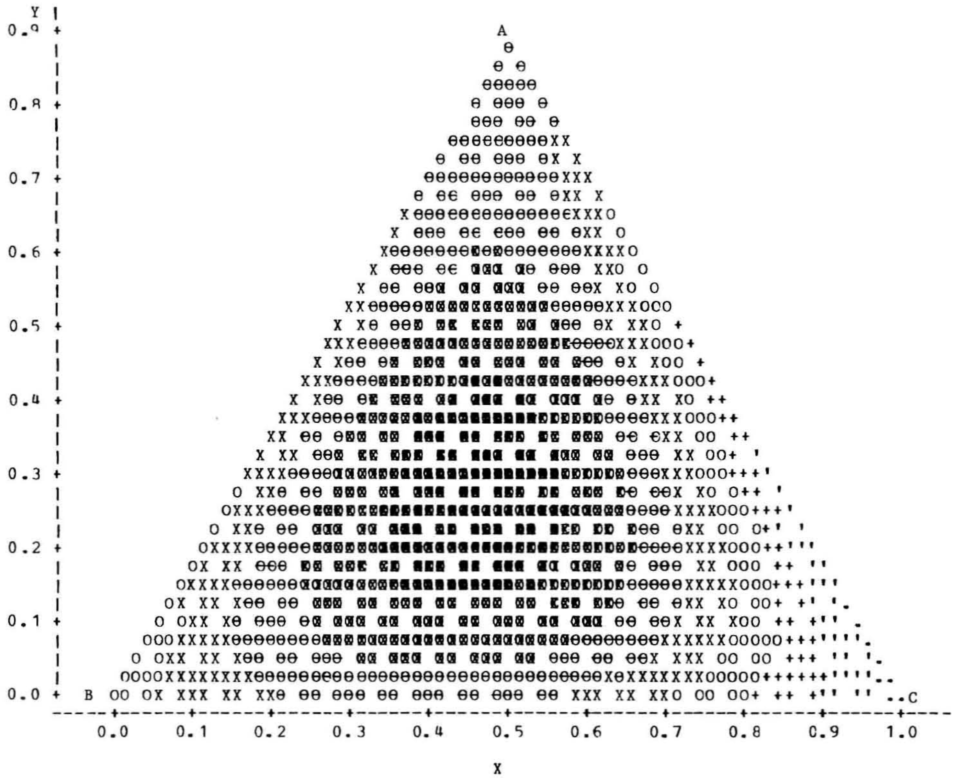

Fig. 5 Contour plot of area of maximum total resolution (▩) between the four aflatoxins. Solvents as in Fig. 1.

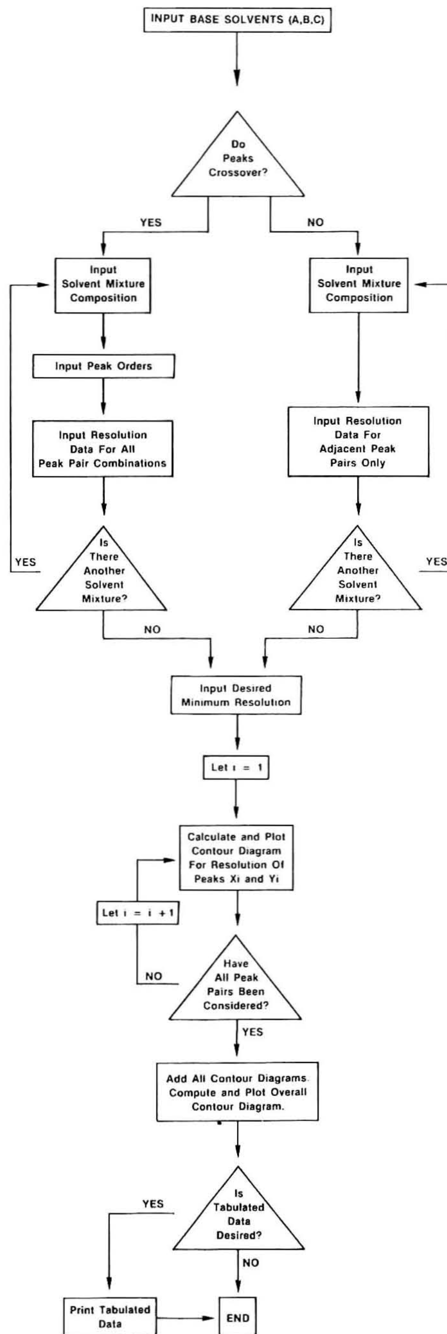

Fig. 6 Flow chart of the procedure used in the present study.

Another criterion is that any pair of the component mixture should be resolved in at least one of the three mobile phases selected, otherwise base line resolution of that pair in the final (optimum) mobile phase may not be possible. The solvents selected therefore, should have different chemical properties, hydrogen bonding, proton-donor or acceptor, dipole-dipole interaction...etc. To achieve that, Synder's solvent groups (11) were used.

THEORETICAL CONSIDERATIONS

Selection of the solvent in thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) is based on the elutropic series. However this series is based on values obtained for pure solvents. Selection of a binary, ternary etc... solvent mixture and predicting the separation results and elution times of the components of a mixture may not be that simple. In reverse and normal phase liquid chromatography elution times of the solute are a function of the properties of the stationary and mobile phases. Snyder (12) gave the following equation for calculating the polarity of a binary solvent mixture:

$$P' = \Phi_1 P_1 + \Phi_2 P_2 \qquad (I)$$

ϕ_1 and ϕ_2 are the volume fractions of solvents 1 and 2, P_1 and P_2 are the polarity of pure solvents 1 and 2 and P' is the polarity of the mixture. This relation does not apply to normal phases where the calculations are more complicated (12).

The relation between retention time and solvent strength is described by the following equation (12):

$$\log K'_1/K'_2 = \alpha A_s (\varepsilon^\circ_2 - \varepsilon^\circ_1) \qquad (II)$$

Where K' is the capacity factor, and ε° is the solvent strength parameter of solvents 1 and 2. A_s is molecular area of adsorbed sample and α is adsorbent surface activity function, and

$$K' = (R_t - R_{to})/R_{to} \qquad (III)$$

It was found that the relation in equation (II) does not always hold (13-15). For example, when two mobile phases (acetonitrile/water and methanol/water)

which have the same solvent strength (calculated according to eq. I) were used to elute the same solutes (naphthalene and biphenyl) on the same solid phase the retention times were not the same (15). A term was later added to eq (II) which accounted for solvent-solute interactions (13,14).

Since different solvents give different selectivities (11,16-18), changing the solvent composition may result in different elution orders depending on the properties of the sample mixture and the solvent chosen. For a mobile phase mixture, solvent strength (polarity) in general determines elution distance of the solutes (i.e. R_t), while mobile phase composition determines its selectivity. The composition of the mobile phase would determine the degree of separation (α), between two adjacent peaks i and ii, where

$$\alpha = K'_{ii}/K'_{i} \qquad (IV)$$

Based on Snyder's theory (12), Saunders (17) presented a graphical representation based on $\varepsilon°$ for selecting a solvent for adsorption liquid chromatography. The application of these graphs is rapid and provides a reasonable first approximation to a solvent mixture appropriate for a given sample. It must be stressed that the results are approximate, and in some cases, the solvent mixture will not be ideal (17).

For a given sample and adsorbent, log K' varies linearly with $\varepsilon°$. This is generally true for K' values between 1 and 10, which is an acceptable working range allowing separation of a component from the mixture, but does not lead to dilution of the sample or long retention times. Solvent strength (polarity) gives a general indication of solute retention but it may not predict the correct retention times (15,16,19).

In their work Glajch _et al_ (4) used eq (I) to select solvents that gave the same K' values. We have used solvent strength to predict approximate retention times, which in turn were used to predict the resolution (R_s) between two adjacent peaks;

$$R_s = (R_{t2} - R_{t1}) \;/\; 1/2(W_1 + W_2) \qquad (V)$$

Where R_t is the time of elution of peak maximum, and W is the baseline width of the peak in units of time.

Also, resolution in liquid chromatography has been defined (16) by the following equation:

$$R_S = 1/4\ (\alpha - 1)\ (N)^{1/2}\ (K'/1 + K') \qquad (VI)$$

where the number of theoretical plates, N is defined as:

$$N = 16\ (R_t/W)^2 \qquad (VII)$$

Note that all the above factors are a function of R_t (equations III-VII)

The three terms in eq. (VI) should be optimized to achieve maximum resolution. However, if the experimental conditions (flow rate, column dimensions and particle size and properties of sample) are kept constant the only parameter effecting separation is the mobile phase. The composition of which will determine not only the retention times of the solutes but also their order of elution. It is important to have a solvent which will give reasonable retention times for all components of the mixture, R_t between 5 - 40 min, in HPLC, and an R_f value of 0.2 - 0.7 in TLC.

The resolution values for HPLC were calculated according to eq. (V). For TLC, resolution was defined as $R_{fn} - R_{f(n-1)}$. We found this to be simple and human error is eliminated from the measurement of spot width. Otherwise a densitometer should be used to scan the spots and calculate R_S as defined in eq. (V).

RESULTS AND DISCUSSION

The basis for statistical data analysis in both cases was the work of Snee (10). However, in the present work a cubic equation was used where nine data points were required for an answer and the tenth point allows for goodness of fit. In their work (4) a quadratic equation was used where seven data points were required for solvent optimization, and three for checking the system. Belinky (5) on the other hand used 17 data points: he used acetonitrile, methanol and water to form four solvent systems (pure acetonitrile, pure methanol, 60% methanol and 70% acetonitrile) from which an optimum mobile phase was selected. This is time consuming when the mixture contains more than four components.

The separation of alfatoxins B_1, B_2, G_1 and G_2 on silica gel TLC plates was used to test the method and to see if the optimum mobile phase selected from the ORM calculations would optimize the resolution between the aflatoxin pairs B_1-B_2, B_2-G_1 and G_1-G_2. As base solvent, chloroform was selected based on literature data (20). The solvent combinations used are listed in Table 2 along with the resolution between the peaks of the adjacent pairs. Note that the resolution between aflatoxin B_1 and B_2 is always equal to or greater than 5.30, no matter what solvent composition is used as the mobile phase. However this is not the case for aflatoxin pairs B_2-G_1 and G_1-G_2. Table 2 also indicates that no solvent combination gives a resolution greater than 5 between the three pairs of aflatoxins. Our aim therefore, is to find a mobile phase which would maximize the resolution between the four aflatoxins, and which would give a resolution value greater than 5.15 between each of the aflatoxin pairs. The contour plots generated from the data in Table 2 is shown in Figs. 1-3. Fig. 1 shows that any solvent combination would give a minimum resolution of 5.15 between aflatoxins B_1 and B_2. Fig. 2 shows (the shaded area) where the minimum resolution between aflatoxins B_2 and G_1 is equal to or greater than 5.15. Fig. 3, the shaded area shows that solvent combination which would produce a minimum resolution of 5.15 between aflatoxins G_1 and G_2. Fig. 4 is th contour diagram for the resolution of the four aflatoxins obtained from the union of the diagrams of the individual aflatoxin pair resolutions Figs. 1-3. The four ▨ in the center of the triangle (Fig. 4) gives the solvent combination where the resolution between each pair of the four aflatoxins is greater than 5.15. The 0 indicate areas of mobile phase combinations which will give a resolution greater than 5.15 between 3-4 aflatoxins. The dotted areas indicate mobile phase compostions which will give a resolution greater than 5.15 for 2-3 aflatoxins. Fig. 5 gives the area of maximum total resolution, i.e. the sum of the resolutions between the four aflatoxins. This is not necessarily the best resolution between each pair. The ▨ in the center of the triangle corresponds to mobile phase compositions which would give maximum resolution. Good correlation was obtained between

predicted and experimental values for the separation of the aflatoxins. Other examples will follow.

Reverse phase C_{18} plates were used for the separation of naphthalene (N), 1-methylnaphthalene (M), 1-ethylnaphthalene (E), and 1,3-dimethylnaphthalene (D). Solvents used and resolutions obtained are listed in Table 3. The contour plot, Fig. 7, predicts an optimal solvent mixture (⊠) containing only methanol and 2-ethoxyethanol. These two solvents, therefore, are mainly responsible for the separation, while acetonitrile does not help. This means that CH_3CN/H_2O (80/20) is a bad choice. If the resolutions obtained are unsatisfactory, the analyst may choose to vary the ratios of CH_3 CN/H_2O or an entirely different organic modifier. Table 5 shows the predicted and experimental resolutions obtained using reverse phase C_{18} plates and different mobile phases of various compositions.

It is also possible to select one solvent (B) which gives better resolution of the components of a mixture than the other two solvents (A & C). The contour plot will show a bias toward solvent (B), Fig. 8. In this case, other solvents should be substituted for A & C. These examples show that the initial selection of the individual mobile phases is an important step which can lead to good resolution using the three organic modifiers.

HPLC results indicated that this solvent selection system can be successfully applied to mobile phase optimization. Peak crossover due to different solvents can easily be handled by this method for both HPLC and TLC. Figs. 9-11, show the separation of a napthalene, biphenyl, anthraquinone, methyl- and ethylanthraquinone mixture. Note the peak crossover in each of the solvents used. Fig. 12 shows the separation using the predicted mobile phase mixture on reverse phase C_8 column.

Although peak crossover occurred in each of the solvents used in HPLC, Figs. 9-11 this was not the case in TLC. Only when 42% THF/water was used did peak crossover occur, (Table 6) as shown by the negative R_s values. This may be due to differences of carbon loading and manufacturing processes of the plates and the columns.

Table 5

Predicted and experimental peak pair resolutions for N - M, M - E and E - D, using reverse phase TLC plates and selected mobile phases from the contour plot.

Selected Mobile Phase	Compound	Predicted Resolution	Experimental Resolution
95% Methanol: 75% 2-Ethoxyethanol	D		
(2:3)	E	8.1	8.1
	M	5.8	5.4
	N	3.5	3.6
95% Methanol: 75% 2-Ethoxyethanol	D		
(3:2)	E	7.9	7.9
	M	5.1	5.0
	N	3.5	4.2
95% Methanol: 75% 2-Ethoxyethanol	D		
(1:1)	E	8.1	7.5
	M	5.4	5.9
	N	3.5	3.5
95% Methanol: 42%	D		
80% Acetonitrile: 4%	E	8.1	7.9
75% Ethoxyethanol: 54%	M	5.7	5.0
	N	3.4	3.5

S T A T I S T I C A L A N A L Y S I S S Y S T E M

NUMEER OF PEAKS WHERE RESOLUTION>3.5

NAPHTHALENE STUDY - TLC -A=95%CH3OH,B=80%CH3CN,C=75%2ETHO

CONTOUR PLOT OF Y*X

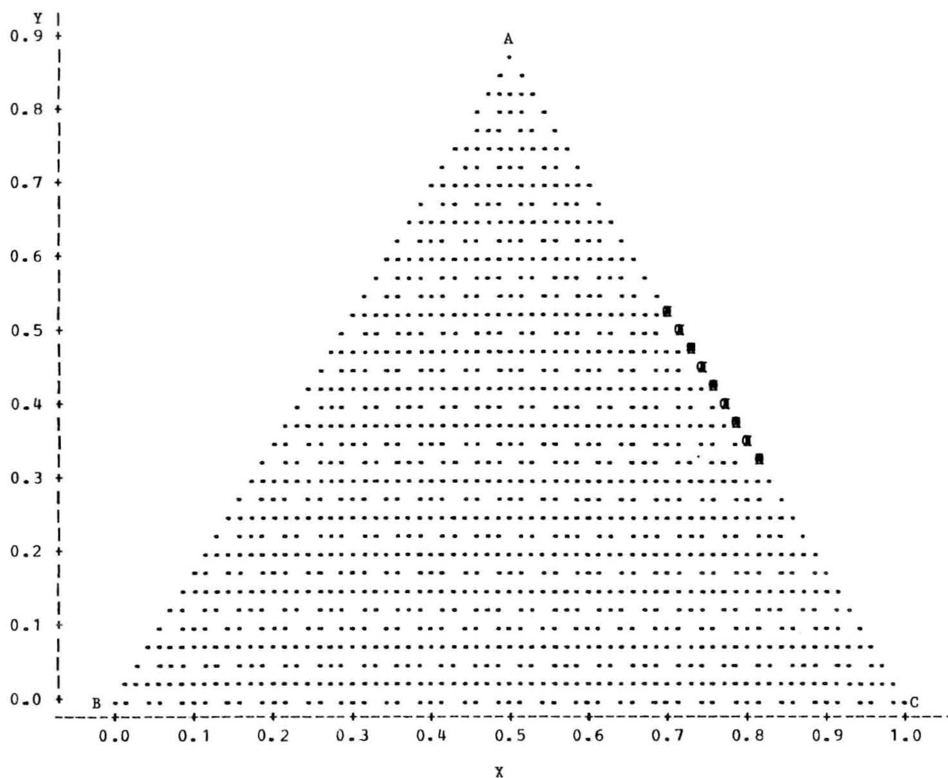

Fig. 7 Contour plot of the naphthalenes N, M, E and D using reverse phase TLC plates and 95% CH_3OH/H_2O (A), 80% CH_3CN/H_2O (B) and 75% 2-ethoxy ethanol/H_2O (C). Shaded circles (■) designate mobile phase composition that would give resolution greater than 3.5.

S T A T I S T I C A L   A N A L Y S I S   S Y S T E M

NUMBER OF PEAKS WHERE RESOLUTION>2.25

ANTHRA & NAPTH-BI - HPLCS - A=64%CH3CN,B=42%THF,C=72%MEOH

CONTOUR PLOT OF Y*X

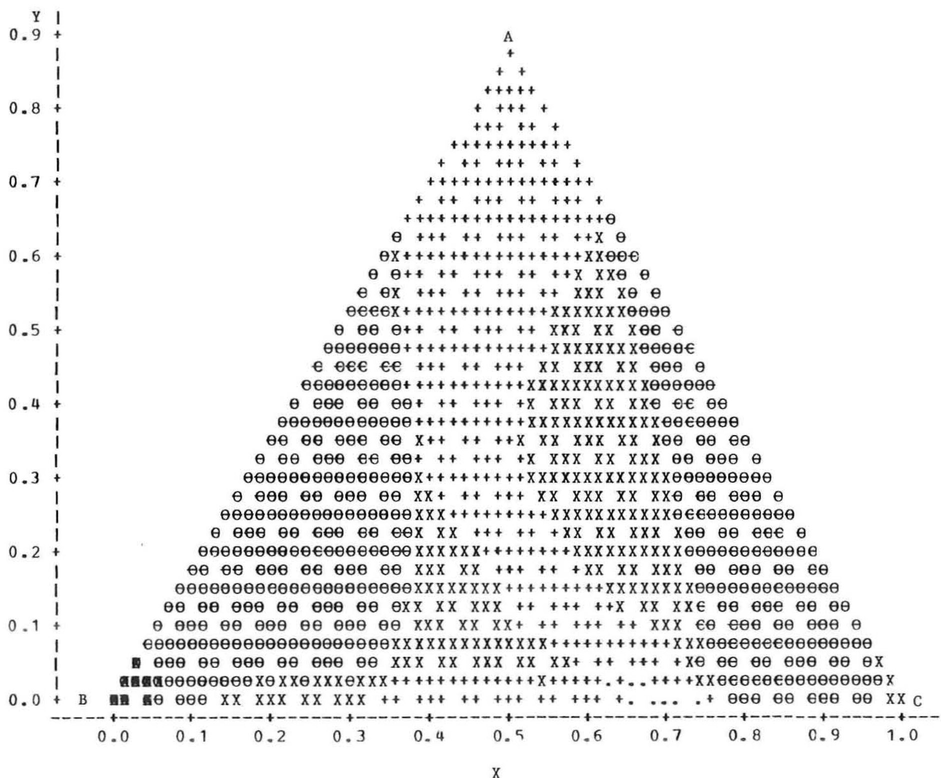

Fig. 8 Contour plot of naphthalene, biphenyl, anthraquinone, methyl- and ethyl anthraquinone, using reverse phase TLC plates and 64% CH_3CN/H_2O (A), 42% THF/H_2O (B) and 72% CH_3OH/H_2O (C). Shaded circles (■) are optimum mobile phase compositions.

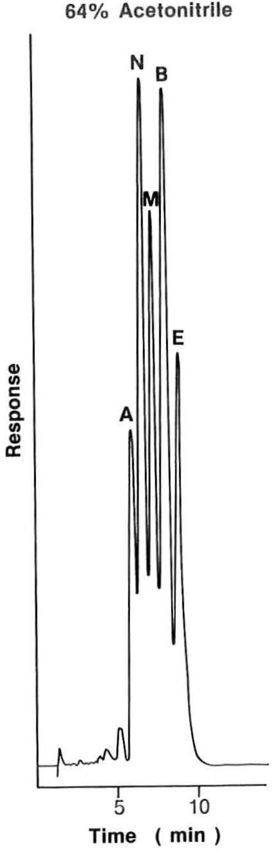

Fig. 9 HPLC separation of anthraquinone (A), naphthalene (N), 1-methyl-naphthalene (M), 1-ethylnaphthalene (E), and 1-3-dimethyl-naphthalene (D) on reverse phase C_8 column using 64% CH_3CN/H_2O, at a mobile phase flow rate of 1.2 ml/min.

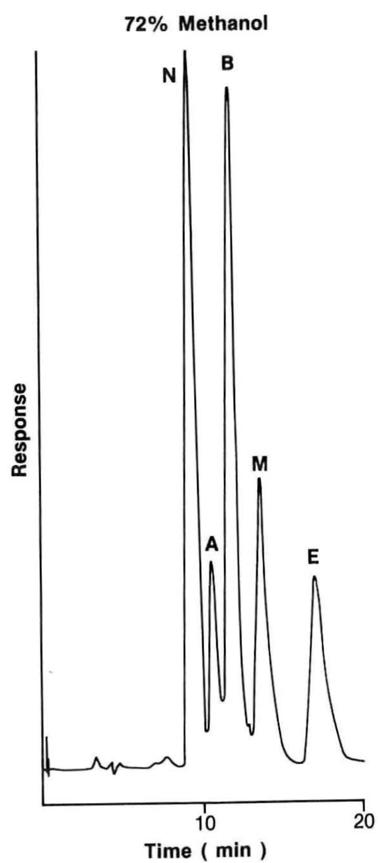

Fig. 10 Same as Fig. 9 but the mobile phase used is 72% CH_3OH/H_2O.

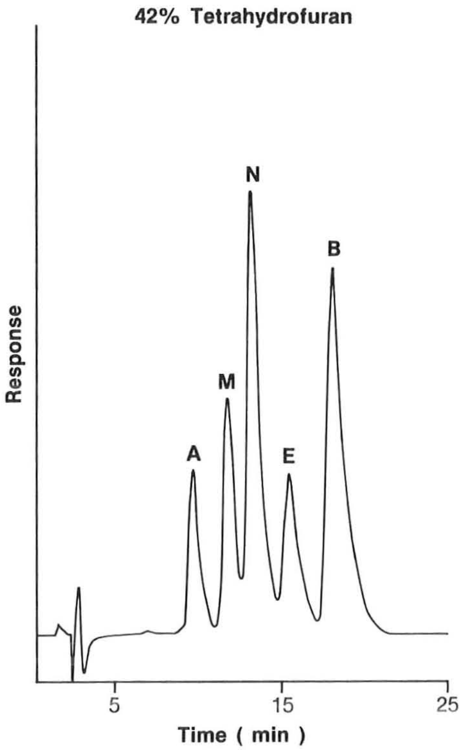

Fig. 11 Same as Fig. 9 but the mobile phase used is 42% THF/H_2O.

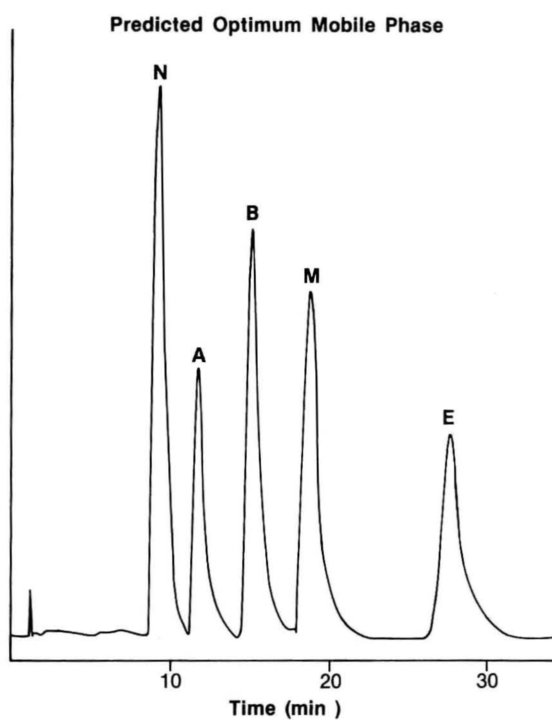

Fig. 12 Separation of A, N, M, B and E on reverse phase C_8 column using a predicted mobile phase of 64% CH_3CN:72% CH_3OH:42% THF (10:67:23), at a flow rate of 1.2 ml/min.

Table 6

Solvent combinations and peak pair resolutions for N, A, B, M, and E, and experimental resolutions obtained using reverse phase C_8 HPLC column and 72% $CH_3OH:H_2O$, 64% $CH_3CN:H_2O$ and 42% $THF:H_2O$.

Solvent	Mobile Phase Ratios									
72% CH_3OH	1.00	0.0	0.0	0.50	0.50	0.0	0.33	0.67	0.16	0.16
64% CH_3CN	0.0	1.00	0.0	0.50	0.0	0.50	0.33	0.16	0.67	0.16
42% THF	0.0	0.0	1.00	0.0	0.50	0.50	0.33	0.16	0.16	0.67
R_s(N - A)	0.8	4.6	2.1	0.4	4.2	5.0	3.7	2.1	4.2	5.5
R_s(N - B)	7.5	6.7	1.7	7.1	4.2	5.8	4.5	5.4	6.2	3.4
R_s(N - M)	9.2	11.7	4.2	9.2	8.8	10.8	5.4	9.2	11.2	10.5
R_s(N - E)	11.7	13.8	3.3	12.1	7.9	10.8	5.4	10.4	12.1	8.8
R_s(A - B)	6.7	2.1	-0.4	6.7	0.0	0.8	0.8	3.3	2.0	-2.1
R_s(A - M)	8.4	7.1	2.1	8.8	4.6	5.8	1.7	7.1	7.0	5.0
R_s(A - E)	10.9	9.2	1.3	11.7	3.7	5.8	1.7	8.3	7.9	3.3
R_s(B - M)	1.7	5.0	2.5	2.1	4.6	5.0	0.9	3.8	5.0	7.1
R_s(B - E)	4.2	7.1	1.75	5.0	0.9	5.0	0.9	5.0	5.9	5.4
R_s(M - E)	2.5	2.1	-0.8	2.9	-0.9	0.0	0.0	1.2	0.9	-1.7

Table 7 shows good agreement between the predicted and experimental resolution of the five components of the mixture using RP-8 TLC plates.

CONCLUSION

The method described here employs statistical data analysis to predict optimum ternary mobile phase compositions in a systematic and straightforward manner in contrast to operator intuition. The initial selection of the three solvents, of which the final mixture is composed, is important and will affect the degree of separation and resolution of adjacent peaks. The method is easily applied to both TLC and HPLC. Good agreement was observed between predicted

Table 7

Predicted and experimental peak pair resolutions for N, A, B, M, and E, using reverse phase C_8 TLC plates and selected mobile phases from the contour plot.

SELECTED MOBILE PHASE	COMPOUND		COMPOUND	PREDICTED RESOLUTION	EXPERIMENTAL RESOLUTION
72% CH_3OH:64% CH_3CN	N	-	A	3.4	4.7
1:9	N	-	B	6.8	7.7
	N	-	M	11.1	12.0
	N	-	E	13.5	14.1
	A	-	B	3.4	3.5
	A	-	M	7.8	7.3
	A	-	E	10.1	9.4
	B	-	M	4.3	4.3
	B	-	E	6.7	6.4
	M	-	E	2.3	2.1
72% CH_3OH:64% CH_3CN	N	-	A	2.7	2.9
16:84	N	-	B	6.9	7.0
	N	-	M	10.8	11.6
	N	-	E	13.3	13.7
	A	-	B	4.1	4.1
	A	-	M	8.1	8.7
	A	-	E	10.5	10.8
	B	-	M	3.9	4.6
	B	-	E	6.4	6.7
	M	-	E	2.5	2.6

and experimental data. To the best of our knowledge, this is the first systematic statistical method of solvent selection for normal and reverse phase TLC.

ACKNOWLEDGEMENT

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APPENDIX 1

```
C                                                                       0001
C PROGRAM PEAKIN:                                                       0002
C       THIS PROGRAM ACCEPTS PEAK RESOLUTIONS FROM UP TO 20 SOLVENT     0003
C       MIXTURES, CORRECTS FOR CROSSOVER IF NECESSARY, AND OUTPUTS A    0004
C       DATA FILE THAT IS PASSED TO A SAS ROUTINE TO ANALYZE THE BEST   0005
C       SOLVENT MIXTURE TO USE FOR MAXIMUM PEAK RESOLUTION OF ALL       0006
C       PEAKS.                                                          0007
C                                                                       0008
C       IF PEAKS DO NOT CROSSOVER FOR DIFFERENT MIXTURES, ONLY          0009
C       RESOLUTIONS FOR ADJACENT PEAKS ARE USED.  IF CROSSOVER DOES     0010
C       OCCUR, ALL PEAK RESOLUTIONS ARE USED                            0011
C                                                                       0012
C       CARD INPUT ORDER:                                               0013
C            TITLE                                                      0014
C            NAMES OF SOLVENTS - 3A8                                    0015
C            CROSSOVER OPTIONS - 'CROSS' OR 'NOCROSS'                   0016
C            # OF PEAKS                                                 0017
C            REPEAT AS NEEDED:                                          0018
C                SOLVENT COMPOSITION - SHOULD ADD TO 1.0 - 3F5          0019
C                IF 'NOCROSS' -                                         0020
C                    RESOLUTIONS OF ADJACENT PEAKS  -  ONE/CARD         0021
C                         RESOL 1 - 2                                   0022
C                         RESOL 2 - 3                                   0023
C                             :                                         0024
C                         RESOL N-1 - N                                 0025
C                                                                       0026
C                IF 'CROSS' -                                           0027
C                    ORDER OF PEAKS - 20I3                              0028
C                    RESOLUTIONS OF ALL PEAKS  -  ONE/CARD              0029
C                         RESOL 1 - 2                                   0030
C                         RESOL 1 - 3                                   0031
C                             :                                         0032
C                             :                                         0033
C                         RESOL 1 - N                                   0034
C                         RESOL 2 - 3                                   0035
C                             :                                         0036
C                         RESOL 2 - N                                   0037
C                             :                                         0038
C                         RESOL N-1 - N                                 0039
C                                                                       0040
```

```
      DOUBLE PRECISION A,B,C                                              0041
      DIMENSION DATA(190,20),ACOMP(20),BCOMP(20),CCOMP(20),NORD(20)       0042
      DIMENSION TITLE(20)                                                 0043
      DATA CROSS/'CROS'/                                                  0044
      INTEGER P1,P2,P11                                                   0045
C                                                                         0046
      READ(5,15) TITLE                                                    0047
15    FORMAT(20A4)                                                        0048
      READ(5,10)A,B,C                                                     0049
10    FORMAT(3A8)                                                         0050
      READ(5,11) OPTION                                                   0051
11    FORMAT(A4)                                                          0052
      COPT=0                                                              0053
      IF(OPTION.EQ.CROSS) COPT=1                                          0054
      READ(5,*)NPEAK                                                      0055
      NPEAK1=NPEAK-1                                                      0056
      NPOINT=0                                                            0057
100   READ(5,12)A1,B1,C1                                                  0058
12    FORMAT(3F5.0)                                                       0059
      IF(A1+B1+C1.LE.0) GO TO 1000                                        0060
      NPOINT=NPOINT+1                                                     0061
      ACOMP(NPOINT)=A1                                                    0062
      BCOMP(NPOINT)=B1                                                    0063
      CCOMP(NPOINT)=C1                                                    0064
      IF(COPT.EQ.1) GO TO 500                                             0065
C                                                                         0066
C INPUT NO CROSSOVER PEAK RESOLUTIONS                                     0067
      DO 150 P1=1,NPEAK1                                                  0068
      P2=P1+1                                                             0069
      NPTR=(P1-1)*NPEAK+(P2-1)                                            0070
150   READ(5,*) DATA(NPTR,NPOINT)                                         0071
      GO TO 100                                                           0072
C                                                                         0073
C INPUT CROSSOVER PEAK RESOLUTIONS                                        0074
500   READ(5,13)NORD                                                      0075
13    FORMAT(20I3)                                                        0076
      DO 550 I=1,NPEAK1                                                   0077
      I1=I+1                                                              0078
      DO 550 J=I1,NPEAK                                                   0079
      P2=NORD(J)                                                          0080
      P1=NORD(I)                                                          0081
      IF(NORD(I).LT.NORD(J)) GO TO 510                                    0082
      P1=NORD(J)                                                          0083
      P2=NORD(I)                                                          0084
510   NPTR=(P1-1)*NPEAK+(P2-1)                                            0085
550   READ(5,*) DATA(NPTR,NPOINT)                                         0086
      GO TO 100                                                           0087
C                                                                         0088
C PRINT TABLE OF RESULTS                                                  0089
1000  WRITE(6,20) TITLE                                                   0090
20    FORMAT('1'//1X,20A4//' DATA ENTERED BY COMPOSITION'//)              0091
      WRITE(6,21)A,(ACOMP(I),I=1,NPOINT)                                  0092
21    FORMAT(1X,A8,20F6.2)                                                0093
      WRITE(6,21)B,(BCOMP(I),I=1,NPOINT)                                  0094
      WRITE(6,21)C,(CCOMP(I),I=1,NPOINT)                                  0095
      WRITE(6,22)                                                         0096
22    FORMAT(/2X,'P1  P2  -  RESOLUTIONS:'/)                              0097
      IF(COPT.EQ.1) GO TO 2000                                            0098
      DO 1050 P1=1,NPEAK1                                                 0099
      P2=P1+1                                                             0100
      NPTR=(P1-1)*NPEAK+(P2-1)                                            0101
      WRITE(6,23)P1,P2,(DATA(NPTR,I),I=1,NPOINT)                          0102
23    FORMAT(2I4,1X,20F6.2)                                               0103
C                                                                         0104
C OUTPUT DATA TO FILE                                                     0105
      DO 1050 I=1,NPOINT                                                  0106
      WRITE(11,24)P1,P2,ACOMP(I),BCOMP(I),CCOMP(I),DATA(NPTR,I)           0107
24    FORMAT(2I4,3F6.2,F10.3)                                             0108
1050  CONTINUE                                                            0109
      STOP                                                                0110
2000  DO 2050 P1=1,NPEAK1                                                 0111
      P11=P1+1                                                            0112
      DO 2050 P2=P11,NPEAK                                                0113
      NPTR=(P1-1)*NPEAK+(P2-1)                                            0114
      WRITE(6,23)P1,P2,(DATA(NPTR,I),I=1,NPOINT)                          0115
      DO 2050 I=1,NPOINT                                                  0116
      WRITE(11,24)P1,P2,ACOMP(I),BCOMP(I),CCOMP(I),DATA(NPTR,I)           0117
2050  CONTINUE                                                            0118
      STOP                                                                0119
      END                                                                 0120
```

APPENDIX 2

```
*****************************************************************************

  SAS ANALYSIS OF SOLVENT MIXTURE RESOLUTION DATA

  CHANGE 'XXX' TO CUTOFF RESOLUTION
  CHANGE 'ASSAY TITLE' TO TITLE OF ASSAY

*****************************************************************************

  DATA SET1;
     INPUT PEAK1 PEAK2 A B C RESOL;
     INFILE PEAK;

  PROC SORT;
      BY PEAK1 PEAK2;

  DATA SET2;
     SET SET1; BY PEAK1 PEAK2;
           X=1.232*A+1.732*C-(1-B)*.732;
           Y=A*.866;
      OUTPUT;
      IF LAST.PEAK2 THEN DO;
         RESOL=.;
         DO A=0 TO 1 BY .02;
            DO B=0 TO 1-A BY .02;
               C=1-A-B;
                  X=1.232*A+1.732*C-(1-B)*.732;
                  Y=A*.866;
                  OUTPUT;
            END;
      END;
  END;

  PROC GLM; BY PEAK1 PEAK2;
       MODEL RESOL=A B C A*B A*C B*C A*B*C/NOINT;
       OUTPUT OUT=SET3 P=RESPRED;

  DATA SET4;
       SET SET3;
       IF RESPRED>XXX THEN GTRES=1;
              ELSE GTRES=0;
       EDGE=GTRES;
       IF A=0 OR B=0 OR C<.01 THEN DO; EDGE=2; END;
       OUTPUT;

  PROC PLOT; BY PEAK1 PEAK2;
       PLOT Y*X=EDGE / CONTOUR=3 S1=' ' S2='#' S3='.'
                               HPOS=80 HAXIS=0 TO 1 BY .1
                              VAXIS=0 TO .9 BY .1;
       TITLE4 GTRES=1 - RESOLUTION>XXX   GTRES=0 - RESOLUTION<=XXX;
       TITLE8  ASSAY TITLE;

  PROC SORT;
       BY A B C PEAK1 PEAK2;

  PROC PRINT;
       VAR A B C PEAK1 PEAK2 RESPRED;

  PROC SORT;
       BY A B C;

  DATA TOTAL;
       SET SET4 END=EOF; BY A B C;
       IF FIRST.C THEN DO; TOTGTRES=0; TOTRESOL=0; END;
       IF RESOL=. THEN DO;TOTGTRES+GTRES; TOTRESOL+RESPRED; END;
       IF LAST.C THEN DO; IF TOTRESOL>0 THEN OUTPUT; END;
       IF EOF THEN DO; IF TOTRESOL>0 THEN OUTPUT; END;

  PROC PLOT;
       PLOT Y*X=TOTGTRES/CONTOUR=8 HPOS=80 HAXIS=0 TO 1 BY .1
                         VAXIS=0 TO .9 BY .1;
       TITLE4 NUMBER OF PEAKS WHERE RESOLUTION>XXX;
       TITLE8  ASSAY TITLE;

  PROC PLOT;
       PLOT Y*X=TOTRESOL/CONTOUR=8 HPOS=80 HAXIS=0 TO 1 BY .1
                         VAXIS=0 TO .9 BY .1;
       TITLE4 SUM OF PEAK RESOLUTIONS FROM EACH MIX;
       TITLE8  ASSAY TITLE;
```

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A MODEL OF LIQUID ADSORPTION CHROMATOGRAPHY INVOLVING SOLUTE-SOLVENT INTERACTION IN THE MOBILE PHASE, ENERGETIC HETEROGENEITY OF THE ADSORBENT, AND DIFFERENCES IN MOLECULAR SIZES OF SOLUTE AND SOLVENTS

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ABSTRACT

A simple model for liquid-solid chromatography (LSC) process with mixed mobile phases has been proposed. According to this model the LSC process is represented by suitable reversible phase-exchange reactions between molecules of solute and solvents and reversible solute-solvent reactions in the mobile phase. These reactions describe the competitive adsorption of solute molecules and formation of solute-solvent complexes in the mobile phase. Analytical equations for the capacity ratio, derived in terms of the model, involve solute-solvent interaction in the mobile phase, differences in molecular sizes of solute and solvents, energetic heterogeneity of the adsorbent and ideality of the

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surface phase. Linear forms of these equations are very convenient for analysing the experimental chromatographic data.

INTRODUCTION

Theoretical and experimental studies of LSC with mixed mobile phases show that this process is determined by many factors. They are :

(a) competitive character of solute and solvent adsorption,
(b) non-specific solute-solvent and solvent-solvent interactions in the mobile and surface phases,
(c) specific solute-solvent and solvent-solvent interactions in the mobile and surface phases,
(d) dissociation of solutes in the mobile phase,
(e) differences in molecular sizes of solutes and solvents,
(f) energetic heterogeneity of the adsorbent and topography of adsorption sites onto surface,
(g) multilayer character of the surface phase and partition effects in LSC process, and
(h) orientation of solute and solvent molecules in the surface phase.

Quantitative estimation of the above effects was discussed by many authors (1-26). The most popular

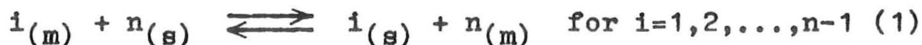
approach to LSC with binary mobile phases has been formulated by Snyder (1,2), which assumes the competitive character of solute and solvent adsorption. This approach involves also energetic heterogeneity of the solid surface (1) and solute-solvent localization effects (3). Recently, Snyder's treatment has been developed for LSC with multicomponent mobile phases (4-6). The LSC model of Snyder (1,2), described exactly in the review of Snyder and Poppe (7), has been considerably enriched by Soczewiński's studies (8, 9). On the other hand, Jaroniec et al.(10-17) described theoretically the LSC process by applying the general theory of adsorption from multicomponent liquid mixtures on solid surfaces and utilizing the fundamental studies of Snyder (1,2) and Soczewiński (8,9). The treatment of Jaroniec et al. involves energetic heterogeneity of the solid (10,12,15), non-ideality of both phases (10,11,13,14), differences in molecular sizes of solutes and solvents (13,14),and solute - solvent and solvent-solvent interactions (16,17). Recent studies of Jaroniec et al.(13,14,26) concern the correlation between adsorption and chromatographic parameters.

In this paper we shall present a general description of LSC process involving the formation of

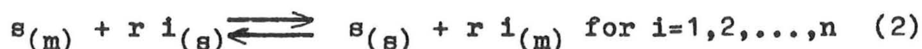
solute-solvent complexes in the mobile phase, energetic heterogeneity of the solid surface, and differences in molecular sizes of solute and solvents.
In this description the most important factors determining the LSC process are considered.

GENERAL CONSIDERATIONS

Let us consider the LSC process for the s-th substance (solute) chromatographed in n-component eluent. The components of the mixed eluent are numbered successively beginning from the most efficient eluting solvent to the weakest solvent. Thus, 1-st solvent is the more efficient one, however, n-th component is the weakest solvent. One of the main assumptions of the proposed model is that concerning the competitive character of solute adsorption. The adsorption process may be represented by the following reversible phase-exchange reactions (10) :



$$i_{(m)} + n_{(s)} \rightleftharpoons i_{(s)} + n_{(m)} \quad \text{for } i=1,2,\ldots,n-1 \quad (1)$$



$$s_{(m)} + r\, i_{(s)} \rightleftharpoons s_{(s)} + r\, i_{(m)} \quad \text{for } i=1,2,\ldots,n \quad (2)$$

where the subscripts (m) and (s) refer to the mobile and surface phases, respectively, "i" and "s" denote molecules of the i-th solvent and s-th solute, and r

is the ratio of molecular sizes of the s-th solute and i-th solvent. The reactions 1 and 2 have been written by assuming the equality of molecular sizes of all solvents, i.e.,

$$w_1 = w_2 = \cdots = w_n = w \tag{3}$$

and inequality of molecular sizes of solute and solvents :

$$w_s \neq w \tag{4}$$

Thus, the parameter r is defined as follows :

$$r = w_s/w \tag{5}$$

The next assumptions are following :

(a) non-specific interactions between molecules of solute and solvents in the mobile phase are neglected,

(b) the surface phase is assumed to be monolayer and ideal,

(c) molecules of the s-th solute form complexes with molecules of 1-st solvent in the mobile phase,

(d) the total number of moles of all solvents in the surface phase is constant and independent upon the presence of solute molecules, because the solute concentration is infinitely low,

(e) molecules of solute and solvents have a spherical shape,

(f) the adsorbent surface may be energetically homogeneous and heterogeneous.

Since, the surface phase is ideal, the adsorption depends upon the global distribution of adsorption sites, however, it is independent upon topography of adsorption sites onto surface (27).

It means that our theoretical considerations, concerning heterogeneous adsorbents, are valid for solids showing random and patchwise distribution of adsorption sites onto surface.

Now, we shall return to the assumption (c). According to this assumption, molecules of the s-th solute form associates with molecules of the most efficient eluting solvent in the mobile phase. However, the silanol groups of the silica surface can compete with complexes in the surface phase. Thus, stronger interactions of molecules of the s-th solute and 1-st solvent with the silica silanols can preclude solute-solvent interactions in the surface phase. Taking into account the possibility of destruction of the solute-solvent complexes in the surface phase by the silica silanols, we assume that these complexes form only in the mobile phase.Moreover, let us assume that one molecule of the solute can bound q molecules of 1-st solvent according to the following reaction :

$$s_{(m)} + q\, 1_{(m)} \rightleftharpoons 1_q s_{(m)} \qquad (6)$$

where $1_q s_{(m)}$ denotes the (q+1)-molecular complex in the mobile phase.

In the next section equations for the capacity ratio will be discussed by using the assumptions (a) - (f). However, in the Appendix we shall discuss the possibility of extension of these equations by applying the additional assumption that molecules of 1-st solvent form p-molecular complexes.

EQUATIONS FOR THE CAPACITY RATIO

Homogeneous surfaces

According to Snyder (1) the capacity ratio for the s-th solute, k_s' , chromatographed in the mixed mobile phase is proportional to the distribution coefficient k_s :

$$k_s' = \beta\, k_s \qquad (7)$$

where

$$k_s = y_s^o / x_s^o \qquad (8)$$

and y_s^o and x_s^o are the total mole fractions of the s-th solute in the surface and mobile phases, respectively, however, β is characteristic for a given adsorbent and independent upon the eluent nature in a good approximation.

The thermodynamic equilibrium constant relating to the reaction between molecules of the s-th solute and 1-st solvent on a homogeneous surface is :

$$K_{1s} = (y_s/x_s)\cdot(x_1/y_1)^r \tag{9}$$

However, the equilibrium constant for the reaction 6 is expressed as follows :

$$L_q = x_q/(x_s\ x_1{}^q) \tag{10}$$

In the above x_s and y_s are the mole fractions of single molecules of the s-th solute in the mobile and surface phases,respectively, x_q is the mole fraction of the complex $1_q s$ in the mobile phase defined as the ratio of the number of complexes $1_q s$ to the total number of molecules in the mobile phase.

The total mole fractions of the s-th solute and all solvents in the mobile and surface phases may be expressed as follows :

$$y_s^o = y_s \tag{11a}$$

$$y_i^o = y_i \quad \text{for } i=1,2,\ldots,n \tag{11b}$$

$$x_s^o = x_s + x_q = x_s + L_q x_s x_1{}^q \tag{12a}$$

$$x_1^o = x_1 + qx_q = x_1 + qL_q x_s x_1{}^q \approx x_1 \tag{12b}$$

$$x_i^o = x_i \quad \text{for } i=2,3,\ldots,n \tag{12c}$$

Equations 7,8,9,11 and 12 give :

$$k'_s = \beta K_{1s} (y_1/x_1)^r/(1 + L_q x_1^q) \qquad (13)$$

or

$$\lg k'_s = \lg(\beta K_{1s}) + r \lg(y_1/x_1) - \lg(1+L_q x_1^q) \qquad (14)$$

For the special values of r , q and L_q equations 13 and 14 give the majority of the well-known relationships. For $L_q = 0$ (absence of solute-solvent interaction) equation 14 gives the expression derived by Jaroniec et al.(13) ; it is :

$$\lg k'_s = \lg(\beta K_{1s}) + r \lg (y_1/x_1) \qquad (15)$$

If 1-st solvent is considerably strongly adsorbed than other solvents, the mole fraction y_1 is close to unity for the whole concentration region except the low concentrations of x_1. Then, equation 15 becomes the most popular expression, called as Snyder - Soczewiński relationship (7) :

$$\lg k'_s = \lg(\beta K_{1s}) - r \lg x_1 \qquad (16)$$

For $r=1$, $y_1=1$ and $q=1$ equation (13) reduces to the expression obtained by Soczewiński (8), Jaroniec and Piotrowska (16) :

$$k'_s = \beta K_{1s}/[x_1(1 + L_1 x_1)] \qquad (17)$$

However, for r=1 (identical molecular sizes of solute and solvents) and $L_q=0$ equation 13 gives :

$$k'_s = \beta K_{1s}(y_1/x_1) \tag{18}$$

Expressing the mole fraction y_1 by (15) :

$$y_1 = K_{1n}x_1/(x_n + \sum_{j=1}^{n-1} K_{jn}x_j) \tag{19}$$

and taking into account the following equalities :

$$K_{jn} = k'_{ns}/k'_{js} \tag{20a}$$

$$k'_{1s} = \beta\ K_{1s} \text{ for } L_q = 0 \tag{20b}$$

we have :

$$1/k'_s = \sum_{j=1}^{n} (x_j/k'_{js}) \tag{21}$$

Equation 21 has been derived by Jaroniec et al.(15) and it is equivalent with Snyder's fundamental assumption (1) :

$$k'_s = \sum_{j=1}^{n} y_j\ k'_{js} \tag{22}$$

where k'_{js} is the capacity ratio of the s-th solute chromatographed in the j-th pure solvent.

Equation 13 may be transformed to the following linear form :

$$y_1{}^r/(k'_s x_1{}^r) = (\beta K_{1s})^{-1} + (L_q/\beta K_{1s})\ x_1{}^q \tag{23}$$

Two special cases of equation 23 are interesting for analysis of the chromatographic data. They are obtained from equation 23 for $r = 1$:

$$y_1/(k_s' x_1) = (\beta K_{1s})^{-1} + (L_q/\beta K_{1s})\, x_1^{\,q} \qquad (24)$$

and for $q = 1$:

$$y_1^{\,r}/(k_s' x_1^{\,r}) = (\beta K_{1s})^{-1} + (L_1/\beta K_{1s})\, x_1 \qquad (25)$$

The mole fraction y_1 appearing in equations 24 and 25 may be evaluated by using the analytical equation 19 or by applying the following relationship (10,13):

$$y_1 = n_1^e/n^s + x_1 \qquad (26)$$

where n_1^e is the adsorption excess of 1-st solvent, which may be measured experimentally, and n^s is the total number of moles in the surface phase. Assuming $y_1 = 1$ (this assumption is frequently used in LSC) equations 24 and 25 reduce to the very simple relations :

$$1/(k_s' x_1) = (\beta K_{1s})^{-1} + (L_q/\beta K_{1s})\, x_1^{\,q} \qquad (27)$$

$$1/(k_s' x_1^{\,r}) = (\beta K_{1s})^{-1} + (L_1/\beta K_{1s})\, x_1 \qquad (28)$$

Equations 27 and 28 are especially convenient for interpretation of the chromatographic data, because they define k_s' in a simple way.

Heterogeneous surfaces

Let us consider a heterogeneous surface showing L types of adsorption sites. The equilibrium constant 9 for the l-th type of adsorption sites is defined as follows :

$$K_{1s,l} = (y_{s,l}/x_s)\cdot(x_1/y_{1,l})^r \qquad (29)$$

Since, the equilibrium constant L_q refers to the mobile phase only, it may be assumed to be independent upon the distribution of adsorption sites onto surface. Thus, combining equations 7,8,11,12 and 29 we have :

$$k'_{s,l} = \beta K_{1s,l}(y_{1,l}/x_1)^r/(1 + L_q x_1{}^q) \qquad (30)$$

where

$$k'_{s,l} = y^o_{s,l}/x^o_s = y_{s,l}/x^o_s \qquad (31)$$

Following Snyder (1) and Jaroniec et al.(10,12), the capacity ratio of the s-th solute on the entire heterogeneous surface is defined as follows :

$$k'_s = \sum_{l=1}^{L} f_l\, k'_{s,l} =$$

$$[\,\beta\, x_1{}^{-r}(1+L_q x_1{}^q)^{-1}]\cdot \sum_{l=1}^{L} f_l\, K_{1s,l}(y_{1,l})^r \qquad (32)$$

where

$$\sum_{l=1}^{L} f_l = 1 \qquad (33)$$

and f_l is the ratio of the number of adsorption sites of the l-th type to the total number of adsorption sites. Using the approximation proposed by Jaroniec et al.(10,12) for the sum appearing in equation 32 we have:

$$k'_s = \beta K_{1s}\, y_1^{r/m} / [\, x_1^{r}(1 + L_q x_1^{q})] \qquad (34)$$

where m is the heterogeneity parameter, which changes from zero to unity, and K_{1s} is an averaged equilibrium constant referring to the entire surface.
Equation 34 for m=1 reduces to equation 13, which has been derived for homogeneous surfaces. However, for $L_q = 0$ it becomes the following relationship :

$$\lg k'_s = \lg(\beta K_{1s}) + (r/m)\lg(y_1/x_1^{m}) \qquad (35)$$

Equation 35 has been derived by Jaroniec et al.(14). The mole fraction y_1 may be calculated according to the relationship 26 or by using the following analytical equation (28) :

$$y_1 = (K_{1n}x_1)^m / [\, x_n + \sum_{j=1}^{n-1} (K_{jn}x_j)^m \,] \qquad (36)$$

Assuming in equation 34 r=1 and L_q=0 and applying the expression 36 for y_1, in which K_{jn} are equal to k'_{ns}/k'_{js}, we have :

$$(1/k'_s)^m = \sum_{j=1}^{n} (x_j/k'_{js})^m \qquad (37)$$

Equation 37 has been derived by Jaroniec et al.(12) and it is equivalent to the following relationship :

$$k'_s = \sum_{j=1}^{n} k'_{js}\, y_j^{1/m} \tag{38}$$

However, assuming in equations 13 and 34 that $y_1 = 1$, we obtain the following expression :

$$k'_s = \beta K_{1s} / [\, x_1^{r}(1 + L_q x_1^{q}) \,] \tag{39}$$

Thus, assumption that the surface phase contains mainly molecules of 1-st solvent, i.e., $y_1=1$, eleminates influence of the energetic heterogeneity on the LSC process. The heterogeneity effects play an important role in LSC with the mixed eluents containing similar solvents ; then $y_1 \neq 1$ in a wide concentration range.

MODEL CALCULATIONS FOR LSC WITH BINARY ELUENTS

The model calculations have been performed for binary eluent "1+2" by using the following equations:

$$k'_s = \left[\left(\frac{x_1}{k'_{1s}(1+L_q)}\right)^{m} + \left(\frac{1-x_1}{k'_{2s}}\right)^{m}\right]^{-1/m} \cdot \frac{1}{(1+L_q x_1^{q})} \tag{40}$$

and

$$k'_s = [\, k'_{1s}(1+L_q) \,] / [\, x_1^{r}(1+L_q x_1^{q}) \,] \tag{41}$$

where

$$k'_{1s} = \beta K_{1s}/(1 + L_q) \qquad (42)$$

Equation 40 has been obtained from equations 36 and 34 with r=1 ,however, equation 41 relates to the re - lationship 39.

Figure 1 shows the theoretical dependences k'_s vs. x_1 calculated according to equation 40 for different parameters m , q and L_q .All curves k'_s vs. x_1 ,presented in Figure 1, have been calculated by assuming equality of molecular sizes of solute and solvents (r=1).Figure 1a shows the k'_s-dependences drawn for $k'_{1s}=1$, $k'_{2s}=3$ (small difference in the elution strengths of both solvents), $L_q=0$ (neglect of the solute-solvent interactions in the mobile phase) and different values of m . The parameter m causes a reduction of the capacity ratio,when m tends to zero. Moreover, the k'_s-curves show a minimum for smaller values of m . The exact discussion concerning the influence of m on the k'_s-curves has been presented in (29).

Figure 1b shows the influence of q on the k'_s - curves. These curves,calculated for $k'_{1s}=1$, $k'_{2s}=3$, m= 1 , $L_q=1$ and q=1,2,4 (the solid lines),have been compared with the curve predicted by equation 21 (the dashed line); they lie above the dashed line,which is predicted by the simplest model of LSC process.

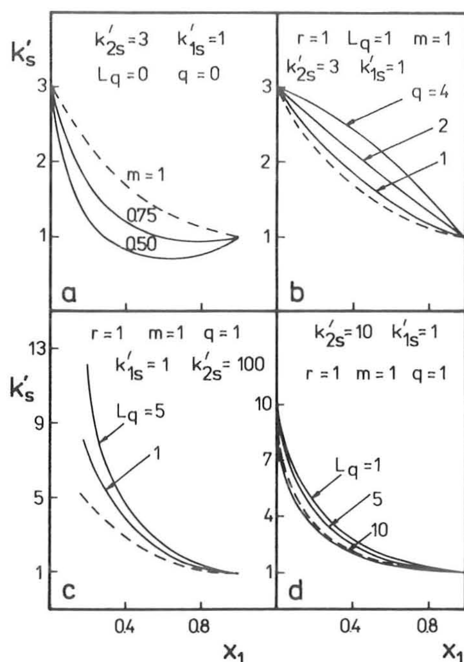


FIGURE 1 - The theoretical dependences k'_s vs. x_1 calculated according to equation 40 for different values of m , q and L_q. The dashed lines have been calculated by using equation 21 for binary eluents.

Figures 1c and 1d show the k'_s-curves calculated according to equation 40 for m=1 (energetic homogeneity of the adsorbent), q=1 (one molecule of 1-st solvent bounds one molecule of the solute) and different values of L_q . The k'_s-curves, calculated for $k'_{2s} \gg k'_{1s}$, lie above the dashed line (c.f., Figure 1c). The distances between the solid lines and the dashed line increase gradually with increasing of L_q . The more complex

behaviour of the k'_s-curves is observed for similar values of k'_{1s} and k'_{2s} (c.f.,Figure 1d).

Figure 2 shows the k'_s-curves calculated according equation 41 for $k'_{1s}=1$ and different values of r , q and L_q .These curves are compared with the Snyder - Soczewiński dependence 16 for r=1 (the dashed lines). Figure 1a presents the typical k'_s-dependences predicted by Snyder-Soczewiński relationship 16 for r = 0.5, 1 and 2 .

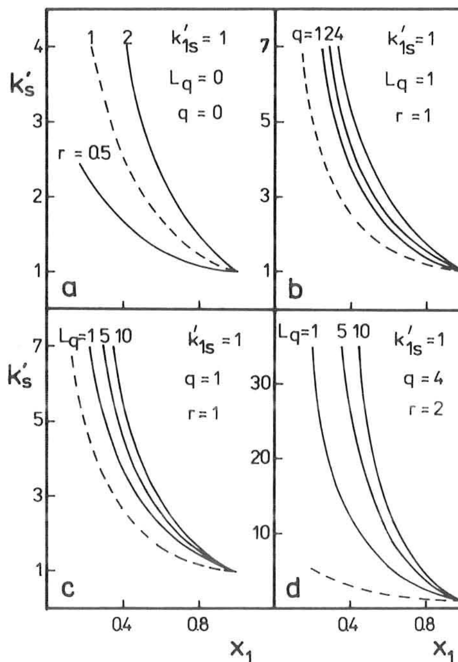


FIGURE 2 - The theoretical curves k'_s vs. x_1 calculated according to equation 41 for $k'_{1s}=1$ and different values of r , q and L_q .The dashed lines have been calculated by using Snyder-Soczewiński equation 16 .

The other parts of Figure 2 show the k'_s-curves calculated for r=1 and different values of q (Figure 2b) and L_q (Figures 2c and 2d).These curves lie above the k'_s-curve plotted according to Snyder-Soczewiński relationship 16 .The k'_s-values,calculated for a given value of x_1 ,increase with increasing of the number of 1-st solvent molecules forming complexes with one molecule of the solute (the parameter q) and solute-solvent interaction energy (it is connected with the parameter L_q).

In Figure 3 the k'_s-curves presented in Figures 2b and 2c have been plotted in the logarithmic scale. In this scale Snyder-Soczewiński relationship 16 is linear.The dependences lg k'_s vs. lg x_1 ,plotted for q=1,2,4 (Figure 3a) ,may be approximated by the straight lines,which show deviations at higher concentrations of x_1 .Similarly, the dependences lg k'_s vs. lg x_1 ,plotted for q=1 and different values of L_q ,are linear in a wide concentration region (Figure 3b). Although,these dependences have been plotted for r=1, their slopes are not equal to that predicted by Snyder-Soczewiński equation 16 .

It follows from Figure 3 that the analysis of the chromatographic data by means of Snyder-Soczewiński relationship 16 creates a difficulty in physicochemical interpretation of its slope, because a good

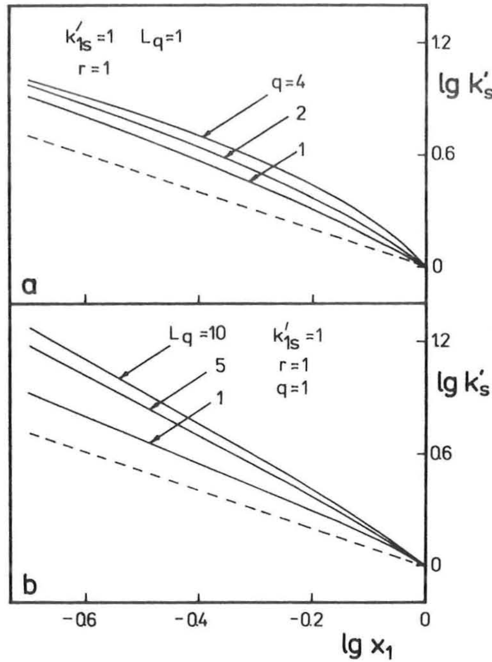


FIGURE 3 - The k'_s-curves ,calculated according to equation 41 for different values of q and L_q,plotted in the logarithmic scale.The dashed lines have been calculated by using Snyder-Soczewiński equation 16 .

linearity of the dependence of lg k'_s vs. lg x_1 is observed for different models of the LSC process.According to Snyder-Soczewiński linear plot 16 the ordinate is equal to k'_{1s} .However, the ordinates of the linear segments of the lg k'_s-plots (the solids lines in Figure 3)are not equal to k'_{1s} .This fact may be very helpfull during interpretation of Snyder-Soczewiński model of the LSC process.

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APPENDIX

The LSC model, representing by the reactions 1 , 2 and 6 , may be extended by assuming that molecules of 1-st solvent form p-molecular complexes in the mobile phase. The equilibrium constant M_p , describing the formation of the complexes in the mobile phase, may be defined in an analogous way to equation 10 :

$$M_p = x_p \,/\, x_1{}^p \qquad (A.1)$$

where x_p is the mole fraction of p-molecular complexes formed by molecules of 1-st solvent in the mobile phase. Taking into account equations A.1 and 12b , the total mole fraction x_1^o may be defined as follows:

$$x_1^o = x_1 + qx_q + px_p \approx x_1 + px_p = x_1 + pM_p x_1{}^p \qquad (A.2)$$

The solution of equation A.2 with respect to x_1 is a function of x_1^o , i.e.,

$$x_1 = X(x_1^o; p, M_p) \qquad (A.3)$$

For two-molecular complexes (p=2), the solution of equation A.2 is the following :

$$x_1 = X(x_1^o; 2, M_2) = [(1+8M_2 x_1^o)^{1/2} - 1\,]/(4M_2) \qquad (A.4)$$

Analytical solutions of equation A.2 are also possible for three- and four-molecular complexes, i.e., p=3 and 4 .

Combining equations 34 and A.3 , we have :

$$k'_s = \beta K_{1s}\, y_1^{\,r/m}\, [\, X(x_1^o;p,M_p)]^{-r} \cdot$$
$$[\, 1 + L_q\, X^q(x_1^o;p,M_p)\,]^{-1} \qquad (A.5)$$

For $M_p=0$ (then p=1) equations A.2 and A.3 give :

$$x_1 = X(x_1^o;1,0) = x_1^o \quad , \qquad (A.6)$$

however, equation A.5 becomes equation 34.

For $y_1 = 1$ we obtain the relationship analogous to equation 39 :

$$k'_s = k'_{1s}\, (1+L_qA^q)\cdot [A/X(x_1^o;p,M_p)\,]^r \cdot$$
$$[\, 1 + L_qX^q(x_1^o;p,M_p)\,]^{-1} \qquad (A.7)$$

where

$$A = X(1;p,M_p) \qquad (A.8)$$

$$k'_{1s} = \beta K_{1s}\, A^{-r}(1+L_qA^q) \qquad (A.9)$$

Assuming in equation A.7 that r=1 , q=1 and p=2 , we have :

$$k'_s = 16\beta K_{1s}M_2^{\,2}\, [\, (1+8M_2x_1^o)^{1/2}-1\,]^{-1}.$$
$$[\, 4M_2 + L_1(1+8M_2x_1^o)^{1/2} - L_1\,]^{-1} \qquad (A.10)$$

For small values of M_2 equation A.10 gives (16,17) :

$$k'_s = \beta K_{1s}\, (x_1^o)^{-1}[\, 1 + (L_1-2M_2)x_1^o\,]^{-1} \qquad (A.11)$$

Now, we consider equation A.5 for r=1 and $y_1 < 1$. It means that the surface phase contains molecules of all solvents. Such situation is observed for solvents having similar adsorption energies. In the case of association of 1-st solvent in the bulk phase, the mole fraction y_1 is given by the expression analogous to equation 36 :

$$y_1 = [K_{1n}X(x_1^o;p,M_p)]^m / \left[x_n^m + [K_{1n}X(x_1^o;p,M_p)]^m + \sum_{j=2}^{n-1}(K_{jn}x_j)^m\right] \qquad (A.12)$$

Combining equations A.12 with A.5 for r=1, we have :

$$k'_s = BK_{1s}K_{1n}\left[x_n^m + [K_{1n}X(x_1^o;p,M_p)]^m + \sum_{j=2}^{n-1}(K_{jn}x_j)^m\right]^{-\frac{1}{m}} \cdot [1 + L_q X^q(x_1^o;p,M_p)]^{-1} \qquad (A.13)$$

For boundary-concentrations, i.e., $x_1^o=1$ and $x_2=x_3=\ldots=x_n=0$, we have :

$$k'_{1s} = BK_{1s}[A(1+L_qA^q)]^{-1} \qquad (A.14)$$

The equilibrium constants K_{jn}, K_{js} and K_{ns} fulfil the following condition :

$$K_{jn} = K_{ns}/K_{js} \qquad (A.15)$$

where

$$k'_{js} = B\,K_{js} \quad \text{for } j=2,3,\ldots,n \qquad (A.16)$$

Since, 1-st solvent forms p-molecular complexes in the bulk phase, the relationship between k'_{1s} and K_{1s} is more complex than that defining k'_{js} by means of K_{js} for $j>1$ (see equations A.14 and A.16).

Taking into account the relationships A.14 and A.16 in equation A.13 ,we have :

$$k'_s = \left\{ \left[\frac{X(x_1^o;p,M_p)}{k'_{1s}A(1+L_qA^q)} \right]^m + \sum_{j=2}^{n} (x_j/k'_{js})^m \right\}^{-1/m} \cdot$$

$$[\,1 + L_q\; X^q(x_1^o;p,M_p)]^{-1} \qquad (A.17)$$

For $n=2$, $M_p=0$ equation A.17 becomes equation 40.

Applying a similar procedure to that described above we can derive the further equations for the capacity ratio by assuming that the mobile phase contains one- , two- and p-molecular complexes of 1-st solvent and they can bound one molecule of the solute. These equations contain many parameters. Therefore, they are little interesting for analysis of the chromatographic data.

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THE STATIONARY PHASE IN THIN LAYER CHROMATOGRAPHY

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ABSTRACT

This review provides an overview of the stationary phases used in thin layer chromatography organized according to the classes of attractive forces between the solute and the stationary phase. Specific examples of recent innovations are included.

INTRODUCTION

Rather than being a unique chromatographic principle, the thin layer approach is really a special technique for applying several different principles. Samples are applied to a stationary phase coated on a support rather than one packed in a column. The solvent then either rises up the stationary phase by capillary action or runs down by gravity. Thin layer chromatography has sometimes been referred to as an "open column" technique, sharing this classification with paper chromatography. The first thin layer separations applied the principles of adsorption chromatography.

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Since then the thin layer technique has been extended to partition chromatogrpahy, ion exchange separation, electrophoresis, and gel filtration.

The advantages of thin layer chromatogrpahy lie in its requirement for only very small samples and, more significantly, in the ease with which compounds on the plate may be detected visually and be quantitated by relatively simple scanning techniques. However when compared with a column procedure the method imposes the constraint on the stationary phase that it must adhere to the surface of the support. This constraint eliminates some stationary phases from consideration, while others that might better be used as pure as possible must have binders added, compounds like calcium sulfate, polyvinyl alcohol, or starch, whose function is to cause the stationary phase to adhere to the support. The binders alter the character of the separation, may prevent desired heating of the plate for activation or solute detection, or may otherwise interfere with solute detection.

Reviewing the character of the thin layer chromatography stationary phase has been done previously in books and articles (1-4). It was decided in this article to focus on the attractive forces between the solute and the stationary phase that are responsible for retarding the movement of the solute. Thin layer electrophoresis and gel filtration are omitted on the basis that the attractive forces between the solute and the stationary phase are of secondary importance, while ion exchange separations are included in spite of arguments that these are not by definition classical chromatography.

ADSORPTION CHROMATOGRAPHY

Adsorption is a complex process with several classes of forces making contributions. These include dispersion forces and electrostatic attraction between ions and ionic charges, dipoles, and induced dipoles. Hydrogen bonding and charge transfer may also take place. Between the solutes and an adsorbing stationary phase several of these forces may be acting concurrently.

Adsorption occurs in a monolayer on the surface of the stationary phase and should be viewed as displacement of solvent rather than as simple attraction to an empty site. In the usual case the surface includes numerous binding sites at which the chemical structures necessary for adsorption are available. These sites vary in terms of binding effectiveness or activity. The first solute molecules tend to bind to the most active site available and once attached will spend a relatively long time there. The more nearly the surface approaches saturation the broader is the range of activities being utilized, and the likelihood of solute binding readily to one of the remaining sites is statistically decreased. Solute is more likely to move on to a new section of stationary phase. These occurrences alter the dissolved/adsorbed equilibrium of the solute. As a result the quality of separations is altered by heavy loading of the adsorbent surface.

The surface area of adsorbent available is an important characteristic. The size of the particles obviously directly effects the the amount of surface area available. Beyond this the roughness of the particle surface is a factor. The more rough the surface, the

greater is the total surface area. A more irregular surface includes more varied binding sites because of the increase in edges and corners. Some portions of the surface, usually described as pores, are infolded and more difficult to reach.

An outstanding reference by L. R. Snyder (5) deals with all aspects of solute-stationary phase interaction in adsorption chromatography.

Electrostatic Interactions

Perhaps the simplest model for a polar adsorbing stationary phase is that of a crystalline array of inorganic ions. The surface has a mix of positive and negative ions arising from the local character of the crystal structure. Molecules with a permanent or inducible dipole orient so as to bind to the surface by electrostatic attraction with the largest dipole producing the strongest interaction. In practice the operation of such a chromatographic system is likely to be more complicated than the model suggests.

a. Alumina

Alumina represents a good example of the ionic crystal type of stationary phase. Alumina is prepared by removal of water by heating from hydrated aluminum hydroxide preparations. A variety of crystalline forms result depending on the starting material and the dehydration process used, and these differ in their chromatographic properties. Broadly we can differentiate mixed oxide-hydroxide preparations, low temperature (200-600°C) aluminas, high temperature (900-1000°C) aluminas, and very high temperature (1100°C)

preparations. The various aluminas differ in their surface area and pore size (5). In general surface area is reduced as the temperature of dehydration is increased. For example the very high temperature product has a relatively low surface area, and is in fact not useful chromatographically. Alumina for thin layer chromatography is generally low temperature with a surface area of 100-250 m^2/g and a grain size of about 60 μm (1). Mixing it with water, spreading it, and activating (drying) it at 110°C leaves some adsorbed water and hydroxide ions still clinging to the surface. Water may be present as capillary water in the pores. Both water and hydroxide ions are adsorbed to the surface in a variety of fashions through dipolar attraction or hydrogen bonding as indicated by infrared studies. Thus there exists a complex variety of binding sites including Al^{+3}, OH^-, and O^{-2}. Heating to 300°C is required to strip off the water, but at this temperature hydroxide ion remains. The oxide and hydroxide sites can be classed as basic or proton accepting sites, while the aluminum ion provides an acidic site. The geometry of the crystal lattice provides further variety in terms of the availability of any specific ion to solute and the nearness and character of the neighbors to that ion. Having inferred this degree of complexity, it is necessary next to say that adsorption on alumina seems to depend primarily on the aluminum and the oxide ions. Activity, hence the energy of adsorption, increases as water is removed from the alumina, and increases further upon heating to temperatures above 800°C, which corresponds to the removal of the hydroxide ions.

Studies by Snyder (6) on the contribution of solute functional groups to adsorption indicate that for most groups the strength of adsorption relates to the basicity, in the sense of hard or electrostatic basicity, of the group. The fact that the energies of adsorption of such groups as -OH, -NH- or -SH are not excessively high infers that hydrogen bonding is not making a significant contribution to adsorption.

The surface of alumina because of the oxide ions is quite basic, being estimated to be approximately pH 12. Acids of pK_a lower than about 13 transfer protons to this surface producing charged conjugate bases that are strongly adsorbed. The use of calcium sulfate as a binder neutralizes the alumina layer eliminating the selective adsorption of such acids.

Finally charge transfer makes a contribution to adsorption on alumina in the case of easily ionizable aromatic molecules, and some small amount of ion exchange has been observed in the separations of inorganic ions.

Hydrogen Bonding

Hydrogen bonds form and break very rapidly yet are of sufficient strength to be ideal contributors to the adsorption process. Since molecules capable of hydrogen bonding are polar, electrostatic attraction of solutes to the stationary phase could be used as the sole explanation of adsorption. The evidence that hydrogen bonding does contribute is based on the observation that molecules capable of such bonding are in fact retained more strongly than would otherwise be anticipated.

a. Polyamide

Probably the best example of hydrogen bonding as a major contributor to the bonding forces between solute and stationary phase is provided by separations on polyamide. Polycaprolactam is generally used, although nylon 6,6 has also been employed. Chromatographic applications on polyamide have been reviewed extensively by Wang with various coauthors (7,8). Polyamide was used in columns as early as 1955 but difficulties experienced in bonding polyamides successfully to a glass plate or any other support held back the use of polyamide for thin layer chromatography. The use of loose layers or binding with starch, polyvinyl alcohol, or cellulose was attempted. However in 1961 the evaporation of a fomic acid solution of polyamide resin successfully deposited the material onto glass (9) providing a useful layer. Later a technique for coating polyamide on a polyester support was also developed (10).

The amide nitrogen can serve as a hydrogen donor and the amide carbonyl can be a hydrogen acceptor in hydrogen bond formation with solutes. Retention of the solute can also involve adsorption by despersion forces or by attraction to the polar bond of the amide structure. Partitioning with the traces of water retained by the stationary phase is also possible. These may make a contribution, but are established as not being the major attractive force (7). Given the very low basicity of the amide nitrogen, it is unlikely to become protonated to provide the charged structure necessary for ion exchange types of solute retention.

The substances separated most successfully on polyamide layers usually contain an -NH- or an -OH group which can donate a hydrogen

TABLE 1. Sample R_f Values on Polyamide and Silica Gel Layers

	Compound	Polyamide	Silica Gel
a.	o-Cresol	0.30	0.26
b.	m-Cresol	0.21	0.19
c.	p-Cresol	0.21	0.20
d.	o-nitroaniline	0.92	0.38
e.	p-nitroaniline	0.70	0.17

a,b,c solvent: benzene
d,e solvent: hexane/acetane (3:1)v/v
data from reference 7

to the amide linkage. Examples include phenols, sulfonic acids, carbohydrates, anilines, nucleosides, nucleotides, imidazols, and dansyl derivatives of amino acids. Perhaps the most convincing evidence of the involvement of hydrogen bond formation is provided by studies of the effect substituting phenols has on the R_f value on polyamide (11, Table 1). An ortho nitro group which provides an internal and competing hydrogen bond, reduces adherance to the polyamide. Substitution of nitro or halogen groups elsewhere on the phenol, increasing the acid strength of the phenol and consequently its ability to hydrogen bond, increase the phenol-polyamide bonding. Finally and most significantly, substituting the phenol in the ortho position, thus sterically interfering with hydrogen bond formation, reduces attraction to the polyamide layer. The larger the substitution the greater the interference. Further support is provided by studies of nitroanilines. Once again if a nitro group is in the ortho

position where it can form an internal hydrogen bond adsorption is reduced, while no similar effect is shown on palcement of the nitro group meta or para.

There is also evidence that the amide proton interacts with the electrons of a double bond. Ethylene urea for example is more strongly retained than is ethyl urea.

When a very polar partially aqueous solvent system is employed polyamide acts like a non-polar surface. For example, gallic esters with a variable length of hydrocarbon chain display decreasing R_f with increasing chain length. However in solvents wherein acetic acid is the sole polar component the pattern reverses (12). This was explained at first by assuming that the acetic acid bound to the polyamide, creating a polar surface.

Nitro groups on solutes may interact specifically with free $-NH_2$ groups at the ends of polyamide chains. For example DNP-amino acids are retained far less well when the stationary phase is acetylated, a process which eliminates the terminal amine groups.

b. Silica Gel

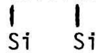
Properties of silica gel were studied early and have been well reviewed (5, 13). Silica gel has been (4) and still is overwhelmingly the most frequently used stationary phase.

Silica gel is prepared by polymerization and dehydration of aqueous silicic acid which is generated by adding acid to sodium silicate. The product of this process is an amorphous porous solid. The amount of surface area can vary over quite a range (200 to more

than 1000 m^2/g) as can the size of the pores. Pores less than 100A in diameter are generally termed small.

A study of the effects of layer thickness and particle size led to an optimization of silica gel characteristics for the separation of small samples (14). This formulation has been described as high performance thin layer chromatography (HPTLC) and has been commercially marketed as precoated plates by Merck.

Chemically the surface of silica gel contains these species: siloxane groups (Si-O-Si), free silanol groups (Si-OH), silanol groups hydrogen bonded to one another



, water (H_2O...H-O-Si), and "capillary" or bulk water. Siloxane groups are unreactive and do not contribute significantly to binding of solutes. Silanol groups are largely free on the large pore gels, at a population density of approximately 4-5 groups per 100 A^2. Most TLC gels have large pores. In the small pore gels a large population of self hydrogen bonded silanol groups are found. In these gels the structure is less orderly.

The hydrogen bonding of water to the silanol groups competes with solute binding. Activation by heating at 150 - 200°C removes this water thereby improving binding characteristics. Sintering by heating to higher temperatures gradually converts silanol groups to siloxane groups with the release of water. Above 400°C the surface area is decreased, and after heating at 1000°C the ability to rebind water is lost. Such a silica has become a hydrophobic surface of siloxane structures. Similarly when silanol groups are chemically

converted to methoxy or trimethylsiloxy groups polar solutes are no longer well retained.

Silanol groups are weakly acidic (aqueous pK 6-8) making them attractive to basic solutes. This is not sufficiently acidic to encourage the idea that bonding of adsorbates is due to attraction to negatively charged ionized silanol groups. The primary bonding forces must be hydrogen bonding or simple electrostatic attraction to the polar hydroxyl group. Studies of the retention of phenols suggest that as with polyamide layers hydrogen bonding is of primary importance.

c. Other Hydrogen Bonding Phases

Other stationary phases capable of hydrogen bonding are encountered. Silicates are used, particularly magnesium silicates and occasionally calcium silicates. Early successes of magnesium silicates included separations of hydroxylated compounds such as carbohydrates. Florisil is a commercial magnesium silicate, and talc (Mg_3 $[Si_4O_{10}](OH)_2$) is occasionally reported as successful for separations. The silicates are different from silica gel in a few important ways. They possess an ionic matrix rather than a largely covalent one. Some resemblance to alumina type adsorbances is therefore expected. Furthermore, while silica gel is weakly acidic, suspensions on magnesium silicate display a pH ranging from 8 to around 10.

The diatomaceous earth preparation usually referred to as Kieselguhr is largely a highly porous silicic acid preparation with a variety of impurities, some of which are removed by processing.

It has a relatively inactive surface as compared with silca gel. Advantage has been taken of this low activity by coating the first 3 cm of a 20 cm plate with Kieselguler as a "zone of inert application" which shortens the time required for application. The rest of the plate was coated with silica gel, and separation did not begin unitl the solutes reached the silica gel (15).

Chitin is a cellulose-like polysaccharide in which the 2-hydroxyl of the glucose monomer has been replaced with an acetylated amine group. It has been used with phenols, amino acids, nucleic acid derivatives, and metal ions, producing separations comparable with those on silica gel, polyamide, or cellulose (16).

Special effects were noted when plates were impregnated with oxalate salts compared to other salts in the separation of aromatic amines (17). These were attributed to hydrogen bond formation between the amines and the oxalate ion.

Van Der Waals Forces

In chromatographic systems involving polar solutes and stationary phases relatively strong electrostatic forces operate between the solute and the stationary phase. However if the solutes are very non-polar, electrostatic forces cannot assist in adsorption. The weak Van der Waals forces must provide the explanation for such separations. A key consideration in application of these attractive forces is the requirement that atoms must approach one another very closely before significant attraction is present. The distance term in the equation for calculating the magnitude of these forces is raised to the sixth power as contrasted to the corresponding calculation

for electrostatic attractions where it is squared. The solute must therefore be able to fit the stationary phase very closely to generate good adsorption. As a result the success of a particular stationary phase is affected by the presence of surface irregularities, the presence or absense of which relates to the original preparation of the adsorbent, especially in terms of heating and mechanical treatment.

a. Reversed Phase Chromatography

A decade or more ago separations dependent on Van der Waals forces were generally performed on silica gel or alumina using solvent systems composed of such non polar liquids as hydrocarbons, ethers, or esters. Recently it has become increasingly popular to use an adsorbent which has been chemically altered to reduce its polarity. Somewhat more polar solvents are then employed and the result is a reversal of the normal chromatographic pattern so that the less polar solutes are more strongly adsorbed. This is termed reversed phase chromatography.

b. Modifying Traditional Stationary Phases

Silica gel can be made less polar by reacting the silanol groups by a process called silanization. Reagents such as alkyltrichlorosilane (18) or dimethyl dichlorosilane (19) convert the hydroxyl groups to ether structures, thus completely deactivating the silica gel with respect to polar or hydrogen bonding adsorption sites. Such phases are commercially available and have been studied for their relative effectiveness (20).

Similarly cellulose has been modified by acetylation of the hydroxyl groups. Such acetyl cellulose is a satisfactory non polar stationary phase, and can be used either by itself or in combination with other supports such as kieselguhr.

Traditionally reversed phased svstems have been created by imoregnating a solid support with a nonpolar liquid. This is discussed later under "Partition Chromatography". The partition approach has the disadvantage that an additional component is now part of the system, introducing problems of saturation and reproducibility in impregnating the layer, solution or evaporation of the inert liquid during chromatography, and extraction of the inert liquid with solute in a preparative procedure.

c. New Stationary Phases

Nonpolar plastics have been utilized for reversed phase stationary phases. Amberlite XAD-2, a polystyrene-divinylbenzene copolymer, and XAD-7, a polymethacrylate,have been employed. These were applied as crushed solid and required large amounts of binder. Of the binders tried, calcium sulfate had the least effect on separations (21). Porapak Q, P, and N, ethylvinyl benzene polymers crosslinked with divinyl benzene, have also been employed. These polymers are porous enough to have a high surface area.(22)

Charge Transfer

The transfer of electrons between solute and adsorbent accounts for some attractive forces observed. Most frequently unsaturated organic compounds have been involved, particularly aromatic molecules.

The need to separate a large variety of polycyclic aromatic structures in environmental and toxicological studies has stimulated efforts to improve chromatographic techniques by incorporating electron acceptors into the stationary phase. Silver nitrate when added to silica gel was observed to retain structures with pi electrons more strongly. More recently organic electron acceptors were employed, including 2, 4, 7-trinitrofluorenone (23,24), 1, 3, 5-trinitrobenzene (23), and picric acid (23,25). Positive results were also attributed to this type of bonding when nitrobenzene and chlorobenzene were added to the stationary phase (26).

Other examples of charge transfer or complexation influencing separations are abundant (3,27). Layers impregnated with boric acid or borate salts have long been used in the separations of carbohydrates and other polyols (23,29). Tungstate complexes have recently also been reported to be useful (30). Dihydroxybile salts have been separated on silica gel impregnated with KH_2PO_4 (31). The addition of $NaHSO_3$ promotes stronger retention of carbonyl compounds. Salts of several metal ions which complex readily including copper, cadmium, manganese, and zinc have been employed to modify chromatographic separations of nitrogenous compounds (32-34).

Chelating agents have been added to stationary phases to analyze mixtures of metal ions. Recent reports mention the use of EDTA (35) and nitrilotriacetic acid (36, 37).

Ligand Exchange

Ligand exchange chromatography is defined in a recent comprehensive review (38) as "a process in which the interaction between

the stationary phase and the molecules to be separated occurs during the the formation of coordination bonds inside the coordination sphere of the complex forming ion". Two classes of separation are described. In the first the complex-forming metal is firmly bound to the stationary phase with the coordination sites probably occupied by solvent molecules. The solute displaces these solvent molecules and becomes a ligand of the metal. The order of elution of solutes then depends on the tightness of the coordinate bond formed. In the second class of separation the solutes are already bound to a metal ion, all solutes complexed to the same metal species. The stationary phase is composed of potential ligands which are free of metal until the solute complex is bound by them. Metals employed include Cu, Co, Ni, Fe, Zn, Cd, Mn, Hg, Ag, UO_2^{+2}, and VO_2^{+2}. These are bound by cation exchangers with carboxylic, phosphoric, iminoacetate, and α-aminoacid groups.

A specific example is provided by the use of layers of cellulose and chelex resin saturated with Co^{+3}, Ni^{+2}, Cu^{+2}, or Zn^{+2}. Separations of diamines, amino acids, and carboxylic acids were performed (39).

PARTITION CHROMATOGRAPHY

In partition techniques the relative rates of movement of the solute in the chromatographic medium result from the partition coefficients between the solvent and a liquid coated onto a stationary support. This presents the system in a convenient fashion, but may allow some oversimplification to mislead the user.

Cellulose

Oversimplification is a clear danger when the stationary phase is cellulose. Cellulose is viewed as serving as the support for water, and partition occurs between the solvent and the water layer. Given the relatively small amount of water taken up by the cellulose, one would be badly misled to view the stationary phase as bearing a close resemblance to the contents of a mountain stream, but would be more accurate to liken it to a container of syrup. Water molecules hydrogen bond to the cellulose alcohol groups or ring oxygen as well as to one another. A solute in such a system might interact either with water or with cellulose, and because of the similarity of the chemical groups it is difficult to distinguish which is actually involved. The system is most effective with polar solutes, particularly those which can form hydrogen bonds, using a relatively polar solvent system. A very non-polar solvent system might well not wet, hence interact with, the stationary phase. It is desirable to include water in the solvent to insure that the water layer remains intact. Anhydrous cellulose serves as an adsorption stationary phase which also has a small ion excahnge capacity (see Ion Exchange-Celluose). As an adsorption stationary phase it is composed of layers of polymer strands tightly hydrogen bonded together. Approached perpendicular to the surface of the glucose rings it can hydrogen bond to free alcohol groups and ring oxygens. However parallel to the rings only Van der Waals attractive forces are possible.

Cellulose was early used in the form of paper sheets. The adaptation of cellulose to thin layer systems was an easy transition, al-

though given the simplicity and low cost of paper chromatography, such a transition may at first seem pointless. However in practice separations on cellulose thin layers often are superior to the corresponding analysis on paper, especially when the plate size is small. Part of the credit for this is due to the uniformity of the coated layers, but the character of the cellulose itself may be of greater significance. Cellulose fibers for thin layer use are generally shorter than would be found in chromatography grade paper sheets, especially in the case of microcrystalline cellulose which has been deliberately hydrolyzed to lower molecular weight structures. It is thought that solute moves rapidly along the fibers resulting in more rapid diffusion of the spots than would occur otherwise, and the shorter fibers mimimize the problem.

Cellulose adheres to a glass surface, so that the problems inherent to introducing a binder into the stationary phase are avoided. In fact, cellulose has occasionally been mixed with other stationary phases to help bind them to the plate. Other advantages are sometimes claimed for such combination. A silica gel - cellulose mixed layer is described, for example, as allowing the employment of inexpensive solvents and permitting a convenient method for stripping for liquid scintillation counting.

Partition chromatography on cellulose may involve other liquid coatings than water. Glycols also adhere to the cellulose to produce a polar stationary liquid phase.

Starch is chemically similar to cellulose and is occasionally used as a stationary phase for the same types of separations as

cellulose. The author has not been impressed with it as offering any advantages over cellulose, and it suffers the disadvantage of not being widely commercially available either for spreading or as a precoated sheet.

Reversed Phase Chromatography

The non-polar character of the stationary phase is achieved here by coating a support with a liquid of very low polarity and volatility. In principle the support does not enter into the solute - stationary phase equilibrium, and in practice silica gel and kieselguhr are most often used. As largely uncharged covalent structures they accept the liquid coating in a reasonable fashion. Silicone or paraffin oils are common examples of the non-polar liquid. The moving phase is usually a mixture of polar liquids. Solvents rich in water often produce very slow runs since the water does not wet the plate. Similarly spray reagents conventionally prepared in water solution may have to be adapted to solvents like methanol or acetone to be effective.

ION EXCHANGE

In the ion exchange technique the stationary phase has charged groups, usually titrable groups maintained in the charged form by appropriate adjustment of the solvent pH. Ions of opposite charge are attracted to these groups and adhere with degrees of firmness that vary primarily with the size and the charge of the ion in simple inorganic examples. When the technique is extended to charged organic molecules, as has been done extensively in the cases of amino

acids, peptides, proteins, nucleotides, and oligonucleotides, adsorption of the molecule to the stationary phase also occurs, requiring that the character of the uncharged portion of the stationary phase also be considered. In the straightforward application of ion exchange, ions charged oppositely to the stationary phase attach at the charged site of the stationary phase by displacing an ion that is already there, but is held less strongly. Later these ions are displaced in order of the tightness of their binding either by low concentrations of ions bound more tightly yet or by high concentrations of ions bound less tightly.

Homochromatography, a technique that has been applied extensively to nucleotide studies, is an interesting variation of the method (40). The ion exchange stationary phase is saturated before analysis with a mixture of non-radioactive nucleotides which array themselves in order of tightness of binding on the plate, generating a series of fronts. Mixtures of radioactive nucleotides under analysis then move to appropriate zones on the plate. Recent papers report a number of variations of the method combining it with electrophoresic analysis and using DEAE cellulose or polyethyleneimine cellulose (41-43).

Cellulose Based Methods

Cellulose itself has been used as an ion exchanger. Its ability to perform in this fashion depends on the presence of carboxyl groups on about 1% of the glucose monomer units (44). As such, however, the capacity is small and in an aqueous solvent the attractive forces are not strong. The glucose primary alcohols may be converted to

TABLE 2 - Cellulose Based Ion Exhangers

Name	Structure substituted on alcohol groups
oxycellulose	-COOH (primary alcohol only)
cellulose phosphate	$-OPO_3H_2$
carboxymethyl cellulose	$-OCH_2\ COOH$
sulfoethyl cellulose	$-OCH_2CH_2SO_3H$
cellulose citrate	$-OOC-C(OH)(CH_2COOH)_2$
diethylaminoethyl cellulose	$-OCH_2CH_2N(CH_2CH_3)_2$
aminoethyl cellulose	$-OCH_2CH_2NH_2$
triethylaminoethyl cellulose	$-OCH_2CH_2N^+\ (CH_2CH_3)_3$
ECTEOLA cellulose	$-O(CH_2-CHOH-CH_2)_n\ OCH_2\ CH_2N(HOCH_2CH_2)_2$
guanidinoethyl cellulose	$-OCH_2CH_2NH\ C(=NH)NH_2$
p-aminobenzyl cellulose	$-OCH_2C_6H_4NH_2$
QAE cellulose	$-OCH_2CH_2N^+\ (CH_2CH_3)_2CH_2CHOHCH_3$

carboxyl groups by oxidizing agents, generating a much higher capacity product called oxycellulose.

A variety of other chemical derivatives of cellulose with exchange properties are produced (45) as is summarized on table 2. These involve replacement of more than one alcohol group with charged groups. The interchain hydrogen bonds involving the alcohol groups that maintain the cellulose in a crystalline, water-soluble state are replaced by electrostatic repulsive forces, To prevent the polymer from dispersing in the solvent as the result of these changes, it is necessary to crosslink the polymer before modifying the side chains.

In order to react groups in the interior of the cellulose, the matrix may be swollen with alkali (Mercerized) before reacting the alcohol groups. As the cellulose dries unaltered alcohol groups re-form hydrogen bonds and attractive interactions made possible by the new substituents occur. The matrix shrinks, and a portion of the new ion exchange sites became unavailable unless the substituted cellulose is reswollen before use by treatment with acid or base. Micro-granular cellulose avoids some of these difficulties. The non-crystalline regions of cellulose are more susceptible to hydrolysis and can be removed to form pores. The crystalline regions are then crosslinked. Substitution of alcohol groups then occurs primarily at the borders of the pores, and a product that may be used without reswelling results.

Cellulosic ion exchangers have been of particular value with biochemical separations, most frequently on columns, but also on thin layer plates. The fact that the stationary phase is hydrophilic rather than hydrophobic, as in the case of more traditional polystyrene based ion exchangers, has avoided problems with denaturation and overly strong retention of the proteins be separated.

Resins

The traditional ion exchange resins used for so long in columns are not inherently well suited to open column methods. Typical poly-styrene based beads do not adhere at all to glass. The beads are very large by camparison with the typical particles coated on plates, the expectation being that ions will penetrate into the very porous bead to interact at interior binding sites. Using the usual binders

to attach the beads to the plate usually inhibits good interaction between ions and the resin. However because so much ion exchange work has been done with resins the temptation to find some means of using them in the open column mode is irresistable. Examples of successful applications are provided by the comparative studies of phenol separations by L. Lepri and coworkers (46) using a variety of ion exchange stationary phases. Cellulose derivates (DEAE-, PEI-, and benzoylated DEAE-) were used, but in addition they employed Dowex 50-X4, Rexyn 102, BioRad AG 3-X4A, and BioRad AG 1-X4. Crushed resin was bound to the plates by mixing 3g resin with 9g microcrystalline cellulose.

Liquid Ion Exchangers

In the mid-sixities the idea of impregnating a support with a liquid ion exchanger for inorganic analysis was explored extensively. Silica gel was usually the support of choice, although other media were tried including a polyvinyl chloride/vinyl acetate copolymer (47). Most interest centered on di-(2-ethylhexyl) hydrogen phosphate (47,48), and on a series of amimes including Amberlite LA-1, N-dodecenyl-(trialkylmethyl)- amine, Primene JM-T, triisooctylamine and Alamine 336 (47,49,50). Cellulose impregnated with polyethyleneimine, called PEI cellulose, has been used by biochemists in a variety of ways.(51)

Other Ion Exchange Media

A wide range of other approaches have attempted to provide an ion exchange stationary phase. For example silanized silica gel has

been impregnated with detergents. The non-polar portions of the detergent associates with the very non-polar support, leaving the charged group available to attract solute ions. Such detergents as sodium lauryletherosulfate, triethanolamine dodecylbenzene sulfonate, and sodium dodecylhydrogen sulfate were studied (52).

Spreading insoluble inorganic ionic material in which a large negative ion attaches cations in an exchangable fashion is also possible . Tungstate, molybdate, or their heteropolyacid anions are useful in this fashion, metal ions having been separated on layers of ammonium molybdophosphate (55) and thorium tungstate (56). Silica gel impregnated with these salts is also successful for ion exchange separations of metal ions. The use of ceric molybdate (57) and lanthanum tungstate (58) have been reported recently. Separations of cations on hydrous ZrO_2 has also been reported (59).

Deacetylating chitin generates a polysaccharide with a primary amine group on each carbohydrate unit. This has been used for the separation of nucleosides and nucleotides (60).

SINTERED THIN LAYER CHROMATOGRAPHY

As a final section, a promising technique for binding the stationary phase to the plate should be mentioned. Mixing the coating with a binder which melts and flows on heating, if carefully done, produces a very tightly bound layer. For example, silica gel is mixed with sodalime or borosilicate glass powder, spread on a plate, and heated from 470-770°C. The resulting plate survives rough handling and can be recycled. Reuse of the plate permits greater reproducibility, especially after recycing a few

times. Problems with visualization reagents that char or corrode organic binders are eliminated.

The use of a fluorescent glass such as zinc silicate allows detection by fluorescence quenching.

The method has been extended to alumina, kieselguhr, florisil, ZnO, MgO, and titania. Using polyolefin in place of powdered glass, one may bind organic and cellulose ion exchangers and other organic phases.

The techniques have been reviewed by Okumura .(61)

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ROD CHROMATOGRAPHY- A NEW APPROACH TO AUTOMATING TLC

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ABSTRACT

A new technique for separating and quantifying materials using thin layer chromatography techniques is described. In this method the separation is done using quartz rods coated with adsorbing materials. Coating material selection and application uniformity are critical to the process.

Rods are spotted at one end with a solution of the unknown and dried. Development is similar to that used for TLC plates. Following development the rods are scanned in an apparatus designed expressly for the purpose which utilizes flame ionization detection. The output from the FID is amplified and integrated. A two pen recorder is used to record the resulting chromatographic and the integration curves.

Examples of applications are given in which such varied materials as olive oil, plasma lipids and surface active agents

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are analyzed. Descriptions of unique and interesting practical applications are given for finger printing, crude oils and the forensic analysis of dyes.

INTRODUCTION

While rod chromatography has been known as a laboratory possibility ever since its discovery and patenting by Unilever in England, it was not until an instrument was built by Iatron Laboratories in Japan that it became a practical reality. This is due to the fact that a carefully designed and constructed mechanism is necessary to carry out the sequential steps in preparing, developing and scanning a rod without considerable time and careful manipulation.

Rod chromatography is relatively unknown in the U.S. but has been well developed in Japan, Europe and Canada. Since many may not be familiar with the technique, this paper will commence with an explanation of the principles and sequence of events involved in carrying out a thin layer chromatography/flame ionization detector analysis (TLC/FID) on a rod.

PRINCIPLES OF OPERATION

Separation in chromatography depends on selective adsorption on some medium. The medium may be a column or coated on another substrate and still adsorb selectively. Thus, plates of glass or

plastic film coated with silica or alumina separate dissimilar components over two dimensions.

Detecting the quantitive presence of the various components is a commonly desired end. A developed TLC plate may be read by a scanning densitometer and some quantitation achieved. A generally accepted quantifying method for other separation techniques such as GC has been a flame ionization detection. How to apply this to a plate separation is a design enigma. Lacking a flame that could scan a plate, the inventor of rod chromatography conceptualized a plate dimensioned to fit a flame and, thus, derived the TLC coated rod.

The basic technique is to use a rod coated with an adsorbing material that will withstand a hydrogen flame briefly. The construction decided upon was a quartz rod 0.9 mm in diameter and 155 mm long. The rod is coated with a 75 micron thick layer of a mixture of powdered glass and alumina or silica and the mixture fired onto the rod at 900°C. About 5 mm are left uncoated on each end for handling.

In use, the sample to be separated is spotted onto the rod about 2 cm from one end using 0.1 to 3 ul of volume containing 5-10 ug of sample. Any solvent that will evaporate at temperatures that do not disturb the sample may be used as the sample carrier. The end of the rod bearing the sample is immersed in the developing solvent in a closed chamber to a depth of about 1.5 cm

Elution is carried out over 15 to 45 minutes, depending on the mobile phase used.

The rod is then dried until free of the developer solvent then passed through a hydrogen flame and scanned from top to bottom at a pre-selected speed. The combustion products are detected and a signal generated proportional to concentration as each eluted spot passes through the flame. Good linearity is found in the 3 to 30 ug sample weight range. The FID was chosen because it detects percent by weight of carbon. Greater accuracy or sensitivity is possible than with optical scanning of spots along with avoidance of problems of color development, wave-length selection for UV scanning or derivatization to generate fluorscence.

Thus, the steps are sample dissolution, sample spotting on a rod, solvent development, drying, flame scanning, curve plotting and peak integration. The tools needed are suitable rods, a suitable spotting method, a uniform and variable speed scanning set-up, an FID and a signal recorder.

Reducing this combination of operations for use as a routine method required the design and construction of a carefully specified and constructed instrument. This was the part contributed by Iatron Laboratories in Japan. Thus, we have, in effect, a black box that takes over after the rods are spotted, developed and dried and does everything else automatically. The rods are an item of supply also produced by Iatron and are, of

course, the heart of the system, and therefore, are carefully made and pretested. A rod is good for up to 300 scans and can therefore be looked upon as reusable. Only an accident or use of a compound that will not burn off without leaving a residue that cumulatively plugs the chromatographic surfaces limits its life. Such things are rare and are usually complex mixtures that perhaps should have been cleaned before analyzing.

INSTRUMENT DESIGN DETAILS

The device used is called the Iatroscan TH-10 and consists of the parts shown schematically in Fig. 1.

The rods are handled ten at a time in a rack for spotting, developing, drying and scanning thus avoiding finger contact with the rods. The rack is driven over a stationary hydrogen flame above which there is a collector electrode,the positive pole of the FID, while the burner sleeve is the negative pole. The rack of rods in indexed sideways from rod-to-rod and returns to the top of each rod while the flame is located between adjacent rods. The FID signal is amplified and fed to a recorder. An integrater is built into the instrument, thus, a two pen recorder is used giving the curves and their integration as a vertical distance plot between horizontal moves from peak-to-peak.

The instrument contains a variety of other features such as automatic zeroing between curves, an air curtain around the hydrogen flame to exclude air borne particles, flow controls for

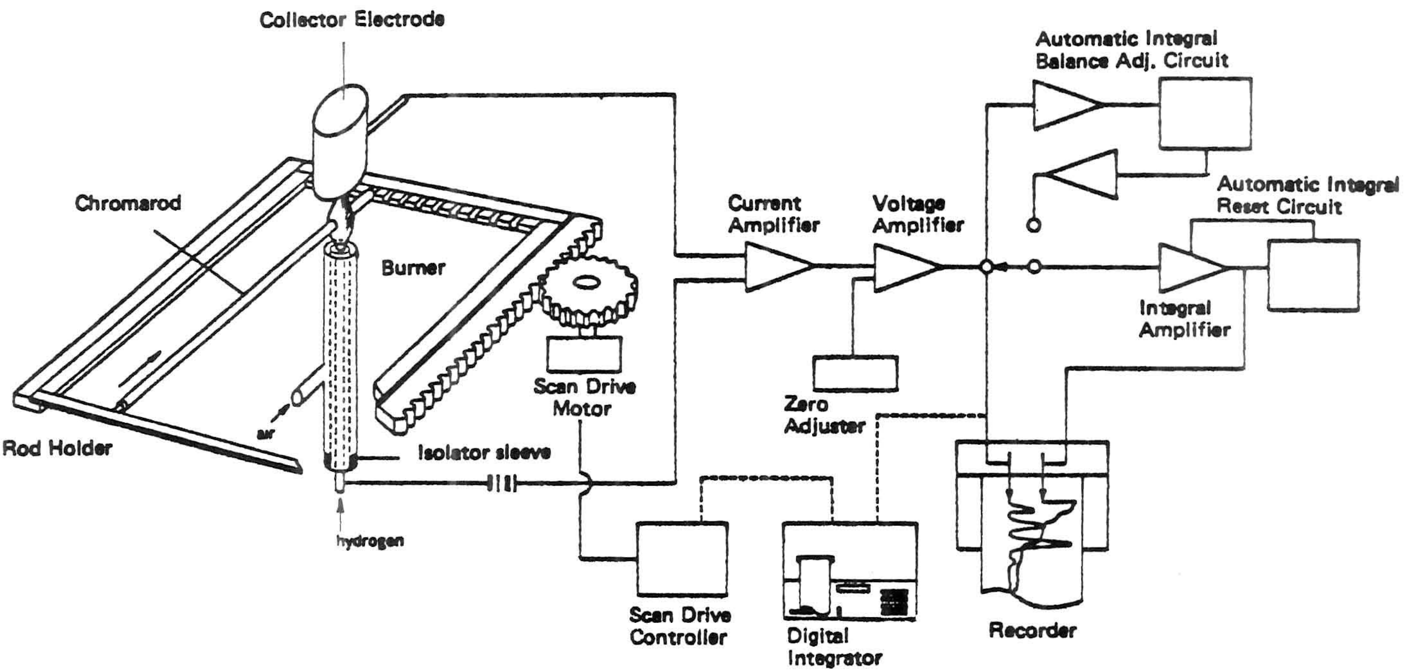


Figure 1

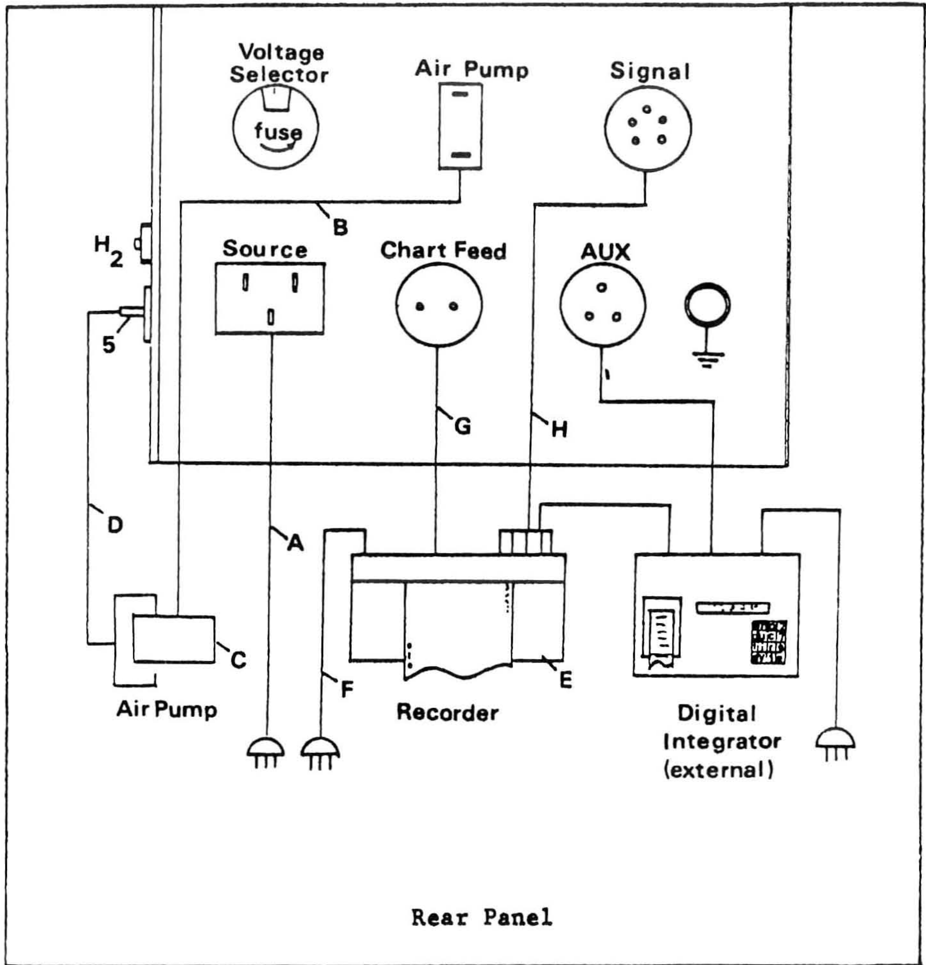


Figure 2

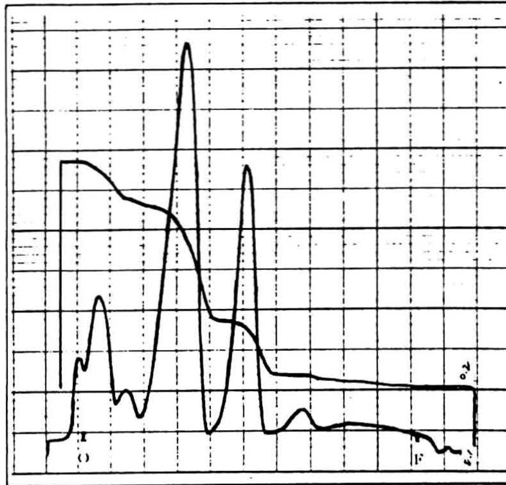
air and hydrogen, signal outputs for remote recording and a chart paper drive to shut off the strip chart recorder paper drive between rods or when a rack of rods is completely scanned. The layout of these features on the back of the instrument is shown in Fig. 2.

APPLICATIONS OF ROD CHROMATOGRAPHY

There have been literally hundreds of curves run successfully using compounds of the widest variety. A number of things that have been done using this technique that eluded TLC and HPLC methodology. However, since they were specialized areas they are simply noted in the references. A series of examples of applications in diverse areas but those of fairly broad interest are given below.

A great deal has been done with natural oils since it is often difficult to separate them and to identify the component compounds. Since a great deal of this work was done in Japan it is not surprising that a great emphasis was placed initially on seed and other food oils, especially those related to products of the sea. Thus, methods (Fig. 3) have been worked out and numerous analyses done on olive oil, coconut oils, sesame oil, peanut oil, shark liver oil, rape oil, soybean oil and safflower oil. The next most active area has been in the analysis of lipids including neutral and phosolipids and has included both those occuring in human systems and other forms of life. Some very interesting research has been done in analyzing complex lipid mixtures from both serum and tissue lipid sources by Shishido at Nipon Roche in Japan (4) and Sipos and Ackman at the Fisheries and Marine Service in Halifax (24). One interesting sideline in the latter group's work was an experiment where a single

● Olive Oil



SAMPLE:
① Olive Oil

CONDITIONS:
Stationary Phase:
CHROMAROD-S
$AgNo_3$
Impregnated
Mobile Phase:
Benzene: Ethyl Ether
97 : 3
Gas Flow: H_2 160ml/min
Air 2000ml/min
Scanning speed: 32sec/scan
Chart speed: 240mm/min

Figure 3

indistinct spot was removed from a TLC plate following plate separation of fluid from a sea mussel. This was applied to a rod and eluted along it into three distinct peaks that were identified., one being a hydrocarbon. Finding the later was a vital point since the research was directed at whether or not mollusks ingest oil from oil spills and it seems they do.

It was in this work that it was learned that rod life could be extended by storage in chromic acid cleaning solution. The normal recommended storage in a humid atmosphere gave a shorter rod life with these complex materials that tended to plug the rod surfaces even after flaming. The cleaning solution overcame this problem.

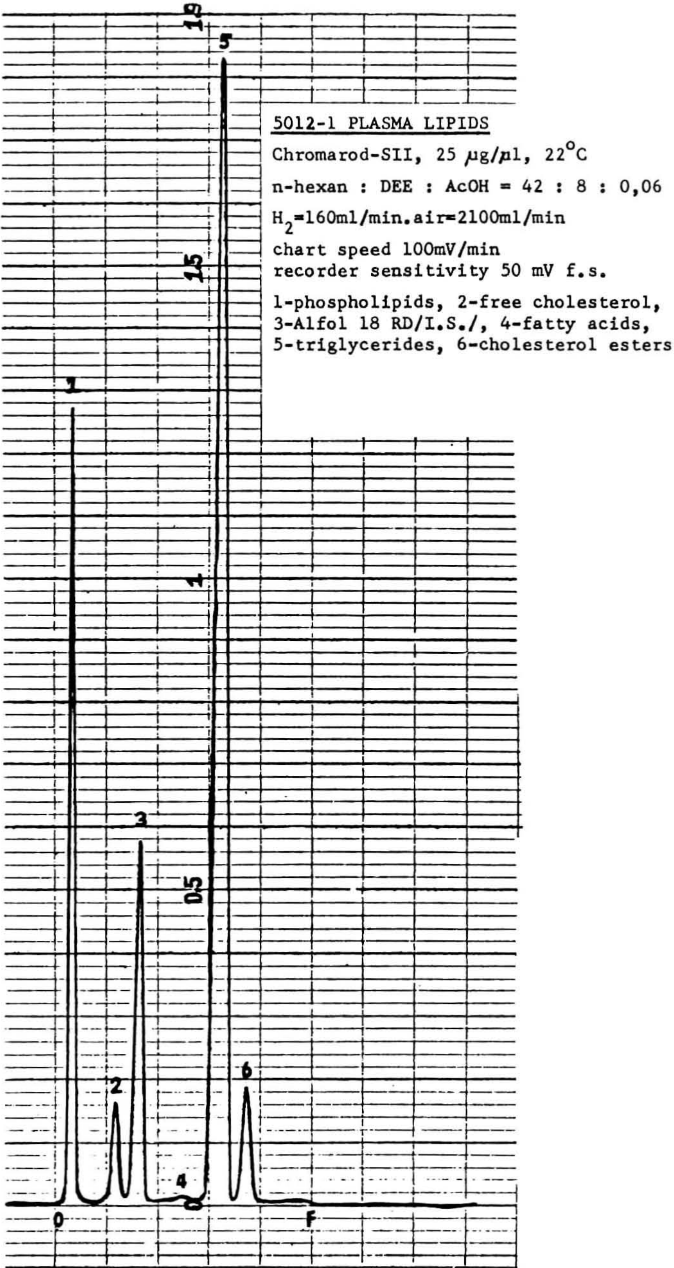


Figure 4

The work in Japan with serum (6)is shown is Fig. 4. This provided an excellent opportunity to evaluate the response in a natural mixture to the spotting quantity on the rod.

A variety of plasma lipid samples were used from patients with various types of hyperlipidema. The amount of of material in the spot is plotted against the cell volume. A mean is drawn in and it shows that the volume percent shoots up sharply below about 0.5 microgram. See Fig. 5.

Much has been done with pharmaceutical compounds (6) on the TH-10. Results have been published in which analyses have been done on psychotropic drugs in which studies were made to relate dosage size with blood content at time intervals. Other analyses have included quantitation of the active component in natural medicants such as saponins from ginseng root and glycyrrhizin from licorice root.

Leaving natural substances, an area with surprising success has been polymer separation and molecular weight distribution determinations. Also, chain length separation is readily accomplished from mixed alkyls used in making synthetic detergents. Following is an example of a polyoxyethylenenonyl phenyl ether. Note the closeness and sharpness of the peaks. It should be noted here that a strip chart recorder with fast response is necessary to catch all the peaks. The common response time of 1/2 second for recorders used in such analyses

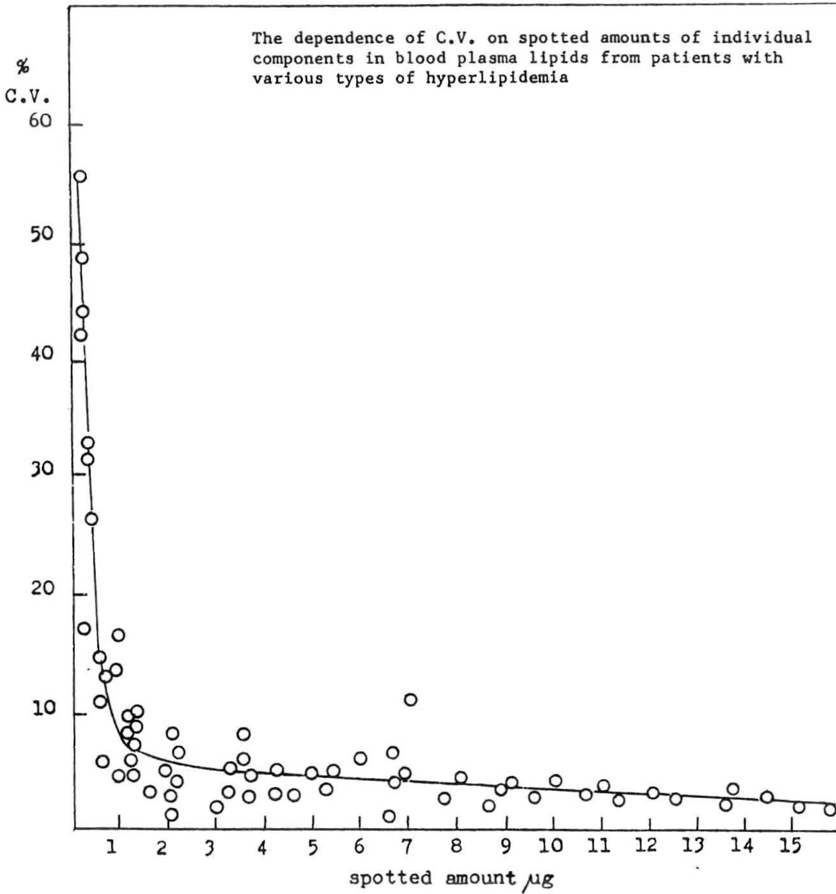
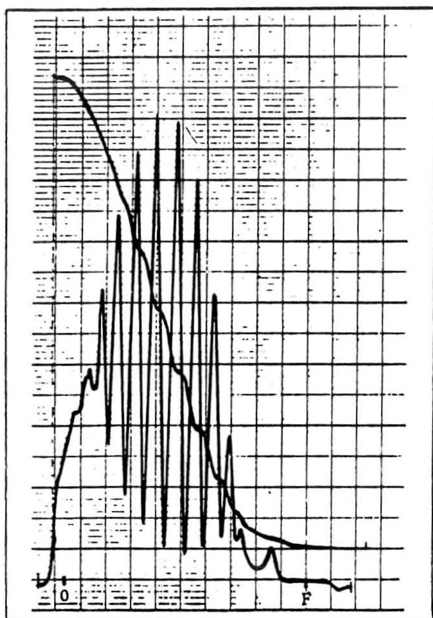


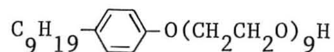
Figure 5

may not be acceptable and a recorder responding full scale in 1/4 second is required. This is shown in Fig. 6.

This description would not be complete without mentioning that rod chromatography, although a unique and independently useful tool, will inevitably be compared with TLC, HPTLC and HPLC. It cannot be claimed at least as yet that it will replace



SAMPLE:
polyoxyethylenenonylphenyl-ether
(EO = 9 added molecules)



CONDITIONS:
Stationary phase:
CHROMAROD-SII
Mobile phase:
Ethyl Acetate: Acetone: Water
70 : 20 : 4
Gas Flow: H_2 160ml/min
Air 2.01/min
Scanning speed: 32sec/scan
Chart speed: 240mm/min

Figure 6

them. It does appear quicker, easier to use, more sensitive and less costly to use repetitively in certain applications. A comparison is made among the plate methods in the Fig. 7. Its limitations preclude the use of volatile compounds which could be handled readily in GC or HPLC and in materials that are substantive to the rod material and develop very low R_f values such as aflatoxins. There are, however, very few such compounds.

One very interesting example of a practical usage that is a forensic application. In detecting forgeries, analysis of ink is often required. The ink may be present as only a short line or

as a dot, such as in a figure or decimal alteration. A figure that had been written as a straight line figure one was believed altered into a seven by addition of a cross bar. To the eye the seven appears as an authetic figure. Since the chances are extremely remote that the same ink would have been used in making the cross bar as used in making the vertical line, an analysis of the inks was desired.

A spot from each line was cut from the paper using a square end syringe needle. This spot was placed in a chloroform and methanol mixture. The resulting ink solution was spotted on the rod then developed in a methanol-hexane mixture. The resulting elutions were scannned and two very dissimlar sets of curves

COMPARISON OF TLC (1) HPTLC (2) AND RTLC

	TLC	HPTLC	RTLC
Plate Size	20 x 20 cm	10 x 10 cm	1 mm x 155 mm
Sample Volume (capiliary application)	1 - 5 µL	0.1-0.2 µL	0.1-3 µL
Diameter of spots	3 -6 mm	1.0 mm	1 -3 mm
Diameter of separated spots	6-15 mm	2-5 mm	N/A*
Solvent migration	10-15 mm	3-6 mm	N/A*
Detection limits	-	-	
Flame Ionization			<1 ng
Absorption	~5 ng	~0.5 ng	
Fluorescence	~0.1 ng	~.01 ng	

(1) ANALYTICAL CHEMISTRY, Vol. 53, No. 2, p.254A, February 1981

(2) From Hezel, U.B. (3)

* Not Applicable

Figure 7

resulted. In this case the differences could be seen qualitatively by eye but the scan quantifies the components and produces curves that can be used as hard evidence. Such curves in the hands of an ink chemist can result in the identification of the ink by manufacturer and by the type applications such as ball point, fountain pen, or office machine ribbon. Thus, pen alteration of office machine produced records can be distinguished, however skilled the forger. Such applications are limited only by the imaginations of those who put rod chromatography to work.

Rod chromatography is in its infancy. The next five years will likely produce a geometric increase in publications in this field expanding its uses into what will likely be routine control uses.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 4(12), 2193-2203 (1981)

DENSITOMETRIC DETERMINATION OF C-18 FATTY ACIDS AS PHENACYL ESTERS FOLLOWING REVERSED-PHASE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

A simple method of quantitatively determining C-18 fatty acids in an aqueous oxidized polyethylene emulsion is presented, utilizing the formation of phenacyl esters in the presence of crown ether as catalyst, followed by separation by reversed-phase thin layer chromatography, and subsequent densitometry of the strongly ultraviolet absorbing separated compounds.

The method has good sensitivity, permitting the detection of as little as 5 nanomoles or less of fatty acids, and is applicable to various biological mixtures and to acids of different chain lengths.

INTRODUCTION

Long chain fatty acids differing only in degree of unsaturation are not readily separated by adsorption chromatography on silica gel (1), but are well suited to separation by reversed-phase partition thin layer chromatography (2). The problem encountered here is that the organically bonded layer cannot withstand the high temperatures necessary for detection of fatty acids by the

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commonly used charring techniques, particularly in the case of the unsaturated, less reactive fatty acids. The method of Gübitz (1), employing p-hydroxybenzaldehyde/sulfuric acid as a reagent has the disadvantage of having a crucial reaction step which is time and temperature dependent. In addition, the sulfuric acid used in the reagent results in a deterioration of the background, due to the degradation of the hydrocarbon chains.

Due to their relatively low reactivity, there are virtually no non-destructive visualization methods applicable to fatty acids detection in thin layer chromatography. The ultraviolet quench technique, which is well suited for densitometric work and requires no destructive reagents, does not apply to free fatty acids, since most fatty acids do not absorb ultraviolet radiation, at least not sufficiently to allow acceptable levels of detection. Formation of suitable ultraviolet absorbing derivatives is an ideal solution, allowing for ready visualization and optimum quantitation of the compounds on a thin layer plate by densitometry.

The formation of phenacyl esters in the presence of a crown ether catalyst, as described by H.D. Durst (3) and applied to liquid chromatography (4), forms the basis for this method. Crown ethers are known for their ability to complex metal salts and aid the dissolution of these salts in non-polar, aprotic solvents (5).

Stoichiometric concentrations of the crown ether are not necessary, thus the phase transfer of carboxylate salts may be catalyzed by crown ethers in molar ratios of from 1:10 to 1:100. A 1:10 ratio was used in this procedure.

The reaction can take place satisfactorily in almost any nonpolar aprotic solvent. The solvent used in this method was benzene, but acetonitrile, cyclohexane, methylene chloride or carbon tetrachloride are reported to be equivalent and can be substituted to meet solubility requirements. Traces of water have not been shown to affect the completeness of reaction, thus rendering rigorous anhydrous conditions unnecessary (3). The reaction takes place rapidly and under extremely mild conditions, giving quantitative yields and good reproducibility. The p-bromophenacyl esters of fatty acids thus obtained exhibit high molar absorptivity and can, therefore, be detected at very low concentrations on a thin layer place with fluorescent indicator. This technique was applied to the quantitative determination of fatty acids presence in polyethylene emulsions.

MATERIALS

α, p-Dibromoacetophenone and 18-Crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane) were obtained from Aldrich Chemical Co.

Stearic acid, oleic acid and linoleic acid of 99.0% or better purity were obtained from Chemical Dynamics.

All organic solvents used were commercial A.R. grade and were used without further purification.

Thin Layer Plates- Whatman KC-18F, reversed phase plates containing a fluorescent indicator excited at 254nm, 20 x 20 cm., 0.25mm thickness (#4803-800) were used.

Instrumentation - Densitometric measurements were performed using a Schoeffel Model SD-300 Spectrodensitometer.

METHODS

Standard Stock Solution - approximately 60mg each of stearic, oleic and linoleic acids are dissolved in chloroform and diluted to 50.0ml.

Alkylating Solution - 555mg of dibromoacetophenone and 53mg of 18-Crown-6 ether are dissolved in 20ml of benzene, yielding a 10:1 molar ratio of alkylating agent/crown ether. This solution may be stored in a refrigerator for two to three weeks.

Sample Preparation - a 10.0ml sample of oxidized polyethylene emulsion was precipitated by the addition of 1.0ml of 6N hydrochloric acid. The precipitate was suction filtered on a coarse porosity sintered glass funnel, repeatedly washed with 25.0ml portions of distilled water, followed by a final rinse of 50% methanol/ water. Following suction-drying to remove residual

water, the precipitate was redissolved in chloroform, transferred to a 100ml volumetric flask and diluted to volume with chloroform.

Alkylation

For the preparation of standard curves, 2.0ml, 3.0ml, 4.0ml, 5.0ml and 6.0ml aliquots of Standard Stock Solution (concentration range of total acids from 0.025mM - 0.075mM) are pipeted into five appropriately marked 10ml volumetric flasks held in specially designed rack for ease of handling.

A 4.0ml aliquot of sample solution (equivalent to approximately 0.05mM total acids) is pipeted into a 10ml volumetric flask.

All solutions are neutralized to a phenophthalein end-point by dropwise addition of 0.5% methanolic potassium hydroxide solution and are then evaporated to dryness under nitrogen in a 45° - 50°C water bath.

Into all flasks,1.5ml of Alkylating Solution are added to suspend residues. Flasks are immersed in an 80°C water bath and heated for 20 minutes with frequent agitation. Solutions must not be allowed to dry out. If necessary, 0.5ml of benzene may be added to all flasks.

At the end of the heating interval, flasks are cooled and diluted to volume with benzene. Solutions are now ready for thin layer chromatography.

Thin Layer Chromatography

Using disposable, open-end capillaries (Drummond Microcaps), 5 lambda (μl) of each standard and three 5-lambda spots of sample solution are applied at two centimeter intervals to a 20 x 20cm Whatman KC-18F reversed phase TLC plate.

When dry, the plate is placed in a filter paper lined chamber, equilibrated for at least 30 minutes, containing the solvent system acetonitrile:chloroform (90:10).

Plates are developed to a distance of 12cm. Development time is approximately 15 minutes. Plates are immediately dried at room temperature while protected from light.

After development and drying, the chromatograms are viewed under short wave ultraviolet light. The fatty acids appear as follows:

Compound	R_f
Behenic acid	0.31
Erucic acid	0.37
Stearic acid	0.43
Oleic acid	0.50
Linoleic acid	0.56
Linolenic acid	0.61
Excess reagent	0.75

The chromatograms are scanned in the reflectance mode using a Schoeffel Spectrodensitometer (Fig. 1). Peak areas of standards and samples are determined, the

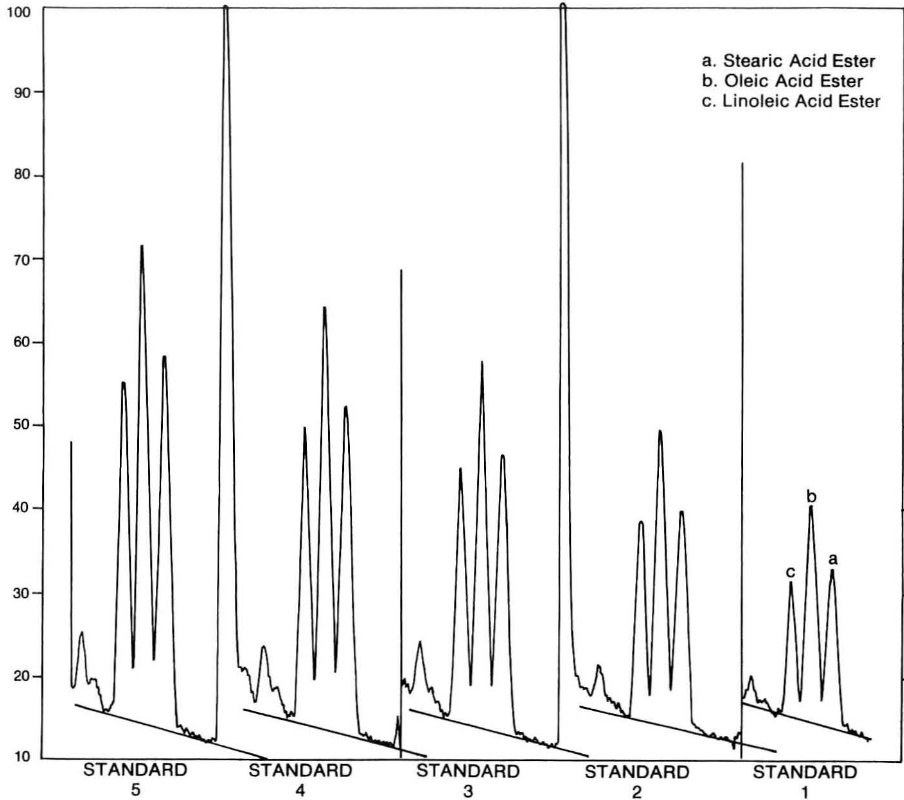

Figure 1. Densitometric Scans for Standard Curves.

average of triplicate scans are plotted vs. corresponding weight of fatty acid in mg., to obtain a standard curve for each acid (Figs. 2, 3 and 4). It is essential for good precision to scan in triplicate.

Fatty acid contents in samples are then calculated using these standard curves.

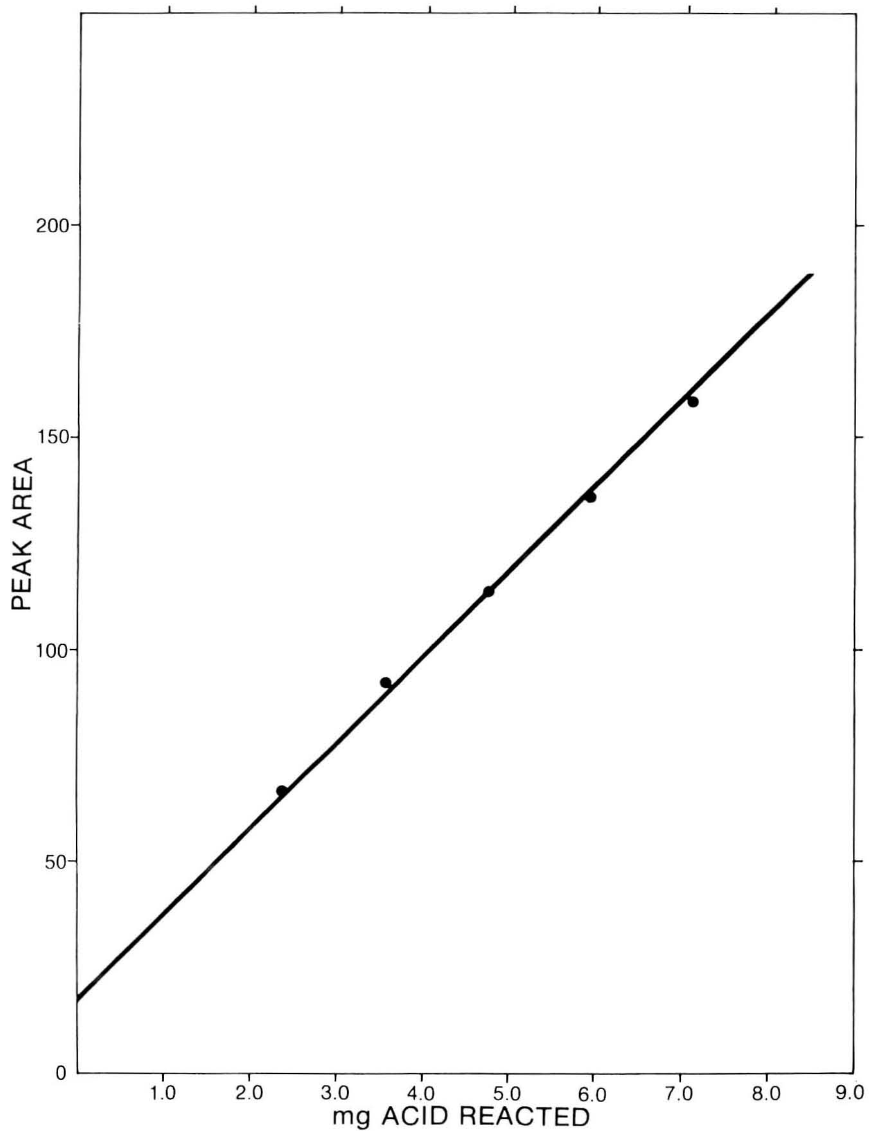

Figure 2. Standard Curve for Stearic Acid.

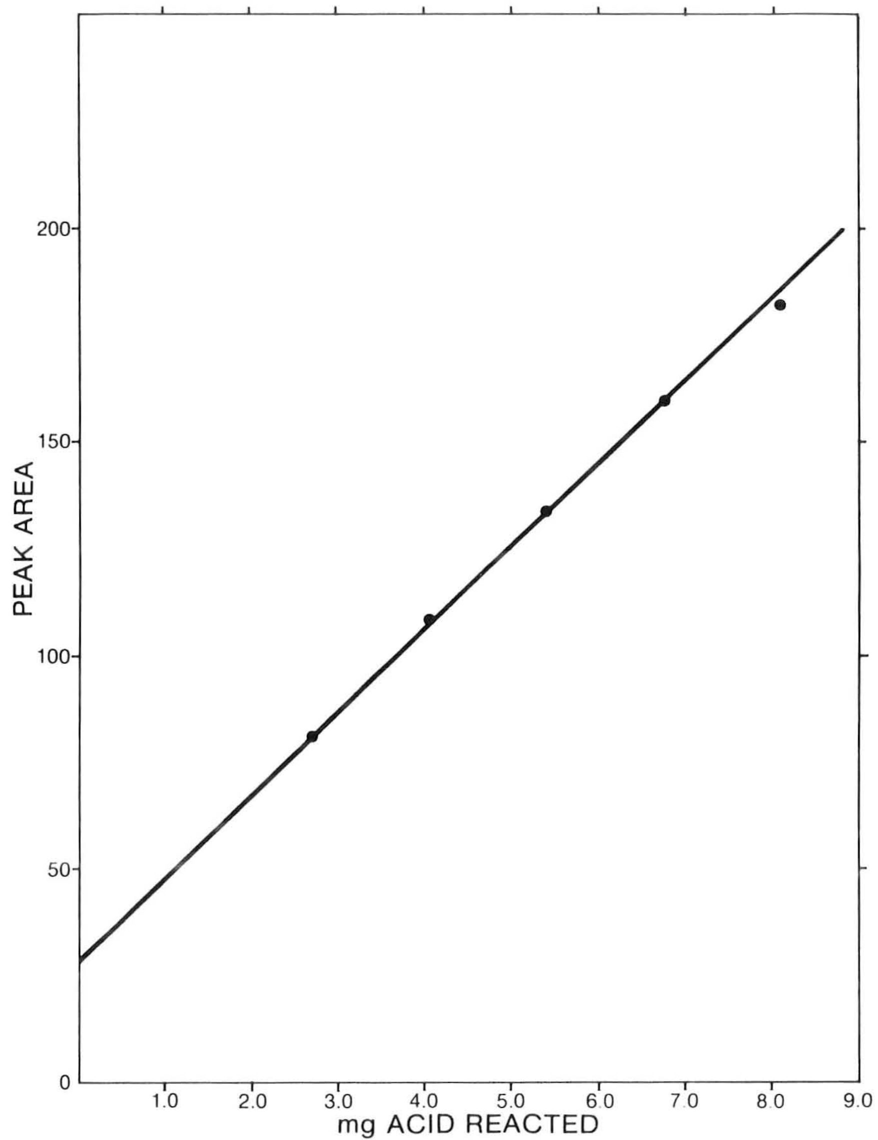

Figure 3. Standard Curve for Oleic Acid.

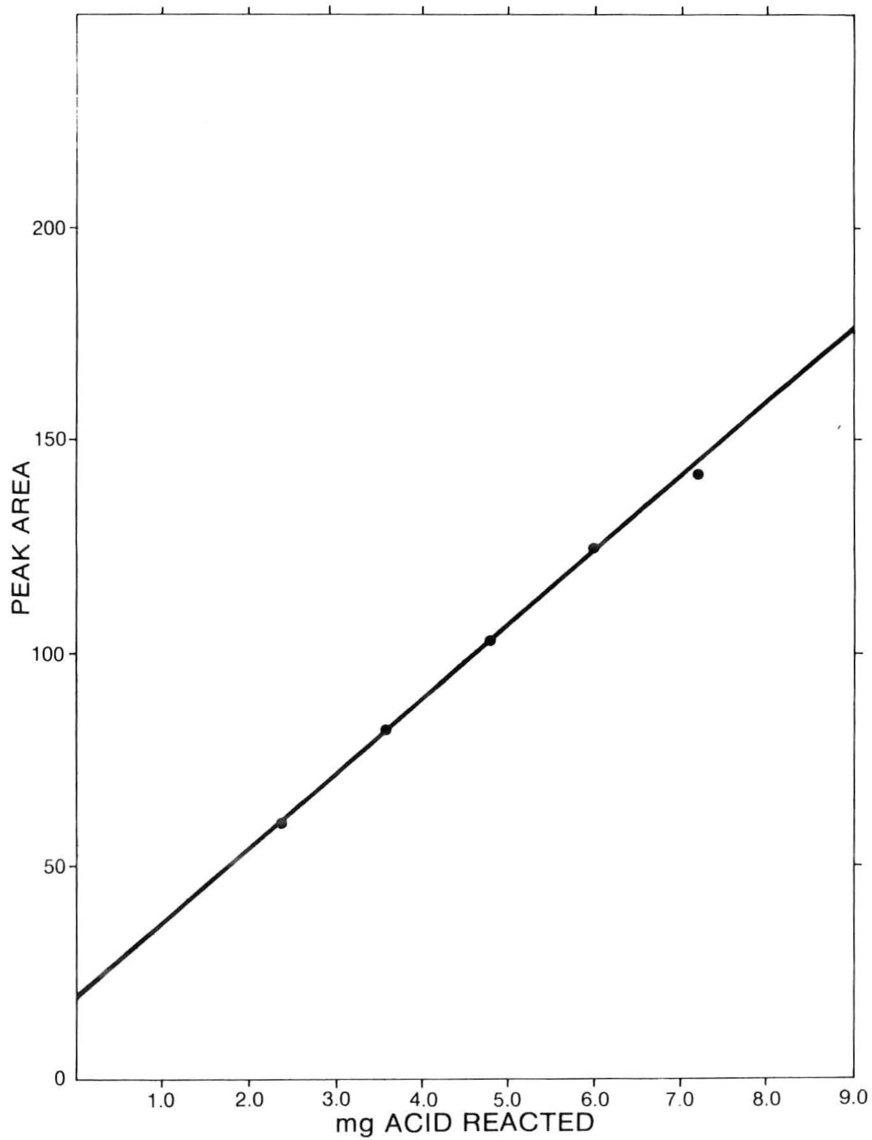

Figure 4. Standard Curve for Linoleic Acid.

CONCLUSIONS

A simple and stable method for simultaneous quantitative determination of 18-carbon fatty acids in a mixture by derivatization, reversed phase TLC separation, and densitometry of the chromatogram has been presented.

The method shows coefficient of variations in the range of 2.0 to 6.5% for the six fatty acids studied. The reproducibility of the method was determined by measuring the peak areas for 20 developed spots of each fatty acid. The Relative Standard Deviation was 1.0 to 2.3%.

The ability to determine fatty acid content in emulsions of the nature described, we believe, make it applicable to a wide variety of biological samples, where fatty acid determinations are considered to be of great diagnostic value. Future work will be conducted in this area.

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THE SEPARATION OF DICHLOROBENZOPHENONE ISOMERS BY CONTINUOUS DEVELOPMENT AND REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

Two thin-layer chromatographic methods are described for 2,4'-dichlorobenzophenone with which 1% of the 2,2'- and 2,3'-isomers can be determined. Continuous thin-layer chromatography on silica gel and conventional reversed-phase thin-layer chromatography were found to be complementary techniques for the separation of the dichlorobenzophenone isomers.

INTRODUCTION

Dichlorinated benzophenones are intermediates in the synthesis of selected fungicides and other agricultural products. In the Friedel-Crafts synthesis for the preparation of these chemicals, small quantities of isomeric impurities are also produced. In our laboratory, the purity of 2,4'-dichlorobenzophenone must be controlled to prevent carry-over of isomeric impurities to the final product, a chemical fungicide.

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Relatively little has been published on the chromatographic separation of these benzophenones. Perhaps the most extensive work has been a gas-chromatographic procedure reported recently by Abraham et al. (1). However, because this procedure is time-consuming and the resolution is insufficient for the determination of minor isomeric impurities, the method is not readily adapted to a real-time control situation.

A variety of TLC systems have been used for the separation of halogenated aromatic compounds (2-6). Applications include the separation of polychlorinated biphenyls (3) and the separation of chlorinated insecticides (4). Tewari and Sharma (5) report the thin-layer chromatographic behavior of a number of chlorinated pesticides in 26 different solvent systems; several chromogenic reagents were investigated in this work. A review of pesticide analysis by TLC has been published by Sherma and Zweig (6).

Despite the widespread use of TLC in the analysis of halogenated fungicides, pesticides, and insecticides, there are few reports on the resolution of isomeric halogenated aromatics by thin-layer chromatography. In an early report, Fishbein (7) separated a number of halogenated derivatives of aniline and benzene using three separate systems. However, with the conditions investigated, the isomeric dichlorobenzenes were not separated.

In this work, simple TLC methods are described for 2,4'-dichlorobenzophenone with which 1% of the corresponding 2,2'- and 2,3'- isomers can be detected.

We had screened a variety of TLC systems according to a previously described procedure (8). Using conventional thin-layer chromatography (i.e., using ascending development in a closed, saturated chamber over a bed length of 15 cm), equal concentrations of the three isomers could be separated. Our original optimized TLC system consisted of silica gel TLC plates and

carbon tetrachloride/benzene (4:1) as the developing solvent. However, the 2,2'- and 2,3'- isomers could not be separated at a level less than 10% of the 2,4'- main component.

Approaches to enhance the resolution of compounds with similar R_f values in conventional TLC are well documented (9-12). These approaches include successive development over two dimensions (10), multiple development (11), and continuous development over the 20-cm plate (12). The first two techniques require repeated operator interaction. All three suffer from solute diffusion problems and, consequently, high detection limits. Perry (13) recently discussed the combination of continuous development TLC with short bed lengths. With this approach, the selectivity of nonpolar solvents can be utilized without excessive spot diffusion. Perry indicated that this form of continuous TLC might be most applicable to difficult separation problems. This technique was investigated for the separation of the dichlorinated benzophenones.

In the last few years, reversed-phase chromatography has been widely applied to the separation of positional isomers of relatively nonpolar compounds. Here, the possibility of utilizing reversed-phase TLC plates to resolve the isomers of dichlorobenzophenone is also reported.

EXPERIMENTAL

Silica gel 60 F_{254} TLC plates (EM Laboratories, Inc., Elmsford, N.Y. 10523), 20 cm x 5 cm with 0.25-mm-thick layer, were used for all of the continuous development TLC separations. A stock solution of the 2,3'- and 2,2'- dichlorobenzophenone isomers was prepared at a concentration of 2.8 mg/ml in methanol. Ten, five, and one percent solutions of these isomers in the presence of the 2,4'- dichlorobenzophenone were prepared from this solution. One-microliter aliquots were applied to the TLC plate.

The equipment needed to perform the continuous TLC with short beds was available commercially (Regis SB/CD TLC chamber, Regis Chemical Company, Morton Grove, Illinois 60053). However, for this work, a convenient screening chamber was designed which could accommodate up to six plates with variable bed lengths and choice of developing solvents.

A conventional TLC chamber (20.7 cm x 8.7 cm) was modified to accommodate continuous development and a variety of bed lengths (Figure 1). The glass chamber was cut to a height of 10.5 cm and fitted with a tight-fitting Teflon lid, 1.3-cm thick. Slits (5.1 mm x 2 mm) were machined 3.0 cm apart in the Teflon parallel to its width. The slits were the proper geometry to just allow insertion of the 5 x 20 cm plates without disruption of the silica gel layer. Glass solvent reservoirs, 6 cm x 2 cm x 10 cm in height, were placed inside the chamber and made a tight seal with the Teflon lid. During development, the chamber was placed in a laboratory hood which had an air flow of approximately 200 ft/min. Under these conditions, the developing solvent traveled up the TLC plate and evaporated when it reached the top

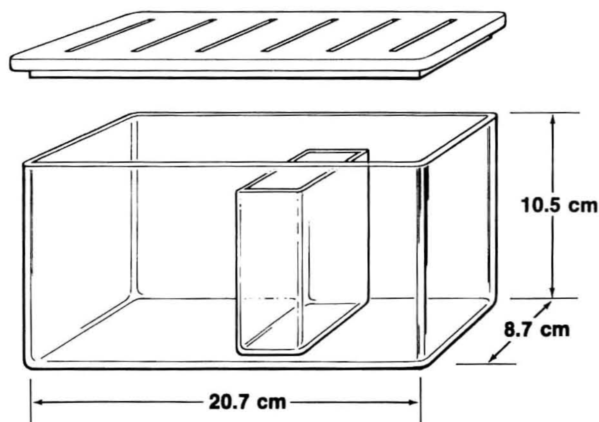

Figure 1: Continuous development TLC chamber.

of the Teflon cover. Bed lengths were varied by changing the depth of solvent in the individual reservoirs. For any depth, the samples were applied 5 mm above the solvent line.

This experimental arrangement proved to be a very efficient and economical system for continuous thin-layer chromatographic screening and optimization. Six plates could be developed simultaneously with six different solvent systems. Bed lengths could be varied continuously up to 8 cm. With this system, the plates could be placed in the chamber at the end of the day; results were available the next morning. The use of individual reservoirs resulted in very little solvent being used.

For optimization of the dichlorobenzophenone continuous TLC system, several solvent systems were prepared by diluting the optimized conventional TLC system, carbon tetrachloride/benzene (4:1), with cyclopentane. The ratio of conventional solvent systems to cyclopentane was varied in the following proportions -- 0:100, 1:100, 2:100, 5:100, and 10:100. Development of the plates was terminated when the more mobile sample zone approached 0.5 cm from the solvent evaporation line. The bed lengths investigated in this work were 4, 5, 6, and 8 cm. The 8-cm plate was allowed to run overnight. Zones were visualized by exposure to short-wavelength ultraviolet light (approximately 254 nm) after a 30-min exposure of the plate to iodine vapors.

In the reversed-phase mode, the TLC plates ($KC_{18}F$, 20 cm x 20 cm, Whatman Chemical Separation, Inc., Clifton, New Jersey 07014) were developed over a 15-cm bed in a conventional TLC chamber. The developing solvents investigated consisted of methanol and water mixtures. The proportion of water was varied from 0 to 25%. The concentrations of benzophenones and the amount applied to the plate were the same as those described above for the continuous development. The separated zones were detected by irradiation with short-wavelength ultraviolet light.

RESULTS AND DISCUSSION

Advantages and characteristics of continuous development over a short chromatographic bed have been discussed in detail by Perry (13). The results obtained with the benzophenone isomers tend to support his general conclusion that this approach to TLC may be used to advantage for select difficult TLC separations. Improved separation of the benzophenone isomers over conventional development on silica gel plates was achieved using the continuous development system. With the utilization of a more nonpolar developing solvent and a short bed length, it was possible to determine 1% of the 2,3'- isomer and 5% of the 2,2'- isomer in the presence of 2,4'-dichlorobenzophenone. This separation was obtained using a ratio of cyclopentane to conventional solvent, carbon tetrachloride/benzene (4:1), of 100:10 and an 8-cm bed length. The mobility of 2,4'-dichlorobenzophenone was intermediate between the two isomers, the 2,3'- isomer being the more mobile. For an overnight development (approximately 16 hrs), the relative mobility of the 2,4'-, 2,3'-, and 2,2'- isomers was 1.00, 1.19, and 0.81, respectively. The 8.0-cm bed was necessary to resolve the 2,2'- and 2,3'- compounds from the large amount of the 2,4'- isomer (28 μg) applied to the plate.

Reversed-phase TLC was found to be complementary to the continuous development TLC for the separation of dichlorobenzophenone isomers. Several reversed-phase systems were investigated. While the 2,3'- and 2,4'- dichlorobenzophenones could not be resolved, complete separation of 2,4'-dichlorobenzophenone and the 2,2'- isomer was obtained using a developing solvent of methanol and water (60:10). The R_f values for the 2,2'-, 2,3'-, and 2,4'- isomers with this solvent system were 0.55, 0.45, and 0.45, respectively. Using this solvent system, we were able to detect 1% of the 2,2'- isomer in the synthetic intermediate of interest; namely, the 2,4'- isomer. The development time was about 60 min.

In summary, a preliminary purity evaluation of 2,4'-dichlorobenzophenone can be made using the two simple TLC procedures reported in this work. The methods are complementary, and both are quite applicable to a production situation. In combination, they can be used to determine whether the synthetic 2,4'-dichlorobenzophenone meets existing specifications of allowable isomeric impurities.

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ASSAY OF THE ENZYMES OF PYRIMIDINE SYNTHESIS BY THIN LAYER ION EXCHANGE CHROMATOGRAPHY AND VIDEO-DENSITOMETRY (CV-TECHNIQUE)

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ABSTRACT

A simple and rapid method is described for the assay of the glutamine-dependent carbamoyl phosphate synthetase and aspartate carbamoyltransferase in minute amounts of liver tissue. From 60-120 µl assay mixtures 10-20 µl aliquots were chromatographed on thin layer ion exchange chromatoplates, and the amino acids were developed with ninhydrin. The intensity of the ninhydrin spots, i.e. the quantity of amino acids formed or consumed, was determined by video-densitometry and the specific activity of the enzymes was calculated.

INTRODUCTION

The activity of enzymes in tissue and cell extracts is usually determined by techniques requiring 1 ml or even a larger quantity of assay mixture and, obviously, a proportional amount of tissue extract. However, the available amount of the tissue sample to be assayed is often limited. In clinical diagnostics, for example, the tissue samples obtained from adult patients as well as that taken at early stages of fetal development often weigh only a few times 10 mg.

In previous investigations we have found (1, 2) that the activity of amino acid-transforming enzymes in rela-

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tively small samples can be determined by the highly sensitive thin layer ion exchange chromatographic and videodensitometric procedure (CV-technique).

Since this technique has been found to be suitable for the kinetic investigation of ornithine carbamoyltransferase as well (2), we thought it would be worth examining other amino acid-transforming enzymes, too.

The present paper describes a method for the assay of aspartate carbamoyltransferase (EC 2.1.3.2) and carbamoyl phosphate synthetase (glutamine-dependent, EC 2.7.2.9.), enzymes participating in the *de novo* synthesis of the pyrimidines. They have an important role in the metabolism of fast growing, e.g. embryonic, regenerating, tumorous, hypertrophic, etc, tissues.

MATERIALS AND METHODS

Preparation of Cytosol Extracts from Liver Cells

CFY rats, weighing 200-250 g, were starved for a day and fed with water *ad libitum*, before the experiments. They were bled under ether anaesthesis. The livers (7-8 g) were rapidly removed and kept on ice until use.The samples were homogenized in a Potter-type tephlon homogenizer at $0^{o}C$ with 3 volumes of 10 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose, 500 µM EDTA and 5 mM dithiotreitol. The homogenate was centrifuged at 15 000 x g for 30 min to remove the debris and mitochondria. The resulting cytosol fraction was concentrated 5-fold on an Amicon Centriflo 50 CF membrane (Oosterhout, The Netherlands) by centrifu-

gation. The protein content of the concentrated samples was determined according to Lowry et al. (4).

Ornithine Carbamoyltransferase

Ornithine carbamoyltransferase was used for the determination of carbamoyl phosphate synthetase activity in a coupled reaction. The enzyme was isolated from bovine liver as described by Marshall and Cohen (5). The specific activity of the purified preparation was 420 mmol citrulline/hr/mg protein.

Determination of Enzyme Activity

For the determination of the activity of the enzymes the substrate and the product/s/ formed in the assay mixture were first separated by thin layer ion exchange chromatography on 20x20 cm chromatoplates (Fixion 50x8, Chromatronix, Palo Alto, CA, USA). The Dowex 50x8 type resin-coated chromatoplates were used either in Na^+ or in Li^+ form. Plates in Li form were prepared by prechromatography in 0.5 M LiCl solution (6). The assay mixtures were incubated at 37°C, then 10-20 µl samples were added to equal amounts of 10 % trichloroacetic acid drops,which had been applied onto the chromatoplate beforehand. The plates were dried by hot air, and were then chromatographed in the solutions descibed below.

For comparison the activity of aspartate carbamoyltransferase was determined also by the colorimetric procedure as described by Prescott and Jones (7), except that deacetylmonoxim-thiosemicarbazide was used instead

of diacetylmonoxim-antipyrine, and the absorption was measured at 520 nm (8).

Solutions Used for Chromatography

For the determination of aspartate and citrulline the chromatoplate was used in Na^+ form. Chromatography was carried out at 50°C in citrate buffer, pH 3.3, composed of 84 g of citric acid, 16 g of NaOH and 5.9 ml of concentrated HCl in 1000 ml final volume (9).

To measure the amount of glutamate chromatography was performed at 50°C on chromatoplates in Li^+ form in a lithium-citrate-formate system composed of 14.1 g of LiCl, 2.3 ml of 85 % formic acid and 8.0 ml of concentrated HCl in a final volume of 1000 ml at pH 2.8 (6).

After chromatography the plates were dried and the amino acids were developed with cadmium-ninhydrin reagent. The reagent was prepared freshly before use by mixing 100 ml of 1 % ninhydrin dissolved in acetone and 20 ml of 1 % cadmium-acetate dissolved in 10 % acetic acid (9).

Quantitative Determination of Amino Acids

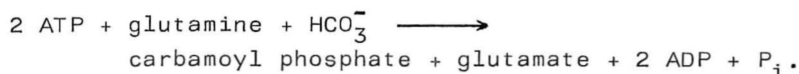
A Telechrom reflexion video-densitometer (3) was used for the determination of the intensity of ninhydrin spots (10). The chromatoplates were placed into the equipment perpendicular to the direction of chromatography.The density of identical amino acid spots of samples, withdrawn from the assay mixture at different time intervals, together with that of the reference spot were taken as 100 % and the intensity of the individual spots was ex-

pressed in percentage values. The molar concentration of the individual amino acid spots was calculated from the percentual density of the reference amino acid (1, 2). The standard deviation of the video-densitometric evaluation of chromatograms was ± 3 %, as determined with lysine (10).

RESULTS AND DISCUSSION

Determination of the Glutamine-Dependent Carbamoyl Phosphate Synthetase Activity

This enzyme catalyzes the following reaction:



$$2\ ATP + glutamine + HCO_3^- \longrightarrow carbamoyl\ phosphate + glutamate + 2\ ADP + P_i.$$

If the assay mixture contained ornithine and ornithine carbamoyltransferase, carbamoyl phosphate was converted into citrulline: carbamoyl phosphate + ornithine ⟶ citrulline + P_i.

The activity of carbamoyl phosphate synthetase can be determined by measuring the increase in glutamate or in citrulline concentrations. When the formation of glutamate was followed, the assay mixture of 120 µl final volume contained 25 mM ATP, 25 mM sodium-hydrogen-carbonate, 50 mM magnesium-sulphate, 50 mM glutamine and tissue extract equal to 12-15 mg of fresh liver, in 50 mM veronal-sodium buffer, pH 7.4. The mixture was incubated at 37°C, and at 30-min intervals 20 µl samples were withdrawn and applied onto a chromatoplate in Li^+ form then it was chromatographed in a lithium-formate

A.)

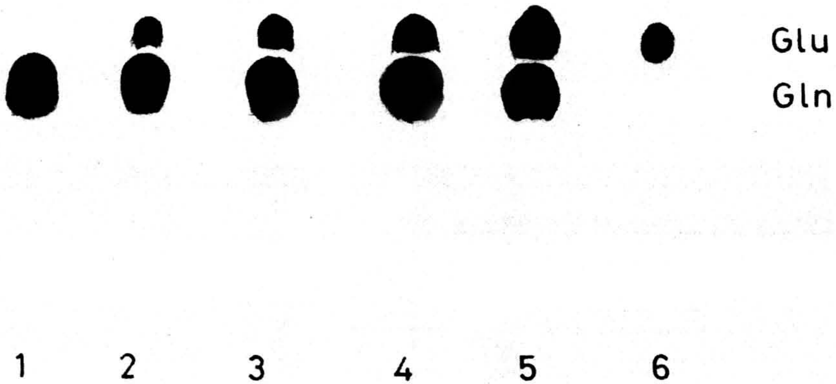


FIGURE 1 - Carbamoyl phosphate synthetase assay by CV-technique.
A./ Formation of glutamate from glutamine.

solvent system as described in Methods. As reference, 25 nmol of glutamate was also run (Fig. 1a). The specific activity of the enzyme measured in 15 liver samples was 430 $\pm$ 32 nmol glutamate/g wet tissue/h.

The glutamate-dependent carbamoyl phosphate synthetase can also be determined by measuring the formation of citrulline (2). In this case the above assay mixture was supplemented with 10 mM ornithine and 10 μl ornithine carbamoyltransferase. 20 μl samples were chromatographed on a chromatoplate in Na^+ form. The amount of citrulline formed in the reaction was measured (Fig. 1b).

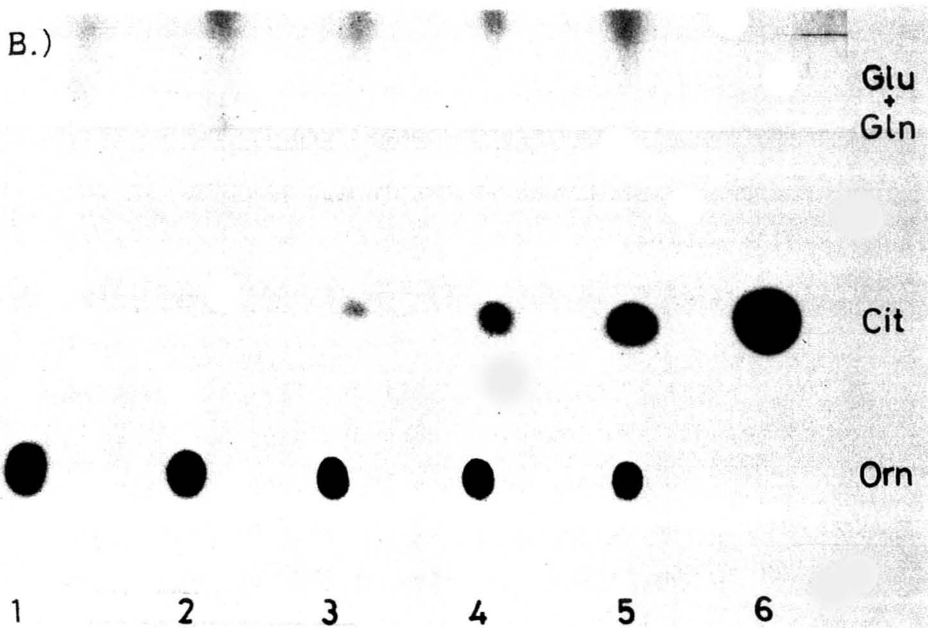


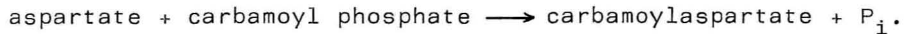
FIGURE 1 - (continued)
B./ Formation of citrulline from ornithine and carbamoyl phosphate. 1 - 5 samples taken in 30 min intervals, 6. reference amino acids. For details see Methods.

The specific activity of the same 15 liver samples as above was 520 ± 60 nmol citrulline/g wet tissue/h. This value is by 17 % greater than that obtained by measuring the glutamate formation. The difference may be explained by the fact that a portion of carbamoyl phosphate is further converted by ornithine carbamoyltransferase present in the liver extract. Hence, the reaction is pulled in the direction of carbamoyl phosphate synthesis. Thus, the activity of carbamoyl phosphate synthetase can be de-

termined more reliably by measuring the amount of glutamate.

Determination of Aspartate Carbamoyltransferase Activity

The enzyme catalyzes the following reaction in the pyrimidine synthesis:



aspartate + carbamoyl phosphate ⟶ carbamoylaspartate + P_i.

The activity of the enzyme was determined by measuring the changes in aspartate concentration. The assay mixture contained liver extract, corresponding to 1-2 mg of fresh liver, 10 mM aspartate and 15 mM carbamoyl phosphate in 20 mM triethanolamine-HCl buffer, pH 7.7, in a final volume of 60 µl. It was incubated at 37°C and at 30-min intervals 10 µl samples were withdrawn and applied onto a chromatoplate in Na^+ form. As reference, 50 nmol of aspartate was also run. A specific activity of 48 ± 3.3 µmol aspartate/g wet tissue/h was determined in the assay carried out with 25 samples of rat liver.

For comparison the activity of aspartate carbamoyltransferase was also determined by spectrophotometry. According to least squares regression analysis, $y = 0.96x + 1.02$, where y is the spectrophotometric activity and x is the activity determined by the CV-technique. The calculated determination coefficient, r^2, was 0.90, which indicates a 90 % correlation between the data obtained with the two procedures.

We may conclude that ion exchange chromatography combined with video-densitometry, i.e. the CV-technique, is

suitable for the assay of the activity of the above two enzymes, and of other enzymes as well (1,2). The sensitivity of the technique is reasonably high. The formation or consumption of even 1-2 nmol of amino acids can be detected by the CV-technique statisfactorily. Enzyme reaction can be assayed in 10-20 µl samples of a reaction mixture of even less than 100 µl final volume, and thus enzymes can be detected in extracts prepared from a few milligrams of tissues.

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SYNTHETIC INORGANIC ION-EXCHANGERS. XX. THIN LAYER CHROMATOGRAPHY OF METAL IONS ON LANTHANUM ANTIMONATE. QUANTITATIVE SEPARATION OF Hg (II) FROM SEVERAL METAL IONS.

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ABSTRACT

The analytical potential of Lanthanum antimonate as an ion exchanger has been explored by thin layer chromatographic (TLC) technique. Binder-free thin layers of Lanthanum antimonate have been explored for several important binary and ternary separations. A TLC method has been developed for quantitative separation of microgram quantities of Hg(II) from several metal ions by using 1,4 dioxane as solvent.

INTRODUCTION

Thin layer chromatography is being used in recent years for inorganic analysis (1). In continuation of our work on TLC studies on thorium phosphate, tungstate, antimonate, ziroconium tungstate (2-4), we report in this paper systematic investigations on TLC behaviour of several metal ions on binder-free thin-layer plates of lanthanum antimonate. Based on studies in HNO_3 (pH-1,2 and 3), butanol, 1,4 dioxane, 1,4 dioxane - HNO_3, some important binary and ternary separations have been achieved. A quantitative method for the separation of Hg(II) from numerous metal ions is recommended.

EXPERIMENTAL

Apparatus

Thin layers of Lanthanum antimonate were prepared on glass plates (20 x 3 cm), which were subsequently developed in several

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solvent systems in jars (25 x 7 cm). For spectrophotometric studies, Spectrophotometer G.S. 865 B of Electronics Corporation of India, Hyderabad, India was used.

Reagents

Chemicals and solvents used in this work were of analytical grade (B.D.H / E.Merck / Pfizer)

Preparation of the Ion-Exchange Materials on thin layer plates.

The ion exchanger, lanthanum antimonate (Sb:La=4.298) was prepared according to the procedure described in earlier paper (5). Each material was then powdered separately and slurried with a little demineralized water in a mortar. It was then spread over the glass with the help of an applicator. Almost uniformly thin layers (0.1 mm thickness) were obtained. The plates were dried and ready for use. These plates gave reproducible R_F values.

Test Solutions and Detection Reagents

The test solutions in general, had a metal concentration of 4 mg/ml (chloride/nitrate/sulphate) Standard spot test reagents were used for detection (6).

Solvent System

The following solvent systems were used in these studies.

1. HNO_3 solution (pH-1,2,3)
2. Butanol
3. 1,4 Dioxane
4. 1,4 Dioxane: 0.1 M HNO_3 (8:2).

PROCEDURE

One or two drops of the test solution were placed on the plates with thin glass capillaries. The spots were allowed to dry and developed in different solvent systems. In each case the solvent was allowed to rise 11 cm. R_T and R_L values were measured as usual after detection.

For quantitative work, a stock solution of Hg(II) (5.1 mg/ml) was prepared by dissolving $HgCl_2$ in 0.1 M HCl. The known amount of

synthetic mixture containing Hg was applied with the help of micro pipette on the line of application. The plates were developed in dioxane system. A pilot plate was run simultaneously to locate the position of Hg by detecting it with yellow ammonium sulphide. The area corresponding to Hg was scratched from the working plate and the mass was extracted with 10 ml 1 M H_2SO_4. The suspended particles of the exchanger were filtered off. The filtrate was collected and Hg(II) was determined spectrophotometrically by dithizone method(8).

RESULTS AND DISCUSSION

The results of our TLC studies reveal that most of the metal ions have appreciable R_F values in nitric acid system. The general trend in R_F values is that these values decrease with increase in

TABLE - I

Binary and Ternary Separations on Lanthanum Antimonate Thin Layer

Solvent system.	Separations achieved R_T - R_L	Time (hours)
0.1M HNO_3	Fe^{3+}(0.0-0-30) - Pt^{4+}(0.72 - 0.90)	
	Fe^{3+}(0.0-0.21) - Ni^{2+}(0.62 - 0.85)	
	Fe^{3+}(0.0-0.21) - Au^{3+}(0.78 - 0.95)	
	Ce^{3+}(0.0-0.0) - Au^{3+}(0.85 - 0.94)	2 hrs
	Ce^{3+}(0.0-0.0) - Hg^{2+}(0.83 - 0.93)	
	Ce^{3+}(0.0-0.0) - Pt^{4+}(0.80 - 0.83)	
	Ce^{3+}(0.0 - 0.0)- Mn^{2+}(0.78 - 0.90)	
	Bi^{3+}(0.0-0.0) - Hg^{2+}(0.89 - 0.96)	
	Bi^{3+}(0.0-0.0) - Au^{3+}(0.82 - 0.95)	
	Bi^{3+}(0.0-0.0) - Ni^{2+}(0.76 - 0.90)	
	Bi^{3+}(0.0-0.0) - Pt^{4+}(0.30 - 1.0)	
	Bi^{3+}(0.0-0.0) - Mn^{2+}(0.71 - 0.90)	
	Cu^{2+}(0.0-0.21) - Au^{3+}(0.68 - 0.84)	
	Cu^{2+}(0.0-0.14) - Hg^{2+}(0.74 - 0.86)	

TABLE 1 (Continued)

Solvent system	Separations achieved R_T - R_L	Time (hours)
$0.01M HNO_3$	Co^{2+}(0.0-0.0) - Pt^{4+}(0.75 - 0.85)	
	Co^{2+}(0.0-0.0) - Ni^{2+}(0.72 - 0.84)	2.5 hrs.
	Co^{2+}(0.0-0.0) - Hg^{2+}(0.85 - 0.92)	
	Co^{2+}(0.0-0.0) - Au^{3+}(0.85 - 0.90)	
	Pb^{2+}(0.0-0.0) - Ni^{2+}(0.80 - 0.89)	
	Pb^{2+}(0.0-0.0) - Pt^{4+}(0.80 - 0.91)	
	Pb^{2+}(0.0-0.0) - Au^{3+}(0.81 - 0.90)	
	Th^{4+}(0.0-0.0) - Hg^{2+}(0.82 - 0.90)	
	Th^{4+}(0.0-0.0) - Pt^{4+}(0.78 - 0.91)	
	Th^{4+}(0.0-0.0) - Au^{3+}(0.80 - 0.92)	
$0.001M HNO_3$	UO_2^{2+}(0.0-0.0) - Hg^{2+}(0.84 - 0.96)	
	UO_2^{2+}(0.0-0.0) - Au^{3+}(0.86 - 0.95)	3 hrs
	UO_2^{2+}(0.0-0.0) - Pt^{4+}(0.78 - 0.98)	
	Ni^{2+}(0.0-0.35) - Hg^{2+}(0.82 - 0.95)	
	Ni^{2+}(0.0-0.25) - Pt^{4+}(0.85 - 0.97)	
Butanol	Zn^{2+}(0.0-0.0) - Au^{3+}(0.71 - 0.85)	10 hrs.
	Al^{3+}(0.0-0.0) - Au^{3+}(0.80 - 0.90)	
	Ni^{2+}(0.0-0.0) - Au^{3+}(0.84 - 0.94)	
	Mn^{2+}(0.0-0.0) - Au^{3+}(0.81 - 0.90)	
	UO_2^{2+}(0.0-0.0) - Ru^{3+}(0.35 - 0.42) - Au^{3+}(0.75 - 0.82)	
	Cu^{2+}(0.0-0.0) - Ru^{3+}(0.26 - 0.35) - Au^{3+}(0.82 - 0.94)	
	Pb^{2+}(0.0-0.0) - Ru^{3+}(0.24 - 0.36) - Au^{3+}(0.81 - 0.92)	
	Bi^{3+}(0.0-0.0) - Ru^{3+}(0.27 - 0.36) - Au^{3+}(0.84 - 0.96)	
1,4 Dioxane : $0.1M HNO_3$ (8:2)	Fe^{3+}(0.0-0.1) - Hg^{2+}(0.85 - 1.0)	8 hrs.
	Pb^{2+}(0.0-0.0) - Hg^{2+}(0.85 - 0.95)	

TABLE - 2

Quantitative Separation of Hg^{2+} from Binary Mixtures

Sl. No.	Mixture taken	Other metal ion added (μg)	Hg^{2+} added (μg)	Hg^{2+} recovered (μg)	Percentage of error
1.	$Cu^{2+}-Hg^{2+}$	Cu^{2+}(7.8)	10.3	10.4	+ 1.0
2.	$Cu^{2+}-Hg^{2+}$	Cu^{2+}(3.9)	5.1	5.2	+ 2.0
3.	$Pb^{2+}-Hg^{2+}$	Pb^{2+}(14.2)	10.3	10.6	+ 3.0
4.	$Pb^{2+}-Hg^{2+}$	Pb^{2+}(7.1)	5.1	5.4	+ 6.0
5.	$Cd^{2+}-Hg^{2+}$	Cd^{2+}(14.2)	10.3	10.1	- 2.0
6.	$Cd^{2+}-Hg^{2+}$	Cd^{2+}(7.1)	5.1	5.2	+ 2.0
7.	$Mn^{2+}-Hg^{2+}$	Mn^{2+}(7.2)	10.3	10.7	+ 4.0
8.	$Mn^{2+}-Hg^{2+}$	Mn^{2+}(3.6)	5.1	4.9	- 4.0
9.	$Co^{2+}-Hg^{2+}$	Co^{2+}(7.6)	10.3	10.0	- 3.0
10.	$Co^{2+}-Hg^{2+}$	Co^{2+}(3.8)	5.1	5.3	+ 4.0
11.	$Zn^{2+}-Hg^{2+}$	Zn^{2+}(8.5)	10.3	10.8	+ 5.0
12.	$Zn^{2+}-Hg^{2+}$	Zn^{2+}(4.3)	5.1	4.9	- 4.0
13.	$Fe^{3+}-Hg^{2+}$	Fe^{3+}(7.3)	10.3	10.5	+ 2.0
14.	$Fe^{3+}-Hg^{2+}$	Fe^{3+}(3.7)	5.1	5.2	+ 2.0
15.	$Bi^{3+}-Hg^{2+}$	Bi^{3+}(13.5)	10.3	10.5	+ 2.0
16.	$Bi^{3+}-Hg^{2+}$	Bi^{3+}(6.8)	5.1	5.3	+ 4.0
17.	$UO_2^{2+}-Hg^{2+}$	U^{6+}(14.6)	10.3	10.8	+ 5.0
18.	$UO_2^{2+}-Hg^{2+}$	U^{6+}(7.3)	5.1	5.3	+ 4.0

pH in HNO_3 system which is a characteristic feature of ion exchange operation. In pure 1,4 dioxane system most of the metal ions except Hg(II) are retained at the base line. This permits quantitative separations of Hg(II) from other metal ions. Tables 1 and 2 show some useful and important binary and ternary separations of metal ions achieved in different solvents.

ACKNOWLEDGEMENT

One of the authors (P.B.C) is grateful to the University Grants Commission, New Delhi for the award of a Junior Research Fellowship.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 4(12), 2229-2232 (1981)

Identification of some closely related Pyrazolin-5-Ones by TLC

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ABSTRACT

A rapid thin layer chromatographic procedure that utilizes neutral solvent system for the separation of 22 closely related pyrazolin-5-Ones on silica gel adsorbent is reported.

INTRODUCTION

Pyrazolin-5-Ones and its derivatives are biologically active compounds and have been used as analgesies[1], antimicrobial agents[2], fungicides[3], herbicides[4], antidiabetics[5], antidiuretics[6], antioxidants[7] and in influenza[8]. They also find many applications in photography as colour couplers[9], sensitizers[10], super sensitizers[11] and developers[12]. Another important commercial use of pyrazolin-5-Ones is as a dye for rayon[13], silk[14], leather[15], rubber[16],polyester[17] and plastics[18].

Recently, Garg and Prakash[19,20] have synthesised some 1-phenyl-3-methyl-4-arylhydrazono-2-pyrazolin-5-Ones (A) as potential antidiabetic agents. As these compounds contain the hydrazono grouping -NH-N=C- , they have found wide applications in synthetic chemistry for the preparation of compounds of most diverse structure and also for the detection of a large number of metal cations.

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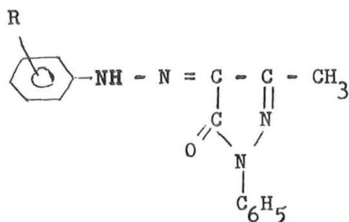

(A)

where, R represents different substituents.

Keeping in view also the medicinal properties of pyrazolin-5-Ones it was now considered worthwhile to study the separation of some closely related 1-phenyl-3-methyl-4-arylhydrazono-2-pyrazolin-5-Ones by TLC.

EXPERIMENTAL

Commercially available silica gel G, TLC plates of size 21.5 x 21.5 cm^2, layer thickness 0.4 mm were used after activation for 24 hrs. All the pyrazolin-5-Ones were synthesised[19] in the laboratory and repeatedly recrystallised with DMF:water mixture before subjecting them to chrometographic separation. All the compounds (I-XXII) in acetone (1 % V/V) were applied by means of a fine class capillary and then put in the developer for the development process. The resolved compounds were visualized by exposing to NO_2 for 40 sec. It is pertinent to note that no tailing was observed in any case except in 4-OC_2H_5derivative where slight tailing was observed. The Rf values obtained were found reproducible in different identical runs.

RESULTS AND DISCUSSION

The TLC data obtained are given in Table 1. The development time for the solvent systems employed was about 45 min. Both the solvent systems used gave satisfactory separation of most of the compounds. The colour of all the spots (I-XXII) was light yellow, darkened on exposure to NO_2. The results show an interesting

Table - 1

No.	R	M.P. °C	R_f x 100		Detection limit (μg)
			A	B	
1	H	132	71	36	2.5
2	2-CH_3	175	51	26	3.0
3	3-CH_3	180	76	32	3.0
4	4-CH_3	137	85	43	3.5
5	2-Cl	185	35	57	2.5
6	3-Cl	135	55	63	2.5
7	4-Cl	140	62	72	3.0
8	2-Br	180	26	29	3.5
9	3-Br	150	30	39	3.5
10	4-Br	145	49	45	2.0
11	2-OCH_3	163	40	40	3.5
12	4-OCH_3	135	62	51	3.5
13	2-OC_2H_5	163	58	24	3.5
14	4-OC_2H_5	150	67	35	2.0
15	2-NO_2	212	38	18	3.5
16	3-NO_2	175	46	22	3.0
17	4-NO_2	170	80	48	3.5
18	2,3-$(CH_3)_2$	210	21	26	2.5
19	2,5-$(CH_3)_2$	188	17	18	2.5
20	3,5-$(CH_3)_2$	193	26	21	3.0
21	2,6-$(Cl)_2$	179	13	11	3.5
22	2,4,6-$(Cl)_3$	227	8	9	2.5

Solvent Composition for compounds I-XXII

(A) Xylene: Chloroform (60 % : 40 %).

(B) Xylene: Chloroform: Acetone (65 %: 25 %: 10 %).

trend in the R_f values. It is observed that in the case of ortho substituted derivative the rate of flow (R_f) of the spot is low whereas meta and para substituents increase the value of R_f in comparison with that of the ortho substituted derivative.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 4(12), 2233-2241 (1981)

A SIMPLE ION EXCHANGE THIN LAYER CHROMATOGRAPHY METHOD FOR THE SEPARATION OF AMINO ACIDS IN CLINICAL SAMPLES

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ABSTRACT

An ion exchange thin layer chromatography method for the separation of 16 amino acids is described. The effect of the pH of buffer on the resolution of the amino acids is discussed. Development in pH 3.3 buffer at 45°C resolved all the amino acids. The method is particularly well adapted to screening metabolic disorders in neonatal children.

INTRODUCTION

A number of metabolic diseases are characterized by the presence of amino acids in the blood and urine, e.g. phenylketonuria (PKU), in which the concentration of phenylalanine is elevated. The semi-quantitative Guthrie-Susi test (1) normally used to detect this condition is based on bacterial growth, and the results require confirmation by another method. The amino acid analyzer

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(2) could be used but this is an expensive approach which can still handle only one sample at a time. The same is true of high performance liquid chromatography. The amino acids are analyzed after derivatization with dansyl chloride (3,4) phenyl thiohydantoin (5) or o-phthalaldehyde/2-mercaptoethanol (6-8) because they are not readily measured by HPLC due to their low response to both UV and fluorescence detection. A recent method (9) was described for the determination (in 6 hours) of phenylalanine using immobilized bacteria and a lactate electrode. Although this method is sensitive it can analyze only one sample at a time and is specific for phenylalanine.

Ireland and Read (10) described a thin layer chromatography (TLC) method for use in neonatal screening to detect excess aminoacidaemia. In this method the extracted blood is spotted on cellulose plates which are then developed in two solvent systems. The plate is throroughly dried and then developed overnight in an ammonia-free atmosphere (a chromatography tank containing a dish of sulfuric acid) in the dark. Lepri et al (11) described a TLC method using impregnated reverse phase plates. However, the resolution of phenylalanine was poor on all reverse phase plates (C_2, C_8 and C_{18}) used.

When ion-exchange thin layer chromatography was introduced (12), basic and aromatic amino acids could be separated on one plate. The technique was soon applied to the detection of metabolic disorders (13-15). We have developed a simple and rapid adapation of this technique which can be used to analyze amino acids in whole blood, serum, or even a blood sample dried on a piece of filter paper.

EXPERIMENTAL

Materials: Strongly acidic cation-exchange resin-coated TLC plates, Fixion 50 x 8, sodium form, were obtained from Chromatronix (Mountain View, CA). All chemicals used were reagent grade. Buffers were prepared in distilled deionized water. Standard amino acid solutions (table 1) were prepared in ethanol/HCl (95:5). Stock solutions were stored in the freezer. Small quanti-

TABLE 1
Concentration of standard amino acids
in ethanol:hydrochloric acid (95:5) solution.

Amino acid	mg/ ml	Amino Acid	mg/ ml
Aspartic acid	1.5	Isoleucine	6
Threonine	28	Leucine	12
Serine	21	Tyrosine	15
Proline	25	Phenylalanine	15
Glutamic acid	12	Lysine	36
Glycine	31	Ornithine	15
Alanine	34	Histidine	14
Valine	20	Arginine	12
Cystine	9	Tryptophan	10
Methionine	5.5		

ties of samples diluted 4-fold will keep for several days in a refrigerator at 4°C. Regular TLC tanks and disposable micropipettes were used.

Eluting Solution: The buffer pH 4.2 in which the amino acids were separated was prepared by dissolving 14.4 g citric acid monohydrate, 8 g NaOH, 11.7 g NaCl and 7.7 ml concentrated HCl (37%) in 1L distilled deionized water.

Spray Reagent: Solution A - 1 g ninhydrin in 100 ml 80% acetone.

Solution B - 1 g cadmium acetate in 150 ml 33% acetic acid

The reagent is prepared by mixing 100 ml of A with 20 ml B to which 1 ml of pyridine has been added. For maximum color development use a freshly prepared solution. Pyridine and cadmium acetate are added to enhance the color of the spots, and to exert a slight buffer effect which counteracts the acidity of the plate.

Extraction of amino acids from fresh blood or serum. 50 µl whole blood or serum are pipetted into a 1 ml vial containing 10 µl ethanol and 5 µl trifluoro-

acetic acid. Mix vigorously, preferably with a sonicator for 5 min, and then centrifuge for 5 min. Spot 10 μl of the supernatant on the plate, allow to dry and then develop for a distance of 10 cm in the buffer described above. The plate is again dried, then sprayed with the ninhydrin reagent and dried once again with a hair dryer on the hot setting. The amino acids will appear as pink spots which can easily be seen in white light.

Extraction of amino acids from dried blood: The filter paper on which the blood sample (50 μl) has dried is cut into small pieces which are placed in a test tube. 100 μl ethanol:HCl (95:5) and 10 μl TFA are added and the tube is allowed to stand for 10 min and then centrifuged. 10 μl of the supernatant is spotted on the plate and the procedure described above then followed.

RESULTS AND DISCUSSION

Effect of pH on the Separation of Amino Acids. When plates are run in aqueous buffer it is possible to change the chromatographic conditions rapidly so that the necessary separation can be achieved. The analyst can change the pH, the ionic strength of the buffer, or both, as needed. In Fig 1 the separation of 16 amino acids on a plate developed in citrate buffer, pH 3.3 at 45°C is shown. Note that all the amino acids are well resolved and can clearly be identified.

When the plate was developed in citrate buffer pH 4.2 at room temperature, (Fig 2B) the amino acids arginine, histidine, lysine, ornithine, phenylalanine, and tyrosine were resolved from each other followed by leucine + isoleucine + methionine as one spot, and then valine. The remainder of the amino acids appeared as one spot at a high R_f. Note that lysine and histidine have changed their order of elution with the change in pH from 3.3 (Fig 2A) to 4.2 (Fig 2B).

At a pH of 5.1 (Fig 2C) lysine and ornithine are not resolved. At higher pH's, such as 6.0 (Fig 2D) it is possible to separate tryptophan from other amino acid. We conclude that, for the resolution of all 16 amino acids the best conditions are pH 3.3 citrate buffer at 45°C (Fig 1).

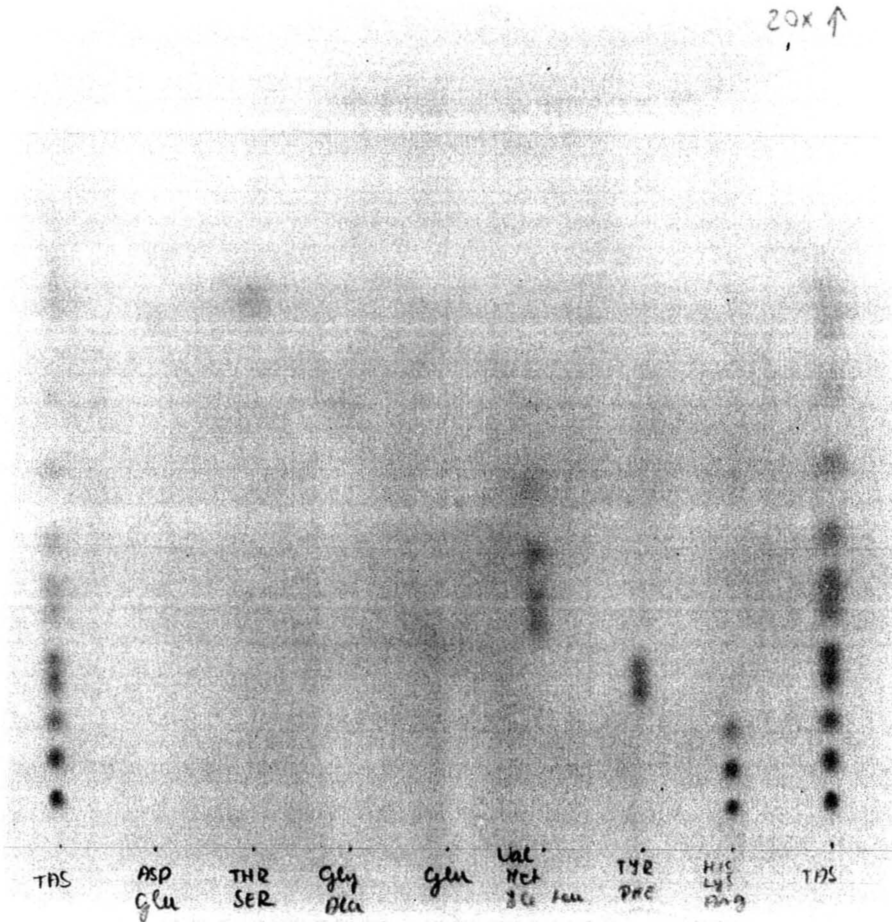

Figure 1. Separation of 16 amino acids in citrate buffer, pH 3.3 at 45°C.

In blood samples from PKU cases the levels of phenylalanine are elevated, and the best conditions to use are buffer pH 4.2 or 5.1 and to develop the plates at room temperature. A comparison of the phenylalanine levels in the blood of a normal child with that from a PKU patient is shown in Fig 3. Note that better results were obtained when serum was used (Fig 4) rather than whole blood. Unlike the Guthrie-Susi test (1) the present method allows the simultaneous

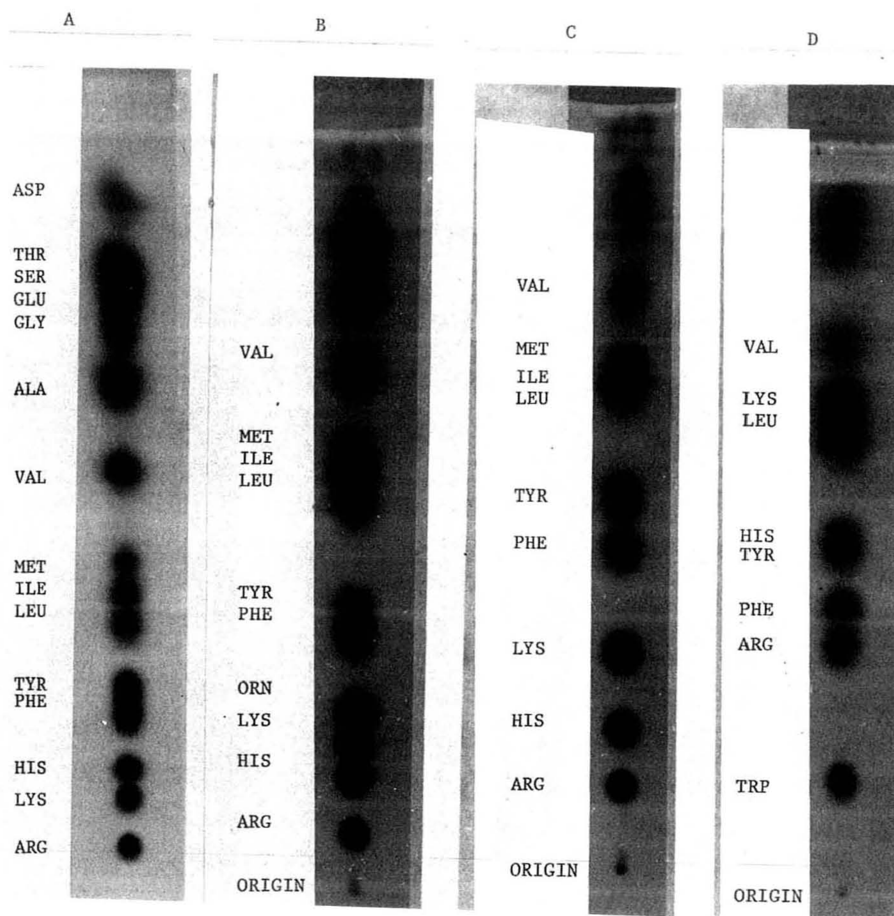

Figure 2. Comparison of the separation of amino acids in a citrate buffer having a pH of 3.3 (A), 4.2 (B), 5.1 (C) and 6.0 (D).

determination of more than one amino acid in more than one sample on the same plate. This is important because it was found by Haltzman et al (16) that false PKU positives can be reduced if a tyrosine determination is performed together with phenylalanine. The use of pH 5.1 buffer allows such a determination (Fig 2C). In a recent review article of screening for inborn errors of

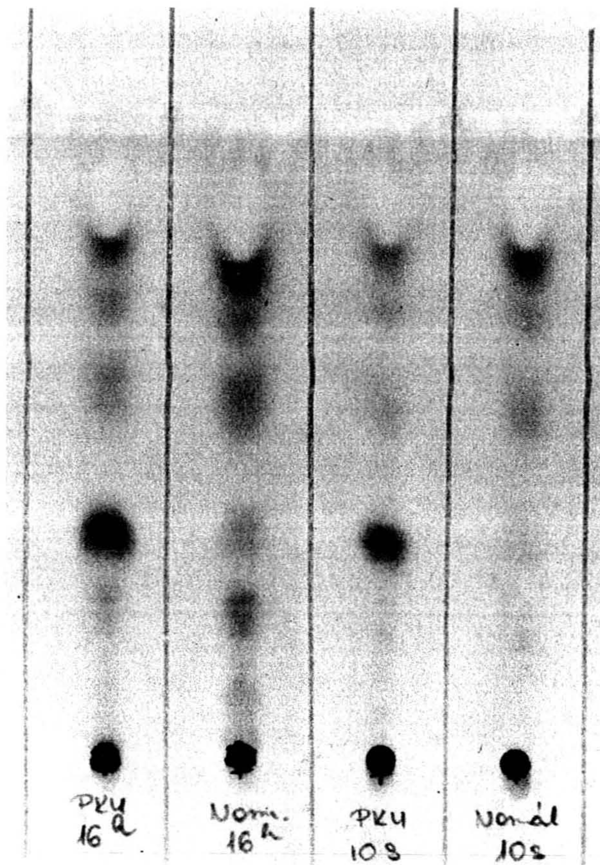

Figure 3. Comparison of phenylalanine levels in the blood of PKU patient and a normal child. See Figure 2(B) for order of elution.

metabolism, Watts (17) suggested that a screening method should be (a) reliable and simple; and (b) produce no false negatives, and few false positives. The test described here is simple, reliable and quantitative (when densitometry is used) and can be used for screening more than one amino acid disorder simultaneously, for example tyrosinaemia, histidinaemia, lysinaemia, and maple syrup urine disease.

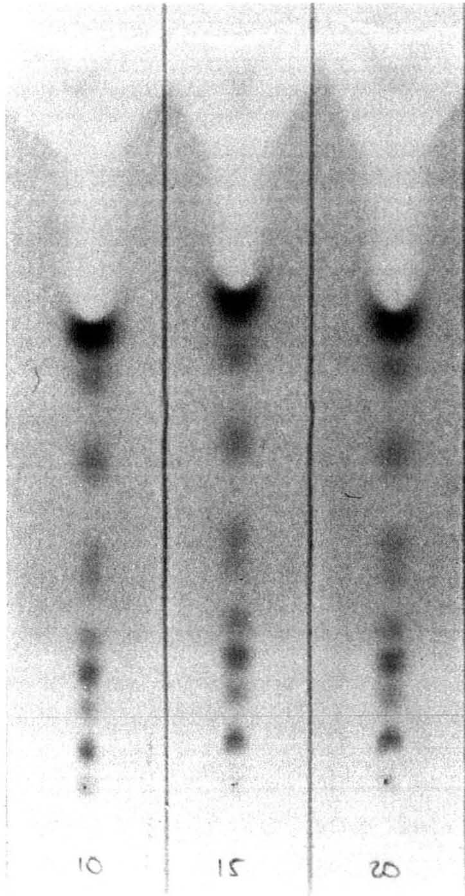

Figure 4. Separation of amino acids in serum using a citrate buffer, pH 4.2 at room temperature. See Figure 2(B) for order of elution.

CONCLUSION

Ion exchange thin layer chromatography using strongly acidic resin-coated plates affords a simple and straight forward method for the separation in a single one-dimensional run in aqueous buffer of the basic and aromatic amino acids. The technique is ideal for use in screening amino acid disorders in the blood and other biological samples. It can be operated with as little as one drop of blood dried on a piece of filter paper.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 4(12), 2243-2244 (1981)

LC NEWS

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JOURNAL OF LIQUID CHROMATOGRAPHY, 4(12), 2245-2246 (1981)

LC CALENDAR

1982

March 28 - April 2	National American Chem. Soc. Meeting, Las Vegas, NV, USA. Contact: A.T. Winstead, Am. Chem. Soc., 1155 Sixteenth St., NW, Washington, D.C., 20036, USA.
April 14 - 16	"12th Annual Symposium on the Anal. Chem. of Pollutants," Amsterdam, The Netherlands. Contact: Prof. R.W. Frei, Congress Office, Vrije Universiteit, P.O. Box 7161, 1007-MC Amsterdam, The Netherlands.
June 28 - 30	"Analytical Summer Symposium," Michigan State Univ., East Lansing, MI, USA. Contact: A.I. Popov, Chem. Dept., Michigan State Univ., East Lansing, MI 48824, USA.
July 12 - 16	"2nd Int'l Symposium on Macromolecules," - IUPAC, University of Massachusetts, Amherst, MA, 01003, USA.
July 19 - 22	23rd Prague Microsymposium on Macromolecules: "Selective Polymeric Sorbents" - IUPAC, Institute of Macromolecular Chemistry, Prague, Czechoslovakia. Contact: P.M.M. Secretariat, Institute of Macromolecular Chemistry, 162-06 Prague 616, Czechoslovakia.
August 15 - 21	"12th Int'l Congress of Biochemistry", Perth, Western Australia. Contact: Brian Thorpe, Dept. of Biochemistry, Faculty of Science, Australian National University, Canberra A.C.T. 2600, Australia.
September 12 - 17	National American Chem. Soc. Meeting, Kansas City, MO, USA. Contact: A.T. Winstead, American Chem. Soc., 1155 Sixteenth St., NW, Washington, D.C., 20036, USA.

1983

March 20 - 25 National American Chem. Soc. Meeting, Seattle, WA USA. Contact: A.T. Winstead, American Chem. Soc., 1155 Sixteenth St., NW, Washington, DC, 20036, USA.

The Journal of Liquid Chromatography will publish announcements of LC meetings and symposia in each issue of the Journal. To be listed in the LC Calendar, we will need to know: Name of meeting or symposium, sponsoring organization, when and where it will be held, and whom to contact for additional details. You are invited to send announcements for inclusion in the LC Calendar to Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P.O. Box 1440-SMS, Fairfield, CT, 06430, USA.

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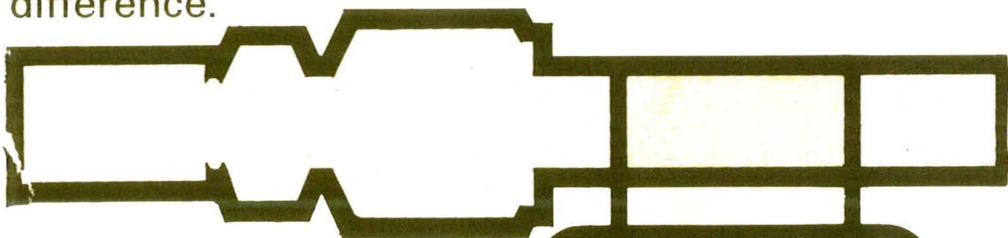

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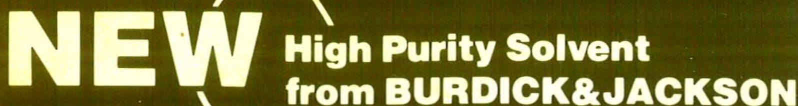

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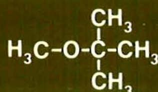

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