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(continued)

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CONTENTS

RELIABILITY OF CHROMATOGRAPHIC RESULTS
IN DETERMINATION OF NARROW MOLECULAR
WEIGHT DISTRIBUTIONS OF POLYMERS

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ABSTRACT

The influence of the procedure for numerical evaluation of chromatograms on the precision of the calculated molecular parameters of chromatographed polymers and the effect of the precision of the measured physical parameters that determine the resulting chromatogram are studied theoretically in this paper. It is shown by using model log-normal and Poisson molecular weight distributions that molecular parameters and polydispersity of polymers with narrow and ultra-narrow molecular weight distributions in particular can be determined by chromatographic separation methods with high reliability which cannot be obtained when other methods are used.

INTRODUCTION

Size exclusion chromatography (SEC), called alternatively gel permeation chromatography (GPC),

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has already become a standard method for characte-
rizing molecular weight distributions (MWD) of po-
lymers. In comparison with classical methods, such
as, e.g., precipitation fractionation and subsequent
measurement of molecular weights of separated frac-
tions,is manifold more rapid and average molecular
weights and MWD are more precise. The precision of
2 - 5 % of molecular weights (1) that is usually
obtained is entirely acceptable for the determina-
tion of molecular weights and polydispersity values
of common polymers. The determination of molecular
weights and polydispersity indices, I, of polymers
with narrow and ultra-narrow MWD requires the use
of high-efficiency chromatography methods. Thanks
to the improvement in the technology of the prepa-
ration of columns for SEC, high efficiencies were
obtained, and thus it was made possible to obtain
in the separation of polymers with narrow MWD re-
sults with higher reliability. The invention of
field-flow fractionation (FFF) has opened further
possibilities of obtaining high selectivity and
high resolution in fractionation of polymers (2,3).

As a result of this progress a question
appears what are the present possibilities and li-
mits of the determination of accurate and precise
values of polydispersity of polymers with narrow
and ultra-narrow MWD. If approximately fifteen
years ago the separation of polymers into individu-
al mers was practically not obtainable and obtain-
ment of the polydispersity index of fractions,
I = 1.2, by classical methods was considered to
be a success, nowadays it is possible to fraction-
ate into individual mers oligomers up to polymeri-

zation degrees ca. 15 - 25 and both to determine
analytically and to obtain preparatively fractions
with polydispersity values lower than I = 1.1. The
question how real molecular parameters and polydis-
persity values calculated from experimental chroma-
tographic data of polymers with narrow and ultra-
narrow MWD are, has, however, not been studied. For
physico-chemical studies of model synthetic poly-
mers and for research of biopolymers in particular,
the answer to this question is of fundamental
significance.

THEORETICAL CONSIDERATIONS

Chromatogram G(V) is a distribution curve in
coordinates of the elution volume and of the detector
response to an instantaneous concentration of an
eluting macromolecular species. The position, the
width and the shape of the chromatogram provide the
information on average molecular weights and MWD of
a polymer sample under separation and on the peak
broadening caused by the injection, the columns and
the detector. All kinds of the data mentioned above
can be obtained by evaluating and interpreting an
experimental chromatogram in a suitable manner. Each
kind of the information obtained will suffer from
the primary error of the measurement of physical
quantities in the mentioned coordinates of the
chromatogram. Let us notice first the coordinate of
the elution volume which can be transformed into the
coordinate of the molecular weight by using a cali-
bration (1). The precision of the determination of
molecular weights and polydispersity index obtain-
able by SEC method can be calculated theoretically.

The following relationship holds between the elution
time and the partition coefficient

$$V_e = V_o + K_{SEC} V_i \qquad (1)$$

and for the relationship between K_{SEC} and the ratio
of the size of the separated macromolecules of sphe-
rical shape to the size of a cylindrical pore it
holds that

$$K_{SEC} = (1 - R/r)^2 \qquad (2)$$

For the macromolecule the relationship can be
written between the radius of an equivalent hydro-
dynamic sphere and the product of $[\eta] \cdot M$, proporti-
onal to the hydrodynamic volume,

$$(R/r)^3 = [\eta] \cdot M / ([\eta] \cdot M)_{excl} \qquad (3)$$

By substituting from the Mark-Houwink equation

$$[\eta] = K M^a \qquad (4)$$

and by rearranging it is obtained

$$(M/M_{excl})^{a+1} = (1 - \sqrt{K}_{SEC})^3 \qquad (5)$$

The calibration curve of a system of columns for SEC
is usually constructed in the coordinates ln M
versus V_e or K_{SEC}. The maximal selectivity
d ln M / d K_{SEC} is obtained in the point of inflex-
ion of the calibration curve for which it holds

$$d^2 \ln M / d^2 K_{SEC} = 0 \qquad (6)$$

Hence it can be written

$$d^2 \ln (M/M_{excl})^{a+1} / d^2 K_{SEC} =$$

$$= -1.5 (1 - 0.5 K_{SEC}^{-0.5}) / (K_{SEC} - \sqrt{K}_{SEC})^2 = 0 \quad (7)$$

The point of inflexion is thus in coordinate $K_{SEC} =$
$= 0.25$ and the slope of the calibration curve at
this point of inflexion is

$$d \ln (M/M_{excl}) / d K_{SEC} =$$

$$= -1.5 / (K_{SEC} - \sqrt{K}_{SEC}) \cdot (a + 1) =$$

$$= 6 / (a + 1) \quad (8)$$

For the obtainable precision of the determination of
the molecular weight expressed in terms of per cents
of the deviation, % M, which depends on the repro-
ducibility of the elution volume or, more precisely,
on the absolute deviation from the correct value,
ΔK_{SEC}, it can then be written

$$\% M = \left| 1 - \exp \left(\frac{6 \; \Delta K_{SEC}}{a + 1} \right) \right| \times 100 \quad (9)$$

Percentage deviation of polydispersity index, % I,
can also be calculated with the aid of Eq. (9). In
this case, ΔK_{SEC} has the meaning of the absolute
precision of the difference in the partition coeffi-
cients that corresponds to the particular value of
polydispersity. Table 1 presents the values of % M

TABLE 1

Influence of the Precision of Elution Volume Measurement on the Precision of Molecular Weights and Polydispersity Indices Calculated from SEC Data at the Maximal Selectivity

Precision of elution volume measurement (% rel.)	Precision of calculated molecular parameters		
	% M	% I at I = 1.1	% I at I = 2.0
1.0	7.30	0.095	0.70
0.5	3.60	0.048	0.35
0.1	0.71	0.009	0.07
0.01	0.35	0.005	0.03

and % I calculated theoretically (for two different values of I) for different values of relative errors of retention volumes, which are actually obtainable with respect to the precision of pumps or sensing elements measuring the flow-rate or the volume of the eluent passed.

For instance, if the elution volume is read with the precision of 0.5 %, which is the precision commonly reached with the use of commercial pumps, the obtainable precision of the determination of molecular weights is comparable with the maximal precision of the measurement obtainable by, e.g., light-scattering or osmometry (1). The precision of the determination of polydispersity, I, by means of SEC exceeds the precision obtainable by absolute methods by an order of magnitude (1).

When the obtainable precision of the detector
response to the eluting solution of a polymer, i.e.,
the error of the reading of physical quantity of the
other chromatogram coordinate is considered, it is
advantageous to start from physical capabilities of
the differential refractometer as the most universal
detector of the polymer concentration in the eluate.
The change in the refractive index of the polymer
solution at a given concentration in comparison with
the refractive index of the pure solvent will be de-
termined by the relationship

$$\Delta R.I. = \frac{dn}{dc} \times c_I \times \frac{w_I}{w_x} \tag{10}$$

The mean value of the refractive index incre-
ment is usually $dn/dc = 0.1$ (ml/g), the concentra-
tion of the polymer solution injected is usually,
$c_I = 0.001$ g/ml, and the ratio of the width of the
injection to the width of the eluting zone should be
$w_I/w_T = 0.05$ (4). The noise of the base-line of a
good quality differential refractometer is 5×10^{-8}
units of the refractive index and hence according
to Eq. (10) the obtainable precision of the detector
response in the vicinity of the maximum of the elu-
tion curve is approximately 1 %.

Approximately identical quantitative conclu-
sions are valid for the evaluation of the results of
the separations obtained by other methods, particu-
larly by highly efficient FFF method. With FFF
Eqs. (1) to (9) are not applicable owing to a sub-

stantially different separation mechanism. Since
theoretical relationship between molecular parame-
ters of separated polymers and retentions in FFF is
not known, an analogous consideration is based on
empirical calibration functions only. But it is
possible to take into consideration comparative
study of FFF and SEC (3) and fractionation power
obtained in FFF (5) and to state on the basis of
these results that the precision of the molecular
parameters calculated from FFF data, determined by
the precision of the coordinate of the elution vo-
lume of the fractogram,will be better than in SEC.

The evaluation of MWD, average molecular weights
and polydispersity index from chromatograms is car-
ried out by using a numerical calculation. The pro-
cedure for numerical processing of experimental
chromatograms and the above precision of physical
quantities that determine the position, the shape
and the width of the chromatogram reflect in the
precision of MWD and molecular parameters of poly-
mers under analysis. This problem was already solved
partially in the preceding papers (6,7), and will
be analyzed in further detail, with particular res-
pect to polymers with narrow and ultra-narrow MWD.

The log-normal distribution, which is a good
approximation of SEC chromatograms of polymers with
narrow MWD, and the Poisson distribution, which is
the most probable distribution of commonly used po-
lystyrene standards prepared by anionic polymeriza-
tion, will be used for theoretical model calcula-
tions.

Molecular weights are calculated from chroma-
tograms according to the following relationship

$$M_w = \frac{\int_{0}^{+\infty} M \; W(M) \; dM}{\int_{0}^{+\infty} W(M) \; dM} \qquad (11)$$

$$M_n = \frac{\int_{0}^{+\infty} W(M) \; dM}{\int_{0}^{+\infty} M^{-1} \; W(M) \; dM} \qquad (12)$$

and polydispersity index is calculated according to the relationship

$$I = M_w / M_n \qquad (13)$$

The following relationship is valid between the chromatogram (fractogram) and the differential distribution

$$W(M) = G(V) \; (\overset{+}{-}dV / dM) \qquad (14)$$

The detector response, i.e., the values of $G(V)$ suffer from the error δ discussed in the preceding text. In order to evaluate average molecular weights, Eqs. (11) and (12) can be written in the following forms

$$M_w = \frac{\int_0^{+\infty} M[G(V) \overset{+}{\underset{-}{}} \delta] \, dV}{\int_0^{+\infty} [G(V) \overset{+}{\underset{-}{}} \delta] \, dV} \tag{15}$$

$$M_n = \frac{\int_0^{+\infty} [G(V) \overset{+}{\underset{-}{}} \delta] \, dV}{\int_0^{+\infty} M^{-1} [G(V) \overset{+}{\underset{-}{}} \delta] \, dV} \tag{16}$$

Log-normal weight MWD is defined by the relationship

$$W(\ln M) = \exp[-(\ln M - \ln M_{max})^2 / 2\sigma^2] / (2\pi\sigma^2)^{1/2} \tag{17}$$

It applies further that

$$M_w = M_{max} \exp(\sigma^2 / 2) \tag{18}$$

$$M_n = M_{max} \exp(-\sigma^2 / 2) \tag{19}$$

$$\ln(M_w / M_n) = \sigma^2 \tag{20}$$

Log-normal differential distribution curve of molecular weights is demonstrated schematically in Fig. 1. It is obvious that it holds

$$\ln M = \ln M_{max} \overset{+}{\underset{-}{}} x\sigma \tag{21}$$

TABLE 2

Values of the Multiple of the Standard Deviation,
Calculated for Relative Values of W(ln M) Function
with Regard to the Maximum from Eq. (23)

W(ln M)/W(ln M_{max}) (% rel.)	5	2	1	0.5	0.2	0.1
x	2.45	2.80	3.03	3.26	3.53	3.72

It can be written further

$$W(\ln M) / W(\ln M_{max}) = \exp(-x^2 / 2) \qquad (22)$$

If readable level of the ratio W(ln M) / W(ln M_{max})
is adjusted to be equal to an assumed error or to
the precision of the reading of the detector respon-
se, then the width of the distribution or integra-
tion limits can be calculated with respect to the
maximum of the distribution curve, in which it makes
sense to read W(ln M) values according to the modi-
fied relationship

$$x = \sqrt{-\ln[W(\ln M) / W(\ln M_{max})]^2} \qquad (23)$$

Values of x, i.e., multiples of the standard devia-
tion, σ , (with regard to the maximum of the distri-
bution curve) that define the range of W(ln M) va-
lues exceeding the level of the adjusted error of the
reading are listed in Table 2.
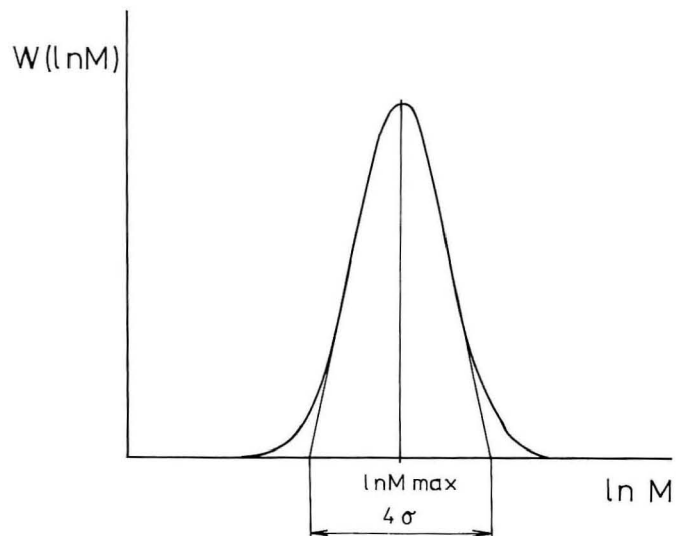
Figure 1. Log-normal distribution curve of molecular weights.

It is obvious that already the range $\pm 3\sigma$ from the maximum is sufficient for the reading of $W(\ln M)$ values provided that the precision of the detector response is ± 1 % of the $G(V_{max})$ value.

Polydispersity index, I, can be calculated by using Eq. (20), e.g., by graphical evaluation of the σ values. Let us calculate what effect the error of the reading of σ will have on polydispersity. For the ratio of the apparent polydispersity index to the correct polydispersity index it then holds

$$I_a = I(\sigma_a/\sigma)^2 \qquad (24)$$

Fig. 2 demonstrates the dependence of the percentage difference between the apparent and the real
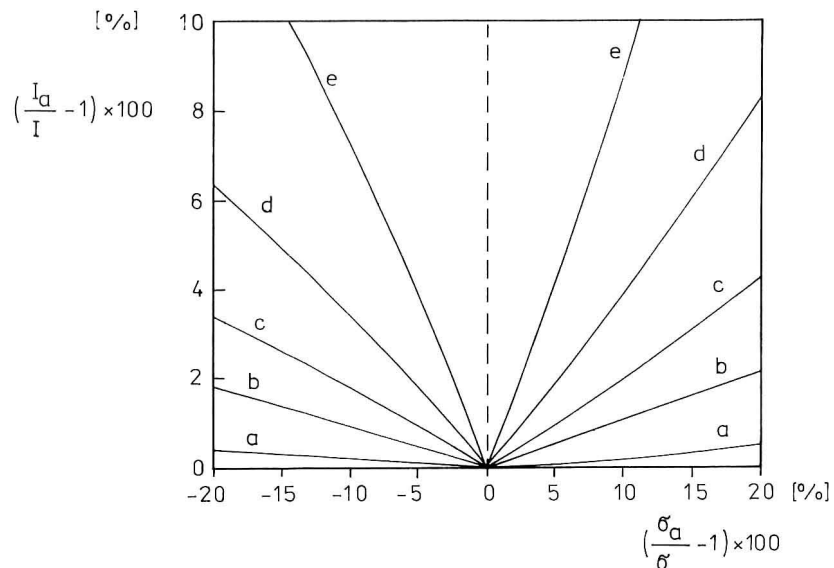
Figure 2. Dependence of the difference between the values of apparent and real polydispersity on the difference between the correct and the apparent values of the read standard deviation of the log-normal molecular weight distribution.

polydispersity values, i.e., $(\frac{I_a}{I} - 1) \times 100$ on the percentage difference between the correct and the apparent values of σ, i.e.,

$(\frac{\sigma_a}{\sigma} - 1) \times 100.$

It is obvious from Fig. 2 that for extremely low polydispersity values, I = 1.01, even the error 20 % rel. of the reading of σ values has practically no effect. σ value can reliably be determined graphically with the precision of ca. 5 % rel., which is the precision satisfactory up to polydispersity values I = 1.2. For higher polydispersity

values a more precise reading of σ values is de-
sirable.

In practice the distribution function is mostly
neither known nor can be predicted and the values
of M_w, M_n or I must be obtained by numerical calcu-
lation from an experimental chromatogram. The proce-
dure for the numerical processing of experimental
data can influence final results. Important parame-
ters of the numerical calculation which must be ta-
ken into consideration are the total width of the in-
terval in which the reading of the peak heights in
the chromatogram is taken and the subsequent calcu-
lation performed and the density of the chromatog-
ram segmentation or the number of the heights read
in the whole width of the chromatogram. These para-
meters and their influence on the calculated molecu-
lar weights were already studied in the earlier pa-
pers (6,7), nevertheless for the purpose of the pre-
sent study it was necessary that general conclusions
of the previous study should be given more preci-
sion within the interval of narrow MWD.

The calculation was made by using Eqs. (11) to
(13) for the log-normal MWD defined by Eq. (17) in
the range of polydispersity values I = 1.01 - 1.50.
The results are presented in Table 3. The width
of the interval was selected so that it was possi-
ble to demonstrate the effect of the interval ex-
pansion into the heights range comparable with ex-
perimental errors of the detection, as they were
calculated in Table 2. The segmentation densities
were selected so that the range of two orders of
magnitude included all of the practical cases. Num-
ber of the points taken for individual cases in

TABLE 3

Dependence of Numerically Calculated Values of Poly-
dispersity of Model log-normal Molecular Weight Dis-
tributions on Different Parameters of the Numerical
Calculation

Segmenta-tion den-sity $\Delta\sigma$	Deviation of numerically calculated va-lues of polydispersity from the given values %				
	Given value of I				
Δ	1.01	1.05	1.10	1.20	1.50
Interval width $M_{max} \pm n\sigma$ with n = 4.0					
1	-0.0001	-0.0006	-0.0016	-0.0054	-0.0334
0.1	-0.0009	-0.0061	-0.0155	-0.0448	-0.2149
0.01	-0.0011	-0.0071	-0.0182	-0.0521	-0.2456
Interval width $M_{max} \pm n\sigma$ with n = 3.5					
1	-0.0007	-0.0045	-0.0117	-0.0353	-0.1810
0.1	-0.0054	-0.0324	-0.0776	-0.2037	-0.8201
0.01	-0.0063	-0.0374	-0.0889	-0.3212	-0.9157
Interval width $M_{max} \pm n\sigma$ with n = 3.0					
1	-0.0043	-0.0265	-0.0649	-0.1764	-0.7514
0.1	-0.0240	-0.1341	-0.3013	-0.7201	-2.4426
0.01	-0.0271	-0.1508	-0.3369	-0.7983	-2.6658

Table 3 is $2 \times (n/\Delta) + 1$, i.e., 9, 81 and 801
points with the interval width $\pm 4\sigma$.

The results presented in Table 3 show clearly
that for the evaluation of narrow MWD the neglec-
tion of the fractions eluting in the interval out-
side the limits of detectability (1 %) has no signi-

TABLE 4

Dependence of Numerically Calculated Values of Poly-
dispersity of Model log-normal Molecular Weight
Distributions on the Error of the Reading of the
Peak Height from the Base-line

Interval width $M_{max} \pm n\sigma$		Segmentation density $\Delta\sigma$		Error of G(V)	Deviation of numerically calculated values of polydispersity from the given values (%)		
					Given value of I		
n_{min}	n_{max}	Δ	(%)		1.01	1.10	1.50
4.0	4.0	1.0	+1		+0.2033	+2.6222	+22.6088
		0.1	+1		+0.1423	+1.6990	+12.4027
3.0	3.0	1.0	+1		+0.0779	+0.8014	+3.7781
		0.1	+1		+0.0266	+0.2004	−0.1921
		1.0	−1		−0.0915	−0.9996	−5.9769
		0.1	−1		−0.0771	−0.8347	−4.9342
3.0	4.0	1.0	0		−0.0029	−0.0581	−0.7398
		0.1	0		−0.0155	−0.2546	−2.3549
4.0	3.0	1.0	0		−0.0015	−0.0085	−0.0452
		0.1	0		−0.0095	−0.0623	−0.3046

ficant influence. Similarly the segmentation density,
provided that it is selected within rational limits,
has no significant influence.

In addition, the influence of experimental
errors of the detector response, i.e., of G(V) va-
lues, was studied with the use of Eqs. (15) and (16).
These parameters have not been studied yet. The re-
sults of the calculation for the log-normal distri-
bution are presented in Table 4.

The data in Table 4 show that not even systema-
tic errors the magnitude of which is equal to the
precision of the reading of G(V) values have any

substantial influence on the calculated polydisper-
sity values, provided that the distributions are
calculated in the boundaries within which the rea-
ding of G(V) values has still been reliable. The
segmentation density of the whole interval has again
only a minor effect. At the same time it is necessa-
ry to be aware of the fact that the error of all of
the G(V) values read, either +1 % or -1 %, depending
on the actual calculation, demonstrates extreme ca-
ses. In practice, this error will fluctuate for va-
rious G(V) values read and it will result in a cer-
tain statistical compensation of the set of all of
the errors. The extreme cases, as calculated and
presented in Table 4, can in practice correspond to
a wrong adjustment of the base-line, which can be
caused by the noise. In such a case the calculated
deviations correspond to the maximal values when
the absolute noise level reaches 2 % rel. of the de-
tector response in the chromatogram maximum.

In practical evaluation of the chromatogram,
the start and the end of the chromatogram reading
need not be at the same distance from the maximum
(in the present case judged from the width of the
interval in both directions from the maximum), which
can be caused by an uncertainty in precise adjust-
ment of the base-line. That is why also this poten-
tial influence was evaluated numerically. The re-
sults are presented in the second part of Table 4.
Even with the difference in the read interval width
$\pm 1\,\sigma$ both to the right and to the left from the ma-
ximum, which exceeds potential errors safely, the
values of polydispersity calculated numerically are
affected in an entirely negligible manner. For

illustration it is possible to compare analogous va-
lues from Table 3.

The Poisson distribution is defined by the re-
lationship

$$N (P - 1) = e^{-r} r^{(P - 1)} / (P - 1) !$$
$$P = 1, 2, 3 \ldots \qquad\qquad (25)$$

For the weight distribution it then holds

$$W(P) = P \cdot N(P) / P_n \qquad\qquad (26)$$

For number and weight averages of the polymerization
degree and for polydispersity it further holds

$$P_n = \sum_0^\infty N(P) \cdot P \qquad\qquad (27)$$

$$P_w = \sum_0^\infty W(P) \cdot P = (\sum_0^\infty N(P) \cdot P^2) / P_n \quad (28)$$

$$I = P_w / P_n = (r + 1) / r \qquad\qquad (29)$$

Model Poisson distribution curve of molecular
weights is illustrated in Fig. 3. In comparison with
the log-normal distribution the Poisson distribution
is in coordinates of molecular weights symmetrical
enough, and its symmetry increases with increasing
value of r. The log-normal distribution, on the other
hand, is strongly asymmetrical in these coordinates;
it is symmetrical in the coordinate of the logarithm
of molecular weight. The Poisson distribution is,
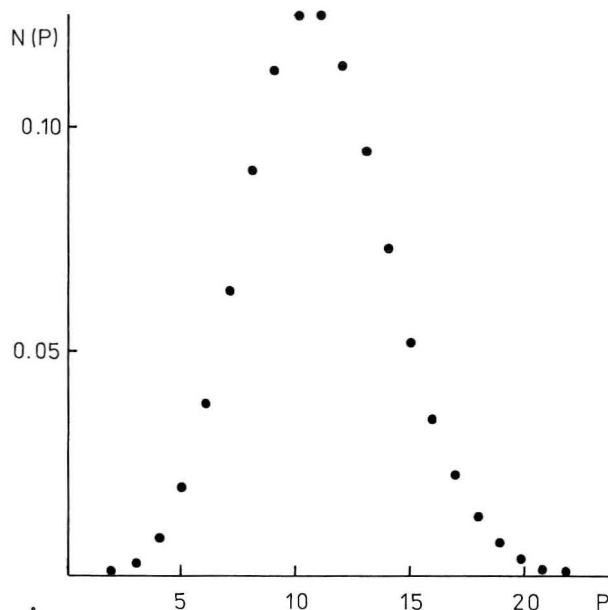
Figure 3. Poisson distribution curve of polymeri-
zation degrees. (r = 10, $I_{theor.}$ = 1.10)

moreover, a distribution function of discrete values
of the polymerization degrees, which, understanding-
ly, is a more correct image of physical reality.

The Poisson distribution, defined by Eqs.
(25) - (28), was used together with Eqs. (15) and
(16) for model calculations analogous to those in
the above case of the log-normal distribution. With
respect to the character of the Poisson distribution
the basic parameters of numerical calculations were
selected in a rather different manner. The initial
values of parameter,r = 10, 30 and 100,correspond to
the values of polydispersity, I = 1.10, 1.033 and
1.01, respectively. The width of the interval of the

TABLE 5

Dependence of Numerically Calculated Values of Poly-
dispersity of Model Poisson Molecular Weight Distri-
butions on the Error of the Reading of the Peak
Height from the Base-line

Given value of r	Interval width P_{min} P_{max}		Error of N(P) (%)	Deviation of numerically calculated from theoreti-cal polydispersity values (%)
10	3	21	0	−0.1951
			+0.5	+0.0112
			−0.5	−0.4077
			+1.0	+0.2115
			−1.0	−0.6270
30	16	48	0	−0.0781
			+0.5	−0.0009
			−0.5	−0.1573
			+1.0	+0.0743
			−1.0	−0.2386
100	72	132	0	−0.0226
			+0.5	+0.0026
			−0.5	−0.0485
			+1.0	+0.0271
			−1.0	−0.0750

numerical calculation, i.e., the minimal and the ma-
ximal values of the polymerization degree, P, were
selected so that the value of the function in the
given point has just exceeded 1 % from the maximal
value of N(P) function. This selection was again
taken with respect to the precision of the reading
of the peak heights in the chromatogram that is usu-
ally reached. In analogy with Eqs. (15) and (16),
the effect of systematic errors of the reading of
N(P) values was investigated. The results are summa-

rized in Table 5. The deviations of polydispersity
values calculated by the procedure suggested above
from the theoretical values calculated according to
Eq. (29) are minimal and their expression in terms
of per cents in Table 5 demonstrates clearly that
with real assumed errors of experimental chromatog-
rams it is possible to reach a high reliability of
the calculation of polydispersity values.

CONCLUSIONS

Numerical calculations of molecular parameters
of model log-normal and Poisson molecular weight dis-
tributions showed clearly the quantitative extent in
which the procedure for the calculation can affect
the accuracy and the precision of the result. With
the particular method of the calculation the manner
in which it is performed does not introduce any sig-
nificant errors into the result.

By analyzing decisive physico-chemical parame-
ters of separations from the viewpoint of their re-
producibility that can be obtained, the limits of
reliability of the calculated molecular parameters
of polymers with narrow and ultra-narrow MWD were
found. It was observed that the existing instrumen-
tal and experimental techniques make it possible to
determine these parameters with a high precision and
reliability. From this viewpoint the chromatographic
methods discussed above exceed the potentialities of
absolute methods, such as light-scattering, osmo-
metry etc. Molecular parameters and polydispersity
of polymers with narrow and ultra-narrow distribu-

tions of molecular weights in particular can be determined entirely reliably, with the precision exceeding by an order of magnitude other methods of characterization that have been used so far.

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LIST OF SYMBOLS

a   exponent of the Mark-Houwink equation

c_I concentration of injected sample

$G(V)$ chromatogram

I polydispersity index

K constant of the Mark-Houwink equation

K_{SEC} distribution coefficient

M molecular weight

M_{excl} excluded molecular weight

M_{max} molecular weight corresponding to the maximum of the chromatogram

M_w weight average molecular weight

M_n number average molecular weight

$N(P)$ number distribution of polymerization degree

dn/dc refractive index increment

P Polymerization degree

P_n number average polymerization degree

P_w weight average polymerization degree

R radius of equivalent hydrodynamic sphere of the macromolecule

r radius of cylindrical pore

$R.I.$ refractive index

V_e elution volume

V_i total pore volume

V_o total interstitial volume

$W(M)$ weight distribution of molecular weight

$W(P)$ weight distribution of polymerization degree

w_I width of the injection

w_x width of the elution curve

x multiple of standard deviation of the
               elution curve

δ error of chromatogram height reading

$[\eta]$ intrinsic viscosity

σ standard deviation of the elution curve

GEL PERMEATION CHROMATOGRAPHY : PROBLEMS CAUSED BY POLYDISPERSITY
IN THE APPLICATION OF THE BENOIT'S UNIVERSAL PARAMETER.

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ABSTRACT

The application of the Benoit's universal calibration
$Ln(\eta).M = f (Ve)$ in GPC is examined through the general problem of
polydispersity, when an attempt is made to represent a mixture of
different macromolecules by a single parameter $(\eta).\overline{M}$. The recently
proposed $(\eta).Mn$ parameter leads to an ambiguous interpretation of
universal calibration ; we try to classify the problems into three
families. For the comparison of linear homopolymers of different
polydispersities only by their peak apex, there is no general form
of universal parameter. In the characterization of heterogeneous
polymers, such as branched polymers or copolymers, $(\eta)_i.\overline{Mn}_i$ is the
form to be used in the GPC calculations. It leads to (η) and $\overline{Mn}$ when
using the GPC-viscometer coupling but $\overline{Mw}$ can only be strictly obtained
by the light scattering coupling. Finally, the apparent polydispersity
caused by instrumental spreading cannot generally be represented by
the $(\eta)_i.\overline{Mn}_i$ parameter.

INTRODUCTION

In the early days of Gel Permeation Chromatography (GPC) (1),
it already appeared that macromolecule separation was not directly
related to molecular weight, but was, rather, dependent upon molecu-
lar size. To take this effect into account, some parameters were
successively proposed :

- the extended chain length and the subsequent Q factor (2).

- the product $(\eta)^{1/3}.M^{1/2}$ (3) where (η) is the limiting viscosity
number and M the molecular weight. Besides their lack of theoretical

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*basis, those parameters do not exhibit the universal character
required for the interpfetation of GPC data. In 1966, Benoît et al
(4, 5) demonstrated the universality of the product (η).M as
a calibration concept. Besides its simplicity, this parameter is
the most suitable with regard to the basic support of the Flory's
theory (6) for linear polymers :*

$$(\eta) = \emptyset' . \frac{R_G{}^3}{M}$$

*where R_G is the polymer radius of gyration in solution, and Einstein's
equation (7) giving the viscosity of spherical particles in suspension :*

$$(\eta) = K . \frac{V}{M}$$

*where V is the particle volume. Analogously, Sadron (8) gave a
definition of the macromolecule hydrodynamic volume V_H :*

$$(\eta) . M = K . V_H$$

*The parameter (η).M is therefore proportional to the equivalent
volume of a macromolecule in a stream and assuming that the GPC sepa-
ration is only based upon size exclusion, it can be directly linked to
the elution volume by a "universal calibration curve". Except for some
highly branched polymers (9, 10), this concept has been widely verified
and is now unanimously accepted.*

*Using this single assumption, we have examined the influence of
polydispersity in the application of universal calibration. This
parameter is not taken into account in the theory but often confuses
the interpretation of GPC data. Problems related to polydispersity were
encountered in three different ways we have successively examined :*

- *comparison of polymers of different polydispersities*

- *polydispersity caused by structural or chemical heterogeneity*

- *polydispersity occuring from instrumental spreading.*

COMPARISON OF POLYMERS OF DIFFERENT POLYDISPERSITIES

General case.

*This is the most frequent problem. It is encountered every time
attempts are made to characterize the behaviour of a macromolecular
sample by a single point of the curve $Ln(\eta).M = f(V_e)$. It concerns
the molecular weight standards required to establish the calibration curve
but mainly the comparison of polymers with different polydisper-
sities using the universal calibration concept. We will assume here
that axial dispersion caused by instrumental spreading is negligible.*

Whereas a single molecular weight M is expected, a set of molecular
weights M_j, having a limiting viscosity number $(\eta)_j$ is practically
considered, the chromatogram spreading out a certain range in elution
volumes. As it is impossible to determine any set of values $(M_j, (\eta)_j,$
$Ve_j)$ corresponding to the monodisperse fraction j for the application
of the universal calibration concept, we have to use a set of mean
values. Some are directly measured, such as :

  - the number and weight-average molecular weights $\overline{Mn}, \overline{Mw}$

  - the peak elution volume Ve_p and, very rarely, the weight-average
elution volume $\overline{Ve}_w$ (11)

  - the limiting viscosity number $(\eta) = \dfrac{\sum_j (\eta)_j C_j}{\sum_j C_j}$

The average (η) being thus stated, the only coherent set of
values is, accordingly (12) : (η), the viscometric average molecular
weight $\overline{Mv}$ and Ve_v elution volume of the molecules j such as $M_j = \overline{Mv}$.
Unfortunately, this only valid solution :

$$ln(\eta).\overline{Mv} = f(Ve_v)$$

is inaccessible by experiment. It is, then, necessary to find the best
approach. Some as :

$$ln(\eta).\overline{Mw} = f(Ve_p)$$

or
$$ln(\eta)\sqrt{\overline{Mn}.\overline{Mw}} = f(Ve_p)$$

are the most widely used. Recently, $(\eta).Mn$ was proposed (13) as the
general parameter of universal calibration ; that leads to the confusion
of considering $(\eta).\overline{Mn}$ to represent the behaviour of a broad distribution.
This form, as any form such as $ln(\eta).\overline{Mx} = f(Ve_p)$ has no theoretical
basis because no average molecular weight associated with the intrinsic
viscosity and the peak elution volume can represent the universal
calibration concept. However, we shall demonstrate that simple forms
can be used for some models of molecular weight distribution : the
Wesslau distribution, and the Schulz distribution.

Case of the Wesslau distribution.

This simple model can be considered as a good approximation of the
distribution curve of some macromolecular samples. It is expressed by :

$$W(LnM) = \frac{1}{\beta\sqrt{\pi}} . exp(- \frac{1}{\beta^2} . ln^2 \frac{M}{M_p})$$

where W(LnM) is the weight fraction, M_p, the peak molecular weight

and β, a function of the distribution width. Average molecular weights are easily obtained by :

$$\overline{Mn} = M_p . exp(-\frac{\beta^2}{4})$$

$$\overline{Mv} = M_p . exp(a\frac{\beta^2}{4})$$

$$\overline{Mw} = M_p . exp(\frac{\beta^2}{4})$$

where a is the exponent of the Mark-Houwink relationship :

$$(\eta) = K\overline{Mv}^a$$

Consequently, we obtain : $M_p = \sqrt{\overline{Mn}.\overline{Mw}}$

By using this peak molecular weight, M_p, and the corresponding peak elution volume Ve_p, the viscosity to be used is : $(\eta)_p = KM_p^a$, which is different from the limiting viscosity number :

$$(\eta) = K\overline{Mv}^a = KM_p^a . exp(a^2.\frac{\beta^2}{4}) = (\eta)_p . exp(a^2.\frac{\beta^2}{4})$$

Accordingly, the simultaneous use of (η), M_p and Ve_p is not suitable. In order to apply (η), which is the only value available, we can write :

$$(\eta)_p . M_p = (\eta) . exp(-a^2.\frac{\beta^2}{4}) . M_p$$

which gives the expression of the average molecular weight $\overline{M}$ to be used :

$$\overline{M} = M_p . exp(-a^2.\frac{\beta^2}{4})$$

or

$$\overline{M} = \overline{Mn}^{\frac{1+a^2}{2}} . \overline{Mw}^{\frac{1-a^2}{2}}$$

This expression can be simplified by introducing the polydispersity index $I = \frac{\overline{Mw}}{\overline{Mn}}$:

$$\overline{M} = \overline{Mn}.I^{\frac{1-a^2}{2}} = \overline{Mw}.I^{\frac{-1-a^2}{2}}$$

Consequently, in the case of the Wesslau distribution, the correct form of the universal calibration is :

$$ln((\eta).\overline{Mn}.I^{\frac{1-a^2}{2}}) = f(Ve_p)$$

As this equation is difficult to apply, we have examined the errors introduced when using the classical approximations $(\eta).\overline{Mn}$, $(\eta).\overline{Mw}$ and $(\eta).\sqrt{\overline{Mn}.\overline{Mw}}$ plotted in Figure 1. The errors are :
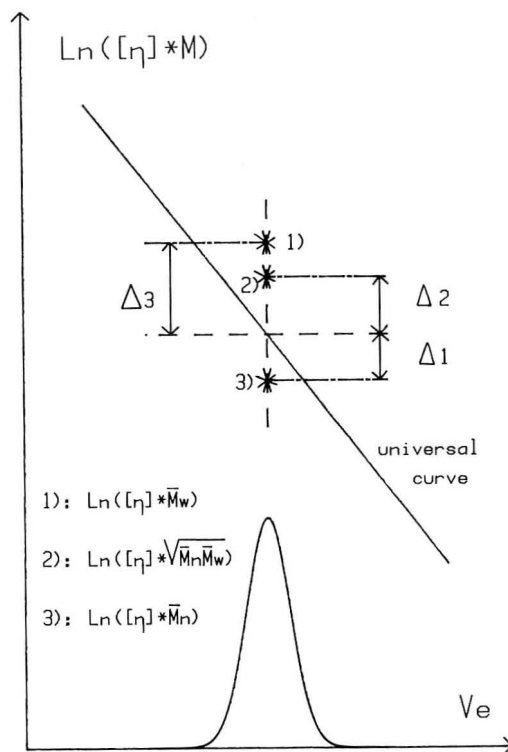
Figure 1 : *Errors introduced by the classical approximations*
in the case of the Wesslau distribution.

for $(\eta).\overline{Mn}$: $\Delta_1 = -\frac{1}{2} lnI.(1-a^2)$

for $(\eta).\sqrt{\overline{Mn}.\overline{Mw}}$: $\Delta_2 = \frac{1}{2} lnI.a^2$

for $(\eta).\overline{Mw}$: $\Delta_3 = \frac{1}{2} lnI.(1+a^2)$

The parameter $(\eta).\overline{Mw}$ obviously appears to be the worst one.
The best approach depends upon the Mark Houwink exponent value a.
when a < 0.7 it is $(\eta)\sqrt{\overline{Mn}.\overline{Mw}}$
when a > 0.7 it is $(\eta).\overline{Mn}$

In addition, $(\eta).\overline{Mn}$ is only strictly exact when a = 1, which
is a limiting form rarely encountered. Finally, we notice that, for

the classical value $a = 0.7$ (i.e.polystyrene in THF), the universal calibration parameter is :

$$[\eta].\overline{Mn}.I^{0,25} \quad or \quad [\eta].\overline{Mw}.I^{-0,75}$$

As an example, let us consider the situation when two polymers of different polydispersities, $I \sim 1$ and $I \sim 2$, having the same peak elution volume, are compared. In using the $[\eta].\overline{Mn}$ parameter, the $\overline{Mn}$ value is found to be approximatly 20% too high. Conversely, when applying the $[\eta].\overline{Mw}$ parameter, an incorrect value of $\overline{Mw}$ about 40% too small is obtained.

Case of the Schulz distribution.

The Schulz distribution (15) is theoretically calculated in classical cases of polymerization or polycondensation. This model can represent the distribution curve of an important portion of macromolecular samples.

Its expression is :

$$W(M) = \frac{1}{\Gamma(1+\nu)} \, \gamma^{\nu+1} M^{\nu}.exp(-\gamma M)$$

where: - Γ is the classical gamma function :

$$\Gamma(n) = \int_{0}^{\infty} e^{-x} x^{n-1} dx$$

- ν and γ are parameters related to the average molecular weights as follows :

$$\overline{Mn} = \nu/\gamma$$
$$\overline{Mw} = (\nu+1)/\gamma$$
$$\overline{Mw}/\overline{Mn} = 1 + 1/\nu$$

The viscosity-average molecular weight $\overline{Mv}$ can be easily calculated (a is the Mark-Houwink exponent) :

$$\overline{Mv} = \frac{1}{\gamma}\left[\frac{\Gamma(1+\nu+a)}{\Gamma(1+\nu)}\right]^{\frac{1}{a}}$$

In GPC, the logarithmic shape of elution chromatograms leads to the peak representation :

$$W(lnM) = \frac{1}{\Gamma(1+\nu)}.\gamma^{\nu+1} M^{\nu+1} exp(-\gamma M)$$

In this case, the weight-average molecular weight $\overline{Mw}$ is located at the peak apex, whatever the values of the two parameters ν and γ may be.

As we have the peak elution volume Ve_p and the peak molecular weight $M_p = \overline{Mw}$, the intrinsic viscosity at the peak apex is :

$$(\eta)_p = K.\overline{Mw}^a$$

When using the limiting viscosity number (η), the average molecular weight $\overline{M}$ to be used is then defined by :

$$(\eta).\overline{M} = K\overline{Mw}^{\,a+1}$$

With the above mentioned $\overline{Mv}$ value, it comes :

$$\overline{M} = \overline{Mw}.(\nu+1)^a.\frac{\Gamma(1+\nu)}{\Gamma(1+\nu+a)}$$

Such a result is not practically easy to use, but calculation shows that the $(\nu+1)^a.\frac{\Gamma(1+\nu)}{\Gamma(1+\nu+a)}$ factor remains, in the current cases, near unity. For example :
- when $\nu = 1$, the Schulz distribution is called the most probable distribution. The mean $\overline{M}$ to be used is :

$$\overline{M} = \overline{Mw}.\frac{2^a}{\Gamma(2+a)}$$

where the $2^a/\Gamma(2+a)$ parameter varying from 1 (for $a = 1$) to 1.06 (for $a = 0.5$).
- $\nu = 2$ corresponds to the vinyl polymerization when termination is only due to radical combination.

Here : $\overline{M} = \overline{Mw}.\frac{2.3^a}{\Gamma(3+a)}$

with $1_{(a=1)} \leqslant \frac{2.3^a}{\Gamma(3+a)} \leqslant 1.04_{(a=0.5)}$

As a result, in the case of the Schulz distribution, the use of the relationship :

$$ln((\eta).\overline{Mw}) = f(Ve_p)$$

is clearly the best one. It never introduces errors greater than a few percent, contrary to the Wesslau distribution.
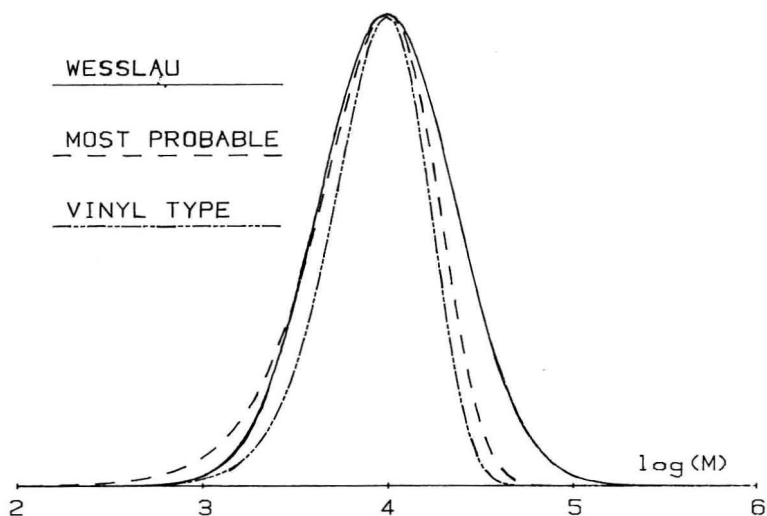
Figure 2 : Comparison of three model chromatograms with the
same peak molecular weight.

As a conclusion of this first part, we have shown the importance
of the molecular weight distribution in the use of the Benoit's cali-
bration concept. Figure 2 is an example of the distributions we have
developed here.

POLYDISPERSITY CAUSED BY STRUCTURAL OR CHEMICAL HETEROGENEITY.

The principle of macromolecule separation according to hydrodynamic
volume leads to a separation of linear homopolymers by molecular weight.
But in the other cases (branched polymers, copolymers, ...) many very
different molecules can exhibit the same hydrodynamic volume.

Accordingly, each fraction in the detector cell has a poly-
dispersity in molecular weight and one detector can only provide a
mean value $\overline{M}_i$ as a function of the elution volume Ve_i. To get more
information on the molecular weight distribution, a second detector
analyzing another polymer property must be coupled to the concentration
detector.

The GPC - viscometer coupling.

This is the case when an on-line viscometer (14) is added to the
GPC instrument to provide the eluant viscosity continuously. We herein

mention the papers of Constantin (16) and Hamielec (13), related to branched polymer analysis. At the elution volume Ve_i, a mixture of different macromolecules M_{i1}, M_{i2}... M_{ij} with the same hydrodynamic volume $((\eta).M)_i$ are present in the detector cell (band broadening is again neglected) :

$$(\eta)_{i1}.M_{i1} = (\eta)_{i2}.M_{i2} = \ldots (\eta)_{ij}.M_{ij} = ((\eta).M)_i$$

As the viscometer measures :

$$(\eta)_i = \frac{\sum_j (\eta)_{ij}.C_{ij}}{\sum_j C_{ij}}$$

that is :

$$(\eta)_i = ((\eta).M)_i . \frac{\sum_j \frac{C_{ij}}{M_{ij}}}{\sum_j C_{ij}}$$

we obtain :

$$((\eta).M)_i = (\eta)_i.\overline{Mn}_i$$

This particular result means that, when measuring continuously the viscosity $(\eta)_i$, the universal calibration involves the number-average molecular weight of each fraction $\overline{Mn}_i$. Consequently, by integrating across the whole chromatogram, only the number-average molecular weight $\overline{Mn}$ can be strictly obtained. But practically, the other average molecular weights are calculated with a non-significant error compared to other experimental errors (16).

Conclusively, $(\eta)_i.\overline{Mn}_i$ and not $(\eta).\overline{Mn}$, appears to be the real parameter of the universal calibration in this case.

The GPC - light scattering coupling.

The problem is different here, the weight-average molecular weight $\overline{Mw}_i$ being continuously determined. The application of the universal calibration must lead to the determination of the limiting viscosity number (17).

With
$$\overline{Mw_i} = \frac{\sum_j M_{ij}.C_{ij}}{\sum_j C_{ij}}$$
and
$$M_{ij} = \frac{((\eta).M)_i}{(\eta)_{ij}}$$

we see that :

$$((\eta).M)_i = \frac{\sum_j C_{ij}}{\sum_j \frac{C_{ij}}{(\eta)_{ij}}} . \overline{Mw}_i$$

That is, dividing $((\eta).M)_i$ by $\overline{Mw}_i$, we arrive at an apparent
viscosity :

$$(\eta)'_i = \frac{\sum\limits_j C_{ij}}{\sum\limits_j \dfrac{C_{ij}}{(\eta)_{ij}}}$$

which is an unusual average viscosity, different from the classical
limiting viscosity number $(\eta)_i$. In replacing $((\eta).M)_i$ by its previously
demonstrated value $(\eta)_i.\overline{Mn}_i$ and using the instantaneous polydispersity
$I_i = \overline{Mw}_i/\overline{Mn}_i$;

We have : $(\dot\eta)'_i = (\eta)_i/I_i$

This simple result shows that the GPC-light scattering coupling
leads to the real intrinsic viscosity $(\eta)_i$ for linear homopolymers
$(I_i = 1)$, but that an apparent viscosity $(\eta)'_i < (\eta)_i$ is obtained for
polymers exhibiting a polydispersity in branching or composition. The
higher the heterogeneity, the greater the difference between the apparent
and the real viscosity.

Multidetection.
The above results lead to the conclusion (13) that the dual GPC-
viscometer-light scattering coupling is the only way to strictly deter-
mine the different structural parameters of complex polymers such as low
density polyethylene. The variations of $(\eta)_i$, $\overline{Mn}_i$, $\overline{Mw}_i$ can then be
measured as a function of the hydrodynamic volume allowing the deter-
mination of the instantaneous polydispersity and, therefore, the
calculation of (η), $\overline{Mn}$ and $\overline{Mw}$ by integrating across the whole chroma-
togram.

POLYDISPERSITY OCCURING FROM INSTRUMENTAL SPREADING.

Finally, we examine the effects of instrumental spreading, hitherto
neglected, that lead to an apparent polydispersity in the detector cell.
In the simple case of a linear homopolymer, at the elution volume
Ve_i, although we would expect a single molecular weight M_i, a mixture of
various macromolecules with close hydrodynamic volumes is present.
For each macromolecule j, the hydrodynamic volume equation gives :

$$(\eta)_{ij} \cdot M_{ij} = K \; V_{Hij}$$

The limiting viscosity number $(\eta)_i$ *can then be written :*

$$(\eta)_i = \frac{\sum\limits_{j} \cdot \dfrac{K \; V_{Hij}}{M_{ij}} \cdot c_{ij}}{\sum\limits_{j} c_{ij}}$$

and using the number n_{ij} *of macromolecules j :*

$$(\eta)_i = K \; \frac{\sum\limits_{j} V_{Hij} \; n_{ij}}{\sum\limits_{j} n_{ij}} \cdot \frac{\sum\limits_{j} n_{ij}}{\sum\limits_{j} n_{ij} M_{ij}}$$

that can be expressed by :

$$(\eta)_i \cdot \overline{M_n}_i = K (\overline{V_{Hn}})_i$$

where $(\overline{V_{Hn}})_i$ *is the number-average hydrodynamic volume. This relation-*
ship, previously demonstrated by Newman et al (18) could lead us to use
the $(\eta)_i \cdot \overline{M_n}_i$ *parameter to take into account the instrumental*
spreading. Unfortunately, except for some particular shape of
spreading distribution, the hydrodynamic volume V_{Hi} *is different*
from the number average $(\overline{V_{Hn}})_i$ *of the mixture. Consequently, the*
average molecular weight $\overline{M}_i$, *deduced from universal calibration, is*
not the number-average $\overline{M_n}_i$, *but a non-classical average near* M_i,
depending upon the spreading function that does not permit the correc-
tion of band spreading.

CONCLUSION

We find a complex situation, especially for the simple comparison
between polymers with different polydispersities in universal calibra-
tion. Using the experimental values of the intrinsic viscosity (η)
and the peak elution Ve_p, *there is no general rule that leads to a*
single average molecular weight $\overline{M}$ *to point out a general universal*
parameter $(\eta) \cdot \overline{M}$. *In the Wesslau distribution :* $\overline{M} = \overline{Mw} \cdot I^{\frac{-1-a^2}{2}}$ *rather*
different from $\overline{Mw}$ *in practice, but in the Schulz distribution* $\overline{M} \simeq \overline{Mw}$.
Conclusively, the knowledge of the distribution shape and the Mark
Houwink exponent are required to select the most suitable universal
parameter.

By contrast, in the analysis of heterogeneous polymers (branched
or copolymers), $(\eta)_i \cdot \overline{M_n}_i$ *is the real information provided by the*

universal calibration and must be used in the calculation of molecular weights. Viscometric coupling specifically gives (η) and $\overline{Mn}$, whereas, with light-scattering coupling only $\overline{Mw}$ is correctly determined. Both detectors are required to achieve the complete characterization of complex polymers.

Finally, $(\eta)_i . \overline{Mn}_i$ cannot be used for the correction of the apparent polydispersity in the detector cell caused by instrumental spreading.

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HYDROPHOBICITY AND RETENTION IN REVERSED PHASE LIQUID CHROMATOGRAPHY

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ABSTRACT

Reversed phase liquid chromatography retention data for several compounds are examined in relationship to their hydrophobicities. Alcohols and various aromatics are used to compare hydrophobicities in aliphatic and aromatic compounds. Capacity factors, k', can be correctly evaluated by using the hydrophobic factors (log P) derived from the hydrophobic fragmental constants. Appropriate solvent mixtures to achieve good separations can be choosen from graphical data.

INTRODUCTION

The behaviour of a solute in a chromatographic system is a constant preoccupation for the analytical chemist. Distribution coefficients of ionizable compounds have been related to capacity ratios in a system using polystyrene gel as packing (1). Retention of dipolar acids on ion-exchange resin has been explained

through ion-ion interaction. Optimization of chromatographic con-
ditions for non-ionizable compounds has been discussed by many
researchers ; solvent effects (2), molecular size effects (3), and
branching effects (4) give useful information for the prediction
of the retention time. Molecular size effects have also been used
to study the influence of alkyl groups on capacity factors (5-8).

Optimization of procedures in reversed phase liquid
chromatograhy, RPLC, requires some knowledge of the separation
mechanism. Various modes of interaction are possible. Non-polar
solid supports give rise to absorption on a surface which may
behave both as if it were a solid and a liquid. Solute retention
can be envisaged as a reversible association between the
hydrocarbonaceous surface and the solute molecule. This type of
interaction is called hydrophobic interaction (9). It is the
result of a repulsion between the chromatographic support and
water as well as between water and the solute : it may be said
that water forces the association between the solute and the
support. The interaction can also be viewed as a partition
between one liquid, the aqueous eluent and a second pseudoliquid,
the solid hydrocarbonaceous support.

Rekker's hydrophobic fragmental constants (10) have been
useful in evaluating hydrophobicities of aromatic as well as
aliphatic compounds. The concept of hydrophobicity is quite
helpful in the development of new drugs. It may also be useful in
the optimization of reversed phase liquid chromatography.

Attempts, based on the hydrophobicities of various alcohols, were made in order to predict the relative retention of theses solutes on a polystyrene gel using methanol-water or acetonitrile-water mixtures as eluent (11). In the present paper, capacity factors of alcohols, fatty acids, and various aromatic compounds such as polyaromatic hydrocarbons, alkylbenzenes and benzoates are related to the hydrophobicities of the molecules. Solvent composition permitting good separations is also discussed in terms of the observed relationship between retention and hydrophobicity.

Hydrophobicity

The hydrophobicity of a solute is conveniently estimated from its partition coefficient, P, between an organic solvent and water. The larger the value of P, the more hydrophobic the substance is. Rekker (10) has shown that

$$\log P = \sum a_i \ F_i \qquad\qquad [1]$$

where a_i represents the number of times a particular fragment "i" is found in a given molecule and F_i is the hydrophobic fragmental constant of fragment "i". Thus a molecule can be visualized as a series of fragments, each of which contributes to its hydrophobic character.

An extensive study of the partition coefficients of various solutes (aromatics and aliphatics) in octanol-water led to the various fragmental constants reported by Rekker (10). Arbitrarily, but based on practical considerations, the log P value of a

given solute as calculated using equation [1] and the F_i values derived from the partition coefficients in octanol-water, is called the hydrophobic factor of this solute. In liquid chromatography, provided the hydrophobic effect is the sole interaction,

$$\log P = A* + \log k'$$

In this equation, A* is a constant and k' is the capacity factor. Thus, a straight line with unit slope should be observed in a system using octanol-water as eluent. It is easily shown that for retention using another eluent,

$$\log k' = a + \beta \log P \qquad [2]$$

where "a" is a constant and the slope β, a characteristic of the eluent.

EXPERIMENTAL

A chromatographic system was assembled from various modules. Pumps were Waters, model 6000 A (Water Associates Inc., Milford, MA 01757), and Altex, model 100 (Altex Scientific Inc., Berkeley, CA 94710). Detectors were Waters differential refractive index detector, Model R401, and a Hitachi spectrophotometer, model 100-20 (Hitachi, Mountain View, CA 94043) equipped with and $8\text{-}\mu\text{L}$ flowthrough cell from Altex. Injector was Altex, model 905-19. Recorders were Brinkmann, model 2541 (Brinkmann Instruments, Inc., Westbury, NY 11590), or Linear Instruments, Model 915 (Linear Instruments Corp. Irvine, CA 92714).

Chemically bonded octadecyl packing was LC 7 from Johns-Manville (Johns-Manville, Denver, CO 80217) packed in our laboratory

TABLE 1

HYDROPHOBIC FACTORS

COMPOUNDS	log P		COMPOUNDS	log P
Polyaromatics			**Alcohols**	
1. Benzene	2.16		17. Butyl Alcohol	0.80
2. Naphtalene	3.18		18. Pentyl Alcohol	1.33
3. Anthracene	4.20		19. Hexyl Alcohol	1.86
4. Pyrene	4.50		20. Heptyl Alcohol	2.39
			21. Octyl Alcohol	2.92
Alkylbenzenes			22. Decyl Alcohol	3.98
			23. Dodecyl Alcohol	5.04
5. Toluene	2.59			
6. Ethylbenzene	3.12		**Fatty Acids**	
7. Isopropylbenzene	3.52			
			24. Hexanoic Acid	1.87
Benzoates			25. Octanoic Acid	2.93
			26. Decanoic Acid	3.99
8. Methylbenzoate	2.15		27. Dodecanoic Acid	5.05
9. Isopropylbenzoate	3.09		28. Tetradecanoic Acid	6.11
10. Butylbenzoate	3.74		29. Hexadecanoic Acid	7.17
11. Isopentylbenzoate	4.15		30. Octadecanoic Acid	8.23
			31. Eicosanoic Acid	9.29
Substitued Benzenes			32. Palmitoleic Acid	6.58
			33. Oleic Acid	7.64
12. Aniline	1.03		34. Linoleic Acid	7.05
13. Phenol	1.54		35. Linolenic Acid	6.46
14. Benzoic Acid	1.79		36. Arachidonic Acid	6.98
15. Chlorobenzene	2.81			
16. Bromobenzene	3.02			

by a balanced slurry method in 19.7 cm long, 3.2 mm I.D. stainless steel column fitted with zero dead volume attachments, Altex, No 250-21, or Swagelok (Crawford Fitting CO., Solon Ohio 44139). Chemicals came from various sources ; the list of the different solutes studied is given in Table 1. Solvents were HPLC grade ; water was first distilled in glass and further purified through a Milli-Q system (Millipore, Corp., Bedford, MA 01730).

RESULTS AND DISCUSSION

Capacity factors (k') were evaluated from at least two repro-
ducible chromatograms for each compound. Various solvent mixtures
were used as eluents. Uric acid, eluting faster than any other
solute, served to measure the dead volume of the column. The
hydrophobic factor, log P, was calculated by summing up Rekkers's
hydrophobic fragmental constants (eq 1) of all fragments in the
molecule ; the values obtained are given in table 1. As observed
in Figures 1 to 3, a good linearity exists between log k' and the
hydrophobicity of the molecule. Equation 2 is generally obeyed ;
least squares analysis shows that the linear model explains 95+%
of the variations observed.

Figure 1 shows the relationship between log k' and log P for
alcohols, polyaromatics, benzoates, alkylbenzenes, benzene deriva-
tives and fatty acids in 50% acetonitrile in water as eluent. All
these compounds, except the fatty acids, lie on the same straight
line. Capacity factors of fatty acids are always lower than those
observed for the other types of compounds having similar hydropho-
bicities. This behaviour might be due to ionization of the acids
in acetonitrile-water mixtures and/or because of the high polarity
of the solutes.

In Figure 2, the variation of capacity factors (log k') as a
function of hydrophobicities is shown for various acetonitrile-
water mixtures. Equation 2 holds for solvent mixtures containing
between 20 and 80% acetonitrile in water, and for non-ionizable
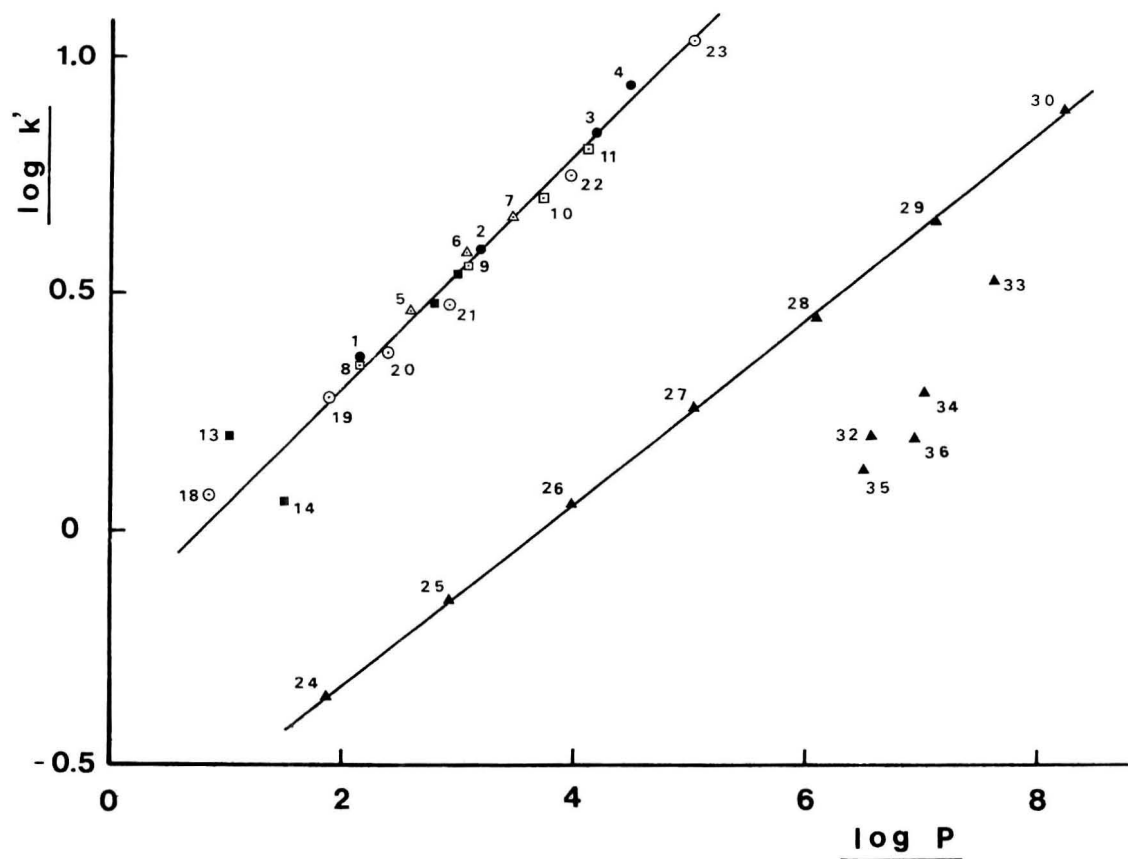
FIGURE 1. Relationship between the logarithm of capacity ratio and hydrophobic factors in 50% $^V/v$ aqueous acetonitrile on octadecyl packing. ⊙ alcohols , ● polyaromatics ; ▣ benzoates ; △ alkyl-benzenes ; ■ benzene derivatives ; ▲ fatty acids. Numbers : see Table 1.
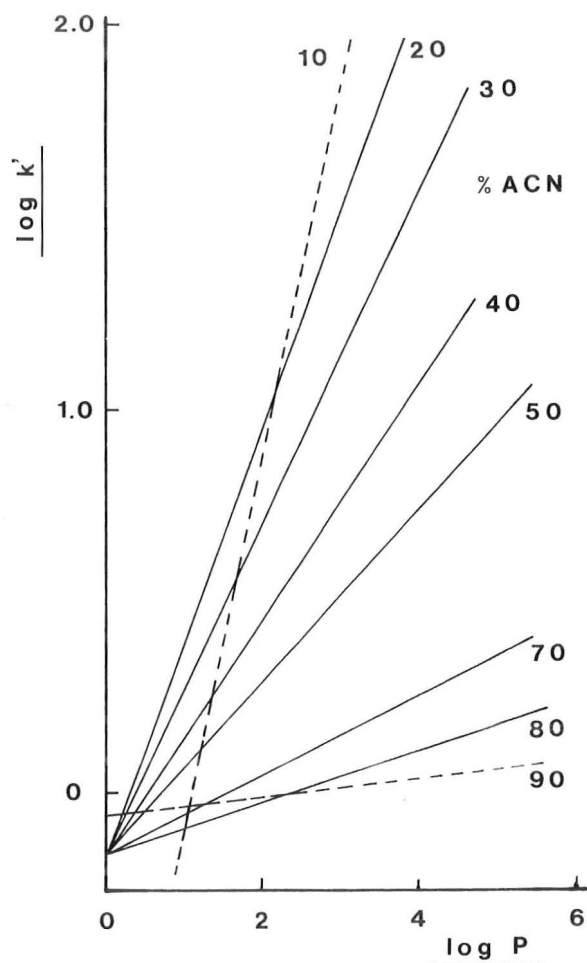
FIGURE 2.

Variation of the capacity ratio (log k') as a function of hydro-
phobicity (log P) in various acetonitrile (% ACN) eluents. N.B.
For clarity in the graphical representation, experimental points
are not indicated on the drawing.
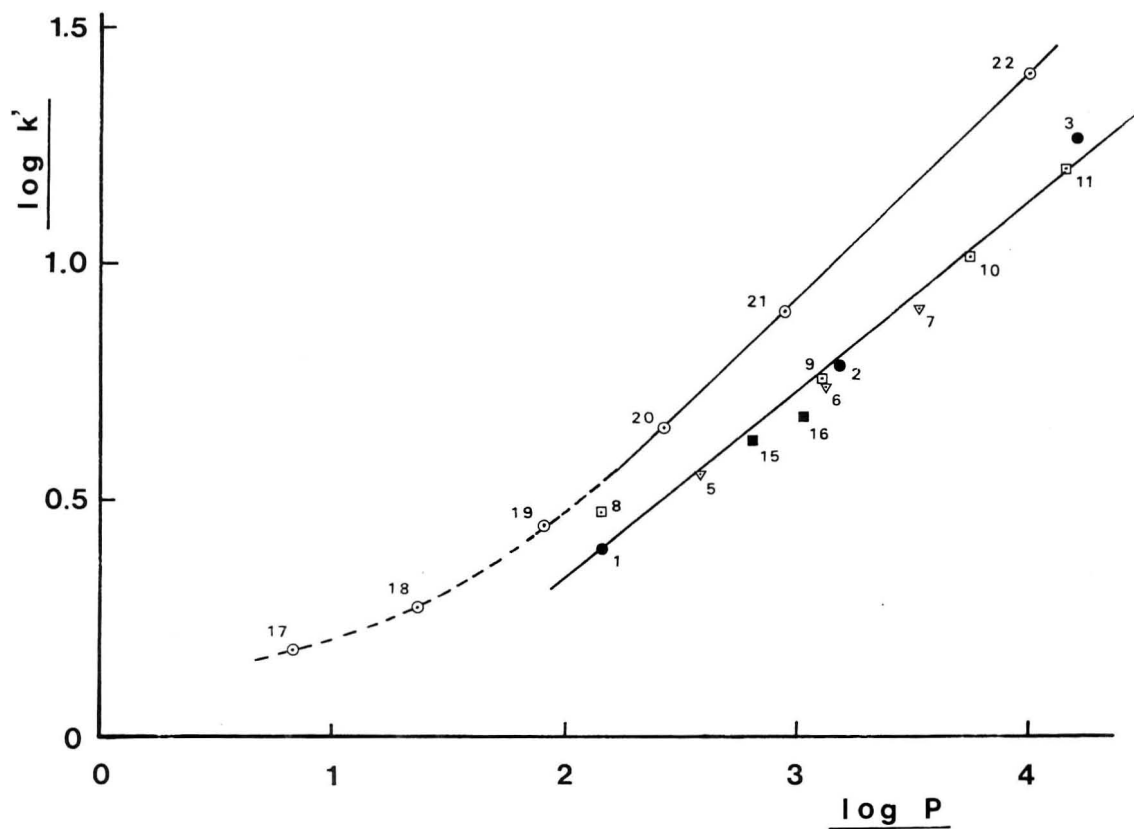
FIGURE 3. Relationship between the capacity ratios (log k') and the hydrophobicity (log P) in 50% aqueous methanol on octadecyl packing. Symbols : see Figure 1.

compounds such as alcohols, polyaromatic hydrocarbons, alkylben-
zenes, benzoates and halogenated benzenes. The convergence of the
family of lines to the same point suggests that constant "a" of
equation [2] is a characteristic of the system itself. The varia-
tion of the slope was predictable from eq. 2. The behaviour at
extreme concentrations, higher than 80% ACN and lower than 20% ACN
in water indicates that the mechanism of retention is more
complex than pure hydrophobic effect. We believe that hydrogen
bonding competes with hydrophobic effects at high acetonitrile
content whereas coating of the packing material with water molecu-
les could be responsible for the behaviour at low acetonitrile
content. A similar observation was made by Horvath (9).

The graph of Figure 3 describes the relationship between log
k' and the hydrophobic factor in 50% aqueous methanol. A linear
relationship is observed for all compounds except for alcohols in
which a curvature is seen at low hydrophobicities. The regres-
sion line for alcohols is also above the line for other com-
pounds. This indicates that hydrophobicity of a given fragment in
aliphatic groups is higher than that in aromatics in methanol-
water solvent mixtures. The study has also been performed at
other MeOH-Water ratios and also in aqueous ethanol and aqueous
tetrahydrofuran. Similar results were obtained with all eluents.
The least squares analysis in the case of the 50% aqueous eluents
are given in Table 2. It is worth mentioning the following obser-
vations from those results :

TABLE 2

Summary of Least Squares Coefficients for log k' vs log P

Solutes*	Eluents (% in water)	log k' = a + β log P		
		a, s_a	β, s_β	R^2**(%)
1 to 23	50% ACN	- 0.182 0.011	0.241 0.035	96.1
acids (24 to 30)	50% ACN	- 0.763 0.002	0.197 0.014	99.9
1 to 16	50% MeoH	- 0.625 .028	0.445 0.095	95.7
alcohols (17 to 23)	50% MeoH	- 0.268 0.025	0.403 0.062	98.5
(19 to 23)	50% MeoH	- 0.444 0.015	0.460 0.045	99.8
1 to 12	50% EtoH	- 0.227 0.015	0.229 0.048	96.0
alcohols	50% EtoH	- 0.277 0.010	0.255 0.030	99.7
1 to 4	50% THF	0.162 0.026	0.109 0.093	90.0
benzene derivatives	50% THF	0.094 0.022	0.154 0.060	90.7
benzoates	50% THF	0.064 .009	0.183 0.030	99.5
alcohols	50% THF	0.005 0.001	0.136 0.004	99.9[8]
acids	50% THF	0.0431 0.0006	0.144 0.004	100%

* Numbers refer to compounds in Table 1.

** R^2 (%) : Percentage of the total variation explained by the model.

i) the negative intercepts in the cases where ACN, MeOH or EtOH
   are used in eluents.

ii) although a linear relationship was observed in aqueous THF,
   the straight lines calculted for the various categories of
   compounds were all different in this solvent.

iii) the high slope of log k' vs log P in methanol as compared
   with the values in the other solvents.

iv) the increase in slope as the mole fraction of organic modi-
   fier increases.

The slopes, β, of the straight lines can be useful in the
choice of the proper solvent mixture for the achievement of a
given separation. Moreover, the regression lines may serve for
the identification of unknown compounds. Figure 4 shows the
slope, β, as a function of the organic content of the eluent. For
any pair of solutes, A and B, equation 2 can be rearranged in
terms of the separation factor, α, to give :

$$\beta = \log \alpha - \log P_a/P_b \qquad\qquad [3]$$

Since hydrophobicity characterises a solute, it becomes easy to
calculate the β value which will achieve a given separation (i.e.
a given value of α). Thereafter, an eluent insuring a value of
β equal to or larger than that calculated from equation 3 can be
chosen from a graph such as that of figure 4. The solvent mixture
so chosen will separate compounds A and B satisfactorily.

The discrepancies observed between the unit slope expected in
octanol and the slopes observed in the various eluents can be
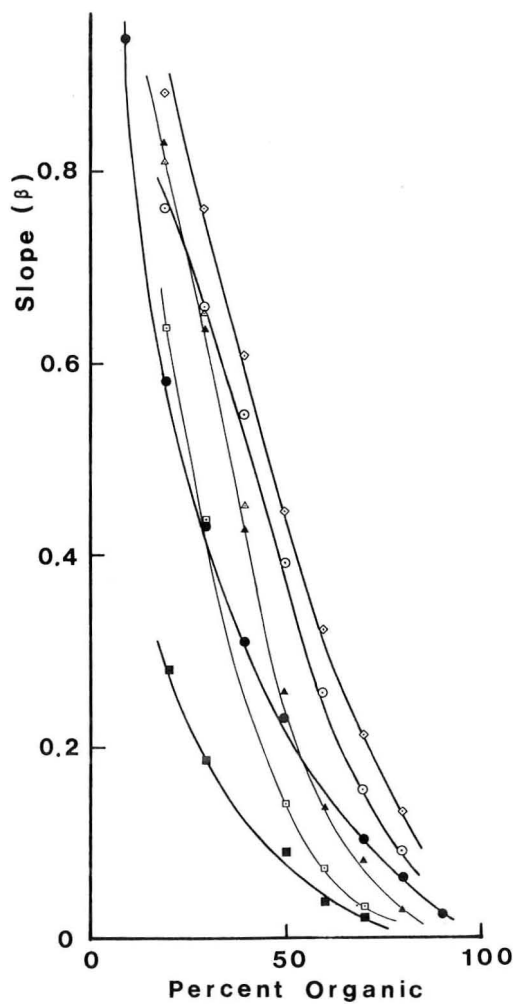
FIGURE 4.

Variation of the slope (β) for various solutes as a function of the organic content (%) in the eluent. ◇ Methanol (alcohols) ; ☉ Methanol (polyaromatics ; ● acetonitrile (alcohols and aromatics); △ ethanol (alcohols) ; ▲ ethanol (aromatics) ; ▣ tetrahydrofuran (alcohols) ; ■ tetrahydrofuran (polyaromatics).

understood if one considers that equation [2] refers to a partition between octanol and water whereas the quantity measured, log k', is due to a partition between an aqueous organic eluent and a pseudoliquid, the hydrocarbonaceous material.

The separating ability observed in this work is similar to that reported by Hoffman and Liao (12) except for methanol which shows a larger separating ability than acetonitrile.

The use of hydrophobicities to link the retention of various compounds in liquid chromatography seems promising. Of the many ways to evaluate hydrophobicity, we have selected Rekker's (10) method. The reasons for this choice are the easiness with which the parameters may be calculated and the fact that the approach does not require additional experimental measurements as do, for instance, Sliwiok, Macioszcyk and Kowalska's relative coefficients (13). The method described in this paper to link the retention data is thus most convenient for routine laboratory work.

Finally, as shown by Murray, Hall and Kier (6), a straight linear relationship exists between log P and the connectivity index, χ. Therefore, a straight line should be observed when plotting log k' vs connectivity indices.

ACKNOWLEDGEMENTS

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REVERSED-PHASE ION-PAIRING LIQUID CHROMATOGRAPHIC
SEPARATION AND FLUORIMETRIC DETECTION OF POLYAMINES

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ABSTRACT

A rapid and specific reversed-phase ion-pairing high perform-ance liquid chromatographic procedure for putrescine, spermidine and spermine is reported. The ion-pairing reagent, heptanesul-fonate, was employed and o-phthalaldehyde and 2-mercaptoethanol were used for on-line post-column derivatization and subsequent fluorescence detection. Experiments were carried out to determine the effects of several variables such as pH, concentration of the aqueous buffer, counter-ion concentration, and the percentage of organic modifier in the moving phase. The minimum detection limits for the polyamines ranged from 120 pmoles for spermine to 12 pmoles for putrescine. The method includes a gradient program which provides complete separation from amino acids and specificity for the three polyamines. The procedure was applied successfully to urine and serum samples.

INTRODUCTION

During recent years there has been a great deal of interest in the physiological and clinical significance of certain polyamines as clinical markers. Urinary and serum polyamines have been re-ported to be useful biochemical indicators of cancer (1-4). It has also been reported that polyamine metabolism may play an important role in the pathophysiology of psoriasis (5-7).

The biologically significant polyamines of interest in this study are putrescine (Pu), spermidine (Sd), and spermine (Sp).

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TABLE 1

Polyamine Structures

Name (Abbrev.)

Putrescine (Pu) $\overset{\oplus}{H_3N}(CH_2)_4\overset{\oplus}{NH_3}$

Spermidine (Sd) $\overset{\oplus}{H_3N}(CH_2)_3\overset{\oplus}{NH_2}(CH_2)_4\overset{\oplus}{NH_3}$

Spermine (Sp) $\overset{\oplus}{H_3N}(CH_2)_3\overset{\oplus}{NH_2}(CH_2)_4\overset{\oplus}{NH_2}(CH_2)_3\overset{\oplus}{NH_3}$

Table 1 shows the structures of these molecules in their protonated forms.

Previous analytical methods for these and other biogenic amines have included gas chromatography (8-10), thin layer chromatography (11), ion-exchange chromatography (12-18), and more recently, reversed-phase high performance liquid chromatography (HPLC) utilizing pre-column derivatization (19-25).

Most of these methods, however, possess certain disadvantages. The gas chromatographic procedures tend to require tedious sample preparations and sometimes lack accuracy. Ion-exchange techniques are also characterized by tedious sample preparations in addition to lengthy analysis times ranging from 60 to 120 minutes.

Reversed-phase HPLC techniques utilizing pre-column derivatization of amines currently appear to be quite popular. Several recent papers have been published describing pre-column fluorimetric derivatization with fluorescamine and o-phthalaldehyde (19, 20), dabsyl chloride (25), and the popular reagent, dansyl chloride (21-24). These techniques also require extensive sample preparation and analysis times and often yield complex chromatograms. A comprehensive review of analytical methods for polyamines in physiological fluids has been published by Seiler (26).

An increasingly popular approach to the separation of ionic species is ion-pairing HPLC. Several excellent reviews of the theoretical aspects of this technique have been published (27-32).

Very recently, Seiler and Knödgen reported the separation of natural
polyamines and their monoacetyl derivatives by ion-pairing HPLC and
post-column derivatization (33). In this paper, we report a rapid,
sensitive, and specific reversed-phase ion-pairing HPLC separation
of putrescine, spermidine and spermine followed by on-line post-
column derivatization and fluorimetric detection. We believe our
method is complementary to that of Seiler and Knödgen (33); it also
includes a detailed study of the retention behavior of the three
polyamines and provides a more rapid alternate analysis for natu-
rally occurring polyamines. In addition, our detection limits for
the polyamines appear to be slightly lower than those reported by
Seiler and Knödgen. The method of detection involves the highly
fluorescent product formed from the reaction of o-phthalaldehyde
(OPT) and primary amines (34-36). A recent review of reaction
liquid chromatography has been published by Frei (37) which outlines
the principles involved in post-column derivatization.

MATERIALS AND METHODS

Apparatus

The chromatographic system used in this separation is illus-
trated in Figure 1. Pumps A and B were Model 6000A units (Waters
Associates, Milford, MA 01757). These were controlled by a Model
660 Solvent Programmer (Waters Associates) and delivered the mobile
phase through a 30 x 0.39 cm (I.D.) μBondapak C_{18} analytical column
(Waters Associates). Samples were introduced into the system by
use of a Model U6K injector (Waters Associates) supplied with a
100 μL sample loop.

Pump C was a Milton-Roy Mini-Pump 106-31 (Waters Associates).
This pump delivered the OPT derivatizing reagent to the column
effluent; the two streams were mixed in an on-line post-column
reaction coil to form the fluorescent derivatives of the polyamines.
The reaction coil was constructed of 305 x 0.023 cm (I.D.) coiled
stainless steel tubing. The resulting fluorophores were then de-
tected in a Schoeffel Model FS 970 Spectrofluoromonitor (Kratos,
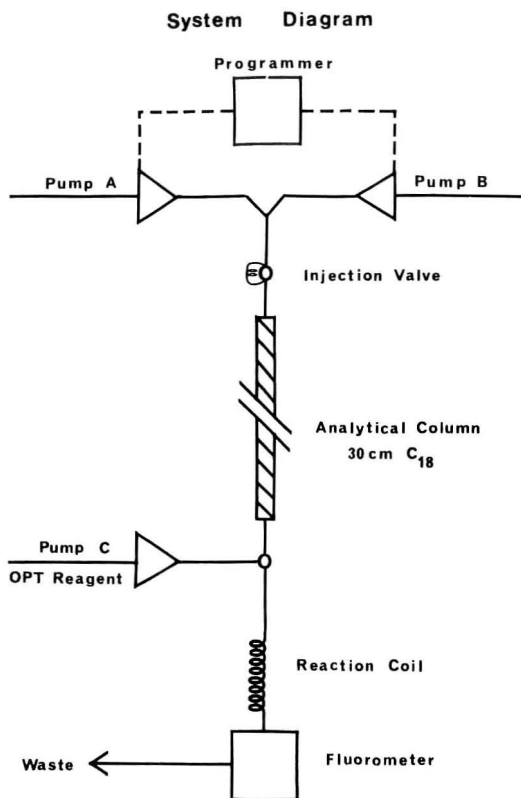
FIGURE 1.   Schematic diagram of the chromatographic system.

Westwood, NJ 07675) with the excitation wavelength set at 340 nm
and a Type 440 emission filter (Kratos). A Spectra-Physics, SP
4100 computing integrator (Spectra-Physics, Santa Clara, CA 95051)
was used to record all chromatograms and perform data reduction.
The post-column "T" connector was a Swagelok fitting (Allentown
Valve & Fitting Co., Allentown, PA 18049).

Reagents and Chemicals

    All polyamines were obtained in the hydrochloride salt forms.
The putrescine salt was obtained from Eastman (Eastman Kodak Co.,

Rochester, NY 14650) and recrystallized from absolute ethanol. The
spermidine and spermine hydrochlorides (Sigma Chemical Co., St.
Louis, MO 63178) were used without further purification. OPT was
also obtained from Sigma Chemical Co. and was used as received.
Reagent grade 2-mercaptoethanol was purchased from Matheson, Cole-
man and Bell (Norwood, OH 45212). Sodium 1-heptanesulfonate was
supplied by Eastman and used without further purification. Reagent
grade tetrahydrofuran was obtained from Aldrich (Aldrich Chemical
Co., Milwaukee, WI 53201). HPLC grade methanol was supplied by
Burdick & Jackson Labs, Inc. (Muskegon, MI 49422). HPLC grade
water was produced by a Milli-Q Reagent Grade Water System (Milli-
pore Corporation, Bedford, MA 01730).

Samples and Sample Preparation

Normal, pooled urine and serum samples were obtained from
local hospitals and were kept frozen at -30°C until needed. In
preparation for analysis, the physiological samples were thawed
and hydrolyzed with an equal volume of concentrated hydrochloric
acid at 100°C for a period of 10-12 hours. Following the hydrolysis,
samples were cooled to room temperature. The pH of the resulting
hydrolysate was adjusted to approximately 4.5 by addition of dilute
base. The total volume of acid and base added to the original
physiological fluid was recorded in order to calculate dilution
factors.

Following adjustment of pH, the hydrolysate was filtered
through a 0.20 μm Nalgene Filter (Sybron Corporation, Rochester, NY
14602) to remove particulate matter. These prepared samples were
then stored at -30°C until assayed.

Standard stock solutions were prepared by dissolving weighed
quantities of the polyamine hydrochloride salts in deionized, dis-
tilled water. Appropriate dilutions of these stock solutions were
made to generate standard solutions at desired concentrations.

Mobile Phases and Derivatizing Reagents

All mobile phases used in this separation were prepared volu-
metrically. Solvent A consisted of 80% aqueous acetate buffer

(0.050 $\underline{M}$ acetic acid adjusted to pH 4.50 with sodium hydroxide)
and 20% methanol. Solvent A was also 1.0 x 10^{-2} $\underline{M}$ in 1-heptane-
sulfonate. Solvent B consisted of 80% aqueous acetate buffer
(0.10 $\underline{M}$ acetic acid adjusted to pH = 4.50 with sodium hydroxide),
18% methanol and 2% tetrahydrofuran.

The OPT derivatizing reagent was prepared by dissolving 800 mg
of OPT in 10 mL of methanol. To this was added 600 μL of 2-
mercaptoethanol. This mixture was diluted to a volume of 1.00 L
with 0.50 $\underline{M}$ aqueous potassium borate buffer at pH 9.00. This
reagent is stable at room temperature for a period of 24 hours.

Chromatographic Procedure

Pumps A and B, controlled by the solvent programmer, delivered
the mobile phase through the analytical column at a rate of
2.0 mL/min. The mobile phase gradient consisted of an initial
isocratic period at 0% B for 10 minutes followed by a linear
gradient to 100% B over a 2 minute period. Pump C delivered the
OPT derivatizing reagent to the analytical column effluent at a
rate of 0.70 mL/min. After passing through the reaction coil where
the derivatization process occurred, the mixture was monitored
fluorimetrically (λ_{ex} = 340 nm; $\lambda_{em} \geq$ 440 nm).

RESULTS AND DISCUSSION

Retention Mechanism

The basis for this separation of the polyamines is ion-pairing
chromatography. This technique allows charged species to be
separated using reversed-phase HPLC. Equation 1 gives a very
simplified representation of the process involved.

$$RNH_3^{+}{}_{(aq)} + HpS^{-}{}_{(aq)} \underset{}{\overset{K}{\rightleftharpoons}} (RNH_3^{+}HpS^{-})_{(org)} \qquad (1)$$
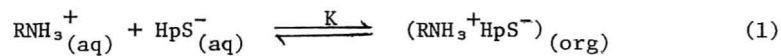
In this example, $RNH_3^{+}{}_{(aq)}$ represents a protonated, positively
charged amine in solution in the aqueous mobile phase. $HpS^{-}{}_{(aq)}$
represents the anionic counter-ion, 1-heptanesulfonate, also in

solution in the aqueous mobile phase. $(RNH_3^+ HpS^-)_{(org)}$ represents the resulting neutral, hydrophobic ion-pair retained in the non-polar organic stationary phase. K is the overall equilibrium constant for the process.

If the equilibrium lies to the left, there is little or no affinity of RNH_3^+ for the non-polar stationary phase, thus little or no retention on the column. However, if the equilibrium is to the right, a neutral, hydrophobic ion-pair is formed which is retained in the non-polar organic stationary phase, thus permitting the separation of the protonated amines.

Retention Behavior of the Polyamine Ion-Pairs

In the process of determining optimum separation parameters, studies of the retention characteristics of the polyamine ion-pairs were performed. This was done by observing the capacity factors (k') of the ion pairs as a function of several variables. These variables included the percent of organic modifier in the mobile phase, the counter-ion concentration, the pH of the aqueous mobile phase buffer, and the acetate concentration of the mobile phase. The results of these studies are presented graphically in Figures 2-5 as plots of k' vs the various variables.

Figure 2 illustrates the change in capacity factors of the polyamine ion-pairs as a function of the percent organic modifier in the mobile phase. The modifier used in all of these studies was a 1:1 mixture of methanol and tetrahydrofuran. The acetate concentration was held at 0.050 $\underline{M}$, the pH was 4.50, and the counter-ion concentration was fixed at 6.0×10^{-3} $\underline{M}$. The retention behavior of the polyamine ion-pairs was as expected. As the percent organic modifier was increased, the capacity factor of each of the polyamine ion-pairs decreased. We believe that there are two reasons for the large difference in the plots for each of the ion-pairs. First, the spermine has four available ionic sites for ion-pairing, while spermidine has only three, and putrescine two. Secondly, spermine contains more methylene groups than spermidine
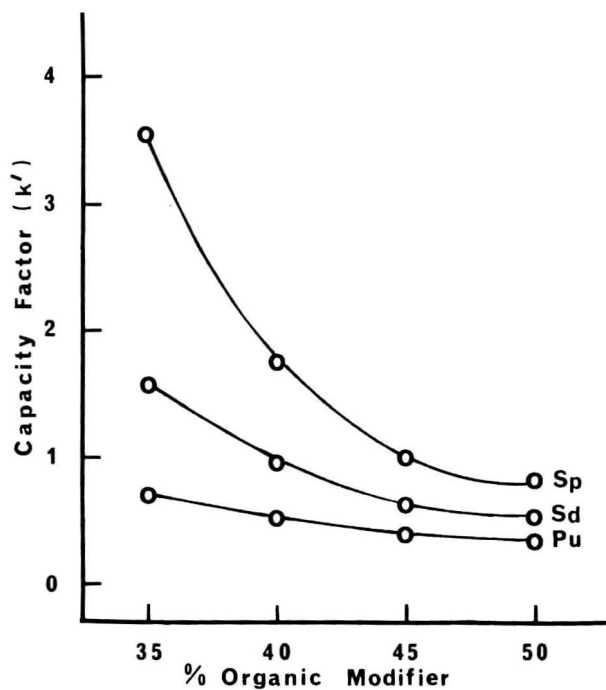
FIGURE 2.   Effect of percent organic modifier (1:1 methanol
             and tetrahydrofuran) on the retention of the polyamine
             ion-pairs (0.050 $\underline{M}$ acetate; pH = 4.50; $[HpS^-]$ =
             6.0 x 10^{-3} $\underline{M}$).

or putrescine. These two factors combine to cause spermine to be
considerably more hydrophobic with a larger retention time than
either spermidine or putrescine.

     Figure 3 illustrates the changes in capacity factors of the
three polyamine ion-pairs as a function of the counter-ion con-
centration. The data were obtained using a mobile phase consist-
ing of 60% aqueous acetate buffer (0.050 $\underline{M}$ acetic acid adjusted
to pH = 4.50 with sodium hydroxide) and 40% organic modifier. As
can be seen from the plots, an increase in counter-ion concentra-
tion results in increased retention. This observation is con-
sistent with published results (31,32) and is reviewed here
briefly.
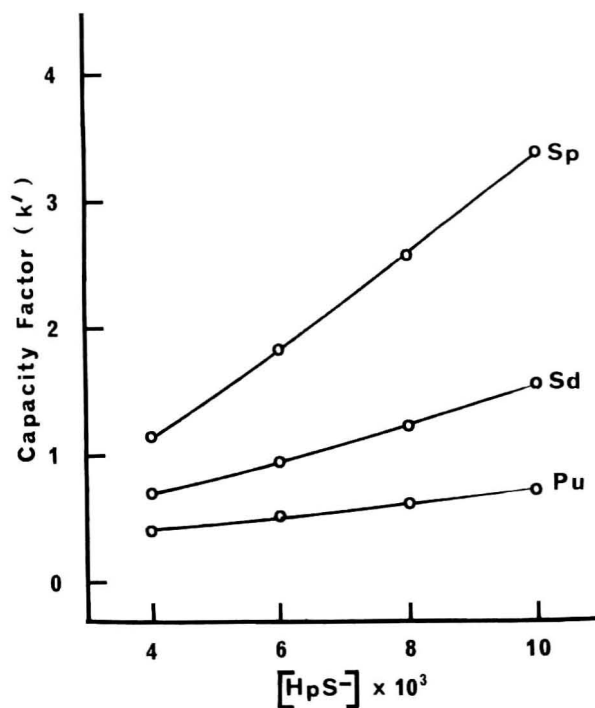
FIGURE 3.   Effect of the counter-ion (HpS⁻) concentration on the
            retention of the polyamine ion-pairs (0.050 $\underline{M}$ acetate;
            pH = 4.50; 40% organic modifier).

     Equation 1 has already described the distribution of the ionic
amine in the mobile phase and the extraction of the ion-pair into
the organic stationary phase. The equilibrium constant for the
extraction into the stationary phase is represented in Equation 1
as K. This extraction constant can be written as:

$$K = \frac{[RNH_3{}^+HpS^-]_{org}}{[RNH_3{}^+]_{aq}[HpS^-]_{aq}} \qquad (2)$$

In order to relate the extraction constant K to the capacity factor
k', the following equations are required (31):
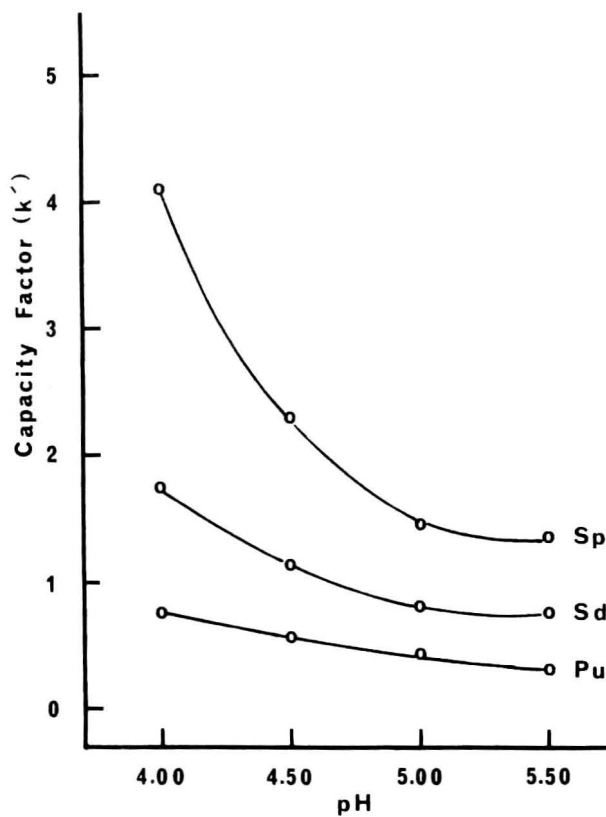
FIGURE 4.   Effect of mobile phase pH on the retention of the
             polyamine ion-pairs (0.050 $\underline{M}$ acetate; $[HpS^-]$ =
             6.0×10^{-3} $\underline{M}$; 40% organic modifier).

$$k' = \frac{[RNH_3{}^+HpS^-]_{org}}{[RNH_3{}^+]_{aq}} \frac{V_S}{V_M} \qquad (3)$$

$$k' = [HpS^-]_{aq} \; K \; \frac{V_S}{V_M} \qquad (4)$$

In Equations 3 and 4, V_M represents the void volume of the chromato-
graphic column and V_S represents the volume of the stationary
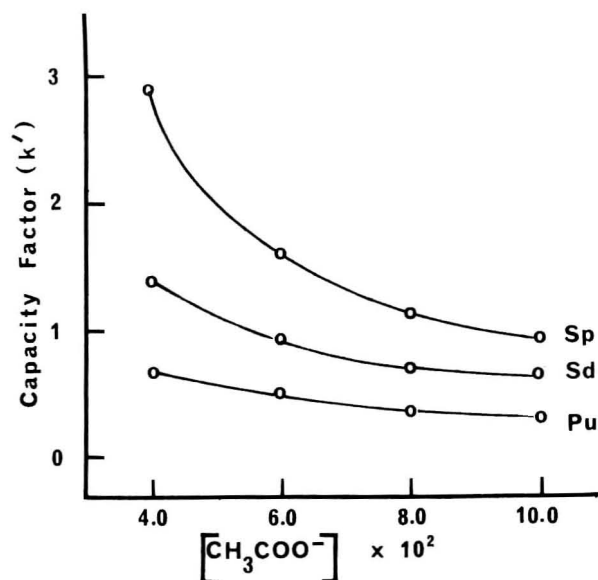
FIGURE 5.   Effect of acetate concentration on the retention of
the polyamine ion-pairs (pH = 4.50; [HpS$^-$] =
6.0 x 10^{-3} M; 40% organic modifier).

phase contained in the chromatographic column. From Equation 4,
it is seen that the capacity factor for the polyamine ion-pairs is
proportional to the counter-ion concentration in the mobile phase.
Figure 3 shows that this is indeed the case.

Figure 4 represents the data obtained in a study of the effect
of the mobile phase buffer pH on the capacity factors of the poly-
amine ion-pairs. In this study the acetate concentration was fixed
at 0.050 M, the percent organic modifier was held constant at 40%, and
the counter-ion concentration was 6.0 x 10^{-3} M. It can be seen
that a decrease in mobile phase pH resulted in an increase in k'
values of the ion-pairs. The reason for this behavior may be due
to the non-equivalent amine groups undergoing a greater degree of
protonation with a resultant increase in ion-pair formation (and
subsequent retention) as the mobile phase pH is decreased.
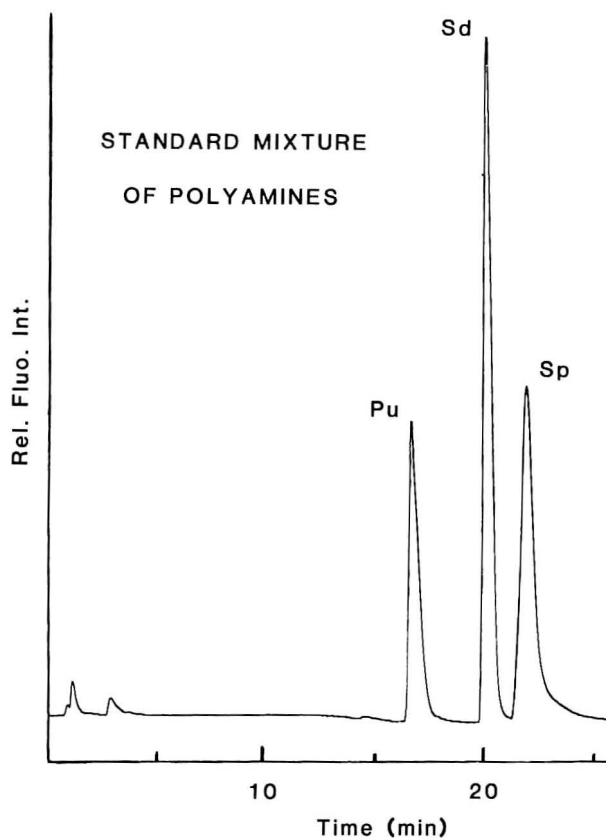
FIGURE 6.   Chromatogram of a standard mixture of the three
             polyamines (1.6 nmol each).

    The final study, shown in Figure 5, illustrates the dependence
of k' of the ion-pairs as a function of the acetate concentration
in the mobile phase. In this series of experiments, the percent
organic modifier was 40%, the aqueous buffer pH was fixed at 4.50,
and the counter-ion concentration was 6.0×10^{-3} $\underline{M}$. It can be
seen that as the acetate concentration increased, the k' values of
the ion-pairs decreased. This behavior was expected, since pre-
vious studies (31,32) have shown that as the ionic strength of the
mobile phase increases, the amount of ion-pair formation with the
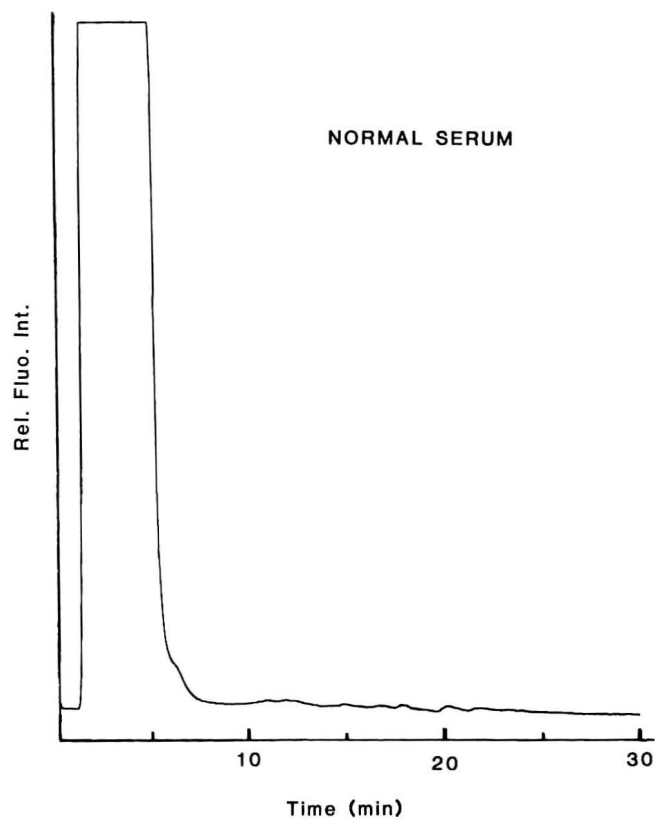
FIGURE 7.   Chromatogram of pooled, normal serum.

counter-ion decreases. This results in a decrease in k' values of
the ion-pairs. The reason for the decrease in the amount of ion-
pair formation is due to the competition of secondary ions in the
ion-pair formation process. In this case, it is likely that the
anionic acetate ion is competing with the HpS⁻ counter-ion to
form ion-pairs with the cationic polyamines.

Figure 6 shows the separation of a standard mixture of the
three polyamines utilizing the previously listed mobile phase and
chromatographic conditions. The chromatogram shows satisfactory
resolution and symmetrical peak shapes. The total analysis time
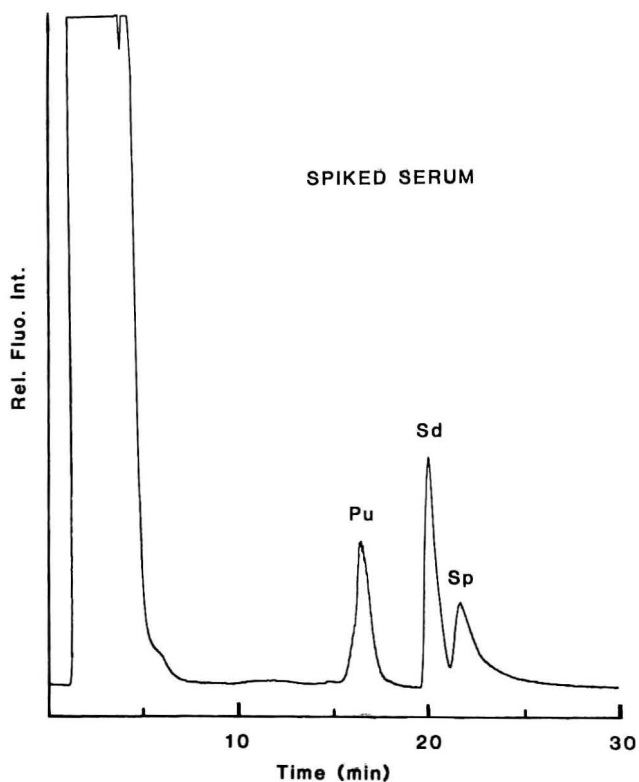
FIGURE 8.   Chromatogram of pooled, normal serum spiked with
             ca. 1 nmol of each polyamine.

was ca. 26 minutes; this is significantly faster than most reported
methods, including the most recently published procedure (33).
The gradient which was used to produce the chromatogram actually
changes three parameters during its operation; the acetate con-
centration is increased, the counter-ion concentration is de-
creased, and the strength of the mobile phase organic modifier is
increased.

Figure 7 represents a chromatogram for a pooled, normal serum
sample. The large peak which elutes early in the chromatogram is
a result of the large quantities of amino acids produced in the
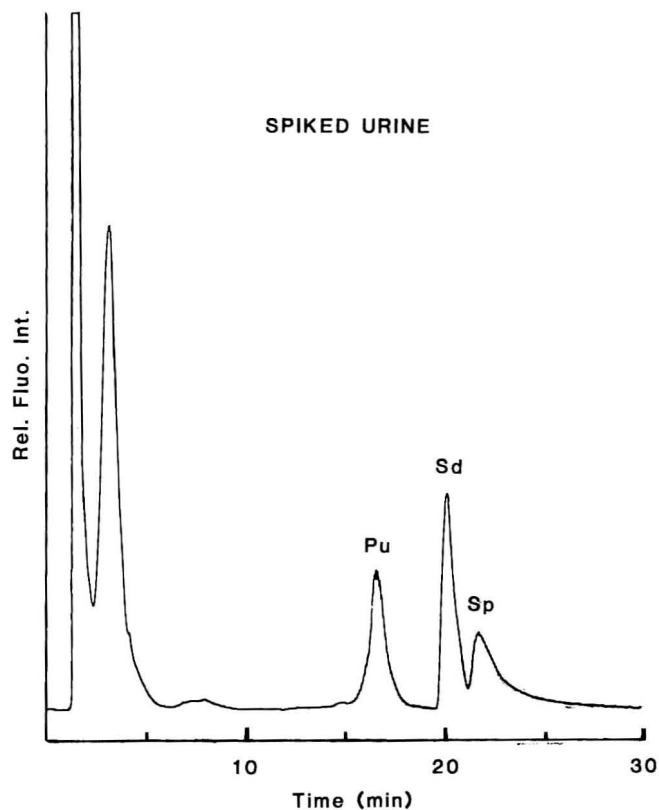
FIGURE 9.   Chromatogram of normal urine spiked with ca. 1 nmol
              of each polyamine.

hydrolysis procedure. Since OPT reacts with primary amines, this
response cannot be eliminated. However, under the chromatographic
conditions used, the polyamines are strongly retained on the column
during the initial isocratic period. The gradient portion of the
program then elutes the polyamine ion-pairs after the interfering
amino acids have passed through the column. Chromatograms for
normal serum and normal urine samples spiked with the three poly-
amines are shown in Figures 8 and 9. These separations illustrate
the non-interference of amino acids when analyzing physiological
fluids for polyamines.

TABLE 2

Linear Ranges and Minimum Detection Limits

Compound	Ret. Time (min)	Min. Detection Limit	Linear Response Range	R^2
Pu	17.0±0.5	1.0 ng (12 pmole)	1.0 to 350 ng	0.999
Sd	20.0±0.5	6.0 ng (40 pmole)	6.0 to 290 ng	0.997
Sp	22.0±0.5	24 ng (120 pmole)	24 to 800 ng	0.998

There is a relatively long time period between the tailing edge of the amino acid front and the beginning of the first poly-amine peak. The experimental parameters reported in this paper were tailored to provide optimum separations for our specific application. This separation method, however, is extremely flex-ible. If samples appear to have a consistently low amino acid content, then the initial isocratic period of the gradient may be shortened to decrease analysis time with no loss of resolution.

The linear ranges and minimum detection limits for each of the polyamines are presented in Table 2. The minimum detection limits listed are for the polyamines in the free form, $R(NH_2)_x$, and not in the protonated or ion-paired state. The linear cor-relation coefficients (R^2) for each of the polyamines indicate good linearity of detector response based on measurement of peak areas.

CONCLUSION

In summary, we have described a new, rapid, sensitive, and specific separation technique for putrescine, spermidine, and spermine utilizing reversed-phase ion-pairing HPLC coupled with on-line post-column derivatization and fluorimetric detection of

the polyamines. Analysis times are significantly shorter than most methods currently available. We are presently using this method to determine polyamines in physiological fluids obtained from psoriatic arthritis patients.

ACKNOWLEDGEMENT

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FRACTIONATION AND CHARACTERIZATION OF INORGANIC
LONG-CHAIN POLYPHOSPHATE BY GEL CHROMATOGRAPHY

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ABSTRACT

Inorganic long-chain polyphosphates were fractionated by
Sephadex G-100 and G-50 columns. Fractions whose average chain
lengths, $\bar{n}$, were 20 - 150 and 10 - 50 were obtained by the G-100
and G-50 columns, respectively. By rechromatography of these
fractions, linear relationship was found for the plots of the
elution volumes vs. logarithms of the $\bar{n}$ values. By the use of
this relationship, chain length distribution analysis could be
performed.

INTRODUCTION

Among condensed phosphates, linear phosphates whose chain
length, n, range from 2 to several ten thousands form one family.
Since these materials contain polyanions which bind metal cations
such as calcium ions, they have found widespread applications in
industry. The binding characteristic of these anions is depen-
dent on the n value of a sample phosphate. The study of the inter-
action between these polyphosphate anions and metal cations (1) is
expected to give clear insight to polyelectrolyte solution chemistry,

because these anions bear quite crowded negative charges on their
molecules. Samples which have various n values will help the under-
standing of the transition from simple electrolyte to polyelectrolyte.

Preparation of pure samples of tri- and tetraphosphates has
been reported (2,3). In order to prepare linear phosphates whose
n value is higher than 4, sodium phosphate glass which has a broad
chain length distribution has been used as a crude material (4).
For a separation purpose, ion-exchange chromatography has been
applied to yield pure samples whose n value is up to about 10 (5).
Since it is quite difficult to prepare the pure samples whose n
value is higher than 10, mixture samples whose chain length distri-
bution is as narrow as possible should be prepared.

Gel chromatography has been applied to fractionations of water-
soluble polymers (6). The aim of the present work is to examine
the applicability of gel chromatography to fractionation of long-
chain polyphosphates. The fractions obtained were analyzed for
their $\bar{n}$ values and chain length distributions.

EXPERIMENTAL

Materials

Sodium phosphate glasses were prepared according to the liter-
ature (4). The $\bar{n}$ values of these crude materials determined by
end group titration method (7) were 113 and 19 for Sephadex G-100
and G-50 systems, respectively. Other reagents were of analytical
grade.

Fractionation Procedure

Sephadex G-100 and G-50 columns whose size was ϕ2.6x95 cm
were used for the preparative purpose. 0.1 M sodium chloride
solution was used as an eluent. Elution flow rate was 0.65 ml/min.
Each 0.5 g of sodium phosphate glass sample dissolved in 10 ml of
the eluent was applied to the gel column and was eluted. The eff-
luent was divided successively into 18-ml fractions and an $\bar{n}$ value
of each fraction was determined by the end group titration method.

Gel Chromatographic Analysis

Sephadex G-100 and G-50 columns whose size was $\phi1.5\times30$ cm were used for the analytical purpose. Eluent contained 0.1 M sodium chloride, 0.01 M sodium acetate and 5×10^{-4} M acetic acid (pH 6). 0.5 ml portions of the fraction samples obtained by the preparative columns were applied to the analytical columns and were eluted. An AutoAnalyzer detector (8,9) was used in order to detect continuously total phosphate concentration in an effluent. It has been reported that the plots of the peak area vs. the amount of polyphosphate give a good linearity (9). Furthermore, it was found that the slopes of the calibration curves are almost equal to each other for the linear phosphate samples whose $\bar{n}$ value is higher than 10 (9).

RESULTS AND DISCUSSTION

Since it took about a half day to accomplish the fractionation with each preparative column, hydrolysis of the sample during elution was examined prior to the fractionation. 0.01 M sample ($\bar{n}$ = 113) solution was allowed to stand at 50°C, and at dayly intervals, a portion of the sample solution was withdrawn to be analyzed by the gel column (Sephadex G-100, $\phi1.5\times30$ cm). No detectable change in elution profile was observed for three days, which ensured the stability of the samples to hydrolysis during fractionation.

In Fig. 1, representative elution profiles of the sodium phosphate glasses obtained with the preparative Sephadex columns are shown.   It can be seen that these samples have a broad distribution. Small peaks appeared at approximately totally permeable volume of the columns may correspond to the cyclic phosphates (mainly trimeta- and tetrametaphosphates). An $\bar{n}$ value of each fraction was determined and some of the fractions were chromatographed with the analytical columns. The elution profiles of the fractions are shown in Figs. 2 and 3. The data of elution volumes, V_e, which were determined from the peak positions of these samples
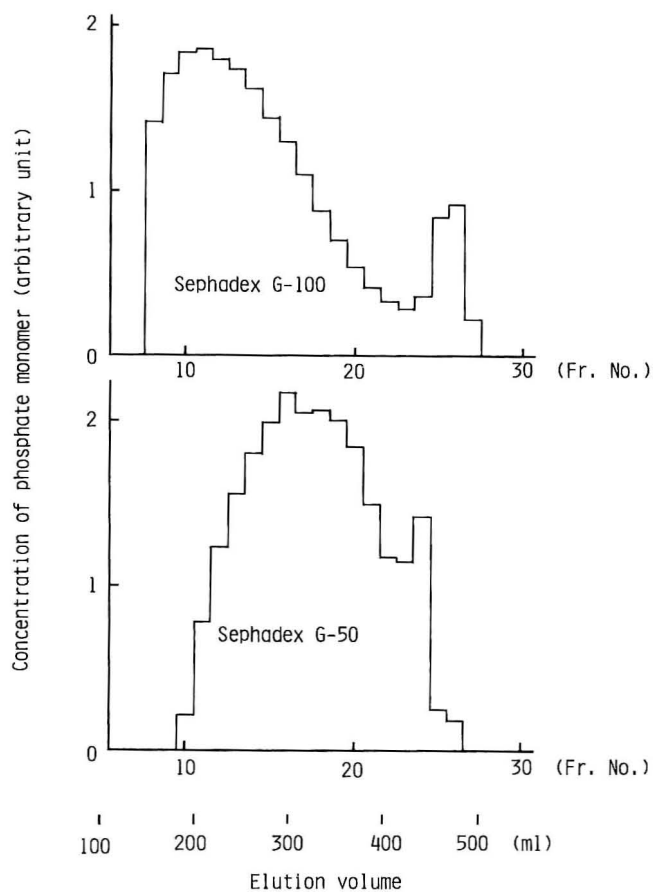
FIGURE 1.

Elution profiles of sodium phosphate glasses.

are given in Tables 1 and 2. It can be seen that linear phosphate mixture whose $\bar{n}$ value is from ca. 10 to ca. 150 can be prepared by the use of the Sephadex G-100 and G-50 columns with 0.1 M sodium chloride solution.

V_e values were plotted against log $\bar{n}$ values (Fig. 4). Good linearity was obtained for both the Sephadex G-100 and G-50 columns.

TABLE 1.

Fractionation of Sodium Phosphate Glass
($\bar{n}$ =113) by the Sephadex G-100 Column.

Fr. No.	$\bar{n}$	V_e (ml)	$\overset{..}{\bar{n}}*$	$\bar{M}_w/\bar{M}_n$
10	157	17.7		
11	128			
12	118	20.8	93	1.1_1
13	101			
14	83	24.9	67	1.1_2
15	65			
16	53	28.6	47	1.1_2
17	41	31.5	36	1.1_4
18	26**	32.8		
19	30	35.5		
20	24	37.2	21	1.1_2
21	19			
22	17	40.5	15	1.1_4
23	16			

** Since this value is considered to be too low, this plot
is omitted in Fig. 4.

TABLE 2.

Fractionation of Sodium Phosphate Glass
(n = 19) by the Sephadex G-50 Column.

Fr. No.	$\bar{n}$	V_e (ml)	Fr. No.	$\bar{n}$	V_e (ml)
11	57		18	20	29.3
12	52	22.4	19	17	
13	45		20	14	33.5
14	37	24.9	21	11	
15	34	25.9	22	9	37.1
16	29	27.9	24*	12	41.2
17	24				

* This fraction contains cyclic phosphates.
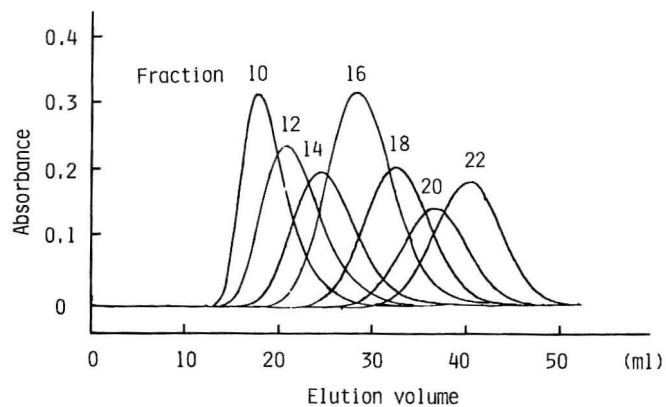
FIGURE 2.

Elution profiles obtained by the Sephadex G-100 analytical column. Each sample fraction was obtained by the Sephadex G-100 preparative column. Fraction numbers are the same as those in Table 1.
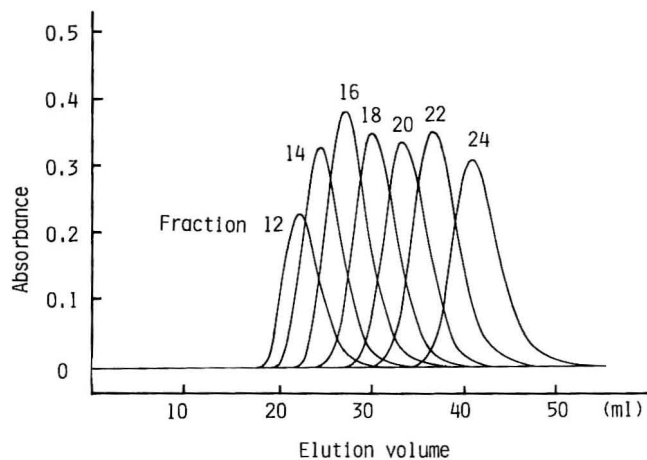
FIGURE 3.

Elution profiles obtained by the Sephadex G-50 analytical column. Each sample fraction was obtained by the Sephadex G-50 preparative column. Fraction numbers are the same as those in Table 2.
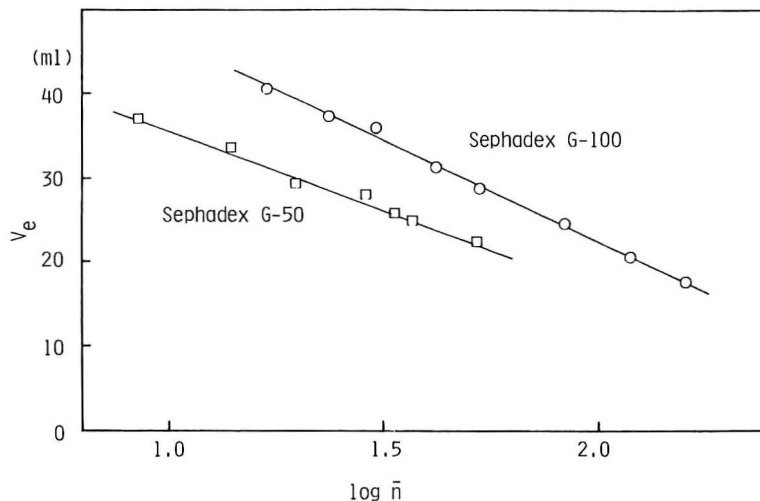
FIGURE 4.

Plots of V_e vs. log $\bar{n}$.

Since a similar phenomenon has been observed for a Sephadex G–25
system (10), it seems worthwhile to correlate the molecular structures
of phosphate polymers in solution to their gel chromatographic
behavior.   Consideration on this point will be discussed elsewhere.

Practically, this relationship between V_e and log $\bar{n}$ is useful
to determine chain length distribution of a phosphate mixture.
The chain length distribution analysis of a phosphate mixture was
carried out by the Sephadex G–100 column using the same procedure
as has been applied to the analysis of dextrans (11).   In Fig. 5,
a representative chain length distribution analysis for fraction 14
in Table 1 is shown.   In Table 1, chain lengths calculated from
the gel chromatographic analysis, $\bar{n}*$, are presented.   The $\bar{n}*$ values
calculated from the chromatographic analysis are not always consistent
with the values determined directly by the end group titration
method.   This discrepancy may be attributed to small dispersion of
the sample phosphate zone in tubes of the detector due to the wall
effect (12).   Elimination of this effect in the detector seems
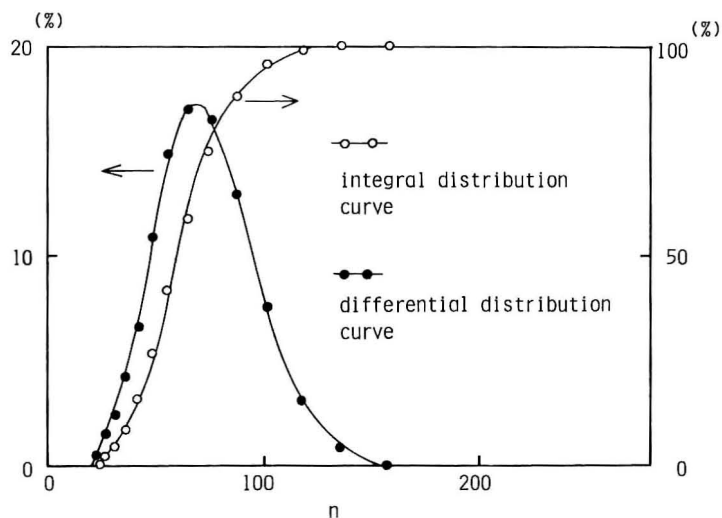
FIGURE 5.

Representative chain length distribution analysis.
Sample: Fraction 14 in Table 1.

necessary to precise determination of the chain length distribution.
Though there remains some problems to be solved in the detector,
the gel chromatography-AutoAnalyzer system is quite useful for the
present purpose, because only a small amount of sample is necessary
for this analysis and the time needed for the analysis is relatively
short. Both the weight average molecular weight, $\bar{M}_w$, and the number
average molecular weight, $\bar{M}_n$, have been calculated from the gel
chromatographic elution curves. The heterogeneity ratio, $\bar{M}_w/\bar{M}_n$,
gives an indication of the efficiency of the fractionation. These
values were calculated to be about 1.1 for all the fractions obtained
(Table 1).

In order to obtain solid samples of phosphates, each fraction
was freeze-dried. Since the elution profiles of the samples obtained
after the freeze-drying procedure were quite the same as those obtained
before the procedure, it was concluded no hydrolysis occured during
the treatment.

ACKNOWLEDGMENTS

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DETERMINATION OF FREE FATTY ACIDS IN NATURAL OILS AND

ALKYD RESINS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Mixtures of free fatty acids in natural oil and alkyd resin samples have been analyzed using a μBondapak Free Fatty Acid column in conjunction with a ternary mobile phase. Variation of the mobile phase composition allows "fingerprinting" as well as quantitation of the fatty acid components. Samples can be analyzed in ten minutes by this method. The results of the application of this technique to the identification of oil sources of fatty acids as well as the production of fatty acids during alkyd resin synthesis are given. Good agreement is observed for fatty acid compositions determined via HPLC with those obtained by gas chromatographic methyl ester analysis.

INTRODUCTION

The development of chromatographic methods for the analysis

of fatty acids has reached a high level of sophistication over

the last two decades. Reviews by Dallas, et. al. (1) and Met-

calfe (2) attest to the magnitude of research effort in this area.

The emphasis placed upon the analysis of these materials derives

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in part from their importance as items of commerce in the fields
of protective coatings, foods, and pharmaceutical products (3).

The most widely applied technique for fatty acid analysis
is gas chromatography (GC) (4,5). Application of this technique
usually requires the formation of volatile derivatives (i.e.
methyl esters) to affect separation. These derivatization pro-
cedures are extremely well developed (6), but do require the
attendant time and expense associated with their use. In addi-
tion, there have been reports in the literature of incomplete
conversion of the fatty acids to their methyl ester derivative (7)
as well as chemical transformation of the acid prior to chromato-
graphic analysis (8).

High performance liquid chromatography (HPLC) of fatty acids
has been developed in recent years as an alternative to the con-
ventional GC approach. Limited success was experienced with this
technique prior to 1975 as noted in the reviews of Cooper and
Anders (9) and Aitzetmüller (10). The last five years have seen
the development of a number of novel separation schemes based
upon the use of bonded phase support materials. These methods
have frequently involved the separation of the methyl esters
(11-16), or the formation of derivatives having chromaphoric (17-27)
or fluorescence (28,29) properties. Impressive separations have
also been recorded using a variety of gradient elution methods
(23-27,30,31) and argentation chromatography has been effective
in segregating unsaturated fatty acids from the aliphatic acids
(11,30,32-34).

Unfortunately, many of the above methods require the forma-
tion of fatty acid derivatives and are prohibitively long. For
example, several gradient separations require an analysis time
of 150 (27) and 250 (23) minutes, respectively. These factors
mitigate against employing some of the above chromatographic
assays in an industrial environment, where rapidly obtained
results are important in identifying oil types, changes in fatty
acid composition, and to monitor reaction kinetics and products.

In 1976, Bidlingmeyer, et. al. (35) reported on the development of a µBondapak Free Fatty Acid column. This column/isocratic mobile phase combination allowed the rapid separation of many of the fatty acids contained in important commercial oils. Since the introduction of this reverse phase column, there have been a limited number of papers describing its use in margarine analysis (36), in the isolation of minor fatty acids in tall oil (37), and a vague report of its use in the coatings industry (38).

In this publication, we will show how the above described column, with appropriate variation of the mobile phase, can be used for the identification and quantitation of fatty acid mixtures derived from industrial oil and alkyd resin samples. Examples will be given which show how unique chromatographic "fingerprints" are obtained for each oil and how these can be used to make semi-quantitative conclusions regarding changes in oil fatty acid composition during alkyd resin synthesis.

EXPERIMENTAL

Apparatus. Chromatographic data were obtained using a Waters Associates (Milford, Mass.) Model ALC 202 liquid chromatograph equipped with a Waters Model 6000 dual reciprocating piston pump and Model U6K injector. Detection of the solutes was facilitated by using a Waters Model R401 differential refractometer. Chromatograms were recorded on a Sargent Model SR recorder. Attenuation settings for the refractometer were typically 4 to 16X yielding suitable responses on the recorder set at a 12.5 mV full-scale range.

A Waters µBondapak Fatty Acid Analysis Column (4 mm i.d. x 30 cm length) was utilized to fractionate the fatty acid mixtures. The mobile phase consisted of a ternary mixture of tetrahydrofuran (THF), acetonitrile (CH_3CN), and water, the sum of these three solvents being equal to 105 volume units (39). The level of tetrahydrofuran was kept constant at 25 volume units to prevent

precipitation of the fatty acids. Retention of the solutes was adjusted by varying the ratio of acetonitrile to water. Addition of two volume units of acetic acid was found to be very beneficial in improving peak symmetry.

Reagents. Mobile phase solvents were obtained from the following sources: acetonitrile (ACS Certified Grade) and tetrahydrofuran (Certified Grade) from Fisher Scientific (Pittsburgh, Pa.) and glacial acetic acid (Reagent Grade) from J. T. Baker Co. (Phillipsburg, NJ). All solvents including distilled water were used without further purification.

Fatty acid standards were obtained from Applied Science Laboratories, Inc. (State College, Pa.), Analabs, Inc. (North Haven, Conn.) and Supelco, Inc. (Bellefonte, Pa.). Commercial samples of fatty acids, oils, and alkyd resins were obtained from Ashland Chemical Co. (Columbus, Ohio), Emery Industries, Inc. (Cincinnati, Ohio), Lilly Industrial Coatings (Indianapolis, Ind.), and Armak Co. (Chicago, Ill.). The saturated fatty acid standards (100 mg) were dissolved in 1.0 ml of tetrahydrofuran, while unsaturated fatty acid standards (25 mg) were dissolved in 0.5 ml of the same solvent. Commercial samples were diluted one to ten by volume in tetrahydrofuran.

Procedure. Samples were injected using a 25.0 µL Precision Sampling Corp. (Baton Rouge, La.), Series B-110, Pressure-Lok liquid syringe. Typical injection volumes were in the range of 0.5-5.0 µL. Eluent flow rates were measured using a Kimax 10.0 ml burette and a Huer stopwatch (1.0×10^{-2} sec. resolution).

Laboratory saponification of the oils and alkyd resins was accomplished using a modification of the procedures of Ast (40) and Metcalfe, Schmitz, and Pelka (41). Two milliliters of vegetable oil were refluxed with 50 ml of saturated KOH/methanol in a 100 ml round bottom flask equipped with a N_2 sparge for 10 minutes. The sample was then removed and placed in an ice bath for two

minutes. Twenty milliliters of distilled water were added to the flask and the pH adjusted to 2 with dilute hydrochloric acid. After the sample was sufficiently cool, 15-25 ml of n-hexane was added to extract the fatty acids. Agitation was then applied to transfer the solutes to the n-hexane followed by separation of the two layers in a separatory funnel. The n-hexane was then removed using gentle heating and vacuum aspiration. Finally, the concentrated acids were redissolved in tetrahydrofuran.

A similar procedure was used to prepare the fatty acids from alkyd resins. In this case, a quantity of 4-5 ml of the resin was used initially. An additional extraction step using ethyl ether was performed after the refluxing stage to remove the unsaponifiable matter. With practice, the above saponification scheme yielded a sample ready for injection in 30 minutes.

RESULTS AND DISCUSSION

Tables I and II summarize the retention behavior of model solutes on the Free Fatty Acid column for the two most frequently used mobile phase compositions in this study. The observed trends in retention volume are identical to those previously reported (35). The retention of fatty acids increases with carbon number for both the saturated and unsaturated acids. As the degree of unsaturation increases, for acids having the same carbon number, their retention volumes decrease correspondingly. This is amply illustrated by the decrease in retention volume in going from stearic acid to linolenic acid.

The substitution of two double bonds into a given fatty acid structure reduces its retention volume by an increment approximately equivalent to that observed in reducing its carbon number by a factor of two. Hence, there is peak overlap between certain saturated and unsaturated moieties, i.e. myristoleic and lauric acid. Such a situation can be partially alleviated by changing the mobile phase composition for the fatty acid mixture being chromatographed.

TABLE I

RETENTION VOLUMES OF SATURATED FATTY ACIDS

Solute	Retention Volumes (ml)[a]	
Caproic Acid (n-hexanoic)	3.8[b]	4.0[c]
Caprylic Acid (n-octanoic)	4.1	4.4
Capric Acid (n-decenoic)	4.5	5.1
Lauric Acid (n-dodecenoic)	5.2	5.9
Myristic Acid (n-tetradecenoic)	6.1	7.1
Palmitic Acid (n-hexadecenoic)	7.2	8.8
Stearic Acid (n-octadecenoic)	9.1	11.2
Arachidic Acid (n-eicosanoic)	11.0	14.3
Behenic Acid (n-docosanoic)	14.0	18.2

[a]Measured from point of injection

[b]Mobile phase composition: 43/37/25 parts by volume acetonitrile/water/tetrahydrofuran

[c]Mobile phase composition: 43/37/25/2 parts by volume acetonitrile/water/tetrahydrofuran/glacial acetic acid

The regularity in these retention trends is of great aid in identifying the individual fatty acids in the oil. Coupled with compositional data from GC results (42,43), oil types can readily be identified. We have used these data along with infrared and nuclear magnetic resonance techniques, to identify unknown solutes.

The addition of acetic acid to the mobile phase not only improves peak symmetry but increases the retention volume of the solutes. The result is usually improved resolution between the chromatographic peaks in the fatty acid mixture. For some cis/

TABLE II

RETENTION VOLUMES OF UNSATURATED FATTY ACIDS

Solute	Retention Volumes (ml)[a]	
Myristoleic Acid (cis-9-tetradecenoic)	5.2[b]	6.2[c]
Palmitoleic Acid (cis-9-hexadecenoic)	5.9	7.5
Palmitelaidic Acid (trans-9-hexadecenoic)	6.0	7.6
Oleic Acid (cis-9-octadecenoic)	7.2	9.1
Elaidic Acid (trans-9-octadecenoic)	6.3	9.4
Linoleic Acid (cis-cis-9,12-octadecadienoic)	6.5	8.1
Linolelaidic Acid (trans,trans-9,12-octadecadienoic)	6.4	8.5
Linolenic Acid (cis,cis,cis-9,12,15-octadecatrienoic)	6.2	7.3
Cis-5-Eicosenoic Acid	7.3	12.2
Erucic Acid (cis-13-docosenoic)	8.1	14.1
Nervonic Acid (cis-15-tetracosenoic)	9.6	18.2

[a]Measured from point of injection

[b]Mobile phase composition: 43/37/25 parts by volume acetonitrile/water/tetrahydrofuran

[c]Mobile phase composition: 43/37/25/2 parts by volume acetonitrile/water/tetrahydrofuran/glacial acetic acid

trans isomer pairs, there is a reversal in elution order observed by the addition of acetic acid.

The factors responsible for the retention volume differences observed for the two mobile phase compositions are difficult to elucidate. Both solvent compositions have similar solubility parameters (15.4 and 15.8 Hildebrands) (44). Undoubtedly, reten-

Figure 1:   Chromatogram of commercially saponified soybean oil
              fatty acids. Identification of acids: (1) linolenic;
              (2) linoleic and palmitic; (3) oleic; (4) stearic.
              Mobile phase composition: 40/40/25, water/acetonitrile/
              tetrahydrofuran by volume.

tion is governed in part by hydrophobicity of the solutes (45,46)

and the relative donor, dipole, and acceptor profiles (47) of the

solvents making up the mobile phase.

An excellent example of the effect of the mobile phase com-

position is in the separation of a soybean fatty acid mixture as

shown in Figures 1-3. Figure 1 is a rather poorly defined

chromatogram showing incomplete resolution of linolenic, linoleic,

palmitic, and oleic acids. Clearly, not even semi-quantitative
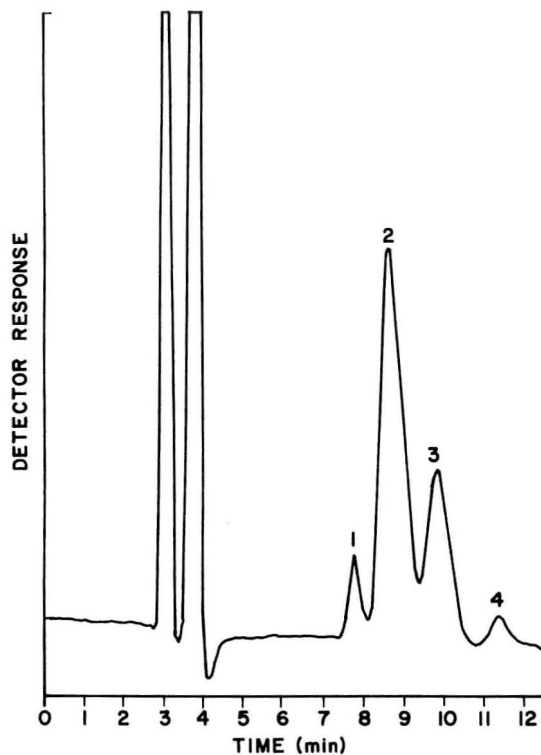
Figure 2:   Chromatogram of commercially saponified soybean oil
            fatty acids.   Identification of acids: (1) linolenic;
            (2) palmitic and linoleic; (3) oleic; (4) stearic.
            Mobile phase composition: 37/43/25, water/acetonitrile/
            tetrahydrofuran by volume.

information can be obtained from this chromatogram developed with

a 40/40/25 mixture of $H_2O/CH_3CN/THF$. By adjusting the water to

acetonitrile ratio slightly, a dramatic improvement in peak reso-

lution is obtained as illustrated in Figure 2. Here there is

sufficient separation of the major fatty acid peaks in the soy-

bean oil to allow conclusions to be drawn as to alteration in com-

position. Further improvement is realized by the addition of

2 parts by volume of acetic acid. This sharpens the peaks suffi-

ciently so as to allow the analyst to see the palmitic acid as a
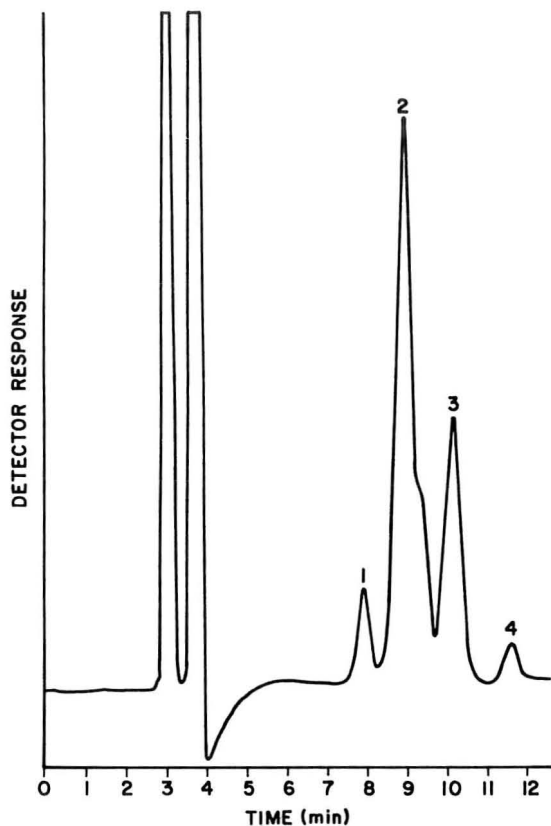
Figure 3:   Chromatogram of commercially saponified soybean oil
             fatty acids. Identification of acids: (1) linolenic;
             (2) palmitic and linoleic; (3) oleic; (4) stearic.
             Mobile phase composition: 37/43/25/2, water/aceto-
             nitrile/tetrahydrofuran/acetic acid by volume.

shoulder on the linoleic acid peak in Figure 3. Similar improve-
ments can be achieved with other oil mixtures by adjusting the
ratio of solvents in the mobile phase (48). In general, we have
achieved the most successful separations using the acidified
solvent mixture.

Oil Results. Figures 4-6 are typical chromatographic profiles
for commercially saponified oils. These were developed using a
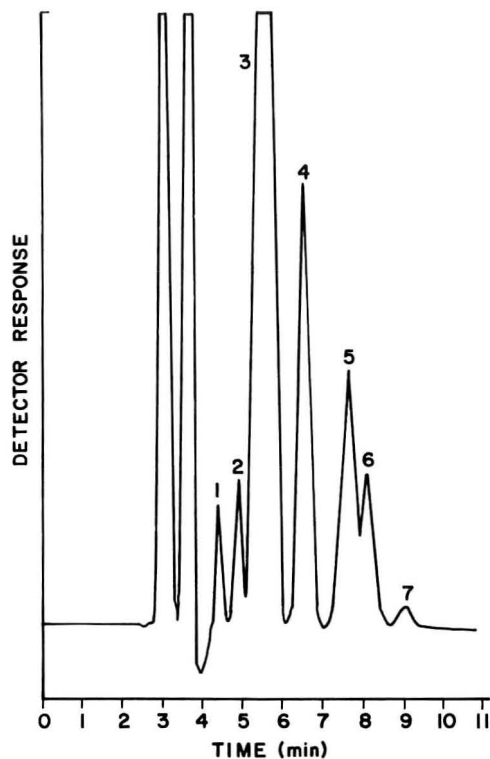
Figure 4:   Chromatogram of commercially saponified coconut oil
            fatty acids. Identification of acids: (1) caprylic;
            (2) capric; (3) lauric; (4) myristic; (5) palmitic
            and linoleic; (6) oleic; (7) stearic. Mobile phase
            composition: 40/40/25, water/acetonitrile/tetrahydro-
            furan by volume.

ternary mobile phase of 40/40/25 parts by volume of acetonitrile/

water/tetrahydrofuran. The peaks appearing at retention volumes

less than 4.0 ml are due to the solvent vacancy effect (49).

Their presence eliminates the possibility of calculating accurate

capacity factors (K') for the fatty acids (50). It is readily

apparent by intercomparing Figures 4-6 that one can identify the

source (oil) of the fatty acid mixture. This can be accomplished

within a 10 minute elution time frame.

    Figure 4 represents a commercially saponified coconut oil

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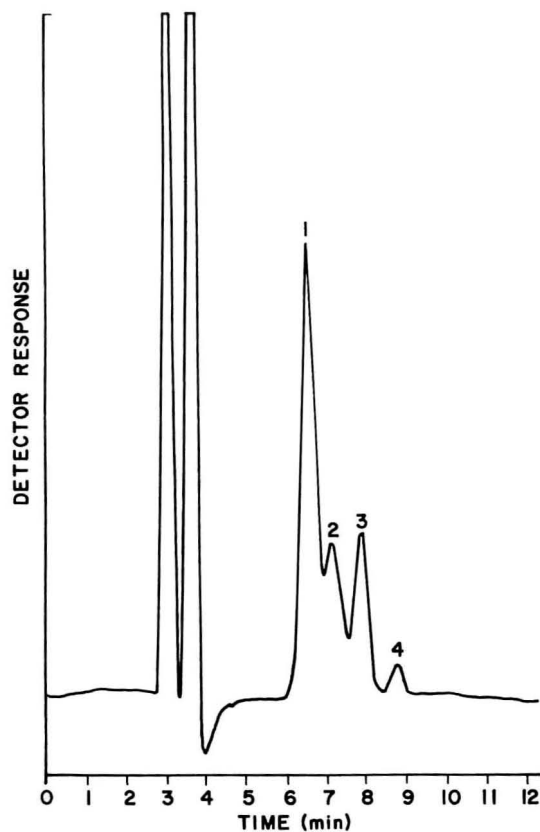
Figure 5:   Chromatogram of commercially saponified linseed oil
            fatty acids. Identification of acids: (1) linolenic;
            (2) palmitic and linoleic; (3) oleic; (4) stearic.
            Mobile phase composition same as in Figure 4.

fatty acid mixture. All of the major fatty acids comprising this

saponified oil are present in the chromatogram, with the exception

of linoleic acid which elutes under the palmitic acid, peak number

5. The elution order of the components in the chromatogram is in

agreement with trends previously discussed. The fused peak pair,

palmitic and linoleic acids, were confirmed by the method of

standard addition with highly purified fatty acid standards. This

type of procedure was used in addition to the previously discussed

methods to identify any ambiguities in the peak elution order.
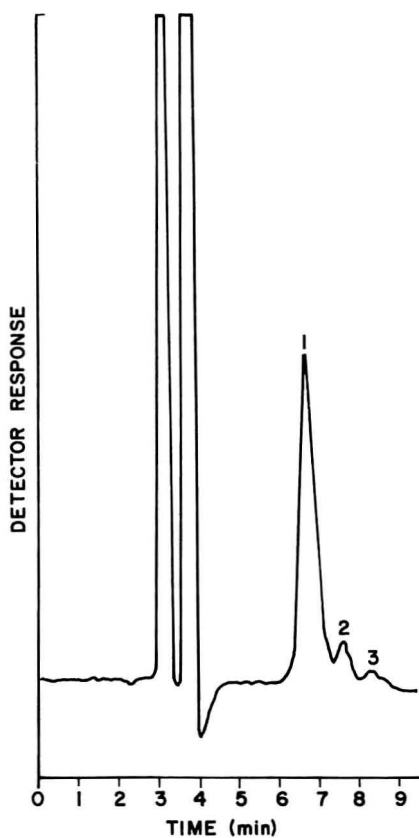
Figure 6:   Chromatogram of commercially saponified tung oil fatty
             acids. Identification of acids: (1) eleostearic,
             linoleic, linolenic, and palmitic; (2) oleic;
             (3) stearic. Mobile phase composition same as in
             Figure 5.

    Figure 5 illustrates the elution pattern for linseed oil,

whose major fatty acid components are unsaturated C_{18} fatty acids.

In this chromatogram, linolenic, the fatty acid having the most

unsaturation, elutes first followed by linoleic, oleic, and the

saturated analogue, stearic acid. The fifth component, palmitic

acid, present to the extent of approximately 6% by weight, is

eluted along with the linoleic acid (16% by weight) (42). The

chromatogram in Figure 6 represents tung oil fatty acids. The

Figure 7:   Chromatogram of laboratory saponified coconut oil
             fatty acids. Identification of acids: (1) caprylic;
             (2) capric; (3) lauric; (4) myristic; (5) palmitic
             and linoleic; (6) oleic; (7) stearic. Mobile phase
             composition: 37/43/25, water/acetonitrile/tetrahydro-
             furan by volume.

fatty acid composition of tung oil is dominated to the extent of

85% (by weight) (51) by eleostearic acid, 9,11,13-octadecatrionoic

acid. Trace constituents, such as linoleic, linolenic, and

palmitic acids all elute under the eleostearic peak.

A chromatogram of a laboratory saponified coconut oil using

a slightly different solvent mixture than was employed on the

previous three chromatograms is shown in Figure 7. The elution
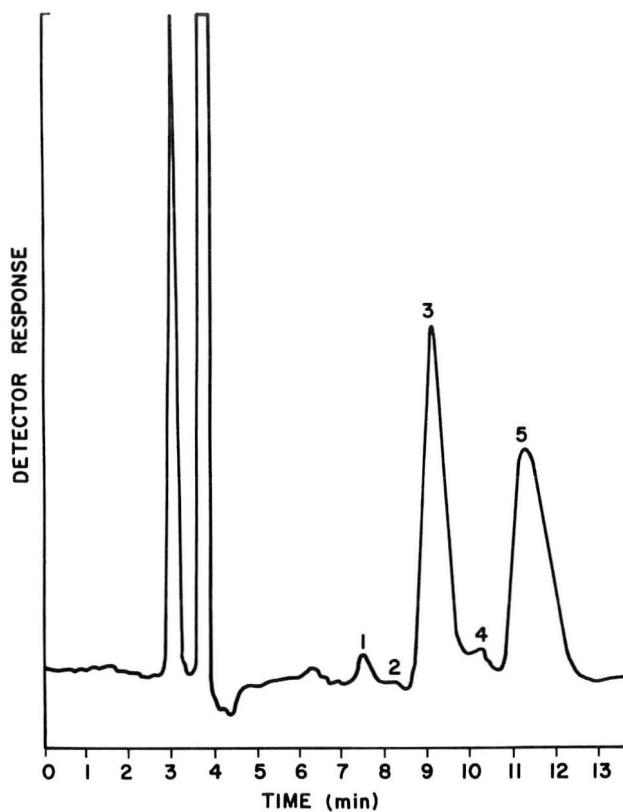
Figure 8:   Chromatogram of a commercial stearic acid sample.
             Identification of acids: (1) myristic; (2) pentadece-
             noic; (3) palmitic; (4) margaric; (5) stearic. Mobile
             phase composition same as in Figure 7.

pattern is identical to that exhibited in Figure 4, with the
exception that the resolution between particular components is
slightly improved by lowering the water content of the ternary
eluent and replacing this reduction in water with an equal volume
of acetonitrile. In general, we have obtained better resolution
for our fatty acid mixtures using a 37/43/25 volume ratio of
water/acetonitrile/tetrahydrofuran than by using a 40/40/25 volume
ratio. The agreement between laboratory and commercial saponified
coconut oil profiles is typical of the results we have obtained
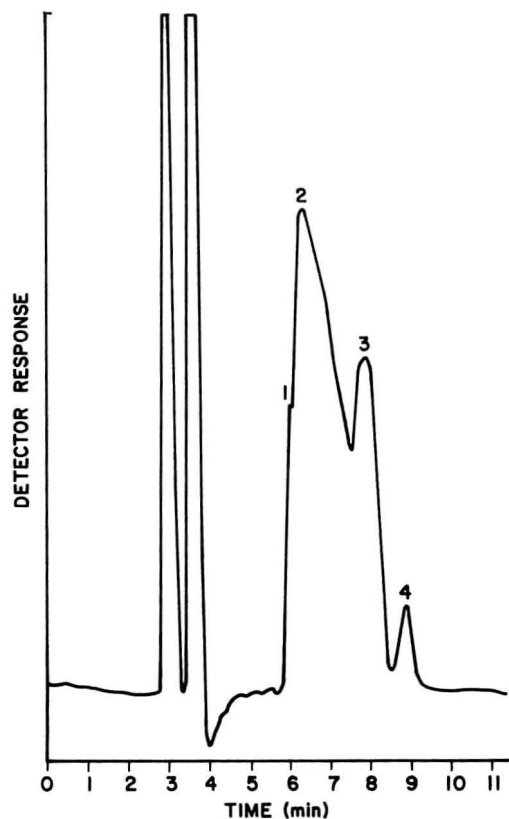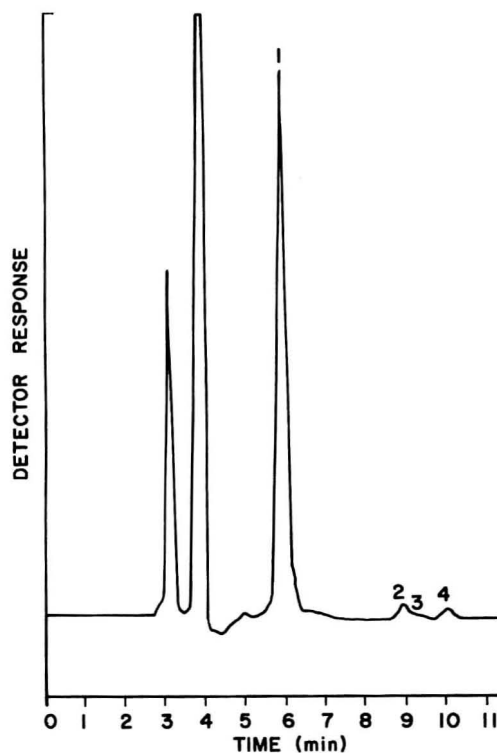
Figure 9:   Chromatogram of commercially saponified castor oil
            fatty acids. Identification of acids: (1) ricinoleic;
            (2) linoleic; (3) palmitic; (4) oleic. Mobile phase
            composition same as in Figure 7.

in this study and lends credence to our saponification/extraction
technique.

The liquid chromatographic method presented here also has
the capability of discerning differences in grades of fatty acids
and in monitoring chemical transformations of fatty acids. Figure 8
is the chromatogram of a commercial grade stearic acid sample.
In this case, there is considerable palmitic acid in addition to
the stearic acid. An additional feature of this chromatogram is
the ability to separate and detect odd-carbon number fatty acids,
such as pentadecenoic and heptadecenoic acid (margaric acid) from
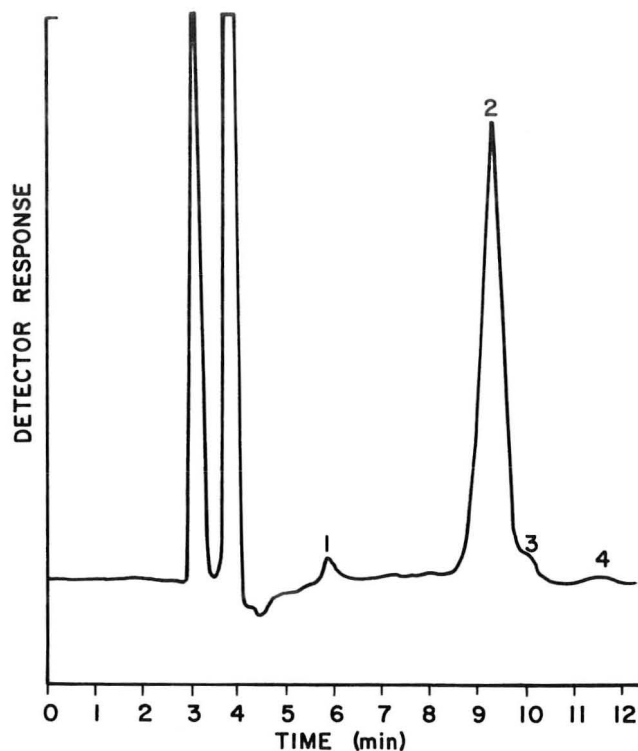
Figure 10.   Chromatogram of commercially saponified dehydrated
             castor oil fatty acids. Identification of acids:
             (1) ricinoleic; (2) 9,11-octadecadienoic and 9,12-
             octadecadienoic; (3) oleic; (4) stearic. Mobile
             phase composition: 37/43/25/2, water/acetonitrile/
             tetrahydrofuran/acetic acid by volume.

the even-carbon number acids. The latter compound is frequently

used as an internal standard in the gas chromatography of fatty

acid methyl esters since it does not occur to any appreciable

extent in natural oil compositions. It would appear from the

results of this chromatogram that it could also serve as an

internal standard in our high pressure liquid chromatographic

assay.

    One particular fatty acid transformation which can be readily

followed using our method is the conversion of castor oil fatty

acids to dehydrated castor oil fatty acids. This reaction involves

the dehydration of ricinoleic acid (12-hydroxy-cis-9-octadecenoic

to a mixture of 9,11 and 9,12-octadecadienoic acids. Production

of the latter acids confers "drying properties" on alkyd resins

containing those acids incorporated in the polymer chain via

esterification. The conversion of castor oil by this process is

of major importance to the coatings industry. Figure 9 is the

chromatogram of commercially saponified castor oil fatty acids.

The ricinoleic acid elutes early due to the presence of the

hydroxyl group in its structure. This retention trend is typical

of fatty acids containing polar functional groups (i.e., hydroxyl,

oxirane) other than the carboxyl moiety. Ricinoleic acid repre-

sents 90% of the fatty acid composition of castor oil; however,

other minor acids are apparent in the chromatogram.

Comparison of Figures 9 and 10 shows a typical conversion

of commercially saponified ricinoleic acid to the mixture of con-

jugated and unconjugated octadecadienoic acids by dehydration. A

small quantity of unconverted ricinoleic acid is apparent as well

as oleic and stearic acids. The broad peak contains both posi-

tional isomers, 9,11-octadecadienoic and 9,12-octadecadienoic

acid.

Alkyd Resin Results. We have experienced no difficulty in apply-

ing the above liquid chromatographic procedure to alkyd resins.

Since these resins are derived from polyesterification reactions

between selected acids, polyols, and fatty acids derived from

natural oils, the elution characteristics of several alkyd resin

constituents were determined on the Free Fatty Acid column. These

multifunctional alcohols and acids are tabulated in Table III. All

of these highly polar compounds eluted with the solvent front,

co-eluting under the vacancy peaks. This confirms their absence

in the fatty acid profile should they have been extracted simul-

taneously with the fatty acids in the n-hexane extraction step.

Figure 11 is the chromatogram of fatty acids derived from a

"pure" alkyd resin containing only soya oil, glycerine, and
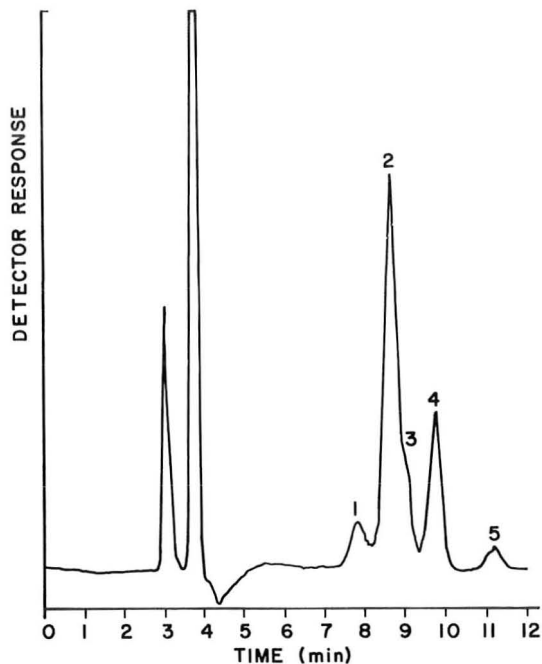
Figure 11.   Chromatogram of laboratory saponified soya-based
             alkyd resin fatty acids.   Identification of acids:
             (1) linolenic; (2) linoleic; (3) palmitic; (4) oleic;
             (5) stearic.   Mobile phase composition same as in
             Figure 10.

phthalic anhydride. The profile of peaks compares very favorably

with the results in Figure 3, using the same solvent system, and

confirms that this is a soybean oil based alkyd resin.

A "non-drying" alkyd fatty acid chromatogram is given in

Figure 12. The general profile compares well with a coconut oil

based alkyd (see Figure 7), with the exception of the ratio of

peaks 5 and 6, which are linoleic and palmitic acid, respectively.

This is due to the small charge of cottonseed oil (3% by weight

of total alkyd ingredients) added along with the coconut oil

(27% by weight of total alkyd) in this alkyd synthesis. The high

linoleic acid content of cottonseed oil combined with the acidi-

TABLE III

ALKYD CONSTITUENTS NOT RETAINED ON
THE FREE FATTY ACID COLUMN

Acids

Fumaric Acid
Maleic Anhydride
Isophthalic Acid
Phthalic Anhydride
Benzoic Acid
Succinic Acid
Terephthalic Acid

Alcohols

Pentaerythritol
Ethylene Glycol
Diethylene Glycol
Mannitol
Glycerol
Propylene Glycol
Trimethylolpropane

fied mobile phase splits the combined palmitic/linoleic acid peak
into a pair of peaks.

Figures 13 and 14 were chromatograms of unknown alkyd resin
samples submitted for analysis. The chromatographic profile and
peak heights strongly suggested a tall oil-based alkyd for both
samples. This is based on the ratio of the two predominant acids
in the chromatogram, linoleic and oleic acid. In actuality, both
resins are tall oil alkyds, containing weight percents of tall
oil of 32 and 35%, respectively.

The chromatograms in Figures 13 and 14 differ substantially
from other published chromatographic data for tall oils on the
Free Fatty Acid column (37). Our peak area ratios correctly
correspond to reported values for tall oils (43), however, there
are many grades of tall oil. It is obvious that the Free Fatty
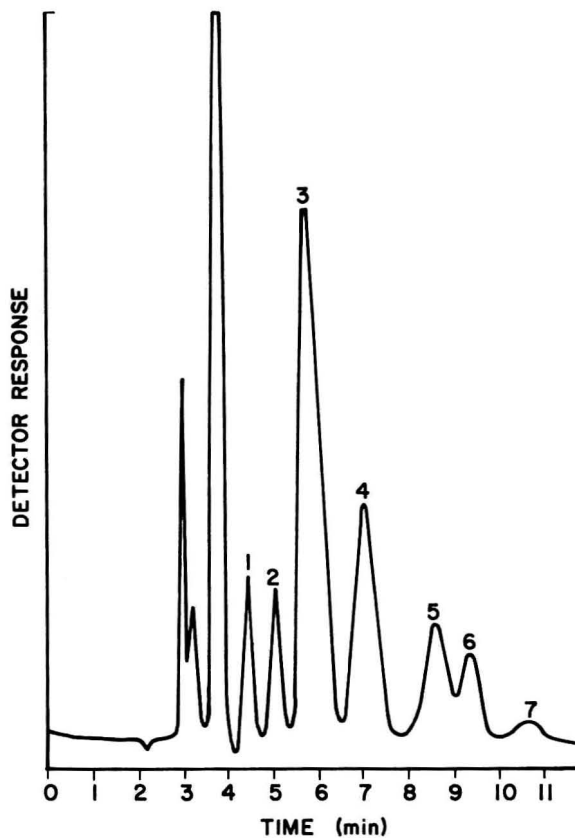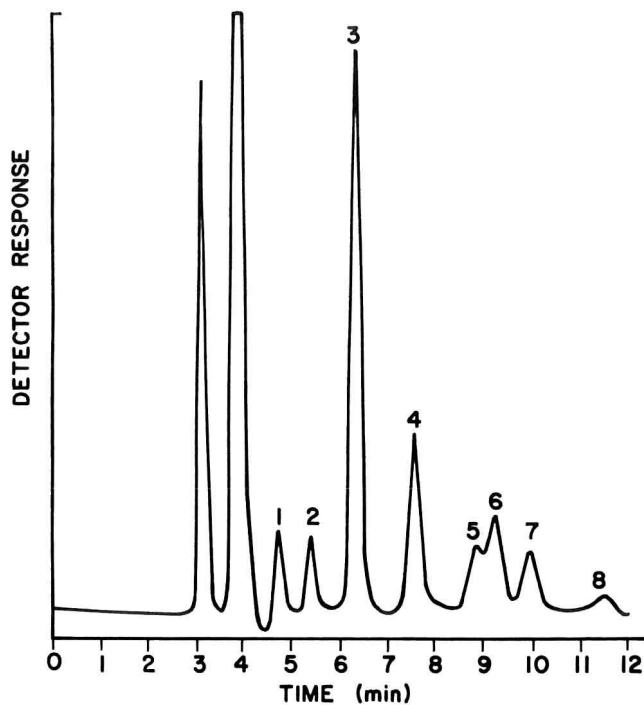
Figure 12: Chromatogram of laboratory saponified non-drying alkyd
            resin fatty acids. Identification of acids:
            (1) caprylic; (2) capric; (3) lauric; (4) myristic;
            (5) linoleic; (6) palmitic; (7) oleic; (8) stearic.
            Mobile phase composition same as in Figure 10.

Acid column is capable of producing chromatograms which show
these differences.

     The last two fatty acid chromatograms, derived from alkyd
resin saponification, are Figures 15 and 16, and represent pro-
files derived from a styrenated-acrylated alkyd and rosin-maleic
ester modified alkyd, respectively. These alkyd resins have been
modified by the addition of vinyl monomers or a maleic anhydride
adduct to the polyester backbone (52). The important component
in the original alkyd composition is the dehydrated castor oil
content which for both resins is approximately 32 weight percent
of the initial reactor charge. However, there is a significant

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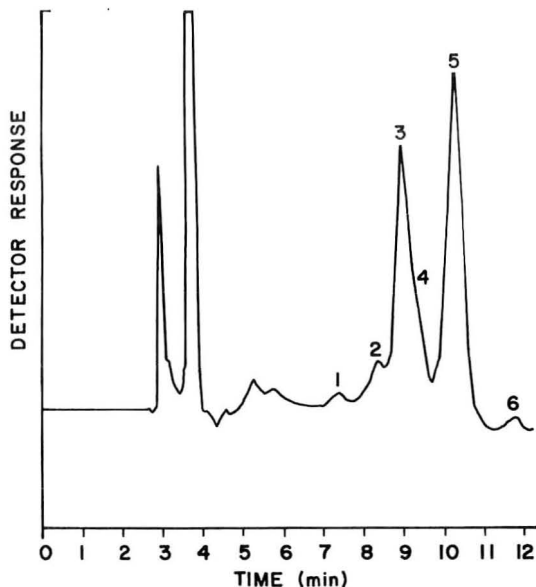
Figure 13:   Chromatogram of laboratory saponified tall oil-based
             alkyd resin fatty acids. Identification of acids:
             (1) unidentified; (2) palmitoleic or linolenic;
             (3) linoleic; (4) palmitic; (5) oleic; (6) stearic.
             Mobile phase composition same as in Figure 10.

difference in how these dehydrated castor oil fatty acids were
derived.

    The fatty acid composition in Figure 15 represents the final
composition in the alkyd resin from a reaction in which dehydrated
castor oil was charged to the reactor initially. Note that there
is a large amount of unconverted ricinoleic acid in the chromato-
gram which would suggest that the supplier of this oil had not
extensively dehydrated the castor oil. This lot of dehydrated
castor oil could have been rejected for synthetic purposes had a
liquid chromatographic analysis been run on the initial dehydrated
castor oil.

    In Figure 16, the dehydrated castor oil fatty acids have been
produced in-situ during the alkyd synthesis. The resultant fatty
acid composition is quite different from the chromatographic pro-
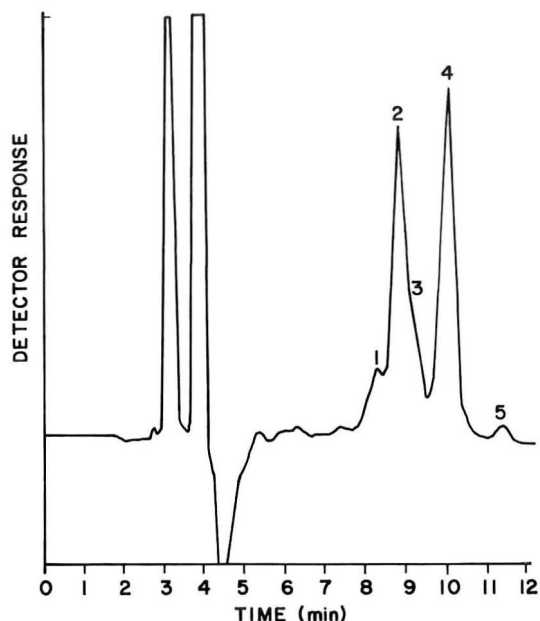
Figure 14: Chromatogram of laboratory saponified tall oil-based
alkyd resin fatty acids. Identification of acids:
(1) palmitoleic or linolenic; (2) linoleic;
(3) palmitic; (4) oleic; (5) stearic. Mobile phase
composition same as in Figure 10.

file in Figure 15. There is a large quantity of unconverted

ricinoleic acid and a number of fatty acids which are normally

not associated with dehydrated castor oil and/or castor oil (see

Figures 9 and 10). One of the acids, undecylenic acid, is pro-

duced commercially by pyrolytic decomposition of castor oil.

This would seem to infer that the high temperatures in the reactor

contribute to the decomposition of castor oil. Hence, the result-

ant polymer will be quite different from that produced in a syn-

thesis based on totally converted castor oil.

Quantitative Aspects. Excellent quantitative results have been

obtained in our laboratory using the Free Fatty Acid column/mobile

phase combinations mentioned above. Chromatographic analysis of
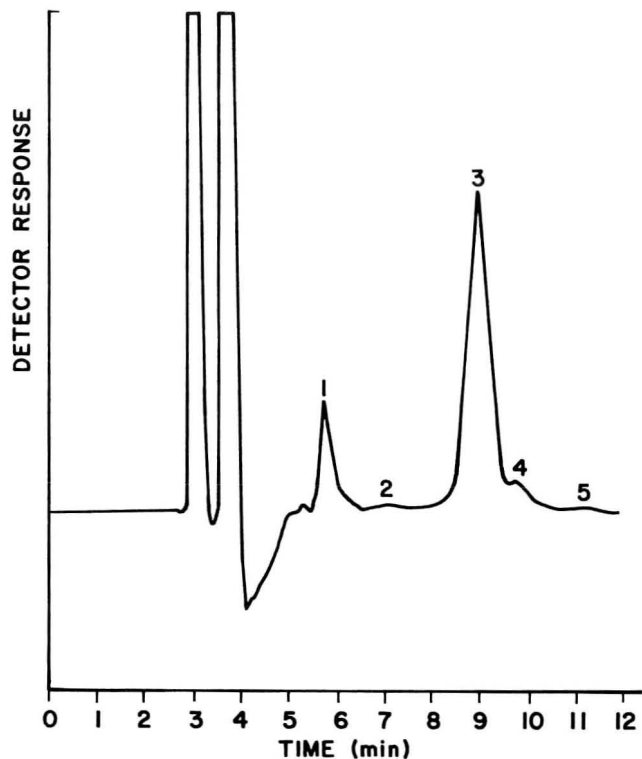
Figure 15: Chromatogram of laboratory saponified styrenated-
            acrylated alkyd resin fatty acids. Identification
            of acids: (1) ricinoleic; (2) myristic; (3) 9,11-
            octadecadienoic and 9,12-octadecadienoic; (4) oleic;
            (5) stearic. Mobile phase composition same as in
            Figure 10.

coconut, soya, and castor oil derived fatty acid mixtures have

yielded agreement for individual component fatty acids within

1-3% of compositions determined by methyl ester gas-liquid

chromatographic analysis. These results have been obtained on

liquid chromatographic peaks which were uncorrected for detector

response. It should be noted that comparisons to reported com-

positions of commercial oils are not entirely significant, since

composition of certain major components in fatty acid mixtures

derived from natural oil sources may vary as much as 5%. The
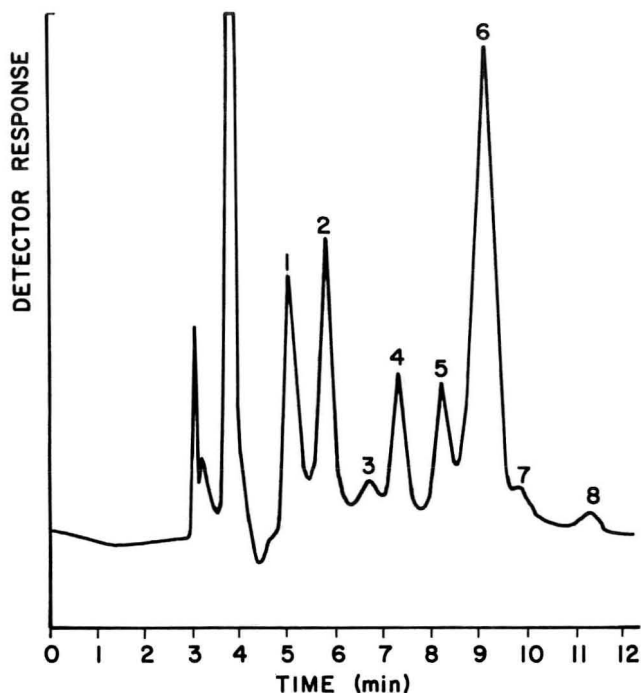
Figure 16: Chromatogram of laboratory saponified rosin-maleic
ester modified alkyd fatty acids. Identification of
acids: (1) undecylenic; (2) ricinoleic; (3) cis or
trans-9-hexadecenoic; (4) myristic; (5) linolenic;
(6) 9,11-octadecadienoic and 9,12-octadecadienoic;
(7) oleic; (8) stearic. Mobile phase composition
same as in Figure 10.

variation is a result of the varied environments under which
such natural oils are derived.

For example, in Table IV uncorrected peak areas have been
normalized for the stearic acid mixture in Figure 8. When this
data is compared to results obtained from GC analysis (53,54),
one can see that typical stearic acid mixtures varied over an eight-
year period. In this context, the comparison of the liquid and
gas chromatographic results is quite good.

Table V affords an actual comparison between liquid and gas
chromatographic results obtained on the same fatty acid mixture.

TABLE IV

FATTY ACID COMPOSITION OF COMMERCIAL
STEARIC ACID SAMPLE

Fatty Acid	HPLC (1976) % Total	GLC (1966) % Total	GLC (1974) % Total
Myristic	1.6	2.5	2.5
Pentadecenoic	0.1	0.5	0.5
Palmitic	48.7	53.0	50.0
Margaric	3.7	2.0	1.5
Stearic	45.9	42.0	45.5

TABLE V

COMPARISON OF FATTY ACID COMPOSITIONS
DETERMINED BY HPLC AND GC

Fatty Acid	HPLC % Total	GC % Total
Caprylic	1.2	0.3
Capric	2.0	1.4
Lauric	58.3	56.0
Myristic	21.3	23.4
Palmitic	7.9	9.5
Linoleic	0.8	0.3
Stearic	1.6	1.9
Oleic	7.1	7.3

The gas chromatograph results were corrected for detector response while the liquid chromatographic data was obtained by normalizing peak heights uncorrected for refractometer response. In general, the agreement is moderately good and would undoubtedly be better if the liquid chromatographic peaks were corrected for detector response. It should be noted that the HPLC analysis took 4-1/2 minutes per sample.

TABLE VI

COMPARISON OF FATTY ACID COMPOSITION OF
COCONUT OIL ANALYZED OVER A THREE-WEEK
PERIOD BY HPLC

Fatty Acid	1st Week % Total	2nd Week % Total	3rd Week % Total
Caprylic	2.2	2.1	2.1
Capric	3.2	3.1	3.4
Lauric	46.1	45.7	45.8
Myristic	20.0	19.8	19.8
Palmitic + Linoleic	15.1	15.2	14.9
Oleic	8.9	9.2	9.2
Stearic	3.8	4.4	4.1
Unidentified Peaks	0.7	0.5	0.7

The stability of this analytical method is indicated by the results presented in Table VI. The compositional data reported for a coconut oil fatty mixture was taken over a three-week time span. Each assay was taken approximately one week apart on the same sample. The agreement for the percentages of fatty acid is usually within 0.1-0.2%, quite acceptable for fatty acid analysis.

In summary, the above column/mobile phase system is capable of rapidly determining the fatty acid composition of commercial oils and alkyd resins. With appropriate adjustment of the mobile phase, quantitation of many of the component fatty acids can be made possible. The results of studies on marine oils, incorporating ultraviolet as well as refractive index detection, will be presented in the near future as well as the analysis of fatty alcohols.

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THE PREPARATIVE SCALE REVERSE PHASE HPLC SEPARATION OF EPIMERIC
ALKALOIDS USING CAMPHORSULFONIC ACID AS AN ION PAIRING REAGENT

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ABSTRACT

A reverse phase paired ion HPLC procedure is described for the
separation of multigram quantities of epimeric alkaloids using
camphorsulfonic acid as the ion-pairing reagent.

INTRODUCTION

In medicinal chemical research it is often necessary to separate
relatively large amounts of epimeric compounds for subsequent structure-
activity studies. With the advent of preparative HPLC, multigram
separations of a great many classes of compounds have become
feasible(1-4). However, there are limitations imposed on this
technology by the paucity of stationary phases available in commercial
preparative scale cartridges, i. e., silica gel normal phase and C_{18}
reverse phase. While many classes of compounds are successfully
separated using these stationary phases, there has been a notable lack
of success with alkaloids. Normal phase chromatography has been

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generally unsatisfactory due to the highly polar and acidic nature of
the adsorbant. While reverse phase HPLC offers some advantage(5-7),
alkaloids tend to exhibit nonideal behavior, resulting in streaking and
poor resolution. Also, the usual reverse phase procedure is of limited
value on the preparative scale due to the rapid deactivation of the
stationary phase that occurs at the high pH required for successful
separations. More recently, ion-paired reverse phase techniques have
been particularly successful for the separation of mixtures of alkaloids
on the analytical scale(8-12). Unfortunately, the very high cost of the
long-chain sulfonic acids used as ion-pairing reagents makes their use
on the preparative scale economically unfeasible.

In order to overcome this disadvantage, we have investigated the
use of commercially available racemic camphor-10-sulfonic acid (CSA) as
an ion-pairing reagent on the preparative scale for the separation of
mixtures of alkaloids. While CSA has proven to be useful on the
analytical scale(13,14), no preparative applications have been reported.
Preliminary trials on reverse phase TLC using ion-pair techniques showed
no measurable differences in separation with either CSA or the long-
chain alkyl sulfonic acids. As representative alkaloids we have chosen
the 9-disubstituted alpha- and beta-3-azabicyclo[3.3.1]nonanes 1 and 2,
which occur in a 70:30 ratio as the products of a Grignard reaction.
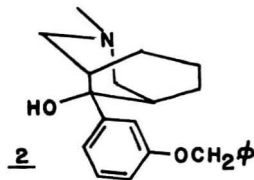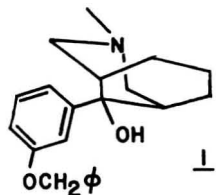
EXPERIMENTAL

Reagents:

The reverse phase TLC plates were Whatman KC18F. Methanol and
ethyl acetate were obtained from J.T.Baker and distilled from glass.
The water was doubly distilled from a glass apparatus. The CSA was
obtained from Aldrich Chemical Co. (Metuchen, NJ) and was purified by
treatment with decolorizing carbon (Norite A) followed by
recrystallization from ethyl acetate. The purified CSA was then
dissolved in water and passed through a flash chromatography column
filled with bondapak C_{18} (Waters Associates, Milford, MA) to remove
nonpolar impurities. The solution was lyophilized and the solid was
recrystallized from ethyl acetate again before use.

Preparation of Mobile Phase:

Two mobile phases were used. One was 60 volume % aqueous buffered
methanol, and the other was 20 volume % aqueous buffered methanol. Both
contained 10 mM CSA, 2% acetic acid, and were buffered to pH 2.5 with
sodium acetate trihydrate. Each was prepared by dissolving the
necessary amounts of CSA, glacial acetic acid, and sodium acetate
trihydrate in the aqueous portions of the mobile phases. These aqueous
solutions and the methanol were filtered separately through 0.45 micron
Millipore filters immediately prior to use and were then mixed to
produce the final mobile phases stated above.

The Apparatus:

The HPLC apparatus used was a Prep/LC 500 unit equipped with a
refractive index detector (Waters Associates, Milford, MA). The column
was a cartridge containing 500 g of bondapak C_{18} reverse phase packing
material also obtained from Waters Associates.
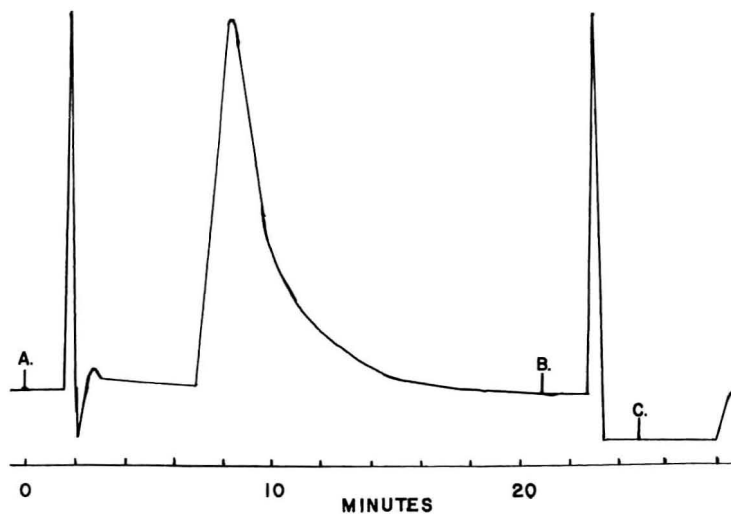
Figure 1. Detector output for the separation of 1 and 2. The chart speed was 2 min/cm at a flow rate of 250 mL/min. Legends: A. Injection of eluent; B. Change to 20 volume % aqueous buffered methanol; C. Restore 60 volume % aqueous buffered methanol.

RESULTS AND DISCUSSION

A typical chromatographic separation of 1 and 2 is illustrated in Figure 1. The best results were obtained using a 60 volume % aqueous buffered methanol mobile phase. After allowing the stationary and mobile phases to equilibrate, the alkaloid mixture was introduced in 10 gram quantities as the CSA salts in a minimum amount of methanol (final volume 25mL). Under these conditions, the alpha epimer eluted first, followed by the beta epimer. We subsequently found that the time and solvent volume needed for elution of the beta epimer could be substantially reduced by converting in one step to 20 volume % aqueous buffered methanol immediately after the alpha epimer had eluted. All of the beta epimer was eluted after two column volumes under these conditions. Using this procedure, four or five successive separations

could be performed before deterioration of the baseline on the
refractive index detector occurred. A stable baseline was restored by
flushing the system with 6 - 8 liters of water to remove the pairing
reagent and buffers, then with 2 liters of 50 volume % aqueous methanol
followed by 2 liters of 100 % methanol. The column was then re-
equilibrated by treatment with 2 liters of 50 volume % aqueous methanol
again, and finally with 60 volume % aqueous buffered methanol. Failure
to remove the 100% methanol from the column before the buffered mobile
phase is introduced results in precipitation of the salts on the column
and subsequent difficulty in restoring it to the functional state. The
column can be used indefinitely without loss of performance under these
conditions.

The alkaloids were recovered by combining homogeneous fractions and
alkalinizing them to pH 10 with sodium hydroxide pellets. The methanol
was evaporated in vacuo and the resulting alkaline aqueous suspension
was subjected to overnight continuous extraction with chloroform. The
organic phase from the extraction was then dried over anhydrous sodium
sulfate and evaporated to dryness, producing essentially quantitative
recovery of the free bases.

The chromatographic efficiency of the preparative scale cartridge
described herein did not even closely approach that exhibited by the
microparticulate HPLC columns presently available for analytical
separations. In fact, $\underset{\sim}{1}$ and $\underset{\sim}{2}$ were easily separable under a variety of
simple conditions using a 10-micron C_{18} analytical column. Because of
this large difference in efficiencies, it is quite difficult to find the
optimal preparative scale chromatographic parameters using an analytical
HPLC system. We have found that for scouting purposes, commercially
available reverse phase TLC plates more realistically approximate the
chromatographic behavior exhibited on the preparative scale. Moreover,

the epimeric purity of the resulting eluent fractions may easily be
checked by the same technique.

The success of the chromatographic separations using this technique
is apparently highly sensitive to the composition of the mobile phase.
For example, the concentration of ion pairing reagent affects the
chromatographic behavior, with higher concentrations of CSA increasing
the efficiency of the column toward the eluents, at least over a limited
range. Thus, mobile phase that was 10 mM in CSA produced much sharper
peaks and improved resolution over mobile phase that was 5 mM in CSA.
While still higher concentrations of CSA may further improve
chromatographic behavior in these systems, they were not investigated.
Using higher concentrations of pairing reagent requires the use of a
buffer, since the lower pH of the more concentrated CSA solution causes
column degradation by loss of covalently bonded stationary phase. In
this study, sodium acetate trihydrate was used to obtain the proper pH
for the mobile phase. Also, the presence of acetic acid was found to be
necessary for a successful separation. However, there were no
differences in the quality of the separations over the range of acetic
acid concentrations investigated (1%-3.5%).

We briefly investigated the influence of pH upon separation. As
one would expect, pH strongly influences chromatographic behavior in
this system. Increasing the pH of the mobile phase from pH 2.5 to 3.0
(or even to pH 2.75) significantly degrades chromatographic performance.
On this basis we may state that at least to a first approximation, the
best chromatographic performance in systems of this kind may be obtained
at the highest millimolar concentration of CSA and the lowest pH of the
mobile phase consistent with column and eluent stability.

We feel that this method offers several advantages for the
separation of alkaloids on the preparative scale. The separation is

complete and reproducible, recovery of the alkaloids is facile and quantitative, and perhaps most importantly, the reagents used are inexpensive and readily available. A singular disadvantage is the expense of the C_{18} stationary phase. While this expense necessitates the rigorous purification of the reagents used in order to insure column longevity, with proper care the column remains functional indefinitely. We are presently investigating the general applicability of this procedure to other classes of alkaloids.

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A RAPID METHOD OF QUANTITATING STEROIDS RESULTING FROM
THE INCUBATION OF GONADAL TISSUES WITH RADIOACTIVE PRECURSORS

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ABSTRACT

A rapid method has been developed for the quantitation of steroid
metabolites resulting from the incubation of specific gonadal cell types or
gonadal tissue with radioactive precursors. The method involves the use of
high performance liquid chromatography (HPLC) for separating the steroids and
a flow-through radioactive detector (Flo-One HP) for quantitating the radioac-
tive ^3H precursor and metabolites in the presence or absence of ^{14}C-
steroid recovery tracers. A comparison is made between the results ob-
tained directly by the Flo-One HP radioactivity detector and the fraction col-
lection method, (counting aliquots from individual fractions in the liquid
scintillation counter). In addition, the results using an electronic stream
splitter in the analysis of a percentage of the effluent directly by Flo-One
HP are evaluated. The remaining percentage is collected in a fraction col-
lector and is used for further analysis (e.g. recrystallization, RIA, further
purification and characterization).

The separation and quantitation of steroids from various tissues has been

routinely done by one of several techniques including direct colorimetric an-

alysis (1), radioimmunoassay (2), gas chromatography (3), and gas chroma-

tography-mass spectrometry (4). These techniques are very sensitive and ac-

curate for tissue studies involving determination of the exact amount of var-

ious steroids present, but for metabolic studies using radioactive precursors,

these techniques are not effective for quantitation. Previously, for the

quantitation and separation of radioactive gonadal steroids resulting from the

incubation of specific gonadal cell cultures or tissues with radioactive

precursors, the major procedure used was a combination of paper and thin layer

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chromatography. This involved the initial extraction of the tissue; and, the extract was initially chromatographed on paper, followed by radioactivity scanning to locate the radioactive zones. The zones containing the radioactivity (5,6) were then eluted. Each of the eluted zones was separated further by thin layer chromatography. The developed thin-layer plates were then scanned for radioactivity, the silica gel removed from the plate and eluted with solvent. The solvent was filtered through a fritted glass filter to remove silica gel before quantitation by recrystallization (until a constant $^{14}C/^{3}H$ ratio was established). This procedure was difficult and time consuming, and required from one to four weeks for completion (7). More recently, with the development of HPLC, the separation of radiolabeled steroids (8-12) has become much more efficient but the quantitation still required the fraction collecting of the HPLC eluent into individual tubes, removing an aliquot from each tube to a scintillation vial, and then counting directly in the scintillation counter (fraction collection method).

This paper describes the use of an HPLC system (8) for the separation of gonadal steroids found in gonadal tissue or specific cell cultures. This HPLC system is interfaced with one of three separate options for the quantitation of the radioactive steroids. First, the eluent from the HPLC can be collected in tubes using a fraction collector with a rapid tube change (fraction collection method). Second, the eluent from the HPLC can be quantitated directly with a flow through radioactive detector (Flo-One HP). This detector permits the direct quantitation of both ^{14}C and ^{3}H labeled steroids used in metabolic studies of gonadal tissues. Third, the eluent from the HPLC is split using an electronic stream splitter with a certain percentage being directed to the flow radioactive detector and the remaining collected in tubes for further analysis (i.e. purification, recrystallization, NMR, GC-mass spectrometry, etc.). The latter two detection system in conjunction with the HPLC permit the separation and quantitation of gonadal steroid (radiolabeled) in less than one hour.

MATERIAL AND METHODS

The Waters (Milford, MA,) HPLC system was employed for all of the studies. This system consists of a U6K injector, one 6000A and one M45 pump, a 450 variable-wavelength detector, a 440 fixed-wavelength (254nm) detector, a 730 Data Module (for plotting and analysis of peak area and retention time) and a 720 Data Controller. A radial-Pak A (reversed-phase permanently bonded 10μm octadecylsilane C_{18} column, 8 cm in length) was used as part of a radial compression system.

A 3μm reversed-phase C_{18} (12% ODS) column, 4.6 mm ID x 15 cm (Custom LC, Houston, Texas) was employed for high-resolution HPLC. The Waters HPLC was equipped with an in-line precolumn filter. A 25 μl Precision Sampling pressure lock syringe, (Supelco, Bellfort, PA.) was used for sample injections.

Highly purified water was obtained by triple glass distillation of de-ionized water in our laboratory. Methanol (glass distilled-Omni Solv, MCB Manufacturing Chemists, Inc., Cincinnati, Ohio, Lot #10M/4) was used. The steroids were from Steraloids (Wilton, N.H.) and the radioactive steroids were obtained from New England Nuclear (Boston, MA.). The buffers and solvents were filtered through a 0.45μm Millipore filter and then degassed.

For the detection of radioactive metabolites one of the following methods was employed. First, the Water's HPLC system was interfaced with a RediRac Fraction Collector, Model 2112 (LKB, Rockville, MD.), with fractions being collected at 0.5 min intervals. A 50 μl aliquot was removed from each fraction, dried in a scintillation vial, 10 ml of scintillation fluid (PPO, 5 gm/liter of toluene) was added. Each sample fraction was counted for 5.0 min in a Packard Liquid Scintillation Counter. Second, the Waters HPLC system was interfaced directly with the Flo-One HP radioactivity detector (0.500 ml flow cell), using Flo Scint II as the nongelling scintillation fluid (Flo Scint II: effluent, 3:1, v/v). The No. 2 pen of the Waters Data Module was connected directly to the output of the Flo-One HP, resulting in a plot of dis-

integrations per 6 sec (either ^{14}C or ^{3}H). The ^{3}H and ^{14}C counts were
determined simultaneously with the Flo-One HP which automatically corrects for
the ^{14}C crossover and prints out the corrected number of dpm of ^{3}H and
[14]C. Third, the calculation and the data obtained from aliquots counted
directly in the Packard Liquid Scintillation Counter were compared to the data
from the Flo-One HP. This was accomplished by splitting the column effluent
after passing through the Fixed Wavelength 440 detector (Water Associates,
Milford, Mass.) with the Electronic Variable Ratio Splitter, Model ES
(Radiomatic Instruments and Chemical Co., Tampa, Florida) 50% at 2-sec inter-
vals.

Human testicular biopsy tissues were incubated *in vitro* with [3]H
pregnenolone by a previously described technique (13). The involves incuba-
tion of teased testicular tissue (20 mg) with a saturating concentration of
[3]H-pregnenolone in 3 ml of incubation medium in an atmosphere of O_2: CO_2
(95:5) at 37°C under constant shaking for 3 hr. At the end of the incubation,
[14]C-labeled recovery tracers and nonlabeled carrier steroids were added. The
metabolites formed and remaining substrate were extracted, dried and injected
into the Water HPLC system using the 3 μm C_{18} (12% ODS column) described ear-
lier. The procedure for incubating the interstitial cell cultures with
radioactive precursors has been described elsewhere (14).

RESULTS AND DISCUSSION

Previously, the separation and quantitation of gonadal steroids involved
several steps (thin layer and paper chromatography) or derivatization (gas
chromatography, colorimetric, gas chromatography-mass spectrometer) of the
steroids was necessary. These techniques were excellent for quantitation of
large amounts of steroids but for radiolabeled steroids used in metabolic
studies these techniques could not be used, because these techniques were not
sensitive for the quantitation of radioactivity. More recently with the de-
velopment of the HPLC for the separation of gonadal steroids (8,9,10),
radiolabeled steroids could be quantitated by collection of the eluent from
the HPLC with fraction collector method. This involved fraction collecting of

the HPLC effluent. Each of the individual tubes could then be quantitated in
a liquid scintillation counter. These methods proved excellent for quantita-
tion, but required a large number of scintillation vials, scintillation fluid,
time to aliquot the samples, and liquid scintillation counting time. This
paper presents the comparison of the fraction collector method with two other
direct method for the direct quantitation of radiolabeled steroids separated
by the HPLC.

COMPARISON OF FRACTION COLLECTION AND DIRECT RADIOACTIVE DETECTION METHODS

A simple method for the quantitation of radioactive steroids after HPLC
is to collect fractions at short time intervals, and to count aliquots in
scintillation vials, in a liquid scintillation counter. The procedure re-
quires sufficiently rapid tube changes so that no more than one drop is lost
between fractions. The results of fraction collecting an HPLC analysis of an
extract of an incubation of interstitial cells with ^{3}H-testosterone is shown
in Figure 1A. Individual fractions were collected every 0.5 min. Two major
peaks and one minor peak are seen. Allowing 5 minutes for counting each frac-
tion, it took 8-10 hours to count 80 fraction obtained from each sample.

A comparison of the fraction collection method with the direct analysis
by interfacing the eluent from the HPLC with a flow-through radioactivity de-
tector (Flo-One HP) is shown in Figure 1 A + B. In Figure 1B, (radioactive
detector) two major and four or five minor peaks are detected and found to be
well resolved from each other. This greater resolution is due to the fact
that the Flo-One HP gives an updated signal to the recorder every 6 seconds.
This data is then plotted on Pen 2 of the Waters Data Module. To obtain
similar results by the fraction collection method, fractions would have to be
collected every 0.1 minute over the 40 min collection period, i.e. 400 tubes.
Thus, the flow-through detector is more rapid and yields greater re-
solution than the fraction collector method. The time and cost of sample an-
alysis is also lower for the flow-through radioactive detector.

The quantitative analysis by the fraction collection method is compared
with the Flo-One HP method in Table I for two similar samples from study of

ELUTION (Minutes)
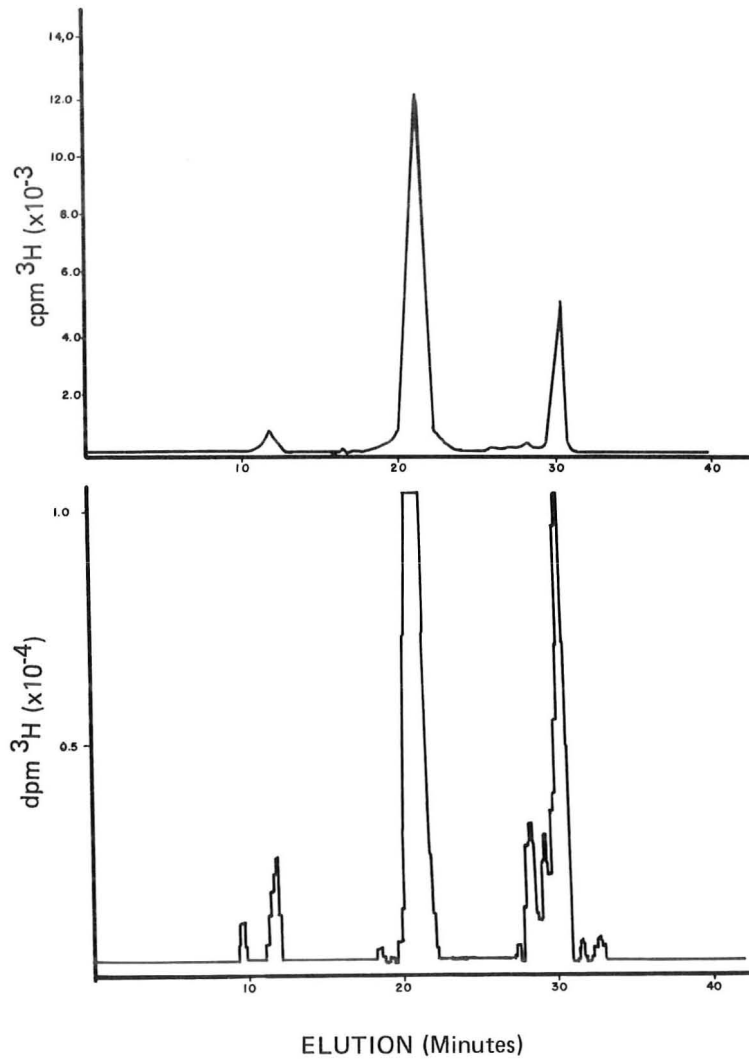
Figure 1: HPLC analysis of an incubate of isolated testicular interstitial cells with ^{3}H–testosterone using a 10μm C$_{18}$ radial compression column eluting with a methanol/water gradient. Conditions: Solvent A = methanol/H$_2$0, (1/1,V/V) and Solvent B = methanol. A linear gradient 2.0 ml/min. 0%B$\rightarrow$30%B, 20 minutes followed by 30$\rightarrow$100%B for the next 20 minutes. The upper panel is a plot of cpm/aliquot of fractions from the Redi Rac 2112 fraction collector, fractions are collected every 0.5 min (1.0 ml each) and count for 5.0 min each in liquid scintillation counter. The lower panel is the actual plot the Flo-One HP radioactivity detector signal for ^{3}H dpm printed out by Pen 2 of the Waters Data Module.

Table I: Comparison of Counts Obtained With Flo-One HP
Flow Radioactive Detector and Fraction Collection Followed by
Liquid Scintillation Counting

Sample #	Peak*#	Flo-One/HP (dpm)	F.C. (dpm)	Flo-One dpm/F.C. dpm
84 F	1	2,860	3,636	0.78
	2	65,400	79,070	0.82
	3	9,040	12,325	0.73
	4	48,628	58,387	0.83
			153,418 dpm	
			(99% recovery)	
			S.D. = 0.045	S.E. = 0.023
84 B	1	2,770	3,614	0.76
	2	39,304	52,727	0.75
	3	18,810	22,872	0.82
			79,213 dpm	
			(91% recovery)	
			S.D. = 0.039	S.E. = 0.021
		TOTAL:	S.E. = 0.039	S.E. = 0.015

* Peak # is the order of elution of the major radioactive peaks shown in Figure 1.

the metabolism of radioactive steriods by interstitial cell cultures. The
overall recoveries from HPLC were 91-99%, and the mean ratio dpm Flo-One/dpm
scintillation counter was 0.784 for the seven peaks (Figure 1) shown on Table
I. Thus, the overall efficiency of the flow-through detector for ^{3}H was 20%
(0.784 x 25% efficiency set on the Flo-One HP) or about 1/2 that of the liquid
scintillation counter. In addition, the standard deviation and error are very
small, indicating that the efficiency for the flow through detector is
maintained for low counting (2860 dpm) as well as high counting (48,628 dpm)
samples. The Flo-One HP radioactivity detector can thus quantitate the
radioactive steroids in the eluate from HPLC in less than one hour per sample
compared to 5-10 hrs for the fraction collection method counting 5
minutes/tube.

ELECTRONIC SPLITTER - ANALYSIS OF A CERTAIN PERCENTAGE OF THE SAMPLE

The major drawback of using only the Flo-One HP and mixing the HPLC effluent with a nongelling scintillation is that the entire sample must be utilized, thus, the sample cannot be used for further analysis. Instead of the use of the liquid scintillation mixing, a solid scintillator could be used. This would allow complete recovery of the sample, but for detectable counts above background 10-20,000 dpm of ^{3}H and 250-400 dpm of ^{14}C would be required. The solid scintillation also has the disadvantage that the radioactive sample may interact with the solid support (usually silica) and slowly bleed from the solid scintillator giving high background levels, or actual bind to the solid scintillation.

Ideally, the system of choice should have high sensitivity (800 dpm for ^{3}H) and allow at least partial recovery of the sample. The following method was used to accomplish this objective. The eluent from HPLC was diverted to an electronic splitter (ES) using microbore tubing. Fifty percent of the eluate was diverted to the fraction collector and the remaining 50% to the Flo-One HP radioactive detector. This method was employed to analyze human testicular biopsy specimens, which were incubated with ^{3}H-pregnenolone for 3 hours. At the end of the incubation, before the samples were extracted, ^{14}C-recovery tracers were added. The samples were extracted and the extract analyzed by HPLC or paper and thin-layer chromatography. The results obtained by each technique were very similar (manuscript in preparation), but HPLC using the conditions shown in Figure 2 was much more rapid. After elution from HPLC, the sample was divided by the ES (50% to fraction collector, 3A and 50% to Flo-One HP, 3B). The results obtained by the fraction collection method, Figure 3 A, were corrected to the entire sample (multiplied times two), with the Flo-One HP graph not corrected to the entire sample with the dpm for only 1/2 of the sample. The plot, Figure 3C, showed the actual histogram for ^{3}H as plotted by the Water Data Module (Pen 2). As can be seen from Figure 3A, B, C, the profiles for the two methods are almost identical for both ^{14}C and ^{3}H. The Flo-One HP corrected the ^{3}H channel for the ^{14}C crossover
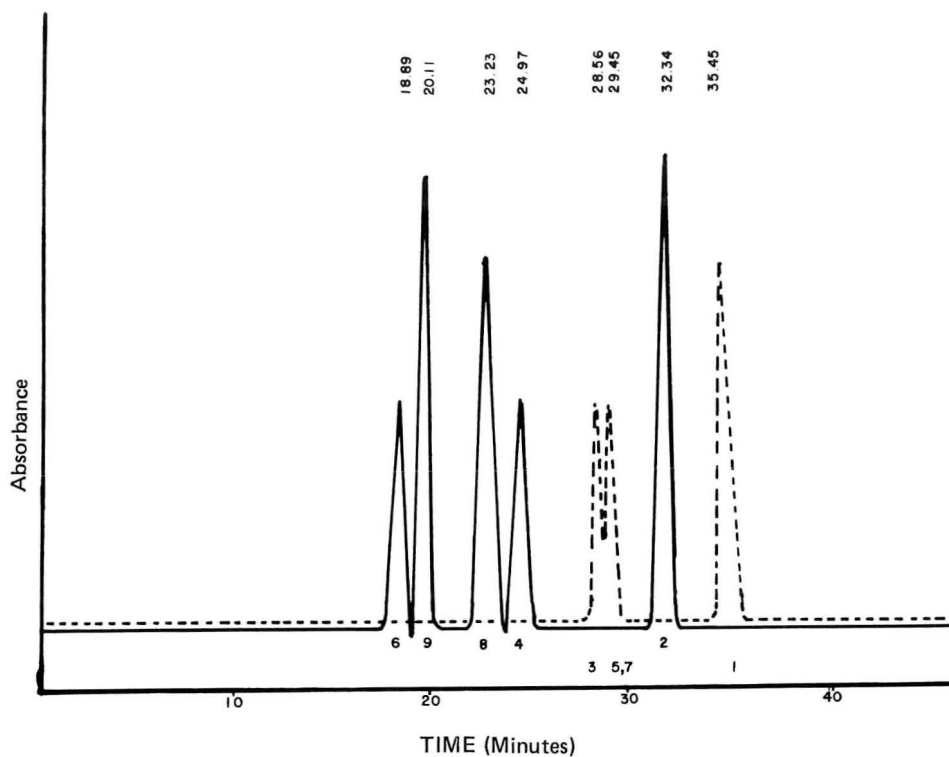
Figure 2: HPLC separation of gonadal steroids obtained by using methanol-/water as the eluent on a 3 μm C$_{18}$ reversed-phase stainless steel column. Solvent A = methanol/water (1/1,v/v) and solvent B = methanol are used. A gradient elution at a flow rate of 1.0 ml/min from 0→10% B over 20 minutes with Waters Curve 6 (linear), followed by Waters Curve 2 to 50% B in the next 10 minutes, and then a linear increase to 100% B in the next 15 minutes is used. Steroid absorbance of the progesterone pathway steroids is are plotted by a solid line; the dashed line represents the pregnenolone pathway steroids. The relative retention times are shown above each peak. The number below each peak refers to the identity of each peak: (1)pregnenolone, (2) progesterone, (3) dehydroepiandrosterone, (4) 17-hydroxyprogesterone, (5) 17-hydroxy-pregnenolone, (6) androstenedione, (7) Δ^5-androstenediol, (8) testosterone, (9) estradiol.
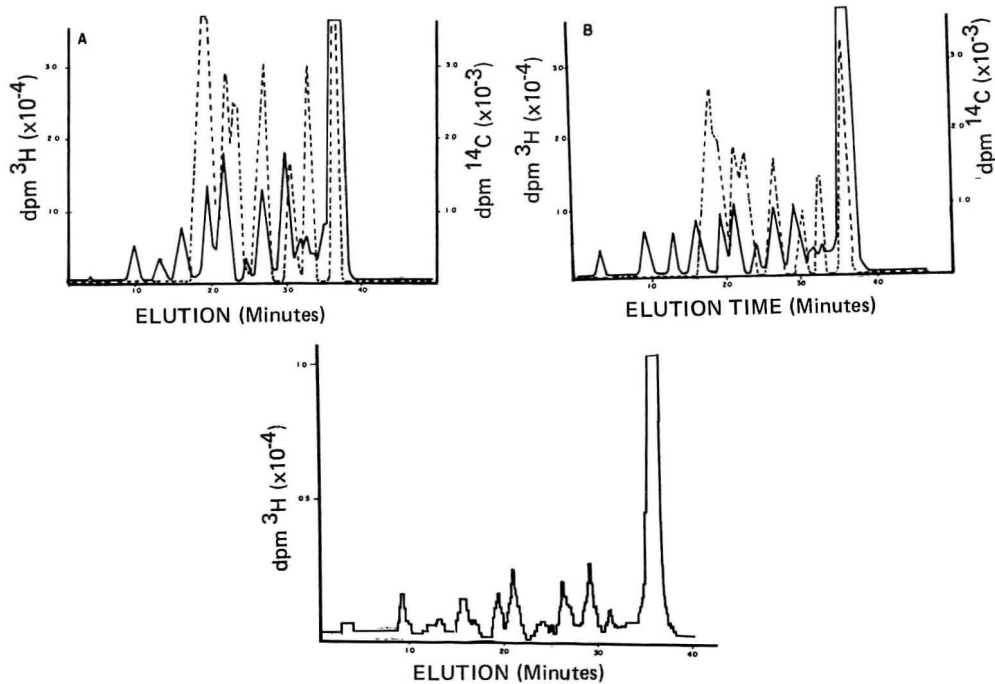
Figure 3: HPLC of a human testicular biopsy specimen incubated with ^{3}H-pregnenolone. At the end of the incubation ^{14}C recovery tracers were added the sample extract with organic solvent, and the steroids were analyzed on the HPLC using condition shown in Figure 2. The eluent from HPLC was split with an electronic splitter, 50% was diverted to fraction collector and 50% was analyzed directly by the flow-through radioactivity detector. Panel A is a plot of the fraction collector number (0.5 minute/fraction) vs. dpm, corrected for the entire sample (multiplied by 2). Panel B shows the direct analysis of the sample by the Flo-One HP radioactive detector, analysis for ^{14}C and ^{3}H being printed every 0.5 minute. The ^{14}C and ^{3}H were then plotted on the same time scale as the fraction collected samples. The dashed line represent ^{14}C and the solid line [3]H. The lower panel is the actual plot of the Water Data Module Pen 2 (10,000 dpm full scale). The time scale is different from panel A and B, since it is a direct reproduction of actual plot (0.50 cm/minute) for ^{3}H dpms.

(number of ^{14}C counts in the ^{3}H channel) and automatically calculated the number of dpm for both ^{14}C and ^{3}H. This feature was advantagous, because a ^{3}H precursor and ^{14}C recovery tracers are used in most gonadal steroid metabolism studies because of the loss of various steroids in the extraction and subsequent purification steps. The overall recoveries for the two method are about the same for ^{3}H and ^{14}C, as shown in Table II with a several different radioactive steroid peaks being analyzed (dpm/peak).

The results in Table II show that the data obtained by using the fraction collection method, compare very well with those obtained with the radioactivity flow detector when the eluate is split in half for each of the two modes of detection. The overall ratio of dpm for the scintillation counting to the radioactivity detector is about one, the efficiency for ^{14}C is 52.42% in the ^{14}C over ^{3}H channel. With the 25% crossover of ^{14}C into the ^{3}H channel the overall efficiency for the complete ^{14}C (52.42 + 25.0%) is 77.42% compared with 90–95% for the scintillation counter. The overall ^{3}H efficiency for the radioactivity flow detector is about 37.19% with a standard deviation of 3.56 and a standard error of 1.26, compared to the 50–60% efficiency for the liquid scintillation counter. Therefore, the radioactive flow detector can be used to quantitate rapidly ^{14}C and ^{3}H simultaneously in a sample and using the electronic splitter a portion (1–99%) of the eluate can collected in fraction collector tubes for further analysis. Eight human testicular biopsy specimens have been analyzed by this procedure. The HPLC and Flo-One HP separation and quantitation method represents a rapid method for studying steroidogenesis in human testes.

In conclusion, radiolabeled steroids from metabolic studies can be separated by HPLC and quantitated directly by the Flo-One HP. In addition, with the use of an electronic stream splitter a certain percentage of the eluate from the HPLC can be diverted to the radioactivity flow detector with the remaining percentage of the sample used for further analyses (recrystallization, derivatization, or further purification by HPLC). These methods result in almost identical quantitative data compared to the previous used frac-

Table II: Ratio of dpm scintillation counter to
dpm on Flo-One HP radioactive flow detector and % efficiency
for several peaks of testicular biopsy sample (50% split)

A. Peak*# ^{14}C	dpm scintillation/dpm Flo-One HP[1]	% eff. ^{14}C[2]
1	1.05	52.5
2	1.27	63.5
3	0.93	46.5
4	0.93	41.5
5	1.15	57.5
6	0.96	48
MEAN = 1.048		52.42
S.D. = 0.138		6.90
S.E. = 0.056		2.81

B. Peak*# - ^{3}H	dpm scintillation/dpm Flo-One HP[3]	% eff. ^{3}H
1A	2.20	44.0
2A	1.89	37.8
3A	1.68	33.6
4A	1.80	35.0
5A	2.20	44.0
6A	1.89	37.8
7A	1.68	33.6
8A	1.80	36.0
MEAN = 1.89		37.19
S.D. = 0.205		3.56
S.E. = 0.072		1.26

1. Ratio of ^{14}C dpm for $^{14}C/^{3}H$ channel.

2. % efficiency for $^{14}C/^{3}H$ channel with 25% ^{14}C in ^{3}H channel.

3. The % efficiency for the radioactivity detector was set at 20%; thus, final % efficiency is 20% times the dpm scintillation/dpm Flo-One HP

* The peak in order of elution from the HPLC and relative to the standards shown in Figure 2.

tion collection method, with only a decrease of 30-40% in counting efficiency for ^{3}H. Finally, by employing these methods the quantitation is obtained immediately compared to the lengthy steps involved in the fraction collection method.

ACKNOWLEDGEMENTS

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DETERMINATION OF CARBAMIC HERBICIDES BY HIGH PERFORMANCE LIQUID
CHROMATOGRAPHY (HPLC). II. CHLORPROPHAM.

by

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ABSTRACT

Two simple, precise, and rapid reversed-phase high performance
liquid chromatographic procedures, External and Internal standard
methods, are described for the determination of chlorpropham in
emulsifiable concentrates. Samples are diluted with methanol and
4-nitro-diphenyl ether is added as the internal standard. Calibration
and quantitation is made with the use of pure chlorpropham, and
results obtained in absolute or relative amounts according to our
needs. Linearity in both cases is achieved from 0.1 to 15 μg of
chlorpropham, equivalent to 10 μL injections of solutions of this
chemical at concentrations of 1×10^{-2} to 1.5 g L^{-1}. Methanol/water
(60/40 V/V) at a flow of 2 mL min^{-1} is used as eluent and retention
times are approximately 4.4 and 5.9 min respectively for chlorpropham
and 4-nitro-diphenyl ether. Detection limit turned out to be 3.9 ng

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of chlorpropham, that is, 10 μL of a solution at 3.9×10^{-4} g L^{-1}.
The Internal standard method improves slightly the confidence limit
and the relative standard deviation with regard to the External
standard method.

INTRODUCTION

Chlorpropham (CIPC), Isopropyl 3-chloro-phenyl-carbamate
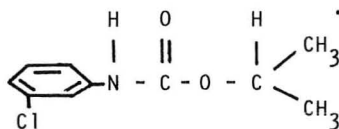
is a pre-emergence non-specific herbicide and inhibitor of sprouting
in ware potatoes.

It presents a low toxicity and is formulated as emulsifiable
concentrate or granules; product and formulated analysis is by
hydrolisis, measuring the carbon dioxide formed or titrating the
liberated 3-chloro-aniline with sodium nitrite (1) and by GLC with
lindane as internal standard (2); a slight decomposition of chlorpropham
occurs with this method. The first two are not specific.

To avoid the above inconveniences, two reversed-phase high
performance liquid chromatographic methods, with external (A) and
internal (B) standards, were developed.

Sparacino and Hines (3) separated some 30 carbamates using both
normal and reverse-phase mode by HPLC, working at the residue level.

MATERIALS AND METHODS

Apparatus

High performance liquid chromatograph Hewlett-Packard 1084 B
equipped with microprocessor, RP-8 chromatographic column and
Millipore filters as described in a previous paper (4).

Reagents

Eluent: Methanol HPLC quality (Merck, Darmstadt)/bidistilled water (60/40 V/V). Filter through appropriate Millipore filters and degas under light vacuum.

For method A

1) External standard solution. 0.2 mg mL^{-1} in methanol/water (65/35 V/V). Weigh approximately 0.050 g of pure chlorpropham (Riedel-de Haën AG, Seelze-Hannover) into a 50 mL volumetric flask, dissolve and dilute to volume with methanol/water 65/35. Dilute 10 mL of this mother solution in a 50 mL volumetric flask with the same solvent. Filter through appropriate Millipore filter into small vial and cap.

2) 40 % EC solution. Weigh approximately 0.150 g of 40 % Emulsifiable Concentrate (EC), (Zeltia Agraria, S.A., Porriño, Spain), into a 50 mL volumetric flask and dilute to volume with methanol/water 65/35. Dilute 10 mL of this solution in a 50 mL volumetric flask with the same solvent. Filter through appropriate Millipore filter into small vial and cap.

3) Working chlorpropham solutions. Weigh 1.1603 g of 40 % EC into a 50 mL volumetric flask and dilute to volume with methanol. Dilute 16 mL of this solution in a 25 mL volumetric flask with methanol (14.852 g L^{-1}). Eleven solutions of decreasing concentration were prepared, starting from the above and diluting to 1:2 with methanol.

For method B

1) Internal standard solution. Weigh 1.125 g of 4-nitro-diphenyl ether (Fluka AG, CH-9470 Buchs, Switzerland) into a 250 mL volumetric flask, dissolve and dilute to volume with methanol.

2) Mixed standard solution. 0.2 mg mL^{-1} chlorpropham and 0.9 mg mL^{-1} 4-nitro-diphenyl ether in methanol/water (65/35 V/V). Transfer 10 mL of the mother External standard solution from method A and 10 mL of the above Internal standard solution to a 50 mL volumetric

flask and dilute to volume with methanol/water 65/35. Filter through appropriate Millipore filter into small vial and cap.

3) Mixed 40 % EC solution. Transfer 10 mL of the 40 % EC solution from method A, at a concentration of 0.150 g 50 mL^{-1}, and 10 mL of the above Internal standard solution to a 50 mL volumetric flask and dilute to volume with methanol/water 65/35. Filter through appropriate Millipore filter into small vial and cap.

4) Mixed working chlorpropham solutions. Weigh 1.1343 g of 40 % EC into a 50 mL volumetric flask and dilute to volume with methanol. Dilute 16 mL of this solution in a 25 mL volumetric flask with methanol (14.519 g L^{-1}). Prepare eleven solutions of decreasing concentration, starting from the above and diluting always to 1:2 with methanol, in 25 mL volumetric flasks. Add to every flask 5 mL of the Internal standard solution and dilute to volume with methanol.

Chromatographic conditions

Eluent: Methanol/water (60/40 V/V), flow 2 mL min^{-1}, column temp. 40ºC, methanol temp. 45ºC, water temp. 80ºC, variable wavelength detector vis.-UV, wavelength 240 nm vs. 430 nm, inject. vol. 10 μL, attn. 2^8.

Calibration and quantitation

Inject 10 μL aliquots of every standard solution, for every method, into chromatograph until variation in standard peak areas is less than 1 %. Adjust detector sensitivity in order to obtain peak heights ca 60-80 % full scale. Calibrate and inject 10 μL of the samples to be analyzed. Results will be obtained in absolute or relative amounts, according to our needs, by automatic integration of the peak areas.

RESULTS AND DISCUSSION

Some previous tests were made to find out how many analyses should be made for every sample and how many injections for every

CHLORPROPHAM
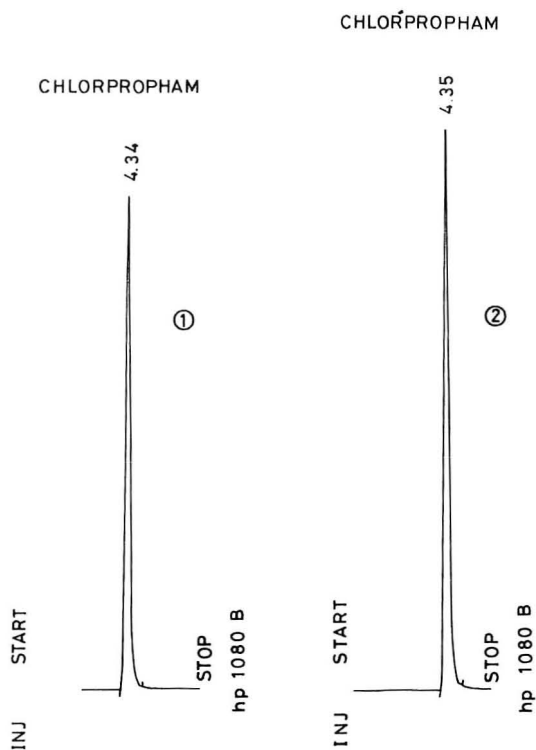
CHLORPROPHAM

Figure 1. Chromatography of pure ① and 40 % EC chlorpropham ② with CH_3OH/H_2O (60/40 V/V) as eluent.

analysis, in every method, in order to maintain a precision lower than 1 % (P = 0.01). One analysis and one injection are enough for the Internal standard method B. One analysis but three injections are necessary for the External standard method A.

The chromatography of pure and 40 % EC of clorpropham, with External standard, is shown in Figure 1. Retention time is approximately the same in both cases: 4.34 and 4.35 min. The concentration of active ingredient in the EC labelled 40 %, mean of 6 determinations, is 41.9 $\pm$ 0.13, s = 0.127, S_r = 0.303.

Scan for chlorpropham was done and it presents a maximum of absorbance at 240 nm and a shoulder at about 280 nm.

Table I. μg of chlorpropham injected and found and its correlation
         coefficient. Accuracy % and precision %.
         ESTD method A.

Correlation coefficient	injected μg	found μg	accuracy %	precision % [a]
	62.229	36.042	42.08	0.56
	31.115	27.404	11.93	0.68
	15.557	16.092	-3.44	1.29
	7.779	8.032	-3.25	0.13
	3.889	4.078	-4.86	0.71
	1.945	1.994	-2.52	0.31
0.9999	0.972	0.994	-2.26	0.77
	0.486	0.493	-1.44	1.02
	0.243	0.240	1.23	1.05
	0.122	0.117	4.10	1.71
	0.060	0.048	20.00	3.18

[a] Standard relative deviation of three determinations

$$\text{accuracy \%} = \frac{\text{injected μg} - \text{found μg}}{\text{injected μg}} \times 100$$

The eleven working clorpropham solutions from method A were
analyzed. Table I summarizes injected and found μg of chlorpropham
as well as accuracy, precision and calculated correlation coefficient
for those numbers in brackets, in which accuracy and precision are
smaller than 5 %. From this Table it is possible to reach the conclusion
that Beer's law is followed, and the optimum working space is found,
with a correlation coefficient of 0.9999, between 0.122 and 15.557 μg
of chlorpropham.
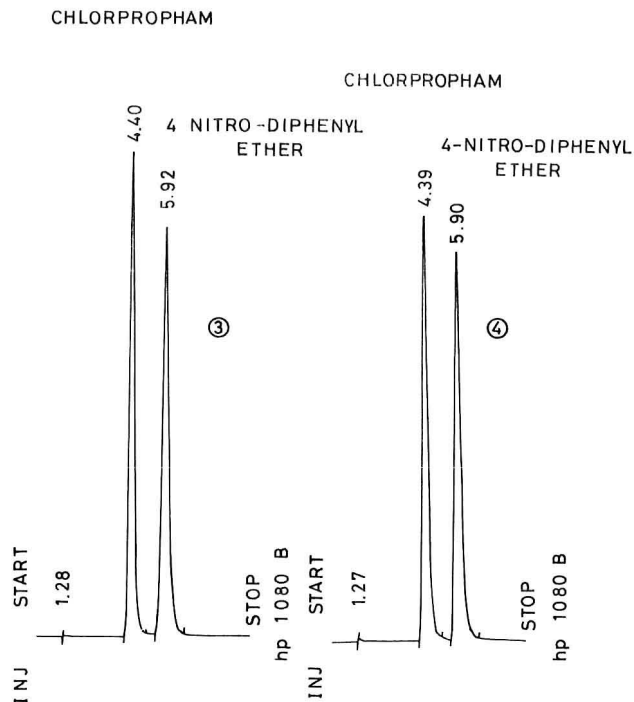
CHLORPROPHAM

Figure 2. Chromatography of pure ③ and 40% E C
chlorpropham ④ with CH_3OH H_2O (60/40
V/V) as eluent and 4-nitro-diphenyl ether
as internal standard.

In spite of the very good repeatability of the automatic
variable volume injector, the introduction of an internal standard,
4-nitro-diphenyl ether, improves the results.

The chromatography of pure and 40 % EC of chlorpropham, with
internal standard, is shown in Figure 2. Retention times are
aproximately the same in both cases: 4.40-5.92 and 4.39-5.90 min.
The concentration of active ingredient in the EC labelled 40 %, mean
of 6 determinations, is 41.3 $\pm$ 0.08, s = 0.079, S_r = 0.191.

The eleven Mixed working chlorpropham solutions from method B
were analyzed. Table II shows injected and found μg of chlorpropham

Table II. μg of chlorpropham injected and found and its correlation
          coefficient. Accuracy % and precision %.
          ISTD method B.

Correlation coefficient	injected μg	found μg	accuracy %	precision % [a]
	59.935	30.592	48.96	2.15
	29.967	25.704	14.23	0.71
	14.984	15.140	-1.04	0.23
	7.492	7.820	-4.38	0.30
	3.746	3.850	-2.78	0.27
0.9999	1.873	1.920	-2.51	0.21
	0.936	0.961	-2.67	1.04
	0.468	0.491	-4.91	2.60
	0.234	0.238	-1.71	2.52
	0.117	0.119	-1.71	4.37
	0.059	0.058	1.69	11.31

[a] Standard relative deviation of three determinations

$$\text{accuracy } \% = \frac{\text{injected μg} - \text{found μg}}{\text{injected μg}} \times 100$$

as well as accuracy, precision, and calculated correlation coefficient
for those numbers in brackets in which accuracy and precision are
smaller than 5 %. From this Table it is possible to reach the conclu-
sion that, although Beer´s law is followed from 0.059 to 14.984 μg,
the optimum working space, with a correlation coefficient of 0.9999,
is only found between 0.117 and 14.984 μg of chlorpropham.

    Lower concentrations of the working chlorpropham solutions
from method A were employed using a signal to noise ratio of 2:1,
to find the detection limit which turned out to be 3.9 ng of

chlorpropham, equivalent to 10 µL of a solution of this chemical at a concentration of 3.9×10^{-4} g L^{-1}.

In conclusion, both methods work satisfactorily in the analysis of EC of chlorpropham, the Internal standard one improves slightly the confidence limit and the relative standard deviation.

Linearity in both cases is achieved from about 0.1 to 15 µg of chlorpropham, equivalent to 10 µL injections of this chemical at concentrations of 1×10^{-2} to 1.5 g L^{-1}.

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THE RAPID DETERMINATION OF INDOMETHACIN
IN 50 µl BLOOD SAMPLES

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ABSTRACT

A method for the determination of indomethacin in 50 µl
samples of rat blood by high-pressure liquid chromatography has
been developed. After addition of sodium acetate buffer (pH=
5.4) and an internal standard (glutethimide), the blood was
extracted twice with heptane containing 1.5% (v/v) isoamyl alco-
hol. The organic solvent was evaporated, the residue dissolved
in methanol, and aliquots (5 µl) injected automatically into
the chromatograph. The separation and quantification of indo-
methacin was achieved on a µBondapak C_{18} column with 66% (v/v)
methanol in water solution at a flow rate of 2 ml/min and
detected at 254 nm wavelength. The analysis was linear for
concentrations ranging from 0.50 to 10 µg/ml indomethacin solu-
tions. The method was tested to determine the concentration of
indomethacin in blood of rats receiving orally 2 mg/kg of indo-
methacin.

INTRODUCTION

Indomethacin [1-(p-chlorobenzoyl)-5-methoxy-2-methylindole-
3-acetic acid], a potent inhibitor of prostaglandin synthesis,
is clinically used as an anti-inflammatory agent as well as a
promoter of constriction of the patent ductus arteriosus in

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premature infants (1,2). In biological research, it is widely
used as a tool to clarify the involvement of prostaglandins in
a number of physiological functions (3).

Several methods for the detection of indomethacin in bio-
logical samples have been described using gas-liquid chromatog-
raphy (4-7), liquid chromatography (8), and mass fragmentography
(9). These methods are sensitive and specific; however, they
have some shortcomings such as the need for high-volume sample
size, use of hazardous solvents, derivatization steps, or expen-
sive detectors.

In our research program, we have the need for a fast,
accurate, and economical method for quantification of indometha-
cin in small-size blood samples. This report presents such a
method using glutethimide as the internal standard and reverse-
phase high-pressure liquid chromatography under isocratic con-
ditions with methanol as the eluant and automatic sample
injection.

METHODS

Apparatus

The analyses were performed in a Waters Associated Liquid
Chromatograph equipped with a Model 440 Absorbance Detector
(254 nm wavelength), a WISP Model 710A Autosampler, and a
Perkin-Elmer Sigma I Data System. The separation of indometha-
cin and the internal standard was achieved using a 4 mm i.d. x
30 cm μBondapak C_{18} column (Waters Associates) with a 66% (v/v)
methanol in water solution at a flow rate of 2 ml/min.

Reagents

All chemicals used were reagent grade. The drug standards
were obtained from Applied Science Laboratories.

Sensitivity and Linearity

Standard solutions of indomethacin in methanol were prepared in concentrations of 0, 1, 2, 4, 6, 8, or 10 μg/ml and containing 50 μg/ml of glutethimide (internal standard). Aliquots (5 μl) of these solutions were injected into the chromatograph.

Recovery

Blood standards were prepared by adding to 50 μl of drug-free blood samples 50 μl of 100 μg/ml glutethimide solution and 50 μl solution containing 0, 1, 2, 4, 6, 8, or 10 μg/ml indomethacin. These standards were extracted as described below. The recovery rates were calculated by comparison with a standard solution of the compounds in methanol.

Extraction Procedure

The 50 μl blood sample, collected in a capillary tube, was added to a screw cap test tube containing 50 μl (100 μg/ml) glutethimide solution and 125 μl sodium acetate buffer (pH=5.4), and the solution was vortexed for 30 seconds. A 5 ml solution of heptane containing 1.5% (v/v) isoamyl alcohol was added; the mixture was vortexed for 2 minutes and centrifuged on a clinical bench centrifuge for 15 minutes. The organic layer was removed to a second test tube. The sample was extracted a second time using another 5 ml of heptane-isoamyl solution. Both organic layers were combined and evaporated to dryness under a stream of nitrogen. The dried extract was reconstituted with 50 μl of methanol, vortexed for 30 seconds, and the solution transferred to the sample vials of the WISP automatic sampler. Each sample (5 μl) was injected automatically into the chromatograph twice at 10-minute intervals to obtain duplicate runs.
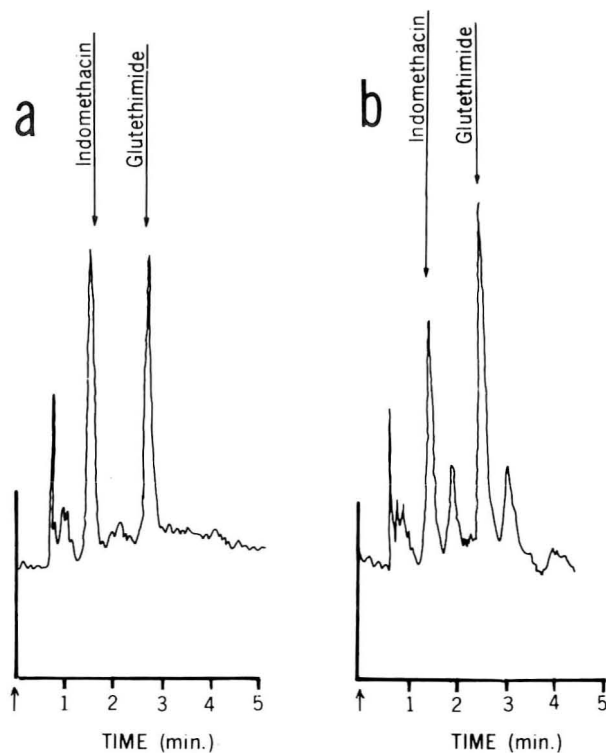
FIGURE 1.   Chromatogram of the extract of (a) rat blood with
            indomethacin and glutethimide added and (b) blood
            from a rat injected with indomethacin and added
            glutethimide.

Indomethacin and Blood Levels in Rats

A group of 6 male Wistar rats 250–300 g in weight was
administered by gastric intubation with 2 mg/kg indomethacin
suspension in peanut oil. Tail vein samples (50 μl) were col-
lected from each rat at 1.0, 1.5, 2.5, 3.5, and 4.5 hours after
drug administration. A parallel set of 2 blood standards per 10
blood samples was prepared by adding to drug-free rat blood 2 μl
(100 μg/ml) of indomethacin and 50 μl (100 μg/ml) of gluthethi-
mide. The blood standards and samples were extracted simulta-
neously. The concentration of indomethacin in the blood samples

was determined using the blood standards as the calibrating solu-
tions. This procedure eliminated the need for correction due to
recovery losses.

Calculations

The peaks on the chromatogram were identified by their
retention times relative to the internal standard. The concen-
tration of indomethacin was calculated on the basis of the ratio
of its peak height to that of glutethimide internal standard.

RESULTS AND DISCUSSION

Sensitivity and Linearity

The detection limit was 12.5 nanograms of indomethacin in
5 µl methanol injected into the chromatograph.

The detector response curve was linear in the range of 0.50
to 10 µg/ml for indomethacin solutions (y = 0.13 + 0.56x; corr.
coeff. 0.993).

Recovery

The recovery rates after extraction from blank blood of
indomethacin and glutethimide were 96±1 and 93±3%, respectively.

Indomethacin Blood Levels in Rats

A characteristic chromatogram of a blood sample from a rat
administered 2 mg/kg indomethacin is shown in Figure 1. The run
was completed in 10 minutes, and interfering peaks were not
observed in the blood samples. The concentrations of indometha-
cin in blood of rats receiving the drug by gavage at a dose of
2 mg/kg are shown in Figure 2.

The simple extraction and the short time required for
analyses of blood samples make this method very economical and
fast for the analyses of a great number of samples. Furthermore,
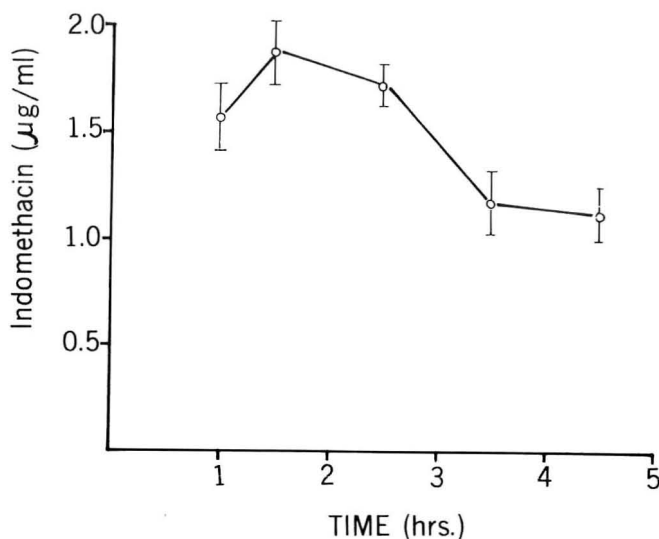
FIGURE 2. Indomethacin concentration in blood of rats receiving
           2 mg/kg indomethacin orally. Each point represents
           the mean value of 6 samples ± S.E.M.

the small volume of sample needed allows for the determination
of complete time-blood level curves for indomethacin in indi-
vidual rats at reasonable times without significant risks to
the animals.

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HPLC ANALYSIS OF THE ISOMERIC THIOETHER
METABOLITES OF STYRENE OXIDE

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ABSTRACT

An efficient separation of the isomeric thioether metabolites
of styrene oxide was achieved under reversed-phase conditions.
The column was eluted isocratically with 15% methanol in buffered
solutions of phosphoric acid-tris-hydroxymethylaminomethane. The
thioether conjugates were separated by class, and the order of
elution was cysteine, cysteinylglycine, glutathione, and N-
acetylcysteine. The effect of pH and buffer salt concentration
on the HPLC separation was examined. Optimal conditions for a
separation were either found at low pH (pH 3 or 4) or neutral pH,
both at a high buffer salt concentration (75mM). The positional
isomers and stereoisomers comprising each amino acid conjugate
sample were separated into two peaks. The variations in $k^{\dagger}$ and α
observed with changes in pH were interpreted as reflecting the
degree of interaction of the ionizable groups in the amino acid
residue and the hydrophobic portion of the molecule. This inter-
action was found to be strongly influenced by the relative stereo-
chemistry of the benzylic carbon center, thus allowing the separa-
tion of diastereoisomeric thioethers.

INTRODUCTION

The reaction of glutathione (GSH) with electrophilic chemicals

constitutes an important part of the detoxication mechanisms

available to many species. Depending upon the reactivity of the

electrophile involved, the conjugation reaction with GSH may

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occur with or without enzyme catalysis (1). The catalytic function is performed by a group of enzymes known collectively as the GSH transferases (2).

The measurement of the enzymatic activity of the GSH transferases is an important parameter as it constitutes an expression of the metabolic potential of a given biological system. This interest in the GSH transferases is reflected in the several procedures which have been developed in order to monitor the activities of these enzymes toward different electrophiles. These assay procedures are based on spectral differences between reactants and products (3), or in the use of radiolabelled substrates, either thiol (4) or electrophile (5). The latter procedure requires separation of excess labelled substrate from the incubation mixture by a solvent partition or by chromatographic procedures such as paper and thin-layer chromatography (tlc). One of the most widely used radioactive assays utilizes styrene oxide as the substrate (5,6). Total levels of enzyme activity are determined by extracting excess oxide from the incubation medium, and the assumption is made that all remaining radioactivity in the aqueous layer represents GSH conjugates. An improvement over the extraction procedure is the use of silica gel tlc (7) which allows distinction among the major metabolites resulting from the in vivo transformation of the GSH conjugates, namely the cysteinylglycine (CysGly), cysteine (Cys) and N-acetylcysteine (mercapturic acid, MA) thioethers (Scheme 1, and Figure 1). This assay is satisfactory for most cases, a major drawback being that it is operationally lengthy.
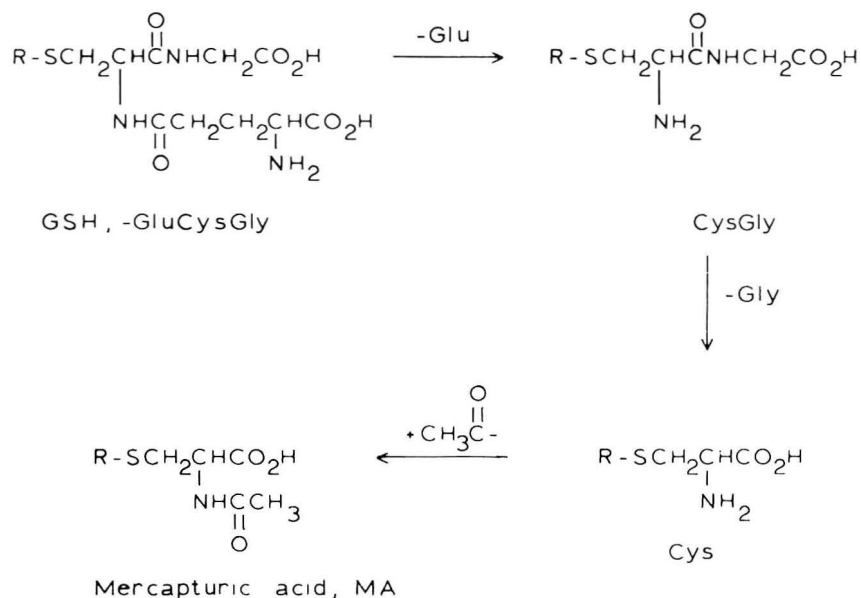
Scheme 1

The use of high-performance liquid chromatography (HPLC) for the analysis of mixtures of GSH conjugates has been reported recently. The system utilizes a reversed-phase column and the separation mechanisms are based on ionic suppression, using acetic acid as modifier (8), or on an ion-pair mechanism with alkylammonium or sulphonate counter ions (9). The HPLC analysis of the conjugates derived from an epoxide substrate has not been previously described in detail. Our interest in the chemical and biological aspects of epoxide metabolism by the GSH transferase system prompted us to explore the separation of the different thioether metabolites derived from the enzymatic conjugation of styrene oxide with GSH in detail.
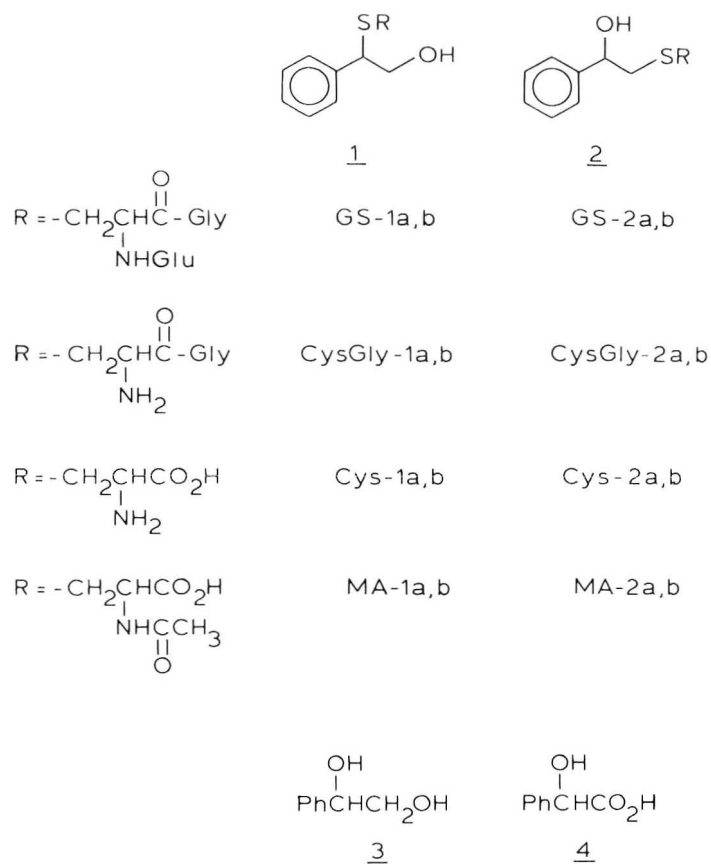
FIGURE 1. Thioether metabolites derived from styrene oxide. Benzylic thioether (1) and benzylic alcohol (2) isomers; a and b denote diastereomeric forms. Also included are styrene glycol (3) and mandelic acid (4).

Our preliminary findings indicated immediate advantages of the HPLC system over the tlc procedure, namely the shorter time required for analysis and the higher resolution achieved under the reversed-phase conditions utilized (10). The superior resolution achieved by HPLC allowed the separation and identifi-

cation of positional isomers resulting from the epoxide ring

opening reaction (Fig. 1, 1 and 2) as well as the presence of

diastereoisomers for each positional isomer (Fig. 1, 1a and 1b).

The initial set of conditions developed has been applied to the

analysis of in vitro and in vivo samples of styrene oxidesulfur

conjugates (10,11). In the present study we explore the effect

of pH and ionic strength on the HPLC separation of the different

thioether metabolites of styrene oxide.

MATERIALS AND METHODS

The thioether compounds were available from previous studies.

The stereochemical assignments, chemical synthesis and structural

properties are the subject of other publications (10,12). The

peaks listed in Table 1 were individually collected and charac-

terized by ^{13}C NMR (10). The presence of diastereomers was implied

from the doubling of signals on the ^{13}C NMR spectra and further

verified by reaction of optically pure styrene oxide with GSH.

The notation used in Figure 1 implies the existence of two diastereo-

isomers for each positional isomer present (i.e. 1 and 2). No

attempts are made in this text to correlate diastereoisomers to

the corresponding optical forms of styrene oxide.

The equipment used consisted of Waters Associates M6000A

pumps, model 440 UV absorbance detector, model U6K injector and a

model 660 solvent programmer. The columns used were μ-Bondapak

C_{18}(0.39 x 30 cm) equipped with a pre-column packed with Corasil

Bondapak C_{18}. The solvent mixtures consisted of 75 mM H_3PO_4

(prepared from 85% H_3PO_4, ca. 5 ml per liter) neutralized to the

indicated pH with tris-(hydroxymethyl)aminomethane (Tris-base).
The pH values were measured with a Corning 125 pH meter equipped
with a Markson combination glass electrode, before addition of
organic solvent.

RESULTS

The use of trialkylammonium-based buffer systems in HPLC was
originally introduced by Rivier (13). This buffering reagent has
proven a most useful tool for the analysis of amino acids and
polypeptides. Our initial work utilizing Rivier's procedure,
which calls for triethylamine as the basic component in the
buffer eluent, produced good results. A recurring problem
however, was the instability of this organic base in air and
light. This decomposition process produces UV absorbing impurities
which must be removed by distillation, preferably immediately
before use. In searching for another, more stable organic base
than triethylamine, we discovered that tris-(hydroxymethyl)-
aminomethane (Tris-base) produced results very comparable to
those obtained with triethylammonium phosphate. The Tris-phosphate
solutions are sufficiently transparent to allow detection at 254
nm, and the chemical stability of Tris-base makes it a desirable
choice for routine analysis. The work described in this study
was conducted with Tris-phosphate buffers although as indicated
above, similar results may be obtained with the triethylamine-
phosphoric acid system.

The separation of the thioether derivatives of styrene oxide
(Fig. 1) was originally accomplished (10) by using a reversed-

phase (μ-Bondapak C_{18}) column and a 75 mM solution of phosphoric acid adjusted to pH 3.1–3.5 with organic base. A methanol gradient provided a clean separation of the desired compounds.

It was clearly established that separation of diastereo-isomeric forms was possible and that the order of elution of the different thioether derivatives was, at pH 3.5, directly proportional to the number of amino acid residues in the molecule, i.e. the order of elution under those conditions was Cys, CysGly and GS. This latter finding suggested that the separation mechanism was strongly influenced by the amino acid residue. Consequently, factors affecting the ionizable portion of the molecule should be reflected in the separation of a given mixture of thioether conjugates. Hence, the most obvious variables to explore were the effect of pH and ionic strength on the HPLC profile. The separation conditions were determined by initially selecting an isocratic solvent combination which provided baseline separation of GSH conjugates 1 and 2 (Fig. 1, GS-1 and GS-2), and then holding the amount of organic solvent constant, but varying the pH from 3 to 7 in one pH unit increments. The lower limit used (pH 3) was based on the observation that at lower pH, decomposition of the benzylic alcohols 2 takes place readily. This was particularly true with 0.1% phosphoric acid solution (pH 2.2), a popular buffering system for polypeptide purification (14).

The chemical (non-enzymatic) reaction of the thiol amino acid-GSH, CysGly, Cys, NAcCys-with styrene oxide produces a mixture of positional isomers consisting of 1 (70%) and 2 (30%) (10,12).
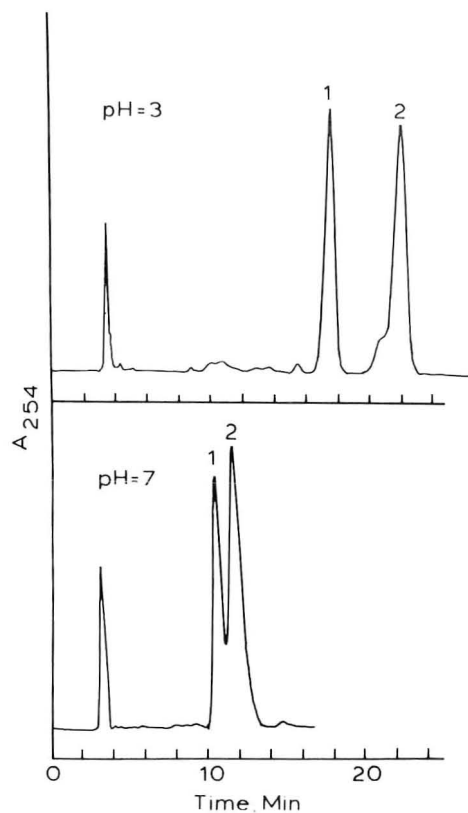
FIGURE 2. Isocratic separation of the glutathione conjugates of
styrene oxide. Conditions were: one μ-Bondapak C$_{18}$ column
(0.39 x 30 cm) eluted at 1 ml/min with 15% MeOH/75 mM H$_3$PO$_4$
buffered with Tris-base, upper trace pH 3; lower trace, pH 7; 0.1
AUFS. Peak 1 is GS-P1 and peak 2 is GS-P2. GS-P1 contains GS-1a;
GS-P2 contains GS-1b, GS-2a, and GS-2b.

The isocratic separation of the GSH conjugates of styrene

oxide, GS-1 and GS-2, at pH 3 and pH 7 is illustrated in Fig. 2.

This sample consists of two positional isomers (GS-1 and GS-2)

for each of which two diastereoisomeric forms (GS-1a,b and GS-

2a,b) are possible. This mixture of stereoisomers is resolved

into two peaks (GS-P1 and GS-P2). The benzylic thioether
diastereoisomers (GS-1a and GS-1b) are clearly resolved emerging
in peaks GS-P1 and GS-P2 respectively, while the benzylic alcohol
diastereoisomers (GS-2a and GS-2b) emerge in the late eluting
peak (GS-P2) together with GS-1b. Optimal conditions for the
separation of these particular glutathione conjugates are at pH 3
or pH 4, both of which provide baseline separation. At pH 5 the
resolution drops drastically and does not deteriorate further
with increasing pH (Table 1).

   The isocratic separation at pH 3 and pH 7 of a mixture
containing all of the thioether conjugates of styrene oxide (Fig.
1) is illustrated in Fig. 3. The cysteine (Csy) and cysteinyl-
glycine (CysGly) samples, as is the case for GSH, are resolved
into two peaks. In these samples, however, the isomeric compo-
sition of the peaks relative to GSH is reversed. In the cysteine
case, the first eluting peak (Csy-P1) contains a benzylic thio-
ether isomer (i.e. Cys-1a) and the benzylic alcohol diastereo-
isomers (Cys-2a and Cys-2b); the remaining benzylic thioether
diastereoisomer (Cys-1b) elutes in the second peak (Cys-P2). The
cysteinylglycine compounds behave similarly with the two peaks
(CysGly-P1 and CysGly-P2) containing an identical isomer distri-
bution as for Cys. It is noteworthy that for each amino acid
conjugate the benzylic thioethers 1a and 1b are consistently
separated. This finding is relevant since this is the predom-
inant positional isomer (80-90%) produced in the enzymatic conjuga-
tion of styrene oxide with GSH (10).
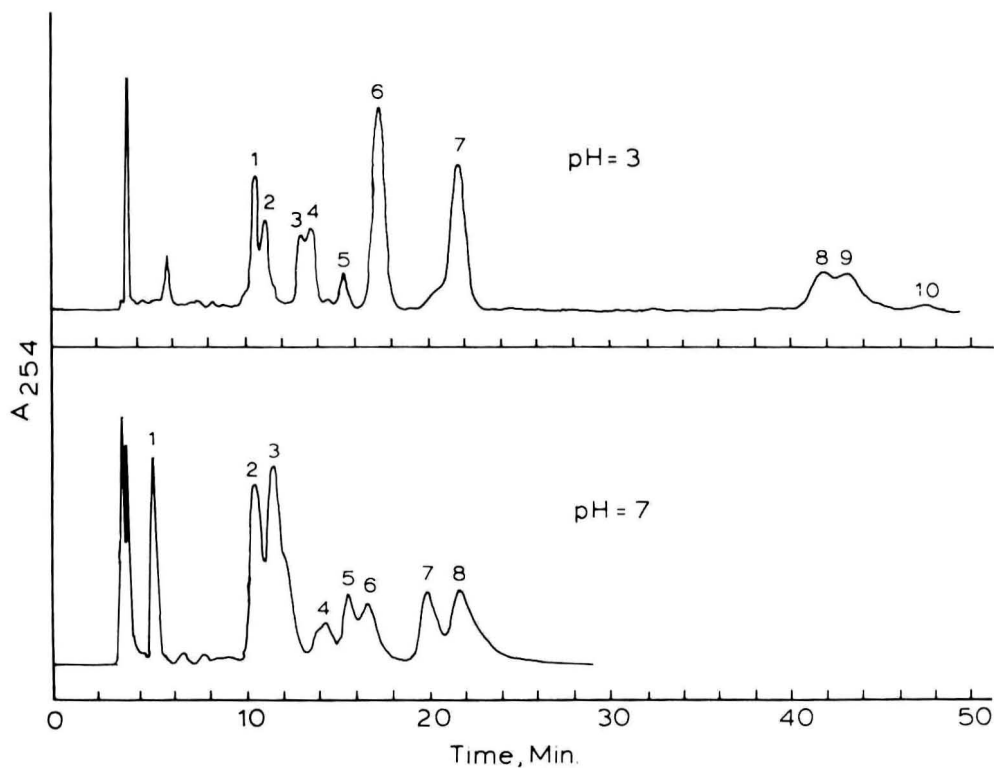
FIGURE 3. Isocratic separation of a mixture of the thioether conjugates of styrene oxide, styrene glycol, and mandelic acid. Conditions as described in Fig. 2. Peak composition: pH 3, 1, mandelic acid; 2, Cys-P1; 3, Cys-P2; 4, CysGly-P1, 5, styrene glycol; 6, GS-P1 and CysGly-P2; 7, GS-P2; 8, MA-P1; 9, MA=P2; 10, MA-P3; pH 7, 1, mandelic acid, 2, Cys-P1 and GS-P2; 3, Cys-P2 and GS-P2; 4, styrene glycol; 5, Cys-Gly-P1; 6, CysGly-P2, MA-P1; 8, MA-P2.

TABLE 1

The Capacity Factor (k^1) as a Function of pH

Sample/pH[a]	3	4	5	6	7	% $\Delta k'$ at pH 7[b]
GS-P1	4.3	3.1	2.4	2.4	2.4	-44
GS-P2	5.7	3.8	2.8	2.6	2.7	-52
Styrene glycol	3.7	3.7	3.5	3.6	3.7	0
CysGly-P1	3.1	2.7	2.6	3.0	4.0	+29
CysGly-P2	4.3	3.6	3.4	3.7	4.3	0
Cys-P1	2.4	2.3	2.3	2.4	2.5	+4
Cys-P2	3.0	2.9	2.8	2.9	3.0	0
Mandelic acid 4	2.2	1.2	0.6	0.7	0.5	-77

a - 75mM H_3PO_4; b - Relative to pH 3.

TABLE 2

The Effect of pH on α for Diastereomeric Pairs
(e.g. GS-1a and GS-1b)

Sample/pH	3	4	5	6	7	3[a]
GSH	1.32	1.22	1.16	1.08	1.12	1.31
CysGly	1.35	1.33	1.3	1.23	1.07	1.37
Cys	1.25	1.26	1.2	1.2	1.2	1.28

a - 10 mM phosphate solution, all others were 75 mM.

The variation of k' (capacity factor) with pH for the
different conjugates is summarized in Table 1.
The changes introduced were not entirely predictable. The GSH
conjugates showed a drastic decrease in retention times (44% and
52%) at pH 7 relative to pH 3. The cysteine compounds were for
the most part unaffected, and interestingly, only one of the
cysteinylglycine peaks (CysGly-P1) showed a significant increase
(29%) in retention. Styrene glycol, as anticipated was not
affected by changes in pH, and mandelic acid exhibited the most
dramatic decrease (77%) in retentivity of all compounds examined.
The effect of pH on the separation factor (α value) for dia-
stereoisomeric pairs is illustrated in Table 2.

TABLE 3

The Effect of Salt Concentration on k'. Buffer Solutions at the
Indicated Acid Concentrations were Adjusted to pH 3
with Tris-base

Sample	10 mM H_3PO_4	75 mM H_3PO_4	%Δk'
GS-P1	3.8	4.3	+13
GS-P2	5.0	5.7	+14
Styrene glycol 3	3.2	3.7	+15
CysGly-P1	2.7	3.1	+15
CysGly-P2	3.7	4.3	+16
Cys-P1	2.1	2.4	+14
Cys-P2	2.7	3.0	+11
Mandelic acid	0.7	2.2	+214

a - Increase at 75 mM H_3PO_4 relative to 10 mM H_3PO_4.

The trend parallels that for the k' values, that is a decreasing
value for α with increasing pH. The notable exception again is
the cysteine compounds for which α remains relatively constant
throughout the tested pH range. For the cysteinylglycine isomers,
a significant decrease in α at pH 7 is consistent with the
increased k' observed for only one of the peaks (Table 1).

The effect of the buffer salt concentration was examined at
pH 3 and it is described in Table 3.
Operating at 10 mM phosphate concentration caused a general
decrease in retention times. This decrease was essentially
constant for all the sulfur compounds and styrene glycol (3);
mandelic acid showed a disproportionate decrease in retentivity.
The changes in retentivity listed in Table 3 are expressed as
percentage increase at the high molarity buffer relative to the
low molarity buffer. It is worth reemphasizing that the salt
concentration had no effect on α, over the ranges tested.
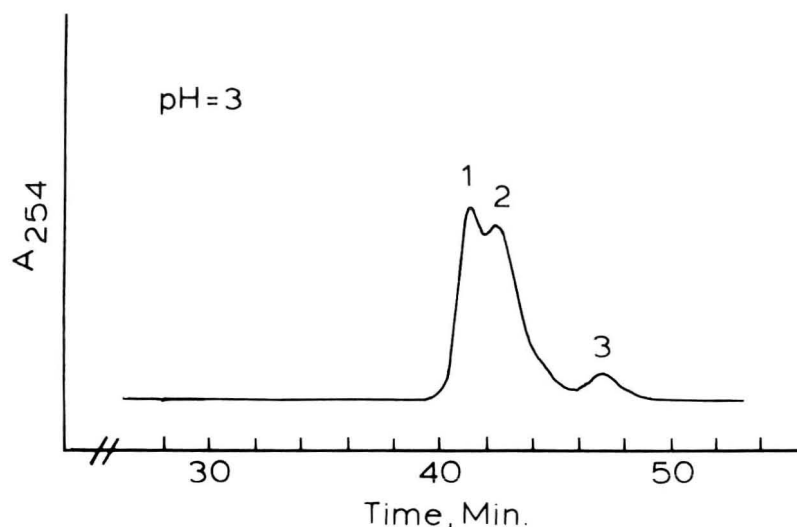
FIGURE 4. Isocratic separation of the mercapturic acid conjugates (MA) of styrene oxide. Conditions as described in Fig. 2 (pH 3). Peak identification: 1, MA-P1; 2, MA-P2; 3, MA-P3. MA-P1 contains MA-1a; MA-P2 contains MA-1b and MA-2b; MA-P3 contains MA-2a.

TABLE 4

The Effect of pH and Salt Concentration on k' for the Mercapturic Acid Isomers

Sample/pH	3	3[a]	4	5	6	7
MA-P1	11.8	10	7.0	5.6	5.5	5.4
MA-P2	12.1		7.5	6.1	6.0	6.0
MA-P3	13.5					

a - 10 mM H_3PO_4, all others 75 mM H_3PO_4.

The chromatographic behavior of the N-acetylcysteine (mercapturic acid) conjugate isomers of styrene oxide is summarized in Table 4.

The effect of pH on k' is similar to that found for the GSH compounds. The best separation is achieved at pH 3 where three

of the four possible peaks are distinguishable on the trace (Fig.
4). At pH 4 only two peaks are resolved, a further drop in k' is
found at pH 5 and these latter values remain unchanged up to pH
7. Interestingly the α obtained for MA-P1 and MA-P2 at pH
4 is not affected by subsequent increases in pH. The isomeric
composition of the resolved peaks is as follows: at pH 3, MA-P1
and MA-P2 contain benzylic thioether isomers (MA-1a and MA-1b);
a benzylic alcohol (MA-2a) elutes in the third peak (MA-P3) while
the other diastereomeric benzylic alcohol (MA-2b) coelutes with
MA-1b in the second peak. The two peaks separated at pH 4-7 each
contain a benzylic thioether diastereoisomer. The location of
the benzylic alcohols (MA-2a,b) was not established. The effect
of buffer concentration on the separation is more pronounced for
this isomer mixture. The resolution obtained at pH 3 is lost
entirely when the phosphate concentration is decreased to 10 mM
(Table 4).

DISCUSSION

The chromatographic behavior of the thioether metabolites of
styrene oxide may be interpreted by a structural model in which
hydrophobic interactions are modulated by equilibria of the
ionizable groups in the molecules. The hydrophobic interactions
are considered to be due largely to the affinity of the least
polar end of the conjugate (phenyl substituent) for the hydro-
carbon bonded-phase of the column. The ionic forms of an amino
acid equilibrium in aqueous solution may be represented as in
Scheme 2.

$$R-CHCO_2H \rightleftharpoons R-CHCO_2^- \rightleftharpoons R-CHCO_2^-$$

$$\underset{^+NH_3}{|} \qquad \underset{^+NH_3}{|} \qquad \underset{NH_2}{|}$$

$$\underline{A} \qquad\qquad \underline{B} \qquad\qquad \underline{C}$$
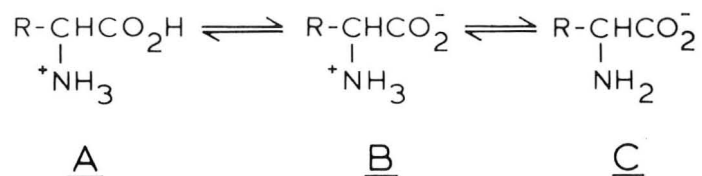
Scheme 2

The relative concentrations of the three ionic forms are pH
dependent, and for the range used in this study (pH 3-7) all
three forms are expected to participate in the separation mechanism.
The pKa values for the carboxyl groups lie between 3. and 3.5 and
those of the amino group are between 8. and 8.5. These values
are anticipated to change slightly due to the presence of organic
solvent.

It has been demonstrated by Kroeff and Pietrzyk that for a
series of dipeptides, differences in k' values for acidic and
basic elements are related to the differences in position of the
charged species relative to the non-polar substituents, and not
to the degree of ionization (pKa) of the amine and carboxyl group
of the amino acids (15). This type of analysis is also applicable
to the interpretation of the separation mechanism(s) of the
compounds involved in the present study. The conjugates are
separated by class, and the order of elution is Cys > CysGly >
GSH which correlates with the relative distance of the charged
groups to the non-polar end of the molecule.

For Cys-1 and Cys-2 the close proximity of the ionic groups
and a slightly lower pKa for the carboxyl group combine to render

these compounds insensitive to pH changes; ionic dissociation is
established at pH 3 and is not drastically affected by increases
in pH. For GS-1 and GS-2 the distance between the participating
groups is greater; the effect of the α-amino acid should not be
as strong, and it apparently allows participation of the carboxyl
group of the Gly residue. This effect is reflected in the k'
where at pH 5 this group is presumably fully ionized and the α-
amino acid is in the zwitterion form (B); consequently the
capacity factor remains essentially constant (Table 1). Kroeff
and Pretrzyk reported minimum values for k' at the isoelectric
point (form B) of a given dipeptide (15).

A similar argument may be used to explain the HPLC behavior
of the N-acetylcysteine compounds (MA) and mandelic acid, where
decreasing k' values parallel the increased ionization of the
carboxyl group with increasing pH (Tables 1 and 4). The effect
of distance is also exemplified in these two cases. The decrease
in k' for mandelic acid is much more dramatic than for MA-1 and
MA-2 which argues for a stronger inhibition by the ionized
carboxyl group in the mandelic acid case. Styrene glycol (3), as
anticipated is not affected by pH (Table 1).

The CysGly samples do not fall in line with the HPLC behavior
observed for Cys and GS derivatives. Capacity factors for these
compounds are maximal at the two ends of the pH range studied
(Table 1). Additionally, a net increase in k' is observed for
one of the peaks, CysGly-P1. A unique feature of the CysGly is
that it has an isolated (Cys) amino group, which in terms of
proximity to the hydrophobic portion of the molecule should be
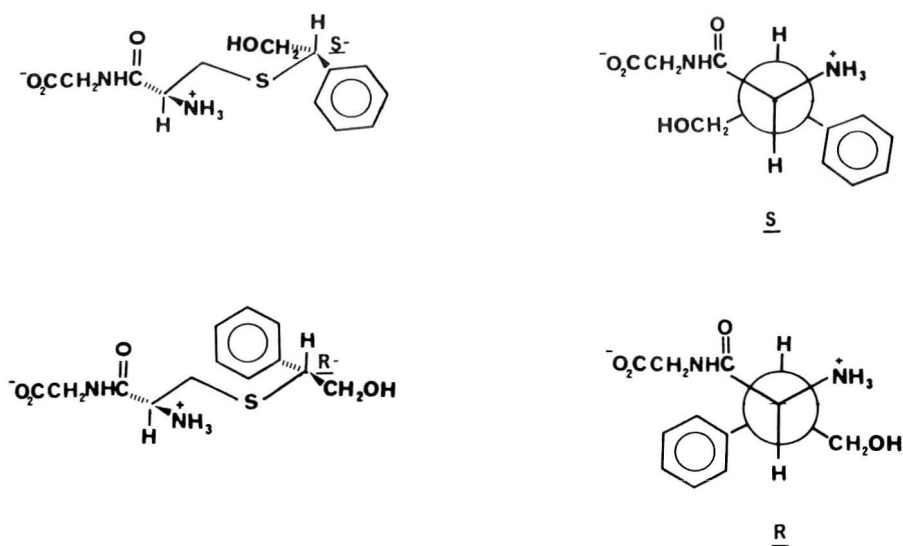
FIGURE 5. Structural projections of the diastereomeric benzylic conjugates of CysGly. The R and S denote the configuration at the benzylic carbon center.

more effective in influencing the separation than the $-CO_2H$. As mentioned earlier, a separation mechanism based only on differences in ionization constants is not plausible. It follows that the anomalous behavior of the CysGly compounds must be primarily explained on the basis of the interaction of the proximate ammonium group with the phenyl ring substituent (Fig. 5). The extent of this interaction will be strongly influenced by the relative configuration at the benzylic center in CysGly-1. From the favorable conformations available to CysGly-1a and CysGly-1b, it is conceivable that for one diastereoisomer the ammonium group is placed on the same side of the molecule as the hydrophobic portion. This diastereoisomer would have a small k' at low pH,

since when the amine is fully protonated the hydrophobic surface
available for binding is decreased. In turn this allows for
participation of the carboxyl group in Gly since the decline in
k' is significant from pH 3 to pH 5 (Table 1). A gradual increase
in k' with increasing pH would be consistent with a decrease in
the concentration of protonated amine and the subsequent increase
in hydrophobic area available for binding to the column. The
other diastereoisomer with the charged group farther away from
the phenyl ring portion, would have a larger k' at low pH as a
result of decreased inhibition by the amine function (relative to
the first diastereoisomer), and since the hydrophobic sites are
more available for binding the effect of the carboxyl group in
the k' is not expected to be as pronounced. An increase in k'
would follow at higher pH for the reason stated earlier. The
chromatographic pattern observed for CysGly-P1 accomodates the
first case described, while CysGly-P2 fits the second model (Fig.
5). Small changes in k' for Cys-P1 and GS-P1 are also suggestive
of this type of conformational control in hydrophobic binding.

    The failure to separate the benzylic alcohol diastereo-
isomers (2) in Cys, CysGly-, and GS-2 conjugates is more likely
related to the increased distance between the ionizable groups
and the chiral center in 2. The fact that partial separation for
MA-2 occurs at pH 3 (Fig. 4) with 75 mM but not 10 mM buffer
indicates a partition mechanism operating on a neutral molecule
(see below).

    The effect of salt concentration is summarized in Table 3.
A net decrease in k' is evident for all compounds at lower ionic

strength buffer. This decrease at 10 mM buffer concentration is nearly constant (13-16%) except for mandelic acid. The α values for pairs of diastereoisomers are not affected (Table 3) by decreased salt concentration. The conclusion is that higher salt concentration favors increased interaction with the column bonded phase by a "salting-out" effect. Of the thioether compounds the mercapturic acids are most strongly affected demonstrating a net loss of separation (Table 4) at lower ionic strength buffer.

From these data one might speculate, that under the conditions used (pH 3-7 and alkylammonium buffer system), the influence of $-{}^+NH_3$ in hydrophobic interactions is stronger than that of $-CO_2H$. A clear example is Cys versus MA; the latter, with a blocked amine group elutes much later and is more susceptible to ionic strength changes. It is possible that by operating at higher pH (pH 9) where the only ionized species would be $-CO_2^-$, the effect of this group in conformational control would be felt; predictably the mercapturic acids would be more strongly affected. It is also possible that the organic base forms ion-pairs with the carboxyl function, and in this way nullifies some of its ionic effect.

The information derived form this study is not limited to thioether metabolites of styrene oxide. For example, in developing an HPLC assay for other sulfur conjugates it is clear that optimal conditions for separation occur at pH 3 or 4. If substrate lability is incompatible with acidic conditions, then working at pH 7 is more desirable than at the intermediate pH 5 or pH 6. At

pH 7 the concentration of zwitterion $\underset{\sim}{B}$ should be less and participation by unionized amine groups becomes more favorable.

For styrene oxide metabolites, an isocratic separation at pH 3 or 4 allows separation of the major metabolites commonly found (11), although the CysGly overlaps with GS under these conditions. However, the presence of CysGly may be inferred by isocratic elution at pH 7; if determined to be present then gradient conditions allow separation of all the compounds at either acidic (pH 3 or 4) or neutral pH.

The analysis described allows the rapid estimation of GSH transferase activities with styrene oxide as substrate and the separation and quantitation of intermediary mercapturic acid derivatives of this alkene oxide, while providing valuable structural and stereochemical information concerning each class of thioether metabolites.

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FRACTIONATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF MICROSOMAL CYTOCHROME P-450 INDUCED BY HEXACHLOROBIPHENYL ISOMERS

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ABBREVIATIONS.

High performance liquid chromatography (HPLC), phenobarbitone (PB), β-naphthoflavone (BNF), hexachlorobiphenyl (HCB), hydrophile-lipophile balance (HLB).

ABSTRACT

High performance liquid chromatography has been employed to fractionate rat liver microsomes under nondenaturing conditions. Selective detection at 405 nm allowed resolution of microsomal heme proteins into three peaks (A, B, and C). Cytochromes in the peaks retain their native property of binding CO after HPLC. Peak-A, first eluting, contains P-450 and is rich in cytochrome P-420. Peak-B is largely hemoglobin and peak-C is a major cytochrome P-450. The ratio of peak-C to A is increased by treatment of rats with phenobarbitone, β-naphthoflavone, 2,3,5,2',3',5'-hexachlorobiphenyl and 3,4,5,3',4',5'-hexachlorobiphenyl as compared to controls. The highest increment in the ratio is observed on feeding 3,4,5,3',4',5'-hexachlorobiphenyl. NADPH cytochrome $\underline{c}$ reductase elutes earlier than peak-C but cytochrome b_5 is not separated from the major cytochrome P-450 peak. The separations obtained are highly reproducible and considerably faster than conventional gel permeation chromatography. The data presented here are very promising in establishing the role of HPLC in the studies of insoluble proteins and enzymes in general and cytochrome P-450 in particular.

INTRODUCTION

The application of high performance liquid chromatography
(HPLC) to the purification of polypeptides and proteins has been
recently reviewed (1). The separation mechanism involves parti-
tion between a silica gel bonded phase and an aqueous eluant.
The highly hydrophobic bonded phase requires in many cases the
use of organic solvents in order to displace the polypeptides
from the column, a clear limitation of this procedure. A recently
introduced bonded phase operates on the principle of molecular
exclusion. These columns have been used for the estimation of
molecular weights of proteins under denaturing conditions (2). A
variation of this latter type involves the use of an ion exchange
phase which was used to further separate a homogenous preparation
of lipoxygenase (3). Most of the studies on protein isolation
and fractionation by HPLC have been confined to soluble proteins
only. In the present study, we have made an attempt to fractionate
by HPLC an insoluble hydrophobic protein which is localized in
the endoplasmic reticulum. Since the mixed function oxidase
system plays an important role in the metabolism of endogenous
substrates (4), xenobiotics (5) and cytochrome P-450 being the
terminal oxidase of microsomal electron transport chain (6), it
was relevant to explore the applications of HPLC to the analyses
of rat liver cytochrome P-450 in control and hexachlorobiphenyl
(HCB) isomers treated rats.

MATERIALS AND METHODS

Charles River CD strain male rats were used in the present
study. The treatment of animals, determination of dose, purity of

HCB isomers, preparation of microsomes and assay of enzyme has been previously described (7) except that β-naphthoflavone was used instead of 3-methylcholanthrene. Protein standards and all other chemicals were obtained from Sigma Chemical Co., St. Louis, MO 63178.

Solubilization of microsomes. The microsomes were solubilized at room temperature in 100 mM potassium phosphate (pH 7.4), 0.2% Lubrol WX, 0.5% sodium cholate, 0.1 mM ethylene diamine tetraacetic acid, 0.1 mM dithiothreitol and 20% glycerol (buffer A) for a period of one hour. The sample size was so adjusted that each time 100 μg protein in 0.05 ml was applied to the column (Fig. 1).

High Performance Liquid Chromatography (HPLC). Buffer and solubilized microsomes were filtered (0.22 μm, Millipore) prior to use. The equipment used consisted of a model 6000A pump, U6K injector, model 440 dual-channel UV absorbance detector, and a model 730 data module (all from Waters Associates, Inc., Milford, MA 01757). Peak absorbance was simultaneously monitored at 405 nm and 280 nm; peak areas were obtained by integration with the 730 data module. The columns used were a Spherogel TSK-3000SW (0.75 x 60 cm, Altex) and, I-125 and I-250 (0.78 x 30 cm each, Waters Associates). The column was equilibrated for one hour and eluted with buffer A isocratically. The flow rate was 0.5 or 1 ml per min as indicated in the figure legends.

RESULTS AND DISCUSSION

Rat liver microsomes are generally solubilized by using a nonionic detergent such as Emulgen-911 in combination with an ionic detergent, sodium cholate. Emulgen-911 is a polyoxyethylene nonylphenol and its strong absorbance at 280 nm makes the monitoring
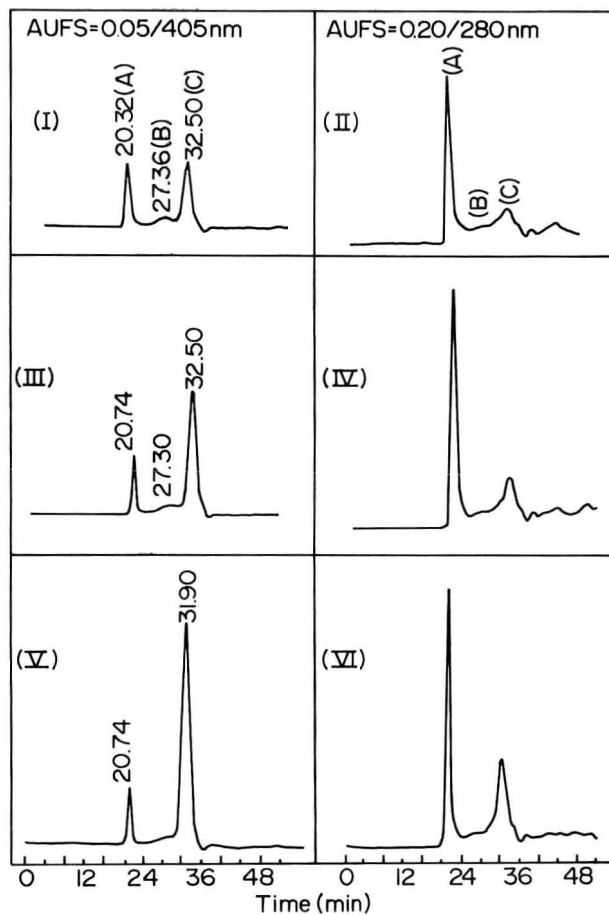
Figure 1.   HPLC of solubilized rat liver microsomes from control
(I and II), 2,3,5,2',3',5'-HCB (III and IV), and 3,4,5,3',4',5'-
HCB (V and VI) treated rats on a Spherogel TSK 3000SW column.
The flow rate was 0.5 ml/min and chart speed 0.25 cm/min.
Chromatogram I, III and V depict absorbance at 405 nm and II, IV
and VI at 280 nm of the respective samples.

of the effluent for protein content impossible.   Lubrol WX, a poly-

oxyethylene alcohol does not have this limitation and its use to

solubilize rat liver microsomes has been documented (8).   For the

HPLC study we chose a combination of sodium cholate and Lubrol WX

which enabled us to monitor the effluent for heme (405 nm) and
protein (280 nm) content. Substituting Emulgen-911 for Lubrol WX
did not change the separation pattern, as determined by absorbance
at 405 nm, a result anticipated from their similar HLB values (9).

As shown in Fig. 1, the rat liver microsomes from control and
treated animals were fractionated into three peaks (A, B, and C)
when monitored at 405 nm. The peaks from HPLC effluent were collected
and their CO binding difference spectra recorded. Peak-B showed a
characteristic absorbance at 420 nm when saturated with CO, thus
establishing its identity as hemoglobin. Peak-A and C did not show
any absorbance when bubbled with CO, however, they showed charac-
teristic absorbance at 420 nm and 450 nm when first reduced with
dithionite and bubbled with CO. These studies demonstrate that
the cytochrome P-450s were able to retain their native property of
binding CO after HPLC analysis. This is noteworthy because cyto-
chrome P-450 is a labile protein in which the heme is not covalently
bound, and it is easily converted to its denatured form cytochrome
P-420 by factors such as temperature, detergents, etc. Peak-C con-
tained most of cytochrome P-450 and no cytochrome P-420. Peak-A
contained cytochrome P-450 and was rich in cytochrome P-420.
Since peak-A eluted at the void volume, the solubilized microsomes
were centrifuged at 167,000 g for 35 minutes. The supernatant was
rechromatographed and no appreciable change in the areas or ratios
of peak-C to A were observed, showing thereby that peak-A is not
undissolved cytochrome.

To study the pattern of induction by various inducers of cyto-
chrome P-450, microsomes from treated animals were solubilized in

TABLE 1. Effect of Phenobarbitone, β-Naphthoflavone, 2,3,5,2',3',5'-HCB and 3,4,5,3',4',5'-HCB on Areas of Peaks-A and C and Their Ratios as Obtained by HPLC Analysis. The conditions are described in Fig. 1. Each value is a mean of three separate determinations.

| Treatment | Area[a] | | Ratio Peak C/A |
	Peak-A	Peak-C	
Control	17.12 (1.5)[b]	27.06 (1.67)	1.58
PB	16.04 (0.25)	59.33 (2.09)	3.70
BNF	17.94 (1.10)	49.97 (2.44)	2.79
2,3,5,2',3',5'-HCB	11.52 (0.23)	55.19 (1.42)	4.79
3,4,5,3',4',5'-HCB	10.43 (2.90)	100.49 (11.50)	9.63

[a] Absorbance at 405 nm.

[b] Standard Deviation in ( ).

buffer A for one hour at room temperature and analyzed by HPLC. Each sample was analyzed three times and mean values of the areas obtained and ratios of peak-C to A are presented in Table 1. The ratio of peak-C to A was 1.58 in control microsomes and increased when rats were treated with various inducers. 3,4,5,3',4',5'-HCB caused the highest increase in the ratio of peak-C to A. 3,4,5,3',4', HCB also increased the area of peak-C 3.7 times as compared to peak-C of control microsomes. Similarly, other inducers such as PB, BNF and 2,3,5,2',3',5'-HCB increased the area of peak-C by a relative factor of 2.19, 1.85 and 2.0 respectively. This trend parallels the one determined by estimating the cytochrome P-450 content by CO-difference spectra of reduced microsomes obtained from control and treated rats (7). Consequently an increase in the area of peak-C by an inducer relative to control may be considered a qualitative index of its potency as an inducer of cytochrome P-450.

TABLE 2. Effect of 2,3,5,2',3',5'-HCB and 3,4,5,3',4',5'-HCB on Specific Activities of Microsomes and Peak-C Obtained After HPLC of Microsomes. Each value is mean of three separate determinations (7). The conditions are described in Fig. 1.

| | Specific Activity nmol Cyt. P-450/mg protein | | Recovery of Cyt. |
Treatment	Microsomes	Peak-C	P-450 in Peak-C(%)
Control	1.02	1.34	78
2,3,5,2',3',5'-HCB	2.34	3.24	88
3,4,5,3',4',5'-HCB	4.31	4.87	99

The capacity of the column is not limited to microgram quantities of protein. Fifty times more protein than used in the initial experiment could be fractionated without any appreciable loss of resolution. Another important feature of the present technique is that peak-C when monitored at 280 nm also showed an increase when microsomes obtained from rat livers treated with 2,3,5,2',3',5'-HCB (Fig. 1, IV) and 3,4,5,3',4',5'-HCB (Fig. 1, VI) were fractionated by HPLC, showing thereby that protein content of peak-C was increased along with heme content leading to the conclusion that inducers increased the de novo synthesis of the associated proteins. These latter experiments were done in triplicate and the specific activities of microsomes and isolated peak-C are presented in Table 2. The specific activities of peak-C showed a modest increase as compared to microsomes indicating the removal of some proteins from the major cytochrome peak. This was further confirmed when sodium dodecyl sulfate polyacrylamide gel electro-
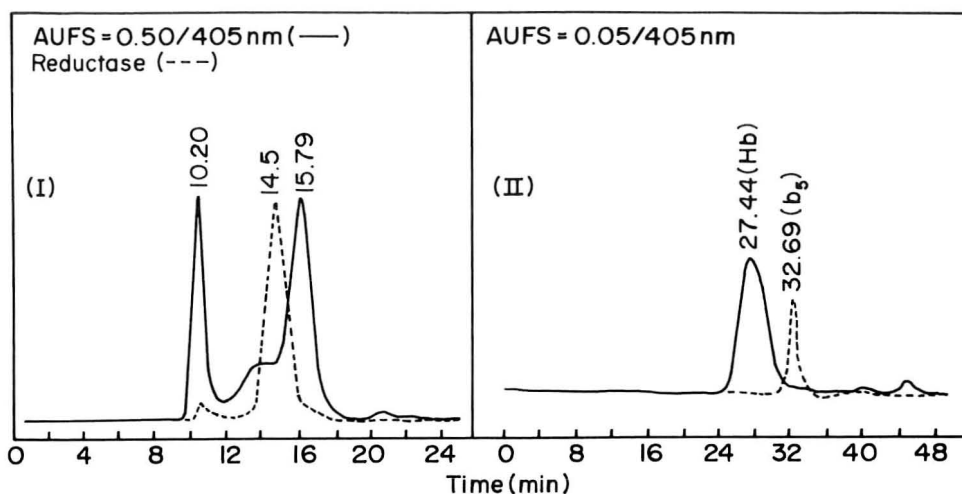
Figure 2. HPLC of solubilized rat liver microsomes on Spherogel
TSK 3000SW column. Chart speed was 0.5 cm/min and flow rate 1.0
ml/min. 0.25 ml Fractions were collected and NADPH cytochrome c
reductase was assayed in each fraction. Hemoglobin and cytochrome
b_5 were chromatographed separately but are shown here in the same
chromatogram (II).

phoresis was performed on liver microsomes and peak-C of control,
2,3,5,2',3',5'-HCB and 3,4,5,3',4',5'-HCB treated rats. Polypep-
tides corresponding to subunit weights of 80,000, 90,000 and 100,000
were absent in peak-C as compared to their respective microsomes.
Since the volumes of the peaks were small, the detergents were not
removed prior to protein estimations, thus affecting the specific
activities reported. As documented in Table 2, the recoveries of
cytochromes in peak-C are excellent.

To study the elution pattern of NADPH cytochrome c reductase,
2.5 mg of microsomal protein was applied to the column. 0.25 ml
fractions were collected and enzyme assays were performed in each

fraction. NADPH cytochrome c reductase eluted at 14.5 minutes at 1 ml/min (Fig. 2, I). These results corroborate well with previous observations (10) that NADPH cytochrome c reductase elutes earlier than cytochrome P-450 when solubilized rat liver microsomes are chromatographed on Sephadex G-200. The column used in the present study operates mainly on the principle of molecular exclusion. The entire operation can be performed in minutes as compared to many hours taken on Sephadex. The binding of substrates to cytochrome P-450 monitored by HPLC is an attractive possibility (11).

Hemoglobin and cytochrome b_5 were obtained from rat liver microsomes by chromatography on DEAE-cellulose (10). Hemoglobin had a retention time of 27.5 minutes at 0.5 ml/min, similar to peak-B (Fig. 2, II). Cytochrome b_5 had a retention time of 32.7 minutes (Fig. 2, II) and did not separate from peak-C. Since the content of cytochrome b_5 is not altered on treatment of rats with the inducers studies (unpublished results), the induction demonstrated by the increase in the area of peak-C is real.

When microsomes obtained from untreated rat testis were solubilized and chromatographed by the procedure described for rat liver, the content of peak-A (Fig. 3) was higher as compared to peak-C. Peak-A contained mostly cytochrome P-420 when analyzed spectrophotometrically and peak-C contained cytochrome P-450 and no cytochrome P-420. This experiment illustrates the contrasting stabilities of cytochrome P-450 from different sources. Rat liver microsomes may be prepared and chromatographed as described here, but under similar conditions the cytochrome P-450 in rat testis is rapidly denatured to cytochrome P-420.
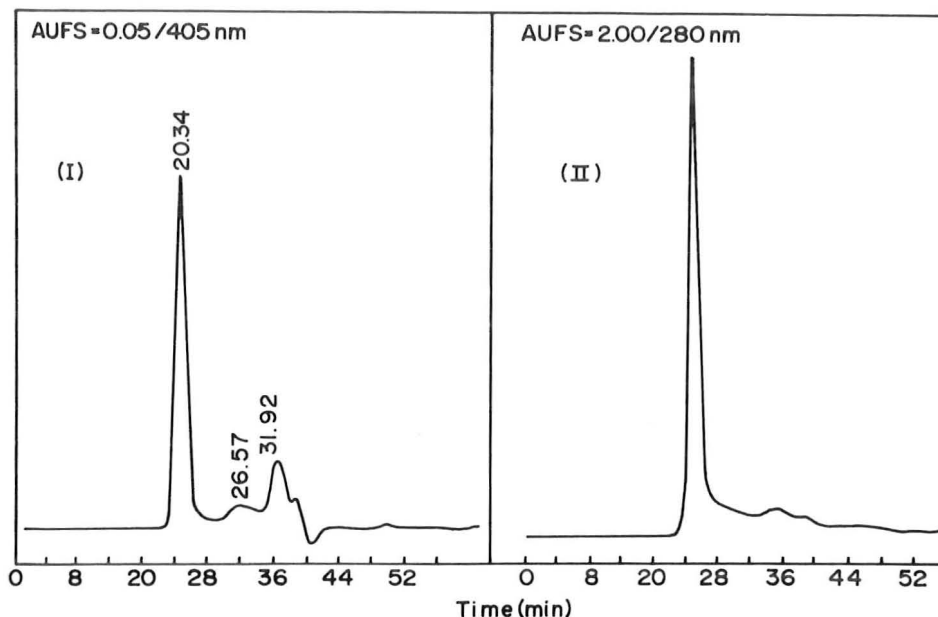
Figure 3. HPLC of solubilized rat testis microsomes on Spherogel TSK 3000SW column. Flow rate was 0.5 ml/min and chart speed 0.25 cm/min. Chromatogram I shows absorbance at 405 nm and chromatogram II at 280 nm.

These studies with rat liver and testis microsomes indicate a very promising role of HPLC in studies of induction, fractionation and denaturation of cytochrome P-450 and extends the application range of HPLC to insoluble proteins. A unique application of this approach is in the study of systems in which cytochrome P-450 content is the limiting factor. The spectral properties of recovered cytochrome P-450, i.e. CO-binding spectrum, are indicative of the structural integrity of the protein (12,13). Reconstitution of the system and demonstration of substrate metabolism are outside the scope of the present manuscript (12,13). This

technique has been successfully employed in our laboratory to monitor the conformational changes in cytochrome P-450 during its conversion to cytochrome P-420 and other applications such as detergent removal are under study.

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AUDIO-VISUAL COURSE REVIEW

TROUBLESHOOTING THE LIQUID CHROMATOGRAPH, AN AUDIO-VISUAL PROGRAM,
by H. M. McNair, F. I. Scott and Associates, P. O. Box 86, Check,
Virginia, 24072, 1981, $250

This audio-visual program is aimed at chromatographers and lab
technicians as a practical step by step introduction to the
selection of operating conditions and recognition of problems
that may exist in the liquid chromatograph. It is in three
parts. Part one deals with operating principles, selection
criteria, and component maintenance (exclusive of the column).
Part two guides the student to understand operation and main-
tenance of liquid chromatography columns. Part three leads
one to diagnose instrument problems.

In the experience of the present reviewer, one is often faced with
the problem of diagnosing trouble in the chromatograph when the
only indication of trouble is a poor chromatogram. This audio-
visual course has been excellently conceived and executed and
leads one directly to the solution of such problems. It is
highly recommended as required reading-viewing for all practicing
liquid chromatographers who do not mind "getting their hands
dirty."

BOOK REVIEW

> "Chromatographic Methods in Inorganic Analysis";
> G. Schwedt; publ. by Dr.Aefred Huthig Verlag,
> Heidelberg, 1981; 226 pp.; $38.00

This volume provides a comprehensive survey of a variety of chromatographic separation systems for a broad range of compounds of the elements, of specialized detection methods, and of practical applications of chromatographic analysis. Chromatographic techniques include LC, TLC, GC, open column chromatography, and paper chromatography.

Details of practical methodology are only superficially covered; however, the numerous tables of applications lead the reader to the original reference for complete descriptions. Applications are generally listed under the chemical elements that are involved; thus, locating a reference for a specific application is a straightforward task. There are 814 references given, including their complete title. A particularly useful feature of this book is the index of references to analysis of the elements, listed in order of their appearance in the periodic table.

This book is highly recommended to the practitioner who must deal on a daily basis with inorganic analysis. It will save him many hours of methods development by leading him directly to the methodology he requires.

JZ

LC NEWS

SAMPLE PREPARATION EDUCATIONAL PROGRAM provides a foundation in modern techniques for efficient sample preparation as required for quality chemical analyses by GC, LC, UV/VIS spectroscopy and others. The program includes a discussion of various laboratory operations (derivatization, filtration, extraction, etc.) and sample preparation methods for selected applications. Also, special sections of reference data, sample preparation abstracts, technical exchange, and a glossary of terms are presented in a binder for easy updating. (19) Zymark Corp., JLC/82/2, 102 South Street, Hopkinton, MA, 01748, U.S.A.

MICRO-COMPUTER-BASED LIQUID CHROMATOGRAPHS DESCRIBED. There are six basic models ranging from simple isocratic to fully automated gradient systems. Also described are: CRT-keyboard, Microcomputer controlled hydraulic system, Choice of injectors, columns and detectors, and performance features. (16) Varian Instruments, JLC/82/2, Box D-070, 10060 Bubb Road, Cupertino, CA, 95014, U.S.A.

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NEW CATALOG doubles as a reference book for chromatographers. This new 300+ page catalog contains applications, useful technical information and handy reference charts, as well as an extensive line of accessories for GC, LC, HPLC, TLC, and GPC. (14) Alltech Associates, Inc., JLC/82/2, 2051 Waukegan Road, Deerfield, IL, 60015, U.S.A.

HPLC PUMP AND DETECTOR CATALOG gives descriptions and specifications for solvent delivery pumps, and a wide variety of LC detectors for measuring UV absorption, fluorescence, refractive index, electrochemical and conductivity. The Model 711 Microprocessor Controller/Lab Computor is also described. (13) The Anspec Company, Inc., JLC/82/2, 122 Enterprise Drive, P.O. Box 7730, Ann Arbor, MI, 48107, U.S.A.

DILUTERS AND DISPENSERS now have high accuracy, low price, great versatility, and at the same time, they offer the economy and simplicity of semi-automated operation. They handle volumes from 2.5 ul to 10 ml with accuracy of 1% or more, depending on sample size. For application flexibility, the diluters and dispensers can handle corrosive, hot and high viscosity fluids. Uses include wet chemistries, RIA, hematology, analytical chemistry, and biochem applications. They can, as an option, be equipped with an electronically actuated hand probe for one-handed operation as far away as three feet from the unit. (12) Hamilton Company, JLC/82/2, P.O. Box 10030, Reno, NV, 89510, U.S.A.

ADVANCED NINHYDRIN REAGENT FORMULA FOR AMINO ACID ANALYSIS is a superior improvement on the popular ninhydrin-DMSO-hydrinantin formulation. It offers the advantage of a superior color response, excellent linearity and remarkable stability. This reagent is prepared easily within minutes and will last for several weeks. (11) Pierce Chemical Company, JLC/82/2, P.O. Box 117, Rockford, IL, 61105, U.S.A.

NEW SERIES OF HPLC PACKING MATERIALS has been developed. Some features of this new material are spherical 10 micron particles, both C8 and C18. It also has extremely good peak symmetry, As 1.2, and controlled batch to batch reproductibility. There is also high selectivity and long term stability. (2) Chrompack Nederland BV, JLC/82/2, P.O.B. 3, 4330 AA Middleburgh, The Netherlands.

HPLC POST COLUMN REACTION SYSTEM for dual reagent derivatization methods. Post column derivatization is the simplest and most convenient method known for enhancing the detectability of compounds separated by HPLC (sensitivity), while also providing selective detection according to compound functional group. This system is the first instrument of its type designed to perform all the steps necessary for dual reagent derivatization. Derivatization using fluorescamine is a well-known method often employed in the analysis of amino acids and peptides. It is a completely self-contained instrument designed for dual reagent derivatization procedures. (17) Kratos Inc, Schoeffel Instrument Div., JLC/82/2, 24 Booker Street, Westwood, NJ 07675, USA.

LC CALENDAR

1982

MARCH 8 - 12: Pittsburgh Conf. on Anal. Chem. and Applied Spectroscopy, Atlantic City, NJ, USA. Contact: Pittsburgh Conf., Inc., P.O. Box 7780-1223, Philadelphia, PA, 19182, USA.

MARCH 28 - APRIL 2: National American Chem. Soc. Meeting, Las Vegas, NV, USA. Contact: A.T. Winstead, Am. Chem. Soc., 1155 Sixteenth St., NW, Washington, D.C., 20036, USA.

APRIL 14 - 16: "12th Annual Symposium on the Anal. Chem. of Pollutants," Amsterdam, The Netherlands. Contact: Prof. R.W. Frei, Congress Office, Vrije Universiteit, P.O. Box 7161, 1007-MC Amsterdam, The Netherlands.

APRIL 18-21: 66th Annual Meeting, Fed. of Am. Soc for Exptl. Biology (FASEB), Louisiana Superdome, New Orleans, LA, USA. Contact: FASEB, 9650 Rockville Pike, Bethesda, MD, 20014, USA.

MAY 16 - 18: LCEC Symposium: "Biomedical Applications of LCEC and Voltammetry", Indianapolis Hyatt Regency. Contact: K. Klippel, LCEC Symposium, P.O. Box 2206, W. Lafayette, IN, 47906, USA.

JUNE 28 - 30: "Analytical Summer Symposium," Michigan State Univ., East Lansing, MI, USA. Contact: A.I. Popov, Chem. Dept., Michigan State Univ., East Lansing, MI 48824, USA.

JULY 12 - 16: "2nd Int'l Symposium on Macromolecules," - IUPAC, University of Massachusetts, Amherst, MA, 01003, USA.

JULY 19 - 22: 23rd Prague Microsymposium on Macromolecules: "Selective Polymeric Sorbents" - IUPAC, Institute of Macromolecular Chemistry, Prague, Czechoslovakia. Contact: P.M.M. Secretariat, Institute of Macromolecular Chemistry, 162-06, Czechoslovakia.

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AUGUST 2 - 5: Int'l Symposium on Ion Chrom, at the 24th
Rocky Mountain Conference, Denver, CO, USA. Contact:
Dionex Corp., 1228 Titan Way, Sunnyvale, CA, 94086, USA.

AUGUST 15 - 21: "12th Int'l Congress of Biochemistry",
Perth, Western Australia.   Contact: Brian Thorpe,
Dept. of Biochemistry, Faculty of Science, Australian
National University, Canberra A.C.T. 2600, Australia.

SEPTEMBER 12 - 17:   National American Chem. Soc.
Meeting, Kansas City, MO, USA. Contact: A.T.
Winstead, American Chem. Soc., 1155 Sixteenth St., NW,
Washington, D.C., 20036, USA.

1983

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

JULY: Third International Flavor Conference, American
Chem. Society, Th Corfu Hilton, Corfu, Greece. Contact:
Dr. S.S. Kazeniac, Campbell Institute for Food
Research, Campbell Place, Camden, N.J.., 08101, USA.

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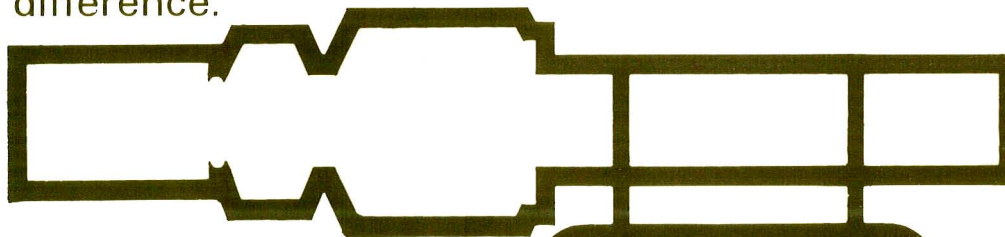
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