JOURNAL OF LIQUID CHROMATOGRAPHY

Special Issue on

THIN-LAYER CHROMATOGRAPHY

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

and

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Fairfield, Connecticut

JOURNAL OF
LIQUID CHROMATOGRAPHY

(continued)

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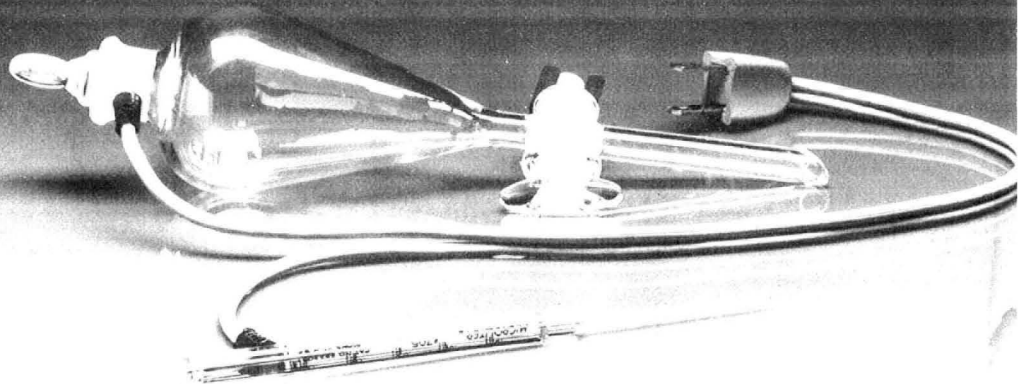
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CONTENTS

PRACTICE, MECHANISM AND THEORY OF REVERSED PHASE
TLC POLYMER FRACTIONATION

Daniel W. Armstrong*, Khanh H. Bui and Richard E. Boehm
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Washington, DC   20057

ABSTRACT

    The use of reversed phase TLC to determine the molecular
weight and molecular weight distribution of poly(methyl
methacrylate), poly(ethylene glycol), poly(ethylene oxide),
poly(tetrahydrofuran), poly(butadiene), poly(isoprene),
poly(α-methylstyrene) and poly(styrene) is demonstrated.   The
mechanism by which fractionation occurs and a theoretical descrip-
tion of the process are given and discussed.

INTRODUCTION

    Among the more basic and important analytical problems in

polymer chemistry is the determination of molecular weights.

There are a variety of primary techniques (i.e., those which

theoretically do not require molecular weight standards; such as

light scattering, osmometry, ebulliometry, cryoscopy, sedimen-

tation, small angle X-ray scattering, etc.) and secondary tech-

niques (i.e., those which require molecular weight standards, such

as viscosity and gel permeation chromatography) available for

these measurements.   Because of its flexibility and efficiency,

gel permeation chromatography (GPC) has become one of the more

1

widely used techniques in polymer analysis. Recently it was
reported that poly(styrene) could be fractionated more efficiently
(than with GPC) using conventional reversed phase TLC and/or LC
(1). This fractionation was the result of selective precipitation
or dissolution of these polymers. In reversed phase TLC a binary
solvent mobile phase was found to change naturally in composition
during development because of selective adsorption of the more
nonpolar solvent by the stationary phase. The changing composi-
tion of the mobile phase, during development, could cause polymer
fractionation if the more adsorbed component of the mobile phase
was a thermodynamically "good" solvent while the less adsorbed
component was a thermodynamically "poor" solvent.

In the present work, this technique is extended to several
more polymers. Further evidence is given for the precipitation
mechanism of separation. Finally, a recently developed theory for
the observed chromatographic behavior of poly(styrene) (2) is
explained in general terms as it applies to the reversed phase TLC
fractionation of homopolymers.

MATERIALS

Whatman KC18F reversed phase TLC plates (5 x 10 cm, 5 x 20 cm
and 20 x 20 cm) were used in the TLC fractionation of
poly(styrene), poly(α-methylstyrene), poly(isoprene),
poly(butadiene), poly(methyl methacrylate), poly(ethylene
glycol), poly(ethylene oxide) and poly(tetrahydrofuran). HPLC
grade methanol, methylene chloride, tetrahydrofuran, dioxan (from

Baker Chemical Co.) and ethylene glycol (from Sigma) were used as received. The following polymer standards were used: (1) poly(styrene) from Waters Associates, mol wt = 2350, mol wt = 17 500 (Mw/Mn = 1.04), mol wt = 35 000 (Mw/Mn = 1.04), mol wt = 110 000 (Mw/Mn = 1.10), mol wt = 390 000 (Mw/Mn = 1.04), mol wt = 3 700 000 (Mw/Mn = 1.20), mol wt = 10 000 000 (Mw/Mn = 1.30). (2) poly(α-methylstyrene) from Polymer Laboratories, mol wt = 19 500 (Mw/Mn = 1.15), mol wt = 87 000 (Mw/Mn = 1.10), mol wt = 760 000 (Mw/Mn = 1.10). (3) poly(isoprene) from Polymer Laboratories, mol wt = 1360 (Mw/Mn = 1.11), mol wt = 3080 (Mw/Mn = 1.08), mol wt = 12 900 (Mw/Mn = 1.08), mol wt = 33 300 (Mw/Mn = 1.05), mol wt = 113 800 (Mw/Mn = 1.05), mol wt = 260 000 (Mw/Mn = 1.07). (4) poly(butadiene) from Polysciences, mol wt = 500 (Mw/Mn = 1.15), mol wt = 1000 (Mw/Mn = 1.2), mol wt = 3000 (Mw/Mn = 1.2), from Phillips Petroleum Company, mol wt = 90 000 (Mw/Mn = 2.7). (5) poly(methyl methacrylate) from Polymer Laboratories, mol wt = 12 000 (Mw/Mn = 1.1), mol wt = 45 200 (Mw/Mn = 1.09), mol wt = 72 000 (Mw/Mn = 1.08), mol wt = 280 000 (Mw/Mn = 1.15), mol wt = 480 000 (Mw/Mn = 1.16), mol wt = 640 000 (Mw/Mn = 1.16). (6) poly(ethylene glycol) from Polymer Laboratories, mol wt = 106, mol wt = 200 (Mw/Mn = 1.09, mol wt = 415 (Mw/Mn = 1.10), mol wt = 630 (Mw/Mn = 1.06), mol wt = 998 (Mw/Mn = 1/06, mol wt = 1580 (Mw/Mn = 1.06), mol wt = 4820 (Mw/Mn = 1.04), mol wt = 9200 (Mw/Mn = 1.08, mol wt = 11 250 (Mw/Mn = 1.07), ml wt = 19 100 (Mw/Mn = 1.09). (7) poly(ethylene oxide) from Polymer Laboratories, mol wt = 25 000 (Mw/Mn = 1.14), mol wt = 40 000 (Mw/Mn = 1.03), mol wt = 73 000 (Mw/Mn =

1.02), mol wt = 150 000 (Mw/Mn = 1.04), mol wt = 280 000 (Mw/Mn =

1.05), mol wt = 660 000 (Mw/Mn = 1.10), mol wt 1 200 000 (Mw/Mn =

1.12). (8) poly (tetrahydrofuran) from Polysciences, mol wt =

2250 (Mw/Mn = 1.10), mol wt = 7600 (Mw/Mn = 1.07), mol wt = 500 000

(Mw/Mn = 1.18).

METHODS

TLC separations were done in an 11 3/4 in long, 4 in. wide and

10 3/4 in. high Chromaflex developing chamber. The reversed phase

plates were not previously equilibrated with the solvent vapor or

specially treated in any way. Methylene chloride-methanol was the

optimum solvent pair in the TLC fractionation of poly(styrene),

poly(α-methylstyrene), poly(isoprene) and poly(butadiene).

Dioxan-ethylene glycol was the optimum solvent pair for

poly(ethylene glycol) and poly(ethylene oxide) although methanol-

ethylene glycol could be used for lower molecular weight polymers

(<25000) as well. Tetrahydrofuran-ethylene glycol was the optimum

solvent pair for poly(methyl methacrylate) and

poly(tetrahydrofuran).

Table I gives the experimental parameters for fractionation of

all polymers. It should be noted that the fractionation behavior

of all polymers is very sensitive to the composition of the mobile

phase in the reservoir (1). The solvent ratios given in Table I

are for the fractionation of the greatest range of polymer molecu-

lar weights. To examine a specific molecular weight

range, one simply alters the volume ratio of the mobile phase (in

TABLE I. Polymers that can be fractionated by reversed phase TLC

Polymer	Solvent Pair	Volume Ratio[c]	Molecular Weight Range[d]	Visualization Method[e]	Development Time	Reference
poly(styrene)	MeCl$_2$:MeOH	(78:22)	10^2 to 5×10^7	fluorescence quench. or I$_2$ vapor	f	1
poly(α-methylstyrene)	MeCl$_2$:MeOH	(81:19)	10^2 to 10^7	fluorescence quench. or I$_2$ vapor	f	this work
poly(isoprene)	MeCl$_2$:MeOH	(82:18)	10^2 to 10^6	spray with 1% I$_2$ in methanol and let yellow background fade	g	this work
poly(butadiene)	MeCl$_2$MeOH	(80:20)	10^2 to 10^6	1% I$_2$ spray as above	g	this work
poly(ethylene glycol)	Dioxan:EG[a]	(60:40)	10^2 to 10^6	I$_2$ vapor	h	this work
poly(ethylene oxide)	Dioxan:EG[a]	(60:40)	10^2 to 1.2×10^6	I$_2$ vapor	h	this work
poly(tetrahydrofuran)	THF[b]:EG[a]	(85:15)	10^2 to 5×10^5	I$_2$ vapor	h	this work
poly(methyl methacrylate)	THF[b]:EG[a]	(75:25)	10^2 to 10^6	1% I$_2$ spray as above	h	this work

a) EG=ethylene glycol b) THF=tetrahydrofuran c) The given volume ratio covers the widest range of molecular weights. One can examine a more narrow range of molecular weights (with greater resolution) by altering this ratio slightly. By increasing the initial concentration of the "poor" solvent one fractionates only lower molecular weight polymers. By increasing the initial concentration of the "good" solvent one fractionates only higher molecular weight polymers. (see Experimental Section). d) In most cases the higher molecular weight should not be taken as an absolute limit. Fractionation of higher molecular weight polymers may possibly occur, unfortunately, higher molecular weight standards were not available at the time of this study. e) When using one of the I$_2$ visualization methods, be sure to remove all traces of the mobile phase from the developed plate (by evaporation or heating in the case of the less volatile solvents) before visualization. f) Closed tank development time = 8 min (5x10 cm plate), 25-40 min (20x20 cm plate). Open tank development times are somewhat longer and dependent on the degree to which the chamber is open. Also, in open chamber development one should start with a 1 to 3% excess of the more volatile solvent. g) Closed tank development time = 8 min (5x10 cm plate), 25-40 min (20x20 cm plate). h) Closed tank development time = 35 min (5x10 cm plate), 3-5 hr (20x20 cm plate).

the developing chamber) by a few percent. For example, if one is
interested in fractionating poly(styrene) only in the molecular
weight range of 10^2 to 10^4 one would use a mobile phase of 70:30
(v:v) methylene chloride to methanol. This technique also results
in increased resolution since one has separated a smaller range of
polymers over the same length plate.

All polymer standards were dissolved in the "good solvent" (~5
mg/ml) and 1µl of this solution was spotted on the TLC plate. The
"good" solvent is the first listed of any given solvent pair (e.g.,
methylene chloride for poly(isoprene), dioxan for poly(ethylene
oxide) or tetrahydrofuran for poly(methyl methacrylate)). Deve-
loped spots were detected by fluorescence quenching, I_2 vapor or
spraying a solution of I_2 in methanol (see Table I). In a few
cases tailing of spots occurred near the origin (in closed
systems). This problem was eliminated by fractionating the poly-
mers in a partially open system (1). If one spots too high a con-
centration of the standard, streaking can occur upon development
(e.g., for poly(isoprene)). This problem is easily avoided by
further dilution of the standards.

RESULTS AND DISCUSSION

For any given polymer there is generally an appreciable number
of "good-poor" solvent pairs. Unfortunately, most of the solvent
pairs are not effective in the reversed phase TLC fractionation of
the polymers. Of critical importance are the relative interac-
tions of the solvents with the stationary phase and the natural

gradient that is produced. For TLC fractionation to occur, the
stationary phase must preferentially interact with the good
solvent thereby enriching the mobile phase in the poor solvent
during development. Ideally, the polymer does not substan-
tially interact with the stationary phase until the mobile phase
reaches a composition which causes precipitation.

Figure 1 illustrates typical fractionations of poly(isoprene),
poly(methyl methacrylate), poly(ethylene glycol/oxide) and
poly(butadiene). Table 1 lists all polymers that can be frac-
tionated using this technique at the present time. After testing
hundreds of possible mobile phases a few trends have become
apparent. Methylene chloride-methanol appears to be the optimum
solvent pair for the TLC fractionation of linear unsaturated
hydrocarbon polymers (i.e., poly(styrene), poly(α-methylstyrene),
poly(isoprene) and poly(butadiene)). For polymers which methanol
is a good solvent (poly(ethylene oxide) for example) ethylene gly-
col seems to be the preferred poor solvent. While ethylene
glycol-containing mobile phases generally give good separations
and spot shapes, development is somewhat slower due to the
increased mobile phase viscosity. Increased resolution often can
be obtained by using vapor unsaturated developing chambers
provided the good solvent (in the binary mobile phase) is more
volatile than the poor solvent (1). The increase in resolution is
due to formation of smaller spots and the elimination of tailing
near the origin. A possible mechanism by which this is
accomplished can be envisioned. The binary mobile phase (in the
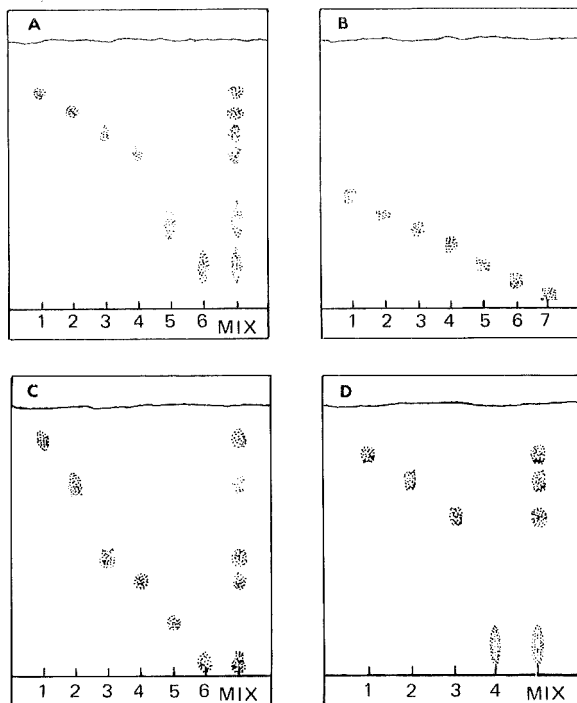
Figure 1: Schematic illustrating typical reversed phase TLC fractionations of:

(A) poly(isoprene) with 81:19 (v:v) methylene chloride:methanol.

$1 = 1.36x \ 10^3; \ 2 = 3.08x10^3; \ 3 = 1.29x10^4;$
$4 = 3.33x10^4; \ 5 = 1.14x10^5; \ 6 = 2.60x10^5$

(B) poly(methylmethacrylate) with 74:26 (v:v) tetrahydrofuran:ethylene glycol.

$1 = 1.2x10^4; \ 2 = 4.5x10^4; \ 3 = 7.2x10^4;$
$4 = 7.6x10^4; \ 5 = 2.8x10^5; \ 6 = 4.8x10^5; \ 7 = 6.4x10^5.$

(C) poly(ethylene glycol/oxide) with 57:43 (v:v) dioxyan:ethylene glycol.

$1 = 4.15x10^2; \ 2 = 9.98x10^2; \ 3 = 4.8x10^3;$
$4 = 9.20x10^3; \ 5 = 2.5x10^4; \ 6 = 2.8x10^5.$

(D) poly(butadiene) with 79:21 (v:v) methylene chloride:methanol.

$1 = 5x10^2; \ 2 = 1x10^3; \ 3 = 3x10^3; \ 4 = 9.0x10^4.$

reservoir of the developing tank) acts as a good solvent.

However, as it travels up the reversed phase plate it becomes

progressively poorer (due to the selective adsorption of the good

solvent). Concurrently, the good solvent evaporates at a greater

rate than the poor solvent. As a result the gradient tends to be

accentuated, precipitation occurs sooner and R_f values are lower.

Because of the gradient, the leading edge of a precipitated

spot is in contact with a slightly poorer solvent than the

trailing edge. When development continues after precipitation,

the mobile phase moving past the spot becomes a somewhat better

solvent. This results in a relatively slow movement of the spot

with the trailing edge being affected more than the leading edge.

This mechanism also explains the somewhat unusual (square or rec-

tangular) spot shapes that often accompany this separation tech-

nique.

The R_f of all reported polymers tends to vary with the log of

their molecular weight (see Figures 2 and 3). For the highest

molecular weight polymers, the relationship becomes nonlinear and

the resolution is not as good (Figure 2). The R_f-log molecular

weight relationship holds for vapor unsaturated development as

well (Figure 3). The slope of the calibration curve varies with

the degree of evaporation allowed. Since standards and unknowns

are generally run on the same plate this poses no inconvenience.

In all cases the separation of polymers is assumed to be due

to a precipitation mechanism. This is somewhat unusual as most

chromatographic separations result from adsorption or partition
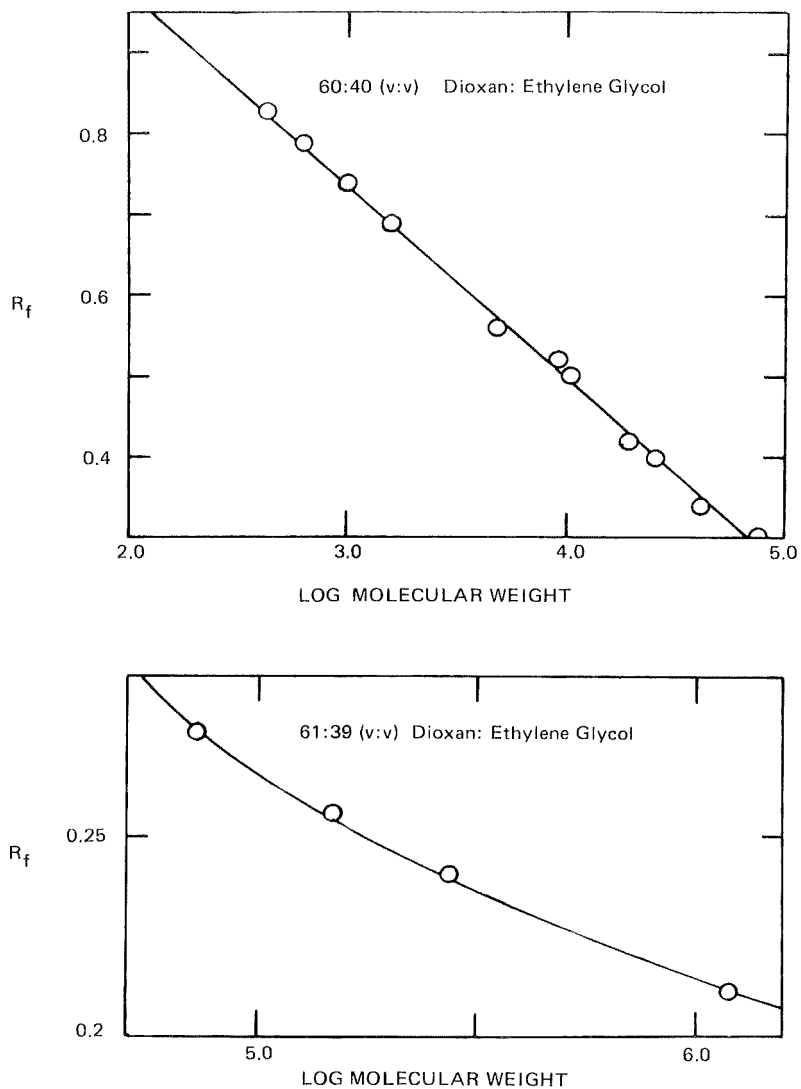
Figure 2:   Plot of R_f versus log molecular weight for
             poly(ethylene glycol/oxide). Note that the rela-
             tionship is linear up to a molecular weight of ~10^5.
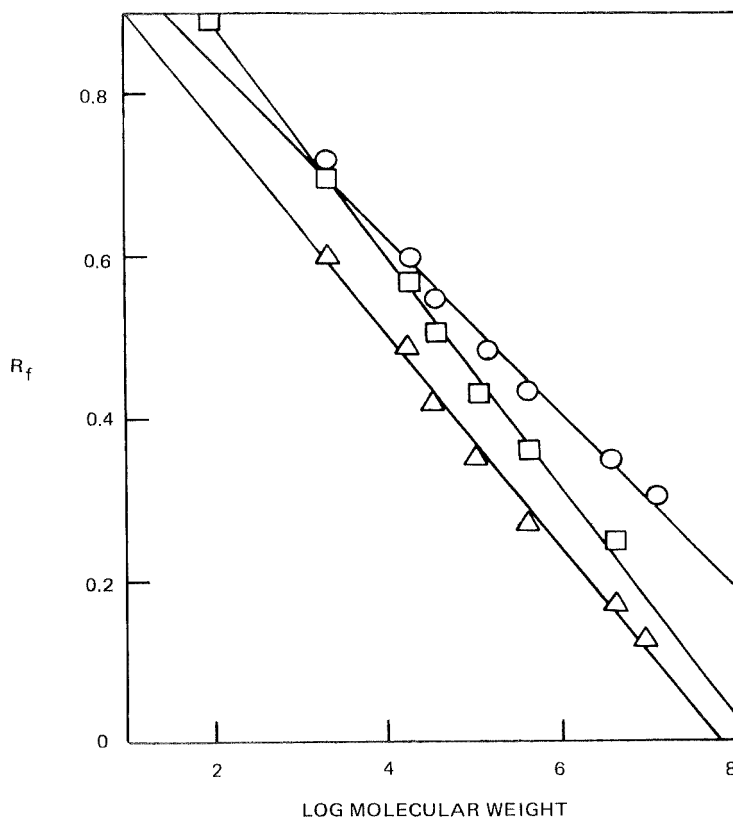
Figure 3: Linear calibration curves for poly(styrene) standards developed in vapor unsaturated Chromaflex chamber.

☐ = 1/2 in, open lid, mobile phase = 80:20 (v:v) $MeCl_2$:OH

◯ = 1 in, open lid, mobile phase = 80:20 (v:v) $MeCl_2$:MeOH

△ = no lid, mobile phase = 80:20 (v:v) $MeCl_2$:MeOH
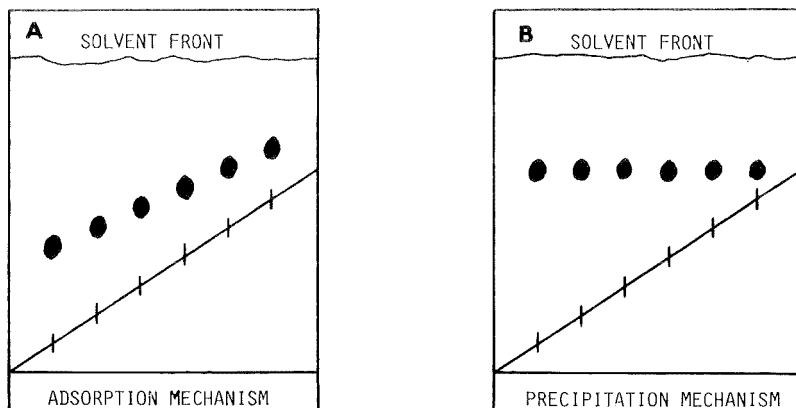
Figure 4:   Schematic illustrating the different TLC development
            patterns expected when a compound's migration beha-
            vior is controlled by (A) adsorption processes or (B.)
            precipitation. In both examples a compound is
            spotted along a line drawn at an angle to the bottom
            of the plate. The compound in chromatogram A has an
            R_f of 0.33. The compound in chromatogram B tends
            to travel with the solvent front until some process
            (e.g., change in mobile phase composition, phase
            ratio, etc.) causes precipitation.

processes. In ideal situations, the mechanism of separation can

be identified with a simple chromatographic test. By spotting a

compound <u>diagonally</u> on a TLC plate instead of along a line

parallel to the bottom edge, one produces a characteristic deve-

lopment pattern (see Figure 4). Figure 4A illustrates the

expected adsorptive chromatographic behavior of a compound (R_f =

0.33) under ideal conditions. In the unusual case where separa-

tion is controlled completely by precipitation, one would expect a

development pattern as illustrated in Figure 4B (i.e., all spots

parallel to the solvent front regardless of "spotting" angle). In

reality, one rarely finds perfect examples of either behavior. In
the more common case of adsorption TLC, changes in solvent com-
position (of binary and higher order mobile phases) and phase
ratio during development can cause deviations from the theoretical
behavior illustrated in Figure 4A. Previous results (1) indicated
that the reversed phase TLC fractionation of polymers might exhi-
bit rarely seen behavior characteristic of the precipitation
mechanism (Figure 4B). Figure 5 illustrates these results for
poly(styrene), poly(isoprene), poly(methyl methacrylate) and
poly(ethylene glycol/oxide). At least two molecular weight stan-
dards are shown in each case (three in the poly(styrene) example).
It is apparent that a polymer of a given molecular weight travels
along the developing plate until the mobile phase reaches the cri-
tical composition where precipitation occurs. If a polymer is
spotted on a TLC plate at a point higher than where the cri-
tical composition occurs, it will not move from the origin (note
the higher molecular weight polymers in Figure 5).

By plotting the initial volume percent of good and poor
solvents in the mobile phase versus polymer R_f value, one can
visualize the effect of solvent composition on polymers of dif-
ferent molecular weights (see Figure 6). Each polymer has a
characteristic sigmoidal curve. The curve lies closer to the
ordinate for higher molecular weight polymers and the variation in
solvent composition needed to change the R_f from 1.0 to 0 becomes
more narrow with increasing molecular weight. The separation bet-
ween any two molecular weight curves for the same polymer is indi-
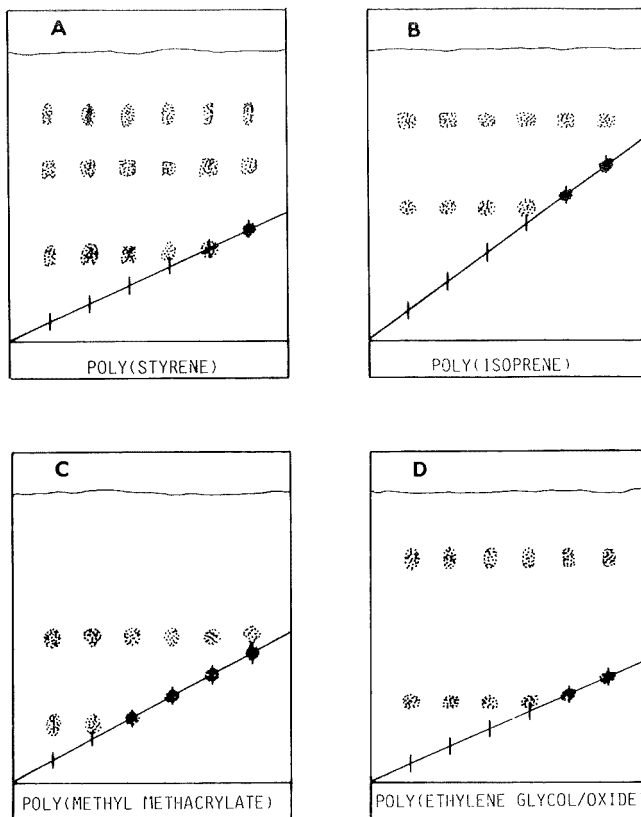
Figure 5:   TLC development patterns indicating a precipitation
             mechanism in the reversed phase fractionation of:

             (A) poly(styrene) with 79:21 (v:v) $MeCl_2$:MeOH
             (top) MW = 2.35×10^3 (middle) MW = 3.5×10^4
             (bottom) MW = 2.7×10^6

             (B) poly(isoprene) with 77:23 (v:v) $MeCl_2$:MeOH
             (top) MW = 1.36×10^3 (bottom) MW = 3.33×10^4

             (C) poly(methylmethacrylate) with 76:24 (v:v)
             THF:ethylene glycol.
             (top) MW = 4.5×10^5 (bottom) MW = 6.4×10^5

             (D) poly(ethylene glycol/oxide) with 59:41 (v:v)
             dioxan:ethylene glycol.
             (top) MW = 9.93×10^2 (bottom) MW = 2.5×10^4
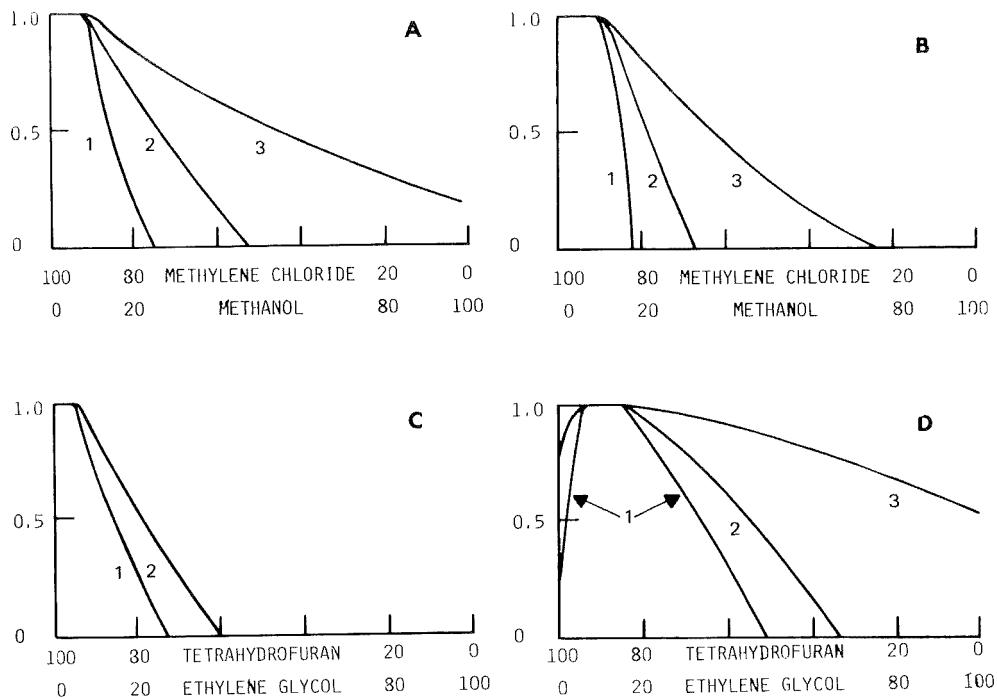
Figure 6: Plots of R_f versus mobile phase composition (volume %). Molecular weights are:

(A) 1 = 9.3×10^4; 2 = 3.0×10^3; 3 = 5.0×10^2
    poly(butadiene)

(B) 1 = 2.6×10^5; 2 = 3.3×10^4; 3 = 1.3×10^3
    poly(isoprene)

(C) 1 = 6.4×10^5; 2 = 1.2×10^4 poly(methyl methacrylate)

(D) 1 = 7.3×10^4; 2 = 9.2×10^3; 3 = 6.3×10^2
    poly(ethylene glycol/oxide)

cative of the resolving power of the technique (i.e., the greater
the distance between two curves, the better TLC resolutions are
obtained). There is an apparent deviation in the behavior of the
higher molecular weight poly(ethylene glycol/oxide) polymers when
the mobile phase contains an excess of the good solvent (Figure
6D). The lower R_f values in this region are thought to be due to
a slight adsorption of the polymer to the stationary phase. This
adsorption may be due to the presence of a small number of resi-
dual silanol groups or possibly to the binder present in the TLC
plate. Regardless of the source of the binding, the presence of a
small amount of the poor solvent, preferentially interacting with
these adsorption sites, releases the polymer. After release, the
behavior of the poly(ethylene glycol/oxide) is analogous to that
of the other polymers (Figures 6A, B and C).

THEORY

A theory has been developed for a chromatographic model system
which accounts for many of the phenomena observed in this work
(2). The model system assumes an equilibrium distribution of iso-
lated flexible polymer molecules between a binary solvent mobile
phase and a planar stationary phase. In the mobile phase the
polymer is assumed to be a spherical gel having a uniform
segment density. On the stationary phase the polymer assumes a
flat, thin cylindrical conformation. For polymers of sufficiently
high molecular weights, there is a critical mobile phase mole
fraction, X_{1mc}, of the more favorable, less polar solvent 1 such

GOOD MOBILE PHASE   POOR MOBILE PHASE
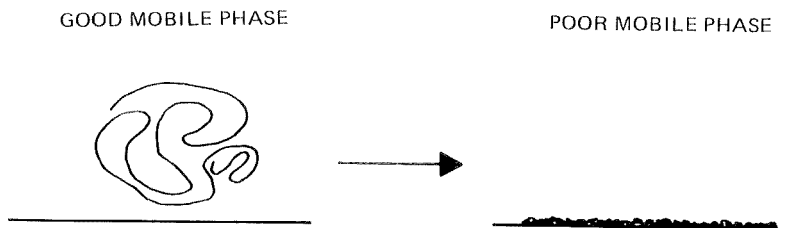
Figure 7:   Illustration of the two theoretically possible con-
             figurations for a high molecular weight polymer in
             reversed phase TLC or LC gradient fractionation.

that when $X_{1m} > X_{1mc}$ the polymer will move at the same rate as

the mobile phase while when $X_{1m} < X_{1mc}$ the polymer is completely

retained by the stationary phase (see Figure 7). The critical

composition is dependent on molecular weight. This dependence

results from the flexibility of the polymer which enables it to

change configuration in response to its environment. As a result

of the X_{1mc}-molecular weight dependence, one can chromatographi-

cally fractionate homopolymers with gradient elution techniques

(2). In the TLC fractionation described in this work, for

example, the mobile phase changes composition continuously during

development as a result of selective adsorption of the better

solvent by the stationary phase. To achieve fractionation via

this method one must carefully choose a good-poor solvent pair

that will interact differently with the stationary phase and pro-

duce the optimum gradient.

   One can obtain the theoretical expression for the capacity

factor of a polymer using the Flory-Huggins lattice model for iso-

lated polymer-solvent systems in both the mobile and stationary
phases, as well as the Bragg-Williams approximation for the
nearest neighbor interactions (2-4). From the expression for the
capacity factor one can obtain equations relating retention time
(in LC) and R_f (in TLC) to polymer molecular weight (vide infra).

$$t_r = t_m + B^{-1}(X_{1mc} - X_{1m(o,o)}) + (| A_1| MB)^{-1} \ell n(| A_1| MBt_m) \quad (1)$$

where t_r = retention time of polymer, t_m = retention time of an
unretained solute, X_{1mc} = critical solvent composition for a
polymer, $X_{1m(o,o)}$ = initial mobile phase composition, B = rate at
which the mobile phase composition varies, A = a constant depen-
dent on solvent-solvent and solvent-polymer interchange energies,
and M = degree of polymerization.

$$R_f = (| A_1| Mk\frac{L}{u})^{-1} \ell n[1 + (| A_1| Mk\frac{L}{u}) exp[| A_1| M(X_{1m(o)}$$
$$- X_{1mc})](1 - R_f)] \quad (2)$$

where: k = rate constant indicative of the change in mobile phase
composition. In the above case the rate law is considered to be
independent of the composition of the better polymer solvent.
$X_{1m(o)}$ = composition of the mobile phase in the reservoir of the
development tank, L = distance advanced by the solvent front and
u = average rate of migration of the solvent front.

Equations 1 and 2 describe analogous processes. The main dif-
ference is that in LC separations, (equation 1) fractionation
occurs by going from a poor to a good mobile phase, while in TLC
(equation 2) fractionation occurs by going from a good to a poor

mobile phase as the chromatogram develops. In both cases the
relationship between the mobile phase composition and the critical
composition controls the elution of a polymer.

Both equations 1 and 2 can be used to predict qualitative
chromatographic trends. The closest correlation between theoreti-
cal and experimental values (of t_r and R_f) is for the higher mole-
cular weight polymers (2). As one can see in Figure 6, the con-
cept of a critical concentration is most accurate for the higher
molecular weight polymers. For lower molecular weight polymers
there can be an appreciable transition range in which the polymer
elution behavior varies over a wide range of solvent compositions
(Figure 6). In these cases one can envision more gradual tran-
sitions from the desorbed to adsorbed state (Figure 8). Predic-
tion of retention times from equation (1) tends to be more
accurate than the prediction of R_f values from equation 2 (2).

GOOD MOBILE PHASE   **TRANSITION REGION**   **POOR MOBILE PHASE**
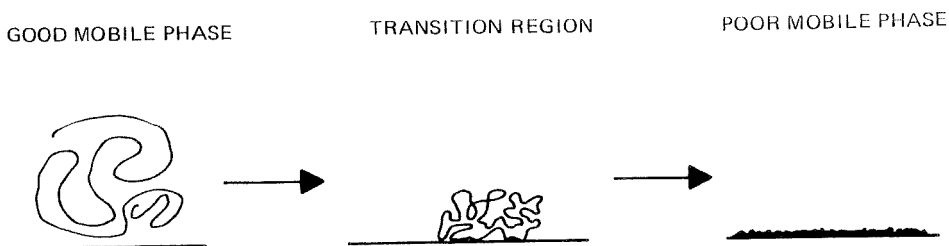
Figure 8:   Illustration of the change of configuration of a
             polymer which occurs in the transition region (i.e.,
             that region where polymers are not completely
             adsorbed or desorbed). The range of the transition
             region increases as a polymer's molecular weight
             decreases.

The major reason is believed to be that the solvent gradient is known and exactly controlled in LC but not in TLC. In addition, TLC is subject to changing phase ratios, wetting phenomena, etc. Despite these deficiencies, the major theoretical trends are consistant with experimental observation.

Equation 2 can be solved directly (2); however, it is somewhat cumbersome and difficult to visualize in terms of experimental results presented here. Fortunately, one can simplify the expression to a more understandable form when analyzing certain special cases. For all but lower molecular weight polymers (where $X_{1m}(o) - X_{1mc} > 0$),

$$(|A_1| M \frac{L}{u}) \exp(|A_1| M(X_{1m}(o) - X_{1mc}))(1 - R_f) \gg 1$$

and equation 2 simplifies to:

$$R_f = \frac{u(X_{1m}(o) - X_{1mc})}{kL} + \frac{u}{|A_1| MkL} \ln(\frac{|A_1| MkL}{u}) + \frac{u \ln(1 - R_f)}{|A_1| MkL} \qquad (3)$$

For moderate to high molecular weight polymers the second and third terms of equation 3 become increasingly small relative to the first term. Therefore, for "high polymers":

$$R_f = \frac{u(X_{1mc}(o) - X_{1mc})}{kL} \qquad (4)$$

When $X_{1m} - X_{1mc}$ is greater than zero (i.e., the mobile phase in the developing chamber is a good solvent), polymer migration will

occur. The extent of this migration can be calculated providing
the gradient (k) and the critical mobile phase composition (X_{1mc})
are known. X_{1mc} increases monotonically with M and asymptotically
approaches a limiting value X_{1mc}^{∞} as M $\rightarrow \infty$. This indicates that
chromatographic resolution should decrease as the molecular weight
increases.

When $X_{1m(o)} - X_{1mc}$ is less than zero (i.e., the mobile phase in
the developing chamber is a poor solvent mixture) then

$$(|A_1| \; Mk\frac{L}{u})exp[|A_1| \; M(X_{1m(o)} - X_{1mc})](1 - R_f) \rightarrow 0$$

for higher molecular weight polymers and $R_f = 0$.

It is apparent that the theoretical description is consistent
with experimental observation. The results of this union are
threefold:

1. The fractionation mechanism is largely understood.

2. A useful polymer fractionation technique can now be uti-
    lized and improved systematically.

3. The use of TLC and LC can now be used as a potentially
    powerful tool in the theoretical and physico-chemico
    study of polymers and their behavior in various environ-
    ments.

ACKNOWLEDGEMENTS

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SEPARATION AND QUANTITATION OF
ANIONIC, CATIONIC AND NONIONIC SURFACTANTS BY TLC

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ABSTRACT

    TLC is a potentially powerful technique for the separation
of surfactants. Reversed phase thin layer chromatography (RPTLC)
can be used to separate entire classes of surfactants (i.e.,
anionics from nonionics from cationics). Conversely, silica gel
can be used to separate individual anionic or cationic surfac-
tants from other similarly charged surfactants. RPTLC can also
be used to separate individual nonionic surfactants. Using two
dimensional TLC (with a special silica gel plate containing a 2.5
cm strip of reversed phase material along one edge) a complex
mixture of surfactants was first fractionated into classes and
then (using the second dimension) into individual components.
Standard scanning densitometry was used for quantitation.

INTRODUCTION

    The analysis of surfactants (e.g., detergents, soaps, etc.)

can be a difficult analytical problem. Surfactants are generally

somewhat soluble in both water and organic solvents. They con-

centrate at interfaces and tend to bind to anything available

(1,2). There are a variety of spectrometric, titrimetric, atomic

absorption spectrometric and ion-selective electrode methods for

the analysis of surfactants (3-11). All of these techniques have

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the characteristic of being selective for certain functional groups. For example both the sodium dodecylsulfate electrode and the methylene blue complex spectrophotometric methods are selective for surfactants with sulfate or sulfonate functional groups. Consequently these techniques give positive responses for a variety of homologous, isomeric and even structurally dissimilar anionic surfactants. Another shortcoming of these techniques is that one class of surfactants cannot be effectively analyzed in the presence of another. The so-called neutralization effect of cationic with anionic surfactants is well documented (12). As a result of these limitations, the analyst has increasingly turned to physicochemical techniques which provide information on the total surfactant content in a sample (13) or to chromatography (14-16). Because most surfactants are nonvolatile without derivatization, LC or TLC methods are often preferred. The use of TLC to separate a mixture of anionic surfactants was recently demonstrated (17). In this work we not only demonstrate the separation of identically charged surfactants from each other but also the TLC separation of the three major classes of surfactants (i.e., anionic, nonionic and cationic).

MATERIALS

Whatman reversed phase TLC plates (KC18F), silica gel plates (K6F) and hybrid Multi-K plates (CS5) were activated at 115°C for two hours before use. Cetyltrimethylammonium bromide (CTAB, Sigma), cetylpyridinium chloride (CPC, Sigma), cetyltrimethyl-

ammonium chloride (CTAC, Pfaltz & Bauer), dodecylamine (DA,

Aldrich), octadecylamine (OA, Eastman), sodium dodecylsulfate

(SDS, Bio Rad), dodecylbenzenesulfonate (DBS, Pfaltz & Bauer),

sodium dioctylsulfosuccinate (SDOS, Aldrich) and sodium laurate

(SL, Pfaltz & Bauer) were recrystallized three times from

ethanol-water before use. The nonionic surfactants Triton X 100

(TX 100, Bio Rad), Surfynol 465 (S 465, Air Products) and Igepol

CO-530 (ICO-530, GAF) were used as received. ICO-530 is

nonylphenoxypoly(ethyleneoxy)ethanol where the hydrophilic

poly(ethyleneoxy)ethanol "head-group" averages five units in

length. TX 100 is dodecylphenoxypoly(ethyleneoxy)ethanol. S

465 is a poly(ethyleneoxy)ethanol (averaging ten units) adduct of

2,4,7,9-tetramethyl-5-decyn-4,7-diol. Gold lable sodium

tetraphenylborate (Aldrich) was used as received. Methanol,

ethanol, methylene chloride and glacial acetic acid (Baker) were

also used as received.

METHODS

All separations were done in a 11 3/4 in. long, 4 in. wide

and 10 3/4 in. high sealed chromaflex developing tank. The

plates were not pre-equilibrated with solvent vapor before use.

Separation of anionic surfactants: 1 μℓ of 0.1 M SDS, SL, DBS

and SDOS was spotted 1 cm from the bottom of a 5 x 20 cm silica

gel plate. The mobile phase consisted of 8:1 (v:v) methylene

chloride:methanol. The addition of very small amounts of acetic

acid to the mobile phase tended to increase the R_f's but did not

affect the resolution. Spots were visualized by exposure to I_2 vapor.

Separation of cationic surfactants: 1 µℓ of 0.1 M CPC, OA, DA and CTAC or CTAB was spotted 1 cm from the bottom of a 5 x 20 cm silica gel plate. The mobile phase consisted of 8:1:0.75 (v:v:v) methylene chloride:methanol:acetic acid. Spots were visualized by exposure to I_2 vapor.

Separation of nonionic surfactants: 1 µℓ of 10% TX 100, ICO-530 and S 465 were spotted on a 5 x 20 cm reversed phase (C_{18}) plate. The mobile phase consisted of 8:2 (v:v) ethanol:2% sodium tetraphenylborate$_{(aq)}$. The purpose of sodium tetraphenylborate was to prevent the spots from streaking. I_2 vapor was used for visualization.

Separation of anionic, cationic and nonionic surfactants: A Whatman CS5, Multi-K, KC18F/K5F 20 x 20 cm plate was pre-developed in ethanol and then activated at 115°C for 2 hours. Each surfactant mixture was spotted (0.5 µℓ) at a point on the reversed phase strip. The entire 20 x 20 cm plate was then developed with 75% ethanol in the direction of the reversed phase strip. Development was stopped when the solvent front was 2 cm from the top of the plate. Under these conditions, all anionic surfactants travel at or very near the solvent front (i.e., $\leq$ 2 cm), all cationic surfactants remain at or near the origin of the reversed phase strip ($\leq$2.5 cm), while the nonionic

surfactants separate between the anionics and cationics. The 20

x 20 cm plate is then cut into three separate sections in a

direction perpendicular to the first development.

The first cut should be 2.5 to 3 cm below the solvent front.

This will isolate the anionic surfactants. The second cut should

be 3 cm above the origin. This will isolate the cationic surfac-

tants. Perpendicular secondary development of the plates con-

taining the cationic and anionic surfactants (after reactivation

of the plates) will give complete separation of these species.

The mobile phases for secondary development are, 8:1 (v:v)

$MeCl_2$:MeOH for the anionic surfactants and 8:1:0.5 (v:v:v)

$MeCl_2$:MeOH:HOAc for the cationic surfactants. If one develops

the entire plate in the second direction without isolating the

anionic and cationic surfactants as indicated, the nonionic sur-

factants tend to spread and coat the silica gel portion of the

plate thereby obscuring all other components. Visualization is

with I_2 vapor.

Quantitation of surfactants: Scanning densitometry was done with

a Shimadzu Model 910 instrument. Surfactants could be detected

directly in the absorbance-reflectance mode at 215 nm. Detection

limits were lower when the developed plate was exposed to I_2

vapor and scanned at 405 nm (in the absorbance-transmittance mode).

RESULTS AND DISCUSSION

One's approach to the TLC separation of surfactants in a

mixture is largely controlled by the charge of the surfactant

head-groups as well as the diversity of the sample. Silica gel

is adequate for the separation of anionic or cationic surfactants

from other identically charged species. Nonionic surfactants are

best separated by reversed phase TLC (RPTLC). Even in RPTLC

nonionic surfactants tend to streak unless a "lipophilic salt"

such as sodium tetraphenylborate is added. Table 1 summarizes

TABLE I

Experimental Conditions and R_f Values of Individually
Separated Anionic, Cationic and Nonionic Surfactants

Compound	Stationary Phase	Mobile Phase	R_f
Anionic Surfactants	a	c	
1. SDS			0.15
2. DBS			0.09
3. SL			0.70
4. SDOS			0.28
Cationic Surfactants	a	d	
1. CTAB			0.21
2. CTAC			0.20
3. CPC			0.27
4. DA			0.42
5. OA			0.55
Nonionic Surfactants	b	e	
1. TX 100			0.54
2. S 465			0.70
3. ICO-530			0.45

[a] Silica Gel   [b] C_{18} reversed phase

[c] 8:1(v:v) $MeCl_2$:MeOH   [d] 8:1:0.75 (v:v:v) $MeCl_2$:MeOH:HOAc

[e] 8:2 (v:v) EtOH:2% sodium tetraphenylborate$_{(aq)}$
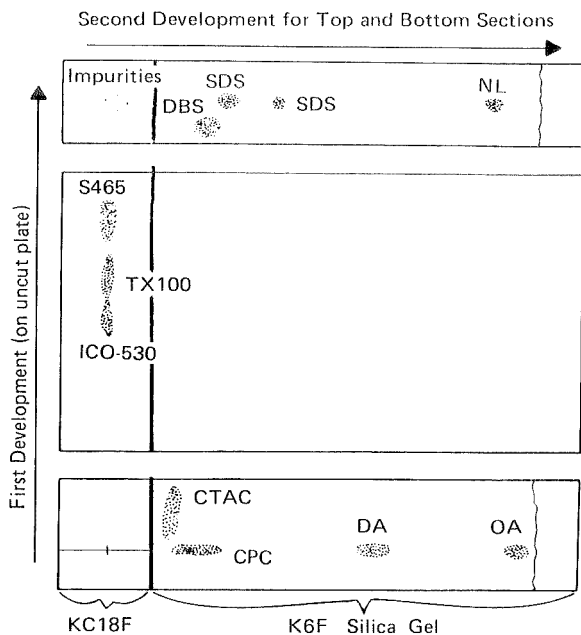
Figure 1: Schematic of a two dimensional TLC separation of
         eleven surfactants on a composit reversed phase-
         silica gel plate. The first development (on the
         reversed phase strip) separated the surfactants
         according to class. Secondary development of the top
         and bottom sections of the plate results in complete
         separation of individual surfactants. SDS = sodium
         dodecylsulfate, DBS = dodecylbenzenesulfonate, NL =
         sodium laurate, S 465 = Surfynol 465, TX 100 = Triton
         X100, ICO-530 = Igepol CO-530, CTAC = cetyltrimethy-
         lammonium chloride, CPC = cetylpyridinium chloride,
         DA = dodecylamine, OA = octadecylamine.

the separation conditions for each class of surfactants. The
R_f's of the cationic surfactants can be altered (i.e., increased)
considerably with a slight increase in the concentration of ace-
tic acid in the mobile phase. The separation of surfactants with
identical hydrophylic head groups (i.e., DA and OA or Tx 100 and
ICO-530) is dependent on the size of the hydrophobic "tail".
Generally the larger the hydrophobic portion of the surfactant,
the greater the R_f.

The analysis of solutions containing surfactants of dif-
ferent charge can be a difficult process because of precipitation
and "neutralization" effects (12). RPTLC, however, can be used
to separate surfactants by class (see Figure 1). A 75% ethanol
mobile phase tends to carry anionic surfactants with the solvent
front and leave cationic surfactants near the origin. Perpen-
dicular secondary development of plate sections near the solvent
front and origin will then separate the anionic and cationic sur-
factants into individual compounds. The secondary development
carries the surfactants from the reversed phase strip into the
silica gel portion of the plate where fractionation occurs
(Figure 1). Secondary development of the whole TLC plate or the
section of plate containing the nonionic surfactants produced
indistinguishable smears over much of the plate.

Quantitation of surfactants by scanning densitometry is a
relatively straight forward process. It is possible to directly
scan untreated spots at wavelengths from 200 to 215 nm. Sen-
sitivity and selectivity can be enhanced by using a variety of
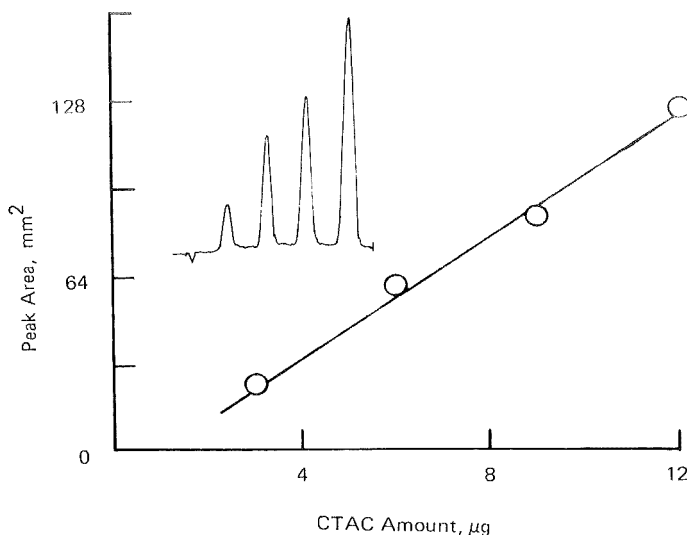
Figure 2:   Calibration plot of peak area versus amount of the
            standard surfactant (CTAC) chromatographed. The
            insert shows the actual peaks obtained from scanning
            densitometery (at 405 nm).

visualization or charring techniques (17, 18). Figure 2 shows a
scan of four CTAC standards (λ = 405 nm after visualization with
I_2 vapor) and the corresponding calibration curve.

It is apparent from the literature that exhaustive chroma-
tographic separations are presently the most effective means of
analyzing complex surfactant mixtures. TLC is shown to be a
highly efficient and inexpensive technique for the analysis of a
variety of surfactant and surfactant mixtures.

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A Review of Radiochromatogram Analysis Instrumentation

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ABSTRACT

The available techniques for the analysis of radiolabled TLC
plates are described and compared, including the newest technique
based on imaging proportional counters. The imaging systems
offer a 100-fold improvement in sensitivity over conventional
chromatogram scanners and can replace much of the analysis cur-
rently being done by plate scraping and scintillation counting.
For most quantitative analysis, imaging systems offer superior
speed and comparable or better accuracy when compared with scin-
tillation counting. Large savings can be realized in sample
preparation time, disposable supply costs, and liquid waste
disposal.

Introduction

TLC is a major analytical tool for both qualitative and quantitative applications. For radioisotope work, qualitative techniques are largely photographic such as autoradiography and spark camera analysis, while the quantitative techniques rely on nuclear detectors such as Geiger or proportional counters and scintillation counting. Often a combination of these techniques is used. The qualitative technique provides the location of the activity, and then scintillation counting is used to obtain accurate quantitative results.

A brief summary of the various radioisotope techniques and their capabilities including sensitivity, resolution, and quantitative accuracy will be discussed. Most of these techniques have been in use for many years, and were described by Touchstone and Dobbins (1). The exception is the radiochromatogram imaging system which has been commercially available for about two years. The techniques used for radioisotope analyses are:

1. Autoradiography - film recording of ionization produced by betas or X-rays. High spatial resolution, low sensitivity, poor quantitation.

2. Spark Camera - a gas filled spark chamber produces light along the ionization track which is then photographed. High sensitivity, poorer resolution than direct autoradiography, poor quantitation.

3. Radiochromatogram scanners - a collimated Geiger or proportional counter is scanned along the TLC lane. Output is generally a plot

of counts vs. position on the lane. Moderate
sensitivity and spatial resolution, quantita-
tion possible but not readily available from
output plot.

4. Scintillation counting — active regions or sec-
tions at regular intervals are removed from the
TLC substrate by scraping or cutting and then
individual samples are eluted and prepared for
standard scintillation counting. Poor resolu-
tion, excellent sensitivity and quantitation.

5. Radiochromatogram imaging systems – an imaging
proportional counter which measures both the
occurrence and location of each radioactive
decay event is placed over the TLC lane. Output
is generally in digital form with both CRT and
paper histogram displays available as well as
numerical peak integration features. High sen-
sitivity, moderate spatial resolution, and
excellent quantitation.

There are other requirements and concerns besides sensiti-
vity, resolution, and quantitation which impact on the choice of
radiochromatogram analysis technique. These include turn-around
time (the time elapsed before results are in hand), labor and
material costs, health hazards, and sample preservation. In
general elapsed time is directly related to sensitivity and so is
longest for autoradiography and shortest for spark cameras and
radiochromatogram imaging systems. However, scintillation coun-
ting may also have a long elapsed time due to the large amount of
preparation time required and counter backlogs. Labor and mate-

Table 1

Comparison of Radiochromatogram Analysis Instrumentation

	Sensitivity	Resolution	Quantitation	Turn-around Time	Cost/Sample	Health Hazard	Sample Preserved
Autoradiography	Poor	Ex.[*]	Poor	Poor	Low	Low	Yes
Spark Camera	Good	Mod.[**]	Poor	Ex.	Low	Low	Yes
Scintill. Counting	Ex.	Poor	Ex.	Mod.	High	High	No
Chrom. Scanner	Mod.	Mod.	Mod.	Mod.	Low	Low	Yes
Chrom. Imaging	Good	Mod.	Ex.	Ex.	Low	Low	Yes

[*]Ex = Excellent

[**]Mod = Moderate

rial costs are modest for all techniques except scintillation counting. To prepare a single TLC lane for counting in 20 1-cm segments may require 30 minutes or more of bench time and $3-5 in expendable vials, scintillation cocktail, and liquid waste disposal. Scintillation counting also presents the worst health hazard since it is the only technique in which there is a signi-

ficant chance of creating airborne silica particles and radioac-
tivity during the scraping or cutting process. And finally, if
sample preservation is required for recovery or further analysis,
any of the techniques except scintillation counting can be used.

    A summary of all techniques and the evaluation critieria
discussed above is given in Table 1.

Quantitative Analysis

    For the quantitative analysis of radiochromatograms, the
choice of techniques is limited to conventional scanners, scrap-
ing and counting, and imaging systems. Conventional scanners and
imaging systems are similar in that they measure the activity in
situ, while scintillation counting requires the destruction of
the original chromatogram. The in situ measurements, while
preserving the chromatogram, are limited in their sensitivity by
the absorption of betas by the chromatographic layer itself. The
counting efficiency for tritium is generally in the range 0.5 - 2
percent depending on the type and thickness of TLC plate used.
For ^{14}C, the efficiency is about 10 times greater. Scintillation
counting can have efficiencies in excess of 30 percent for
tritium. Conventional scanners suffer from the further ineffi-
ciency that they view a given portion of the chromatogram for
only a small fraction of the total analysis time. They, as well
as scintillation counters, perform a sequential analysis of the
chromatogram so that for the same total counting time a scanner
will generally have 50 times less sensitivity than scintillation
counting. An imaging system views all portions of the chromato-
gram for the entire analysis time thereby recouping most of its

efficiency disadvantage when compared with scintillation counting.

In general, conventional scanners and imaging systems have similar characteristics except that imaging systems are 20-100 times more sensitive. Available scanners also suffer to some extent from being an older generation of equipment designed before sophisticated electronics and microprocessors were available. The imaging systems, therefore, have a considerable advantage in computational power which allows the user, with a few simple commands, to integrate the counts in peaks of interest, to obtain relative percents of total activity for each peak, and to automate the analysis of large numbers of similar chromatograms.

The quantitative limitations of scraping and scintillation counting and imaging systems are difficult to compare. The major limitations in scintillation counting are in the preparation of the samples. Errors can arise from a desire to scrape the minimum number of zones thus degrading the resolution of the chromatogram or missing some important areas of background or contaminants.

By contrast, the major quantitative limitation with imaging systems is often counting statistics due to the lower counting efficiencies. A 1000 disintegrations per minute (dpm) tritium spot would produce about 5 counts per minute (cpm) in the imaging system so that a 20 minute analysis would produce a total count of 100 with a statistical uncertainty of 10 percent. A 20 minute analysis in a scintillation counter would yield at least 6×10^3 counts with a statistical uncertainty of less than 2 percent. This error is much less than the average error introduced by the scraping process. Also, if the scraping were in zones of 1 cm (rather poor resolution), as many as 15 samples would be gene-

rated from a 15 cm plate development, and the total counter time
required would be 5 hours for 20 minute counts and 1.25 hours for
5 minute counts from a single chromatogram.   If all the
samples were scintillation counted within the 20 minute span
alotted for the imaging analysis, the 1000 dpm spot would produce
400 counts in 1.3 minutes, a statistical precision of 5 percent.

Because quantitative accuracy and spatial resolution are
related in practical work, it is important to compare these
characteristics. For tritium, imaging system resolution is about
2 mm or equivalent to 75 or more scraping zones on a standard TLC
plate.   For ^{14}C, the resolution is degraded somewhat, as
explained below, to 3-5 mm or at least 30 scraping zones.   In
practice, users rarely scrape this many zones, and in each case
it is important to assess what impact not having this resolution
could have on the precision of the analysis.   Often, the poten-
tial impact is important enough that some less quantitative, but
higher resolution technique, such as autoradiography or conven-
tional scanners, is used to validate the scraping and scintilla-
tion counting analysis.

A direct comparison of the quantitative results from scra-
ping and scintillation counting and imaging system analysis was
made by Baird et al.(2).   Duplicate samples of carcinogen
($[^3H]$benzo($\underline{a}$)pyrene) metabolite separations were run and
analyzed by the two methods (Figure 1).   The scintillation
counting results are shown above and the imaging system results
below.   The percentage of each metabolite calculated from the two
methods of analysis agree to better than the 10-15 percent
sample-to-sample variation found by scintillation counting alone.
Thus, the quantitative results from the imaging system are at
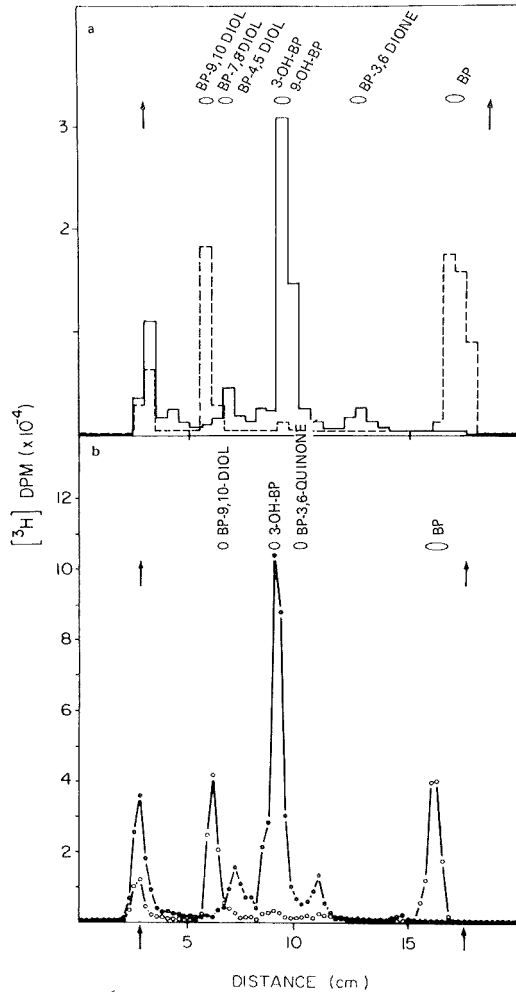
Figure 1   A comparison between scraping and scintillation
             counting (top) and the imaging system (bottom).
             The open and filled circles correspond to the
             dashed and solid lines, respectively. The imaging
             system analysis time was 20 minutes.

least as accurate as the experimental reproducibility in this
type of work.

Overall, the radiochromatogram imaging system offers super-
ior quantitative performance over the conventional technique of
scraping and scintillation counting. It is faster, has better
spatial resolution, and is less costly to use. However, scintil-
lation counting may be the preferred technique with samples of
such low activity that the required imaging system analysis time
is longer than the scraping and preparation time. Typically,
this point is reached with total activities below 500-1000 dpm of
tritium which would require 0.5-1 hour analysis times with the
imaging system. In most laboratory experiments, the imaging
system will handle 90 percent or more of the work load with an
average analysis time of 5-15 minutes per lane.

Radiochromatogram Imaging Systems

The functional characteristics of imaging systems have been
discussed above. In this section, a more detailed explanation of
the underlying principles is presented. Generally, these systems
consist of three separate parts: an imaging proportional counter,
a CRT and associated electronics for storing the data from the
counter and presenting a display of the chromatogram, and a
terminal for controlling the operation of the instrument and
printing the final results.

The new element in this system is the imaging proportional
counter. It is instructive to first review the principles of a
standard proportional counter, Figure 2. The counter has a gas
volume, usually circular or rectangular in cross-section, with a

GAS VOLUME

ANODE WIRE

X-RAY

INITIAL
IONIZATION
CLOUD

+ V

INSULATOR
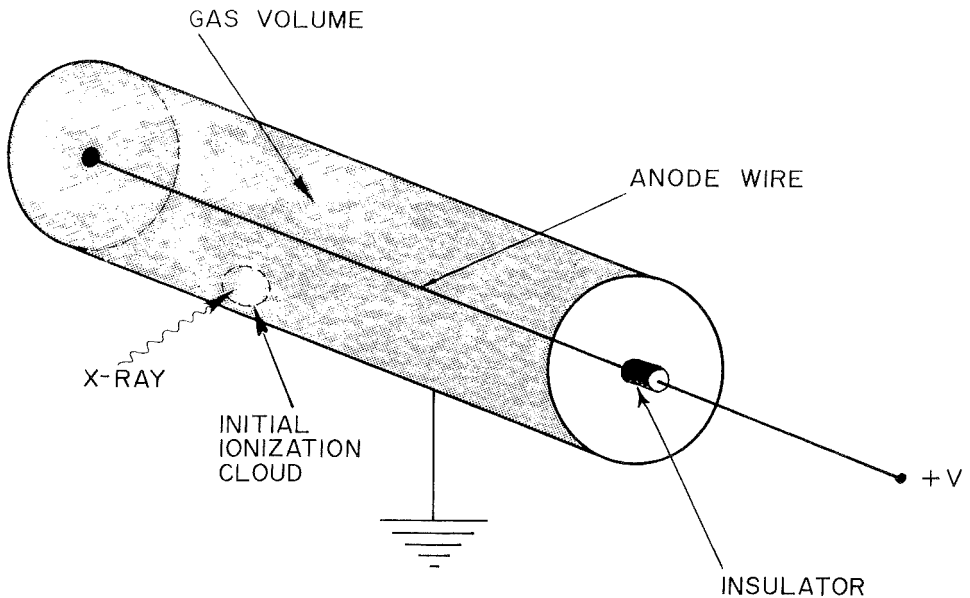
Figure 2   Schematic drawing of a conventional proportional
                counter.

high voltage anode wire running through it.   Radiation, such as
X-rays or beta particles, ionizes the gas and produces free
electrons which are accelerated toward the anode wire.   When the
electrons enter the very high electric field near the wire
surface, they are accelerated to sufficient   energy to produce
further ionization of the gas. This increase in the   number of
electrons produces a pulse on the anode wire of sufficient magni-
tude (1000 electrons or more) to sense with standard electronic
amplifiers.

     In counters designed to detect X-rays or high energy beta
radiation, the gas volume is completely sealed, and the radiation
enters through a window made of plastic, beryllium, or other

RESISTIVE ANODE
GAS VOLUME

IMAGING DETECTOR

TLC PLATE

PREAMPLIFIERS   L   R

POSITION COMPUTATION   L/L + R

STORE IN MEMORY
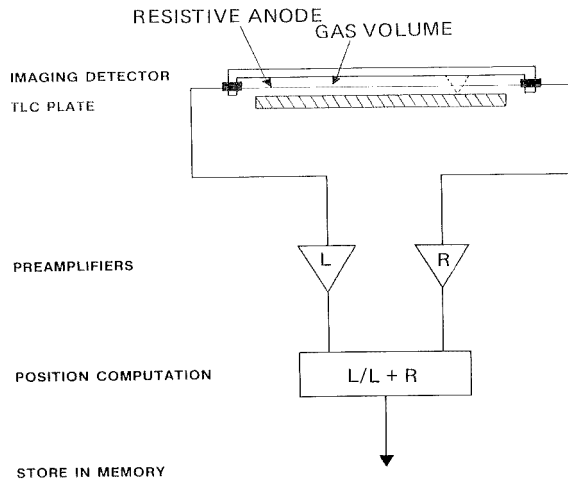
Figure 3   Schematic drawing of a resistance anode imaging
                proportional counter, its electronics, and the
                logical flow of event data.

material. In the case of low energy alpha and beta particle
detection, the entrance aperture must be windowless, and gas lost
to the surroundings must be continually resupplied to the detec-
tor volume. Many gases can be used, but the most common are
mixtures of noble gases and hydrocarbons. A mixture of 90% argon
and 10% methane (called P-10) is widely used and can be readily
obtained from most gas suppliers.

     To add imaging capability to the counters, the design must
be altered to provide an electronic signal which varies as the
position of the incident radiation varies over the sensitive area
of the detector. One such scheme for obtaining position informa-
tion in one dimension is shown in Figure 3. The metal anode of
Figure 2 is replaced by a resistive anode made of a carbon coated

quartz fiber. A preamplifier is attached at both ends instead of only one end. When a pulse of electrons is collected at the resistive anode, it behaves like a current divider. Part of the pulse flows toward each end with the ratio of the two parts of the pulse determined by the amount of resistance between the original collection point and each of the preamplifiers. The two amplified pulses are then converted to a digital result on a pulse height scale, and the quotient shown is computed to give a numerical result for the position. A digital image can then be built up in a computer memory by adding 1 count to the total stored in the memory location which corresponds to a particular interval along the anode.

The resolution which can be achieved with this (or other) imaging scheme is on the order of 0.2-0.5% of the total detector length. For radiochromatograms, the detector length used is 25 cm, and the data is stored in 256 locations in the computer memory corresponding to 1 mm intervals. However, the main limitation on the resolution in practice is due to the finite depth of the detector and the omnidirectional nature of the radiation emanating from the sample. The dashed lines in Figure 3 illustrate two possible paths of betas radiated from the same location on the TLC plate. The betas will produce ionization along their paths, and the detector will measure the centroid of this ionization. In general, the spread (or defocussing) will be comparable to the detector depth for high energy betas, and will decrease as the energy and penertrating power of the beta decreases. Thus for P-32, the resolution will be limited to the detector depth of 5 mm, while for ^{14}C it will improve to about 3 mm, and for tritium it is 1-2 mm.
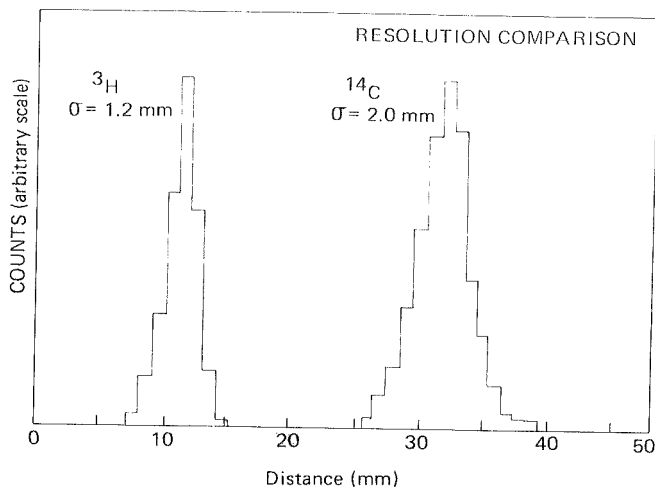
Figure 4   A resolution comparison between tritium and ^{14}C.
The spots analyzed had intrinsic diameters of 1-2 mm.

The relative resolution performance for tritium and ^{14}C is shown in Figure 4. Radioactivity was spotted on a TLC plate with a micropipette, and then analyzed using an imaging system (Bioscan, Inc. BID SYSTEM 100). The spot sizes are 1-2 mm in diameter, so the actual detector resolution is somewhat better than the gaussian fit parameters shown.

Several groups (2,3,4,5) have investigated applications of imaging proportional counters to quantitative radiochromatogram analysis, and their work may be consulted for details.

A Radiochromatogram Imaging System Application

Radiochromatogram imaging techniques when combined with careful preparative and TLC techniques give excellent results.

As an example, the work of Bougnoux, Hoffman, and Herberman (6) as part of a study of membrane metabolism changes associated with the cytotoxicity of human peripheral blood cells acting against human tumor cells is cited.

The principal advantages of the radiochromatogram imaging system in this work are its quantitative accuracy, its sensitivity, and the very small amount of operator attention required. Plate scraping and scintillation counting would have been used if the imaging system were not available, and the researchers then estimate that the scraping and sample preparation would limit the rate at which experiments were performed and analyzed. With the availability of the imaging system, the time and effort required for accurate quantitative analysis is not a significant part of the total laboratory effort.
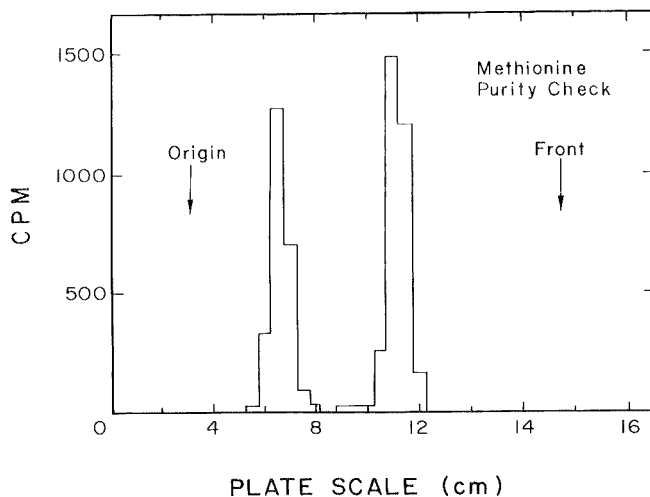
Figure 5   TLC separation of [Methyl-^{3}H]-L Methionine and
                  principal impurity Methionine sulfoxide.

The first step in the careful procedures worked out for
these separations is the reanalysis of the labeled reagents. An
example is shown in Figure 5 of tritium labeled methionine used
in kinetic studies of phospholipid methylation. In storage, the
methionine (peak at 11.0 cm) is slowly oxidized to methionine
sulfoxide (peak at 6.5 cm). The radiochromatogram imaging system
required only 1 minute to acquire this data, and another minute
of operator time at the terminal produced the numerical analysis
that the sample is 43% inactive sulfoxide and 56% methionine.
The total tritium activity on the plate is approximately 7×10^5
dpm.

The TLC separations of both neutral lipids and phospholipids
are shown in Figure 6. The lipids were labeled with ^{14}C arachi-
donic acid. The TLC was done on Silica gel G plates using
hexane/diethyl ether/acetic acid (60:40:1) to separate the neu-
tral lipids (top) and chloroform/methanol/acetic acid/water
(50:25:7:3) to separate the phospholipids (bottom). The success
of the TLC techniques is complemented by the imaging system which
gives a full display of the entire chromatogram. The user is
able to verify instantly the success of his experiments by noting
the symmetry of the peaks and the low background between peaks.
When scraping and scintillation counting are used in similar
circumstances, the tendancy is to scrape only one or two regions
in the vicinity of each standard in order to reduce preparation
and counting time. Often the resolution of the scraping proce-
dure is inadequate to judge the extent of background smearing,
peak asymmetry, and spurious peaks which might provide clues to
problems in the experimental procedure.

The data shown in Figure 6 were obtained in a 5 minute
analysis of each chromatogram. The neutral lipid separation
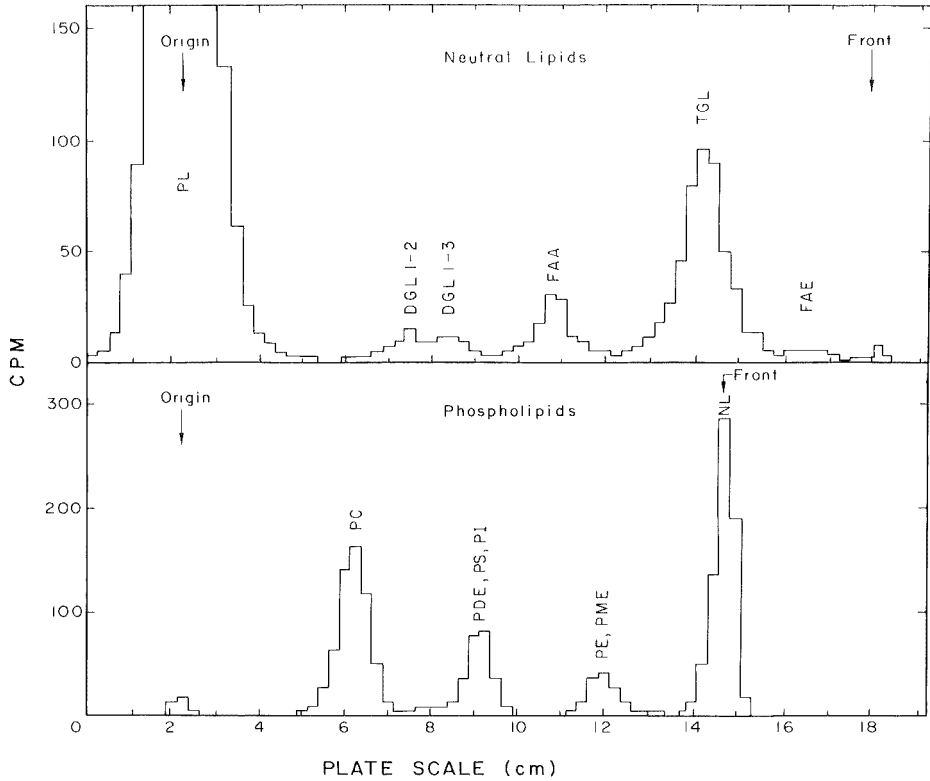
Figure 6   TLC separations of neutral lipids (top) and phos-
                  pholipids (bottom) labeled with ^{14}C-Arachidonate.

(top) had a total of 5 x 10^4 dpm on the lane with approximately

81% of the activity in the phospholipids which remain at the

origin.   Of the remaining 19% labeled neutral lipid, 57% is in

the triglyceride peak (TGL).   The phospholipid separation (bot-

tom) is from a different cell type and had a total of 2 x 10^4 dpm

on the lane with 38% in the neutral lipids at the front.   A total

of 54% of the activity is in the three major phospholipid peaks.
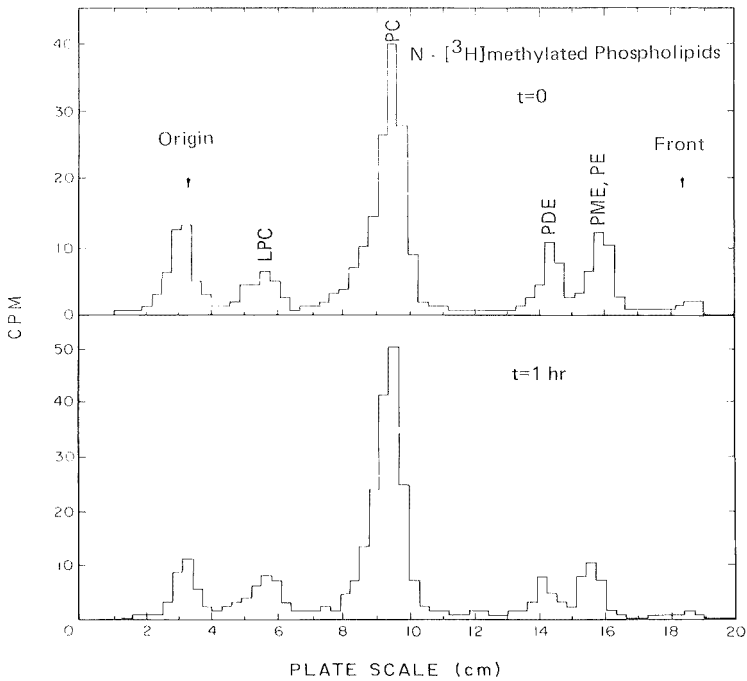
Figure 7   TLC separations of N-methylated phospholipids.   The

                t=0 (top) data were obtained immediately after

                washing the cells to remove the [Methyl-^{3}H]-L

                Methionine labeling compound.   The t=1 hour

                (bottom) data were obtained from cells harvested

                one hour after washing.

    Another part of this work involves studies of the kinetics

of phospholipid methylation.   A technique using tritium labeled

methionine has been worked out in which cell lipids can be

labeled almost exclusively at the three methylation sites associ-

ated with the amine.   The cells are washed so that all free label

is removed, and then the progress of methylation can be monitored at successive times by measuring the relative amounts of the monomethyl (PME), dimethyl (DME), and trimethyl (PC) phospholipids. An example of such a kinetics study is shown in Figure 7. Even though the unmethylated (PE) and monomethyl derivatives are not separated chromatographically, the unmethylated substance is not labeled and a complete separation of labeled methylated compounds is achieved. In this example the change after 1 hour is small but easily detected with the radiochromatogram imaging system. The PME and PDE derivatives continue to be methylated and are transferred into the PC peak. At the start (top) PME is 17% of the total and PDE is 14%. After 1 hour (bottom), PME is 13% and PDE is 10%. These results were obtained with a 15 minute analysis of each lane and a total tritium activity of approximately 6.5×10^4 dpm on each lane.

With the imaging system, quantitative results are reproducible to better than 1% from analysis to analysis so that these small changes can be measured reliably. Although scintillation counters give equally reproducible results, it is doubtful whether the entire process of plate scraping (or cutting of plastic backed plates) and sample elution can be performed reliably enough to measure such small changes.

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SEPARATION OF PROSTANOIDS BY ONE–DIMENSIONAL THIN–LAYER CHROMATOGRAPHY

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ABSTRACT

We have developed a simple and rapid method for separation of 7 prostanoids and arachidonic acid by one-dimensional thin-layer chromatography.   For this separation we employ commercially prepared thin-layer plates that have a preadsorbant (celite) area to which samples are applied.   Due to the inert characteristics of the celite no separation occurs until the sample reaches the preadsorbant-silica gel junction.   Since all the material moves with the solvent front as a sharp, narrow band to the preadsorbant (celite)-silica gel boundary, a high resolution of the separated compounds is achieved.   The time required to apply one sample to the preadsorbant (celite) area is considerably less (2 min) than that required for application of a sample to silica gel (10 min).   This method is suitable for the separation of major prostaglandins (PGE_2, $PGF_{2\alpha}$), the major enzymatically formed metabolites of these prostaglandins, and the stable, nonenzymatically formed product of thromboxane A_2 (TXB_2).

INTRODUCTION

To investigate the metabolism of arachidonic acid and prostanoids, thin-layer chromatography (TLC) on silica gel is used widely for the separation and identification of a number of prostaglandins (PG) and prostaglandin-like compounds.   Many TLC systems have been described (1-5) in which a variety of

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prostanoids can be separated. According to the conventional
method for TLC, samples are dissolved in small volumes (20 –
30 µl) of organic solvents which are applied to the silica gel in a
successive series of aliquots (1 – 5 µl) overlaid at the origin.
This process is extremely time consuming since the solvent must
be removed completely between each application of a portion of
the sample. Moreover, with a small volume, it is difficult to
remove completely the compounds from the tubes. We have
developed a technique for the separation of 7 major prostanoids
and arachidonic acid by one–dimensional TLC in which we employ
commercially available thin–layer plates with a preadsorbant
(celite) area. With this method samples can be applied rapidly
and very high resolution of the compounds is achieved. We
developed this method to evaluate the formation of prostanoids in
human endometrial stromal cells, which are maintained in
monolayer culture in the presence of [^{14}C]arachidonic acid.
These cells biosynthesize and metabolize prostanoids and secrete
the products into the culture medium. This method has proved to
be advantageous for these studies and will undoubtedly be
applicable to a number of systems in which high resolution and
quantification of prostanoids is desired.

MATERIALS AND METHODS

        Silica gel G preadsorbant thin–layer plates were purchased
from Analtech, Newark, DE, USA, and were used without an
activation or washing procedure. Prostanoid standards were gifts
from Dr. John E. Pike, Upjohn Company, Kalamazoo, MI, USA.
All solvents were of analytical grade from scientific supply
houses. Glass tubes were siliconized using Aquasil (Pierce
Chemical Company, IL, USA.)

        The TLC plate was scored into 7 lanes (25 mm each). Each
sample, in 150 µl chloroform:methanol (2:1, by vol), was applied
with a micro–selectapette (Clay Adams, Parsipanny, NJ, USA) to
the preadsorbant (celite) layer on an area (625 mm^2) 5 mm above
the bottom edge of the plate and 5 mm below the preadsorbant

(celite)-silica gel boundary. To minimize oxidation of the compounds, the origin areas of the TLC plate were exposed to continuous nitrogen flow during the application process by use of a device supplied by Sindco Corp., Miami, FL, USA. The plates were placed into a plate conditioning apparatus (Analtech, Newark, DE, USA) and developed in a solvent system chloroform:methanol:acetic acid:water (95:5:1:0.2, by vol) to a height of 16 cm above the preadsorbant (celite)-silica gel limit. After removal of the plate from the TLC chamber, the solvent was evaporated under a stream of air for 5 min and thereafter the chromatogram was exposed to iodine vapor.

In a typical experiment human endometrial stromal cells were maintained in monolayer culture in the presence of [^{14}C]arachidonic acid (Amersham, Arlington Heights, IL, USA). At the end of the incubation period the medium (4 ml) was collected and acidified with 2 ml acetic acid (1N) and then extracted with 30 ml chloroform:methanol (2:1, by vol); the organic phase was removed and evaporated at room temperature under a stream of nitrogen. The residue was subjected to silicic acid column chromatography and the bulk of free [^{14}C]arachidonic acid was collected in the first fraction (13 ml chloroform). In the second fraction (chloroform:methanol, 10:1, by vol) PGs were obtained and the organic phase was concentrated at room temperature under a stream of nitrogen to approximately 0.5 ml. Ten µg each of PGF$_{2\alpha}$, PGE$_2$, 15-keto-PGE$_2$, 15-keto-PGF$_{2\alpha}$, 13,14-dihydro-15-keto-PGE$_2$ (PGEM), 13,14-dihydro-15-keto-PGF$_{2\alpha}$ (PGFM), thromboxane (TX) B$_2$ and arachidonic acid were added as a mixture in 50 µl ethanol and the evaporation process was completed. The compounds were dissolved in 0.15 ml chloroform:methanol (2:1, by vol) and separated by TLC as described. After visualization of the standards and evaporation of the iodine, the areas of the chromatogram corresponding to the various compounds were scraped from the plate and eluted with 6 ml chloroform: methanol (2:1, by vol). The extracts were evaporated at room temperature under a stream of nitrogen and radio-

activity was assayed in 16 ml Liquiscint (National Diagnostics, Somerville, NJ, USA) by liquid scintillation spectrometry.

RESULTS AND DISCUSSION

The positions of different prostanoids and arachidonic acid on a chromatogram after one-dimensional preadsorbant (celite)- silica gel chromatography are given in Fig 1. For purpose of
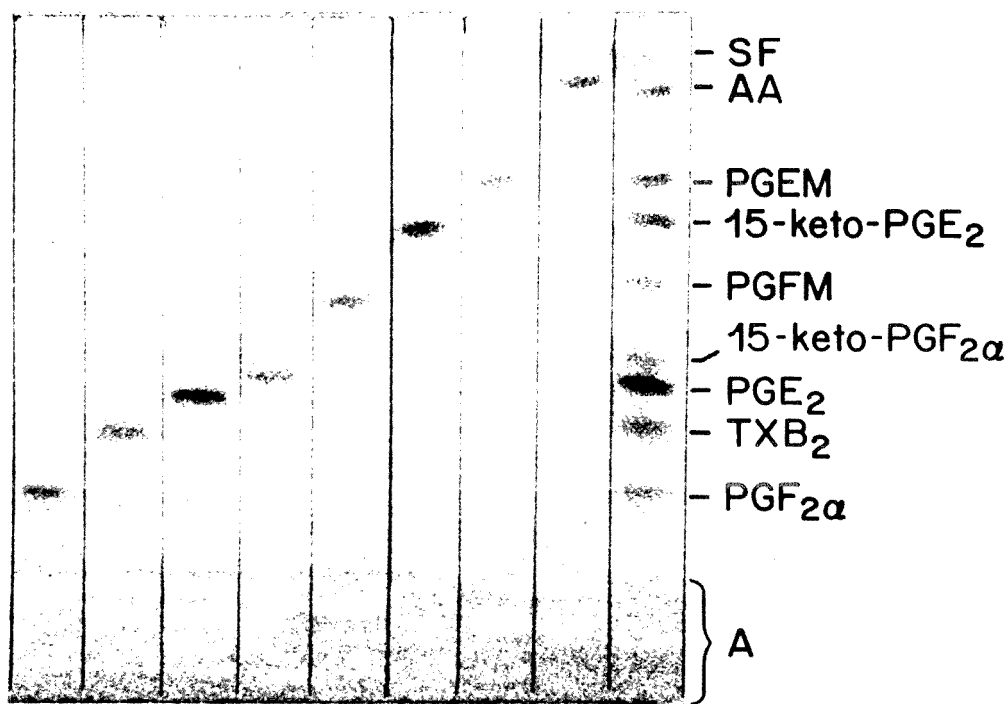
Figure 1. Prostanoid standard chromatogram after iodine staining. The compounds were applied either separately or as the mixture. Ten µg each of prostaglandin (PG)$F_{2\alpha}$, thromboxane B_2 (TXB$_2$), PGE$_2$, 15-keto-PGF$_{2\alpha}$, 13,14-dihydro-15-keto-PGF$_{2\alpha}$ (PGFM); 15-keto-PGE$_2$; 13,14-dihydro-15-keto-PGE$_2$ (PGEM); and arachidonic acid (AA) were applied on the preadsorbant (celite) area. A = preadsorbant (celite) layer; SF = solvent front.

TABLE 1

Mobility of Separated Prostanoids and Arachidonic Acid

Compound	Mobility (expressed as R_f)
$PGF_{2\alpha}$	0.11
TXB_2	0.20
PGE_2	0.25
15-keto-$PGF_{2\alpha}$	0.32
PGFM	0.46
15-keto-PGE_2	0.59
PGEM	0.69
Arachidonic acid	0.89

illustration the scored lanes are 20 mm wide. The mobilities of the compounds, expressed as R_f values, are shown in Table 1. For computation of R_f values, the preadsorbant (celite)-silica gel boundary was taken as the origin.

Each standard is separated distinctly and application of the compounds as the mixture does not lead to alterations in R_f values. In the TLC system used the mobility of 6-keto-$PGF_{1\alpha}$ [the more stable, nonenzymatically formed product of prostacyclin (PGI_2)] is the same as that of PGE_2 (not shown). However, if separation of these two compounds is desired, they may be eluted from the silica gel with chloroform:methanol (2:1, by vol), and separated by TLC in a solvent system of chloroform:isopropanol:ethanol:formic acid (45:5:0.5:0.3, by vol) as described previously by Goswami et al. (4).

An example of the results obtained with the application of the methods described is shown in Fig. 2. Human endometrial stromal cells maintained in monolayer culture were incubated with [^{14}C]arachidonic acid for 24 h to evaluate the biosynthesis of radiolabeled prostanoids. Since the radioactivity in the medium extract may be limited, it is necessary to apply the entire extract residue on TLC to achieve accuracy when assaying radioactivity by liquid scintillation spectrometry. The conventional means of
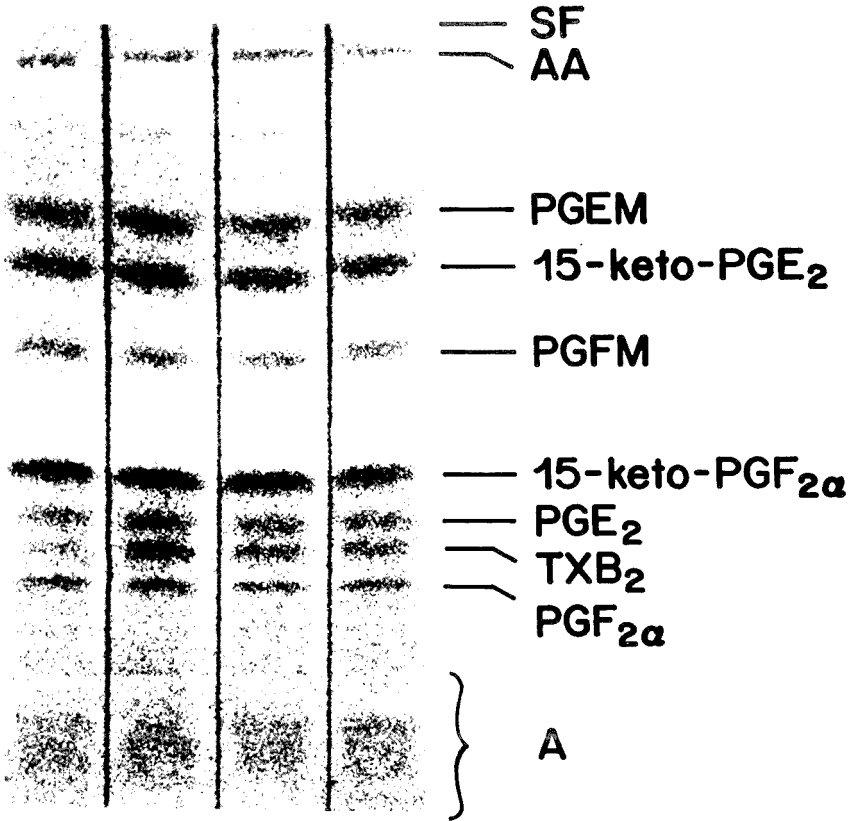
Figure 2. Prostanoid chromatogram of human endometrial stromal cell monolayer culture medium extracts after iodine staining. The culture medium was extracted, the standards were added and the compounds were applied to the preadsorbant (celite) area in 0.15 ml chloroform:methanol (2:1, by vol) as described in the text. For abbreviations see legend of Fig. 1.

sample application on TLC is very time consuming because of the necessity for complete solvent removal between repeated application of aliquots of the sample in a single band at the origin. Moreover, the capacity of the silica gel is limited. Using the method we describe here the spotting time per sample was 2 min compared to 10 min with conventional methods. By virtue of the characteristics of the preadsorbant (celite) layer, all of the applied material moves with the solvent front as a sharp band to the preadsorbant (celite)-silica gel boundary and no separation occurs until the sample reaches the silica gel layer. For this reason, sample application in a single band is not necessary. The method we describe provides for a more rapid means of sharp separation of most major prostanoids and arachidonic acid and is applicable to many systems in which identification of these compounds by TLC is to be achieved.

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EFFECT OF DILUENT ON THE STRUCTURE

OF THE ADSORPTION LAYER IN SYSTEMS OF

THE TYPE DILUENT + POLAR SOLVENT-SILICA

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ABSTRACT

The adsorption behaviour of solvents whose molecules differ in polarity, functional groups and shape and the effect of the less polar diluent was investigated. The surface excess was determined for various combinations of diluents and polar solvents using the modified thin-layer frontal chromatography technique.

INTRODUCTION

Adsorption in liquid-solid systems is important not only from the viewpoint of the phenomenon itself, but also owing to its fundamental role in the description of disperse systems /1/. Differences in the opinions about the orientation of molecules in the adsorption layer and

its structure are reflected by the use of various models
in the theory of retention mechanism in liquid chromato-
graphy /2-4/. Recently, Ościk et al./5/ concluded that
ketones form multilayers when adsorbed from heptane
solutions and monomolecular layers in benzene solutions.
Therefore, it seemed   worthwhile to investigate the
adsorption behaviour of other solvents whose molecules
differ in polarity, functional groups and shape; the
effect of the less polar diluent was also investigated.
For this purpose, the surface excess $\Gamma_i^{/n/}$ was determined
for various combinations of diluents and polar solvents,
using the modified thin-layer frontal chromatography
technique /6/.

EXPERIMENTAL

The chromatographic experiments were carried out
according to the procedure described in an earlier
paper /6/ with a recently introduced modification /7/.

RESULTS   AND   DISCUSSION

Four polar solvent of class B and AB according to
Pimentel and McClellan and belonging to various homolo-
gous series were chosen: diethyl ether, diethyl ketone,
methyl ethyl ketone and n-pentanol-1. The four solvents

have similar molar volumes. As diluent, both nonpolar
/heptane, hexane, cyclohexane, decalin/ as well as
weakly polar solvents were chosen /benzene, chloroform,
methylene chloride/. The point of the excess isotherm
was determined for 1 M concentration of the polar
solvent which should correspond in most cases to mono-
molecular coverage. If the monomolecular adsorption
model is correct /t = 1/, then the molar fraction of the
solvent in the adsorption layer should not exceed unity
/$X_i \leqslant 1$/. The value of X_i was calculated from Everett's
equation:

Assuming t = 1 we have

$$X_i^{\sigma} = \frac{X_i^l + a_j^o \; \Gamma_i^{/n/}}{1 - /a_i^o - a_j^o / \Gamma_i^{/n/}}$$ /1/

where $a_{i,j}^o$ are the areas covered by the given molecules
in the monolayer

$\quad$ $\Gamma_i^{/n/}$ – surface excess concentration

$\quad$ t   – number of layers

$\quad$ X_i^l – molar fraction of "i" in the bulk phase.

If the calculations for given values of $a_{i,j}^o$ result
in $X_i^{\sigma} > 1$ then the assumption of monomolecular adsorption
was false. For many simple systems the condition t = 1 is
fulfilled, however, for others t > 1 is required.

The surface excess values obtained are given in
Table 1.

TABLE 1

Values of the surface excess $n_i^{e}/v/$ for some solvent
systems at $C_i^{l}= 1$ ML^{-1}. Adsorbent: silica gel, $a_s=500$ m^{2}g^{-1}

Diluent	Di-ethyl ether	Di-ethyl ketone	Methyl-propyl ketone	n-pentanol-1
Heptane	0.94	1.59	1.95	0.83
Hexane	0.82	0.99	1.33	0.80
Cyclohexane	0.66	1.10	1.16	0.71
Benzene	0.46	0.76	0.94	0.64
Decalin	1.00	1.14	1.32	0.81
Carbon tetra-chloride	0.51	0.67	0.81	0.55
Dichloromethane	0.31	0.33	0.49	0.57
Chloroform	0.27	0.32	0.42	0.53

The calculations according to eq.1 should be preceded
by the choice of the values of the molecular areas $a_{i,j}^{o}$.
Two methods were applied in the present study. In the
first the calculation were based on the formula proposed
by McClellan and Harnsberg /8/

$$\omega_{ads} = 2.567 . v^{0,687}- 10.336$$

To calculate the molecular area from this formula it
is sufficient to know the molar volume of the component:
ω_{liq} denotes the area of the molecule assuming spherical
shape and dense hexagonal packing. In the second method
the molecules were considered as cylinders of cross-
section area equal to 0.22 nm^2 /calculated from molecular
parameters; Van der Waals radii etc./ and length proport-
ional to the chain length, in accordance with the equation

$$0.22 \times H \times N_A = \tilde{V} \qquad\qquad /3/$$

where N is the length of the cylinder, N_A - Avogadro number and $\tilde{V}$ - molar volume.

The areas of 1 mole of adsorbate calculated by the two methods are given in Table 2 for flat and perpendicular orientation relative to the adsorbent surface.

From the molecular excess values $n_i^{\sigma}/v/$ /assuming that $n_i^{\sigma}/v/ = n_i^{\sigma}/n/$ and specific surface area of silica equal to 500 $m^2g^{-1}/$ the molar fraction of the polar component in the adsorption layer was calculated for various possible values of $a_{i,j}^{o}$, /relative to 1 mole/.

TABLE 2

Areas occupied by one mole of molecules in the monolayer $a_{i,j}^{o}$, $\times 10^5$ m^2mol^{-1}

Solvent	a_i^o, a_j^o calculated		
	from /eq.2/	for cylindrical shape	
		flat orientation	vertical orientation
Heptane	3.73	3 29	1 27
Hexane	3.29	2 82	1.27
Cyclohexane	2.93	2.05	1.40
Benzene	2.49	2.05	1 40
Decalin	3.88	4.10	2 80
Carbon tetra-chloride	2 67		
Dichloromethane	1.89		
Chloroform	2.29		
Di-ethyl ether	2.84	2 35	1.48
Di-ethyl ketone	2.88	2 46	1 48
Methyl-propyl ketone	2.89	2.69	1.48
n-Pentanol-1	2.95	2.67	1 27

TABLE 3

a. Molar fraction X_i^{σ} of polar solvents in the adsorption
   layer /calculated for a_i^0, a_j^0 from eq./2// assuming
   monolayer adsorption /t = 1/

Diluent	Di-ethyl ether	Di-ethyl ketone	Methyl-propyl ketone	n-pentanol-1
Heptane	0.72	1.04	1.20	0.67
Hexane	0.62	0.72	0.91	0.62
Cyclohexane	0.49	0.74	0.78	0.53
Benzene	0.33	0.50	0.60	0.43
Decalin	0.76	0.84	0.93	0.67
Carbon tetra-chloride	0.38	0.47	0.55	0.40
Dichloromethane	0.20	0.21	0.28	0.32
Chloroform	0.21	0.24	0.29	0.35

b. Molar fraction X_1^{σ} of polar solvent in the adsorption
   layer /calculated for cyllindrical molecular shape
   and flat adsorption of polar solvent and vertical
   adsorption the diluent/

Diluent	Di-ethyl ether	Di-ethyl ketone	Methyl propyl ketone	n-pentanol-1
Heptane	0.73	1.50	2.66	0.76
Hexane	0.62	0.80	1.35	0.71
Cyclohexane	0.48	0.85	1.04	0.59
Benzene	0.33	0.55	0.79	0.51
Decalin	0.57	0.66	0.83	0.57

c. As in b/ calculated for cyllindrical molecule
   shape and flat adsorption of polar solvent and flat
   adsorption the diluent

Diluent	Di-ethyl ether	Di-ethyl ketone	Methyl-propyl ketone	n-pentanol-1
Heptane	0.49	0.73	0.96	0.53
Hexane	0.48	0.57	0.81	0.54
Cyclohexane	0.44	0.49	0.86	0.53
Benzene	0.31	0.49	0.68	0.47
Dekaline	0.46	0.52	0.62	0.42

The molar fractions calculated for various assumptions
are given in Table 3 a,b,c.

The simplified procedure /various geometrical
models of molecules/ is commonly used when no general
solution is available; it may provide approximate
information about the role of the investigated parameters
in the phenomena studied.

It follows from the calculations /Table 3a/ that
the use of molecular areas based on eq.2 results in
molar fractions larger than unity for methyl-n-propyl
ketone and in same cases also for diethyl ketone. This
seems to indicate that the monomolecular model of
adsorption is not valid in these cases. On the other
hand, the molar fraction of ether is smaller than
unity for all diluents which is in accordance with the
monomolecular adsorption model.

In the second model cylindrical shape of the mole-
cules was assumed. In the case of polar solvents flat
adsorption was assumed while for diluents /hexane,
heptane, benzene, cyclohexane, dekaline/ two extreme
cases i.e., vertical or flat adsorption were considered.
The results are presented in Table 3b,c. Flat adsorption
of polar solvent and vertical adsorption of the diluent
results in data which can be interpreted by multilayer
adsorption /mole fraction smaller than unity is obtained
for t $>$ 1/. On the other hand, assumption of flat

configurations for both polar and nonpolar molecules
leads to calculated values of X_i^{σ} which are in agreement
with the monomolecular adsorption model.

The conclusions emphasize the role of estimated
sitting areas of adsorbed molecules. For instance, the
results indicating bilayer adsorption in ketone solut-
ions and monolayer adsorption in ether solutions may
lead to explication of some anomalies in the behaviour
of ethers in comparison to ketones when used as eluents
in liquid-solid adsorption chromatography. Owing to
the limited number of experimental data analysed, only
general conclusions could be formulated.

Acknowledgement

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EVALUATION OF MODIFIED VALENCE MOLECULAR CONNECTIVITY
INDEX FOR CORRELATIONS OF CHROMATOGRAPHIC PARAMETERS

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ABSTRACT

Twenty five data sets of different chromatographic
parameters for barbituric acid derivatives taken from
the literature were correlated with the modified valence
connectivity index. The results show that more significant
correlations are obtained using the standard valence con-
nectivity index for liquid chromatography techniques.

INTRODUCTION

Molecular connectivity indices (1) are widely used

in structure-activity relationship analyses in medicinal

chemistry (2). There were also numerous attempts to apply

these topological parameters for a description of chro-

matographic behavior of different classes of compounds

(3 - 13). It was found, that the topological indices are

very useful in describing the interaction between member

molecules of one family and the stationary phases (11).

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We investigated the relationship between first
order valence connectivity indices of barbiturates and
chromatographic parameters for different chromatographic
techniques (14). The best correlations were found for
HPLC the worse were those for TLC.

Gas chromatographic retention data for the same
group of compounds were correlated with molecular con-
nectivity parameters by Stead et al. (15). They intro-
duced the modified first order valence connectivity
index $^1\chi_N^v$ and claimed significantly better correla-
tion than that obtained with the standard valence con-
nectivity values $^1\chi^v$. The main difference between
these parameters lies in the calculations of connecti-
vity term for unsaturated and cyclic substituents,
while the calculation rules for saturated chain substi-
tuents are identical.

We wanted to study applications of this modified
parameter for the correlations of chromatographic data
of barbiturates in other techniques of chromatography
and to compare the results with those obtained for the
original valence connectivity indices.

CALCULATIONS

The values of the modified first order valence con-
nectivity index $^1\chi_N^v$ were calculated according to the
original indications (15) and are listed in Table 1.

TABLE 1

Modified connectivity indices of barbiturates

Barbituric acid	$^1\chi_N^v$
1. 5-allyl-5-ethyl	4.5130
2. 5,5-diallyl	4.7903
3. 5-allyl-5-isopropyl	4.8958
4. 5-ethyl-5-crotyl	5.2340
5. 5-allyl-5-isobutyl	5.3679
6. 5-allyl-5-/1-methylpropyl/	5.4338
7. 5-vinyl-5-/1-methylbutyl/	5.4606
8. 5-allyl-5-n-butyl	5.5130
9. 5-allyl-5-/2,2-dimethylpropyl/	5.6595
10. 5-allyl-5-/1-methylbutyl/	5.9328
11. 5-ethyl-5-/1-methylbuten-1-yl/	5.9401
12. 5-allyl-5-/cyclopenten-1-yl/	6.3050
13. 5-methyl-5-phenyl	6.5269
14. 1,5-dimethyl-5-/cyclohexen-1-yl/	6.6992
15. 5-ethyl-5-/cyclohexen-1-yl/	6.8655
16. 5-ethyl-5-phenyl	7.0866
17. 5-ethyl-5-/cyclohepten-1-yl/	7.3655
18. 5-allyl-5-phenyl	7.3659
19. 1-methyl-5-ethyl-5-phenyl	7.4810
20. 1-phenyl-5,5-diethyl	8.0423

Standard first order valence connectivity indices $^1\chi^v$
were calculated as described previously (14).

The chromatographic data for barbituric acid deri-
vatives were taken from the literature (16 - 23). The
following parameters were used for the correlations:
R_F and R_M for paper chromatography (PC) and thin-layer
chromatography (TLC), retention indices (I) and reten-
tion times (t_R and log t_R) for gas chromatography (GC)
and capacity factors (k´ and log k´) for high perfor-
mance liquid chromatography (HPLC).

The correlations were carried out by the least
squares method and the significance of correlation
coefficients was evaluated by Student´s test.

RESULTS

Table 2 reports statistical data: correlation
coefficients (r), significance levels (α) and number
of compounds in data sets (n). The results of compa-
risons between correlations using $^1\chi_N^v$ and $^1\chi^v$
values are presented in Table 3.

CONCLUSION

From the results presented in Tables 2 and 3 it
is clear that the modified valence connectivity para-
meter $^1\chi_N^v$ yields better correlations of chromato-
graphic data only for the gas chromatography techni-

TABLE 2

Results of linear regression analysis

Technique	Data set	Reference	n	y	$^1\chi^v$		$^1\chi^v_N$	
					r	$\propto$	r	$\propto$
	1		12	R_F	0.6356	0.05	0.2024	0.6
				R_M	0.6259	0.05	0.1890	0.6
PC	2	16	16	R_F	0.6297	0.01	0.1832	0.5
				R_M	0.6186	0.02	0.1742	0.6
	3		14	R_F	0.7535	0.01	0.4880	0.1
				R_M	0.7386	0.01	0.4609	0.1
	4	9	15	R_F	0.7394	0.01	0.6480	0.01
				R_M	0.8411	0.001	0.7857	0.001
	5	16	30	R_F	0.3671	0.05	0.1123	0.6
TLC				R_M	0.3721	0.05	0.0908	0.7
	6	17	14	R_F	0.6058	0.05	0.5086	0.1
				R_M	0.6158	0.02	0.4922	0.1
	7	18	18	R_F	0.4107	0.1	0.3573	0.2
				R_M	0.4148	0.1	0.1715	0.5
	8	19	16	t_R	0.6333	0.01	0.6880	0.01
				lg t_R	0.7051	0.01	0.9453	0.001
GC	9	20	17	t_R	0.7120	0.01	0.8899	0.001
				lg t_R	0.8470	0.001	0.9458	0.001
	10	21	19	I	0.7176	0.001	0.9479	0.001
	11		14	k'	0.6513	0.01	0.1529	0.6
		22		lg k'	0.7426	0.01	0.1864	0.6
HPLC	12		14	k'	0.6800	0.01	0.1749	0.6
				lg k'	0.7372	0.01	0.2297	0.5
	13	23	15	k'	0.7801	0.01	0.5886	0.05
				lg k'	0.8722	0.001	0.7638	0.01

TABLE 3

Evaluation of $^1\chi_N^v$ vs $^1\chi^v$ values

		Number of correlations			
		PC	TLC	GC	HPLC
$^1\chi_N^v$ better than $^1\chi^v$		-	-	5	-
$^1\chi_N^v$ worse than $^1\chi^v$		6	8	-	6

que and its application for liquid chromatography data of barbituric acid derivatives cannot be justified.

The advantage of $^1\chi_N^v$ over $^1\chi^v$ values for the correlations of GC data may reflect some specific features of separation processes, different for gas and liquid phases, although their exact nature and general application of $^1\chi_N^v$ values for other classes of compounds remain to be clarified.

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THERMODYNAMIC APPROACH TO TLC WITH MIXED MOBILE PHASE.
DETERMINATION OF PARAMETERS CHARACTERIZING TLC SYSTEMS

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ABSTRACT

An equation, derived in terms of the thermodynamic for-
mulation of TLC with mixed mobile phase, has been discus-
sed.The parameters of this equation,describing solute-
solvent interactions in the mobile phase and phase-exchan-
ge equilibrium between molecules of both solvents,are
determined by using its linear forms.These linear rela-
tionships have been examined by applying the TLC data for
eight different binary mobile phases.

INTRODUCTION

In 1965 (1) one of the authors of this paper propo-
sed the thermodynamic description of TLC with mixed mobile
phase.This treatment has been developed theoretically (2-
6) and widely examined by using the TLC data (7-10).The
first theoretical considerations (1) were presented for
multicomponent eluents and energetically homogeneous so-
lid surfaces.Although,the fundamental equation resulting
from this treatment has been derived for non-ideal phases,
only its simplified version reiering to ideal mobile and

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surface phases was used for analysing the experimental
TLC data.The extensions of the theoretical treatment (1)
have been made for regular eluents (3) and energetical-
ly heterogeneous adsorbents (5,6).

The fundamental equation derived by Ościk (1) for
TLC with binary mobile phases and energetically homo-
geneous adsorbents may be presented in the following
form (4-6) :

$$R_M = R_{M1}x_1^s + R_{M2}x_2^s + A(x_1^s - x_1^l) \qquad (1)$$

where

$$x_1^s + x_2^s = 1 \quad ; \quad x_1^l + x_2^l = 1 \qquad (2)$$

and R_M , R_{M1} and R_{M2} are the R_M-values of a given so-
lute chromatographed in mobile phases "1+2" , "1" and
"2" ,respectively; x_i^s and x_i^l for i=1,2 are the mole
fractions of the i-th solvent in the surface and mobile
phases,respectively; A is a constant characterizing so-
lute-solvent interactions in the mobile phase (2).
Equation 1 contains the mole fraction x_1^s ,which may be
evaluated from the excess adsorption data or may be cal-
culated by using a theoretical equation (5,6).

In this paper we shall discuss the problems connec-
ted with evaluation of the parameters appearing in equ-
ation 1 .Moreover,we shall propose two linear forms of
equation 1 ,which are very convenient for determining
these parameters.The above linear forms will be examined
by using the experimental TLC data.

THEORETICAL

Firstly, we consider the determination of the ad-
sorption parameters from TLC data and the excess adsorp-
tion isotherm. Then, the mole fraction x_1^s may be evalu-
ated by means of the well-known relationship (6) :

$$x_1^s = n_1^e/n^s + x_1^l \qquad (3)$$

where n_1^e is the adsorption excess of 1-st solvent and
n^s is the total number of moles of the adsorbed compo-
nents in the surface phase.

Equations 1 and 3 give :

$$R_M = D\, x_1^l + R_{M2} + (C/n^s)n_1^e \qquad (4)$$

where

$$D = R_{M1} - R_{M2} \qquad (5)$$

and

$$C = A + D \qquad (6)$$

The constant D is the difference of the R_M-values of a
given solute chromatographed in 1-st and 2-nd solvents,
and it is known from experiment. Thus, equation 4 may be
transformed to the following linear form :

$$R_M - D\, x_1^l = R_{M2} + (C/n^s)n_1^e \qquad (7)$$

The constant C/n^s may be calculated from the slope of
the linear dependence $(R_M - D\, x_1^l)$ vs. n_1^e. Since the value
of n^s may be evaluated from the excess adsorption isot-

herm n_1^e vs. x_1^1 (11), the relationship 7 leads for evalu-
ating the constant C .However,the constant A may be cal-
culated by means of equation 6 .Concluding these consi-
derations, we can draw that equation 4,correlating the
chromatographic and adsorption data,makes possible the
determination of the parameters A and n^s .

The parameter A may be determined directly from
the chromatographic data.For this purpose the mole frac-
tion x_1^s is evaluated by means of the following equation:

$$x_1^s = K_{12}x_1^1/(x_2^1 + K_{12}x_1^1) \qquad (8)$$

where K_{12} is the equilibrium constant defining the
phase-exchange reaction between molecules of 1-st and 2-nd
solvents.Equation 8 is the well-known relationship (4)
defining the mole fraction x_1^s for the adsorption model
involving ideality of both phases, energetic homogeneity
of the adsorbent surface and equality of molecular sizes
of both solvents.

The constant K_{12} may be approximated by :

$$K_{12} = 10^{-\overline{D}} \qquad (9)$$

where $\overline{D}$ is the arithmetical average of the D-values
for different chromatographed substances.Applying the
approximate equation 9 for calculating K_{12} ,equation 1
contains only one unknown parameter; it is A .This para-
meter may be easily calculated from TLC data (7-10).
This method for calculating the parameters K_{12} and A
from TLC data was used up-to-date (7-10).

In this paper, two linear forms of equations 1 and 8 are proposed for determining the parameters K_{12} and A . Combining equations 1 and 8 we have :

$$G(x_1^l) = \frac{x_1^l \ (1 - x_1^l)}{R_M - Dx_1^l - R_{M2}} = [C(K_{12}-1)]^{-1} + C^{-1} \ x_1^l \qquad (10)$$

$$G'(x_1^l/x_2^l) = \frac{x_1^l}{R_M - Dx_1^l - R_{M2}} = \frac{1}{C(K_{12}-1)} + \frac{K_{12}}{C(K_{12}- 1)} \cdot \frac{x_1^l}{x_2^l} \qquad (11)$$

Equations 10 and 11 make possible the calculation of K_{12} and A directly from the experimental dependence R_M vs. x_1^l .In the next section of this paper we shall examine these equations by using TLC data.

RESULTS AND DISCUSSION

For the purpose of examination of equations 10 and 11 the TLC data for the selected chromatographic systems were taken from the literature (8-11).These data were measured for different substances chromatographed in eight different binary eluents on silica gel and aluminium oxide at 297 K.The other details concerning the TLC measurements are given in the references (8-11).

The TLC data for some methyl derivatives of naphthalene and polycyclic hydrocarbons chromatographed in dichloroethylene/carbon tetrachloride and dichloroethylene/n-heptane on silica gel were analysed by means of equations 10 and 11.The numerical values of the parameters K_{12} and

TABLE I

The parameters K_{12} and A for solutes chromatographed in binary eluents on silica gel at 297 K.

Substance	Equation 10		Equation 11	
	K_{12}	A	K_{12}	A
Mobile phase : dichloroethylene/carbon tetrachloride				
pyrene	3.70	-0.17	3.81	-0.16
3,4-benzopyrene	3.62	-0.23	5.61	-0.12
naphthalene	3.99	-0.11	3.33	-0.16
2,3-dimethylnaphthalene	3.91	-0.28	3.87	-0.29
2,3,5-trimethylnaphthalene	3.46	-0.44	2.50	-0.65
Mobile phase : dichloroethylene/n-heptane				
pyrene	1.95	-1.16	1.18	-1.69
anthracene	1.68	-0.60	1.49	-0.93
naphthalene	1.84	-1.33	1.56	-1.98
1-methylnaphthalene	1.63	-1.66	2.00	-1.06
2-methylnaphthalene	1.96	-0.85	1.57	-1.50

A are summarized in Table I. It follows from this table that both linear forms obtained from equations 1 and 8 predict similar values of the adsorption parameters . In Figure 1 the linear dependences 10 and 11 are presented for naphthalene chromatographed in dichloroethylene/carbon tetrachloride on silica gel. The experimental points plotted according
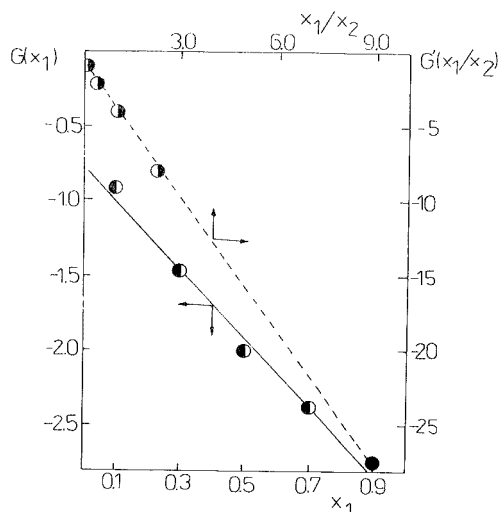
FIGURE 1 . Linear dependences 10 (the solid line) and 11
(the dashed line) for naphthalene chromatographed in
dichloroethylene/carbon tetrachloride on silca gel at 297K.

to the dependence 10 are distributed proportionately on
the straight line $G(x_1^1)$ vs. x_1^1 ,whereas,in the case of the
dependence 11 these points are compressed at lower concen-
trations of x_1^1. This distribution of the experimental
points on the straight lines 10 and 11 may be useful to
select the suitable method for determining the parameters
K_{12} and A .For instance : if we have TLC data ,in which
the R_M -values for high concentrations of x_1^1 were measured
with a small preciseness, the method utilizing the rela-
tionship 10 seems to be better for their interpretation
than the method basing on equation 11 .

TABLE II

The parameters K_{12} and A calculated according to equation 10 for solutes chromatographed in binary eluents.

Substance	Eluent and Adsorbent	K_{12}	A
quinoline	benzene/cyclohexane	12.00	0.82
6-methylquinoline	on aluminium oxide	12.37	0.95
2,6-dimethylquinoline	at 293 K	12.00	0.83
isoquinoline		11.97	0.44
acridine		12.66	0.81
5,6-benzoquinoline		12.67	0.49
naphthalene	benzene/n-heptane	13.63	0.31
diphenyl	on silica gel at	11.98	0.38
chrysene	297 K	13.14	0.48
anthracene		12.08	0.42
acenaphthene		11.55	0.26
pyrene		12.40	0.44
diphenyl	trichloroethylene/	13.78	0.51
naphthalene	n-heptane on silica	13.61	0.36
chrysene	gel at 297 K	14.04	0.62
anthracene		13.78	0.42
pyrene		14.49	0.53
acenaphthene		14.11	0.36

In Table II the parameters K_{12} and A ,evaluated according to equation 10 , are summarized for some quinoline derivatives chromatographed in benzene/cyclohexane on aluminium oxide and for polycyclic hydrocarbons in benzene/n-heptane and trichloroethylene/n-heptane on silica gel.For these chromatographic data equation 10 gives better results than the relationship 11 .Figure 2 shows the linear relationship 11 for some substances chromatographed in benzene/cyclohexane on aluminium oxide.It follows from this figure that equation 10 gives good representation of the data measured for the systems from Table II.

The equilibrium constants K_{12} ,given in Table II, are greater than those summarized in Table I.It means that differences of the elution strengths,characterizing the binary eluents from Table II,are greater than those for the eluents from Table I .Although,the constant K_{12} is evaluated
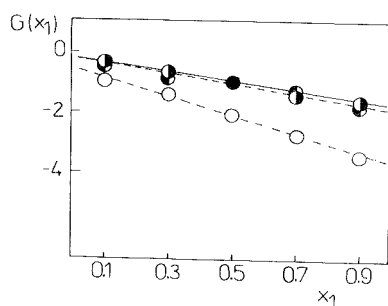
FIGURE 2. The linear dependence 10 plotted for isoquinoline (◐),acridine (◑) and 5,6-benzoquinoline (○) chromatographed in benzene/cyclohexane on aluminium oxide at 293 K.

from the TLC data measured for a given solute,its depen-
dence on the kind of solute is rather small.This result
is physically correct,because according to the theory the
constant K_{12} characterizes the phase-exchange reaction
between molecules of both solvents.Therefore,it is charac-
teristic for a given binary eluent.However,the constant A
characterizes interactions of a given solute with solvents
in the mobile phase,and it depends on the nature of solute
and solvents.

TABLE III

The parameters K_{12} and A calculated according to equation
11 for solutes in binary eluents on silica gel at 297 K.

Substance	Eluent	K_{12}	A
phenol	chloroform/carbon	1.35	−0.57
2,4-dichlorophenol	tetrachloride	1.65	−0.60
2,6-dichlorophenol		1.64	−0.52
3,4-dichlorophenol		1.22	−0.53
pyrene	chloroform/n-heptane	2.68	−0.61
anthracene		2.16	−0.42
chrysene		2.36	−0.96
3,4-dichlorophenol		2.15	−1.43
naphthalene	trichloroethylene/	2.10	−0.06
2,3-dimethylnaphthalene	carbon tetrachlo-	2.70	−0.36
2,4-dichlorophenol	ride	2.63	−0.22
2,6-dichlorophenol		2.06	−0.16

In Table III the parameters K_{12} and A ,evaluated according to equation 11, are summarized for solutes chromatographed in chloroform/carbon tetrachloride, chloroform/ n-heptane and trichloroethylene/carbon tetrachloride on silica gel at 297 K.

For systems summarized in Table III equation 11 gives slightly better results than equation 10. The values of K_{12} for chloroform/carbon tetrachloride, chloroform/n-heptane and trichloroethylene/carbon tetrachloride on silica gel are analogous to those characterizing dichloroethylene/ carbon tetrachloride and dichloroethylene/n-heptane on this same adsorbent (c.f.,Tables I and III).

In Table IV the average values of K_{12} are compared for the systems summarized in Tables I-III.The first column of this table contains the average values of K_{12} calculated according to equation 9 .The second and third columns of this table contain the $\overline{K}_{12}$ -values obtained by averaging the K_{12}-values for different solutes chromatographed in a given eluent/adsorbent system.It follows from Table IV that the differences in the values of $\overline{K}_{12}$,predicted by equations 9 and 10 or 11 ,are greatest for binary eluents containing a solvent of very small elution strength,for instance : n-heptane.Then,the measurements of R_M-values in the pure eluent with a greater preciseness is very difficult. Application of inaccurate R_M-values ,measured for different solutes in a pure solvent, in equation 9 predicts an inaccurate value of $\overline{K}_{12}$ .Therefore,this $\overline{K}_{12}$-value differs from those evaluated by means of equations 10 and 11 .

TABLE IV

The average values $\overline{K}_{12}$ evaluated according to equations 9 , 10 and 11 for the chromatographic systems studied.

System	$\overline{K}_{12}$ (eqn 9)	$\overline{K}_{12}$(eqn 10 and eqn 11)	
dichloroethylene/ CCl_4	2.48	3.74	3.82
dichloroethylene/n-heptane	4.33	1.81	1.56
benzene/cyclohexane	12.60	12.28	-
benzene/n-heptane	6.74	12.46	-
trichloroethylene/n-heptane	5.24	13.97	-
chloroform/CCl_4	1.98	-	1.47
chloroform/n-heptane	5.17	-	2.34
trichloroethylene/CCl_4	1.86	-	2.37

Concluding,we can draw that equations 10 and 11 seem to be more suitable for evaluating the $\overline{K}_{12}$ -value than equation 9, because they utilize the R_M-values measured at different compositions of the binary eluent.

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CHROMATOGRAPHY OF AMINO ACIDS
ON REVERSED PHASE THIN LAYER PLATES

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ABSTRACT

The separation of 19 amino acids was studied on reversed phase thin layers, including C_{18} chemically bonded silica gel, impregnated silica gel, and acetylated cellulose. Normal aqueous-organic solvents, aqueous micellar solutions, and reversed micellar solutions were tested as mobile phases. The only practical system that provided a reversal in migration sequence compared to silica gel and cellulose included a C_{18} layer impregnated with HDBS, and this reversal was apparently due to an ion exchange mechanism.

INTRODUCTION

In an earlier paper (1), separations of 18 amino acids were compared on silica gel, cellulose, and ion exchange thin layers. This paper extends the study of amino acid separations to reversed phase (RP) TLC, including chemically bonded C_{18} silica gel, impregnated silica gel, and acetyl cellulose layers, and conventional aqueous-organic and micellar mobile phases. Comparisons among these systems and with the adsorption, normal-phase partition, and ion exchange systems studied earlier (1) are reported.

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EXPERIMENTAL

Standard solutions of individual amino acids and mixtures
were prepared at a concentration of 500 ng/µl of each compound
in water.   Initial zones were applied to the precoated thin layer
plates (20 x 20 cm) specified below using 1 µl Drummond Microcap
micropipets.

Plates were developed in standard, rectangular glass chambers
that were lined with paper and pre-equilibrated with mobile phase
for at least 10 minutes before inserting the spotted layer.   Plates
were used as received from the manufacturer, with no pretreatment.
In general, development was carried out to a point 15 cm above the
origin line.   The chromatograms were oven dried at 100°C, sprayed
with 0.1% ninhydrin in acetone, and heated again for 5 minutes or
longer to detect the amino acids as colored spots on a white
background.

Layers were impregnated with the surfactants HDBS and CTAB
by attaching a clip to the top of the plate and dipping into
ethanolic solutions contained in a metal Thomas-Mitchell dip tank
(Arthur H. Thomas Co.).   After 5 minutes of soaking, the plate
was removed and placed in a hood until dry.

RESULTS AND DISCUSSION

RP Systems with Conventional Mobile Phases

Table 1 lists amino acids studied and their R_F values in 12
different chromatographic systems.   System A consisted of a What-
man KC_{18} chemically bonded reversed phase plate developed with
n-propanol-water (7:3 v/v).   This mobile phase was chosen as
optimum for amino acid TLC on KC_{18} plates after evaluation of 45
different 2-, 3-, and 4-component solvent mixtures containing
water, acetonitrile, methanol, ethanol, n-propanol, formamide,
THF, DMSO, pyridine, methyl cellosolve, acetic acid, hydrochlo-
ric acid, heptane, hexane, isopropanol, t-butanol, acetone, or
methylene chloride.   Solvent proportions were chosen to provide

TABLE 1

R_F x 100 Data in RP Systems

	Systems											
	A	B	C	D	E	F	G	H	I	J	K	L
Cystine	NV	14	NV	NV	NV	NV	NV	NV	NV	NV	ND	10
Cysteine	NV	ND	18	5.5	12	13	NV	78	NV	62	ND	ND
Arginine	0.60	13	3.6	17	19	16	67	54	79	21	ND	17
Histidine	6.3	12	21	5.5	15	11	43	33	36	32	25	11
Serine	30	26	37	52	35	36	83	78	71	80	59	44
Asparagine	31	ND	31	47	24	21	NV	78	NV	80	ND	ND
Glutamine	44	ND	33	50	33	35	83	78	71	80	59	ND
Threonine	47	30	37	49	47	45	85	78	64	80	56	47
Alanine	50	32	36	51	42	41	85	78	64	80	48	47
Aspartic acid	53	25	41	44	29	29	85	78	79	80	ND	23
Proline	59	24	27	42	55	51	83	78	NV	66	NV	ND
Glutamic acid	61	30	41	50	42	40	83	78	79	80	ND	44
Valine	66	44	42	51	75	71	79	78	50	62	44	54
Methionine	69	47	46	50	75	71	79	78	64	52	44	58
Isoleucine	71	49	47	50	63	60	79	77	50	45	40	54
Tyrosine	72	49	50	59	71	66	79	78	64	61	50	58
Leucine	72	52	49	52	77	72	79	71	64	40	40	58
Phenylalanine	72	52	50	49	77	71	62	60	71	35	40	54
Tryptophan	74	54	54	51	69	70	47	46	71	29	40	50

ND = No data
NV = Not visualized

The systems are described in the text.

similar mobile phase strengths (p') as defined by Snyder (2).
Both nonaqueous and aqueous mixtures were tested, but water was
found to be necessary to move many of the compounds from the
origin and to prevent streaking. Other mobile phases that
provided good resolution with compact spots included pyridine-
water (8:2 v/v), pyridine-H_2O-THF or DMSO (16:4:1 v/v), pyridine-
water-acetic acid (16:4:1 v/v), n-propanol-water-THF (16:4:1 v/v),
t-butanol-H_2O (7:3 v/v), isopropanol-water (7:1 v/v), and
methylene chloride-isopropanol-water (3:6:1 and 4:5:1 v/v). All
of these mobile phases and almost every other one containing water
gave the same sequence of migration as shown for System A in
Table 1, although individual R_F values differed somewhat. Figure 1
illustrates a typical separation carried out on a KC_{18} layer.

The sequence of R_F values on KC_{18} silica gel (System A) was
identical to that found when Whatman K6 silica gel was developed
with the same solvent, n-propanol-water (7:3 v/v) (System B, Table 1).
The absence of order reversal on the reversed phase layer indicated
that perhaps the same separation mechanism was operating both on
conventional silica gel and on chemically bonded silica gel [and,
apparently, on fibrous and micro-crystalline cellulose, which also
gave the same migration sequence (1)].

To determine if the chemically bonded RP layer was unique, the
amino acids were developed with n-propanol-water (7:3 v/v) on an
Analtech reversed phase plate containing a long-chain hydrocarbon
impregnated support layer. As seen in Table 1 (System C), the
migration sequence was again generally unchanged, indicating a
similar separation mechanism. Cystine and cysteine were difficult
to detect at the 500 ng level on both reversed phase layers.

An RPTLC plate designated OPTI-UP Cl2 (3), containing a
layer of silica gel chemically bonded with C_{12} rather than C_{18}
groups, was developed with n-propanol-water (7:3 v/v), and
the same general migration sequence was obtained (System D,
Table 1).

Two bonded reversed phase acetyl cellulose layers (4) were
also developed with the same solvent. These were Baker Flex 10%
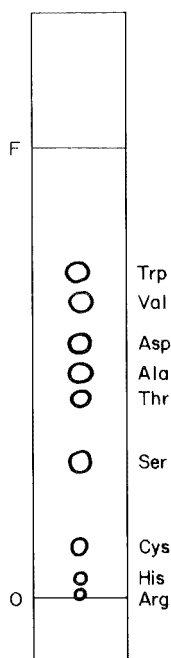
Figure 1.   Separation of a 9-component amino acid mixture
            (500 ng each) on a Whatman KC_{18} thin layer
            developed with n-propanol-water (7:3 v/v) in a
            lined, pre-equilibrated chamber. F = solvent
            front, O = origin.

acetylated cellulose (plastic-backed) (System E) and Analtech
20% acetylated cellulose (glass-backed) (System F). Again, the
same general sequence of migration was observed for these two
systems. Spotting of samples was difficult on the acetylated
cellulose layers. On the 10% acetylated layer, water solutions
remained beaded on the surface of the origin for some time
before finally being absorbed into the layer. Water solutions
could not be spotted on the 20% acetylated layer, so standards
were prepared in 96% ethanol containing 0.1 N HCl.

     Development times for a 15 cm run with n-propanol-water
(7:3 v/v) in Systems A-F were as follows:

(A) KC_{18} 3.5 hours
(B) Silica gel   3.5 hours
(C) Hydrocarbon impregnated silica gel 5.0 hours
(D) C_{12} 7.5 hours
(E) 10% Acetylated cellulose   3.5 hours
(F) 20% Acetylated cellulose   4.0 hours

The ninhydrin reagent produced purple, red, and tan spots on
silica gel and KC_{18} chemically bonded silica gel plates, while
all spots appeared some shade of purple on cellulose (1). The
intensity of colors produced by the ninhydrin reagent for 500 ng
of the amino acids varied in the order, silica gel > KC_{18} >
cellulose. The general diffuseness of developed zones varied in
the opposite order, cellulose > KC_{18} > silica gel. On the 10%
acetylated cellulose layer, all compounds that were detected
appeared as faint tan zones. For detection on 20% acetylated
cellulose, ninhydrin was dissolved in ethanol rather than in
acetone because spraying with the latter solvent caused the
layer to peel. Again, all detected spots appeared as a faint
tan color.

Mechanism of Separation of RP Layers

The similar order of migration of amino acids on silica gel,
cellulose, 10% and 20% acetylated cellulose, C_{18} and C_{12} chemical-
ly bonded silica gel, and hydrocarbon-impregnated silica gel
indicated that a similar mechanism might be operative in all of
these systems. The possibility of a mechanism based on solubility
in the alcohol-water mobile phase was considered, but attempts
to correlate R_F values with amino acid solubility in alcohol-
water mixtures were not successful. For example, Nozaki and Tan-
ford (5) reported that solubilities of tyrosine, leucine, phenyl-
alanine, and tryptophan in 60% ethanol-water were 0.02, 0.62,
1.23, and 1.40 g/100 g, respectively, but R_F values for all of
these compounds on the KC_{18} reversed phase layer were 0.72-0.74.
In addition, histidine has a higher solubility than asparagine

(0.5 compared to 0.2 g/100 g), but the R_F value of histidine was 0.063 and of asparagine 0.31.

Further studies were done to check the possibility of separations due to a precipitation mechanism, which sometimes occurs when binary or higher order mobile phases are used and concurrently fractionated during development. Single amino acids were spotted across different thin layer plates at an angle to the bottom edge. If the distance of migration of the amino acids was totally dependent on precipitation from the mobile phase, the final position of all spots, regardless of the location of spotting, would be a horizontal line parallel to the solvent front ($0°$ angle)(6, 7). If no fractionation of the mobile phase occurs and a classic adsorption or partition mechanism was operative then all spots would move an exact distance dictated by their R_F. Hence, one would expect the developed spots to lie along a slanted line with a theoretical angle that could be predicted from the angle of the origin line and the R_F value of the spotted compound. The results of these experiments are shown in Table 2.

The developed spots did not lie on a line parallel to the solvent front ($0°$), but were usually somewhere between this and the theoretical line. The results on silica gel were similar to those on the reversed phase media, indicating a similar mechanism that was possibly some combination of adsorption, partition, and/or solubility. However, an adsorption mechanism is unexpected on the chemically bonded C_{18} layers because of the 10%–12% carbon loading and the fully covered (capped) silanized silica gel particles (8). The migration sequences in Table 1 and the results in Table 2 indicate that the mechanism, whatever it is, is similar on silica gel and chemically bonded and impregnated RP layers, and that it is not conventional reversed phase partition and solubility-based.

Indeed, one should realize that the definitions of "reversed" phase" and "normal phase" are based on ideal or nearly ideal systems. For example, reversal is easily obtained when separating a nonpolar, hydrophobic substance such as anthracene from a relatively

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TABLE 2

Results of Study of TLC Mechanism on Silica Gel and RP Layers

Layer*	Amino Acid (R_F)	Angle of Spotting (°)	Predicted Angle (°)	Experimental Angle (°)
Silica gel	Serine (0.26)	20	17	13
	Alanine (0.32)	14	11	9
	Tryptophan (0.54)	14	8	5
C_{18}	Serine (0.30)	20	17	12
	Alanine (0.50)	15	10	8
	Leucine (0.72)	21	9	3
	Tryptophan (0.74)	15	6	4
C_{12}	Serine (0.52)	21	8	8
	Leucine (0.52)	21	8	8
Analtech RP	Serine (0.37)	19	14	10
	Leucine (0.49)	19	10	9.5

* All developments with n-propanol-water (7:3 v/v)

polar, hydrophilic substance such as sodium picrate   (9).
Unfortunately, in real life many separations cannot be easily pre-
dicted solely on the basis of polarity. The amino acids are clas-
sic examples of amphiphilic compounds having hydrophobic and hydro-
philic parts, the ability to hydrogen-bond, to act as acids or
bases, etc. Further complicating the picture is the fact that the
structure or nature of the stationary phase (particularly the
reversed phase) is not fully understood. Thus, a reversed
stationary phase may appear hydrophobic and essentially deacti-
vated to a nonpolar solute such as anthracene, but may not to
an amino acid. The results of this work indicate that the
reversed phase separation of amino acids (and probably a host
of other compounds) is a complicated process that can not be
explained by traditional idealized notions of "reversed" or
"normal" phase TLC.

RP Systems with Micellar Mobile Phases

Pseudophase chromatography, in which micellar or cyclo-
dextrin solutions are used as mobile phases in TLC or HPLC, has
been described by Armstrong and coworkers (9-15). Amino acids
were studied on polyamide thin layers using a reversed micellar
mobile phase containing sodium dioctylsulfosuccinate, and R_F
values for 20 compounds were reported (11). In general, the
more polar amino acids had the highest R_F values, while the less
polar ones had lower R_F values. Since this behavior is charac-
teristic of reversed phase TLC, it was decided to evaluate
pseudophase TLC on C_{18} layers (15) for amino acid separations.

Development on a KC_{18} plate with a mobile phase containing
0.015 M SDS (sodium dodecylsulfate) micelle-forming surfactant
produced the R_F values shown in Table 1 (System G). Sodium
chloride (0.5 M) was also included in the mobile phase to
retain the binding of the layer in the totally aqueous solution.
Little resolution of the compounds was obtained, and severe
streaking occurred for the zones with R_F values greater than

0.78, which accounted for the majority of compounds. Many compounds were not detected below a level of 2 µg, which was the amount of each acid spotted to obtain the data in Table 1. The plate required 20 minutes of heating rather than 5 minutes to produce spots of reasonable intensity with ninhydrin, and the spot colors were orange and green in some cases in addition to the usual tan and purple produced with conventional mobile phases on the C_{18} layer. Apparent sequence reversals were noted for some compounds compared to non-micelle developments on KC_{18} layers, but results were difficult to assess because of the trailing mentioned above. For example, alanine had a lower R_F than phenylalanine when developed with propanol-water, but a relatively higher R_F with the SDS mobile phase. A 12 cm development with SDS required 2 hours. Virtually identical results as those just described for SDS were obtained when development was carried out with a mobile phase consisting of saturated aqueous CTAB-H_2O (1:19 v/v) also containing 0.5 M NaCl (System H, Table 1). CTAB (cetyltrimethylammonium bromide) is also a commonly used micelle-forming surfactant.

The reversed micellar surfactant sodium dioctylsulfosuccinate, which was used by Armstrong to develop amino acids on polyamide (11), was tested as a mobile phase [1.3 M DOSS in cyclohexane-water (50:4 v/v)] on C_{18} layers. The data in Table 1 (System I) show that, with a few exceptions, R_F values were all in the range between 0.64 and 0.71. The mobile phase was very viscous, and a 7 cm development required 26 hours. Attempts to lower the viscosity of the mobile phase by dilution resulted in streaked zones. The plate required 20 minutes of heating before 500 ng zones were reasonably intense; a standard array of purple and tan spots was produced, except for a few compounds that were orange. Zones were generally round and compact rather than streaked as with the micelle mobile phases. The resolution obtained was not as good as that reported (11) using this mobile phase with a polyamide layer.

RP Systems with Micellar Impregnation

Lepri et al. (16) have reported the TLC of amino acids on Merck C_2, C_8, and C_{18} plates impregnated with dodecylbenzene-sulfonic acid (HDBS) and developed with 1 M acetic acid + 0.2 M HCl in methanol-water (1:1 v/v). Our results with this system using Whatman C_{18} layers are shown in Table 1 (System J). Plates were impregnated by dipping into a 4% solution of HDBS in 96% ethanol; development for 15 cm required 2.5 hours. Again, many compounds were not detected at levels below 2 µg, and plates required 20 minutes of heating to produce reasonably intense spots even for this amount. Although the acids with higher R_F values were badly streaked, this system gave the most evidence of a reversal of R_F values compared to those on C_{18} silica gel, cellulose, and silica gel with conventional aqueous-organic solvents. Although R_F values were different than those reported by Lepri et al. (16), the general sequence of migration for the common acids studied was similar. However, spots were not nearly as tight as illustrated in the figures of Lepri et al. (16). Development was also carried out with HDBS incorporated into the mobile phase instead of being impregnated into the plate. In this case, the mobile phase appeared to "demix", and all of the spots appeared in a narrow band at or very near the solvent front with virtually no resolution.

When the mobile phase was changed to contain 1 M acetic acid + 0.2 M HCl in methanol-water (7:3 v/v) (Table 1, System K), R_F values on the HDBS impregnated layer were generally lower, and streaking was much less of a problem. A development of 7 cm required 55 minutes. R_F values were in the same order as reported by Lepri et al. (16), but the spots were still not as compact as those pictured by these workers. The migration sequence was again reversed compared to C_{18} silica gel, cellulose, and silica gel.

The migration sequence on the HDBS-impregnated layer closely paralleled that which was found earlier (1) for Fixion

{ = octadecylsilane group

♦ = endcapped silanol

-OH = free hydroxyl group
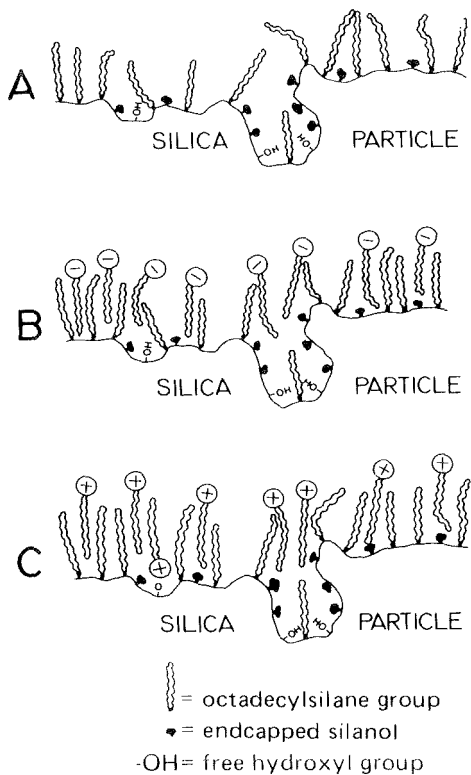
Figure 2.   Schematic diagrams of (A) the surface of a C_{18}
              reversed phase particle, (B) a reversed phase
              particle impregnated with an anionic surfactant,
              and (C) a reversed phase particle impregnated
              with a cationic surfactant.   Stationary phase B
              can behave as a cation exchanger and phase C as
              an anion exchanger.   Surfactant counterions and
              solvent molecules residing in the bonded layers
              are not shown.

strong acid cation exchange layers. This suggests that the
mechanism involved on these layers may be cation exchange
with the impregnated sulfonic acid. This was confirmed by
chromatographing the amino acids on a KC_{18} layer impregnated
with CTAB from a 10% solution of this compound in 96% ethanol.
Development was with methanol-water (9:1 v/v) + 1 M acetic
acid + 0.2 M HCl (System L, Table 1). The results with CTAB
were very different from those with HDBS, with the migration
order for the amino acids being generally, but not uniformly,
reversed. The impregnated surfactant probably caused an anion
exchange mechanism on the CTAB-impregnated layer. Figure 2
illustrates the formation of cation-exchange and anion-exchange
layers by impregnation of KC_{18} with HDBS and CTAB, respectively.

Conclusions

     The only reversed phase system that was found to provide
a reversal of migration sequence compared to silica gel, cel-
lulose, and C_{18} silica gel as well as relatively compact spots
and reasonable development time included a C_{18} layer impregnated
with dodecylbenzenesulfonic acid. The charge of the impregnated
surfactant head groups undoubtedly plays an important role in
this system, and the reversal of migration is very likely due
to an ion exchange mechanism. This system would, therefore, be
useful in helping confirm the presence of an unknown amino acid
in a sample. However, TLC on reversed phase layers has no ad-
vantages compared to adsorption, normal phase partition, or
Fixion ion-exchange TLC (1) for analyses of mixtures of amino
acids. The migration sequences on reversed phase layers are
generally the same as on cellulose and silica gel, and an
adsorption and/or partition mechanism may be operative on these
RP layers. These results indicate that it is sometimes
impossible to predict the relative separation behavior of many
compounds (e.g., amino acids on the basis of chemical notions of
"normal" or "reversed" phase chromatogarphy.

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THIN-LAYER CHROMATOGRAPHIC SEPARATIONS OF AMINO ACIDS
ON STANNIC TUNGSTATE

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ABSTRACT

Thin-layer chromatography of 24 important amino acids in aqueous and mixed solvent systems has been performed on stannic tungstate ion-exchange material. Results of these studies reveal that the stannic tungstate thin-layers offer promising potentialities for the separation of amino acids. The various solvent system which have been studied, acetone-formic acid-water and ethylacetate-formic acid are found to be most useful. It is interesting to note that DL-3,4 dihydroxyphenylalanine (DHPA*) has been selectively separated from a mixture of a number of amino acids in ethylacetate formic acid systems. Moreover, specific separations of DL-methionine has been achieved from a synthetic mixture of other amino acids chromatographed. Aspartic acid and glutamic acid which belong to mono-aminodicarboxylic acid type have been sharply separated from each other in n-butanol- acetic acid and acetone-formic acid-water systems. A large number of other important and difficult ternary and binary separations have also been practically achieved.

INTRODUCTION

Papers impregnated with inorganic ion-exchange material have been widely used for the separation of

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metal ions (1-5). Very limited studies have been made
for the separation of organic compounds on such type of
ion-exchange papers. However, titanium arsenate and zir-
conium phosphate papers have been used for the separation
of few amino acids (6-9) and alkaloids (10). Thin-layers
of pure inorganic ion-exchange material such as stannic
antimonate (11) and stannic arsenate (12-13) have been
found useful for the separation of metal ions. The use
of such layers without any binder makes it easier to have
a clear interpretation of the mechanism of the separation.
A survey of literature revealed that almost no work has
been reported for the separation of organic compounds on
thin-layers prepared from inorganic ion-exchange mate-
rials. It is therefore, worthwhile to explore the impor-
tance of the layers of inorganic ion exchangers for the
systematic separation of organic compounds. Stannic tung-
state thin-layers have been tried because this material
has been found to be quite stable in acids, bases and
other organic solvents and possess excellent separation
potentialities (14). Amino acids especially, have been
chosen for the chromatographic studies because of their
biomedical, physiological and pharmaceutical importance.

EXPERIMENTAL

Reagents and Chemicals

        Stannic chloride pentahydrate (Poland), sodium
tungstate (Reidel, Germany), n-butanol, dioxane, acetic
acid, formic acid (B.D.H., England), pyridine, acetone
(E. Merck Darmstadt) were used. All other chemicals and
solvents used were of analytical grade from B.D.H.,
England.

Apparatus

A thin-layer chromatography (TLC) Desaga (Germany) applicator was used to prepare thin-layers on 20 x 20 cm glass plates. Large mouth (Toshniwal) chamber were used for the development.

Micro-Capillary Pipettes were used for the spotting purposes.

Detector

Ninhydrin solution (0.2%) in n-butanol saturated with water was used for the detection of amino acids on TLC plates.

Preparation of Ion-Exchange Material and Thin-Layer Plates

Stannic tungstate was prepared by mixing 0.05M solutions of stannic chloride and sodium tungstate in the volume ratio of 1:1 at pH = 1 and digesting the resulting precipitate at room temperature for 24 hours. The precipitate was filtered under suction and completely dried in an oven at 40 ± 4°C. The material so obtained was cracked in DMW (demineralized water) and then placed in IMHNO$_3$ for 24 hours to convert it to the H$^+$ form. The material was washed with DMW to remove excess acid and finally dried at 40°C. Ten grammes of stannic tungstate granules thus obtained were mixed in about 5 ml of distilled water and slurry was made by grinding the mixture vigorously in a glass mortar for a long time. This step proves to be very much important for the complete adhesion. The grinding of the granules must be complete and slurry should be in the form of a fine paste without any solid particles being left. The slurry was then spread over the clean glass plates with

the help of an applicator to give 0.10 mm thick layers.
The plates were ready for use after drying at room
temperature.

Procedure

        Approximately 0.04 ml of test solutions of amino
acids were applied with the help of glass capillary on
the plates. After drying the spots the plates were
developed in various solvent systems and solvents were
allowed to ascend upto 12 cm in all the cases from the
point of application.

RESULTS AND DISCUSSION

        Results of these studies reveal that stannic
tungstate thin-layers offer promising potentialities
for the systematic separation of amino acids. The
major advantage of using stannic tungstate layer is that
'ion-exchange' and 'adsorption' take place simultaneous-
ly. As a result compact and well defined spots are
obtained. It is clear from tables (1-7) that a large
number of binary and ternary separation of amino acids
are possible on thin-layers of stannic tungstate. The
various solvent systems which have been studied,
acetone-formic acid-water; η-butanol- acetic acid- water
and ethyl acetate - formic acid systems are found to be
most useful for the separation of amino acids. It is
very interesting and worthwhile to note that DL-3,4
dihydroxyphenylalanine (DHPA*) has been selectively
separated from the mixture of a number of amino acids in
ethylacetate-formic acid. An striking feature emerges
when pure dioxane; dioxane-nitric acid are used as deve-
lopers. In this most of the amino acids remain at the
point of application except DL-methionine which behaves

TABLE - I

R_F Values of Amino Acids on Stannic Tungstate Layers.

COMPOUND	SOLVENT SYSTEM	
	A (4.30 hr)	B (5.15 hr)
DL-Alanine	0.53	0.87
DL-2 Amino n-butyric acid	0.74	0.70
L-Arginine Monohydrochloride	0.78	0.38
DL-Aspartic acid	0.86	0.85
L-Cystine HCl	0.54	0.73
L-Cystine	0.48	-
DL-3,4 Dihydroxyphenylalanine	-	0.20
L-Glutamic acid	0.51	0.56
Glycine	0.55	0.69
L-Histidine HCl	0.95	0.67
L-Hydroxyproline	0.87	0.85
L-Leucine	0.52	0.40
DL-Isoleucine	0.63	0.84
DL-Nor Leucine	0.85	0.62
L-Lysine Mono HCl	0.48	0.85
DL-Methionine	0.71	0.52
L-Ornithine HCl	0.39	0.23
DL-Phenylalanine	-	-
L-Proline	0.32	0.45
DL-Serine	0.82	0.52
DL-Threonine	0.88	0.84
DL-Tryptophan	0.50	-
L-Tyrosine	0.53	0.25
DL-Valine	0.63	0.62

A = n-butanol saturated with water: acetic acid (3:1)
    system;
B = acetone: formic acid: water (2:2:1).

TABLE - II

R_F values of Amino Acids on Stannic Tungstate Layers.

COMPOUND	SOLVENT SYSTEM	
	C (5.50 hr)	D (5.00 hr)
DL- Alanine	0.86	-
DL-2 Amino n-butyric acid	0.72	0.66
L-Arginine Monohydrochloride	0.48	0.50
DL-Aspartic acid	0.55	0.85
L-Cystine HCl	-	0.72
L-Cystine	0.75	0.76
DL-3,4 Dihydroxyphenylalanine	0.39	0.33
L-Glutamic acid	0.85	0.98
Glycine	0.52	0.52
L-Histidine HCl	-	0.60
L-Hydroxyproline	0.68	0.44
L-Leucine	0.86	0.77
DL-Isoleucine	0.53	0.45
DL-Nor Leucine	-	0.56
L-Lysine Mono HCl	0.92	0.85
DL-Methionine	0.57	0.24
L-Ornithine HCl	-	0.53
DL-Phenylalanine	0.62	0.77
L-Proline	0.95	0.59
DL-Serine	0.48	0.85
DL-Threonine	-	0.60
DL-Tryptophan	0.80	0.45
L-Tyrosine	0.45	-
DL-Valine	0.79	0.72

C = n butanol: acetic acid: water (5:4:1);

D = ethylacetate: formic acid (6:4).

TABLE - III

R$_F$ Values of Amino Acids on Stannic Tungstate Layers.

COMPOUND	SOLVENT SYSTEM	
	E (6.45 hr)	F (6.30 hr)
DL-Alanine	0.0	0.0
DL- 2 Amino n-butyric acid	0.0	0.0
L-Arginine Monohydrochloride	0.0	0.0
DL-Aspartic acid	0.0	0.0
L-Cystine HCl	0.0	0.0
L-Cystine	0.10	0.0
DL-3,4 Dihydroxyphenylalanine	0.0	-
L-Glutamic acid	0.0	0.22
Glycine	0.0	0.0
L-Histidine HCl	0.0	0.13
L-Hydroxyproline	0.23	0.0
L-Leucine	0.0	0.0
DL-Isoleucine	0.0	0.0
DL-Nor Leucine	0.0	0.35
L-Lysine Mono HCl	0.0	0.0
DL-Methionine	0.40	0.15
L-Ornithine HCl	0.0	0.0
DL-Phenylalanine	0.0	0.0
L-Proline	0.0	0.0
DL-Serine	0.0	0.0
DL-Threonine	0.0	0.46
DL-Tryptophan	0.0	-
L-Tyrisine	0.18	0.0
DL-Valine	-	0.0

E = Dioxane;   F = Dioxane + 0.1M HNO$_3$

TABLE - IV

R_f Values of Amino Acids on Stannic Tungstate Layers

COMPOUND	SOLVENT SYSTEM	
	G (5.15 hr)	H (5.45 hr)
DL-Alanine	0.61	0.62
DL-2 Amino n-butyric acid	0.87	0.85
L-Arginine Monohydrochloride	0.80	0.53
DL-Aspartic acid	0.50	0.67
L-Cystine HCl	0.72	0.87
L-Cystine	-	0.67
DL-3,4 Dihydroxyphenylalanine	0.96	0.95
L-Glutamic acid	0.63	0.67
Glycine	0.86	0.84
L-Histidine HCl	0.74	0.20
L-Hydroxyproline	0.91	-
L-Leucine	0.45	0.59
DL-Isoleucine	0.68	0.86
DL-Nor Leucine	0.77	0.42
L-Lysine Mono HCl	0.33	0.76
DL-Methionine	0.82	0.78
L-Ornithine HCl	0.47	0.35
DL-Phenylalanine	0.86	0.61
L-Proline	0.74	0.78
DL-Serine	0.38	0.86
DL-Threonine	0.78	0.56
DL-Tryptophan	0.55	0.71
L-Tyrosine	-	-
DL-Valine	0.89	0.91

G = Acetic acid + formic acid + water (4:3:2)

H = Ethylalcohol + ethylacetate + n-butanol (3:4:2)

TABLE - V

R_F Values of Amino Acids on Stannic Tungstate Layers

COMPOUND	SOLVENT SYSTEM	
	I (5.45 hr)	J (5.00 hr)
DL-Alanine	0.70	0.62
DL-2 Amino n-butyric acid	0.84	0.85
L-Arginine Mono HCl	0.41	0.69
DL-Aspartic acid	0.78	0.41
L-Cystine HCl	0.61	0.80
L-Cystine	0.84	0.85
DL-3,4 Dihydroxyphenylalanine	0.95	0.92
L-Glutamic acid	0.77	0.29
Glycine	0.86	0.67
L-Histidine HCl	0.61	0.65
L-Hydroxyproline	—	0.86
L-Leucine	0.86	0.49
DL-Isoleucine	0.24	0.76
DL-Nor Leucine	0.56	0.58
L-Lysine Mono HCl	0.85	0.84
DL-Methionine	0.92	—
L-Ornithine HCl	0.85	0.94
DL-Phenylalanine	0.47	0.69
L-Proline	0.60	0.20
DL-Serine	0.59	0.53
DL-Threonine	—	0.72
DL-Tryptophan	0.85	0.45
L-Tyrosine	0.45	0.76
DL-Valine	0.77	0.86

I = Ethylacetate + Pyridine + Water (2:1:2);
J = acetone + ethanol + water (6:1:3).

TABLE – VI

Separations Actually Achieved On Stannic Tungstate Thin Layers in Important Solvent Systems

Solvent Systems: Acetone : Formic acid : Water

"Ternary Separations"   In Mixture of Amino Acids

(I) DL-Alanine (10.4-11.3) — DL-3,4 DHPA*(3.0-3.6) — L-Leucine (5.0-5.2)
(II) DL-Alanine (10.7-11.2) — DL-3,4 DHPA* (2.8-3.1) — L-Proline (5.8-6.1)
(III) DL-Alanine (10.5-10.8) — DL-3,4 DHPA*(1.0-1.8) — DL-Serine (5.4-6.3)
(IV) DL-Alanine (10.7-11.4) — DL-3,4 DHPA*(2.4-2.9) — DL-Methionine (8.8-10.2)
(V) DL-Alanine (7.0-7.4) — DL-3,4 DHPA*(3.0-3.2) — L-Cystine HCl (7.8-8.1)
(VI) DL-Alanine (10.5-10.7) — DL-3,4 DHPA*(0.0-0.0) — L-Glutamic acid (7.0-7.2)
(VII) L-Tyrosine (0.0-0.0) — DL-Alanine (8.6-9.3) — DL-Methionine (6.6-7.1)
(VIII) DL-Aspartic(10.5-10.7) — DL-3,4 DHPA*(0.0-.00) — L-Glutamic acid (7.0-7.5)
acid

"Binary Separations"

(I) L-Tyrosine (0.0-0.0) — 2 Amino n-Butyric acid (7.8-8.2)
(II) L-Tyrosine (1.2-1.4) — DL-Valine (7.9-8.3)
(III) L-Tyrosine (1.7-2.0) — DL-Aspartic acid (9.5-9.8)
(IV) L-Tyrosine (1.8-2.3) — L-Hydroxy Proline (9.3-10.0)
(V) DL-3,4 DHPA* (0.0-0.0) — L-Lysine Mono HCl (10.9-11.2)
(VI) DL-3,4 DHPA* (0.0-0.0) — DL-2 Amino n-Butyric acid (8.0-8.3)
(VII) L-Ornithine HCl (1.5-1.9) — DL-2 Amino n-Butyric acid (9.4-9.8)
(VIII) L-Ornithine HCl (1.0-1.2) — DL-Valine (8.8-9.0)
(IX) L-Ornithine HCl (0.00-0.00) — DL-Threonine (10.2-10.4)
(X) DL-3,4 DHPA* (0.0-0.0) — DL-Threonine (9.4-9.8)
(XI) DL-3,4 DHPA* (0.0-0.0) — L-Lysine Mono HCl (8.3-8.7)
(XII) L-Arginine Mono HCl (5.0-5.3) — DL-Alanine (10.3-10.7)
(XIII) L-Arginine Mono HCl (4.8-5.1) — DL-2 Amino n-Butyric acid (9.3-10.7)
(XIV) L-Arginine Mono HCl (4.3-5.2) — L-Lysine Mono HCl (9.3-10.1)
(XV) L-Arginine Mono HCl (3.8-4.0) — DL-Threonine (9.5-9.9)

DHPA* = dihydroxyphenylalanine

TABLE - VII

List of Important and Difficult Ternary and Binary Separations Achieved on Stannic Tungstate Layers

Solvent System: Butanol : Acetic acid : Water

"Ternary Separations"

(I)   L-Proline (10.6-10.8) — DL-3,4 DHPA* (3.7-4.0) — L-Hydroxy Proline (8.2-8.5)
(II)   L-Proline (10.2-10.6) — DL-3,4 DHPA* (2.8-3.2) — DL-Aspartic acid (5.8-6.2)
(III) Glycine (7.5-7.9) — DL-3,4 DHPA* (3.0-3.3) — L-Proline (10.8-11.0)
(IV)   L-Proline (9.7-10.1) — DL-3,4 DHPA* (0.0-0.0) — DL-Isoleucine (6.5-6.7)
(V)   L-Proline (10.4-10.6) — DL-3,4 DHPA* (0.0-0.0) — DL-Methionine (5.6-6.0)
(VI)   L-Proline (10.6-11.1) — DL-3,4 DHPA* (0.0-0.0) — DL-Phenyl alanine (5.7-6.3)

"Binary Separations"

(I)   L-Proline (11.0-11.2) — L-Tyrosine (7.0-7.6)
(II)   L-Proline (10.8-11.0) — DL-Serine (6.0-6.2)
(III)   L-Proline (11.2-11.5) — DL-Isoleucine (7.0-7.3)
(IV)   L-Proline (10.0-10.3) — DL-3,4 DHPA* (0.00-0.00)
(V)   L-Proline (9.8-10.2) — L-Arginine Mono HCl (5.8-6.0)
(VI)   L-Proline (11.5-11.7) — DL-Aspartic acid (6.8-7.3)
(VII)   L-Lysine Mono HCl (10.8-11.1) — L-Tyrosine (6.0-6.6)
(VIII) L-Lysine Mono HCl (10.8-11.3) — DL-Serine (6.3-6.9)
(IX)   L-Lysine Mono HCl (10.8-11.2) — L-Arginine Mono HCl (6.0-6.4)
(X)   L-Lysine Mono HCl (9.5-9.8) — DL-Aspartic acid (7.8-8.0)
(XI)   L-Leucine (9.8-10.0) — DL-Serine (6.0-6.2)
(XII)   L-Lucine (10.0-10.3) — L-Tyrosine (5.4-5.6)
(XIII) DL-2 Amino n-Butyric acid (7.8-8.1) — DL-Serine (5.8-6.0)
(XIV)   DL-2 Amino n-Butyric acid (8.0-8.3) — DL-Tyrosine (5.6-5.8)
(XV)   DL-2 Amino n-Butyric acid (8.6-9.0) — DL-DHPA* (0.0-0.0)
(XVI)   DL-2 Amino n-Butyric acid (9.5-9.7) — L-Arginine Mono HCl (5.8-6.3)

DHPA* = dihydroxyphenylalanine

in a peculiar way. As a result of this specific separa-
tion of DL-methionine has been selectively achieved
from a synthetic mixture of a number of amino acids.
Aspartic acid, glutamic acid both belonging to monoamino-
dicarboxylic acid type have been sharply separated from
each other in n-butanol: acetic acid (3:1) and acetone:
formic acid: water (2:2:1) systems. Furthermore, certain
separations of important and difficult pairs of mono-
aminomonocarboxylic acid types such as glycine-leucine;
leucine-DL-serine; alanine-serine; alanine-leucine; DL-
serine- DL-isoleucine; leucine-threonine; DL-serine-DL-
valine; leucine-isoleucine; DL-Norleucine and also valine
from DL-threonine have been conveniently achieved utiliz-
ing stannic tungstate layers. Distinct separations of
heterocyclic amino acids from one another have been
obtained. Thus separation of tryptophan from hestidine
and hydroxyproline have been realized in many systems
i.e. n-butanol- acetic acid-water; acetic acid-formic
acid-water; n-butanol saturated with water-acetic acid
and also n-butanol-ethylalcohol-ethylacetate solvent
systems. It is interesting to observe in the case of
heterocyclic amino acids. The R_F value increases with
the increase in molecular weight of the amino acids. In
ethylacetate-ethylalcohol-n-butanol and ethylacetate-
pyridine-water systems. R_F value decreases in the
following sequence -

         Tryptophan   Histidine   Hydroxyproline
while the order of R_F values are reversed in acetone-
ethylalcohol-water system i.e. with the increase in
molecular weight of amino acids R_F value decreases.
Thus chromatography of amino acids on thin-layers of
stannic tungstate offers a large number of important
and difficult separations of amino acids.

ACKNOWLEDGEMENTS

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SOME ADVANCES IN APPLICATION OF TLC
TO DIAGNOSTIC TOXICOLOGY

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Introduction

During this last year we have progressed in our abilities to make diagnostic toxicological analyses by TLC. Two which I will present are the analysis for Lincomycin and Slaframine.[1] The former, a drug which is very bad for horses, and the latter, a naturally occurring mycotoxin which has had a very large effect on animals this last year.[2] In addition to cases involving closer pastures, this last year alfalfa pasture and hay and grasses have been involved.

The second part of my discussion will have reference to TLC as a reactive system to do chemistry. Our speakers have discussed substrate (absorbent) and optimizing solvents for separation and Dr. Touchstone has discussed application of the advances to actual situations.

Reagents & Apparatus

Finnigan GC/MS 4000; TLC plates, .25mm; silica gel normal phase (E. Merck, Brinkmann, Chicago, IL) (Whatman, Clifton, NJ) and C^{18} reverse phase (Whatman, Clifton, NJ); solvents: ethanol, acetic acid, chloroform, methanol, toluene, ethyl acetate all Nanograde[R] or equivalent (Mallinckrodt, St. Louis); Millipore[R] Q water, (Millipore Corporation, Cambridge, MA); vanillin, ACS reagent, (Fisher, Pittsburgh, PA); sulfuric acid and ammonium hydroxide - ACS reagent (Fisher); TLC equipment, tanks (Brinkmann Instruments, Chicago, IL); short base TLC PMD developing system (Regis Chemical, Chicago, IL); micropets (Fisher Scientific); UV light (Ultra Violet Products, Inc., San Grabriel, CA); 800 scanner (Kontes, Vineland, NJ); quantitative and volumetric glassware (Kontes, Vineland, NJ).

Experimental

A.   Lincomycin

1.   standard was obtained from U.S. Pharmocapedia, Rockbridge, MD. Samples of feed were spiked with

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microgram quantities, extracted with methanol, defatted
with pet ether after 10% water was added. (50g/100 ml
methanol).

2. the pH is adjusted to 2 with 2NHCl and the volume is made
200 ml with water and the aqueous layer is extracted 2
times with 100 ml CH_2Cl_2.

3. The pH is adjusted to about 12 with 1 ml 60% KOH (NaOH)
and the aqueous layer is extracted 2 times with 100 ml
CH_2Cl_2.

4. The CH_2Cl_2 layer is concentrated to dryness.

5. The extract is redissolved in 90/10 $CHCl_3$/methanol an
aliquot equal to 0.1, 0.5, 1 gram of sample is spotted on
TLC.   Normal feed samples contain 800 ppm as a feed
additive.

6. The plate is developed in 80/20 $CHCl_3$/methanol. Multiple
developments or the short base PMD developing chamber may
be used to separate bands in difficult samples.

7. After developing the plate is sprayed with 5% vanillin in
methanol and 20% sulfuric acid in methanol (wt%). The
plate is gently heated to observe Lincomycin (yellow
spots).

8. To quantitate samples the plates are heated until red
brown spots develop which are stable and amenable to TLC
direct analysis.

B. Slaframine

1. Slaframine standards may be obtained from H. P. Broquist,
Vanderbilt University. The amine is derivatized[1] with
pyridine and acetic anhydride if the underivatized
standard is obtained. (If supplied as the picrate
acidification and partition into CH_2Cl_2 is necessary
before conversion to the acetylated product).

2. 50 grams of sample are extracted as in the Lincomycin
procedure. Spikes are done as above also.

3. Thin layer analysis is done as above in the Lincomycin
procedure. Except visualization is done by Iodoplatinate
or iodine fumes.

4. Sulfuric acid methanol has been used to visualize the
spots, but so far quantitation is done by GLC.

Results

Canary yellow spots are produced on TLC by the vanillin
sulfuric acid spray and gentle heating. The spots turn brown
with continued heating and become stable for densitometeric
quantitative analysis. Tenths of micrograms are visible by this
procedure and TLC.

The Rfs of the Lincomycin is 0.5 in 80/20 chloroform
methanol and .8 in 65/30/1 reverse phase solvent[1] (for C^{18}
Whatman plates). The Rf in 60/40/1 (methanol/water/NH$_4$OH) is
0.5. Microgram quantities may be detected. The Rf of acetylated
Slaframine is .5 in 6/2/1 - toluene/ethyl acetate/acetone.

Microgram quantities of Slaframine may also be visualized by
iodine and 10 nanograms by fluorescence quenching.

Discussion & Conclusions

Levels of detection for mycotoxins in tissues have been
greatly improved by the use of reverse phase and normal phase as
reaction surfaces to allow resolution of interfering bands from
the bands of interest. Clean up of samples with preliminary
steps using the combination of cleanup steps has been successful
in allowing analysis of tissues for poisoning levels of
fluorescent mycotoxins. These specific techniques will be
featured in later papers in complete form. However, the real
challenge of TLC has been in analyzing compounds w/o fluorescence
or "real handles". (Figure 1) Lincomycin has no uv absorption or
fluorescence and is very polar. Reverse phase systems require a
basic solvent media. Partition using the very polar basic
functional group allows cleanup. Visualization by vanillin
allows qualitative and quantitative analyses by thin layer
chromatography.

Analysis for Slaframine has a similar difficulty. It is
even more easily decomposed. (Figure 2) Acetylation of this
compound greatly improves its stability and allows detection by
TLC and iodometry. Mass spectrometeric analysis may be used for
confirmation. So far mass spectrometric confirmation of
Lincomycin is still being developed. A desorption technique may
be required.

It is important to recall when applying TLC methods and
optimizing solvents that in the real world samples probably won't
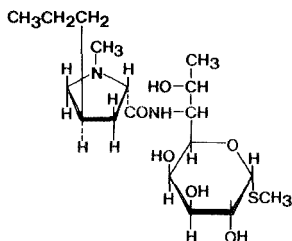
Lincomycin mol. wt. 406.56

$C_{18}H_{34}N_2O_6S$

Figure 1. Lincomycin structure

C$_{10}$H$_{10}$N$_2$O$_2$
mol. wt. 198

mol. wt. 240
Acetyl Slaframine
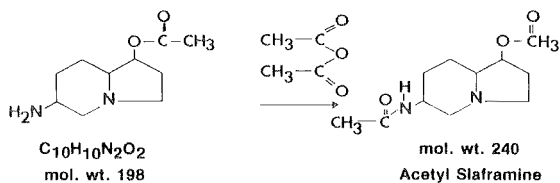
Figure 2. Slaframine derivatization

perform reversibly and predictions from pure standards and
solvent systems are just that - predictions.

Multiple developments in a linear or a two dimensional
fashion allow bands to separate which otherwise might be
inhibited by "junk" which co-partitions and forms a matrix for
chromatography. The short bed PMD system of Regis Chemical
Company also allows experimental separations of difficult to
observe bands by optimizing solvent to absorbent and junk to
analyte. Once qualitative knowledge of the analyte is available,
methods using multiple linear developments and/or optimization of
a cleanup can be used to make quantitative analysis possible.

Conclusions

Thin layer chromatography can be used as an experimental
reaction system to detect and optimize the analysis of any
analyte. The advent of predictable (high performance) normal and
reverse phase and fluorescent quenching has made such techniques
widely used. The use of cleanup procedures, functional groups,
and chemical reaction will make thin layer analysis of any
analyte possible.

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A SIMPLE MICROASSAY FOR GLUTAMIC ACID DECARBOXYLASE

BY ION EXCHANGE THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

A simple radioisotopic method is described for the determination of glutamic acid decarboxylase in biological materials. It is based on the direct determination of γ-amino-butyric acid produced by the incubation of glutamic acid decarboxylase with the radiolabelled substrate. Separation is achieved by thin-layer chromatography on Dowex 1-acetate coated plastic strips. The assay is linear for GABA production with both time and enzyme concentration. The method was also used to determine the presence of glutamic acid decarboxylase in bovine subretinal intercellular fluid and retina.

INTRODUCTION

Glutamic acid decarboxylase (GAD) catalyzes the decarboxylation of

L-glutamic acid to form γ-amino butyric acid (GABA). GABA has been

recognized as a major inhibitory neurotransmitter in the mammalian

central nervous system (1,2). GAD is also implicated in pathological

states such as Parkinson's disease, Huntington's disease and

schizophrenia (3-6).

In view of the significance of GAD in the central nervous system,

there has been a great deal of interest in developing new assay methods

based on the measurement of either of the products formed. However,

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none of the methods developed so far are easy or convenient for the
determination of GAD in biological materials. The CO_2 evolution
methods (7,8) are non specific, time consuming and not practical with
large number of samples obtained during enzyme purification. On the
other hand, the ion exchange column methods, based on GABA determina-
tion, (9-11) are specific and more sensitive than CO_2 evolution
methods, but in general are lengthy, tedious and subject to
interferences such as changes in pH or ionic strength. GAD
determination based on the measurement of NADH formation after coupling
GABA to GABA-transaminase and succinate semi-aldehyde dehydrogenase
(12), is limited by the availability of enzymes in adequate purity and
the effect of activators or inhibitors present in crude extracts on the
coupling reaction. Recently, HPLC has also been used to determine GAD
activity in biological materials (13,14).

   In this paper, we report a simple, sensitive and specific
microassay for determining GAD activity in crude brain and retinal
extracts. The method is based on the separation of GABA from glutamic
acid by thin-layer chromatography (TLC). We have also used this
technique to look for the presence of GAD in subretinal intercellular
fluid which separates neural retina from retinal pigment epithelium.

                              EXPERIMENTAL

Materials

   L-[3,4-³H]-glutamic acid, (40 Ci/mmole) was obtained from New
England Nuclear (Boston, MA, USA). γ-Amino-[2,3-³H]-butyric acid (60
Ci/mmole) was obtained from Amersham (Arlington Heights, IL, USA). ATP,
unlabelled glutamic acid, GABA, α-ketoglutaric acid and succinic acid

were obtained from Sigma (St. Louis, MO, USA). Ethyl acetate was

obtained from Eastman Kodak, (Rochester, N.Y.). Plastic sheets

precoated with Ionex SB-Ac were obtained from Brinkmann (Westbury, NY,

USA). Prior to use, 1.2 x 9.0 cm strips were cut and equilibrated with

0.05% acetic acid for 30 min as recommended by Devenyi (15).

Preparation of Crude Homogenate

Bovine brain and retina were homogenized with 20mM potassium

phosphate, pH 6.8, 10mM mercaptoethanol and 1mM EDTA. The homogenates

were sonicated for one min and centrifuged at 26,000 x g for 60 min at

$4^{o}C$. The supernatant obtained in each case was used as the enzyme

source. Bovine subretinal intercellular fluid was provided by Dr. Y.

Lai (University of St. Louis) and was used without any further

treatment.

Enzyme assay and thin-layer chromatography

The incubation mixture (total volume 50 μl) contained 50 mM

potassium phosphate, pH 6.8, 1mM EDTA, 0.5mM pyridoxal phosphate, 5mM

$[^{3}H]$-glutamic acid (800-2,000 cpm/nmole). The reaction was initiated

by the addition of enzyme, incubated at $37^{o}C$, and stopped by rapid

cooling in an ice bath. Immediately after cooling, 5 μl of the incuba-

tion mixture was spotted as a streak one cm from the bottom of the strip

and dried. Ascending TLC was carried out using ethyl acetate/water

(8:92). After the solvent had migrated about seven cm from the origin,

the strips were removed and dried. Reference strips were also run with

unlabelled glutamic acid and GABA under identical conditions. Spots

corresponding to glutamic acid and GABA were cut and placed in scintil-

lation vials. In cases where reference strips were not run, the strips

were cut into one cm pieces and placed in separate vials.

Amino acids were eluted from the resin by adding 3.0 ml of scintillation fluid (ACS) and 0.5 ml of 20% formic acid as recommended by Himoe and Rinne (16). Recovery of radiolabelled glutamic acid and GABA from the strip using this method was 81 ± 2% (mean ± S.D., n=5), and 83 ± 3% (mean ± S.D., n=5), respectively.

Other methods

Protein was determined by the dye binding method using bovine serum albumin as a standard (17). The α-keto acids were detected by UV absorbance, and the pyridine – acetic anhydride staining method (18). GAD determination by reversed-phase HPLC was performed as described (13).

RESULTS

Separation of Glutamic Acid and GABA

Figure 1 shows the separation of unlabelled glutamic acid from GABA with ethyl acetate/water (8:92) obtained in 30 min. The contents of the assay mixture do not significantly affect the separation of glutamic acid and GABA. In three different runs, the R_F values for glutamic acid and GABA using this solvent system were found to be 0.06 ± 0.01 and 0.95 ± 0.02, respectively. The inclusion of 0.05% acetic acid in the solvent mixture does not affect the mobility of GABA, while the R_F value of glutamic acid increased to 0.09. On the other hand, inclusion of 1% pyridine in the solvent system did not change the R_F values of either glutamic acid or GABA. Also, the presence of 1% trichloroacetic acid in the assay mixture does not interefere with the migration of glutamic acid and GABA. Thus, the system appears to be insensitive to pH.

FIGURE 1: Separation of glutamic acid and GABA. A 2 µl aliquot
containing 4 µg of glutamic acid and GABA in 50 mM phosphate buffer, pH
6.8 was spotted. After development, the strip was dried, sprayed with
0.2% ninhydrin in butanol/acetic acid (95:5) and spots visualized after
brief exposure at 70°C.
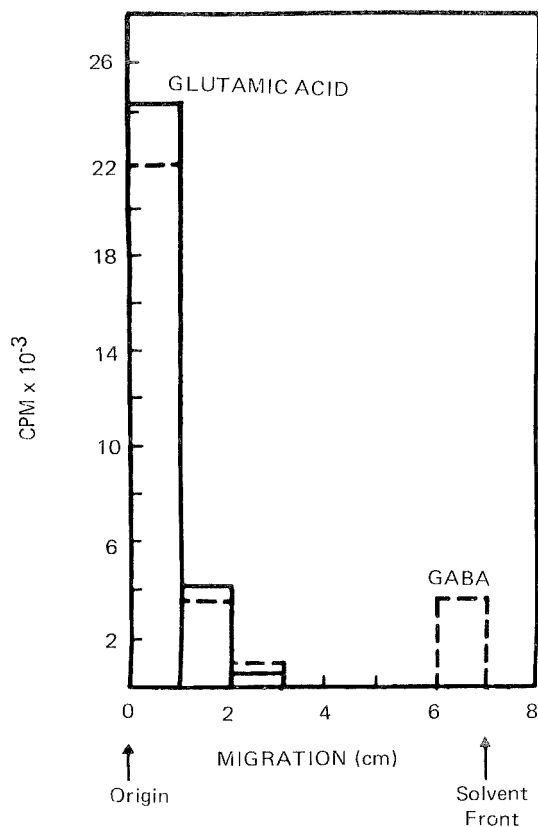
FIGURE 2:   Separation of [^{3}H] GABA (----) from [^{3}H]-glutamic
acid.   After development, the strip was dried, cut into one cm pieces
and radioactivity counted as described in the text.   The assay mixture
was incubated with 180 µg of crude enzyme preparation at 37°C for 60
min.   The control (——) contained the assay mixture mixed with enzyme at
37°C followed immediately by cooling to 0°C.   The composition of
the assay mixture was the same as described in Materials and Methods.

Figure 2 shows the separation of GABA formed from radiolabelled glutamic acid by the GAD present in crude brain homogenate. Glutamic acid remained near the origin, and GABA formed by the enzymatic reaction moved near the solvent front resulting in a complete separation of the two components. No significant amount of GABA was formed during the time when the enzyme assay mixture was immediately cooled and spotted. In order to insure the elimination of non enzymatic formation of GABA, the radiolabelled glutamic acid was incubated at 37°C for 2 h with the assay mixture except that the addition of enzyme was omitted. As shown in Figure 3, the chromatogram revealed only the presence of glutamic acid and there was no formation of GABA when the enzyme was eliminated from the assay mixture.

Relationship of GABA Synthesis with Time and Protein Concentration

A crude enzyme preparation of bovine brain was used to test the linearity of the assay with time and protein concentration. The linear relationship of GABA synthesized with respect to time is shown in Figure 4A and has a correlation coefficient of 0.994. The amount of GABA synthesized was also linear with respect to protein concentration of crude brain extract (Figure 4B) and has a correlation coefficient of 0.997.

Levels of GAD in Retina and Subretinal Fluid

The assay described above was used to look for the presence of GAD in subretinal intercellular fluid and retina. As shown in Table 1, retina revealed significant GAD activity, while subretinal intercellular fluid showed negligible activity. The production of GABA was dependent on the amount of crude retinal extract used. The specific activity of the enzyme was determined to be 0.108 $\pm$ 0.005 from three different experiments.
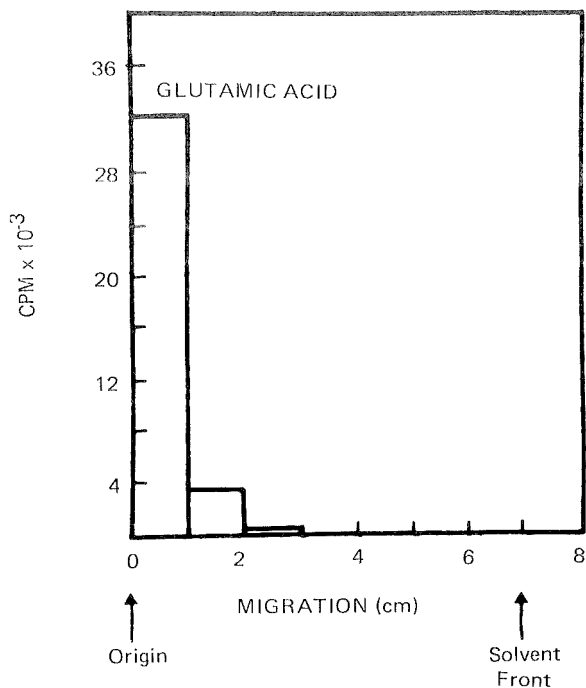
FIGURE 3: Migration of [3H] glutamic acid. The assay mixture without
enzyme was incubated with the radiolabelled substrate at 37°C for
2 h. The composition of the assay mixture was the same as described in
Materials and Methods.
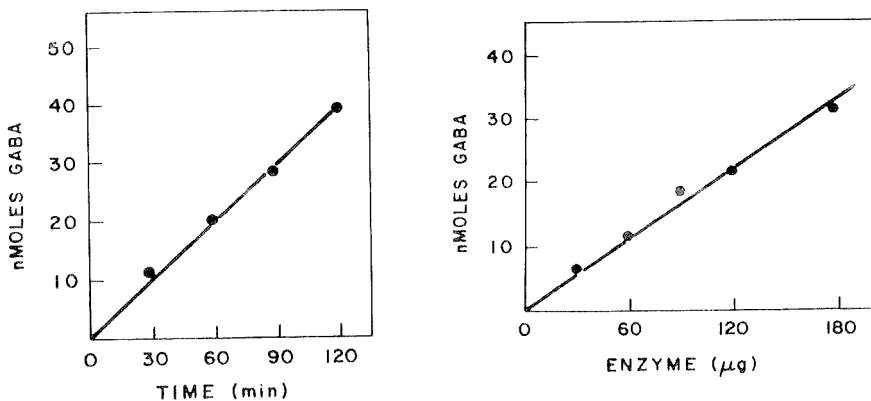
FIGURE 4: Linear relationship of GABA synthesis with time and protein
concentration. A) Assay performed at 37°C with 96 µg of crude
enzyme protein for various incubation times. B) Assay performed at
37°C for 60 min with different concentrations of enzyme. For
additional experimental details, see Materials and Methods.

TABLE 1

Levels of GAD in Bovine Retina and Subretinal Intercellular Fluid

Tissue	Protein Conc.	nMoles of GABA Formed/h	Specific Activity
Retina	300 µg	32.50	0.108
Retina	300 µg	31.02	0.103
Retina	450 µg	51.20	0.113
Subretinal Inter- Cellular Fluid	300 µg	1.25	0.004

Specific activity is defined as µ moles of GABA formed/h/mg protein.

DISCUSSION

Determination of GAD by TLC offers many advantages over other similar methods such as high voltage paper electrophoresis (19,20) ion exchange chromatography (9-11) or HPLC (13,14). The identity of GABA as the only product formed under the present GAD assay conditions has been determined by reversed-phase HPLC (13). Also, the formation of non-specific metabolites such as α-ketoglutarate or succinate as observed in non-neural tissues (21) does not interfere with the present assay. Under identical conditions these metabolites move slow (R_F, 0.02) in comparison to GABA (R_F, 0.95).

The present method measures GABA formation directly and is simpler to use than ion exchange chromatography or high voltage paper electro-phoresis. The enzyme activity determined by this method in brain or retinal homogenates was equivalent to the activity obtained by reversed-

phase HPLC (13). This assay is also more economical and sensitive than

the CO_2 evolution method since the present method uses tritium

labelled glutamic acid. Under the present conditions of assay, it is

possible to detect GAD activity present in 1 μg of crude extract using

tritium labelled glutamic acid (2,000 cpm/nmole). Also, unlike ion

exchange chromatography, separation of glutamic acid from GABA by the

present TLC method is not affected by changes in pH or ionic strength of

the assay mixture.

In summary, we feel the assay is sensitive, simple, specific, rapid

and multiple samples can be run under identical conditions. This method

should be useful in determining low concentration of enzyme as

ordinarily found in cells in culture and in studying the effect of

various metabolites on the specific production of GABA by the GAD

reaction.

ACKNOWLEDGEMENTS

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TLC SEPARATION OF SOME CARBAMATES ON METAL SALT IMPREGNATED LAYERS

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ABSTRACT

A suitable TLC separation scheme for the carbamates - Carbaryl, Bendiocarb, Carbafuran, Baygon, Ziram, Zineb, Aldicarb, MIPC, BPMC, on silica gel plates impregnated with 1 % zinc acetate and using the developer system of benzene-ethyl acetate (50:10) has been worked out.

INTRODUCTION

Kirchner (1) has reviewed the work on TLC separation of carbamates upto 1971, a reference to which shows that impregnation technique has not been used to affect the separation of this class of compounds. Van Hoof and Heyndrickx (2) in 1974 reported the TLC separation of carbamates after their Hydrolysis and Coupling with NBD-Cl. Guley and Karakaya (3) separated seven carbamates on plain silica gel and alumina plates while Davis

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(4) in 1979 separated some carbamates on high
performance TLC plates. Recently Schmid (5), and
Tewari and Ranjeet Singh (6) reported the sepa-
ration of this class of compounds on plain silica
gel plates.

This paper presents an improved TLC separation
and identification scheme for nine closely related
carbamates on impregnated plates.

EXPERIMENTAL

The TLC plates (thickness 0.5mm) were prepared
by spreading a slurry of a mixture of silica gel
G (50g) and varying amounts of metal salt solution.
The plates were activated at $60^{\circ}C$ for 24h. All the
reagent used were of Analytical grade.

The carbamates were supplied by (Rallis India
Ltd., Bayer India Ltd., Union Carbide Bhopal etc.)
and were used after crystallisation. All carba-
mates, except zineb and ziram, were prepared in
Acetone, while zineb and ziram were prepared in
pyridine.

The solution of carbamates was applied to the
layer by means of micropipettes manufactured by
Clay Admas (U.S.A.). After development the plates
were sprayed with suitable reagents. In case of

Carbaryl, Bendiocarb, Carbafuran and Baygon the
spots were located by a saturated solution of
ceric sulphate in 60 % H_2SO_4 and rest by iodine
vapours.

The various impregnants tried were: zinc
acetate, zinc sulphate, cadmium acetate, manganese
acetate. The most suitable solvent system was
found to be Benzene-ethylacetate (50:10). No
change in hR_f value was observed when mixture of
carbamates was applied.

RESULTS AND DISCUSSION

The hR_f values obtained for carbamates on
different plates are given under (Table 1). From
this data it is apparent that the best separation
is obtained on 1 % zinc acetate-impregnated plate.
On this plate the spots are not only well
separated but also the size of the spots is
minimal.

Further, a comparison of the hR_f values on
plain silica gel plate with those on zinc acetate-
impregnated plate shows that the hR_f value is
slightly decreased on impregnated plate and the
tailing is considerably reduced. This suggests
that there is rather weak interaction between the

TABLE 1

Carbamate	hR$_f$ ^{a}					Detection limit (ng)
	On plain silica	zinc acetate	Zinc sulphate	Cadmium acetate	Manganese acetate	
Carbaryl	55	48	51	53	38	224
Bendiocarb	57	53	53	56	43	448
Carbafuran	45	43	37	55	40	224
Baygon	47	40	41	51	38	448
Ziram	88ST	82	83ST	87	87ST	14
Zineb	22T	10	11ST	10	12	112
Aldicarb	23	22	22	-	20	7
MIPC	62	57	58	56	56	28
BPMC	68	62	61	60	61	56

a - hR$_f$ values are average of two or more identical runs, 10 cm in 25 minutes

ST - Slight tailing

T - Tailing.

carbamate and the impregnant. The exact nature of this interaction is being investigated.

ACKNOWLEDGEMENT

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THIN LAYER CHROMATOGRAPHIC SEPARATION OF SOME INORGANIC IONS ON SULPHA DRUG IMPREGNATED LAYERS

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ABSTRACT

Binary, ternary, quaternary and hexanary separations schemes for Co(II), Ni(II), Th(II), Fe(II), U(V), V(IV), Cu(II), As(III), Pb(II), Cd(II), Hg(II), Ag(I), Sb(III), Se(IV), Sn(IV), Ti(IV), Mo(VI), Pd(II) and Pt(IV) have been worked out by using sulphaguanidine as an impregnant on silica gel thin layer and by using the solvent system Isopropanol, ethyl acetate, acetic acid, water, DMF in the ratios (60:30:5:10:5) and (60:30:5:10:3). pH Metric studies have been made to establish the formation of complex between sulphaguanidine and the different metal ions to decide the nature of bonding and to determine their stability constants in an attempt to correlate it with chromatographic behaviour.

INTRODUCTION

Sulpha drugs are the N-substituted compounds of sulphanilamide ($H_2NC_6H_4SO_2NH_2$) which are the drugs of proved therapeutic importance. Sulpha drugs are known to form complexes with different metal ions. Gulko and coworkers[1] have reported the complexation of sulphaguanidine with palladium and also studied the stability of the palladium sulphaguanidine complex. Narang and Gupta [2] synthesised and characterised the Cu(II) chloride complexes with different sulpha drugs namely - sulphanilamide, sulphaguani-

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dine, sulphathiazole, sulphamerazine and sulphapyridine,
while Chaturvedi and coworkers [3] reported the sulphagu-
anidine complexes with few mono and bivalent metal ions.
Recently, the remarkable TLC separation of various sulpha
drugs was achieved in this laboratory [4] by using diffe-
rent metal salts as impregnants. Reference to literature
shows [5] that TLC separation of different inorganic ions
has been attampted by many workers but little attention
has been paid to impregnation technique for the separation
of inorganic ions. Since the chromatographic behaviour
has been shown [6-12] to be influenced by the addition of
a complexing material either as an impreghant or as a cons-
tituent of the developer system, it was considered worth-
while to try different sulpha drugs as impregnants for the
TLC separation of metal ions. The different sulpha drugs
tried as impregnants were – sulphasomidine, sulphaguani-
dine, sulphathiazole, sulphaphenazole, sulphamethizole,
sulphadimidine and sulphadiazine. From these 0.5 % sul-
phaguanidine impregnation showed the best separation po-
tentiality. For understanding the role of impregnant, po-
tentiometric studies were carried out to determine the
stability constants of the complexes formed between the
impregnant and the metal ions. Results of these studies
are presented in this paper.

EXPERIMENTAL

The TLC plates (thickness 0.5 mm) were prepared by
spreading, by means of a Stahl type applicator, a slurry
of 50 g of silica gel (B.D.H.) and 0.50 g of sulphagua-
nidine in 100 ml of distilled water-alcohol (70:30) mix-
ture and the plates were dried for 24 hours at $60\pm1^{o}C$.

The inorganic ions (0.1 % W/V solution in water) were
applied to the layers using glass capillary and the chro-

matograms were eluted at a constant temperature $(30 \pm 1^{\circ}C)$
with a mixture of Isopropanol-ethyl acetate-DMF-acetic
acid-water (60:30:5:5:10), solvent A and (60:30:3:5:10)
solvent B. After development the plates were sprayed with
the suitable spraying reagent. A freshly prepared solution
of $K_4Fe(CN)_6$ (1 % W/V in 2 % HCl) was used as a visualiz-
ing reagent in case of Fe, U, V, Cu and Ti while for oth-
er metal ions a freshly prepared solution of yellow ammo-
nium sulphide was used.

RESULTS AND DISCUSSION

The hR_f values of different metal ions on silica gel
layers and on layers impregnated with 0.5 % sulfaguani-
dine in solvent systems-Isopropanol-ethyl acetate-DMF-ace-
tic acid-water (60:30:5:5:10) solvent A and (60:30:3:5:10)
solvent B, are given under TABLE-1. A perusal of the data
given in this table shows talling to a greater extent (de-
noted by G in table 1) for metal ions on plain silica gel
plate, while all the metal ions are well separated on 0.5%
sulphaguanidine impregnated plates. Hence, 0.5 % sulfagu-
nidine was considered to be a suitable impregnant for the
separation of metal ions. Based on hR_f values and the
analytical, biochemical and industrial importance of the
metal ions separated on sulfaguanidine impregnated pla-
te, some binary, ternary, quaternary and hexanary sepa-
rations of metal ions have been achieved by using solve-
nt systems A and B(tables 2-5). No change in hR_f values
has been observed when mixtures of metal ions were
developed.

The pH-metric method of Calvin and Bjerum [13,14]
was used to determine the stability constants of metal-
sulphaguanidine complexes. The values of stability con-
stants thus obtained with metals Fe, V, Cu, Pb, Se, Sn,

TABLE — 1

hR$_f$ Values of Metal Ions on Silica Gel Layer
Impregnated with 0.5 % Sulfaguanidine

Metal ion		Plain silica gel	hR$_f$		Detection Limit (ug)
			Solvent A	Solvent B	
Co(II)	(chloride)	51G	51	53	2.5
Ni(II)	(sulphate)	40G	29	40	5.0
Th(II)	(nitrate)	13G	10	13	7.0
Fe(II)	(sulphate)	27G	17	24	2.5
U(V)	(pentaoxide)	85	81	83	4.0
V(IV)	(sulphate)	44	04	23	5.0
Cu(II)	(sulphate)	75	65	67	2.5
As(III)	(oxide)	39	32	34	5.0
Pb(II)	(acetate)	52G	45	48	5.0
Cd(II)	(nitrate)	64	57	60	5.0
Hg(II)	(chloride)	96	90	94	1.0
Ag(I)	(nitrate)	74G	60	72	2.5
Sb(III)	(chloride)	18	11	12	2.5
Se(IV)	(dioxide)	45	41	47	4.0
Sn(IV)	(chloride)	37	24	37	5.0
Ti(IV)	(chloride)	40	21	32	5.0
Mo(VI)	(ammonium)	16	10	14	2.5
Pd(II)	(chloride)	84	84	80	2.0
Pt(IV)	(chloride)	74	74	72	2.5

TABLE — 2

Binary Separation of Metal Ions

Metal ions separated		Solvent system employed
Fe(II)	U(V)	A
Fe(II)	Cu(II)	A
Fe(II)	Mo(VI)	B
U(V)	Se(IV)	A
As(III)	Se(IV)	A
As(III)	V(IV)	A
Cu(II)	Ag(I)	A
Pt(IV)	U(V)	A
Sb(III)	Fe(II)	A

TABLE - 3

Ternary Separation of Metal Ions

Metal ions separated			Solvent system employed
Ag(III)	— Cd(II)	— Hg(II)	B
As(III)	— Pb(II)	— Cd(II)	A
Sb(III)	— Sn(IV)	— Se(IV)	A
Cd(II)	— Cu(II)	— Hg(II)	A
Pb(II)	— Sn(IV)	— Ti(IV)	B
Pd(II)	— Pt(IV)	— Th(II)	A

TABLE - 4

Quarternary Separation of Metal Ions

Metal ions separated				Solvent system employed
Ag(I)	— Cd(II)	— Sn(IV)	— Sb(III)	B
Hg(II)	— Pd(II)	— Pt(IV)	— Ag(I)	A
U(V)	— Ti(IV)	— Th(II)	— V(IV)	A
Pd(II)	— Cu(II)	— Sn(IV)	— Fe(II)	A
Hg(II)	— U(V)	— Ag(I)	— V(V)	A
Hg(II)	— Ag(I)	— Pb(II)	— Sb(III)	A
Se(IV)	— Sn(IV)	— Ti(IV)	— Mo(VI)	B

TABLE - 5

Hexanary Separation of Metal Ions

Metal ions separated						Solvent System employed
Pd(II) —Pt(IV)	—Cd(II)	—Pd(II)	—Ti(IV)	—Mo(VI)		A
U(V) —Cu(II)	—Co(II)	—Ni(II)	—Fe(II)	— V(IV)		A
Hg(II) —Ag(I)	—Se(IV)	—As(III)—Sn(IV)	—Sb(III)			A

In tables (2-5) solvents A and B stand for :

    A - Isopropanol-ethyl acetate-acetic acid-water-
       DMF (60:30:5:10:5)

    B - Isopropanol-ethyl acetate-acetic acid-water-
       DMF (60:30:5:10:3)

TABLE - 6

Formation Constants of Metal Complexes of Sulphaguanidine

Metal ion	$\log k_1$	$\log k_2$	$\log \Delta hR_f$	$\dfrac{\log \Delta hR_f}{\log k}$
Fe(II)	6.99	6.78	1.00	0.143
V(IV)	8.75	–	1.60	0.1828
Pb(II)	4.15	–	0.845	0.2036
Cd(II)	2.75⊗	–	0.845	0.307
Se(IV)	5.66	–	0.602	0.106
Sn(IV)	5.27	–	1.1139	0.211
Mo(VI)	4.50	–	0.778	0.1728
Pd(II)	5.42‡	4.38‡	0.00	0.000
Cu(II)	6.77	6.75	1.00	0.1477

⊗ The value taken from the work of Chaturvedi
and coworkers [15].

‡ The values taken from the work of Gulko and
coworkers [1].

and Mo-sulphaguanidine complexes are summarised in the
Table 6. The value of stability constant for Cd-sulpha-
guanidine complex has been taken from the work of
Chaturvedi and coworkers [15] while that of Pd-sulpha-
guanidine complex has been taken from the work of
Gulko and coworkers [1].

To find out the relationship, if any, between chro-
matographic behaviour of different metal ions on sulpha-
guanidine impregnated plate and the stability constants
of their complexes, plots of $\log k$ versus $\log \Delta hR_f$(Fig.1)
and of $\log k$ versus $\dfrac{\log \Delta hR_f}{\log k}$ (Fig.2) were drawn. The

values of $\log \Delta hR_f$ and $\dfrac{\log \Delta hR_f}{\log K}$ are also given in
Table 6. Although no simple correlation could be
obtained between ΔhR_f and the stability constant, it
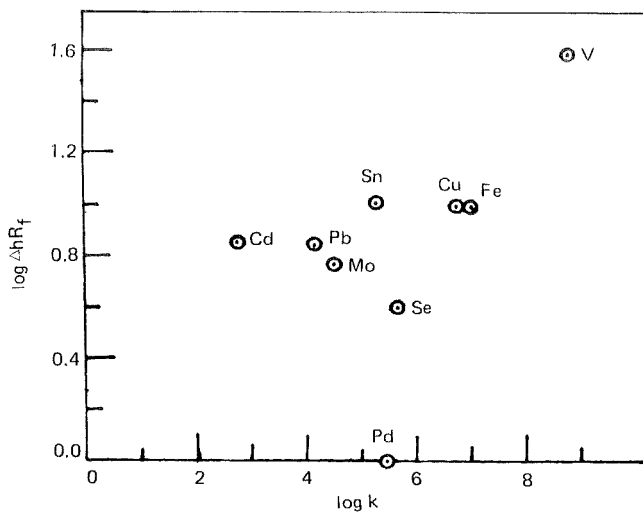
Fig. 1. Relationship between log k and ΔhR_f.
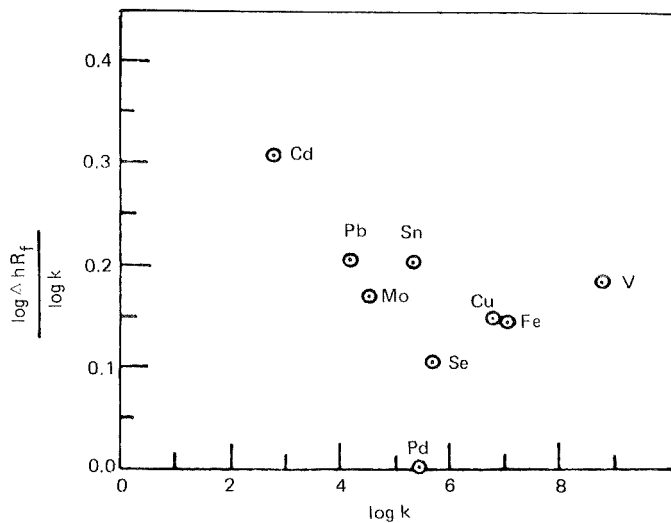
Fig. 2. Relationship between log k and $\dfrac{\log \Delta h R_f}{\log k}$.

can be seen that, in general, ΔhR_f increased with an increase in k suggesting thereby that complex formation between sulphaguanidine and the metal did influence the movement on the sulphaguanidine impregnated plate but it was obviously not the sole factor influencing movement and hence the absence of any correla ion between k and hR_f.

ACKNOWLEDGEMENTS

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CHROMATOGRAPHIC ANALYSIS OF SOME 3d METAL COMPLEXES

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ABSTRACT

Several quaternary mixtures of 3d transition metal ions
(Ti(IV), Mn(II), Fe(III), Co(II), Cu(II) and Zn(II) complexed
with p-diethylaminoanil of anthraceneglyoxal (DEAAnG) alone
and alongwith thiourea (TU) have been resolved by TLC, PC and
EC and identified using migration rate and spectroscopic (i.r.)
correlations. The TLC method showing the best resolution has
been used to separate quaternary mixtures of complexes quanti-
tatively; coloured compounds have been estimated spectrophoto-
metrically.

INTRODUCTION

Diverse chromatographic methods have been employed in
the microanalysis of 3d metal ions as such and as their simple
complexes with organic ligands and a few references[1-8] on
chromatography of mixed ligand complexes are also available.

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However, no chromatographic studies have yet been made on simple complexes of ketoanils obtained from anthraceneglyoxal and mixed ligand complexes, involving ketoanils and thiourea as ligands. Stable distinguished colours of several 3d metal complexes with DEAAnG, a typical ketoanil of the series alone and alongwith thiourea aroused our interest to undertake this work. Although all the three chromatographic methods could be used to resolve various quaternary mixtures, the TLC method showing the best resolution has been used in the quantitative analysis of mixtures of complexes; chromatogram fragments have been estimated spectrophotometrically.

The reḆationship between characteristic infrared frequencies of DEAAnG (primary ligand) in complexes and their migration rate has also been studied.

EXPERIMENTAL

Synthesis of p-diethylaminoanil of antraceneglyoxal and complexes

p-Diethylaminoanil of anthraceneglyoxal was synthesized by the reported method[4]. Simple complexes were prepared by mixing DEAAnG with metal chlorides in stoichiometric ratios in alcohol or acetone medium. Solids were isolated either by evaporating the reaction mixture or by increasing its pH with NaOH solution (in case of Ti(IV) complex only). Products were finally washed with ether. Mixed ligand complex were prepared by mixing simple complexes and thiourea in stoichiometric ratios in acetone. Reaction mixtures were evaporated on water bath and solids were washed with water and ether successively. All complexes were dried in hot air oven. Products were recrystallized from acetone and finally dried over anhydrous calcium chloride at reduced pressure. Chemicals used in the synthetic work were B.D.H. or S.M. Laboratory reagents.

Analysis and Physical Measurements

I.R. spectra of the compounds were recorded in CsI
medium on a Perkin Elmer grating infrared spectrophotometer
model-577. Conductometric measurements were made with Thoshniwal
conductivity bridge using a dip-type cell. Elemental analysis
was performed by routine micro analytical methods at C.D.R.I.,
Lucknow (India). Total and ionic chlorine and metal contents
were estimated at N.R.E.C. College, Khurja. Optical density
of the solutions were measured with Bausch and Lomb Spectronic-20
spectrophotometer.

Preparation, Loading and Development of TLC Plates, PC and EC
Paper Strips

Silica gel G (BDH) mixed with starch binder (19;1, w/w)
was used to prepare 0.1 cm layers on 3x10 cm glass plates. A
home built apparatus[6] was used to spread the aqueous slurry
on the plates. Sample solutions prepared in EtOH or Me_2CO were
applied with fine capillaries on dry plates and development was
in rectangular glass chambers with ground-in-lids by the
ascending technique. The solvent front was allowed to migrate
for a fixed distance of 6-8 cms and development time was noted.
For quantitative work 10x15 cm plates were loaded with known
volumes of standard sample solutions by micro pipettes.
Chromatogram fragments were scrapped and eluted with $EtOH/Me_2CO$.
Optical densities of the eluates were determined at the λ max
of their solutes and concentrations of the complexes were then
deduced from their respective calibration curves (optical
density v/s concentration) prepared under similar conditions
of solvent and temperature.

In paper chromatography Whatman No.1 3x15 cm filter
paper strips were loaded with fine capillaries in 2-3mm spots
and developed in cylindrical glass jars, already saturated with

solvent. Time of development was noted for 6-8 cms travelled
by the solvent front.

Electrochromatography was carried out in vertical type
apparatus. Whatman No.1 paper strips (3x35 cm) were loaded at
the centre by fine capillaries. Loaded strips were hanged with
both ends in the electrode solvents and apparatus was closed.
At saturation stage constant voltage was applied and after
2-35 hrs chromatograms were taken out and dried.

No locating agent was needed as spots were visible
in day light.

RESULTS AND DISCUSSION

Formulae of the complexes (Table-1) were deduced from their
conductance and analytical data. Comparison of infrared spectra
of ligands with their corresponding complexes revealed perturbation
in C=O (1755 cm^{-1}), C=N (1695 cm^{-1}) frequencies of DEAAnG and C=S
(1085 cm^{-1}) frequency of thiourea during complexation indicating
their participation in coordination. Appearance of some new bands
corresponding to M-O, M-N and M-S stretching also supports this
inference. Metal-chloride coordinate bonding in complexes has also
been identified. Considerable disturbance in the frequencies of
1:4 disubstitution (820 cm^{-1}) and aromatic C=C (1605, 1515, 1450cm^{-1})
of DEAAnG in complexes shows conjugation in the principal ligand,
leading to the change of benzenoid structure to quinonoid while
ligand interacted with metal ions. A typical complex structures
are shown in figures.

$Cu^{(II)} \cdot DEA\ AnG$

$$\left[\begin{array}{c} An \cdot C = CH \cdot N = \langle \rangle = N^{+}\tilde{S}(C_2H_5)_2 \\ \mid \qquad\qquad\qquad \backslash \\ O \qquad\qquad\qquad\quad \\ \mid \qquad\qquad\qquad\quad \\ Cl \cdot Cu \cdot Cl^{Cl \cdot} Cu\text{-} Cl \\ \mid \qquad\quad \mid \\ H_2O \qquad\ H_2O \end{array} \right]$$

$Cu^{(II)}$ · DEA AnG · (TU)

$$
\left[
\begin{array}{l}
An - C = CH - N = \langle\ \rangle = N^{+S}(C_2H_5)_2 \\
\quad\ \ | \\
\quad\ \ O \\
\quad\ \ \ \backslash \qquad\qquad\qquad\ _{NH^2} \\
\quad Cl \cdot Cu \leftarrow S = C < \\
\quad / \quad \backslash \qquad\qquad\ \ _{NH^2} \\
\ \ Cl \quad\ Cl \\
\quad \backslash\ \ / \qquad\qquad\ _{NH^2} \\
\ Cl - Cu \leftarrow S = C < \\
\quad /\quad \backslash \qquad\qquad\ _{NH^2} \\
An \cdot C = CH - N \cdot \langle\ \rangle = N^{+S}(C_2H_5)_2
\end{array}
\right] \ .20\ H_2O
$$

$An = \bigcirc\bigcirc\bigcirc$

Chromatographic data obtained by migrating individual complexes revealed the separation of various quaternary mixtures of simple and mixed ligand complexes by TLC and PC, however, EC could resolve only ternary mixtures. Although, complex spots showed different migration rates in several solvents, only the solvents which could give best resolution of diverse complex mixtures have been noted in Table-2. Each complex migration has been found to be independent on the presence of others and on plate and paper size, however thickness of paper or layer has diverse effect on it.

The stretching frequency (γ) values (Table-1) of carbonyl and azomethine groups of primary ligand DEAAnG which are highly metal sensitive have been correlated with migration rates (Table-2)of the complexes in solvents giving best separation. TLC & EC migration rates of simple and mixed complexes in their mixtures are opposite to the orders in the values of γ (C=O). But migration rates of simple complexes in PC and EC and of mixed ligand complexes in TLC and PC which form identical orders are opposite to the sequence in γ (C=N). New chromatographic and spectroscopic (i.r.) correlation useful in making certain identification of resolved components form an interesting feature of the present study.

Although, various quaternary and ternary mixtures of simple and mixed ligand complexes could be resolved and identified (using chromatographic and spectroscopic correlations)

TABLE - 1

M.P. MOLAR CONDUCTANCE, ANALYSIS AND PRINCIPAL IR FREQUENCIES OF COMPLEXES

COMPLEX	M.P.	ΛM (Solvent/electrolytic nature)	Elemental Analysis(%) Calcd.			
			C	H	N	Cl (ionic)
$[Ti(DEAAnG)_2Cl_2].2Cl$	175	155.22 (EtOH/1:2 electrolyte)	65.69 (65.48)	5.05 (4.92)	5.89 (5.68)	747 (7.38)
$[Mn(DEAAnG)_2(H_2O)_2]2Cl.5H_2O$	110	121.44 (Me$_2$CO/1:2 electrolyte)	61.66 (61.82)	6.12 (6.17)	5.53 (5.65)	7.01 (7.30)
$[Fe(DEAAnG)_2Cl_2].Cl$	140	70.33 (EtOH/1:1 electrolyte)	67.65 (67.55)	5.20 (5.35)	6.07 (6.15)	3.84 (3.92)
$[Co(DEAAnG)_2].2Cl$	110	162.87 (EtOH/1:2 electrolyte)	70.12 (70.25)	5.39 (5.60)	6.29 (6.53)	7.97 (7.88)
$[Cu_2(DEAAnG)Cl_4(H_2O)_2]$	125	7.37 (MeCN/non-electrolyte)	45.54 (45.20)	4.09 (4.35)	4.09 (4.33)	– –
$[Zn(DEAAnG)_2].2Cl.4H_2O$	140	189.20 (Me$_2$CO/1:2 electrolyte)	64.43 (64.55)	5.78 (5.75)	5.78 (5.90)	7.33 (7.50)
$[Fe(DEAAnG)(TU)Cl_3]$	130	13.20 (Me$_2$CO/non-electrolyte)	52.39 (52.60)	4.52 (4.52)	9.05 (9.12)	– –
$[Co(DEAAnG)_2(TU)_2].2Cl.8H_2O$	180	235.11 (Me$_2$CO/1:2 electrolyte)	54.64 (54.42)	6.07 (5.95)	9.44 (9.22)	5.98 (6.06)
$[Cu_2(DEAAnG)_2(TU)_2Cl_4].20H_2O$	135	37.60 (Me$_2$CO/non-electrolyte)	42.04 (42.25)	6.22 (6.12)	7.00 (7.15)	– –
$[Cd(DEAAnG)_2(TU)_2].2Cl.6H_2O$	210	220.22 (Me$_2$CO/1:2 electrolyte)	53.84 (53.60)	5.65 (5.55)	9.30 (9.20)	5.89 (6.02)

(Found)					Infrared frequencies (cm^{-1})						
Cl (Total)	M	C=O	C=N	C=C	1:4 Di-substitution	C=S	M–N	M–O	M–S	M–Cl	M–Cl–M
14.94 (15.08)	5.04 (5.19)	1630	1615	1515 1460	760	–	565	470	–	270	–
7.01 (7.30)	5.42 (5.28)	1715	1610	1515 1465	800	–	575	285	–	–	–
11.54 (11.44)	6.05 (6.10)	1705	1615	1515 1470 1450	800	–	560	470	–	300 280	–
7.97 (7.88)	6.62 (6.50)	1700	1665	1500 1470	810	–	530	450	–	–	–
20.72 (20.66)	18.54 (18.62)	1690	1615	1520 1475 1450	795	–	515	475	–	320	–
7.33 (7.50)	6.75 (6.60)	1620	1610	1520 1470	800	–	555	295	–	–	–
17.22 (17.00)	9.03 (8.98)	1725	1670	1610 1515 1460	820	1080	460	380	215	280 260	–
5.98 (6.06)	4.96 (5.00)	1720	1660	1605 1510 1460 1450	815	1075	530	445	280	–	–
9.21 (9.10)	8.24 (8.40)	1735	1620	1590 1510 1460 1445	790	1080	470	210	–	370	255
5.89 (6.02)	9.34 (9.50)	1725	1660	1610 1515 1450	815	1075	465	260 245	220	–	–

TABLE - 2

Resolving Solvents for Different Mixtures

Complexed Ligand(s)	Ions in Mixture	Resolving solvent	Chromatographic method of separation
DEAAnG	Cu(II) or Ti(IV) + Mn(II) or Fe(III) + Co(II) + Zn(II) (0.30) (0.38) (0.48) (0.52) (0.65) (0.75)	Me_2CO	T.L.C.
DEAAnG and T.U.	Cu(II) + Cd(II) + Co(II) + Fe(III) (0.08) (0.43) (0.50) (0.64)	n-BuOH-C_6H_5(1:1)	T.L.C.
DEAAnG	Cu(II)+/or Fe(III) + Ti(IV) + Mn(II) + Zn(II) (0.13) (0.22) (0.33) (0.45) (0.95)	$CHCl_3$	P.C.
DEAAnG	Fe(III) + Ti(IV) + Cu(II) + Mn(II) (0.00) (0.10) (0.24) (0.73)	Iso-BuCOMe	P.C.
DEAAnG and T.U.	Fe(III) + Cd(II) + Co(II) + Cu(II) (0.13) (0.21) (0.51) (0.92)		P.C.
DEAAnG	Co(II) or Fe(III) + Ti(VI) or Mn(II) + Zn(II) (1.6) (1.4) (2.2) (2.1) (3.0)	AcOH-$\frac{N}{10}$HCl(1:1)	P.C.
DEAAnG and T.U.	Co(II) + Fe(III) or Cu(II) + Cd(II) (1.0) (1.8) (2.1) (3.0)	AcOH-$\frac{N}{10}$HCl(7:3)	P.C.

Values given in parenthesis are the R_F values.

TABLE - 3

Maximum Quantities of Complexes resolved by T.L.C. in their various mixtures

Mixture resolved	Spot colour	λmax	Weight of Complex applied on plate (μ)	Weight of Complex recovered (μ)	Error (%)
$[Cu_2(DEAAnG)Cl_4(H_2O)_2]$	Violet	470	65.00	64.50	-0.77
$[Mn(DEAAnG)_2(H_2O)_2].2Cl.5H_2O$	Pink	440	7.60	7.50	-1.30
$[Co(DEAAnG)_2].2Cl$	Reddish-Brown	420	46.00	45.75	-0.54
$[Zn(DEAAnG)_2]2Cl.4H_2O$	Gray	460	26.80	27.00	+0.75
$[Ti(DEAAnG)_2Cl_2].2Cl$	Blue	480	73.00	73.50	+0.68
$[Fe(DEAAnG)_2Cl_2].Cl$	Grayish-Brown	590	65.00	65.25	+0.38
$[Co(DEAAnG)_2].2Cl$			46.00	45.75	-0.54
$[Zn(DEAAnG)_2].2Cl.4H_2O$			26.80	27.00	+0.75
$[Cu_2(DEAAnG)_2(TU)_2Cl_4].2OH_2O$	Yellow	360	56.00	56.25	+0.45
$[Fe(DEAAnG)(TU)Cl_3]$	Reddish-Brown	460	59.50	59.25	-0.42
$[Co(DEAAnG)_2(TU)_2].2Cl.8H_2O$	Pink	430	21.60	21.75	+0.69
$[Cd(DEAAnG)_2(TU)_2].2Cl.6H_2O$	Pinkish-Gray	580	20.25	20.25	0.00

by all the three said methods (Table - 2), the quantitative
separation, however, could only be achieved by TLC. The choice
of TLC to PC and EC also lies in its quick resolution, compacted-
ness of spots and high effectiveness of the solvents in it.
Errors and limit of maximum separation have also been found out
in several typical mixtures (Table-3). Several solvents showing
vide difference in migration rates of the complexes indicated
good possibility of their separations but owing to large diffusion
and trailing effects they could not be used.

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CHROMATOGRAPHIC BEHAVIOUR OF 48 METAL IONS ON TBP IMPREGNATED

SILICA GEL - G LAYERS

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ABSTRACT

The separation potential of TBP impregnated silica gel-G for 48 metal ions has been explored in a number of solvent systems. The effect of the degree of impregnation of TBP has been studied and some important binary and ternary separations were achieved.

INTRODUCTION

In 1958, Winchester(1) first combined the favourable features of high molecular weight extractants with a chromatographic technique and reported the use of di(2-ethyl hexyl) phosphoric acid loaded on Al_2O_3 for separating a number of rare earths. Dilute HCl was used as a mobile phase. Since then reversed-phase chromatography has become very popular and hundreds of papers have been published using high molecular weight amines, substituted quaternary ammonium salts, heterocyclic amines(2) and tetra substituted pyrazole(3) as stationary phases. In this paper, we described the study of the effect of tri n- butyl phosphate concentration on plates loaded with silica gel-G. HCl and HNO_3 have been used as eluants.

We have tried to improve the separations by using some complexing acids such as tartaric acid, citric acid and oxalic acid.

MATERIALS AND METHODS

Apparatus

TLC apparatus (Toshniwal, India) was used to prepare thin layers on glass plates (20x3 cm). The plates were developed in glass jars (20x6 cm).

Reagents

Silica gel-G, tri n-butyl phosphate and benzene were all of analytical grade from B.D.H. England. Other chemicals were of AnalaR grade.

Test solutions and Detectors

Test solutions, 0.1M of chlorides, nitrates or sulphates of cations were prepared in a little amount of the corresponding acids. Conventional spot test reagents were used for detection perposes(4).

Preparation of TBP impregnated thin layers

The slurry was prepared by mixing silica gel-G with conductivity water in the ratio of 1:3 with constant shaking for about 5 min. This slurry was immediately coated on the clean glass plates with the help of an applicator and uniform thin layers ($\sim$ 0.15 mm thick) were obtained. The plates were first dried at room temperature and then in an electric oven for 2 hrs. at $100 \pm 5^{\circ}C$. These plates were stored in an oven at room temperature. Silica gel-G layers were then impregnated with tri n⁻ butyl phosphate in benzene. Benzene was evaporated by heating the plates in an electric oven at $90 \pm 5^{\circ}C$ for 1 hr. The plates were then stored in an oven at room temperature and used as such for chromatography.

Procedure

The sample solution was loaded (1 or 2 Spots) on TBP impregnated silica gel-G plates with the help of glass capillaries and the spots were allowed to dry at room temperature. The solvent ascent was always 11 cm. The R_L and R_T were measured after detection.

Solvent systems

The following solvents were used:

S_1 1N HCl
S_2 1N HNO$_3$
S_3 1N H$_2$SO$_4$
S_4 0.1M Oxalic Acid
S_5 0.1M Tartaric Acid
S_6 0.1M Citric Acid

RESULTS

The R_f values for only those cations which give compact spots were taken for plotting the figures. Intrestingly, most of the

TABLE 1

Binary Separations Achieved Experimentally

Solvent	% TBP Impregnation	Separations Achieved Metal ion (R_T-R_L)	Time
S_1	20	$Tl^{3+}(0.00-0.00) - Ga^{3+}(0.50-0.60)$	1h.30m.
		$In^{3+}(0.00-0.10) - La^{3+}(0.55-0.85)$	
		$Mo^{6+}(0.10-0.25) - Te^{4+}(0.70-0.95)$	
		$Mo^{6+}(0.00-0.20) - UO_2^{2+}(0.30-0.62)$	
		$Cs^{+}(0.10-0.35) - K^{+}(0.90-1.00)$	
		$W^{6+}(0.00-0.00) - UO_2^{2+}(0.20-0.40)$	
		$W^{6+}(0.00-).00) - Cr^{3+}(0.36-0.64)$	
		$Ti^{4+}(0.00-0.06) - Ce^{3+}(0.35-0.65)$	
S_1	40	$W^{6+}(0.00-0.00) - Se^{4+}(0.80-1.00)$	1h.40m.
		$Mo^{6+}(0.00-0.20) - Se^{4+}(0.80-1.00)$	
		$Th^{4+}(0.85-1.00) - UO_2^{2+}(0.00-0.10)$	
		$Fe^{3+}(0.08-0.30) - VO^{2+}(0.45-0.55)$	
S_2	20	$Zr^{4+}(0.00-0.08) - La^{3+}(0.50-0.90)$	1h.40m.
		$Ti^{4+}(0.00-0.28) - La^{3+}(0.58-0.88)$	
		$Y^{3+}(0.21-0.47) - La^{3+}(0.58-0.88)$	
S_2	Zero	$Zr^{4+}(0.00-0.20) - Th^{4+}(0.50-0.55)$	40m.
		$Zr^{4+}(0.00-0.05) - Pr^{3+}(0.60-0.90)$	
S_3	20	$Th^{4+}(0.00-0.35) - Y^{3+}(0.85-0.95)$	2h.
		$Pb^{2+}(0.00-0.00) - Fe^{3+}(0.90-1.00)$	
		$Ti^{4+}(0.00-0.30) - Mn^{2+}(0.70-1.00)$	
		$Nb^{5+}(0.00-0.10) - VO^{2+}(0.80-1.00)$	
S_4	20	$Mo^{6+}(0.00-0.10) - Fe^{3+}(0.60-0.80)$	1h.50m.
		$Ti^{4+}(0.00-0.05) - Zr^{4+}(0.22-0.36)$	
		$Pr^{3+}(0.15-0.50) - Ce^{3+}(0.62-0.85)$	
S_5	20	$Au^{3+}(0.00-0.00) - Pt^{4+}(0.90-1.00)$	1h.50m.
		$Sn^{4+}(0.00-0.35) - Sn^{2+}(0.50-0.80)$	
S_6	20	$UO_2^{2+}(0.00-0.30) - Fe^{3+}(0.75-1.00)$	1h.50m.
		$Hg^{2+}(0.00-0.15) - Cd^{2+}(0.70-1.00)$	
		$Hg^{2+}(0.00-0.10) - Zn^{2+}(0.72-1.00)$	

TABLE 2

Ternary Separations Achieved Experimentally

Solvent	% TBP Impreg- nation.	Separations Achieved Metal ion (R_T–R_L)	Time
S_1	20	Bi^{3+}(0.10–0.20) – Ir^{3+}(0.35–0.55) – Pd^{2+}(0.85–1.00)	1h.30m
S_2	20	Pb^{2+}(0.00–0.00) – Fe^{3+}(0.40–0.60) – Pd^{2+}(0.70–0.90)	1h.40m
S_2	Zero	Zr^{4+}(0.00–0.00) – UO_2^{2+}(0.52–0.72) – Pd^{2+}(0.94–1.00)	40m
S_4	20	Sn^{4+}(0.00–0.15) – Fe^{3+}(0.30–0.60) – Cd^{2+}(0.80–0.90) Sn^{4+}(0.00–0.15) – Fe^{3+}(0.50–0.70) – Pd^{2+}(0.80–1.00)	1h.50m
S_6	20	Pb^{2+}(0.00–0.00) – Ti^{4+}(0.40–0.70) – VO^{2+}(0.80–1.00)	1h.50m

separations were achieved on 20% TBP impregnated silica gel-G layers. The important binary and ternary separations are summarized in Table 1 and 2.

DISCUSSION

Figs. 1a and 1b show the effect of impregnation on the R_f values of the metal ions in 1MHCl and 1N HNO_3. It is apparent that as we increase the impregnation, the R_f value generally decreases. However, in the case of Ag^+, Pb^{2+}, Sn^{2+} and Zr^{4+}, the R_f values are uniformly low in both the solvents at different degrees of impregnation. This shows a definite interaction of silica gel-G with the cations in question.

Figs. 2 and 3 refer to the R_f values of different metal ions in 1M HNO_3 and 0.1M citric acid. Even at the low concentration of 0.1M, the complexing properties of citric acid are apparent and very few metal ions show an R_f value of less than 0.1.

Figs. 4a, b and c explain the effect of pk_1 of the acids used as solvents, on the R_f values of metal ions. In almost all cases, the curve passes through a maximum or a minimum. This is because of the two factors which are affecting the R_f values i.e., the p_H and the complex formation.

Percentage impregnation of TBP in Benzene

———•———•——— 1N HCl
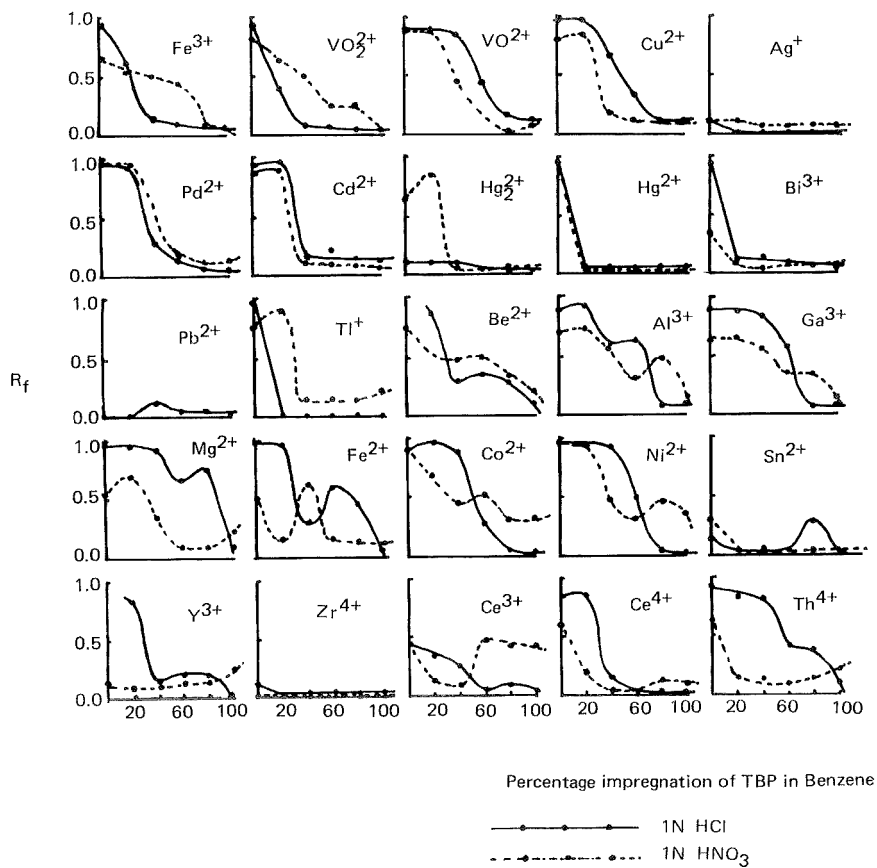
— — •——•——•— •••• 1N HNO$_3$

FIGURE 1a. Plot of R_f Vs. percentage impregnation of TBP.

Percentage impregnation of TBP in Benzene
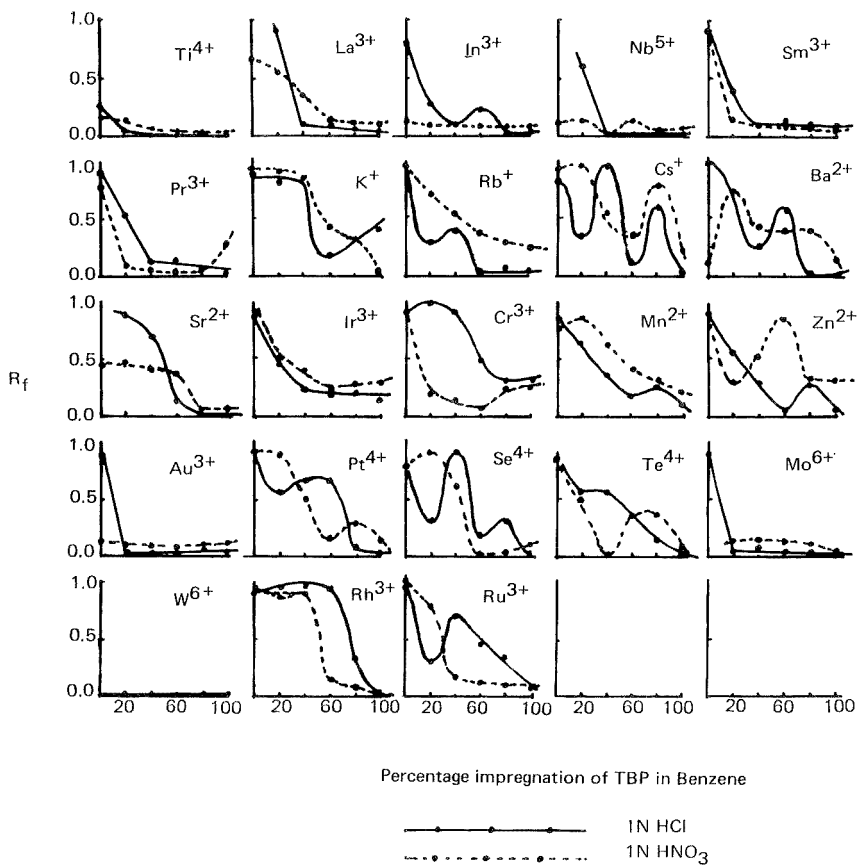
1N HCl
1N HNO$_3$

FIGURE 1b. Plot of R_f Vs. percentage impregnation of TBP.
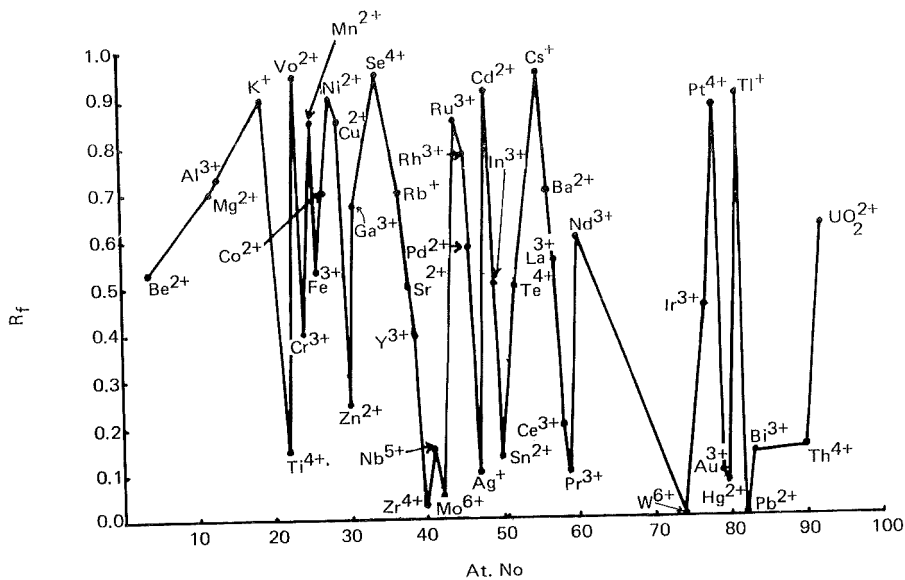
FIGURE 2.   Plot of R_f Vs. atomic number in 1N HNO_3 (20% impregnation of TBF).
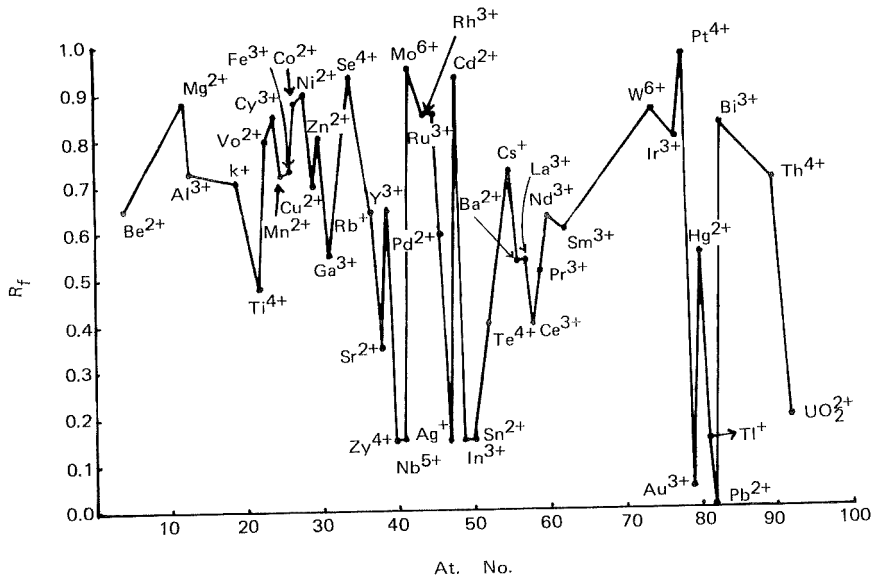

FIGURE 3.   Plot of R_f Vs. atomic number in 0.1N citric acid (20% impregnation of TLF).

TABLE 3

Precipitation of Cations in the Solvents Used

Solvent	Cations which precipitate	Cations which do not precipitate
S_1	Hg_2^{2+}, W^{6+}, Ag^+, Tl^+	Pb^{2+}, Ti^{4+}, K^+, Ce^{3+}, La^{3+}, Mn^{2+}, VO^{2+}, Fe^{3+}, Nb^{5+}, Be^{2+}, Cs^+, Rb^+, Cd^{2+}, Ba^{2+}, Cr^{3+}, Au^{3+}, Al^{3+}, Sn^{4+}, Ni^{2+}, Sr^{2+}, Rh^{3+}, Hg^{2+}, Se^{4+}, Cu^{2+}, Ru^{3+}, Ce^{4+}, Mo^{6+}, Th^{4+}, Pd^{2+}, Y^{3+}, Zr^{4+}, Mg^{2+}, Te^{4+}, Bi^{3+}, Co^{2+}, Ga^{3+}, Sn^{2+}, UO_2^{2+}, Pd^{3+}, Ir^{3+}
S_2	W^{6+}, Mo^{6+}	Pb^{2+}, Ti^{4+}, K^+, Ce^{3+}, La^{3+}, Hg^{2+}, Mn^{2+}, VO^{2+}, Fe^{3+}, Nb^{5+}, Be^{2+}, Cs^+, Rb^+, Cd^{2+}, Ba^{2+}, Cr^{3+}, Au^{3+}, Al^{3+}, Sn^{4+}, Ni^{2+}, Sr^{2+}, Rh^{3+}, Hg^{2+}, Se^{4+}, Cu^{2+}, Ru^{3+}, Ce^{4+}, Th^{4+}, Zr^{4+}, Mg^{2+}, Te^{4+}, Ag^+, Bi^{3+}, Co^{2+}, Ca^{3+}, Sn^{2+}, UO_2^{2+}, Tl^+, Nd^{3+}, Ir^{3+}, Pd^{2+}, V^{3+}
S_3	Pb^{2+}, La^{2+}, Sn^{4+}, W^{6+}, Sr^{2+}	Ti^{4+}, K^+, Ce^{3+}, La^{3+}, Hg^{2+}, Mn^{2+}, VO^{2+}, Fe^{3+}, Nb^{5+}, Be^{2+}, Cs^+, Rb^+, Cd^{2+}, Cr^{3+}, Au^{3+}, Al^{3+}, Ni^{2+}, Hg^{2+}, Se^{4+}, Cu^{2+}, Ru^{3+}, Ce^{4+}, Ro^{6+}, Th^{4+}, Zr^{4+}, Mg^{2+}, Te^{4+}, Ag^+, Bi^{3+}, Co^{2+}, Ga^{3+}, Sn^{2+}, UO_2^{2+}, Tl^+, Nd^{3+}, Ir^{3+}, Pd^{2+}, Y^{3+}

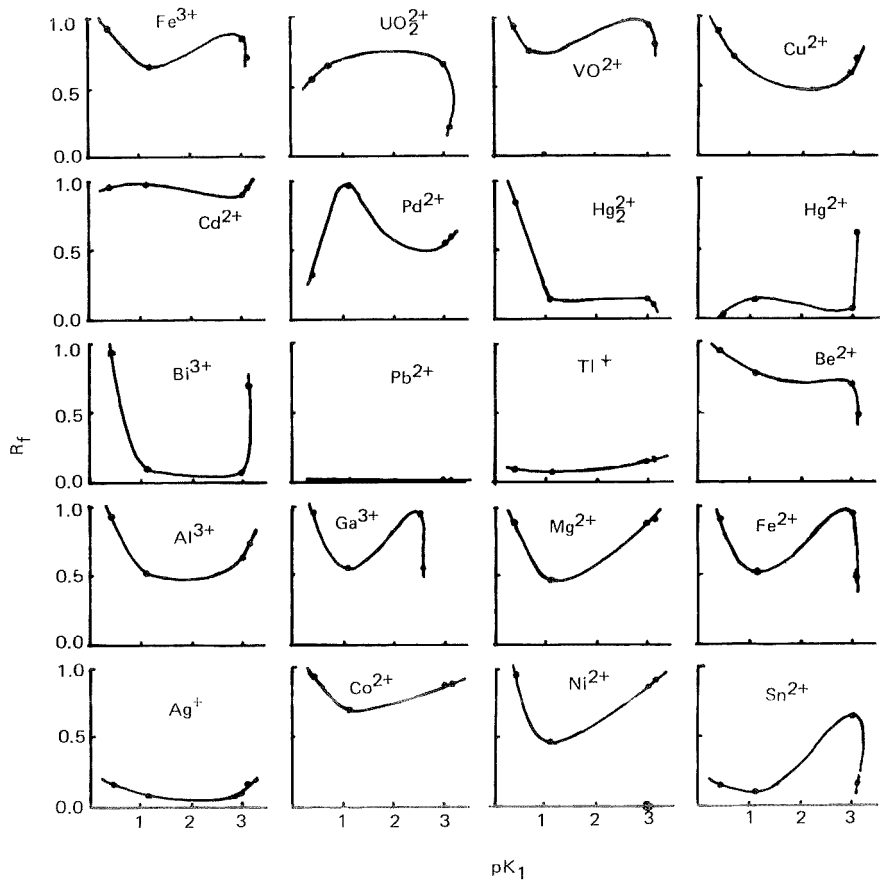|		
S_4	Pb^{2+}, Ce^{3+}, La^{3+}, Y^{3+}, Cd^{2+}, Sr^{4+}, Sr^{2+}, Cu^{2+}, Ce^{4+}, Th^{4+}, Te^{4+}, Ag^+, Bi^{3+}, Co^{2+}, Id^{3+}, Pd^{2+}, Hg_2	Ti^{4+}, K^+, Mn^{2+}, VO^{2+}, Fe^{3+}, Nb^{5+}, Re^{2+}, Cs^+, Rb^+, Ba^{2+}, Cr^{3+}, Au^{3+}, Al^{3+}, Ni^{2+}, $W6+$, Rh^{3+}, Hg^{2+}, Se^{4+}, Ru^{3+}, $Mo6+$, Zr^{4+}, Mg^{2+}, Ga^{3+}, Sn^{2+}, UC_2^{2+}, Tl^+, Ir^{3+}
S_5	Th^{4+}, Te^{4+}, Bi^{3+}	Pb^{2+}, Ti^{4+}, K^+, Ce^{3+}, La^{3+}, Mn^{2+}, VO^{2+}, Fe^{3+}, Nb^{5+}, Re^{2+}, Cs^+, Rb^+, Cd^{2+}, Ba^{2+}, Cr^{3+}, Au^{3+}, Al^{3+}, Sn^{4+}, Ni^{2+}, $W6+$, Sr^{2+}, Rh^{3+}, Hg^{2+}, Se^{4+}, Cu^{2+}, Ru^{3+}, Ce^{4+}, $Mo6+$, Zr^{4+}, Mg^{2+}, Ag^+, Co^{2+}, Ga^{3+}, Sn^{2+}, Tl^+, Nd^{3+}, Ir^{3+}, Pd^{2+}, Y^{3+}, UC_2^{2+}, Hg_2
S_6	Th^{4+}	Pb^{2+}, Ti^{4+}, K^+, Ce^{3+}, La^{3+}, Mn^{2+}, VO^{2+}, Fe^{3+}, Nb^{5+}, Be^{2+}, Cs^+, Rb^+, Cd^{2+}, Ba^{2+}, Cr^{3+}, Au^{3+}, Al^{3+}, Sn^{4+}, Ni^{2+}, $W6+$, Sr^{2+}, Rh^{3+}, Hg^{2+}, Se^{4+}, Cu^{2+}, Ru^{3+}, Ce^{4+}, $Mo6+$, Zr^{4+}, Mg^{2+}, Te^{4+}, Ag^+, Bi^{3+}, Co^{2+}, Ga^{3+}, Sn^{2+}, UC_2^{2+}, Tl^+, Nd^{3+}, Y^{3+}, Ir^{3+}, Pd^{2+}, Hg_2

FIGURE 4a.
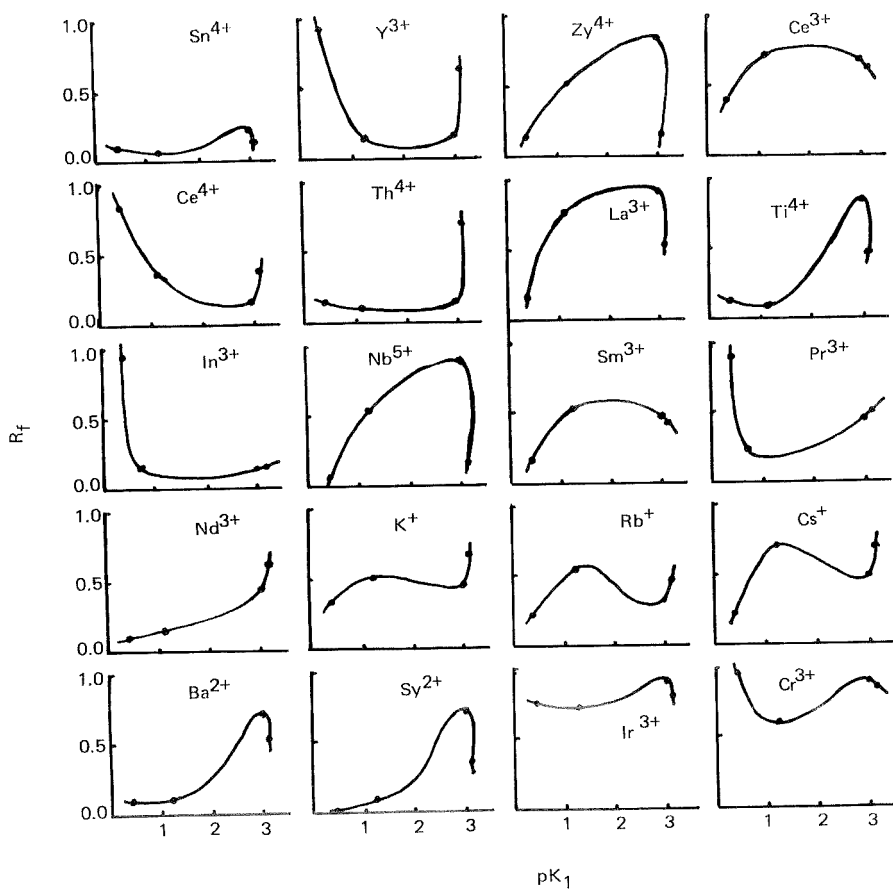
FIGURE 4 b.
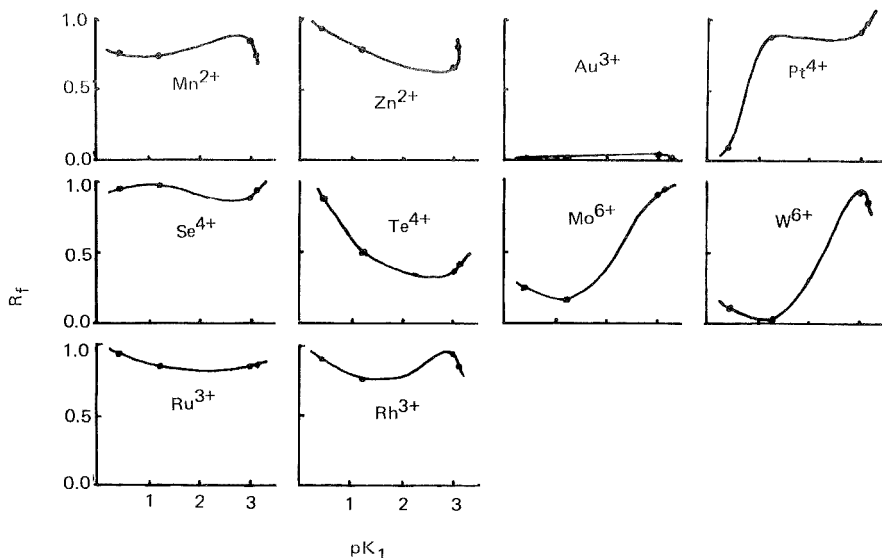
FIGURE 4c.

Plot of R_f Vs. pk_1.

A plot of R_f Vs. atomic number in 1N HNO_3 on 20% TBP impregnated layers (Fig. 5) shows some interesting results. Almost all the transitional metals fall on a straight line. This indicates that in case of transitional metals, the R_f is proportional to the atomic number which may be attributed to their similar chemical nature.

Table 1 summarizes the separations achieved. Thus the separations of $Cs^+ - K^+$, $Fe^{3+} - VO^{2+}$, $Y^{3+} - La^{3+}$, $Zr^{4+} - Th^{4+}$ and $Ti^{4+} - Zr^{4+}$ with the help of simple eluants are interesting and show the advantage of reversed phase chromatography. This is confirmed by the ternary separations described in Table 2.

Table 3 shows that most of the cations which have low R_f values precipitate in the solvent system concerned. There are, however, a few exceptions e.g., Ce^{3+}, La^{3+} and Pd^{2+} in solvent S_4 amd Th^{4+} in solvent S_6. These ions precipitate in the solvent systems but show a high R_f on TBP impregnated silica gel-G layers. This is probably due to the fact that the TBP impregnated silica gel-G immobilizes
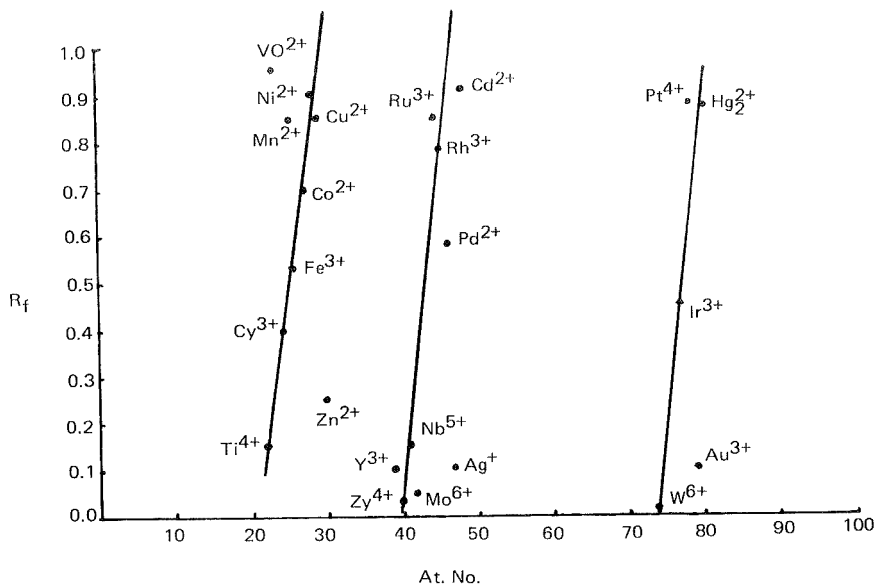
FIGURE 5.   Plot of R_f Vs. atomic number of transitional
                    metal ions in 1N HNO_3 (20% impregnation of TBP).

the precipitating oxalate, tartarate and citrate ions and hence
increases the R_f value of the metal ions.

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Analysis of Saturated and Unsaturated Phospholipids
in Biological Fluids

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ABSTRACT

Cupric acetate (3% in 8% phosphoric acid) as a charring agent
reacts only with unsaturated phospholipids while cupric sulfate
(10% in 8% phosphoric acid) reacts with both saturated and unsaturated
phospholipids. Thus, the amount of saturated phospholipid in a zone
on a thin layer chromatogram (TLC) can be calculated by the difference
in reactivity . An evaluation of methods shows that direct application
of biological samples to TLC for separation and quantitation of phos-
pholipids is reproducible. The use of these techniques for a number
of different samples is described.

INTRODUCTION

The literature has indicated that there was no universally
accepted method for either separation or quantitation of phospholipids
(PL) in biological fluids, particularly amniotic fluid. The problem
was complicated by the fact that in spite of the availability of syn-
thetic phospholipids, many investigators used natural sources without
realizing that these are mixtures of saturated and unsaturated
phospholipids. Thus, reports of quantitation of "dipalmitoyl"
lecithin may in reality be erroneous since the reference material
reportedly used was from natural sources. The methodology for determ-
ination of the individual phospholipids separated in a zone on a thin

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layer chromatogram is tedious, involving extensive detailed analysis
(1-3).

     The experiments described here present methodology to quantitate
the unsaturated and saturated phospholipids separated on thin layer
chromatograms. The traditional analysis of phospholipids in amniotic
fluid has failed to consider that both the saturated and unsaturated
homologues separate together on TLC. It has been reported that in
living sperm there are varying amounts of saturated and unsaturated
phospholipids (4-6). Gluck, et al., in their earlier reports indicated
that as much as 30% of the lecithin in amniotic fluid is of the un-
saturated homologue (7). Using the differentiation provided by reaction
with cupric acetate and cupric sulfate (8) it has been possible to
determine the ratio between these two classes in the phospholipids of
anmiotic fluid and seminal fluid . The method also results in a value
for the true"saturated" lecithin in various fluids. The determination
of saturated lecithin in amniotic fluid has been shown by Torday et al.,
(9) to be a more reliable indication of lung maturity in perinates.

     The cupric acetate reagent has been shown to react only with the
unsaturated PL (8). The cupric sulfate reacts with both the saturated
and unsaturated species. By interpolation on the respective standard
curves and by difference the amounts of the two species can be deter-
mined.

     Results of the use of this method for a number of biological
samples are presented.

                              MATERIALS

     The synthetic phospholipids lysolecithin (LL), sphingomyelin (S),
dipalmitoyl lecithin (L), phosphatidylserine (PS), phosphatidylino-
sitol (PI), phosphatidylethanolamine (PEA), phosphatidylglycerol (PG),
n-monomethyl phosphatidylethanolamine (PEN) and n-dimethyl phosphat-
idylethalomine (PENN) were purchased from Avanti Biochemicals,
Birmingham, Alabama. These were dissolved in chloroform-methanol
(1:1) at the concentration of 100 ng/μL. The purity of these reference
substances was verified by chromatography in various mobile phases.
For preparation of standard regression lines, 2-8 μL of standard were
applied to the chromatoplate.

     Whatman LK-5 layers (20 x 20 cm), 250 u thick, with preadsorbent
zone, were scored on a Schoeffel scoring device to give 1 cm lanes.
These were washed by continuous development overnight in chloroform-
methanol (1:1). Development was carried out in standard size tanks.
Copper (II) sulfate (anhydrous, Baker AR) was made as a 10% solution

in 8% phosphoric acid (8). Copper (II) acetate (monohydrate, Baker
AR) was made as a 3% solution in 8% phosphoric acid (10).

   The chromatograms were scanned in a Kontes Fiber Optic Scanner
(Model 800) using a white phosphor disc. A Hewlett Packard model
3385A integrator provided the means of quantitation.

   The scanning was carried out in the transmission mode using
double beam operation. Scanning in the transmission mode gave higher
results for the individual peaks than did scanning in the reflectance
mode. This is in agreement with previous reports where transmittance
was better than reflectance in scanning of substances separated on
thin layers (11).

                METHODS for TLC and QUANTITATION

   For evaluation of methodology presently in use for separation of
PL from amniotic fluid the flow sheet in Figure 1 was followed.
Labelled dipalmitoyl phosphatidyl choline was added to the sample and
equilibrated at 4° overnight. Phosphatidyl choline, L-α dipalmitoyl-
1 -^{14}C) (0.01 mCi) 1000 m Ci/m Mol was obtained from New England
Nuclear. This was dissolved in toluene-methanol (1:1) to give a
solution of 2.22 x 10^7 dpm in 5 ml. Aliquots of this were taken for
the determination of recovery at each step of the flow sheet.
For 2.5 ml of amniotic fluid 25 µl of this stock solution was used.
Recovery was based on counts in aliquots taken from the extracts in
each step.

   Amniotic fluids were analyzed as soon as received or kept frozen.
Samples were not allowed to remain at room temperature but kept in ice
for transport to the laboratory. They should be immediately frozen
otherwise the phospholipid levels diminish. Seminal fluids were
analyzed within the hour of collection. The samples (both amniotic
fluid and seminal samples) were not centrifuged but applied directly
to the preadsorbent zone of the LK5 layers. The application of untreated
amniotic fluid was performed using a 25 ul microcapillary. Seminal
fluids were diluted 1:1 with water and 10 ul was applied to the layer.
During application a warm air blast from a hair dryer facilitated
evaporation. The samples were applied across the lanes within the
middle third of the application area with drying between each appli-
cation of amniotic fluid until 50 ul (last month) or 100 ul (earlier
gestation) volumes of amniotic fluid had been applied. Standards for
reference are applied on other lanes and only alternate lanes are used.
The samples were applied in duplicate, one each on different halves of
the 20 x 20 plate. When many samples were to be analyzed, duplicates
were applied to each of two whole plates.

After drying, the layers are predeveloped twice with 1:1 chlor-oform-methanol to the interface of the preadsorbent zone. This serves to extract the phospholipid from the sample and deposit it as a line on the starting point of the chromatogram. The developing solvent was chloroform-ethanol-triethylamine-water (30:30:34:8) (8). Development was allowed to proceed until the mobile phase front had reached 2 cm. from the top of the plate. This usually required 1-1.5 hours.

In the mobile phase described, cardiolipin (diphosphatidyl glycerol) migrates near phosphatidyl glycerol (R_f0.70 vs. R_f0.64). In order to verify the lack of cardiolipin in amniotic fluid, aliquots of the same sample were subjected to TLC on Analtech H plates in the mobile phase, chloroform-methanol-acetic acid-water (60:14:13)2) (12). In this system, PG shows R_f of 0.60 vs. that of cardiolipin of 0.85. Eight amniotic fluids showed no detectable cardiolipin, however, all of the seminal fluids contained this substance.

After development, the chromatogram was dried in air, then in an oven at 170° for 2 min. to remove residual solvent. The plate was cut in half with a glass cutter, or two 20 x 20 cm plates are used if multiple samples were separated. One half was sprayed with the cupric acetate reagent and the other half sprayed with the cupric sulfate reagent. The chromatograms were sprayed until throughly wet. The cupric acetate plate is allowed to dry in air for 5 min., heated at 110° for 5 min., then heated in an oven at 180°for 10 min. The cupric sulfate plate is allowed to dry in air for 5 min., heated in an oven for 5 min. at 120°C, then heater at 170° C for 10 min.

The chromatograms are then scanned by densitometry. They should be scanned within the hour or stored in the dark until scanned.

From the standard curve set up by the scanning of serial amounts (2.0 - 8.0 μg) of phospholipid, the amounts of the individual phospho-lipid can be interpolated. The amount of the " unsaturated" phospholipid is obtained from the equivalent integrated curve (cupric acetate spray).

The amount of the "saturated" phospholipid is determined by use of the cupric sulfate curve followed by subtraction of the amount of the unsaturated phospholipid since the cupric sulfate reacts with both. Actual amounts can then be plotted against gestational age to give a working curve in the case of amniotic fluids.

RESULTS and DISCUSSION

Early in assessment of the assay of phospholipids in amniotic fluids as described by Gluck (and the many variations), it became
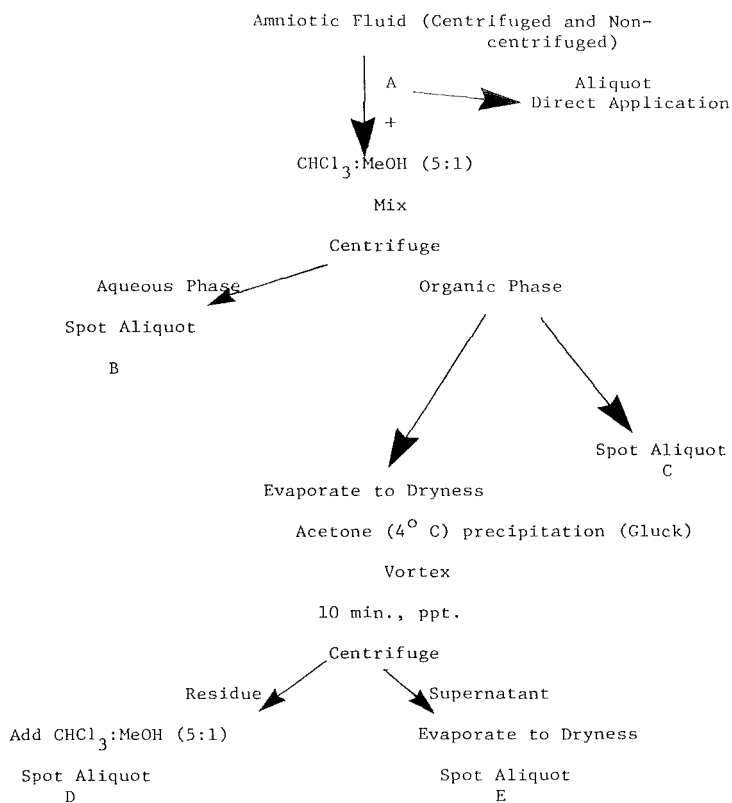
Amniotic Fluid (Centrifuged and Non-
centrifuged)

A ─────────────▶ Aliquot
Direct Application

+

CHCl$_3$:MeOH (5:1)

Mix

Centrifuge

Aqueous Phase   Organic Phase

Spot Aliquot

B

Spot Aliquot
C

Evaporate to Dryness

Acetone (4° C) precipitation (Gluck)

Vortex

10 min., ppt.

Centrifuge

Residue   Supernatant

Add CHCl$_3$:MeOH (5:1)   Evaporate to Dryness

Spot Aliquot   Spot Aliquot
D   E

Figure I   Scheme for Evaluation of Extract Procedures

apparent that the quantitative aspects of PL determination have been
neglected. Using DPL-^{14}C (see methods) a study of the distribution of
DPL-^{14}C in the various fractions of the procedure (see Fig. 1) presently
in use was performed. As much as 35% of added radioactivity was lost
to the precipitate (A) from the amniotic fluid when centrifugation was
included in the first step. The supernatant contained 65% of the
counts. These results are in agreement with those of Oulton (13).
The extraction of the phospholipid form the amniotic fluid by chloroform-
methanol (5:1) (D) was quantitative. The distribution of the radio-
activity between the supernatant and the precipitate as the result of the
acetone precipitation was variable. There was from 6-28% of the
radioactivity in the supernatant (E). This result may be due to the
difficulties related to the reproducible removal fo the acetone after

TABLE I

Reactivity of Phospholipids Toward Charring Reagents

		Character [a]	Acetate [b]	Sulfate [c]
1.	Dimyristoyl PC[c]	s	−	+
2.	Dipalmitoyl PC	s	−	+
3.	Distearoyl PC	s	−	+
4.	1-Palmitoyl-2-oleoyl PC	U	+	+
5.	Dioleoyl PC	U	+	+
6.	Dilinolenyl PC	U	+	+
7.	Dilinoleoyl PC	U	+	+
8.	Lecithin (beef heart)	mix	+	+
9.	Phosphatidyl ethanolamine	mix	+	+
10.	Phosphatidyl serine (bovine brain)	mix	+	+
11.	Phosphatidyl inositol (bovine heart)	mix	+	+

a.   Saturated (s) or unsaturated (u)
b.   Reaction with cupric acetate as described
c.   Reaction with cupric sulfate
d.   Phosphatidyl choline

centrifugation as well as possible evaporation of the acetone although
the sample was kept at 0^Othroughout the procedure. The precipitate (F)
thus contained variable amounts of the PL. Table 1 shows the results
obtained when various synthetic phospholipids were subjected to charring
with the two reagents. Unsaturated phospholipids reacted with both
the cupric acetate and cupric sulfate reagents. The saturated PL
reacted only with the copper sulfate reagents. Also indicated is the
presence of both saturated and unsaturated material in purified material
from natural sources. Manufacturers literature usually show that the
supplied material is not a single entity.

    Evaluation of the amounts of "saturated" versus "unsaturated"
lecithin after chromatographic separation and differentiation with the
cupric acetate and cupric sulfate sprays showed that 33% of the lec-
ithin in (F) was "unsaturated" and the remainder was present as
"saturated" lecithin. With different amniotic fluids the proportions
were different. Gluck et al., (7) reported that there were both un-
saturated as well as saturated lecithins in the acetone precipitate.
The lecithins in the supernatant (E) of the acetone precipitation step
showed both saturated and unsaturated species. Since the classical ace-
tone precipitation procedure for separation of the saturated from
unsaturated phospholipid called for cooling to -60^OC and $- 20^O$ C,
there appears to be no basis for use of the acetone precipitation step
of the various methods, unless there is present in the lecithin fraction
of the chromatograms a substance which can account for the surface
activity present in excess of that expected from the phospholipid.

    Recovery of Lecithin from Amniotic Fluid and from the Sorbent
of the TLC.

    Evaluation of the TLC was performed by determination of recovery
of the DPL-^{14}C (see methods) added to 1) amniotic fluid before aliquot
applications, 2) to amniotic fluid after application and 3) directly to
the preadsorbent layer. The results showed that the preadsorbent layer
retained little if any of the lecithin; the recovery of radioactivity
from the lecithin area of chromatogram was over 95% in each of the
three instances. Less than 0.3% of the added radioactivity was re-
covered from the preadsorbent area of the chromatogram.

    Quantitative Aspects of Densitometry of TLC of PL.
Reproducibility of use of the charring reagents as described in Methods
evolved from the finding that heating of the TLC plate in a convention
oven was erratic. This is a major problem since time and temperature
are critical to reproducibility. Furthermore, it is difficult to
reproduce the conditions of spraying from plate to plate. The problem
was solved by pre-heating the chromatograms at 110° C for 5 min. in one
oven to remove the water of the spray before the charring in a second
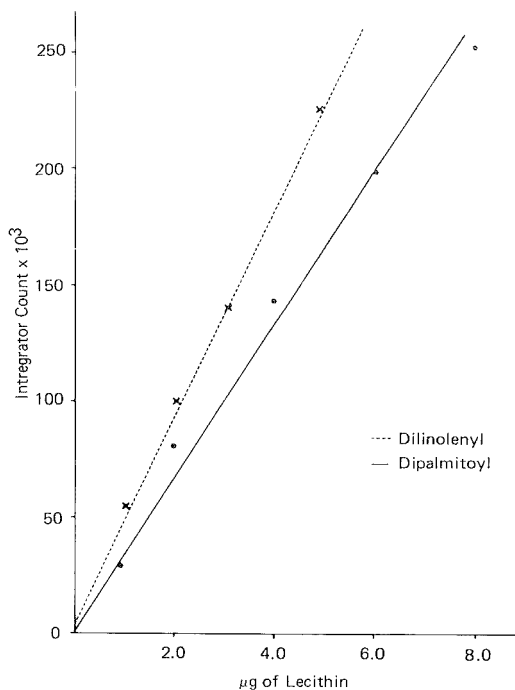
Figure   II   Standard Curve for Saturated and Unsaturated
                Lecithins
                For Lecithin Curve : y=29.214x-0.375
                (n-28)   R-0.984

oven at the higher temperature. The latter oven was modified to include
a metal baffle between the door and the oven in order to prevent heat
loss when the door was opened. It contained a slit , the dimensions of
which permitted a 20 x 20 cm. thin layer plate to be inserted in the
oven without a gross drop in temperature. This improvement of tech-
nique resulted in reproducibility of quantitation of the charring of the
phospholipids and other materials.

    Figure 2 shows the calibration curves for dipalmitoyl lecithin
and dilinolenyl lecithin. The slopes of these curves are reproducible.
(See legend of the figure for evaluation of linear regression). The
curve for the first was obtained using cupric sulfate as reagent. The
curpic acetate has never been found to react with dipalmitoyl lecithin
or other saturated PL. If a reaction is seen with the cupric acetate
reagent it must be concluded that an unsaturated PL is present.
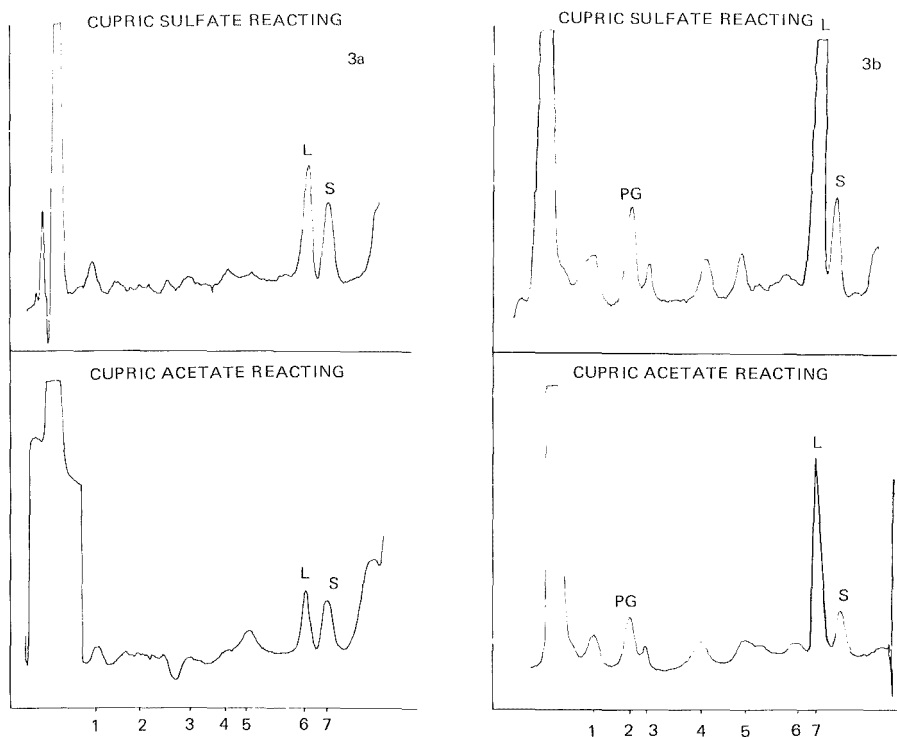
Figure III   Scans of chromatogram of amniotic fluid of
             early pregnancy a) and late pregnancy b)
             after cupric acetate and cupric sulfate detection.

Replicate samples of amniotic fluid were applied to a number
of plates and reproducibility of the method determined from day to day
and within the day for the quantitation of lecithin.   Day to day (n=80)
reproducibility gave a coefficient (CV) of variation of 9.7%, within day
(n=45) the CV was 6%, within a plate (n=9) CV was 3.6%.   These results
were obtained with amniotic fluids carried through the entire proce-
dure including scanning on the densitometer.   It was found that without
the pre-washing of the plates the backgrounds after charring were not
reproducible, varied widely, and gave erratic results even in within day
experiments.

Figure 3 shows composite scans of the chromatograms obtained
after charring with cupric acetate and with cupric sulfate reagents for
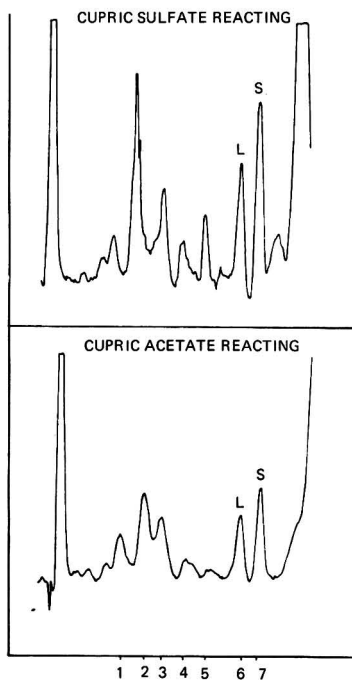amniotic fluids from early (A) and (B) late pregnancy.   It appears that

Figure   IV   Scans of chromatograms of seminal fluid after
               cupric acetate and cupric sulfate reactions.

in early stages of pregancy little PL other than L and S are present in
appreciable amounts. Figure 4 shows the scans from a sample of sem-
inal fluid carried through the same procedure. There is a larger
proportion of sphingomyelin in these samples and more recent evidence
indicates that the greater proportion of spingomyelin is found in the
seminal plasma, while other PL are derived from the sperm. By corre-
lating the integrated areas of these scans, information can be obtained
regarding the relative amounts of percent unsaturated and saturated
species in each of the separated phospholipid areas of the chromatograms.

     Figure 5 shows the results of correlating the amount of
"disaturated" lecithin found in 60 amniotic fluids with the gestational
age. As indicated in this figure, the "disaturated" lecithin shows a
sharp increase in concentration in the period after 34 weeks.
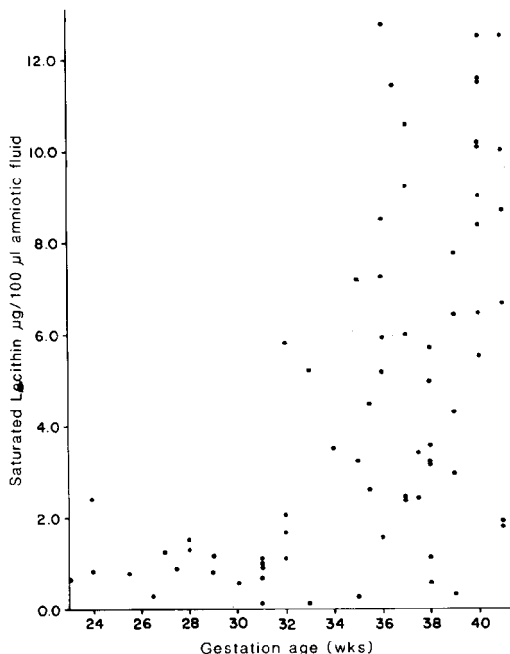
Figure   V   Scattergram resulting from correlating
               gestational age with amount of "disaturated"
               lecithin

     A differentiation was made of the amounts of unsaturated and
saturated PL in various commercially available samples and some bio-
logical tissues.   Table II shows the amounts found in these sources.
The relative amounts of saturated verses the unsaturated lipids varied
widely depending on the source.   The nature of the constituents in the
saturated as well as the unsaturated fractions are unknown.   It has
been shown in earlier work on amniotic fluid by Gluck et al., (7)
and by Selivonchick et al., (2) that the lecithin fraction contains
both saturated and unsaturated compounds.   The method described gives
a reproducible, simple means of separating the phospholipids and
determining the amount of unsaturation of each.

     There has been some controversy over the validity of the use of
cold acetone precipitation in methodology of phospholipid determination.
A number of studies have shown that the acetone precipitated lecithins

TABLE II

Phospholipids in Biological Samples

Amount in Applied Sample (ng)

Source	Lecithin		Sphingomyelin		Phosphatidylglycerol	
	S^a	U^b	S	U	S	U
Egg Lecithin	120	40	ND^c	ND	ND	--
Bovine Heart Plasmalogen	275	150	ND	--	ND	--
Mouse Serum 10 ul	3950	800	280	105	ND	--
Seminal Fluid Human, 5 ul						
1.	700	150	63	630	ND	--
2.	350	100	43	187	ND	--
3.	350	100	ND	402	ND	--
4.	1150	250	ND	1200		
Amniotic Fluid 100 ul						
1. 23 weeks	670	270	ND	600	ND	--
2. 32 weeks	1680	720	ND	865	ND	--
3. 35 weeks	3220	780	ND	585	ND	--
4. 38 weeks	3850	1800	ND	335	ND	--
5. 40 weeks	9750	3750	ND	140	1650	690
6. 40 weeks	6900	2630	ND	950	1250	725
7. 42 weeks	5520	2480	ND	230	1250	720

a   S = saturated   b   U= unsaturated   c   ND= not detected

are not solely composed of saturated molecules (14, 15). In our
earlier work, we found saturated as well as unsaturated phospholipids
in the acetone precipitate as well as the supernatant. This is in
agreement with Torday et al., (9). There also has been disagreement
as to the advisability of centrifuging the amniotic fluid prior to ex-
traction. The fact that amniotic fluids contain different concentrations
of protein in various samples may in a way explain why there is a vari-
ation in the results from different laboratories. Centrifugation of the
amniotic at different speeds as shown here results in loss of phospho-
lipids. Different concentrations of protein ("debris") will only serve
to magnify the variability of error if it is considered that protein
binding of the phospholipid is the factor. For this reason, centri-
fugation was omitted form the present mehtod for both the amniotic and
seminal fluids.

    The method described is reproducible as illustrated by the
calibration curve. However, it is recommended to use internal or ex-
ternal standards for each chromatogram to compensate for normal
analytical variability. This practice should be observed in any an-
alytical chromatographic procedures. It has been shown in each step
of the procedure that quantitative evaluation and real, reproducible
data result. The procedure may serve to provide further data in phos-
pholipid metabolism.

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

MAY 2-6:   VIIth International   Symposium On Column Liquid Chromatography, Baden-Baden, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, Varrentrappstrasse 40-42, D-6000 Frankfurt (Main), West Germany.

MAY 30 - JUNE 3: International Conference on Chromatographic Detectors, Melbourne University.   Contact: The Secretary, International Conference on Chromatographic Detectors, University of Melbourne, Parkville, Victoria, Australia 3052.

JUNE 1-3:   The Budapest Chromatography Conference, Budapest, Hungary.   Contact: Dr. T. Devenyi, Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary or Dr. H. Issaq, Frederick Cancer Research Facility, P.O.Box B, Frederick, MD, 21701, USA.

JULY: 3rd Int'l. Flavor Conf., Amer. Chem. Soc., The Corfu Hilton, Corfu, Greece.   Contact: Dr. S. S. Kazeniac, Campbell Inst. for Food Research, Campbell Place, Camden, NJ, 08101, USA.

JULY 17-23: SAC 1983 International Conference and Exhibition on Analytical Chemistry, The University of Edinburgh, United Kingdom. Contact: The Secretary, Analytical Division, Royal Society of Chemistry, Burlington House, London W1V OBV, United Kingdom.

AUGUST 29 - SEPTEMBER 2: 4th Danube Symposium on Chromatography & 7th I'nt'l. Sympos. on Advances & Applications of Chromatography

in Indudtry, Bratislava, Czech. Contact: Dr. J. Remen, Anal.
Sect., Czech. Scientific & Techn.   Soc., Slovnaft, 823 00
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OCTOBER 1-5:   15th International Symposium on Chromatography,
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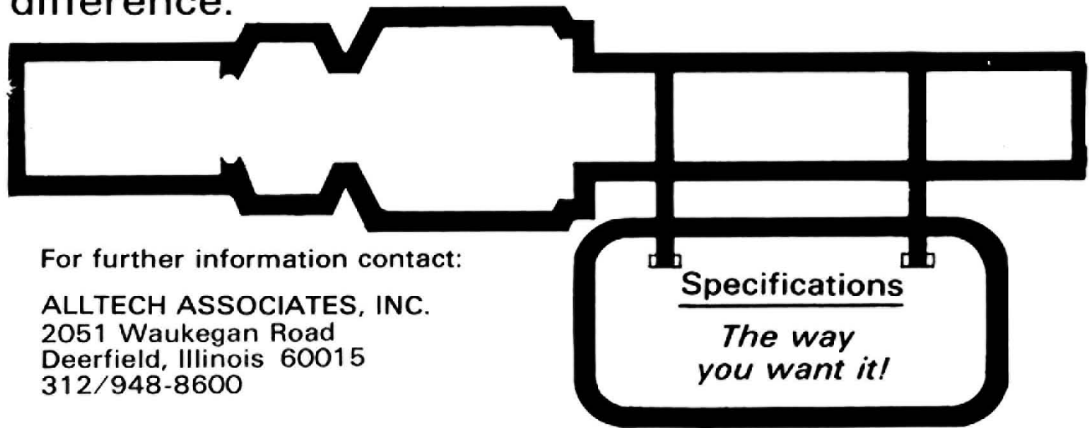
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