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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(14), 2697-2710 (1984)

THE INFLUENCE OF THE SPECIFIC SURFACE AREA OF ADSORBENT UPON THE OPTIMIZATION OF THE PROCESS OF ADSORPTION THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

An important aspect of the influence of specific surface area of adsorbent on R_M values of substances obtained in the process of adsorption thin-layer chromatography has been presented in the paper. The experiments have been conducted with four adsorbents, each having different specific surface area, that is, from 50 to 500 m^2/g and with the use of mixed binary solvent mobile phases of benzene-aliphatic alcohol type. It has been proved that R_M values of investigated substances of group B change regulary according to the specific surface area of adsorbent for individual concentrations of mobile phase. The relationship may be described by means of square trinominal. The parameters of trinominal may be tabulated and the differentiation of the parameters for particular substances is observable at the same time. The relationship in question may be utilized to calculate R_M values of studied substances on any adsorbent with known specific surface area.

INTRODUCTION

In chromatographic investigations great attention is paid to adsorbents with different surface structure, in their monographs Geiss (1) and Snyder (2) denoted much attention to the importance of microporous structure of adsorbent in the process of thin-layer chromatography.

The interest in the role of specific surfabe area of adsorbent in thin-layer chromatography is still current due to the increasing importance of this method as a pilot technique for determining the optimum mixture separation conditions on analitical and preparative scale (3 - 7). The previously observed linear relationships of the difference of R_M values of substances for pure components of mixed binary solvents mobile phases from the specific surface area of adsorbents are not to convenient to use in the routine process of optimization of separation conditions. In the present paper an analysis of relationships between R_M values of substances and specific surface area of adsorbent with the preservation of equal concentrations of mobile phase on adsorbents with different size of specific surface area has been made.

MATERIALS AND METHODS

In order to solve the problem in question the measurements of R_F (R_M) values of model substances have been made by means of the method applied in adsorption thin-layer chromatography. Silica gel f-my Merck (7) with different specific sur-

face area (s) have been used as adsorbent, that is, 500; 400; 150 and 50 m^2/g. Polycyclic compounds, the molecules of which contained heteroatoms have been used as chromatographed substances. All studied substances under experiment showed electrodonor properties and constant dipole moment. The chromatografphed substances were:

Substances	Pimentel and McClellan classification (8)
phenol	AB
acridine	B
quinoline	B
6-methylquinoline	B
7-methylquinoline	B
8-methylquinoline	B

As mobile phases were used binary mixtures of N(B)-AB type solvents, that is, benzene-methanol, benzene-ethanol and benzene-propanol.

The way in which the chromatographic process was conducted and the visualization of the substances under investigations have been presented in many earlier publications (9-12). The results of the experiments performed have given in a form of diagrams of R_M values according to the size of specific surface area of adsorbent vs R_M (Figs. 1-4).

RESULTS AND DISCUSSION

Molecular interactions in chromatographic process are of very complex nature. It is known that R_M values of chro-

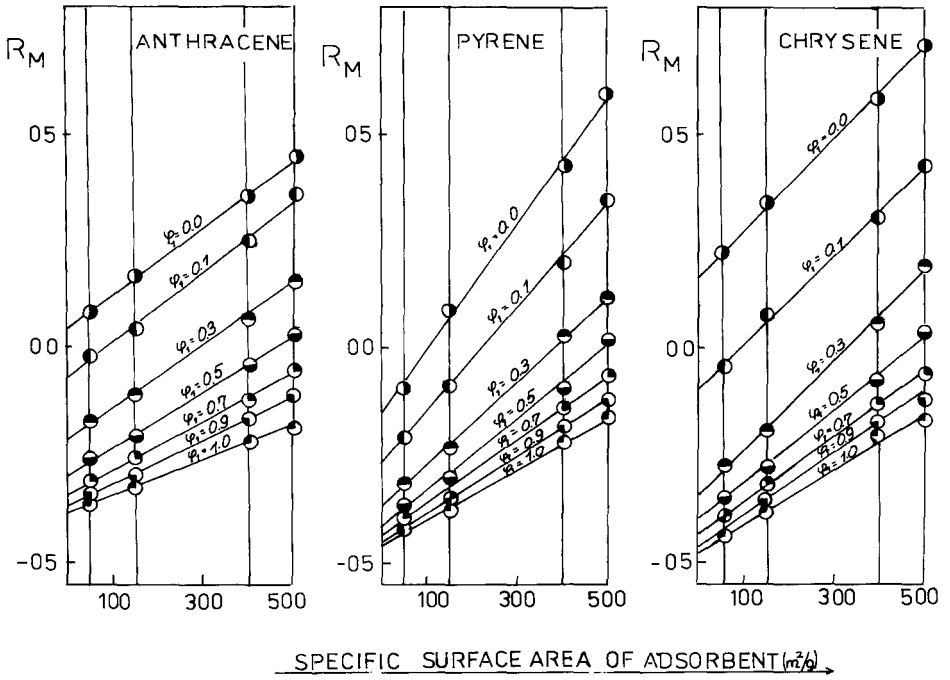


FIGURE 1. Linear relationship R_M=f s for nonactive chromatographic substances. Mobile phase: methylcycloheksane-benzene.

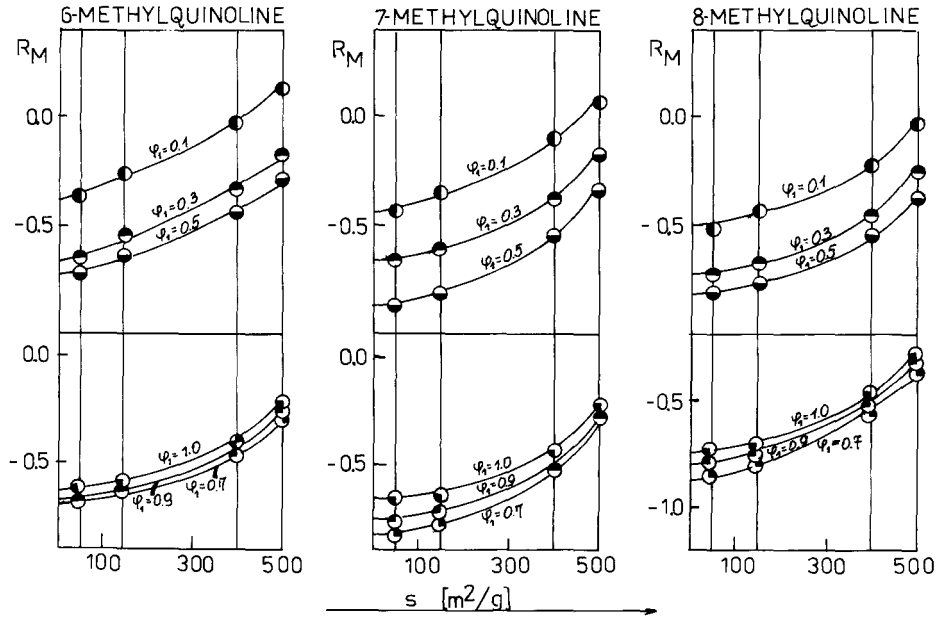


FIGURE 2. Relationship R_M=f s for active solutes. Mobile phase: benzene-methanol.

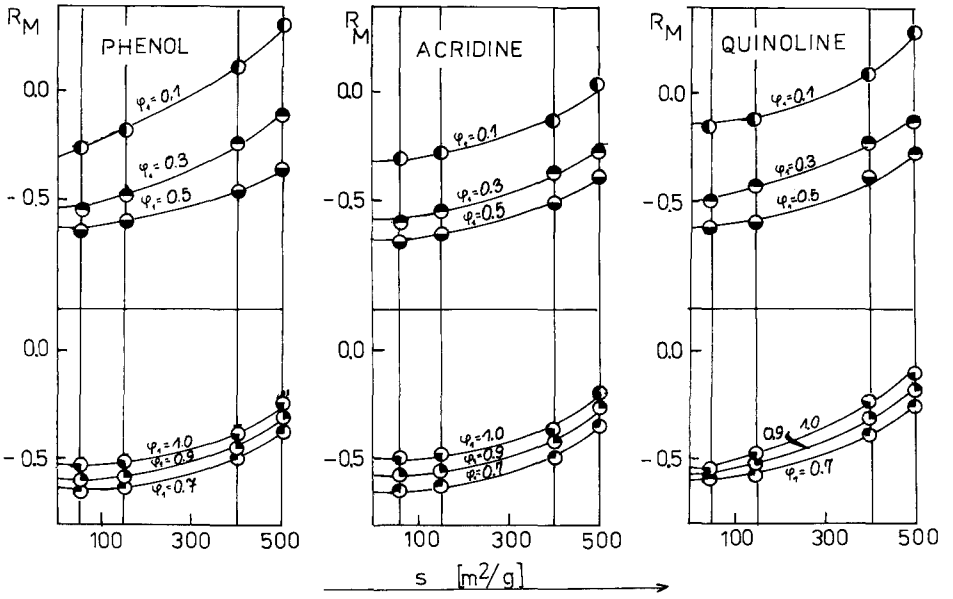


FIGURE 3. Relationship R_M=f s for active solutes. Mobile phase: benzene-ethanol.

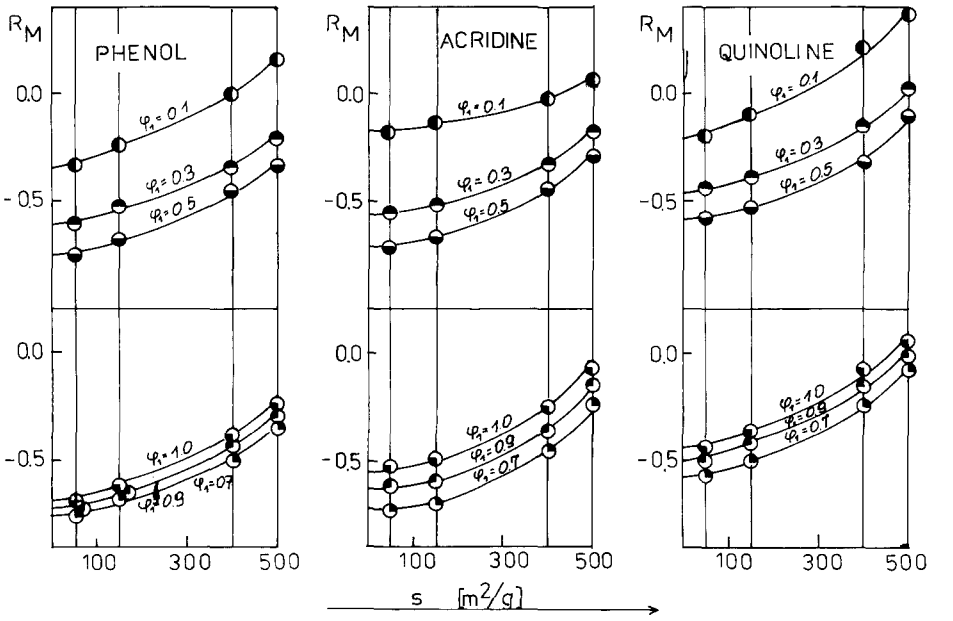


FIGURE 4. Relationship R_M=f s for active solutes. Mobile phase: benzene=propanol.

matographed substances depend upon the kind of investigated substances, property of solvents constituting mobile phase and property of adsorbent. In the paper an attention has been paid to the size of specific surface area of adsorbents and to R_M values of chromatographed substances which are connected with them. In earlier works (13) there has been discussed a problem of relationships between R_M values of chromatographed substances and the specific surface area of adsorbent for substances which exhibit neither electrodonor nor electroacceptor properites (class N) and do not posses significant dipole moment. In the case of such substances in systems of benzene-hydrocarbon (class N-N) and benzene alcohol (class N B - AB types). The relationship between R_M values of these substances in these mobile phase in given as

$$R_M = as + b \qquad 1$$

Values for a and b are chosen in a numerical way. In the paper we deal with the relationship between R_M values and the specific surface area of adsorbent for substances having electrodonor properties and belonging to groups A and AB according to Pimentel and McClellan. In this case diagrams R_M vs s are not straight lines but they have the shape of parabola. This relationship is given as:

$$R_M = as^2 + bs + c \qquad 2$$

Graphical relationships of R_M vs s have been shown in Figs. 2-4. The parameters a,b and c of equation 2 depend upon the kind of chromatographed substance and applied

mobile phase. For each chromatographed substance there have been calculated and tabulated the values of parameters a,b and c according to equation 2 for individual mobile phase compositions. The parameters have been presented in Tab. I-III. Parameter a denotes specific molecular interactions within chromatographic system. Certain regularities may be observed in systems under investigations. The slighest differences between parameters a for individual substances appear together with greater concentrations of alcohol in mobile phase. For quinoline and its methyl derivatives the values of parameter a decrease with the increase of molecular weight of alcohol which is a more active component of mobile phase. It follows from the data in Tab. I-III that the values of parameters a and b greatly depend upon the size of the molecular area of chromatographed substances A_s and upon the configuration of free electrons within the molecule of a given substance. Though acridine and quinoline belong to the same group of compounds according to Pimentel and McClellan classification, we may cleary see the differences in the values of the above mentioned parameters. It is caused by the differences in configurations of free electrons within the molecules of the compound in question.

The values of parameters a and b are also greatly different in the case of quinoline and its methyl derivatives. Though the value of adsorption energy of methyl group on SiO_2 is nearly zero. The differences in the configuration of free electrons in the molecules of substances under in-

Table 1

Parameters a,b and c (eq 2). Mobile phase: benzene-methanol

Solute	volume fraction of methanol						
	0.0	0.1	0.3	0.5	0.7	0.9	1.0
	values of parameter $ax10^4$						
phenol	0.025	0.017	0.012	0.005	0.020	0.019	0.019
acridine	0.052	0.006	0.014	0.010	0.010	0.013	0.013
quinoline	0.032	0.004	0.017	0.021	0.025	0.022	0.022
6-methylquinoline	0.003	0.008	0.011	0.013	0.022	0.021	0.019
7-methylquinoline	0.023	0.016	0.023	0.024	0.034	0.036	0.023
8-methylquinoline	0.025	0.021	0.022	0.020	0.028	0.032	0.037
	values of parameter b						
phenol	0.100	0.057	0.052	0.055	0.000	0.004	0.001
acridine	-0.083	0.066	0.024	0.020	0.020	-0.003	0.003
quinoline	-0.022	0.100	0.017	0.011	-0.025	-0.021	-0.021
6-methylquinoline	0.091	0.065	0.046	0.023	-0.024	-0.019	-0.014
7-methylquinoline	0.016	0.029	-0.014	0.004	-0.058	-0.074	-0.017
8-methylquinoline	0.010	0.018	0.018	-0.015	-0.037	-0.065	-0.093
	values of parameter c						
phenol	0.120	-0.500	-0.730	-0.840	-0.890	-0.880	-0.880
acridine	0.610	-0.530	-0.730	-0.750	-0.720	-0.650	-0.640
quinoline	0.910	-0.410	-0.850	-0.770	-0.800	-0.730	-0.700
6-methylquinoline	0.980	-0.410	-0.670	-0.740	-0.720	-0.700	-0.650
7-methylquinoline	0.940	-0.460	-0.690	-0.890	-0.870	-0.800	-0.740
8-methylquinoline	0.460	-0.570	-0.740	-0.820	-0.850	-0.790	-0.740

Table II

Parameters a,b and c (eq.2). Mobile phase: benżene - ethanol.

Solute	volume fraction of ethanol						
	0.0	0.1	0.3	0.5	0.7	0.9	1.0
	values of parameter a						
phenol	0.025	0.016	0.008	0.011	0.012	0.020	0.023
acridine	0.052	0.002	0.008	0.011	0.019	0.023	0.024
quinoline	0.032	0.019	0.004	0.012	0.010	0.004	0.008
6-methylquinoline	0.003	0.009	0.007	0.013	0.014	0.008	0.012
7-methylquinoline	0.023	0.023	0.002	0.027	0.030	0.030	0.013
8-methylquinoline	0.025	0.016	0.014	0.014	0.017	0.019	0.025
	values of parameter b						
phenol	0.100	0.048	0.053	0.006	-0.064	-0.043	-0.057
acridine	-0.083	0.047	0.032	0.011	-0.031	-0.047	-0.065
quinoline	-0.022	-0.013	0.054	0.012	0.019	0.064	0.051
6-methylquinoline	0.091	0.027	0.044	0.015	0.007	0.043	0.026
7-methylquinoline	0.016	0.002	0.072	0.013	-0.005	-0.036	0.013
8-methylquinoline	0.010	-0.017	-0.003	-0.006	-0.026	-0.021	-0.065
	values of parameter c						
phenol	0.120	-0.320	-0.570	-0.650	-0.640	-0.600	-0.530
acridine	0.610	-0.320	-0.620	-0.710	-0.670	-0.580	-0.490
quinoline	0.910	-0.170	-0.520	-0.640	-0.620	-0.600	-0.580
6-methylquinoline	0.980	-0.260	-0.600	-0.710	-0.660	-0.600	-0.560
7-methylquinoline	0.940	-0.210	-0.550	-0.660	-0.660	-0.620	-0.550
8-methylquinoline	0.460	-0.320	-0.620	-0.700	-0.710	-0.620	-0.500

Table III

Parameters a,b and c (eq.2) . Mobile phase: benzene-propanol.

Solute	volume fraction of propanol						
	0.0	0.1	0.3	0.5	0.7	0.9	1.0
	values of parameter a						
phenol	0.025	0.018	0.015	0.016	0.022	0.018	0.012
acridine	0.052	0.007	0.012	0.017	0.026	0.027	0.012
quinoline	0.032	0.011	0.015	0.019	0.019	0.010	0.007
6-methylquinoline	0.003	0.009	0.010	0.007	0.004	0.017	0.002
7-methylquinoline	0.003	0.022	0.017	0.016	0.013	0.013	0.017
8-methylquinoline	0.025	0.017	0.012	0.012	0.007	0.001	0.000
	values of parameter b						
phenol	0.100	0.014	0.002	0.001	-0.021	0.002	0.025
acridine	-0.083	0.011	0.017	0.000	-0.034	-0.039	0.025
quinoline	-0.022	0.062	0.027	-0.006	-0.007	0.045	0.057
6-methylquinoline	0.091	0.022	0.030	0.038	0.074	-0.004	-0.020
7-methylquinoline	0.016	-0.023	-0.008	-0.004	0.019	0.018	0.031
8-methylquinoline	0.010	0.001	0.015	0.012	0.057	0.094	0.013
	values of parameter c						
phenol	0.120	-0.360	-0.610	-0.760	-0.760	-0.720	-0.690
acridine	0.610	-0.180	-0.560	-0.720	-0.720	-0.630	-0.550
quinoline	0.910	-0.210	-0.460	-0.580	-0.530	-0.500	-0.440
6-methylquinoline	0.980	-0.060	-0.460	-0.550	-0.570	-0.450	-0.400
7-methylquinoline	0.940	-0.140	-0.420	-0.540	-0.500	-0.500	-0.560
8-methylquinoline	0.460	-0.260	-0.640	-0.700	-0.720	-0.670	-0.650

vestigations are seen in the values of their dipole moments and that has an influence on the differentiation of parameters a and b of multinominal.

The smaller is the molecule of alcohol which is a more active component of mobile phase the smaller are the differences in the values of parameter a (from the concentration $\varphi_1 = 0.7$ of volume fraction). The regularities are most distincly observed in the case of benzene-methanol mobile phase. It is understood beoouse of the fact that mathanol is the solvent of the greatest elution strenght among the solvents we used. For benzene-propanol system the relationship is not maintained due to the significantly lower elution strength of the mobile phase.

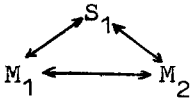
In the investigated systems the smallest values of parameter a appear for acridine. It is also seen that for phenol the values of parameters a and b greatly differ from the value of these parameters for remaining solutes. It may be explained by the different ability of phenol to make hydrogen bonds with the remaining substances of group B.

The values of parameter c denote molecular interactions at specific surface area of adsorbent equal to 0 ($s=0$) in a given chromatographic system, that is, the interactions of the type: chromatographed substance S_1 - - component "1" of mobile phase (M_1) and chromatographed substance (S_1) - component "2" of mobile phase (M_2). In a graphical form, the interactions may be shown as follows:

Table IV

Experimental and theoretical R_M values of solutes on different adsorbent.

s m^2/g	volume fraction of the stronger component of mobile phase (φ_1)													
	0.0		0.1		0.3		0.5		0.7		0.9		1.0	
	R_M^{exp}	R_M^{th}	R_M^{exp}	R_M^{th}	R_M^{exp}	R_M^{th}	R_M^{exp}	R_M^{th}	R_M^{exp}	R_M^{th}	R_M^{exp}	R_M^{th}	R_M^{exp}	R_M^{th}
solvent system: benzene-methanol, chromatographed substance - acridine														
500	1.51	1.50	0.05	0.05	-0.25	-0.25	-0.39	-0.40	-0.37	-0.37	-0.34	-0.34	-0.30	-0.30
400	1.12	1.11	-0.17	-0.17	-0.40	-0.40	-0.50	-0.52	-0.48	-0.48	-0.45	-0.45	-0.42	-0.42
150	0.74	0.60	-0.41	-0.42	-0.63	-0.65	-0.67	-0.70	-0.65	-0.67	-0.62	-0.61	-0.60	-0.61
50	0.63	0.63	-0.50	-0.49	-0.69	-0.70	-0.73	-0.73	-0.70	-0.71	-0.64	-0.63	-0.64	-0.64
solvent system: benzene-ethanol, chromatographed substance - quinoline														
500	1.59	1.60	0.24	0.24	-0.15	-0.15	-0.28	-0.28	-0.27	-0.27	-0.18	-0.18	-0.11	-0.11
400	1.33	1.33	0.08	0.08	-0.24	-0.24	-0.40	-0.40	-0.38	-0.38	-0.28	-0.28	-0.24	-0.24
150	1.02	0.94	-0.02	-0.02	-0.43	-0.43	-0.60	-0.59	-0.56	-0.57	-0.51	-0.51	-0.48	-0.48
50	0.94	0.91	-0.17	-0.17	-0.50	-0.52	-0.62	-0.63	-0.62	-0.61	-0.59	-0.59	-0.56	-0.55
solvent system: benzene-propanol, chromatographed substance - phenol														
500	0.98	1.00	0.16	0.16	-0.22	-0.22	-0.34	-0.34	-0.33	-0.33	-0.28	-0.28	-0.27	-0.27
400	0.66	0.68	-0.22	-0.02	-0.36	-0.36	-0.48	-0.50	-0.50	-0.50	-0.44	-0.44	-0.40	-0.40
150	0.28	0.32	-0.25	-0.29	-0.53	-0.57	-0.68	-0.72	-0.72	-0.74	-0.69	-0.72	-0.62	-0.63
50	0.16	0.18	-0.35	-0.35	-0.63	-0.60	-0.77	-0.76	-0.75	-0.76	-0.73	-0.72	-0.70	-0.68



The values of parameter c change with the composition of mobile phase due to the fact that systems we investigated are non-ideal. The diagrams of relationships c vs φ_1 correspond in their shapes to relationships R_M vs φ_1; but the values of parameter c are in each case lowre then the corresponding R_M values.

In table IV there have been shown R_M values for chosen chromatographed substances obtained experimentally and calculated from equation 2. As it is seen from the given data there exists satisfactory agreement between theoretical and experimental data. The connection of the values of parameter a,b and c with physico-chemical quantities being characteristic of chromatographic system will allow us to avoid the series of tiring experimental measurements and to obtained greater universality of chromatographic data.

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EVALUATION OF SYSTEM ERRORS IN TLC-DENSITOMETRY WHEN USING THE STREAKING TECHNIQUE

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ABSTRACT

The errors that contribute to the total variance of the automated streaking technique are examined. These results are compared with computer optimized and zig-zag scanning approaches. The error due to inhomogeneous sample distribution is shown to be the most important.

INTRODUCTION

Quantitative Thin Layer Chromatography (TLC) is a legitimate supplement or alternative to High Performance Liquid Chromatography (HPLC) (1). This is especially true with pharmaceutical analyses where the sample matrix is often complex and cannot be injected directly onto expensive columns.

Within the past few years, the art of TLC densitometry has made significant advances. Coupled with high performance TLC (2, 3, 4) excellent performance capabilities are now attainable.

One of the chief limitations of densitometry is the relatively narrow dynamic range of sample mass (5). It is well known that non-uniform

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distribution of the sample within the layer causes nonlinear results. To some extent, this difficulty can be overcome by one of three approaches: streaking the sample and scanning the center of the streak; scanning with a small beam in a zig-zag manner; or controlling the positioning of the beam by computer. Each of these approaches has its advantages. For the streaking technique, there is less variation in sample distribution ; zig-zag scanning can overcome differences in spot shape; and the computer controlled system can optimize scanning position.

This paper evaluates the factors that contribute to the overall system error when using an automated streaking device, and compares them with what is attainable by the computer controlled and zig-zag scanning approaches.

EXPERIMENTAL

Sample solutions were prepared in methanol at the following levels: Caffeine (J. T. Baker, USA), 0.30 mg/ml and 2.50 mg/ml; theophylline (J. T. Baker, USA), 0.28 mg/ml; Sunset Yellow (Nederlandsek Kleurstafindustrie, Holland), E110, 1.0 mg/ml; Patent Blue, E131, 1.0 mg/ml. All samples were applied with a Camag Linomat III. Streaks were 10 mm wide and applied at a rate of 50 mm/µl and 5 sec/µl. Conventional 20 x 20 cm TLC plates were purchased from Merck (Alltech Associates, USA) coated with Silica Gel 60F. The 10 x 10 cm high performance plates were purchased from Merck and also coated with Silica Gel 60F. High performance plates were developed three centimeters in a 4" x 4" x 3" chamber, while the conventional plates were developed six cm in a Camag Twin Trough Chamber. Caffeine linearity, and sample volume studies were accomplished on conventional Merck plates and developed a distance of 10 cm. The two dye samples were developed with isopropyl alcohol:ammonia, 75:25, v/v, without preequilibration; while caffeine and theophylline samples were developed with chloroform:methanol,

90:10, v/v, after 15 minutes of preequilibration in a paper-lined TLC chamber. Developed streaks were scanned using a Schoeffel SD3000 (Schoeffel Instrument Corp., Westwood, NJ, USA) densitometer with a 5 mm beam. The Sunset Yellow streaks were scanned at 500 nm, the Patent Blue at 650 nm, and the caffeine and theophylline at 280 nm.

RESULTS AND DISCUSSION

Contributions to the total variance of any TLC process has been described by Ebel (6). The system variance, σ^2 is the sum of the square of the sample application error, σ_v; the chromatography error, σ_c; the measurement error, σ_m; and the positioning error, σ_p.

$$\sigma^2 = \sigma_c^2 + \sigma_p^2 + \sigma_v^2 + \sigma_m^2$$

The measurement error can be estimated by scanning the same spot repeatedly without recentering the beam before each scan; whereas, the positioning error can be obtained by repetative scanning of the same spot after reoptimizing the beam. The sample application and chromatography errors are not as easily established. Determination of the sample application error has been done by assuming that under highly efficient conditions, the chromatographic error is zero (7). Because the chromatography parameter is usually the largest and most difficult to control, this assumption is tenuous.

The chromatography error is related to the physical and chemical aspects of each particular system. However, as far as the densitometric process is concerned, the two most important manifestations of chromatographic variance are the shape of the developed spots and the distribution of the sample within the plate matrix. Because of the open bed aspect of TLC, lateral sample diffusion is often a significant factor in causing spot shape variation and different shaped spots can also yield different responses (8). Also, because of the previously mentioned narrow sample mass dynamic range, variations in

sample distribution can cause significant error (5). Accordingly, the chromatographic variance, σ_c^2, is more accurately described by a spot shape variance, σ_s^2, and a sample distribution variance, σ_d^2. Thus, the total variance becomes:

$$\sigma^2 = \sigma_m^2 + \sigma_p^2 + \sigma_v^2 + \sigma_s^2 + \sigma_d^2$$

In comparing different densitometric modes, each of these errors must be considered.

With computer controlled optimization, the beam positioning error is negligible (9).

$$\sigma^2_{computer} = \sigma_m^2 + \sigma_v^2 + \sigma_d^2 + \sigma_s^2$$

Small beam, two dimentional scanning (zig-zag) devices effectively eliminate variations due to both position and spot shape (8). In addition,

$$\sigma^2_{zig\text{-}zag} = \sigma_m^2 + \sigma_v^2 + \sigma_d^2$$

to some extent the problem of non-uniform sample distribution is not as significant when using a small beam scanned in two dimensions. Whereas, the detector response for a long narrow beam gives an average reading which may or may not reflect the amount of sample present, a smaller beam is not as adversely effected. However, due to the relatively short dynamic range inherent with densitometry, even small beams may not overcome this problem.

The ability of the automated streaking device to minimize the sample volume, beam position, and spot shape errors, was demonstrated by applying 16 µl of a similar 0.25 mg/ml caffeine in methanol solution and 20 µl of a similar 0.20 mg/ml caffeine solution. Since the second solution is exactly 80% of the first one with respect to concentration, equal amounts of caffeine can be applied only if the automated streaking device is able to deliver accurate volumes of sample. Table 1 shows that four streaks of each solution

TABLE I

Densitometric Responses for Different Volume of Solution

	4 µg Caffeine		2 µg Caffeine	
	20 µl	16 µl	20 µl	8µl
x	59.2 x 10^4 area	59.5 x 10^4 area	40.6 x 10^4 area	39.8
SD	2.1	1.2	2.0	1.1
RSD	3.5%	2.0%	4.9%	2.8%

gave equivalent densitometric results according to the null hypothesis when scanned at 280 nm, where the probability of the difference between the two means being greater than zero is less than 60%. Also, 8 µl of 0.25 mg/ml caffeine solution was compared with 20 µl of 0.1 mg/ml caffeine solution. Similarly, the amount of caffeine delivered to the plate by both solutions was the same when scanned, where the probability of the difference between the two means being greater than zero is only 75%.

The automated streaking device is also capable of delivering linear amounts of caffeine solution. As seen in Table 2, the linearity is the same by streaking different concentrations (0.05 to 0.25 mg/ml) of analyte at fixed volume (20 µl), as that obtained by applying changing volumes (4-20 µl) of a fixed concentration (0.25 mg/ml) solution.

If a sample application error were significant, it would be a function of the total volume of sample applied, since the chromatographic measurement and positioning errors are the same in both systems. Clearly, there is no apparent difference in how the same amount of a sample is applied, when the center of the streak is scanned. This conclusion is reenforced by the results obtained when streaking equivalent linear amounts of caffeine from a fixed volume while varying sample concentration versus varying volumes of a fixed concentration solution (Table 2).

Additionally, because the beam position was not optimized during these measurements, the σ_p error is eliminated.

TABLE 2

Linear Regression Analysis of Densitometric Scans of Equivalent Amounts of Caffeine Streaked from Different Volumes of Solutions

	Volume Change[1]	Concentration Change[2]
x mean	2.5%	2.5%
y mean	43.2×10^4 area units	42.5×10^4 area units
correlation coefficient	0.993	0.974
relative y-intercept	20.7%	14.8%
intercept	9.0×10^4 area units	6.3×10^4 area units
slope	1.4×10^5 area units/µg	1.4×10^5 area units/µg

[1]Applied by streaking 4, 8, 12, 16, and 20 µl of 0.25 mg/ml caffeine solution.

[2]Applied by streaking 20 µl of 0.05, 0.1, 0.15, 0.20, and 0.25 mg/ml caffeine solution.

The major errors from automated streaking are related to the chromatographic factors, σ_s and σ_d. With streaking, as long as the solvent front travels uniformly, the shape of the center of the streak will not contribute significantly to the overall variance. Further, shape distortions are much easier to observe with streaks than with spots. Streaks that are distorted or not perpendicular to the flow of solvent should be quantitated. When this is done, only the sample distribution error contributes measurably to chromatographic variance. Thus,

$$\sigma^2_{\text{streaking}} = \sigma_m^2 + \sigma_d^2.$$

Precision data for sight replicate streaks of four different compounds are listed in Table 3. The two dyes are large ionic compounds, with low diffusion coefficients, and are therefore less subject to sample inhomogeneity, *i.e.*, σ_d^2 is small. On the other hand, theophylline and caffeine are more prone to diffusion and this is reflected in slightly larger RSD values.

TABLE 3

Standard Deviation of Samples Applied by the Streaking Technique

Compound	RSD, % (peak height)
1. Patent Blue, E-131	
HPTLC, σ	1.9
TLC, σ	1.6
2. Sunset Yellow, E-110	
HPTLC, σ	1.6
TLC, σ	1.6
3. Theophylline	
HPTLC, σ	2.5
TLC, σ	2.3
4. Caffeine	
HPTLC, σ	2.6
TLC, σ	1.1

In actual practice, contributions to the total variance for all three modes of analysis may be simplified. When samples are applied manually with 0.5 µl microcapillaries, the relative application error is less than 1%. Also, the electronics in densitometers keeps the measurement error around 0.2-0.6% (9). This leaves to the spot shape, and sample distribution errors as the main sources of imprecision. As described above, the variance from automated streaking and zig-zag scanning will mainly depend upon the sample distribution error; while the variance from computer controlled optimization will be related to both spot shape and sample distribution. These two factors remain the Achilles heel of densitometry. However, when any of the above densitometric approaches is coupled with a modern high performance TLC system, percent variations of 1-3% can be expected.

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Reversed Phase Thin Layer Chromatography of Amino Acids

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The use of reversed phase layers for the thin layer chromatography of amino acids is described. Only when a modifier was added to the mobile phase was clear separation of the amino acids achieved. Ion paring with trifluoroacetic acid overcame problems with streaking and poor separation on C_2 or C_{18} reversed phase layers. All amino acids could not be separated with a single mobile phase. Thus, three different combination of acetonitrile-0.4% trifluoroacetic acid were used to separate eighteen amino acids with derivatization. No derivative was required.

The separation of the amino acids by thin layer chromatography (TLC) has been the subject of continuing investigation since the early review of Pataki (1). Biou et al., (2) recently reported the use of two dimensional separation of dansylated amino acids. This solved two problems: detection and better separation, but altogether it is time consuming. The present trend appears to be toward derivatization with dansyl chloride (3), or phenylthiohydantoin (4) for high performance liquid chromatography. Macek et al., (4) and Lepri et al., (6) used reversed phase layers for separation of derivatized amino acids.

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Sherma et al., (7) reported difficulties with the use of reversed phase layers for separation of the amino acids and reported long developing times with the mobile phases that were used.

MATERIALS

Whatman LKC18 and LKC2 layers (20 x 20 cm) were scored into 10 mm lanes with a Schoeffel scoring apparatus. The plates were used directly from the shipping container. All solvents were Omnisolve from EM Sciences (Gibbstown, N.J.). Water was deionized and filtered of organic matter. All amino acids were obtained from Sigma Chemical Co. (St. Louis, MO) and made as 1μ G/1 μL solutions in water. The detection reagent was ninhydrin (0.2% in acetone) sprayed on the chromatogram and heated in an oven at 110° for several minutes.

The mobile phases were acetonitrile with 15 to 25 percent of 0.4% trifluoroacetic acid in water made daily.

METHODS

The sample of amino acid was applied to the preadsorbent area of the plate usually in 5 μG aliquots. After linear development to 2 cm from the top of the plate, the plate was dried at ambient conditions, then dried in an oven at 170° for 2 minutes to remove solvent. Then the chromatogram was sprayed uniformly with the ninhydrin reagent. This was followed by heating at 110° in an oven for several minutes or until the reacting zones appeared.

RESULTS

Table 1 shows the R_f values for the listed amino acid in the noted mobile phases. The group in the upper part of the table would migrate with higher R_f in mobile phases of higher water content. The results obtained with the mobile phases not containing the trifluoroacetic acid were poor with tailing and in general poor resolution. The bands obtained with C_2 reversed phase layers were tighter than those shown by the C_{18} layers.

Table 1

R_f x 100 Values for Amino Acids on Reversed Phase Layers
Mobile phase (Acetonitrile-0.4% TFA)

Amino Acid	85:15		80:20		75:25	
	C_2	C_{18}	C_2	C_{18}	C_2	C_{18}
Valine	50	44	74	71		
Leucine	62	60	88	89		
Isoleucine	59	56	80	84		
Phenylalamine	68	67	90	97		
Tyrosine	56	50	78	82		
Alanine	22	12	41	35		
Proline	43	37	63	62		
Threonine	20	10	38	31		
Glutamine	13	6	30	25		
Glycine	11	4	23	15		
Glutamic Acid	8	0	25	18		
Aspartic Acid	4	0	17	12		
Serine			20	0	34	0
Cysterine			59	0	74	0
Cystine			6	0	10	0
Asparagine			23	0	37	0
Histidine			3	0	7	0
Arginine			4	0	7	0

The development time for both the C_2 and C_{18} chromatograms was 30 minutes, which is somewhat shorter than many separations previously reported. The use of C_{18} layers for separation of the group in the lower

Table II

Resolution of Pairs Difficult to Separate on C_2 layer

	85:15	80:20
Leucine	62	88
Isoleucine	59	80
Theronine	19	38
Serine	8	20
Glycine	11	23
Alanine	23	41

portion of the table did not result in any movement although there was movement in the C_2 layer.

Table II shows the resolution of the three pairs of amino acids that have been traditionally hard to separate (6). Leucine-isoleucine, threonine-serine and glycine-alanine are readily separated in the mobile phases listed. The methods described present a rapid means for separating the common amino acids.

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Analysis of Plasmalogens by In Situ Reaction on Thin Layer Chromatograms

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INTRODUCTION

An "in situ" hydrolysis method that attacks only the enol ether double bond of plasmalogen is described. Alkyl and unsaturated phospholipids are not affected. Treatment of the plasmalogen on thin layers of silica gel with solutions of trichloroacetic acid in dilute hydrochloric acid result in more complete hydrolysis of the vinyl-ether linkage. After development of the chromatogram differential quantitation using copper sulfate charring (1) and densitometry will give the amount of vinyl-ether lipid present in the sample. The results of the method with ethanolamine plasmalogen are presented.

Plasmalogens are the alk-1-enyl glycero-ether classes of phospholipids. They are widely distributed in nature in animal and anaerobic bacterial cells. Almost 20% of the phospholipids in the central nervous system of the human adult are plasmalogens. The myelin sheath phospholipids are 30% plasmalogen.

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The plasmalogens are in highest concentration in the nervous tissue and in striated muscle of the heart (32%). Adipose tissue contains a 1:4 ratio, plasmalogen to phospholipid. These proportions vary within each tissue class and from species to species. Ethanolamine plasmalogen varies from a low of 23% in the retina of the frog to 75% of the phospholipid in the sciatic nerve of the rat. It is found in high concentrations in seminal fluid (2). Methods for the identification and quantitation of the vinyl-ether linked lipids particularly the plasmalogens have been reported and widely used. These have been compiled by Kates (3) and reviewed by Horrocks and Sharma (4). These methods usually depend on the susceptibility of the enol ether bond to electrophyllic attack. Treatment of alk-1-enyl-glycerolipids with fumes from hydrochlorice acid produced aldehydes (5). Hack and Ferrans (6) hydrolyzed the vinyl part of the plasmalogens on thin layer chromatograms with mercuric chloride, followed by Schiff's reagent to detect aldehydes formed. However, we have found that the mercuric chloride method resulted in reaction with some unsaturated phospholipids to give several reaction products. Dinitrophenylhydrazine was used to derivatize the aldehyde released by acid hydrolysis. The hydrochloric acid and phenylhydrazine are made up in a single solution (7). Goldfine et al., have used 90% acetic acid to hydrolyze plasmenyl-ethanolamine (8). A conparision of the present method with some of the conventional methods indicated that evaluation of the methodology of phospholipid determination is needed. The present method was developed in order to avoid time consuming hydrolysis followed by gas chromatography or other analytical procedures and enable quantitation by densitometry.

MATERIALS AND METHOD

The phospholipids used in this study are synthetic except the plasmalogens which were obtained from natural sources as indicated. Phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine plasmalogens were obtained from Avanti Biochemicals (Birmingham, AL) as were

the synthetic materials. A sample of ethanolamine plasmalogen was obtained from Supelco (Bellefonte, PA) through the courtesy of Dr. Lloyd Whiting. A mixture of plasmalogens from beef heart was obtained from Dr. Howard Goldfine (University of Pennsylvania). Solutions were dissolved in chloroform or benzene at 0.5 ug/ul concentration.

Whatman LK-5 (Clifton, NJ) 20x20 cm silica gel layers, 250u thick with a double thick preadsorbant area were used. The plates were scored into 10 mm lanes with a Schoeffel scoring device, then washed overnight by continuous development in a 1:1 $CHCl_3$: MEOH solution. The plates were allowed to completely dry and stored in a Camag Trockengestell until used. Glass distilled solvents were EM Sciences chromatographic grade. The mobile phase was chloroform:ethanol:triethylamine:water, (30:34:30:8). Trichloroacetic acid (TCA), dinitrophenylhydrazine, cupric sulfate, mercuric chloride and Schiff's reagent were of reagent grade. The cupric sulfate solution was prepared as a 10% (w/v) solution in 8% v/v phosphoric acid. The TCA reagent was a solution of 2% TCA-8% HCl (1:1). The TCA solution must be made fresh.

Samples (1-2 ug) were applied in duplicate as a streak across the middle of the preadsorbent area. Alternate lanes were used on each of two plates. One lane of each pair or plate was treated with 25 ul of the TCA reagent over the still wet sample. This was allowed to react for 10 minutes at ambient temperature before drying with forced warm air from a hair dryer at low temperature setting. The chromatogram was then predeveloped twice to the preadsorbent/sorbent interface with $CHCl_3$-MEOH, (1:1). This procedure extracted the sample and deposited it as a narrow band at the juncture. Following development, the solvent was evaporated completely from the plate which was then developed in an unlined chromatagraphy tank with the mobile phase until the solvent front reached 2 cm from the top of the plate.

The chromatogram was allowed to dry at ambient temperature, then placed

on a warm surface to remove residual solvent and finally dried in an oven at 180°C for two minutes. When cool, the chromatograms were sprayed until saturated with the 10% cupric sulfate in 8% phosphoric acid (w/v). The plate was kept at ambient temperature for 5 minutes then placed in a 120°C oven for 5 minutes to remove a major portion of the water. Charring was performed at 170°C for 10 minutes in an oven.

The chromatograms were scanned on a Kontes Model 800 densitometer in a double beam mode using the 440 mm filter. The densitometer was interphased with a Hewlett Packard 3385A integrator recorder. Quantitation was performed by interpolation on calibration curves prepared from standards of each compound. By differentiation between the treated and untreated lanes the plasmalogen can be quantitated since the area of the plasmalogen zones decreased in the case of the treated lane.

Mercuric chloride solution 0.05 M (50 ul) was applied to the sample in the pre-adsorbent region of the plate by a method modified from Hack and Ferrans (6) followed by chromatographic development.

The method using simultaneous cleavage with acid and hydrazone formation was that of Shipski and Barclay (7). A solution of 0.4% dinitrophenylhydrazine in 2 N HCl was sprayed on the developed chromatogram. This was heated in an oven at 110 ° for a few minutes. Yellow zones denoted the formation of the hydrazone of the released aldehyde. The method for hydrolysis of plasmalogens using fumes from concentrated hydrocloric acid was that of Horrocks (8).

RESULTS AND DISCUSSION

The trichloroacetic acid -hydrochloric reagent appears to react specifically with the vinyl ether linkage since saturated and unsaturated

TABLE 1

Hydrolysis of Phospholipids

Phospholipid	TCA/HCl	$HgCl_2$	HCl Fumes
Dileoyl PC	NR	3 products	Destroyed
Dilinolenyl PC	NR	Destroyed	Destroyed
Dipalmitoyl PC	NR	NR	NR
Dimyristyl PC	NR	NR	NR
Dipalmitoyl PEA	NR	NR	NR
1-palmitoyl-2-oleoyl PS	NR	NR	Reaction
PC (brain)	several products	several products	several products
PEA (heart)	several products	several products	several products

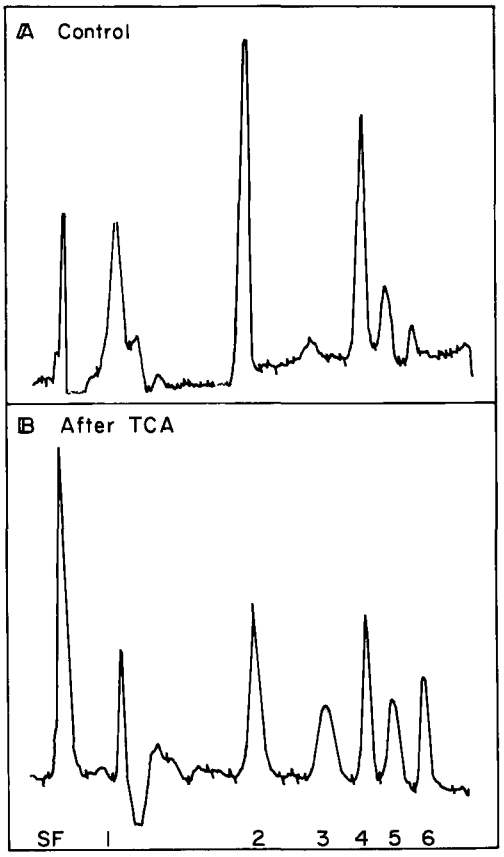
PEA = Phosphatidyl ethanolamine

PC = Phosphatidylcholine

PS = Phosphatidyl serine

NR = No Reaction

analogues were not affected. Table 1 shows the effect of hydrolyzing phospholipids obtained from the various sources. The plasmalogens that are available commerically are natural rather than synthesized preparations and therefore are a mixture of plasmalogen and a related phospholipid, e.g. phosphatidyl choline is 30% plasmalogen, 70% phosphatidyl choline. The results for the beef heart extract, which has a mixture of phosphatidyl choline plasmalogen and phosphatidyl ethanolamine plasmalogen are shown in Figure 1. The decrease in the area of peaks and the appearance of peaks at a



Legend Figure 1

Result of Treatment of Beef Heart "Plasmalogens" with the Trichloroacetic-Hydrochloric acid Reagent

SF. solvent front

1. Phosphatidylglycerol area
2. Phosphatidylethanolamine
3. lysophosphatidylethanolamine
4. phosphatidylcholine
5. sphingomyelin
6. lysophosphatidylcholine

new position indicates the presence of plasmalogens in this sample since the peaks in the untreated chromatogram are not present in the chromatogram obtained after TCA/HCl treatment. The new peaks had R_f corresponding to the reference lysophospholipid. The method has been applied to seminal fluid and the presence of plasmalogen readily indicated. Further evidence of the specificity of the TCA/HCl method is seen when it was compared with the $HgCl_2$ method of Owens (6) on synthetic phospholipid standards. The TCA/HCl or the $HgCl_2$ was applied directly to the samples. There was no reaction for any of the samples exposed to the TCA/HCl. However when the $HgCl_2$ method was used, three new products could be seen on the chromatogram of dioleoyl phosphatidyl choline and there was complete destruction of the dilinolenyl phosphatidyl choline. Since fatty acid side chains of phospholipids could be any one or a mixture of these the $HgCl_2$ would not be useful since it appeared to attack any unsaturated linkage.

The acid cleavage with hydrazone formation method of Skipski et al.(7) could not be evaluated by densitometry since the resulting background was too great and non-uniform. The method utilizing fumes from hydrocholoric (5) acids was not reproducible and low yields were obtained.

The presence of plasmalogens and unsaturated phospholipids in seminal fluid is generally known (9, 10, 11). The methods used for their assay are tedious and include two dimensional chromatography as well as hydrolysis and gas chromatography. Plasmalogen was readily shown by the present method.

Results of a comparison of methods are listed in Table 2. These methods were applied to ethanolamine plasmalogen furnished by Dr. Lloyd Whiting. This is a relatively pure preparation. The present method resulted in a 90.3% hydrolysis of the sample. TCA alone gave only 68% and the 5% HCl alone was only 72% effective. When the chromatogram was placed above fuming HCl for 5

TABLE 2

Table II Hydrolysis of Ethanolamine Plasmalogen*

Method	Number of Experiments	% Hydrolyzed**
Present Method	6	90***
2% TCA	5	68
5% HCl	6	72
HCl fumes	9	20

* Supplied by Dr. Lloyd Whiting, Supelco Inc.

** Represented as percentage of total sample hydrolyzed, the true amount of plasmalogen present not being known. The literature gives a value of 85% for this preparation.

*** Standard error of the mean 4.89

minutes, there was 20% hydrolysis or complete destruction of the sample and the results were not reproducible.

The hydrolysis of the vinyl ether to an aldehyde was verified with Schiff reagent using a modification of the procedure described by Hack and Ferrans (6). Following development and drying, the chromatogram was sprayed lightly with Schiff's reagent. After the color development the plate was sprayed with dilute sodium bisuefite solution. The adehyde reacting zone remained unchanged while the other zones and background were bleached.

The method described provides reproducible results with a minimum amount of manipulation for the in situ reaction, followed by separation and direct densitometric quantitation of plasmalogens.

The addition of this method to the previously reported methods (1) for the differentiation of the saturated and unsaturated fatty acid side chains permits in situ analysis of phospholipids. In this way, a direct densitometric method is provided for saturated, unsaturated and the vinyl ether analogues of the various classes of phospholipids.

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DETERMINATION OF CHLORPYRIFOS AND ITS METABOLITE 3,5,6-TRICHLORO-2-PYRIDINOL IN TAP WATER AND BANANAS BY QUANTITATIVE TLC ON PREADSORBENT SILICA GEL

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ABSTRACT

Chlorpyrifos insecticide and its metabolite TCP were determined in tap water and banana samples by TLC of extracts on preadsorbent silica gel layers, detection with silver nitrate reagent, and densitometric scanning. Cleanup steps were required for the fruit sample extracts. Recovery of chlorpyrifos from tap water at 5 ppb averaged 87.5% and from banana at 0.05 ppm was 84.6%. Recovery of TCP from water at 5 ppb averaged 84.0% and from banana at 0.05 ppm was 86.8%. The sensitivity and precision of the method were shown to be adequate for routine residue analysis.

INTRODUCTION

Chlorpyrifos (Dursban) [O,O-diethyl O-(3,5,6-trichloro-2-pyridyl)-phosphorothioate] is an organophosphate pesticide that is used for the control of many insect pests in rice, vegetable and fruit crops, turf and ornamental plants, and animals (1, 2). Chlorpyrifos has been determined in bananas, peaches, and cottonseed by gas chromatography with a phosphorus selective flame photometric detector and in meat, milk, and lima and snap beans by electron-capture GC (2). The metabolite of chlorpyrifos (3,5,6-trichloro-2-pyridinol; TCP) was determined in bananas and meat by electron-capture GC as the trimethylsilyl derivative (2). HPLC on a cyano-bonded column was used to

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determine chlorpyrifos in polymeric pellet formulations, and a reversed phase ODS column was employed for the analysis of natural water after trace enrichment on a C_{18} Sep Pak cartridge (3).

Thin layer chromatography coupled with densitometric scanning has recognized advantages of high sample throughput, simplicity, versatility, and selectivity for the quantitative analysis of a wide variety of samples (4), including pesticide residues (5-7). With preadsorbent plates, sample application can be carried out rapidly, and the spotting area automatically produces sharp, narrow bar- or streak-shaped zones of constant size, even though different sample volumes are used. Accurate, precise, and sensitive densitometry requires that initial zones of samples and standards have small, uniform dimensions (8).

No studies on the TLC of chlorpyrifos have been reported. Described below are quantitative TLC determinations of this insecticide and its metabolite in tap water at 5 ppb and in banana pulp at 0.05 ppm. Preadsorbent silica gel layers are used for resolution and silver nitrate chromogenic reagent for detection prior to reflectance scanning. Water extracts were analyzed without clean-up while banana extracts required purification by solvent partitioning and column chromatography.

EXPERIMENTAL

Standard chlorpyrifos and 3,5,6-trichloro-2-pyridinol (TCP) were obtained from the Dow Chemical Co. (Midland, MI). Stock standard solutions were prepared in acetone at a concentration of 0.500 g/100 ml and diluted 1.00:100 to give solutions of 50.0 ng/µl.

Analyses were carried out on channeled 20 x 20 cm Analtech Uniplates containing a 48 mm high preadsorbent spotting area along the bottom below the analytical layer of silica gel G. The layer was scored into 19 channels 0.9 cm in width. The plates were prewashed with methylene chloride-methanol (1:1) and dried before use. The principles and practice of preadsorbent TLC were described in a recent manual (9).

Volumes of standards between 1.00 and 20.0 µl (100-1000 ng) were applied by spotting the preadsorbent areas of the lanes using a 25 µl Drummond Dialamatic microdispenser. Application was confined to an area 3 mm below the layer interface and 5 mm above the bottom edge of the plate. The spots were allowed to air dry completely after application.

Plates were developed for a distance of 10 cm beyond the layer interface in a paper lined, saturated (10 min.) rectangular glass TLC chamber with hexane-chloroform (80:20) for chlorpyrifos or hexane-acetone-methanol-glacial acetic acid (60:30:10:0.2) for TCP. Chromatograms were air dried in a fume hood and then dipped for 20 seconds in silver nitrate detection reagent prepared from 8 ml of $AgNO_3$ stock solution (20 g/100 ml water) + 200 ml acetone + 20 ml H_2O + 12 ml concentrated ammonium hydroxide. The NH_4OH is added last with stirring to avoid cloudiness. The dipped plate was dried in a dark hood for 30 minutes and then irradiated for 20-30 minutes by a Hanovia 679A 176W germicidal lamp (UV source) placed 40 cm above the layer and set at 4 amps.

Zones were scanned with a Kontes Model 800 fiber optics scanner in the single beam transmission mode using the B head (8 mm light beam length) and the white phosphor (440 nm peak, 300 nm band width). Peaks were drawn and areas reported by a Hewlett-Packard Model 3390A recorder/integrator coupled to the scanner. All zones were scanned twice, and calibration curves were calculated from the average peak areas by a linear regression program run on a Commodore 64 minicomputer.

Actual analyses were demonstrated using fortified pesticide-free tap water and banana samples. The 50.0 µg/ml standard solutions of chlorpyrifos and TCP were diluted 1.00:10.0 to prepare spiking solutions of 5.00 µg/ml. One ml of chlorpyrifos spiking solution was added to 1.00 liter of tap water, which was shaken vigorously for 5 minutes. The resultant sample contained 5.00 ppb of the pesticide. The water sample was placed into a 2 liter separatory funnel and extracted with 50 ml of hexane. The (lower) water phase was drained into a second separatory funnel, and the hexane layer was dried by

passage through Whatman phase separation (PS) paper and collected in a 250 ml beaker. The extraction of the water was repeated twice and the hexane extracts combined in the beaker. The hexane was evaporated to 3-5 ml under a stream of nitrogen gas on a warm hot-plate, transferred quantitatively by rinsing with hexane to a 12 ml calibrated centrifuge tube, and evaporated to 0.500 ml. Thirty µl of extract (representing 300 ng if recovery is 100%) was spotted along with bracketing standards (100-1000 ng) and an extract from blank (unfortified) water on the same plate. After development, detection, and scanning, the amount of pesticide in the sample was calculated by interpolation of the average of the sample zone areas, minus the blank area, if any, from the calibration curve using a Commodore 64 computer program. The recovery was calculated by comparison to the theoretical 300 ng amount. The spiking and extraction of TCP were performed in the same way except that 2 ml of conc. HCl and 170 g of benzene-washed NaCl were added to the 1 liter water sample, and three 50 ml portions of benzene were used in place of hexane.

To demonstrate use of the TLC method with a sample requiring cleanup, banana pulp was spiked at 0.05 ppm and analyzed by the procedure described as chlorpyrifos Method I in Volume II of the FDA Pesticide Analytical Manual (2). The fortified sample (10.0 g) was extracted in a blender with acetone, the extract filtered, and the filtrate evaporated. The residue was partitioned into hexane and cleaned up by elution with hexane through a 15% water deactivated silica gel column. The eluate was further purified by elution with acetonitrile-benzene (1:1) through a charcoal-MgO-Celite (1:2:4 w/w) column, as described in the FDA PAM, Volume I (10). The eluate was evaporated to dryness, taken up in 50.0 µl of acetone, and a 30.0 µl aliquot was spotted with bracketing standards for TLC. The theoretical value was 300 ng for 100% recovery. Banana pulp spiked with TCP at 0.05 ppm (10.0 g) was analyzed by chlorpyrifos Method III in Volume II of the FDA PAM (2). The fortified sample was extracted with methanol and the extract chromatographed on an acidic alumina column (activated

at 130° C with conc. HCl-H_2O (40:60) as eluent. Eluted TCP was partitioned into benzene, followed by sodium bicarbonate partioning, acidification with HCl, and partitioning into benzene. The solution was concentrated to 50.0 µl and a 30.0 µl aliquot was analyzed by TLC. Again, the theoretical value was 300 ng.

RESULTS AND DISCUSSION

The R_F value of chlorpyrifos was 0.58 using hexane-chloroform (80:20) in a saturated chamber. TCP had an R_F of 0.24 in this mobile phase, which was below the optimum range of 0.3-0.7 recommended for accurate and precise densitometric quantification (9). Therefore, hexane-acetone-methanol-glacial acetic acid (60:30:10:0.2), which gave an R_F of 0.52 for TCP and 0.90 for chlorpyrifos, was used for determination of TCP. The zones were in the form of sharp, narrow streaks across the lanes with both mobile phases. The concentration of acetic acid was very critical in the TCP mobile phase and may require variation between 0.1 and 0.3 ml with different layers and temperature/humidity conditions to obtain an R_F value in the 0.3-0.7 range.

Silica gel G was chosen because polymer bound layers gave a dark background when sprayed with $AgNO_3$ detection reagent and exposed to UV light (11). The pesticide and metabolite were detected as dark brown zones on a white or yellow-white background. Zones should be scanned immediately after detection, if possible, but plates could be stored up to 2 hours in the absence of light without significant darkening of the background. The maximum contrast between the zones and the background was obtained after an irradiation period of 20-30 minutes. The sensitivity limits were approximately 100 ng for chlorpyrifos and 25-50 ng for TCP. Other reagents for detection of phosphate pesticides (TCQ + $MgCl_2$), phenols (diazotized *p*-nitroaniline, diazotized sulfanilic acid, potassium ferricyanide), and organochlorines (*o*-tolidine) either gave no reaction or less satisfactory sensitivity or contrast between the zones and background with the compounds.

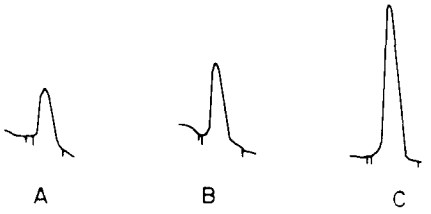

FIGURE 1. Typical densitometer scans of 200 ng (A) and 400 ng (C) standard chlorpyrifos zones and fortified tap water extract (B) chromatographed on the same layer. The sample represented 86.7% recovery (300 ng theoretical) when its area was interpolated from the calibration curve calculated from all standards chromatographed in parallel with the sample. An attenuation setting of X5 was used.

Calibration curves between 100 and 1000 ng for chlorpyrifos and 50 and 1000 ng for TCP typically had linearity (R) values of > 0.99. To correct for variations in slope and intercept values, bracketing standards always were chromatographed on the same plate with samples. Duplicate scan areas of a given zone usually agreed within 1%. Precision of the TLC was tested by developing, detecting, and scanning eight 500 ng samples of TCP applied to adjacent lanes on a single plate. The coefficient of variation (RSD) of the peak areas was 4.0%, which is acceptable reproducibility for densitometry in trace analysis.

Recoveries from tap water using the procedures described above were 77.8%, 91.3%, 94.0%, and 86.7% for four separate samples fortified with 5.00 ppb of chlorpyrifos. Figure 1 illustrates a typical scan of 200 and 400 ng standard zones and the sample zone from the latter analysis. Recoveries of TCP from tap water fortified at the same concentration were 87.2% and 80.8% for two samples. Figure 2 shows a scan of a 300 ng standard TCP zone and an extract zone with a theoretical value of 300 ng. A small zone usually appeared in the blank at the R_F value of TCP, the area of which was subtracted from the sample area before calculation of the percentage recovery.

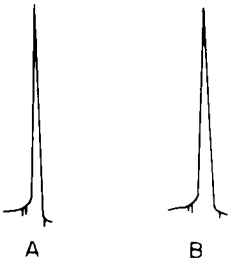

FIGURE 2. Densitometer scans of 300 ng TCP standard (A) and fortified tap water extract (B) zones representing 80.8% recovery (300 ng theoretical) at attenuation X7.

Since the combined selectivity of the TLC separation and the detection reagent allowed the tap water samples to be analyzed without cleanup, banana pulp was chosen to demonstrate application of the method to a more complex sample. The extraction and cleanup procedures described in detail in the FDA PAM (2) and outlined above for determination by FPD-GC were used with an additional carbon column cleanup step (11). No extraneous zones that interfered with scanning of the chlorpyrifos zone were present on the chromatogram of the final acetone solution. Recovery from a sample fortified at 0.05 ppm was 84.6%. The methanol extraction and alumina column and solvent partition cleanup procedures described for the electron capture GC determination ot TCP (2) provided a sample of adequate purity for TLC quantification. Recovery of a sample fortified at 0.05 ppm was 86.8%. Blank values were minimal in both cases. These accuracy (recovery) values obtained for water and banana are well within the range considered acceptable for residue analysis at the low ppb and ppm level, and the recoveries from banana samples compared favorably with those reported by the RPA (2) for determination by GC.

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DETERMINATION OF OCTADECYLAMINE IN WATER BY QUANTITATIVE HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

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ABSTRACT

Octadecylamine (ODA) was isolated from water by solvent extraction with ethylene dichloride or trapping on a micro Chromosorb column. The ODA in the extract or column eluent was chromatographed on a high performance silica gel layer, detected by spraying with ninhydrin, and quantified by reflectance densitometry. Recovery at 3 ppm averaged 81.3% using extraction and 94.0% with the column. Recovery at 0.3 ppm was 94.2% using the column procedure.

INTRODUCTION

Octadecylamine (ODA) is a corrosion-inhibiting boiler water additive used in the preparation of steam that will contact food. The Code of Federal Regulations for Food and Drugs (21:173.310) limits its level in steam to 3 ppm. Currently used analytical methods for the determination of ODA in steam condensate are usually based on extraction with an organic solvent followed by reaction of the amine with methyl orange or salicylaldehyde and colorimetry (1-3). These methods are not selective for ODA in the presence of other amines and bases. ODA has also been determined in boiler water by an indirect method involving formation

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of an amine-chromate complex, extraction into nitrobenzene, and atomic absorption spectrometry of the Cr in the extract (4).

The method reported in this paper is direct and selective since it includes resolution of ODA by thin layer chromatography. Isolation of ODA from water was achieved by solvent extraction and by a more simple micro-column trapping/elution procedure patterned after work by Schwartz (5). Adequate recoveries from spiked water were demonstrated at the tolerance level (3 ppm) for the extraction and column procedures and also at 0.3 ppm for the latter. Other permitted boiler water additives were shown not to interfere with the analysis.

EXPERIMENTAL

Materials and Solutions

Octadecylamine (stearylamine) was purchased from the Life Sciences Group of ICN Pharmaceuticals, Inc., Plainview, NY, and recrystallized from absolute ethanol. A stock spiking solution was prepared in absolute ethanol at a level of 3.00 mg ODA/ml (heating was required). Dilutions with ethanol were made to give a spiking solution containing 0.500 mg/ml and a TLC standard of 60.0 ng/µl.

Procedures

TLC analyses were carried out on 10 x 20 cm Whatman LHPK preadsorbent high performance silica gel TLC plates scored into 19 channels. The plates were prewashed by development with methanol-methylene chloride (1:1) before use.

Standards (5.00 to 20.0 µl; 300 to 1200 ng) and samples (5.00 or 20.0 µl) were applied by streaking broadly across the preadsorbent areas of the lanes using a 25 µl Drummond Dialamatic microdispenser. The spotting area was completely air-dried, and

plates were developed for a distance of 5.5 cm beyond the preadsorbent-silica gel interface in a paper-lined, vapor-saturated rectangular glass HPTLC chamber with the mobile phase n-butanol-glacial acetic acid-water (3:1:1). Development required about 1 hour.

Plates were dried with a stream of warm air from a hair dryer for 5 minutes, sprayed uniformly (but not heavily) with a 2 mg/ml ninhydrin solution in acetone, and heated for 5 minutes at 100° C in a ventilated chromatography oven.

ODA zones were measured with a Kontes Model 800 filter optics scanner in the single beam, reflectance mode using the white phosphor disk (440 nm peak, 300 nm band width) and the 8 mm source beam length to match the lane width of the divided plates. Peak areas were integrated with a Hewlett Packard Model 3390A calculating integrator/recorder coupled to the scanner. Calibration curves were calculated and samples on the same plate interpolated by means of a linear regression program run on a Commodore 64 minicomputer.

Deionized water was fortified at 3.00 ppm by adding 6.00 ml of the 0.500 mg/ml ODA solution to 994 ml of hot (50-60° C) deionized water and shaking vigorously for 5 minutes. One hundred ml (containing 0.300 mg of ODA) was placed in a 250 ml separatory funnel, and 2 ml of acetate buffer solution was added. The buffer was prepared by mixing 3.7 g of sodium acetate, 6.3 g of potassium chloride, and 12.5 ml of glacial acetic acid and diluting to 50 ml with water. ODA was extracted with three 10 ml portions of ethylene dichloride by shaking vigorously for 5 minutes and allowing the layers to separate for 10 minutes each time. The three extracts were combined in a 50 ml beaker and reduced to a volume of about 10 ml on a warm hot plate under a stream of nitrogen gas. The solution was quantitatively transferred to a calibrated 13 ml centrifuge tube and the volume further reduced to a value between 7 and 8 ml. Duplicate 20 µl aliquots were applied to a plate along with the series of bracketing standards. The theoretical amount of ODA in the sample zones for 100% recovery was 750 to 857 ng, depending upon the exact volume of extract in the tube. Percentage recovery was calculated by comparing the actual and theoretical ODA concentrations.

To prepare fortified samples for evaluation of column extraction, 100 µl of the 3.00 mg/ml ethanolic ODA stock solution (measured with a syringe) was dissolved in 100 ml and 1000 ml of hot distilled water. The resultant solutions were 3.00 ppm and 0.300 ppm, respectively. A "large volume" Pasteur pipet (Fisher No. 13-678-8) was plugged with a 0.25-0.3 g wad of glass wool and 2 ml of a water slurry of Chromosorb 102 styrene-divinylbenzene polymer (80-100 mesh) (20 g/100 ml water) was added followed by a layer of sand. The resin was purified by washing with methanol and acetone before suspending in water.

A funnel was attached to the top of the column by 2 cm of 5/16 in. id Tygon tubing, and the 100 ml or 1000 ml water sample passed through by gravity. The funnel was removed and nitrogen gas blown through from the top until the column was totally dry. ODA was eluted into a small vial with 2 ml of acetone, the acetone was evaporated under a stream of nitrogen and the residue dissolved in 3.00 ml of acetone by pipet. Duplicate 5.0 µl portions were applied to a plate with the bracketing standards. The theoretical amount of ODA in the sample zones for 100% recovery was 500 ng for both fortification levels.

RESULTS AND DISCUSSION

ODA appeared as a tight red band with an R_F value of 0.70 after development with the butanol-acetic acid-water mobile phase and detection with ninhydrin. An orange-yellow band formed across the entire width of the layer at R_F 0.50 (probably due to solvent demixing), but this did not interfere with scanning of the ODA zones. After exposure to the atmosphere for 24 hours, the ODA bands faded and the background acquired a yellow or purple tint. Many other mobile phases were evaluated, but all caused diffuse or non-linear (bell-shaped) zones. Fluorescamine gave equal visual sensitivity (ca. 250 ng) for detection of ODA, but peak areas were not as large for the fluorescent zones produced by this reagent.

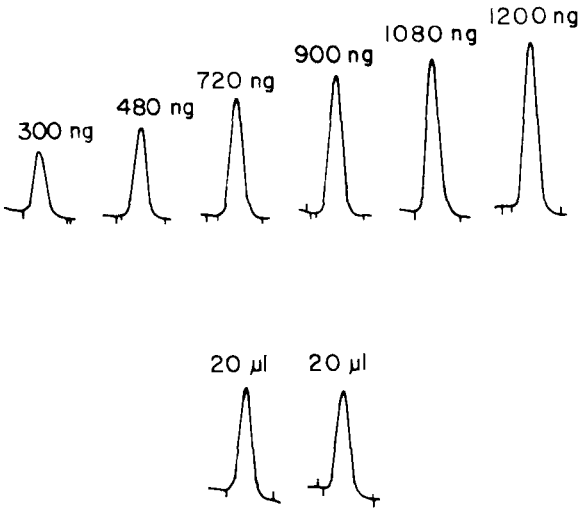

Figure 1. Scans of 300-1200 ng of octadecylamine and duplicate 20 µl extract samples representing a recovery of 71.5% using the Kontes Model 800 scanner and Hewlett Packard Model 3390A integrator with attenuation X7.

Figure 1 illustrates typical scans of a series of ODA standards and duplicate 20 µl solvent extract samples on the same plate. The calibration curve calculated from the standard peak areas had a linearity correlation coefficient of 0.996. Although slope and y-intercept (area) values were relatively consistent from plate to plate, samples and standards were always chromatographed in parallel to correct for variations in these parameters. Reflectance scanning produced larger peaks with more even baselines compared to scanning in the transmission mode.

Recoveries of ODA from eight separate samples fortified at 3.00 ppm and analyzed using the ethylene dichloride extraction procedure (1) ranged from 68.3 to 103% with a mean of 81.3%. The reproducibility (percent difference) of the duplicate samples in each analysis averaged 4.20% with a range of 0.66 to 10.8%. Addition of 20 g of sodium chloride to a water sample before extrac-

tion in order to increase the partition coefficient of ODA in favor of the organic solvent compared to water resulted in a recovery of 85%, an insignificant improvement compared to the results without salt.

Five other boiler water additives that are permitted to be used in combination with ODA under FDA regulations were tested for possible interference in the analysis in case they might be co-extracted with ODA. The compounds were spotted separately at the 1 μg level and chromatographed and detected as described above. Neither trisodium nitriloacetate nor diethylaminoethanol were detected with ninhydrin. Hydrazine produced a light purple zone with R_F 0.53, morpholine a purple-brown streaking zone with R_F 0.57, and cyclohexylamine a very faint purple-brown zone with R_F 0.38. None of these would interfere with scanning of ODA.

The main purpose of this research was to improve the published (1) FDA analytical procedure for ODA in water by substituting a more selective TLC determination for solution spectrometry. However, in the course of this work the Chromosorb column trapping/elution procedure described above was found to be simpler and give better recovery and sensitivity compared to solvent extraction. Two samples fortified at 3.00 ppm gave average recoveries of 92.8 and 95.3%, and a single sample fortified at 0.300 ppm gave a recovery of 94.2%. Columns eluted with blank (nonfortified) samples gave no TLC spots. A larger column with more capacity combined with a greater acetone elution volume might be required to analyze samples containing sorbable organic impurities in addition to ODA by the method. The pH of the water sample must be high enough to assure that the ODA is not in its salt form. This general approach, as devised by Schwartz (5), should be applicable to the recovery of a variety of unionized organic compounds from water samples.

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isolation approach and helping with preliminary experiments to demonstrate its applicability to the analysis of water for ODA. Dr. Charles Warner of the FDA, Washington, DC, provided advice and information on boiler water analysis.

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SEPARATION OF PROSTAGLANDINS E_1 AND E_2 AND OTHER MAJOR EICOSANOIDS BY UNIDIMENSIONAL ARGENTATION THIN LAYER CHROMATOGRAPHY

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ABSTRACT

Prostaglandins (PG) E_1 and E_2 are important regulators of biologic functions, and can express different biological effects. In thin layer chromatography (TLC) systems which separate these compounds, comigration of other major eicosanoids is a problem. This paper describes a TLC system using a mobile phase of chloroform/methanol/acetic acid/H_2O (90:7.5:5:0.8) that separates PGE_1 and PGE_2, as well as other major eicosanoids, including dihomogammalinolenic acid (DHLA), the immediate fatty acid precursor of PGE_1.

INTRODUCTION

PGE_1 and PGE_2 have similar biological actions, but do have different effects on some cell functions. For example, PGE_1 inhibits whereas PGE_2 does not influence or increases aggregation of human platelets (1-3). In addition, PGE_1 but not PGE_2 reduces production of the lymphokine macrophage migration inhibitory factor (4). Furthermore, PGE_1 and PGE_2 express quantitative differences in their ability to resorb bone (5). Several thin-layer chromatography (TLC) techniques have been used to separate PGE_1

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and PGE_2 (6-11). We have examined the mobile phases used and find that they do not separate the major cyclooxygenase products of arachidonic acid (AA) from PGE_1 and PGE_2. This is a potential source of error if the major eicosanoids are to be quantified by bioassay or examined by scintillation counting or autoradiography. We therefore developed a simple TLC technique that separates PGE_1, PGE_2 and thromboxane B_2 (TxB_2), PGD_2, 6-Keto-PGF_{1a} (stable metabolite of prostacycline), and the immediate precursor of the 1 series eicosanoids, dihomogammalinolenic acid (DHLA).

MATERIALS AND METHODS

All chemicals were of high performance liquid chromatographic grade (Fisher Chemicals, King of Prussia, PA). Glass chromatography tanks (28 cm x 27 cm x 8 cm) with glass lids (Scientific Manufacturing Industries, Emeryville, CA) were used. Whatman LK5 silica-coated TLC plates (Whatman Chemical, Clifton, NJ) were scored into 2 cm channels with a 1 cm unused channel on each side. Plates were washed overnight in a mobile phase of chloroform/methanol (90:5), and air dried. They were coated with a 10% solution of silver nitrate (Kodak Chemicals, Rochester, NY) in distilled water and ethyl alcohol (14:86, v/v) in a 10" x 14" metal pan, air dried, activated in an oven (Boekel, Philadelphia, PA) at 110^{O}F for 30 min, and then cooled at room air. Plates wrapped in aluminum foil and an air tight plastic bag could be kept for at least 1 week before use. All unlabeled eicosanoids were purchased from Sigma Chemical Co. (St. Louis, MO). Eicosanoids (5-10 ug) were spotted in the middle of the preadsorbant zone with a 20 ul (Rainen Instrument Co, Woburn, MA) or a 50 ul pipette (Hamilton Co., Reno, NV). After spotting, the eicosanoids were allowed to air dry, and were then run in the appropriate mobile phase until the solvent zone had reached 1 cm from the top of the plate. The plates were air dried, and then

dried at 110°F for 10 minutes. They were cooled at room air, and then sprayed with 3% cupric acetate (3 gm) in phosphoric acid/water (8:92, v/v) and air dried. Charring at 180°F for 10 minutes was often helpful in improving resolution of the bands.

RESULTS

Various solvent systems that have been used to separate PGE_1 and PGE_2 are listed in Table 1.

Although the TLC systems referenced in Table 1 separate PGE_1 from PGE_2, other major eicosaniods comigrate thus diminishing the utility of these systems (Table 2). However, separation of PGE_1,

TABLE 1

Solvent Systems Used to Separate Eicosanoids

TLC System	Ref.	Solvent Composition by Volume
SI	Present paper	chloroform/methanol/acetic acid/H_2O (90:7.5:5:0.8)
SII	12	ethyl acetate/2,2,4-trimethylpentane/acetic acid/methanol/H_2O (110:10:30:35:100)
SIII	6	ethyl acetate/2,2,4-trimethylpentane/acetic acid/H_2O (100:30:10:100)
SIV	6	ethyl acetate/2,2,4-trimethylpentane/acetic acid/H_2O (90:50:20:100)
SV	13	chloroform/methanol/acetic acid (90:5:5)
SVI	14	chloroform/methanol/acetic acid/H_2O (95:5:1:0.2)
SVII	9	0.0025 M phosphoric acid/acetonitrile with 0.2 M NaCl (52:48)
SVIII	8	organic (upper phase) of ethyl acetate/2,2,4-trimethylpentane/acetic acid/H_2O (110:50:20:100)
SIX	8	chloroform/methanol/acetic acid (80:10:10)
SX	15	ethyl acetate/isooctane/ethanol/acetic acid/H_2O (35:10:3:0.1:0.1)
SXI	11	ethyl acetate/methanol/acetic acid (100:10:1)
SXII	11	chloroform/methanol/acetic acid/H_2O (90:9:1:0.65)
SXIII	11	benzene/1,4-dioxane/acetic acid (20:20:1)
SXIV	11	ethyl acetate/acetone/acetic acid (90:10:1)

TABLE 2

Mobile Phases Used to Separate PGE_1 and PGE_2 But With Known Comigration of Major Eicosanoids[1]

TLC System	Ref.	Comigrating Eicosanoids
SII	12	TxB_2 and PGD_2
SIII	6	multiple tailing
SIV	6,11	PGE_1 and PGE_2 blur
SV	13	TxB_2 and PGD_2
SVI	14	6-Keto-PGF_{1a} and PGE_2
SVII	9	PGE_1 and PGD_2
SVIII	8	TxB_2 and PGD_2
SIX	8	TxB_2 and 6-Keto-PGF_{1a}
SX	15	6-Keto-PGF_{1a} tails
SXI	11	6-Keto-PGF_{1a} and PGF_{2a}, PGE_2 and PGD_2
SXII	11	PGE_2 and 6-Keto-PGF_{1a}
SXIII	11	6-Keto-PGF_{1a} and PGF_{2a}
SXIV	11	AA tailing

1 Comigration established as noted in References or by argentation technique as described in Materials and Methods.

PGE_2 and TxB_2, PGD_2, 6-Keto-F_{1a}, PGF_{2a}, AA and DHLA was achieved with chloroform/ methanol/acetic acid/H_2O (90:7.5:5:0.8) (Solvent I) as shown in Fig. 1. The mobilities of the compounds, expressed as R_f values, are shown in Table 3 with the preabsorbant-silica gel boundary as the origin. Development time is approximately 60 minutes. The method is reproducible (R_f standard error of the mean .5%) and reliable.

DISCUSSION

The method presented here allows separation by TLC of the monoenoic and dienoic PGE compounds and their immediate fatty acid precursors DHLA and AA respectively. Thus, metabolism of both PGE precursors may be studied simultaneously. In addition, the same system accommodates separation of the major cyclooxygenase products of AA: TxB_2, 6-Keto-F_{1a}, PGD_2, PGF_{2a}. Other investigators have separated PGE_1 from PGE_2. However, as shown here, comigration of other major eicosanoids with PGE_1 and PGE_2 occurs with several of the solvent systems used. Use of the Solvent I, a mobile phase of chloroform, methanol, acetic acid and water allows separation of PGE_1 from PGE_2 as well as separation of other major eicosanoids. This system is not only useful in

TABLE 3

Mobility of Separated Eicosanoids

Compound	Mobility (expressed as R_f)
PGF_{2a}	0.12
TxB_2	0.28
PGD_2	0.32
PGE_2	0.38
6-Keto-PGF_{1a}	0.51
AA	0.58
PGE_1	0.62
DHLA	0.83

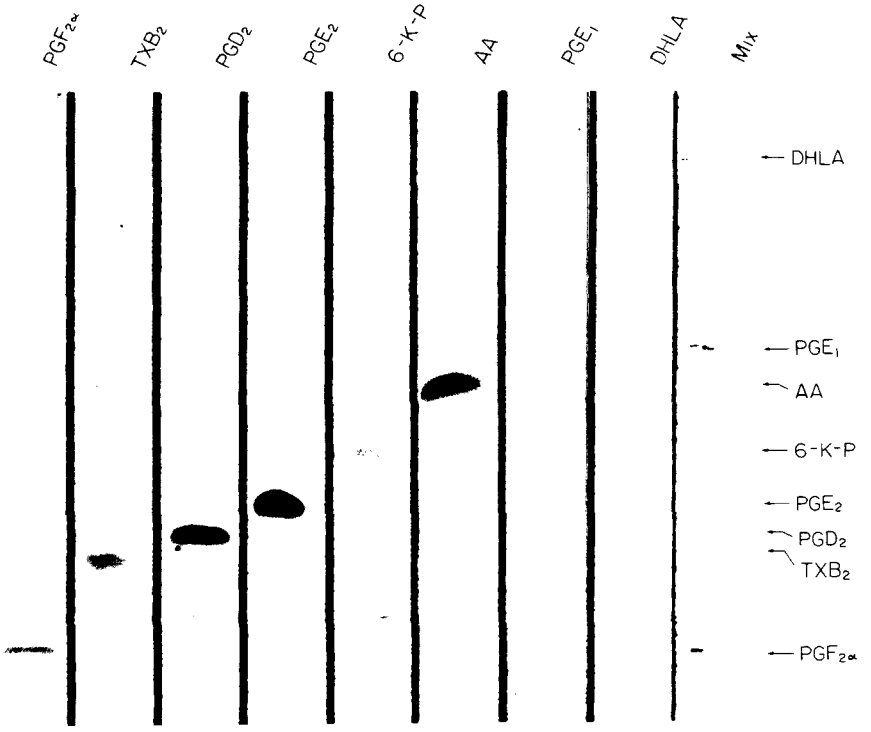

FIGURE 1

Chromatogram showing eicosanoid separation with a mobile phase of chloroform/methanol/acetic acid/H_2O (90:7.5:5.0:0.8). 6-Keto-PGF_{1a} is abbreviated as 6-K-P.

examining AA and its metabolites, but may also be used in experiments where DHLA and PGE_1 are to be examined.

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NEUTRAL LIPID CLASS FRACTIONATION AND FURTHER SEPARATION OF SIMPLE NEUTRAL GLYCOLIPIDS BY OPTLC

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ABSTRACT

In lipid preparation from biological sources extraction and chromatographic methods are widely used. The use of the OPTLC method is introduced in the present paper. The method is suitable for the class separation of the neutral fraction of a total lipid extract with a single isocratic run. With a step or an exponential gradient the simple neutral glycolipids can be separated. Either elution can be performed on a 10 x 20 cm plate on 12 parallel samples. The chromatograms were evaluated by densitometric scanning after staining with orcinol-H_2SO_4 reagent.

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INTRODUCTION

The lipid separation procedures of a given biological sample usually start with an indispensible extraction step to prepare crude Total Lipid Extract (TLE) largely free of non--lipid contaminants (1). In the work up of TLE various chromatographic steps can be used, e.g. classical and High Performance column and layer chromatographic methods. A new layer chromatographic technique, Overpressured Thin Layer Chromatography (OPTLC) and an instrument have been developed in Hungary (2). Detailed studies of the method were published (3,4). Application areas of the technique have included a wide range of biologically important substances (5,6,7,8).

Of modern layer chromatographic techniques only HPTLC has been widely applied in lipid separations. In these applications usually a given, purified lipid class was separated on a HPTLC plate (9,10). An exception to this rule was a communication (11) which separated gangliosides of total lipid extracts. Unfortunately no sequel to this appeared, which might be attributed to that;

- only one of the most polar lipids, gangliosides, were separated, sulphatides are not mentioned in the article,
- the method is extremely time consuming (3 developments of 10 hours in all, not counting the drying periods),
- the separation on a 20 x 20 cm TLC plate does not give the possibility of the determination of other, non-separated lipid classes.

In lipid analysis the OPTLC technique has not been applied up to now. This is a report of our experiments on the separation of the Neutral Lipid Fraction (NLF) of TLE by OPTLC.

The method proved suitable for the class separation of the neutral lipids in a simple, fast isocratic run with chloroform (C). The simple neutral glycolipids can be further separated with a gradient run using either a step from pure chloroform to chloroform, methanol (M), water (W) mixture (C : M : W = 65 : 25: 4) or a delayed exponential gradient of M in C (final concentration approximately C : M = 1 : 1).

EXPERIMENTAL

Reagents and Materials

All the solvents used were HPLC grade purchased from E. Merck, Darmstadt, F.R.G., the water used was prepared according to Gurkins method (12), from water double distilled from glass and sterilized. DEAE Sephadex A-25 was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Staining reagents were prepared from analytical grade chemicals. The HPTLC plates used were purchased from E. Merck, Darmstadt. TLE was prepared from human lymphocytes.

Apparatus

Centrifuge: Janetzki K70 MLW, Leipzig, G.D.R.

Incubator: LP507/1 Labor MIM, Esztergom, Hungary.

Sample applicator: CAMAG Nanomat, CAMAG, Muttenz, Switzerland.

OPTLC system: Chrompres 10 Overpressured Layer Chromatograph, Labor MIM, Esztergom, Hungary. Instead of its eluent pump two HPLC pumps: Waters M-45, Waters Associates Inc., Milford, Mass., USA and Beckman 112 SDM, Beckman Instruments Inc., Berkeley, Calif., USA and a home made gradient mixing chamber were used.

Spectrophotometric densitometer: Opton KM3, Opton Feintechnik GmbH., Oberkochen, F.R.G.

Data system: HP 3354 Lab. Automat. System, Hewlett Packard, Avondale, Calif., USA.

Methods

Sample preparation

The preparation of TLE and the isolation of the NLF was described elsewhere (13). The crude NLF was freed from alkali labile components by incubation. The dried NLF was dissolved in 15 ml 0.1M methanolic KOH and incubated at 37°C for 2 hours. The sample was then neutralized with cc.HCl, evaporated in vacuo. The lipid compounds were taken up in 2 ml of C : M = 85 : 15 mixture. Average samples were prepared from 10^{10} lymphocyte cells.

Plate pretreatment

All plates used were precleaned by running them in methanol and dried. The OPTLC technique requires that the edges of the plates should be sealed, preferably after scraping off a strip (2-4 mm) of sorbent, by impregnating with IMPRES 1. For single dimensional development 3 sides of the plate were sealed and solvent dispersion troughs were scraped into the plates 14, 17 and 20 mm from the lower edge of the plates. Three parallel lines were scraped (and impregnated) into the plates perpendicular to the development direction (from the sample application height to the top) to insure straight fronts. For two dimensional development all four sides were impregnated and teflon solvent dispersion troughs were used.

OPTLC system

The block diagram of the instrument is shown in Figure 1. This

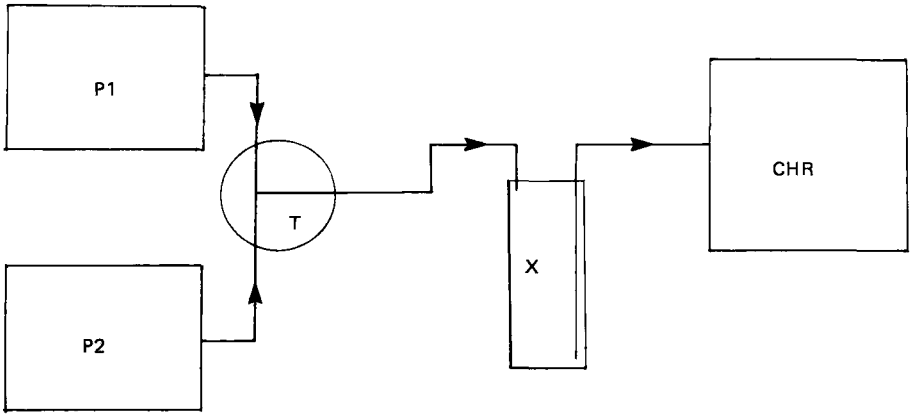


Figure 1. Block diagram of the OPTLC instrument used

P1 = pump 1

P2 = pump 2

T = T-junction

X = mixing chamber

CHR = chromatography chamber

system was used because it gives reproducible liquid flows and gradient profiles at low flowrates necessary to achieve approximately 1.00 cm/min velocity on the plate, which is optimal for elution (6,14).

For isocratic elution one pump (P1) was directly connected to the inlet valve. For the step gradient the two pumps (P1,P2) were connected with a T junction (T) before the inlet valve to make possible quick eluent change over. The exponential gradient was formed with a home made mixing chamber (X). This was a 5 ml closed container equipped with a magnetic stirrer with an inlet at the top and an outlet at its bottom. 2.5 ml of starting solvent (C) was placed in the container into which the strong

solvent was continuously pumped with vigorous stirring. To delay the start of the exponential gradient pumping was switched from C to M (P1 to P2).

OPTLC chromatography and densitometry

Sample application was 2-500 nl/spot, depending on the separation aims, with Pt-Ir capillaries.

For single dimensional development usually 12 samples were applied to a plate at 30 mm from the lower edge and at least 20 mm from the perpendicular edges of the plate.

Development conditions were;

run	isocratic	step grad.	exp. grad.
eluent	C	C/C:M:W	C/C,M
eluent flow (ml/min)	0.15	0.15	0.20
start pressure (bar)	30	30	30
pillow pressure (bar)	12	12	12
development distance (cm)	17	17	17
gradient start (at cm)	-	13	7

For two dimensional development two spots were applied to a plate at 30-30 mm from the corners. The first development of both spots was 7 cm with chloroform. The plate was dried, rotated 90° and one of the spots rechromatographed in the second dimension 4 cm with C : M : W = 65 : 25 : 4 eluent.

The staining was achieved by immersion of the plate into orcinol-H_2SO_4 reagent and heating at 100°C for 2-3 minutes.

Densitometric conditions in remission mode were:

wavelength: 560 nm

slit: 3.5 x 0.1 mm

scanning speed: 50 mm/min

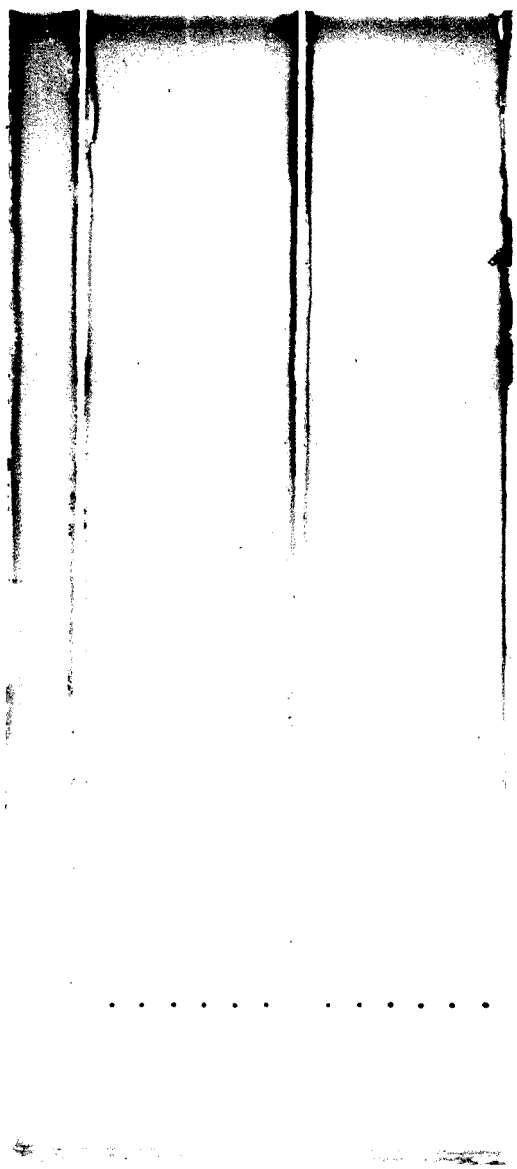
A/D frequency: 2 Hz

RESULTS AND DISCUSSION

Summarizing the experimental results the OPTLC technique is suitable for the separation of lipid samples. In our method the NLF of TLE can be applied and separated in a single isocratic run into lipid classes with different polarities. For qualitative analysis the migration distances (md) and Rf values can be used, which are highly reproducible, as can be seen in Figure 2. and in Table 1.

Figure 2. shows an OPTLC plate with 12 parallel runs (a) and the densitogram of one lane (b). The photograph clearly demonstrates the usefulness of the isocratic OPTLC run for the fractionation of the neutral lipid classes. These results are supported by the numerical data, migration distances (md), Rf values, their averages and the standard deviations (SD), summarized in Table 1. In the class separation of neutral lipids (NL) we aimed at leaving the NGL class at the start, while eluting all the other less polar classes. As can be seen (md of NGL) very small or no movement was achieved and caused a relatively large deviation (SD of NGL).

As sample quantity is frequently severely limited, as in our case of analysing blood cell lipids of leukemic patients, it was necessary to extract as much information as possible from one sample. For this purpose the step gradient elution was evolved making possible the separation of the simple neutral glycolipids after the above mentioned class fractionation on the same plate. Although the use of a second, water containing, eluent gives rise to severely curved β and δ fronts, they do not make impossible the evaluation of the spots in or near them. At the same time the intraclass sepa-



(a)

Figure 2. OPTLC separation of the neutral lipid classes. 2.a plate, 2.b densitogram of one lane, NGL = neutral glycolipids, Ch = cholesterol, Glyc = glycerides, ChE = cholesterol esters. Plate Merck HPTLC Si 60, 17 cm elution with chloroform.

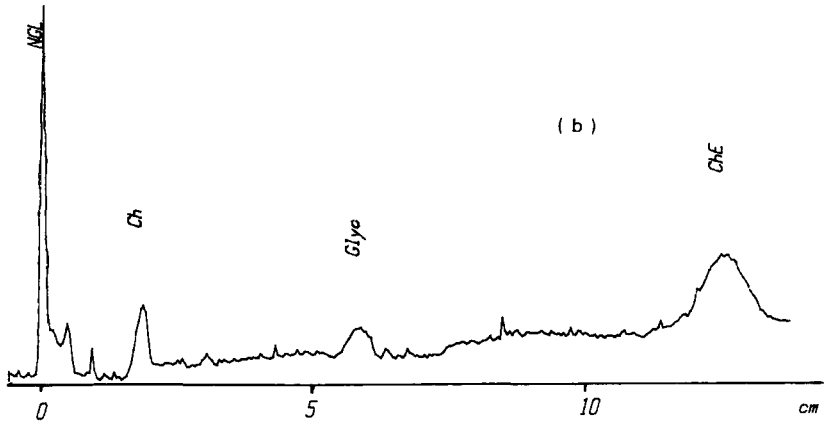
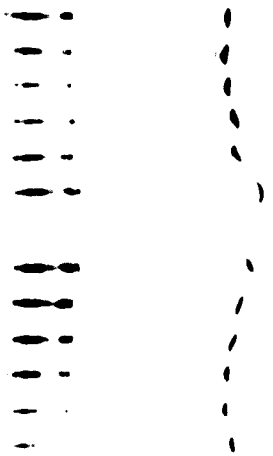


Figure 2 (continued)

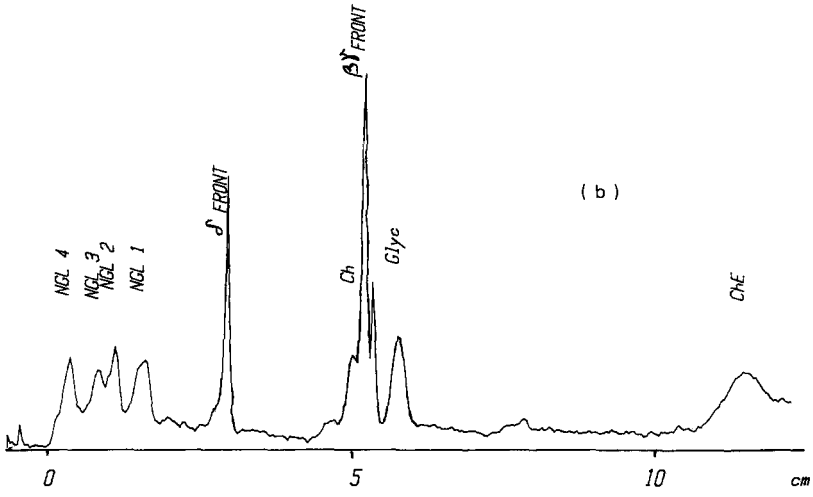
Table 1. Migration distances (md in cm) and Rf values of neutral lipid classes separated by OPTLC.
NGL= neutral glycolipids, Ch= cholesterol, Glyc= glycerides, ChE= cholesterol esters, SD= standard deviation

Class	NGL		Ch		Glyc		ChE	
No.	md	Rf	md	Rf	md	Rf	md	Rf
1.	0.05	0.003	0.37	0.109	1.12	0.329	2.31	0.688
2	0.00	0.000	0.35	0.109	1.09	0.329	2.33	0.688
3	0.00	0.000	0.34	0.100	1.09	0.321	2.31	0.679
4	0.05	0.003	0.35	0.103	1.10	0.318	2.31	0.679
5	0.05	0.003	0.38	0.115	1.13	0.332	2.31	0.682
6	0.05	0.003	0.37	0.112	1.15	0.338	2.34	0.688
7	0.05	0.003	0.36	0.109	1.12	0.329	2.35	0.691
8	0.00	0.000	0.36	0.106	1.13	0.332	2.36	0.694
9	0.05	0.003	0.34	0.100	1.15	0.338	2.50	0.735
10	0.05	0.003	0.37	0.109	1.14	0.335	2.53	0.744
11	0.05	0.003	0.38	0.112	1.17	0.344	2.48	0.729
12	0.05	0.003	0.38	0.112	1.20	0.353	2.46	0.723
Average	0.04	0.002	0.36	0.110	1.13	0.333	2.38	0.700
SD	0.023	0.0014	0.015	0.0048	0.033	0.0095	0.084	0.024



(a)

Figure 3. OPTLC separation of simple neutral glycolipids. 3.a plate, 3.b densitogram of the neutral glycolipids. Plate Merck HPTLC Si 60, 13 cm elution with chloroform, then 4 cm with chloroform:methanol:water=65:25:4. NGL 4 = tetrahexosyl ceramide, NGL 3 = trihexosyl ceramide, NGL 2 = dihexosyl ceramide, NGL 1 = monohexosyl ceramide, Ch = cholesterol, Glyc = glycerides, ChE = cholesterol esters.



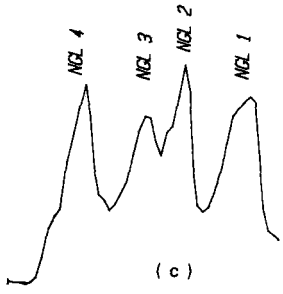


Figure 3 (continued)

Table 2. Migration distances (md in cm) and Rf values of simple neutral glycolipids separated by OPTLC
NGL 4= tetrahexosyl ceramide, NGL 3= trihexosyl ceramide, NGL 2= dihexosyl ceramide, NGL 1= monohexosyl ceramide, SD= standard deviation

NGL / No.	NGL 4		NGL 3		NGL 2		NGL 1	
	md	Rf	md	Rf	md	Rf	md	Rf
1	0.25	0.088	0.80	0.281	1.05	0.368	1.50	0.526
2	0.25	0.088	0.70	0.246	1.00	0.351	1.50	0.526
3	0.25	0.078	0.70	0.219	1.00	0.313	1,50	0.469
4	0.25	0.086	0.70	0.241	1.00	0.345	1.50	0.517
5	0.30	0.102	0.70	0.237	1.00	0.339	1.55	0.525
6	0.30	0.100	0.75	0.250	1.05	0.350	1.60	0.523
7	0.25	0.088	0.75	0.263	1.00	0.351	1.50	0.526
8	0.20	0.071	0.65	0.232	1.00	0.357	1.45	0.518
9	0.25	0.089	0.70	0.250	1.05	0.375	1.50	0.536
10	0.30	0.107	0.70	0.250	1.05	0.375	1.55	0.554
11	0.30	0.107	0.75	0.268	1.10	0.393	1.55	0.554
12	0.30	0.107	0.80	0.286	1.15	0.411	1.55	0.554
Average	0.267	0.0926	0.725	0.2520	1.038	0.3607	1.521	0.528
SD	0.033	0.0119	0.045	0.0197	0.048	0.0158	0.040	0.023

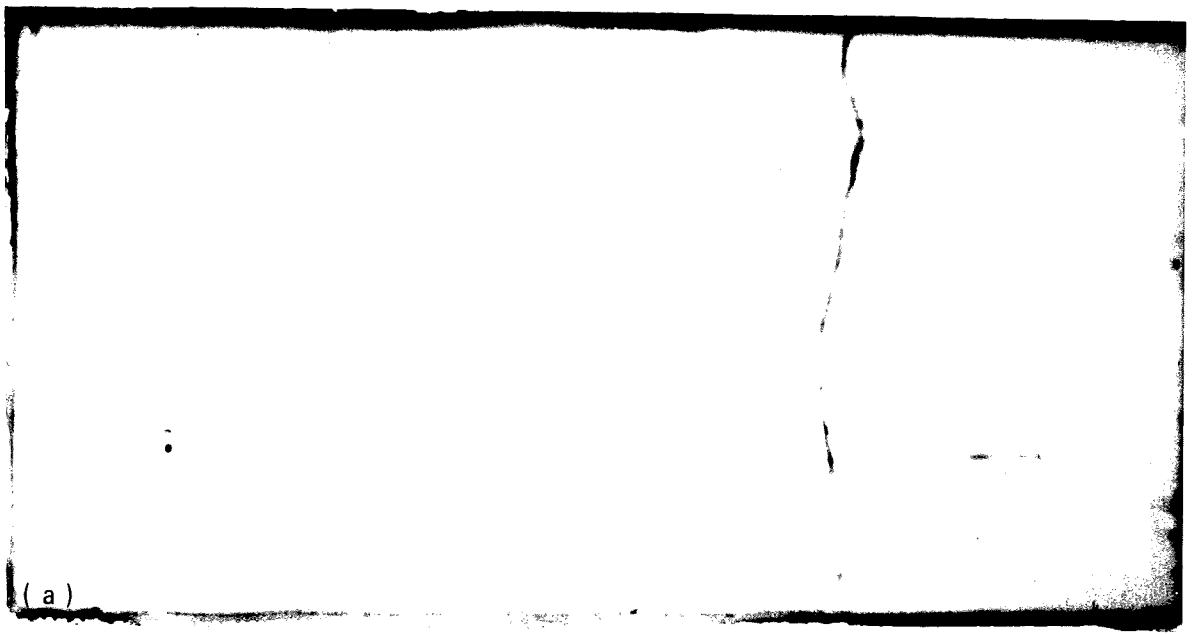


Figure 4. Two dimensional OPTLC separation of neutral lipid classes and simple glycolipids on one plate. 4.a plate 4.b the densitogram of the class separation, 4.c the densitogram of the glycolipid separation. Plate Merck HPTLC Si 60, elution: in the first dimension 7 cm with chloroform, the second dimension 4 cm with chloroform:methanol:water=65:25:4 for one spot.

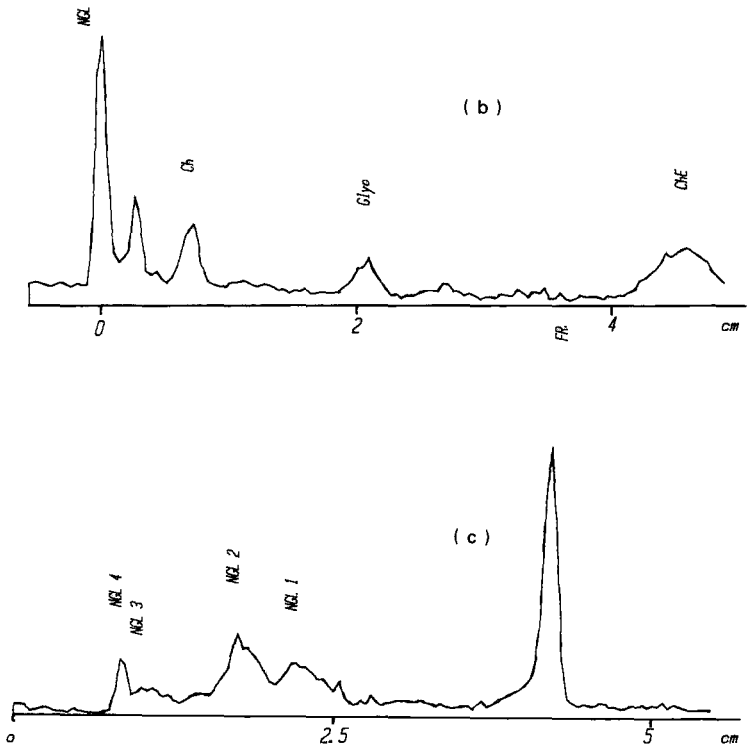


Figure 4 (continued)

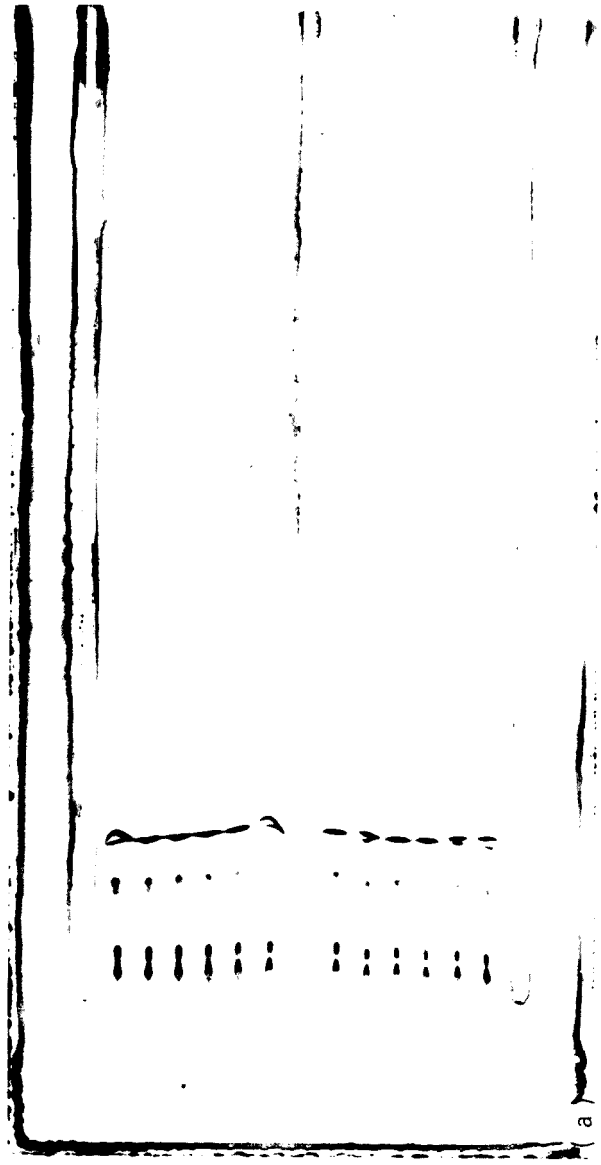


Figure 5. Gradient OPTLC separation of neutral lipids. Plate Merck HPTLC Si 60, elution 7 cm chloroform, then 7 cm exponential gradient of methanol in chloroform to 50 % methanol.

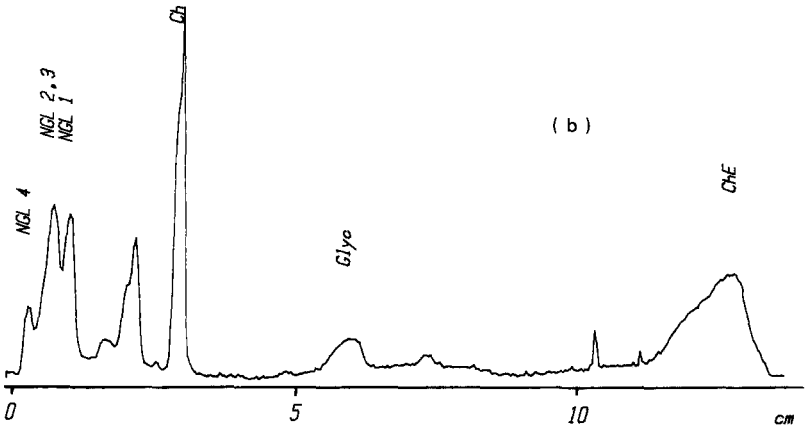


Figure 5 (continued)

ration of simple neutral glycolipids is very good and reproducible as can be seen in Figure 3. and Table 2.

The plate (Fig.3.a.) shows the complete class and NGL intraclass separation. Figure 3.b. is the densitogram of one lane of this plate, while insert (Fig.3.c.) shows the densitogram of the NGL separation. Table 2. gives the corresponding data for the intraclass separation.

A more frequently used way to perform two separations of a single sample is two dimensional development. Figure 4. shows how it can be done using OPTLC. Development for both intra and interclass separations can only be achieved if two spots are applied, and only one sample can be analysed per plate. As this method does not give better resolution but has significant drawbacks, so it was discarded.

The exponential gradient with methanol, which gave good results on normal silica plates in our preliminary experiments (15) was also examined (Fig.5.). This gradient gives good separation in the NGL class but coelutes the classes with

medium polarity.

In conclusion the adaptation of the new lipid separation with the step gradient OPTLC method, affords a rapid, labour and cost effective way to separate the neutral lipid classes from the neutral lipid fraction of the total lipid extract with the possibility of simultaneously separating the simple neutral glycolipids too of very small unique samples.

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CLASS FRACTIONATION OF ACIDIC GLYCOLIPIDS AND FURTHER SEPARATION OF GANGLIOSIDES BY OPTLC

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ABSTRACT

The use of the OPTLC method has been extended to the separation of the acidic fraction of the total lipid extract derived from a given blood element. This newly developed method is suitable for the interclass separation of sulphatides and gangliosides and further intraclass separation of gangliosides on the same TLC plate with step gradient development. The elutions can be performed on 10 x 10 cm (or larger) HPTLC plates with 13 parallels on each one. The chromatograms were stained either with orcinol-H_2SO_4 to show class separation (in this case only a single isocratic elution was performed) or with resorcinol-HCl reagent to visualize the ganglioside intraclass

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separation. The chromatograms were evaluated by spectrodensitometric scanning and the reproducibility of the separation was determined.

INTRODUCTION

Separations of lipids generally and especially of glycosphingolipids are usually carried out by various chromatographic methods. For preparative work classical column and layer chromatographic techniques are used. For analytical purposes HPLC (1) and mainly HPTLC (2) procedures are employed. HPTLC plates have been used for the analysis of various, prepurified ganglioside mixtures using one (3,4,5) and two (6,7) dimensional development with various solvent mixtures.

A new layer chromatographic technique, Overpressured Thin Layer Chromatography has been developed in Hungary (8). This technique was introduced into lipid analysis in our previous paper (9), where the basic theoretical and application aspects of OPTLC were cited and discussed.

The present paper describes the application of OPTLC to the separation of acidic glycosphingolipids.

The method was developed on mixtures of pure ganglioside and sulphatide standards and then applied to the separation of the purified acidic fraction of the total lipid extract (TLE) derived from blood elements (e.g. plasma, lymphocytes, granulocytes of normal and leukemic individuals).

Class separation of acidic glycolipids namely sulphatides and gangliosides can be performed by a single isocratic run with chloroform - methanol (C : M = 70 : 30) mixture. The intraclass separation of gangliosides, in the presence of sulphatides, is accomplished by a step gradient run. The first

segment of the gradient profile is the class separation (see above) followed by a consecutive isocratic development with chloroform - methanol - 0.25% KCl in water (C : M : KCl/W = 55 : 36 : 9) mixture.

EXPERIMENTAL

Reagents and materials

All the solvents used were HPLC grade purchased from E. Merck, Darmstadt, F.R.G., the water used was prepared according to (10), from water double distilled from glass and sterilized. DEAE Sephadex A-25 was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Staining reagents were prepared from analytical grade chemicals, the HPTLC plates used were purchased from E. Merck, Darmstadt, F.R.G. TLE was prepared from human blood elements. Sulphatides (bovine) and GT1b were purchased from Supelco Inc., Supelco Park, Bellefonte, PA, USA. Other ganglioside standards (GM3, GM2, GM1, GD1a, GD1b) were the kind gift of Professor Shimon Gatt, Hadassah Medical School, Dept. of Biochem., Jerusalem, Israel.

Apparatus

Centrifuge: Janetzki K70 MLW, Leipzig, G.D.R.
Incubator: LP 507/1 Labor MIM, Esztergom, Hungary.
Sample applicators: special 1 microliter volume syringe, MTA KKKI, Budapest, Hungary and CAMAG Nanoapplicator, CAMAG, Muttenz, Switzerland.
OPTLC system: Chrompres 10 Overpressured Layer Chromatograph, Labor MIM, Esztergom, Hungary. Instead of its eluent pump a

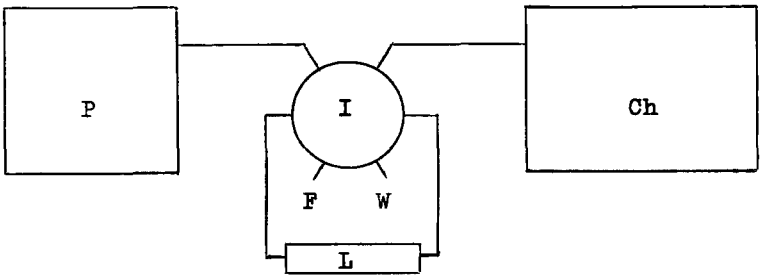


Figure 1. Block diagram of the OPTLC system used.

Ch = OPTLC Chamber

P = Pump

I = Injection valve

L = Loop

F = Filling port

W = Waste

HPLC pump; Beckman 112 SDM, Beckman Instruments Inc., Berkeley Calif., USA, an injector; Altex 210 with a 3ml loop, Beckman Instruments Inc., Berkeley, Calif., USA were used. The block diagram of the system can be seen on Figure 1.

Spectrophotometric densitometer: Opton KM3, Opton Feintechnik GmbH., Oberkochen, F.R.G.

Data system: HP 3354 Lab. Automat. System, Hewlett Packard, Avondale, Calif., USA.

Methods

Sample preparation

Standard glycolipid sample; a mixture was prepared from bovine sulphatides and pure individual gangliosides (GM3, GM2, GM1, GD1a, GD1b, GT1b).

Glycolipid samples from blood elements; the preparation of which has been described elsewhere (11).

Plate pretreatment

All plates used were precleaned by running them in methanol and dried. The OPTLC technique requires that the edges of the plates should be sealed, preferably after scraping off a strip (2-4 mm) of sorbent, by impregnating with IMPRES 1. 3 sides of the plates were sealed and solvent dispersion troughs were scraped into the sorbent layer 14, 17 and 20 mm from the lower edge.

OPTLC and densitometry

Sample application was 200 nl/spot in the case of the standard mixture, with the CAMAG nanoapplicator. Usually 13 samples were applied to a plate at 30 mm from the lower edge and at least 20 mm from the perpendicular edges of the plate. An indicator solution was applied onto the first and last sample spots to help us control the run, also to serve as a reference in calculating migration quotients (relative retention; see later). In the case of glycolipid samples of blood elements 1 microliter was spotted onto the plate. Appropriate sample volumes, for 1 microliter application, were adjusted on the basis of preliminary OPTLC runs.

Development (step gradient) conditions were;

segments	No1	No2
eluents	C:M=7:3	C:M:KCl/W=55:36:9
eluent flow (ml/min)	0.1	0.1
start pressure (bar)	20	20
pillow pressure (bar)	14	14
development distance (cm)	7	14 (overrun)

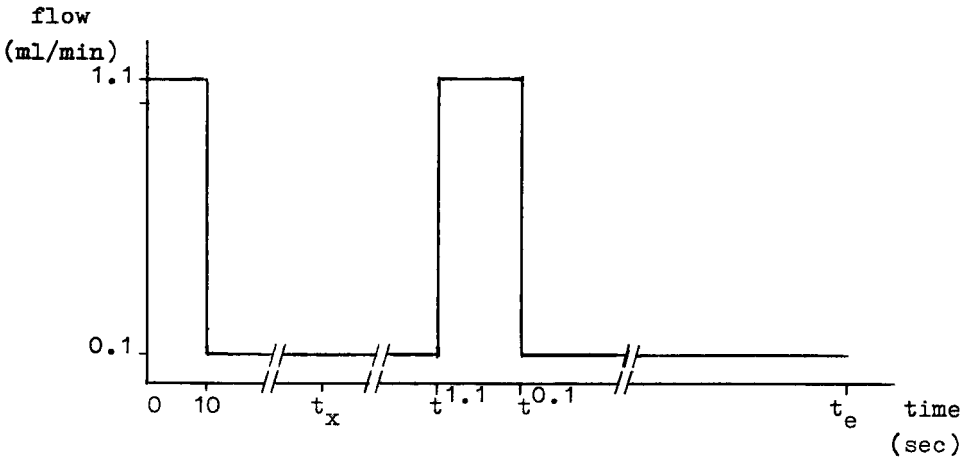


Figure 2. The flow profile employed.

t_x = time of the valve switching

$t^{1.1}$ = t_x + 3'45''

$t^{0.1}$ = t_x + 4'

t_e = time of run end

The step gradient used was the sharp changeover from isocratic segment No1 to No2. The first eluent was pumped by the SDM, the 3 ml volume loop of the injector was filled with the second, No2. eluent. The changeover was accomplished by switching the valve from load to inject. The volume of the connecting tubing from the valve to the plate was taken into calculation.
To achieve ideal dispersion of the eluents, a flow program was used (Figure 2.). The first eluent was pumped until the indicator spots migrated 1.5 cm on the plate, at this moment (t_x) the valve was switched to inject. 3'45'' after switching the

flow was increased to 1.1 ml/min and decreased to 0.1 ml/min 15'' later. The flowrate was maintained at 0.1 ml/min, till the end of the run, when the indicator had migrated 7.0 cm. Staining for class separation, was by immersion of the plate into orcinol-H_2SO_4 reagent and heating at 100°C for 2-3 minutes, for ganglioside separation, by spraying with resorcinol-HCl reagent and heating at 100°C for 15 minutes.
Densitometric conditions in remission mode were:

wavelength:	525 nm
slit:	3.5 x 0.1 mm
scanning speed:	10 mm/min
A/D frequency:	2 Hz

RESULTS AND DISCUSSION

Summarizing the experimental results the OPTLC technique is suitable for the separation of lipid samples. We can now separate the constituent classes of either fraction (neutral and acidic) of the total lipid extract.

In our present paper we described the new separation of the acidic fraction of TLE. The class separation of sulphatides and gangliosides can be achieved by a single isocratic run within 7 minutes, as shown in Figure 3.

As can be seen the resolution is very high between the two classes and the migration of the gangliosides is very small.

Our main purpose was the intraclass separation of the gangliosides in the presence of interfering sulphatides. As we were not interested in the sulphatides at all, the overrun capability of the OPTLC system could be exploited. The

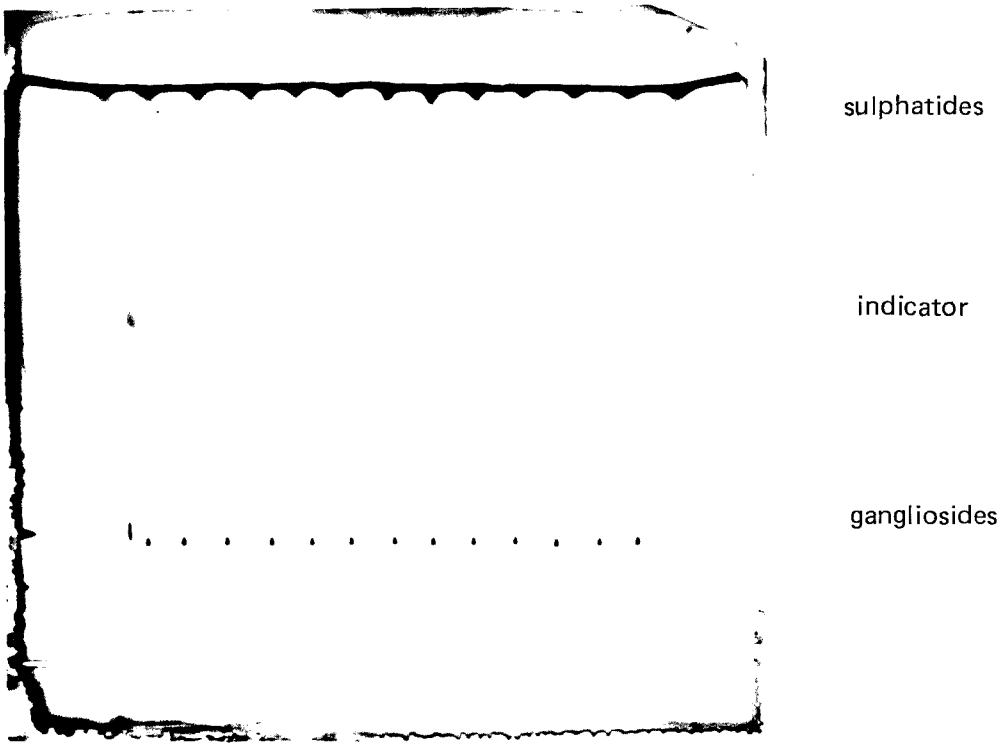


Figure 3. OPTLC separation of acidic glycolipid classes. Plate; Merck HPTLC Si 60. Eluent; C : M = 7 : 3 . Development 7 cm. Staining reagent; orcinol-H_2SO_4. Samples: 1 & 13; 0.2 µl standard mixture + 0.2 µl indicator solution, 2 - 12; 0.2 µl standard mixture.

sulphatides migrate immediately behind the β front of the first eluent and are eluted from the plate before being reached by the second eluent, which gives excellent separation of the individual gangliosides (Figure 4.). As in such cases, overrun and double development, the conventional R_f has no meaning, it was necessary to find a source of reference for

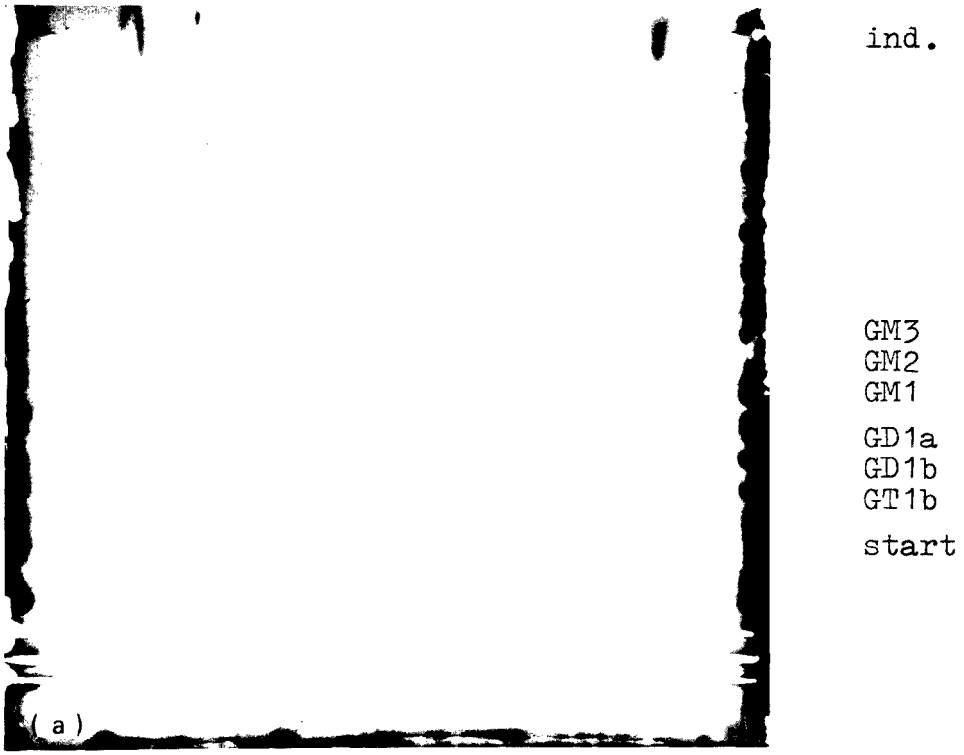
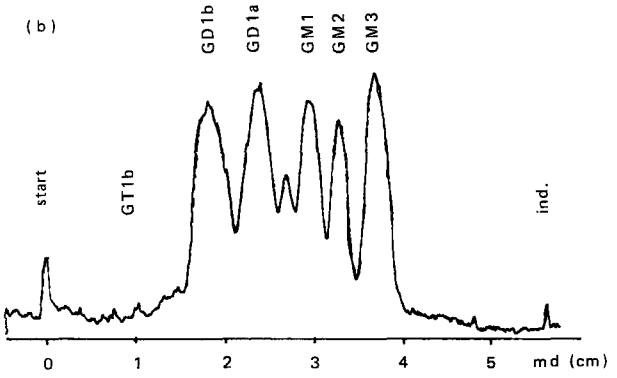


Figure 4. OPTLC separation of acidic glycolipid standards.

4.a. Photograph of the chromatographic plate. Plate; Merck Si 60, staining with resorcinol-HCl. Sulphatides were overrun, see development conditions in text. The samples are the same as for Figure 3.



4.b. A typical densitogram of the plate (lane No7) is shown in Figure 4.a.

standardizing and later characterizing the development process. In our first experiments we developed the plates based on elution time. The migration speed of the front, during the first elution, could be easily measured, and we calculated an approximate development time (equal to the first elution) for the second, overrun. Because of the differences between the commercially available plates (even from the same box), this time based development was not reproducible, so we adapted a different procedure. We selected an indicator, methyl-red, which exhibited very low mobility in C:M=7:3 mixture (R_f=0.2) and has a mobility somewhat higher then all the gangliosides in C:M:KCl/W=55:36:9 mixture.

For the characterization of the separation process we defined the migration quotient (mq) as:

$$mq = \frac{md_G}{md_I}$$

where: md is the migration distance measured for the compound denoted by the subscript,
G subscript stands for gangliosides and
I subscript stands for the indicator.

The quality of the separation method is demonstrated by the numerical data; migration quotients (mq), their averages ($\overline{mq}$), standard deviations (SD) and relative SD (rSD), calculated from the densitograms.

The intraplate reproducibility of the method is demonstrated in Table 1. by the data derived from the densitograms of the plate shown in Figure 4. As the standard deviations and relative standard deviations of the migration quotients show the method is highly reproducible. The relatively high SD-s for GT1b are caused by the small migration distances (the average of which is 8.4 mm), which are influenced more significantly by the variations of sample application.

The interplate reproducibility is shown in Table 2., for nine plates. During the mapping of ganglioside patterns the standard mixture was applied in three places, onto lane 1 and 13 with indicator and to lane 7 without. The rest of the lanes were used for real samples. For the interplate reproducibility studies the center lanes (No7) were used. The migration distance of the indicator was calculated from the averages of the 1. and 13. lanes. Using our standardization method for the development adequate reproducibility can be achieved between plates, but it is advisable to put standards on every plate.

As the results show, the method is highly accurate, reproducible and selective. The method is being used for mapping of ganglioside patterns of plasma, lymphocytes and gra-

Table 1. Intra plate reproducibility of the OPTLC separation of gangliosides

Lane No.	MIGRATION QUOTIENTS (mq)						
	GT1b	GD1b	GD1a	GM1	GM2	GM3	Ind.
1	.16	.19	.23	.3	.35	.42	1
2	.13	.19	.23	.3	.35	.43	1
3	.14	.2	.24	.31	.37	.44	1
4	.14	.2	.25	.32	.37	.45	1
5	.14	.2	.24	.3	.36	.43	1
6	.12	.19	.24	.31	.36	.44	1
7	.12	.21	.26	.32	.37	.44	1
8	.12	.21	.25	.31	.37	.43	1
9	.12	.2	.25	.31	.36	.44	1
10	.12	.19	.23	.31	.37	.43	1
11	.12	.19	.23	.31	.37	.44	1
12	.12	.18	.22	.3	.36	.43	1
13	.08	.15	.23	.3	.37	.45	1
$\overline{mq}$	.13	.19	.24	.31	.36	.44	1
SD.	.02	.01	.01	.01	.01	.01	0
rSD (%)	14.69	7.63	4.15	2.24	1.98	2.02	0

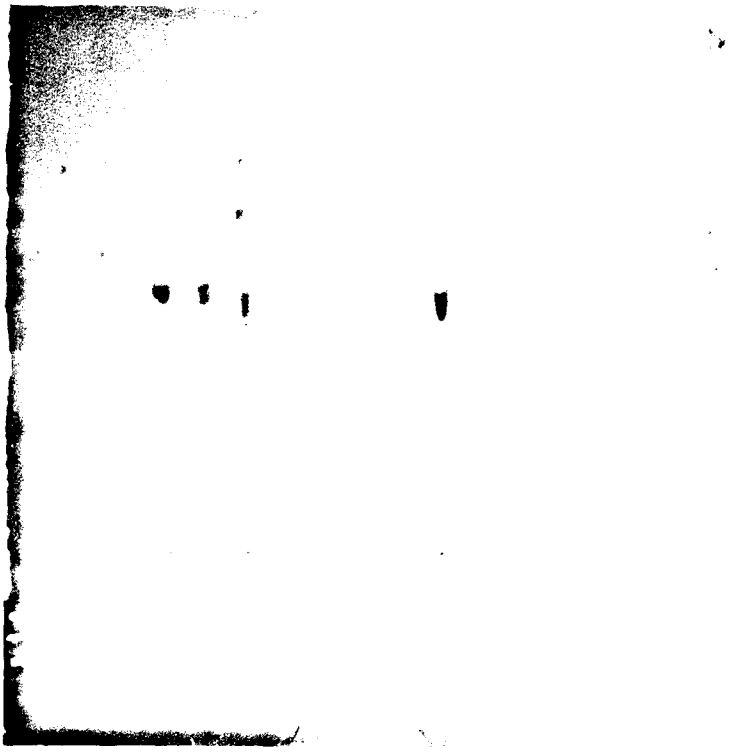


Figure 5. OPTLC separation of gangliosides of acidic glycolipids derived from blood elements.

OPTLC conditions are the same as for Figure 4.

Samples: 1, 7, 13; standard mixture + indicator,
2; MCL plasma,
6; AML plasma (in blast crisis),
3; AMMoL lymphocyte,
AMMoL = Acute Myeloid Monoblastoid L.
CLL = Chronic Lymphoid Leukemia
ALL = Acute Lymphoid Leukemia
4, 5, 9, 10; CLL lymphocyte,
8; ALL lymphocyte,
11; normal granulocyte,
12; blank.

Symbols used: MCL = Mast Cell Leukemia
AML = Acute Myeloid Leukemia

Table 2. Inter plate reproducibility of the OPTLC separation of gangliosides

Lane No.	MIGRATION QUOTIENTS (mq)						
	GT1b	GD1b	GD1a	GM1	GM2	GM3	Ind.
1	.15	.17	.2	.29	.35	.42	1
2	.12	.18	.2	.28	.34	.43	1
3	.1	.18	.2	.28	.35	.43	1
4	.14	.17	.19	.27	.34	.42	1
5	.15	.22	.27	.35	.42	.51	1
6	.11	.21	.25	.31	.37	.46	1
7	.12	.19	.21	.28	.37	.43	1
8	.1	.2	.26	.35	.39	.47	1
9	.12	.19	.23	.27	.36	.44	1
$\overline{mq}$	.12	.19	.22	.3	.37	.45	1
SD.	.02	.02	.03	.03	.03	.03	0
rSD (%)	13.96	9.7	13.69	11.01	7.17	6.22	0

nulocytes of patients with different leukemias. The publication of the results of these experiments is in progress. Here we present one chromatographic plate of selected samples, to show real life application (Figure 5).

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COMPARISON OF TWO COPPER REAGENTS FOR DETECTION OF SATURATED AND UNSATURATED NEUTRAL LIPIDS BY CHARRING DENSITOMETRY

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ABSTRACT

The intensity of staining of each of four classes of neutral lipids (monoacylglycerol, diacylglycerol, triacylglycerol and fatty acid methyl ester) is shown to be dependent on the number or amount of carbon-carbon double bonds (C=C) when using 3% cupric acetate in 8% phosphoric acid. In contrast, staining with 10% cupric sulfate in 8% phosphoric acid is relatively independent of the number or amount of C=C.

INTRODUCTION

The quantitation of lipids by charring densitometry is a well established tool in use in thin-layer chromatography. One of the more widely used reagents is cupric acetate (3% in 8% phosphoric acid). Since its introduction by Fewster, Burns and Mead (1 (personal communication from R. Kopp)), there has been evidence which demonstrates that unsaturated lipids will char to a greater degree than saturated ones (2 - 4), but a quantitative study of a particular lipid class, by varying amounts of unsatur-

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ation, has not been previously done. Description of the extent of charring have either been evaluated by + or - (2,3) or a qualitative scale of 1 - 10 (4).

Our aim in this paper is to present a quantitative examination of the extents of charring, as monitored by densitometry, of lipid species with varying degrees or amounts of unsaturation. We have compared the intensity of staining, under identical charring conditions, of neutral lipids with cupric acetate (1) and cupric sulfate (2,3) reagents.

MATERIALS

Reference standards containing 25% each monoacylglycerol (MAG), diacylglycerol (1,2-DAG + 1,3-DAG), triacylglycerol (TAG) and fatty acid methyl ester of stearin, olein, linolein and linolenin were obtained from Nu Chek Prep (Elysian, MN). Fatty acid methyl esters quantitative mixtures (FAME) were obtained from both Nu Chek Prep and Alltech Associates, Inc. (Deerfield, IL). Whatman (Whatman Inc., Clifton, NJ) LK-5, 250u, 20x20 cm TLC plates, with a preadsorbent area, were scored to obtain 1 cm lanes, developed with chloroform-methanol 1:1 (v/v) and activated at 110°C for 60 min. All solvents were glass distilled or nanograde and chemicals were reagent grade.

METHODS

Thin-Layer Chromatography

Aliquots of quantitative mixtures, dissolved in chloroform

TABLE 1

FAME QUANTITATIVE MIXTURES

Mixture	Composition	mmoles C=C/gm
1	20% each of 16:0, 18:0, 20:0, 22:0 and 24:0	0.00
2	50% each of mixture 1 and mixture 3	1.45
3	20% each of 16:0, 16:1, 18:0, 18:1 and 18:2	2.91
4	1% 14:0, 4% 16:0, 3% 18:0, 45% 18:1, 15% 18:2, 3% 18:3, 3% 20:0, 3% 22:0, 20% 22:1 and 3% 24:0	3.51
5	20% each of 20:0, 20:1, 20:2, 20:3 and 20:4	6.24
6	25% each of 18:2, 18:3, 20:4 and 22:6	12.0

(neutral lipid mixture of MAG, DAG (1,2- + 1,3-), TAG and fatty acid methyl esters) or hexane (FAME), containing 0.25, 0.5, 1, 2 or 4 µg were streaked 1 cm below the silica gel-preadsorbent area boundary. The composition of the FAME quantitative mixtures and the calculated amounts of C=C (mmoles/gm = sum of (wt lipid/MW x no. C=C/wt total lipids)) are given in Table 1.

Every third lane was left empty as a reference during densitometry. Neutral lipids were resolved by development with chloroform/methanol (47:3) to 1.5 cm above the origin, dried *in vacuo* at room temperature for 20 min followed by development with hexane/diethyl ether/acetic acid (62:13:0.75) (5) to 2.5 cm from the

top of the plate. The FAME quantitative mixtures were spotted, dried in vacuo and developed with hexane/diethyl ether (47:3) (6). The minimal volume of sample streaked was 5 µl. Following development, plates were dried in vacuo at 100°C for 30 min. Nitrogen was admitted into the oven when releasing the vacuum and was used when spotting the samples.

Charring Densitometry

Plates were sprayed, until glistening wet (but not running with liquid), with either 3% cupric acetate in 8% phosphoric acid (1) or 10% cupric sulfate in 8% phosphoric acid (2,3). They were then air dried for 5 - 10 min, heated to 120°C for 2 min and charred at 170°C for 9 min (for neutral lipid mixtures) or for 5 and 5 or 9 min respectively (for FAME). These times were chosen so that the plate background remained white (LK-5 plates contain an organic binder which can darken if plates are heated too long or at temperatures > 170°C). Quantitation of the lipids was accomplished by densitometry using a fiber optic scanner (Kontes, Model 800, Vineland, NJ) equipped with a 440 ± 150 nm filter and an integrator (Hewlett Packard, Model 3390A, Avondale, PA) which gave numeric area responses. These were linear in the range tested and area/pg was determined by averaging the values calculated for each level of lipid species (DAG was the sum of the 1,2- and 1,3- species). The upper limit of linearity varied with the area of the lipid band. MAG bands were very tight (1.5 x 10 mm) and linearity was good only to 0.5 - 1 µg while fatty acid

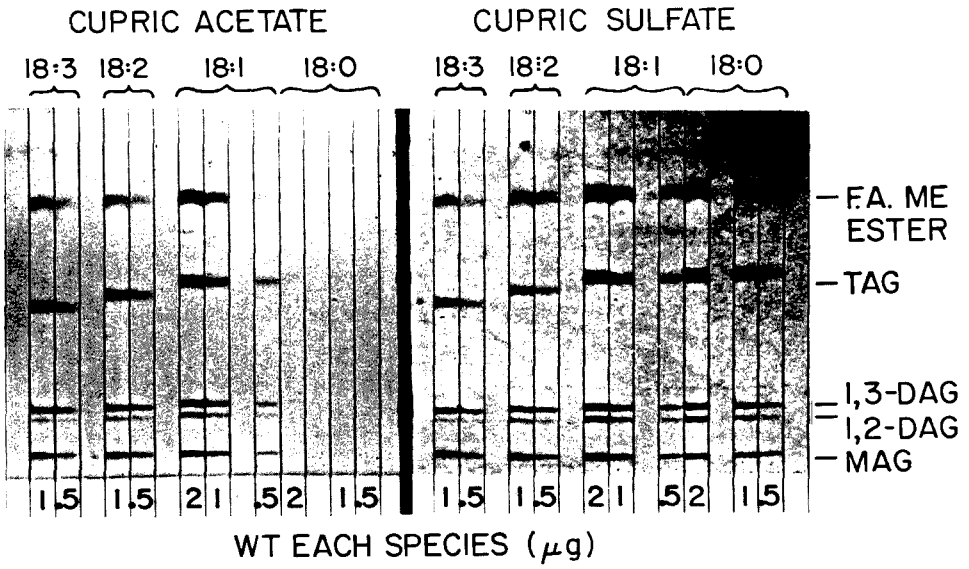

FIGURE 1. Two representative plates stained with cupric acetate and cupric sulfate reagents. Lipids were separated (5) and stained as described in the Methods (2 min at 120°C and 9 min at 170°C). F.A. ME ESTER = fatty acid methyl ester.

methyl esters were broader (5 x 10 - 7 x 10 mm) and linearity was maintained to 3 - 4 μg.

RESULTS AND DISCUSSION

A comparison of the staining intensity of a series of neutral lipids, each containing an 18 carbon acyl chain with 0, 1, 2 or 3 carbon-carbon double bonds (C=C), is shown in Figs. 1 and 2. All four saturated species were barely detectable (significant area levels were obtained at >1 μg) after charring with the cupric acetate reagent. Staining increased as the number of C=C

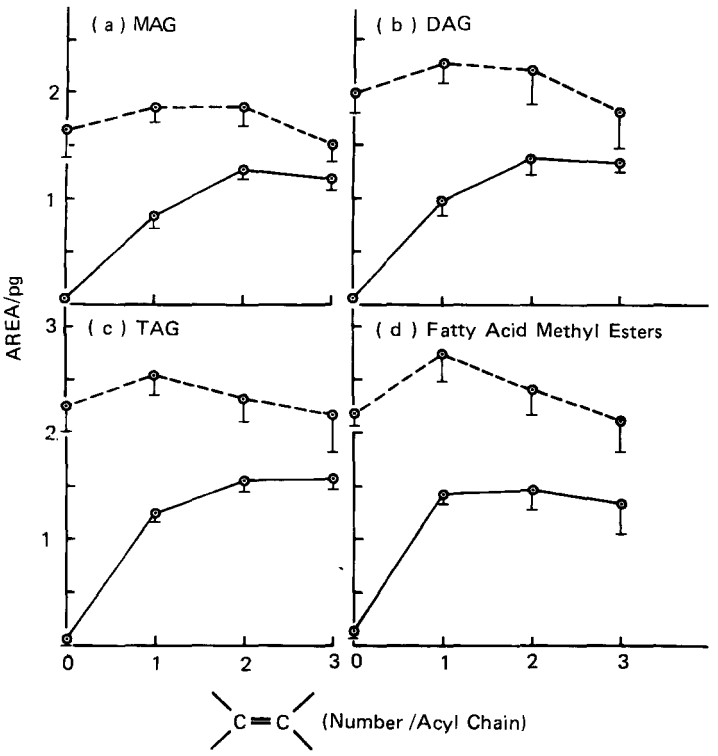

FIGURE 2. Staining of neutral lipids with cupric acetate and cupric sulfate reagents. The solid line is the amount of stain detected with cupric acetate reagent and the dashed line is the amount with cupric sulfate reagent. The values are averages of four plates, two each of separately weighed neutral lipid mixtures of MAG, DAG, TAG and fatty acid methyl ester. Sample standard deviations, with corrections for bias, are indicated (n = 4).

increased. For MAG, DAG and TAG maximal staining was acheived with the linoleate species whereas maximal staining of the fatty acid methyl esters was acheived with methyl oleate. In contrast, the cupric sulfate reagent was able to stain lipids strongly, fairly independent of the degree of unsaturation. However, there

appears to be a slight, but general, increase in staining of the mono and di unsaturated species as compared to the saturated and tri unsaturated species. Paired t tests indicate that these differences are generally significant ($p < 0.05$) when comparing 18:0 - 18:1, 18:1 - 18:3 and 18:2 - 18:3. There was no difference between 18:0 - 18:2 lipids ($p > 0.05$) (except for the fatty acid methyl esters ($p < 0.05$)). With the cupric sulfate reagent, the extent of staining of the different classes was: fatty acid methyl esters = TAG > DAG > MAG. With the cupric acetate reagent maximal staining was approximately equal for all four classes.

The FAME quantitative mixtures also showed increases in staining with increases in C=C using the cupric acetate reagent (Fig. 3a) and relatively small changes using the cupric sulfate reagent with 9 min of charring (Fig. 3b) However, if plates were charred for only 5 min, then there was usually a general decrease in the response using either reagent. In particular, with the cupric sulfate reagent the relative staining of the saturated fatty acid methyl esters decreased about two fold.

These results clearly demonstrate that the cupric acetate reagent will yield an intensity of staining which increases with increasing amounts of unsaturation (Figs. 2 and 3a). The cupric sulfate reagent apparently chars all lipids, within a particular class, to about the same extent providing that the plate is heated for a sufficient length of time.

Plates stained with the cupric sulfate reagent have been observed to vary in response, from plate to plate (Fig. 2 and

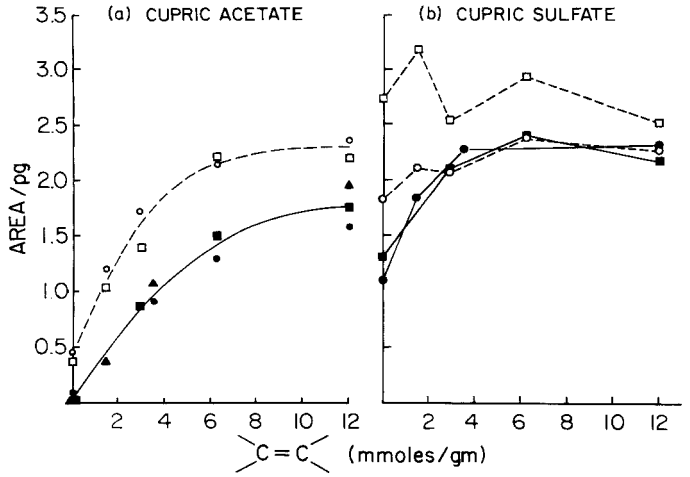

FIGURE 3. FAME stained with cupric acetate and cupric sulfate reagents. Samples were applied to plates, developed (6), stained and quantitated as described in the Methods. Plates were charred at 170° for 5 min (closed symbols, solid lines) or for 9 min (open symbols, dashed lines). Each symbol represents values from a single plate.

3b), but by including standards in each run we have found that they are internally consistent (7). TLC of naturally occurring phospholipids (phosphatidylinositol, phosphatidic acid, phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine), yielded average responses, on five separate plates, of 1.10 $\pm$ 0.09, 1.07 $\pm$ 0.07, 1.11 $\pm$ 0.14, 1.08 $\pm$ 0.07 and 1.06 $\pm$ 0.17 area x 10^6/nmole Pi and were linear up to 5 nmoles Pi (approximately 4 µg phospholipid). Sphingomyelin consistantly gave responses which were higher, about 1.7 area x 10^6/nmole Pi.

ACKNOWLEDGMENTS

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DETERMINATION OF NEOMYCIN COMPONENTS BY THIN LAYER CHROMATOGRAPHY WITH VIDEODENSITOMETRY

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ABSTRACT

A method was developed for determination of the three neomycin components, neomycin A, B and C using silica gel thin-layer chromatography with detection with p-dimethylamino benzaldehyde-ninhydrine reagent and videodensitometry. The method was used to monitor the composition in different stages of production. The results obrained were compared with that of biological determination.

INTRODUCTION

Neomycin, which is an aminoglycoside type antibiotic

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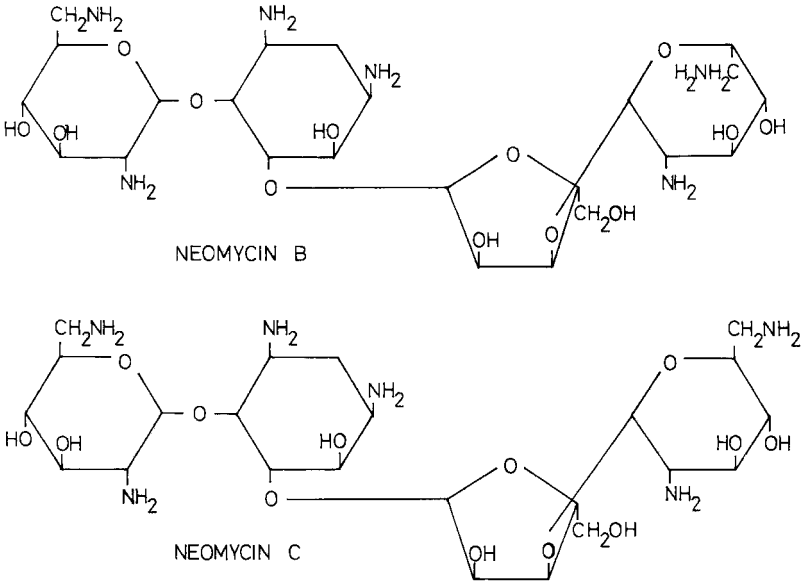

Figure 1.: Structure of neomycins

produced by Streptomyces fradiae, consists of three main components, neomycin A, B and C having very similar structure (Fig. 1.)

The neomycin, as antibiotic, is used for treatment of infections caused by Gram positive and Gram negative bacteria. This antibiotic inhibits the protein synthesis /2./. The neomycin components are not equivalent from therapeutical point of view, having different toxicity. Because of it the knowledge of the exact composition is essential. It is particularly important to determine the neomycin

B and C content, because the C component has twice ototoxicity than does the B component. Because of its biological and commercial interest, methods have been devised for the determination of the composition most often based on gas-liquid chromatography /3,4,5/.

Quantitativ determination of the neomycin components by GLC is difficult, because derivatization procedures are required for the analyses. /6/ This paper describes a simple procedure employing thin layer chromatography with videodensitometry for the direct determination of the components. The method is shown to be accurate and sensitive to µg amounts of neomycin B and C.

EXPERIMENTAL

Reagents and Materials :

Whatman K5 silica gel thin layer plates (20 x 20 cm) were used Neomycin A (neamine), B and C components, as standards were prepared by column chromatographic method in BIOGAL, and Neomycin B was from WHO. p-Dimethylaminobenzaldehyde (DABA), ninhydrine, cyclohexane, ethanol and pyridin were purchased from

REANAL (Hungary). For detection of spots the chromatogram was air dried and sprayed using a solution of p-DABA (500 mg) and ninhydrin (200 mg) in a mixture of cyclohexane : ethanol : pyridine /3 : 7 : 1/.

Apparatus :

The spots were quantitated with a TELECHROM OE 976 (CHINOIN, BUDAPEST) type videodensitometer in reflexion mode. Rectangular, heavy-wall glass tanks 30 cm x 10 cm x 28 cm were used as developing chambers.

Thin-layer chromatography :

Stock solution of Neomycin B (WHO) standard was prepared at the 6 mg/ml level in water and 0,5 - 2,5 µl aliquots were applied to layers with Hamilton microsyringe. Stock solutions of samples were prepared at the 50 mg/ml level in water and 2 µl aliquots for determination of Neomycin B and 10 µl aliquots for determination of Neomycin C were applied to layers with Hamilton microsyringe. Plates were developed at room temperature for a distance of 10 - 15 cm beyond the origin line, which was located 2 cm above the bottom of the plate

TABLE 1.

R_f values and spot colors of Neomycin components on Whatman K5 layers developed with water : ethanol (7:3v/v) containing 2,5 M NH_4Cl.

Component	R_f	Color
Neomycin A	0,42	purple
Neomycin B	0,32	purple
Neomycin C	0,16	purple

using water : ethanol = 7 : 3 containing 2,5 mol NH_4Cl as solvent. For detection the plates sprayed with DABA reagent were placed in a 110° oven for 15 minutes.

RESULTS AND DISCUSSION

Among the studied mobil phases the water : ethanol /96 %/ = 7 : 3 containing 2,5 M NH_4Cl proved the best for separation of Neomycin components. R_f values and spot colors are shown in Table 1. Differences among R_f values would allow this method to detect and quantitate the components. As seen in Table 1. the components were detected as purple spots, which color is the best for videodensitometric determination with Telechrom

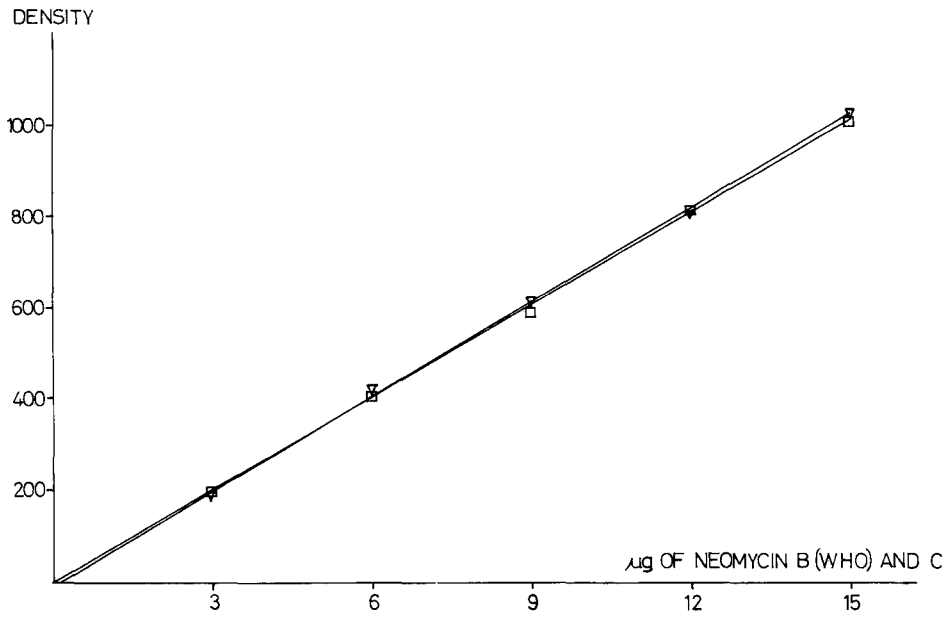

Figure 2.: Calibration curves of neomycin B and C components

neomycin B-ninhydrin-complex: a=401,8 b=1,5r = 1.00
neomycin C-ninhydrin-complex: a=402,2 b=4,7r = 1.00

OE 976. DABA reagent was the best for this purpose. The calibration plot for neomycin B and C was linear in 0-15 µg range (Fig.2.). Calibration curves of B and C components were identical having the same slope. This makes possible to determine the content of C component using neomycin B /WHO/ as standard (Fig.2.).

Calibration curves were quite reproducible in terms of slope and linarity from plate to plate, but standards should always be run on the same plate with samples to obviate the effects of any variations when using the method.

To check reproducibility, eleven 10 µg spots of Neomycin B /WHO/ were spotted across separate K 15 plates, the plates were developed, and the spots were detected and videodensitometrically measured. The relative standard deviation was ± 5,7.

On the basis of the result of thin-layer chromatography of numerous factory samples, the relative amounts of neomycin C was estimated in the percentage of the sum of Neomycin B and C.

The neomycin B and C display different activity against Bacillus pumilis, used in routin test, the activity of neomycin B is twice as much as that of neomycin C. The quantitative results of thin-layer chromatography-videodensitometry give preliminary informations about the probable biological activity.

This method was also used for the control of the steps and modifications of the technology and the results were compared to the biological activity.

This method was also used for the control of the

TABLE 2.

Thin-layer chromatography - videodensitometric determination (v.d.) - biological activity (b.a.)

N° of sample	T L C - v.d. neomycin B %	neomycin C %	neoC x 100 / neoB + Neo C	b.a. NU/mg
1.	12	10	45	160
2.	29	13	31	350
3.	38	12	24	420
4.	52	15	22	570
5.	58	14	19	590
6.	62	12	16	650
7.	65	11	14	650
8.	68	9	12	710
9.	69	9	12	710
10.	69	10	13	710

steps and modifications of the technology and the results were compared to the biological activity. Neomycin B and C content of the samples, the relativ amount of neomycin C and the biological activity of each sample (NU/mg) are summarized in Table 2.

SUMMARY

Thin-layer chromatography with videodensitometry provides rapid preliminary information on the quality of the product. Advantages of this method include simplicity, high sample through put, and the ability to analyse multiple samples at the same time under indentical conditons and to process

standards in parallel.

Precision and accuracy are shown to be satisfying from analytical point of view.

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THIN LAYER CHROMATOGRAPHY OF METAL IONS COMPLEXED WITH ANILS(VII) DETECTION, SEPARATION, AND DETERMINATION

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ABSTRACT

Dark colored chelates of p-dimethylaminoanil of 3-benzoyl-methylglyoxal bidentate ligand with Sb(III), Mn(II), Fe(III), Co(II), Ni(II), Cu(II), ZrO(II), Y(III), La(III), Pr(III), Nd(III) Sm(III), Gd(III) and Dy(III) have been chromatographed on starch bound silica gel thin layers. New correlations of I.R. with R_f (resolving solvent) have been used to ascertain the colored spots.

Among various mixtures resolved qualitatively a few typical ones have been alanysed quantitatively. Errors in determinations and maximum separation limits have also been deduced.

INTRODUCTION

The mixtures, component compounds of which have high solubility in acidic and alkaline media, are generally resolved by liquid-liquid extraction and chromatographic methods, and are estimated volumetrically or colorimetrically. In such case gravimetric method of analysis fails even in the presence of masking agents and on controlling pH. The colored chelates of Sb(III), Mn(II), Fe(III),

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Co(II), Ni(II), Cu(II), ZrO(II), Y(III), La(III), Pr(III), Nd(III), Sm(III), Gd(III) and Dy(III) with p-dimethylaminoanil of 3-benzoylmethylglyoxal[1](DMABG) having high solubility in common solvents under acidic and alkaline conditions have been, therefore, analysed qualitatively as well as quantitatively by thin layer chromatography in continuation to our work[2-7].

The use of R_F and infrared spectral correlations in identification of mixture components is the most interesting feature of these studies.

EXPERIMENTAL

Preparation of Solutions and TLC Plates :

Complexes isolated[8] as solids were dissolved in known quantities in their solvents to prepare the standard solutions.

Glass plates (16x3 cm and 16x10 cm) were coated with silica gel freed from iron and chloride ions and mixed with starch as binder (24:1, w/w) to prepare layers of 0.1 cm thickness by self designed apparatus[9]. Gel coated plates were dried at $\sim$ 100°C for 2-3 hrs in an oven. Dry loaded plates were developed in rectangular glass jars with ground-in-lids by ascending technique.

Loading and Development of TLC Plates :

For qualitative studies one or two drops of the test solutions were placed on 16x3 cm plates with thin glass capillaries. After drying the spots development was done in different solvents and the ascent was fixed as $\sim$ 10 cm in all cases. However, for quantitative analysis mixtures of varying concentrations of components were spotted on the plates of 16x10 cm with the help of micro pipette. After development plates were dried in oven and chromatogram fragments were scrapped and eluted with ethanol. Elutes were reduced to 5ml and optical densities were measured at their λmax. Elute concentrations were deduced from their respective

TABLE-1

SPOT COLOUR, λmax, VISIBLE, I.R. FREQUENCIES AND R_F OF COMPLEXES

Complex	Spot colour	λmax (visible) nm	Infrared frequencies cm^{-1}		R_F							
			C=O	C=N	CH_2Cl_2	$CHCl_3$	CCl_4	C_6H_6	BuOH	C_6H_6-BuOH (1:2)	C_6H_6-BuOH (1:1)	C_6H_6-BuOH (2:1)
$Sb(DMABG)_2Cl_3.6H_2O$	Pink	350	1600	1560		0.03	0.25		0.00	0.97	0.98	0.99
$Mn(DMABG)_2Cl_2.H_2O$	Yellow	356	1610	1520		0.00	0.96		0.00	0.99	0.99	0.99
$Fe(DMABG)_2Cl_3.6H_2O$	Brown	356	1600	1520		0.00	0.00		0.00	0.96	0.98	0.99
$Co(DMABG)Cl_2$	Light brown	350	1610	1550		0.00	0.00		0.99	0.98	0.99	0.99
$Ni(DMABG)_2Cl_2.2H_2O$	Light brown	365	1610	1570		0.02	0.26		0.09	0.46	0.98	0.99
$Cu(DMABG)Cl_2.4H_2O$	Light brown	342	1600	1550	No mig-	0.00	0.96	No mig-	0.00	0.99	0.98	0.99
$(ZrOCl_2)_2(DMABG).9H_2O$	Pink	350	1590	1560	ration	0.07	0.27	ration	0.40	0.99	0.99	0.36
$(Y(DMABG)Cl_3)_2.2H_2O$	Canary yellow	348	1630	1580		0.04	0.96		0.99	0.93	0.99	0.99
$La(DMABG)_2Cl_3$	Canary yellow	345	1610	1570		0.00	0.98		0.00	0.99	0.99	0.99
$(Pr(DMABG)Cl_3)_2$	Yellow	355	1610	1580		0.00	0.10		0.08	0.98	0.99	0.99
$(Nd(DMABG)Cl_3)_2.5H_2O$	Light brown	365	1610	1580		0.04	0.98		0.00	0.96	0.99	0.99
$(Sm(DMABG)Cl_3)_2.2H_2O$	Light brown	345	1610	1580		0.00	0.14		0.57	0.97	0.98	0.99
$(Gd(DMABG)Cl_3)_2.2H_2O$	Canary yellow	345	1610	1580		0.00	0.98		0.00	0.95	0.99	0.98
$Dy(DMABG)_3Cl_3$	Yellow	360	1610	1570		0.00	0.99		0.00	0.99	0.99	0.99

Room temperature = 30 $\pm$ 2°C

TABLE - 2

METAL COMPLEX MIXTURES WITH RESOLVING SOLVENTS

Metal Ions in Complex Mixtures	Resolving Solvent
Co(II) or Fe(III) + Sb(III) or Ni(II) or Zr(IV) + Mn(II) or Cu(II) (0.00) (0.01) (0.24) (0.26) (0.26) (0.95) (0.96)	CCl_4
Sb(III) + Zr(IV) + Co(II) or Mn(II) or Fe(III) or Ni(II) or Cu(II) (0.76) (0.85) (0.98) (0.99) (0.99) (0.98) (0.99)	EtOH
Sb(III) or Mn(II) or Fe(III) or Cu(II) + Ni(II) + Zr(IV) + Co(II) (0.00) (0.00) (0.01) (0.02) (0.09) (0.40) (0.99)	BuOH
Ni(II) + Zr(IV) + Sb(III) or Mn(II) or Fe(III) or Co(II) or Cu(II) (0.38) (0.62) (0.99) (0.99) (0.99) (0.99) (0.99)	Aq.BuOH
Zr(IV) or Sb(III) + Ni(II) + Co(II) or Mn(II) or Fe(III) or Cu(II) (0.24) (0.25) (0.62) (0.97) (0.99) (0.98) (0.99)	AcOH-BuOH (1:1, v/v)
Zr(IV) + Co(II) or Ni(II) + Sb(III) + Mn(II) or Fe(III) or Cu(II) (0.33) (0.50) (0.54) (0.83) (0.99) (0.98) (0.99)	Aq.BuOH-AcOH (1:1, v/v)
Zr(IV) + Mn(II) + Sb(III) or Ni(II) or Fe(III) or Co(II) (0.68) (0.88) (0.95) (0.97) (0.98) (0.99)	Aq.BuOH-AcOH-$CHCl_3$ (5:5:3, v/v)
Ni(II) or Co(II) or Sb(III) + Zr(IV) + Mn(II) or Fe(III) or Cu(II) (0.36) (0.41) (0.41) (0.50) (0.96) (0.98) (0.99)	Aq.BuOH-$CHCl_3$ (5:2, v/v)
Mn(II) + Fe(III) + Sb(III) or Zr(IV) + Ni(II) (0.00) (0.08) (0.34) (0.38) (0.98)	$CHCl_3$-Hexane-BuOH (1:1:1, v/v)
Fe(III) or Co(II) or Cu(II) + Zr(IV) or Sb(III) + Mn(II) or Ni(II) (0.01) (0.00) (0.01) (0.18) (0.16) (0.99) (0.99)	$CHCl_3$-Hexane-BuOH (1:2:1, v/v)
Ni(II) or Mn(II) or Fe(III) or Cu(II) + Co(II) + Sb(III) or Zr(IV) (0.00) (0.02) (0.04) (0.06) (0.11) (0.20) (0.32)	$CHCl_3$-Hexane-BuOH (1:1:2, v/v)

Ni(II) or Sb(III) or Zr(IV) + Co(II) or Cu(II) or Fe(III) or Mn(II) (0.64) (0.81) (0.84) (0.94) (0.95) (0.97) (0.98)	MeOH
Zr(IV) + Ni(II) + Cu(II) or Sb(III) (0.82) (0.88) (0.96) (0.98)	Me_2CO
Sb(III) or Zr(IV) + Ni(II) + Mn(II) or Fe(III) or Co(II) or Cu(II) (0.10) (0.15) (0.33) (0.96) (0.98) (0.99) (0.99)	AcOH-BuOH (1:2, v/v)
Sb(III) + Ni(II) + Mn(II) or Fe(III) or Co(II) or Cu(II) or Zr(IV) (0.28) (0.54) (0.97) (0.99) (0.99) (0.99) (0.99)	AcOH-BuOH (2:1, v/v)
Dy(III) or Sm(III) + La(III) or Pr(III) + Nd(III) or Gd(III) or Y(III) (0.40) (0.44) (0.57) (0.56) (0.93) (0.99) (0.99)	AcOH-BuOH (1:1, v/v)
Gd(III) or Nd(III) or Pr(III) + La(III) or Sm(III) or Dy(III) + Y(III) (0.05) (0.06) (0.07) (0.13) (0.13) (0.15) (0.99)	$CHCl_3$-Hexane-BuOH (1:1:1, v/v)
La(III) or Nd(III) or Gd(III) or Dy(III) + Pr(III) + Sm(III) + Y(III) (0.00) (0.00) (0.02) (0.00) (0.08) (0.57) (0.99)	BuOH
Pr(III) + Sm(III) + Y(III) or La(III) or Nd(III) or Dy(III) or Gd(III) (0.16) (0.36) (0.98) (0.98) (0.99) (0.99) (0.99)	AcOH-BuOH (1:2, v/v)
Sm(III) + Dy(III) + Y(III) or La(III) or Pr(III) or Nd(III) or Gd(III) (0.28) (0.34) (0.98) (0.98) (0.99) (0.99) (0.99)	Aq-BuOH-$CHCl_3$ (5:2,v/v)

R_F Values of complexes are given in parenthesis.

calibration curves prepared under similar conditions of temperature and solvent.

Physical Measurements

Infrared spectra of complexes were recorded on Perkin Elmer-621 infrared spectrophotometer in Nujol mull in full range. Optical density measurements on the complex solutions were done by Bausch & Lomb Spectronic-20 spectrophotometer.

RESULTS AND DISCUSSION

TLC data (table-1) of individually migrated complexes in CH_2Cl_2, $CHCl_3$ and CCl_4 evidently show an adverse effect of solvent polarity on R_F. Almost all complexes have shown high migration in C_6H_6-BuOH mixtures than their component solvents. In the absence of any chemical reaction of migrating compounds with the solvents, this abnormality could only be attributed to azeotropic properties of mixture solvents. Migration of each complex has been found to be independent on the presence of others and on plate size but layer thickness has adverse effect on it.

Among various solvents used those giving differential migrations of individual complexes and could resolve their diverse ternary and quaternary mixtures have been noted in table-2 alongwith R_F values. All the mixtures were qualitatively resolved but only a few typical ones could be tried for quantitative analysis. Maximum limit of separations of different mixtures is exhibited by the quantities of each mixture components resolved (Table-3). Errors determined in each estimation (Table-3) show the precision of this method. In BuOH-AcOH mixture solvents trailing effect was observed but it did not obstruct separations.

Stretching frequencies of metal sensitive azomethine and carbonyl groups of DMABG in complexes have been correlated with their R_F values in almost all the resolving solvents. These

Table-3

QUANTITATIVE ANALYSIS DATA ON TYPICAL MIXTURES

Mixture	Complex applied on plate (μg)	Complex recovered (μg)	Error (%)
$Sb(DMABG)_2Cl_3.6H_2O$	90.50	90.00	-0.55
$(ZrOCl_2)_2(DMABG).9H_2O$	60.40	60.00	-0.66
$Co(DMABG)Cl_2$	69.75	70.00	+0.36
$Cu(DMABG)Cl_2.4H_2O$	35.25	35.00	-0.71
$(Pr(DMABG)Cl_3)_2$	49.75	50.00	+0.50
$(Nd(DMABG)Cl_3)_2.5H_2O$	39.75	40.00	+0.63
$Dy(DMABG)_3Cl_3$	35.00	35.00	0.00
$(Y(DMABG)Cl_3)_2.2H_2O$	35.25	35.00	-0.71
$La(DMABG)_2Cl_3$	30.00	30.00	0.00
$(Gd(DMABG)Cl_3)_2.2H_2O$	50.25	50.00	-0.50

relationships of ν(C=N) and ν(C=O) with R_F of components of diverse mixtures in their resolving solvents have been used in the identification of chromatogram fragments.

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THIN LAYER AND PAPER CHROMATOGRAPHIC SEPARATIONS OF d-BLOCK CATIONS COMPLEXED WITH ANILS

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ABSTRACT

Diverse binary, ternary and quaternary mixtures of Cr(III), Mn(II), Fe(III), Co(II), Ni(II), Cu(II), Zn(II), Cd(II), Hg(II) and Au(III) d-block cations complexed with p-dimethylamino- and p-diethylamino- anils of thiophenglyoxal have been separated by thin layer and paper chromatographic techniques. But quantitative separations have been done by thin layer chromatography, on account of wide difference in migration rates and high compectedness of complexes on gel layers. Chromatogram fragments visualised as such have been estimated spectrophotometrically.

INTRODUCTION

On account of rapid and better separability of organic compounds by paper and thin layer chromatography their metal complexes are chosen instead of metal ions as migrating species. Long persisting dark colours of organometal compounds leading to their self visualisation without any locating agent also justify their use in chromatographic analyses. Chromatographic analyses

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of cations complexed with variety of organic ligands[1-3] including ketoanils[4-8] are well documented. Thin layer and paper chromatographic separations of groups II, III and IV transitional basic radicals and their mixtures with Au(III) complexed with ketoanils including p-dimethylamino- and p- diethylamino anils of thiophene-glyoxal (abbreviated as DMATG and DEATG, respectively) unknown hitherto have been described in the present communication. Coloured complexes resolved by TLC have been estimated spectrophotometrically.

EXPERIMENTAL

Synthesis of Ketoanils and their Complexes :

Both ketoanils, DMATG and DEATG were prepared by condensing equimolar amounts of corresponding amine with 2-thiopheneglyoxal in chloroform; solvent was driven off at $\sim 60^{\circ}C$ under reduced pressure. Residue was washed with small volume of ether several times and purified by recrystallization from chloroform.

Complexes were synthesized[10] by mixing metal chlorides and ketoanils in stoichiometric proportions in acetone-water or ethanol medium. Reaction mixtures were refluxed or concentrated and were left for crystallization. Dark crystalline products washed with ether and dried ($\sim 60^{\circ}C$) were purified by recrystallization from methylcyanide, chloroform or dioxan.

Preparation, Loading and Development of TLC plates and P.C. Paper strips:

Silica gel G (BDH) mixed with starch (E. Merck, Darmstadt, G.F.R.) as binder (19:1, w/w) was used to prepare layers of 0.10cm thickness on glass plates of 18x3 cm and 18x10 cm sizes with a home-built apparatus[11]; coated plates were dried at $\sim 100^{\circ}C$ in an oven. For the qualitative analysis 18x3 cm plates were loaded with sample solutions by fine capillaries but for quantitative analysis known volumes of standard solutions prepared by

dissolving directly weighed quantities of complexes (and ligands) in methylcyanide, chloroform, acetone or alcohol were applied with micro pipette on 18x10 cm plates. While developing the plates in rectangular glass chambers by ascending technique migration of solvent front was kept constant at 8cms. On the chromatograms complexes were visualised as such. For quantitative estimations elutes of scrapped chromatogram fragments were made to 5.0 ml volume and their optical densities were determined spectrophotometrically at λmax of solutes. Elute concentrations were deduced from respective calibration curves prepared under similar conditions of temperature and solvent. For spectrophotometric measurements Bausch & Lomb spectronic-20 instrument was used.

In paper chromatography Whatmann No.1 15x3 cm paper strips loaded with the help of glass capillaries in 2-3 mm diameter spots were developed in cylindrical glass chambers by ascending technique. Solvent front was migrated to a constant distance of 8-10 cms.

Chemicals used in the synthetic work were BDH laboratory grade reagents. Analytical reagents were used in the chromatographic work.

RESULTS AND DISCUSSION

To look at the separation possibilities all the complexes were migrated individually on both, gel layers and paper strips, in several pure and mixture solvents. R_F values (Tables-1 & 2) obtained by migrating the complexes in their mixtures are conciding with R_F values of individually migrated spots. Interestingly, abnormally higher, migrations of almost all the complexes in benzene-pyridine mixtures having solvents ratios from 4:1 to 1:2 (v/v) in both chromatographic methods, than those in benzene or pyridine may be attributed to the substitution of chloro and/or aquo monoligands by pyridine in the coordination zone of metals.

Although, both the chromatographic (TLC and PC) methods are

TABLE - 1

QUALITATIVE SEPARATION OF COMPLEXED CATIONS OF DIFFERENT GROUPS

Metal Ions in complex mixture	Ligand in complex mixture	Spot Colour	λmax visible (nm)	TLC		PC	
				R_F	Resolving solvent	R_F	Resolving solvent
Hg(II)	DMATG	Pink gray	410	-	-	0.21	C_6H_6
Cu(II)		Dirty yellow	600			0.00	
Cd(II)		Buff	520			0.99	
Hg(II)	DEATG	Pink gray	390	0.85	MeCN	-	-
Cu(II)		Pink	490	0.02			
Cd(II)		Yellow gray	680	0.99			
Cr(III)	DMATG	Pink gray	395	0.18	C_6H_5N-C_6H_6 (1:9, v/v)	0.98	MeCN-H_2O-Proponal (3:5:1, v/v)

Fe(III)		Yellow	360	0.00		0.71	
Cr(III)	DEATG	Brown	370	0.78	AmOH	0.99	C_6H_6-$CHCl_3$ (2:1, v/v)
Fe(III)		Brown	355	0.95		0.15	or C_6H_6-Dioxan-H_2O (12:3:1, v/v)
Mn(II)	DMATG	Pink gray	525	-	-	0.15	C_6H_6-$CHCl_3$ (2:1, v/v)
Co(II)		Pink gray	375			0.00	
Ni(II)		Yellow	405			0.09	
Zn(II)		Brown gray	410			0.99	
Mn(II)	DEATG	Gray	410	0.76	BuOH	-	-
Co(II)		Gray	470	0.81			
Ni(II)		Pink gray	390	1.00			
Zn(II)		Yellow	510	0.86			

TABLE - 2

QUALITATIVE SEPARATION OF COMPLEXED Au(III) AND OTHER CATIONS

Metal ions in complex mixture	Ligand in complex mixture	TLC		PC	
		R_F	Resolving solvent	R_F	Resolving solvent
Au(III)	DMATG	0.42	Aq. BuOH-AcOH-$CHCl_3$ (5:2:5, v/v)	-	-
Cu(II)		0.00			
Ni(II)		0.85			
Zn(II)(or Cr(III))		0.93(0.95)			
Au(III)	DMATG	0.00	C_6H_5N-C_6H_6 (3:7, v/v)	-	-
Zn(II) (or Cd(II))		0.65(0.64)			
Co(II)		0.79			
Fe(III)(or Cr(III))		0.95(0.96)			
Au(III)	DMATG	-	-	0.00	CCl_4
Hg(II)				0.09	
Co(II)				0.19	
Ni(II)(or Cr(III) or Fe(III)orZn(II) or Cd(II))				0.99(0.99)	
Au(III)	DMATG	-	-	0.15	BuOH-CCl_4-MeCN (3:3:1, v/v)
Cu(II)				0.50	
Fe(III)				0.69	

Cr(III)(or Co(II)orZn(II) or Cd(II) or Hg(II))				0.90(0.97 or 0.98)	
Au(III)	DEATG	0.06	MeCN	-	-
Co(II)(or Mn(II) or Hg(II))		0.84			
Fe(III)		0.92			
Ni(II)(or Cd(II) or Zn(II) or Cr(III))		0.98(or 0.99)			
Au(III)	DEATG	0.01	AmOH	-	-
Cu(II)		0.66			
Cr(III)(or Cd(II) or Hg(II))		0.78(0.77 or 0.79)			
Mn(II)(or Co(II) or Zn(II) or Fe(III))		0.90(0.89 or 0.88 or 0.95)			
Au(III)	DEATG	-	-	0.00	CCl_4
Mn(II)				0.07	
Ni(II)(or Cd(II))				0.15(0.18)	
Cr(III)(or Fe(III) or Co(II) or Zn(II))				0.99(0.99)	
Au(III)	DEATG	-	-	0.00	C_6H_6
Hg(II)(or Fe(III))				0.09(0.10)	
Co(II)				0.20	
Cd(II)				0.99	

Spot colours of Au(III)-DMATG and Au(III)-DEATG complexes are brown and orange, and λmax values are 420 nm and 400 nm, respectivcly.

TABLE - 3

QUANTITATIVE SEPARATION OF TYPICAL MIXTURES

Complex mixture	Weight of complex applied on plate (μg)	Weight of complex recovered (μg)	Error (%)	Resolving solvent
$Hg(DEATG)Cl_2.H_2O$	10.08	10.00	-0.8	MeCN
$Cu(DEATG)Cl.(OH)$	10.08	10.00	-0.8	
$Cd(DEATG)Cl_2$	17.60	17.50	-0.6	
$Cr(DMATG)Cl_3.4H_2O$	35.07	35.00	-0.2	C_6H_5N-C_6H_6 (1:9, v/v)
$Fe(DMATG)_2Cl_3$	60.12	60.25	+0.2	
$Mn(DEATG)_2Cl_2.2H_2O$	19.84	20.00	+0.8	BuOH
$Co(DEATG)Cl_2$	30.06	30.00	-0.2	
$Ni(DEATG)_2Cl_2$	22.80	22.75	-0.2	
$Zn(DEATG)Cl_2.2H_2O$	8.80	8.75	-0.6	
$Au(DEATG)Cl_3.3H_2O$	19.84	19.75	-0.5	AmOH
$Cu(DEATG)Cl(OH)$	10.08	10.00	-0.8	
$Cr(DEATG)Cl_3.4H_2O$	30.06	30.25	+0.6	
$Fe(DEATG)_2Cl_3$	30.06	30.25	+0.6	
$Au(DEATG)Cl_3.3H_2O$	9.92	10.00	-0.8	MeCN
$Co(DEATG)Cl_2$	30.06	30.00	-0.2	
$Fe(DEATG)_2Cl_3$	60.12	60.00	-0.2	
$Ni(DEATG)_2Cl_2$	22.80	23.00	+0.9	

Formulae of complexes have been reported in reference '10'.

equally effective in the qualitative separations of transitional basic radicals of II, III and IV groups (Table-1) and various mixtures of Au(III) with Cr(III), Mn(II), Fe(III), Co(II), Ni(II), Cu(II), Zn(II), Cd(II) and Hg(II) (Table-2), TLC giving better

quantitative separations than PC was applied in the quantitative analyses of typical mixtures. Maximum quantities of complexes in their mixtures resolved by this method have been noted against them in each mixture set (Table-3). Errors in estimations evidently show the high precision of the present method of TLC.

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REGULAR CONTRIBUTED PAPERS

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INVERSE OPTIMAL FILTERING METHOD FOR THE INSTRUMENTAL SPREADING CORRECTION IN SIZE EXCLUSION CHROMATOGRAPHY

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ABSTRACT

The Kalman filter based techniques are adapted to solve the most general form of Tung's integral formula, i.e. when a non-uniform, non-symmetric calibration model is employed to correct chromatograms obtained in size exclusion chromatography from instrumental broadening errors. Through this method, the inverse smoothing of a chromatogram contaminated with measurement noise of known statistics is optimally performed by minimizing the estimation error variance. The method is numerically very "robust", improves the signal to noise ratio, provides good validation checks, and does not involve any previous parameter estimation procedure.

INTRODUCTION

Most of the methods of correction for instrumental broadening in size exclusion chromatography are based on the deterministic integral equation by Tung (1):

$$z(t) = \int_{-\infty}^{+\infty} u(\tau).g(t,\tau)\, d\tau \qquad (1)$$

where t,τ : both represent elution time or elution volume;
$z(t)$: is the baseline-corrected chromatogram;

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$g(t,\tau)$: is the unit mass (or normalized) detector response $g(t)$, for a truly monodisperse polymer species of retention time τ; and

$u(t)$: is the corrected chromatogram.

With different degrees of success, numerous techniques have been proposed for solving Eqn.(1), but most of them introduce one or both of the following simplifications into the instrumental spreading function:

a) $g(t,\tau)$ is adopted uniform, i.e. independent of the mean retention volume τ, e.g. (1,2,3,4,5,6,7); and

b) $g(t,\tau)$ is considered Gaussian, e.g. (8,9,10).

When the first simplification is adopted, the problem reduces to that of a deconvolution. In the case of the non-uniform Gaussian assumption, the variance is normally considered mean retention volume dependent. In some cases, e.g. (9,10), $u(t)$ is obtained not through a direct numerical solution of Eqn. (1), but after elaborate analytical procedures.

Among the few works that have attempted the direct solution of Eqn. (1) with no assumptions on $g(t,\tau)$ are those of Chang and Huang (11) and Ishige, Lee and Hamielec (12). According to a comparison of different techniques in (13,14), the best numerical method so far was that of (12).

The problem in hand is, in fact, a special case of the much more general one of input estimation or inverse filtering. Typically, a measurement signal must be corrected when the transducer frequency response is not flat over the whole signal frequency spectrum. For example, the inverse filtering of a ventricular pressure record is considered in (15); and the recuperation of seismic responses in oil prospection work is studied in (16, 17 and 18). In these last three publications, different adaptations of the Kalman filter (with or without smoother) were proposed and implemented to solve an inverse filtering problem.

In the present work, the feasibility of the use of inverse optimal smoothers for size exclusion chromatography broadening correction is demonstrated. The method has many characteristics in common with the mentioned works (16, 17 and 18). Nevertheless, it will be described here with some detail; with particular emphasis on the special features of the problem.

THEORY

The System Model

The application of the Kalman filter techniques requires a system description by means of a linear state-space stochastic model that, in our case, will adopt the following discrete single-input single-output form:

$$\underline{x}(k+1) = F(k)\ \underline{x}(k) + \underline{b}(k)\ w(k) \tag{2a}$$

$$z(k) = \underline{h}^T(k)\ \underline{x}(k) + v(k) = y(k) + v(k) \tag{2b}$$

where $k = 0,1,2,\ldots$: is the independent discrete time;

$\underline{x}(k)$: is the state vector;

$w(k)$ and $v(k)$: are zero-mean, Gaussian distributed white random sequences of variances $q(k)$ and $r(k)$, respectively;

$F(k)$: is, in general, a time-varying matrix; and

$\underline{b}(k), \underline{h}^T(k)$: are, in general, time-varying vectors.

The discrete stochastic version of Eqn. (1) can be written:

$$z(k) = \sum_{k_o=-\infty}^{+\infty} g(k,k_o).u(k_o) + v(k) \tag{3}$$

The time-varying calibration $g(k,k_o)$ can be considered as a set of discrete system impulse responses, with the impulses applied at times k_o. Note that in order not to introduce time shifts between the measured and the corrected chromatogram, the system must be assumed non-causal. This means that the response will start to appear before the application of the impulse, normally taken to occur at the maxima or at some mean value. Fig. 1a

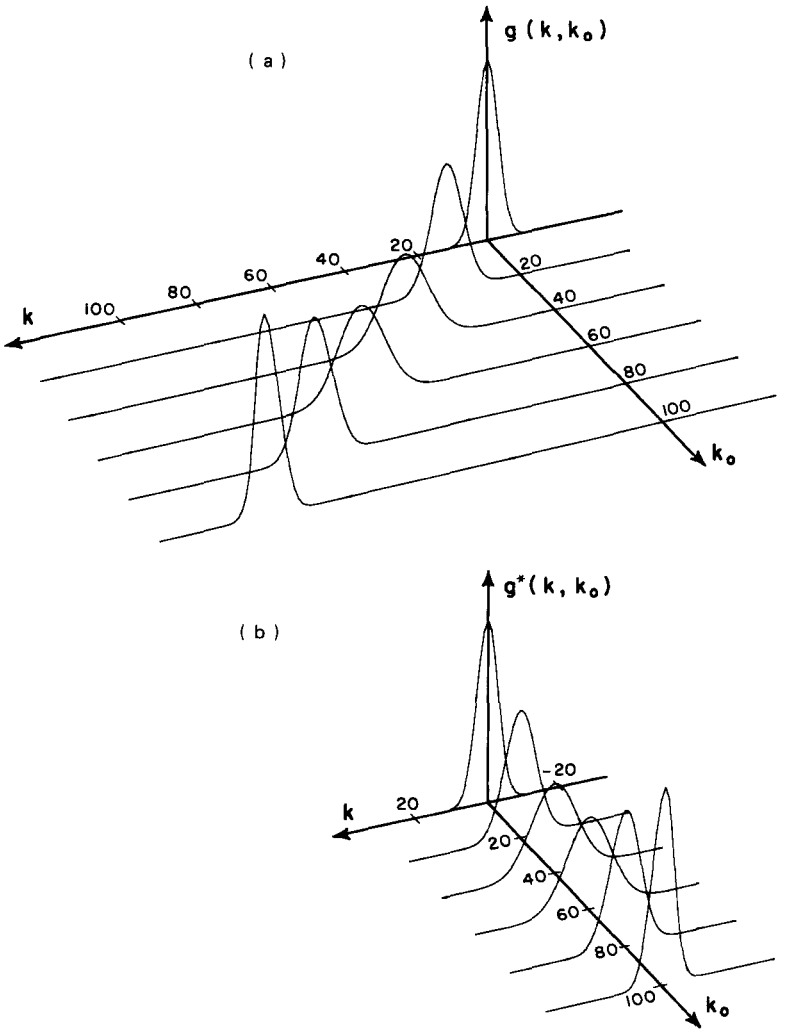

FIGURE 1 : A time-varying spreading function (a); and its corresponding g* function (b).

represents a time-varying impulse response, with the impulses applied at different k_o's . In what follows, it will be assumed that irrespective of k_o , all the responses have a finite number

of non-zero elements. Consider now some transformations to Eqn. (3) that will allow us to obtain the system state-space model in a straightforward fashion, and without calculations. As illustrated by Fig. 1b, let us first define the function $g^*(k,k_o)$, such that

$$g^*(k-k_o,k_o) = g(k,k_o) \tag{4}$$

Call $-c$ and d the lower and upper limit of k with non-zero values of $g^*(k,k_o)$, respectively. Then Eqn. (3) yields:

$$z(k) = \sum_{k_o=k-c}^{k_o=k+d} g^*(k-k_o,k_o).u(k_o) + v(k) \tag{5}$$

and with $i=k-k_o$,

$$z(k) = \sum_{i=-c}^{d} g^*(i,k-i).u(k-i) + v(k) \tag{6}$$

The lower part of Fig. 2 shows a non-causal flow-diagram representation of Eqn. (6), where p^{-1} indicates the backshift operator such that $p^{-1}[u(k)] = u(k-1)$. The instantaneous set of weights g^* of Fig. 2 can be obtained from the successive row vectors $\underline{h}^T(k)$ of the following matrix H^*, where the rows $\underline{h}^T(k)$ extend at least to the calibration limits of the chromatographic column set.

$$H^* = \begin{bmatrix} \cdot & \cdot & \cdots & \cdot & \cdot \\ \cdot & \cdot & \cdots & \cdot & g^*(-c,k-d) \\ \cdot & \cdot & \cdots & g^*(-c+1,k-d) & g^*(-c,k-d+1) \\ \cdot & \cdot & & \cdot & \cdot \\ \cdot & \cdot & \cdot & \cdot & \cdot \\ g^*(d,k-d-1) & g^*(d-1,k-d) & \cdots & g^*(-c+1,k+c-2) & g^*(-c,k+c-1) \\ g^*(d,k-d) & g^*(d-1,k-d+1) & \cdots & g^*(-c+1,k+c-1) & g^*(-c,k+c) \\ g^*(d,k-d+1) & g^*(d-1,k-d+2) & \cdots & g^*(-c+1,k+c) & g^*(-c,k+c+1) \\ \cdot & \cdot & & \cdot & \cdot \\ \cdot & \cdot & & \cdot & \cdot \end{bmatrix} \tag{7a}$$

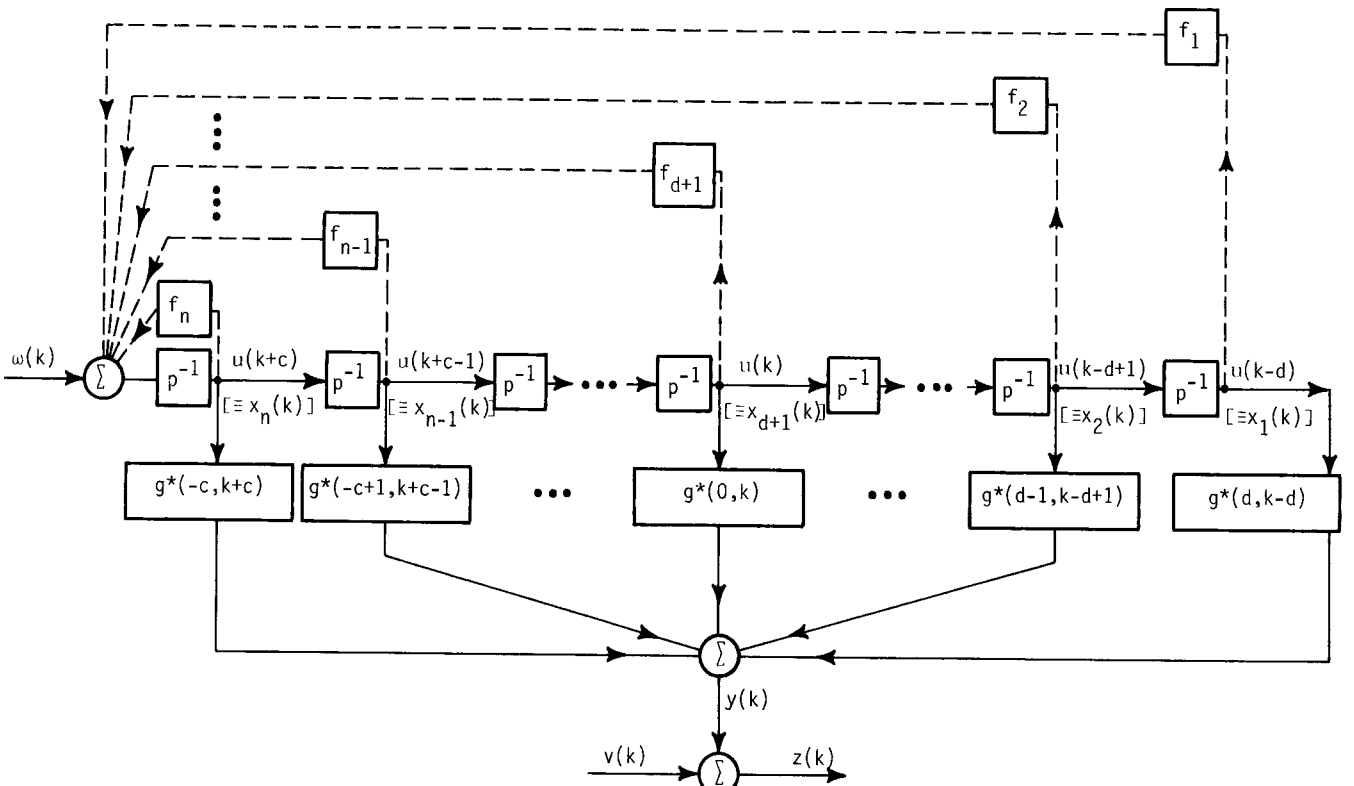

FIGURE 2 : System block-diagram from which the required state-space representation is obtained.

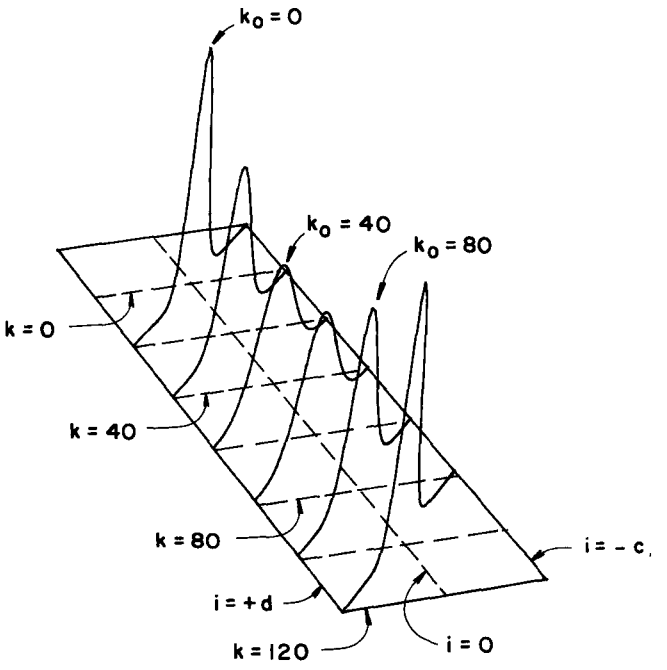

FIGURE 3 : H* matrix corresponding to the spreading function of Fig. 1, showing its 45° "diagonals".

or:

$$H^* = \begin{bmatrix} \vdots \\ \underline{h}^T(k-1) \\ \underline{h}^T(k) \\ \underline{h}^T(k+1) \\ \vdots \end{bmatrix} = \begin{bmatrix} \vdots & \vdots & & \vdots \\ h_1(k-1) & h_2(k-1) & \cdots & h_n(k-1) \\ h_1(k) & h_2(k) & \cdots & h_n(k) \\ h_1(k+1) & h_2(k+1) & \cdots & h_n(k+1) \\ \vdots & \vdots & & \vdots \end{bmatrix} \tag{7b}$$

Note that the successive 45° "diagonals" of H* are made up of the elements of the individual impulse responses. This is illustrated in Fig. 3. Let us define now $n = c+d+1$ state variables x_i $(i=1,\ldots,n)$ to coincide with the successive values of the in-

put, as indicated in Fig. 2. The system model output equation, which is equivalent to Eqn. (2b), may thus be written:

$$z(k) = \underline{h}^T(k).\underline{x}(k) + v(k) \tag{8}$$

where $\underline{h}^T(k)$ is time-varying according to Eqn. (7).

The state equation itself allows the specification of the spectral characteristics that can be simultaneously assigned to all state variables, and consequently to $u(k)$ which coincides with $x_{d+1}(k)$. These spectral characteristics are imposed by filtering the white noise input $w(k)$ through an autoregressive operator, as indicated in the upper half of Fig. 2. Note that the order of this autoregressive operator is equal to the system order (which is normally relatively high). For this reason, a very satisfactory pre-filtering operation could, if desired, be implemented. The state equation, which is equivalent to Eqn. (2a), will have the following structure:

$$\begin{bmatrix} x_1(k+1) \\ x_2(k+1) \\ \vdots \\ x_{n-1}(k+1) \\ x_n(k+1) \end{bmatrix} = \begin{bmatrix} 0 & 1 & 0 & \cdots & 0 & 0 \\ 0 & 0 & 1 & \cdots & 0 & 0 \\ \vdots & \vdots & \vdots & & \vdots & \vdots \\ 0 & 0 & 0 & & 0 & 1 \\ f_1 & f_2 & f_3 & \cdots & f_{n-1} & f_n \end{bmatrix} \begin{bmatrix} x_1(k) \\ x_2(k) \\ \vdots \\ x_{n-1}(k) \\ x_n(k) \end{bmatrix} + \begin{bmatrix} 0 \\ 0 \\ \vdots \\ 0 \\ 1 \end{bmatrix} w(k) \tag{9}$$

Note that matrices F and $\underline{b}$ are constant and in the controllable canonical form. The last row of F (that we shall call vector $\underline{f}$), allows the specification of the stated spectral characteristics of $u(k)$. When $\underline{f} = \underline{0}$, then $u(k)$ will be a white noise. The inclusion of non-zero elements in $\underline{f}$ will, in general, transform $u(k)$ into a "coloured" random sequence.

The Inverse Optimal Smoother

Under the assumption that the system is exactly represented by Eqns. (2), the "best" linear estimate of the state $\underline{x}(k)$ that

can be obtained from the noisy measurements $\{z(k)\ ;\ 0 < k < M\}$ is given by the output $\hat{\underline{x}}(k/M)$ of the optimal smoother. With $\hat{\underline{x}}(k/M)$ we shall indicate the "conditional estimate of $\underline{x}$ at time k given the measurements z up to time M". For general references see (19,20). The smoother is optimal in the sense that at each time k, the mean square error associated with the estimate $\hat{\underline{x}}(k/M)$:

$$E\{[\underline{x}(k) - \hat{\underline{x}}(k/M)]^T[\underline{x}(k) - \hat{\underline{x}}(k/M)]\}$$

is smaller than that achieved by any other linear estimator. Furthermore, if we also make the fairly common assumption that the initial state and the two random sequences satisfy Gaussian probability distributions, then the mean square error is less than that achieved by any other estimator, linear or non-linear. Fig. 4 illustrates the optimal smoother structure. The first stage corresponds to the Kalman filter and includes:

a) The discrete Riccati equation:

$$\dot{\Sigma}(k+1/k) = F\ \Sigma(k/k)\ F^T + \underline{b}\ q(k)\ \underline{b}^T \tag{10a}$$

$$\Sigma(k/k) = \Sigma(k/k-1)\ \{\ I - \underline{h}(k)\ [\underline{h}^T(k)\ \Sigma(k+1/k)\ \underline{h}(k) + r(k)]^{-1}\ \underline{h}^T(k)\ \Sigma^T(k/k-1)\} \tag{10b}$$

where: $\Sigma(k/k)$ is the estimation error covariance matrix, i.e.

$$\Sigma(k/k) = E\ \{[\underline{x}(k) - \hat{\underline{x}}(k/k)]\ [\underline{x}(k) - \hat{\underline{x}}(k/k)]^T\} \tag{10c}$$

b) The remaining algorithm:

$$\underline{\ell}(k+1) = \Sigma(k+1/k)\ \underline{h}(k+1)\ [\underline{h}^T(k+1)\ \Sigma(k+1/k)\ \underline{h}(k+1) + r(k+1)]^{-1} \tag{11a}$$

$$\tilde{z}(k+1) = z(k+1) - \underline{h}^T(k+1)\ F\ \hat{\underline{x}}(k/k) \tag{11b}$$

$$\hat{\underline{x}}(k+1/k+1) = F\ \hat{\underline{x}}(k/k) + \underline{\ell}(k+1)\ \tilde{z}(k+1) \tag{11c}$$

with

$$\hat{\underline{x}}(0/0) = \bar{\underline{x}}_o + P_o\ \underline{h}(0)\ [\underline{h}^T(0)\ P_o\ \underline{h}(0) + r(0)]^{-1}\ [z(0) - \underline{h}^T(0)\ \bar{\underline{x}}_o] \tag{11d}$$

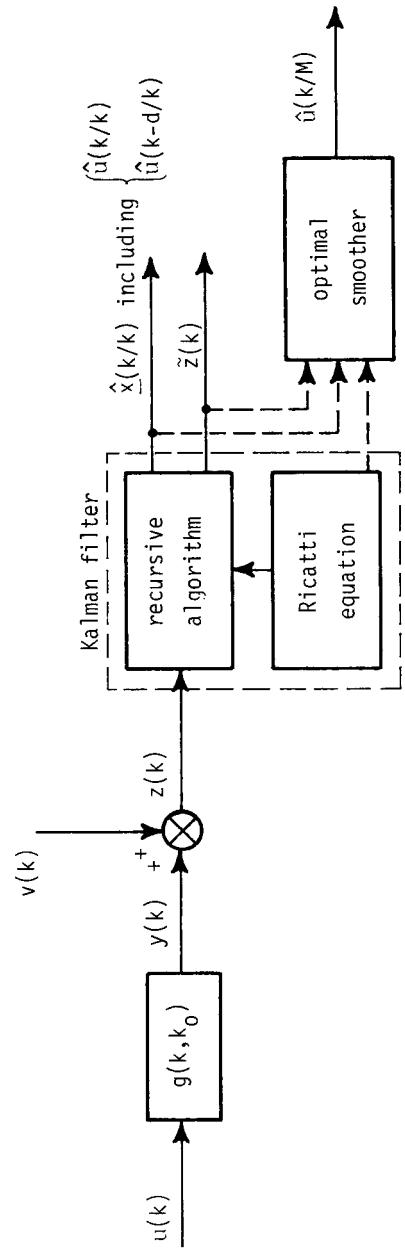

FIGURE 4 : Schematic representation of the optimal filter-smoother.

where: $\bar{\underline{x}}_o$ and P_o : are the mean and covariance matrix of $\underline{x}(0)$, respectively;

$\underline{\ell}(k+1)$: is the filter gain; and

$\tilde{z}(k+1)$: is the innovations sequence.

In the second stage, the fixed-interval smoother algorithm taken from (19):

$$\underline{s}(k) = \underline{h}(k)\ r^{-1}(k)\ \underline{h}^T(k) \tag{12a}$$

$$\underline{\lambda}(k-1) = \left[I - \Sigma(k/k)\ \underline{s}(k)\right]^T \left[F^T(k)\ \underline{\lambda}(k) - \underline{h}(k)\ r^{-1}(k)\ \tilde{z}(k)\right] \tag{12b}$$

$$\hat{\underline{x}}(k/M) = \hat{\underline{x}}(k/k) - \Sigma(k/k)\ F^T(k)\ \underline{\lambda}(k) \tag{12c}$$

with

$$\underline{\lambda}(M) = \underline{0} \tag{12d}$$

is solved backwards in time.

Note that even though the filter section provides the estimate $\hat{\underline{x}}(k/k)$, and the smoother section the estimate $\hat{\underline{x}}(k/M)$, we are really only interested in the element (d+1) of these vectors, i.e.

$$\hat{x}_{d+1}(k/k) = \hat{u}(k/k) \tag{13a}$$

or

$$\hat{x}_{d+1}(k/M) = \hat{u}(k/M) \tag{13b}$$

Clearly, $\hat{u}(k/M)$ is a better estimate than $\hat{u}(k/k)$, but in the first case, a higher computational cost must be paid. The variance of the estimation error associated with $\hat{u}(k/k)$ is the element (d+1,d+1) of $\Sigma(k/k)$, and is automatically provided by the filter. The variance of the estimation error corresponding to $\hat{u}(k/M)$ must be especially calculated however [see (19)], but is always lower than that of the filter.

Because of the very special structure of the system model, the filter section inherently includes a suboptimal smoother: the so called fixed-lag smoother. In fact, if this lag is limited to

d, a suboptimal estimate (better than $\hat{u}(k/k)$) is produced, without extra calculations. In effect, since

$$x_1(k+d) = x_2(k+d-1) = \ldots = x_d(k+1) = x_{d+1}(k) \tag{14}$$

then,

$$\hat{x}_1(k+d/k+d) = \hat{x}_{d+1}(k/k+d) \tag{15}$$

and therefore,

$$\hat{x}_1(k+d/k+d) = \hat{u}(k/k+d) \tag{16}$$

In other words, the filter estimate $\hat{x}_1(k+d/k+d)$ is the fixed-lag smoothed estimate of $u(k)$, and the corresponding estimation error variance is element $(1,1)$ of $\Sigma(k+d/k+d)$. Note also that by artificially increasing the system dimension n (with an appropriate inclusion of zeroes on the left hand side of matrix H^*), the suboptimal smoother lag is also increased. When $c > d$, the lag can be increased to c by filtering $z(k)$ backwards in time.

The Algorithm Adjustment

The following parameters must be set in the given algorithm:

a) *The last row of matrix F (row vector $\underline{f}$)*

Only two cases will be considered: $\underline{f} = \underline{0}$ and $\underline{f} = (0\ 0 \ldots 1)$. In the first case, $u(k)$ is assumed a white noise process; in the second a "random walk" process. By assuming $u(k)$ a white noise, the greatest flexibility in its estimation is provided; and one could, for example, recuperate delta functions when analyzing monodisperse samples. When a polidisperse sample is analyzed, then a smoothing effect (that in general improves the numerical results) may be obtained if $u(k)$ is considered a random walk. As explained below, the other advantage of assuming $\underline{f} = (0\ 0 \ldots 1)$ is related to the mean of the innovations sequence.

b) *The initial conditions $\underline{\bar{x}}_o$ and P_o*

In practice, it has been found adequate to choose $\underline{\bar{x}}_o = \underline{0}$ and $P_o = I$; and to solve the Riccati equation with $\underline{h}^T(0)$ until

steady state conditions are reached. Then, the chromatogram above the baseline may be directly processed. In the examples of the following section, this procedure was adopted in all cases.

c) *The measurement noise variance r(k)*

This value can be estimated from the noise that normally contaminates the detector baseline before and after the polymer peak. It will be hereafter considered constant, of value r .

d) *The input variance q(k)*

Consider first some ways of estimating q(k) when $\underline{f} = \underline{0}$. In this case, the state variables are assumed white processes with a variance:

$$\sigma^2_{x_{i(k)}} = q(k-n-1+i) \quad ; \quad (i = 1,2,\ldots,n) \tag{18}$$

($\sigma_{a(k)}$ will denote the variance of a(k), and $\overline{a}(k)$ its mean value). It is also easy to show that:

$$\sigma^2_{z(k)} = \sum_{i=1}^{n} h^2_i(k)\, q(k-n-1+i) + r \tag{19}$$

Eqn. (19) has no solution because $\sigma^2_{z(k)}$ is unknown. Even if this function could be estimated, Eqn. (19) is of the same type of Eqn. (3) (which we are trying to solve), and therefore is still of no practical use unless some simplifications are added. The simplest situation is to consider w(k) stationary, and the spreading function uniform. Under these circumstances, an estimate for a constant value of q may be obtained from Eqn. (19) as follows:

$$q = \frac{\sigma^2_z - r}{\sum_{i=1}^{n} h^2_i} \tag{20a}$$

with

$$\sigma^2_z = \frac{\sum_{i=1}^{M} [z(k)]^2}{(M-1)} \tag{20b}$$

Eqn. (20) in general overestimates q because $u(k)$ is a highly correlated sequence instead of a white noise. Nevertheless, Eqn. (20) may provide an initial guess of q that can be useful if properly handled. In case of a non-uniform spreading function, the denominator of Eqn. (20a) could correspond to the impulse response at an intermediate retention time.

Consider now $w(k)$ non-stationary (i.e. q variable with k). This assumption has been found essential for particularly ill-conditioned cases. In fact, if r is accurately estimated, the optimal performance of the filter-smoother is produced when the exact $q(k)$ is utilized. Note that $\underline{f} = \underline{0}$ implies $w(k) = u(k+c+1)$. Thus, if $u(k+c+1)$ can be somehow estimated, then one may simply write

$$q(k) = [\hat{u}(k+c+1)]^2 \tag{21}$$

For example, $\hat{u}(k+c+1)$ in Eqn. (21) could be the smoother solution obtained with a constant q. Alternatively, the following approximate formula (that may be also derived from Eqn. (19) assuming no spreading), has been found to provide satisfactory results:

$$q(k) = C\,[z(k+c+1)]^2 \tag{22}$$

where C is an appropriately chosen positive constant. For $C = 1$, $q(k)$ will be, in principle, underestimated for $u(k) < z(k)$ and overestimated when $u(k) > z(k)$. The estimates of $q(k)$ based on Eqns. (21) or (22) have little statistical significance because they are obtained from single values of $u(k+c+1)$ or $z(k+c+1)$. This means that sudden changes in these functions will be reflected on the estimate $q(k)$. A simple remedy is to smooth $u(k+c+1)$ or $z(k+c+1)$ through an averaging filter in order to keep the shape of these curves while eliminating the undesirable variations.

Consider now the estimation of $q(k)$ when $\underline{f} = (0\ 0 \ldots 1)$. It may be shown that when $w(k)$ is assumed stationary, the equivalent formula to Eqn. (20) is:

$$q = \frac{\sigma^2_{\Delta z(k)} - 2r}{\sum_{i=1}^{n} h_i^2} \tag{23a}$$

with

$$\sigma^2_{\Delta z(k)} = \frac{\sum_{i=1}^{M} [\Delta z(k)]^2}{(M-1)} \tag{23b}$$

A simple expression for $q(k)$ can be developed from the fact that $w(k) = u(k+c+1) - u(k+c) = \Delta u(k+c+1)$, and therefore:

$$q(k) = [\Delta\hat{u}(k+c+1)]^2 \tag{24}$$

For the reasons given above, but particularly in this case, it is preferable to employ averaged versions of $\Delta\hat{u}^2$ instead of $\Delta\hat{u}^2$ as such. The following equation was found adequate:

$$q(k) = C' \frac{\sum_{i=-a}^{a} [\Delta\hat{u}(k+c+1+i)]^2}{(2a+1)} \tag{25}$$

where C' is an adjustable gain and $(2a+1)$ is the number of points averaged at each step. Clearly, here again, an iterative procedure that estimates $q(k)$ from $\Delta\hat{u}(k)$, and then $\hat{u}(k)$ and $\Delta\hat{u}(k)$ from the filter-smoother, will normally provide the best results. The results of the filter-smoother are not too sensitive to its adjustment, and relatively crude estimates of the shape of $q(k)$ are normally sufficient for satisfactory results. For example, in certain cases, Eqn. (25) provides a smooth $q(k)$, with a shape which is similar to that of $z(k)$. In such cases, and even when $\underline{f} = (0\ 0 \ldots 1)$, an estimate of $q(k)$ may be directly obtained from the simpler relationship of Eqn. (22). This simplification is conveniently utilized in Examples 2 and 3 below.

Even though the covariance matrices $\Sigma(k/k-1)$ and $\Sigma(k/k)$ depend on the individual values of r and $q(k)$, the filter or

the smoother estimate of $u(k)$ is a function of the $q(k)/r$ ratio only. In a very ill-conditioned problem, the results become sensitive to deviations of this ratio from its correct value. In more relaxed situations however, relative gross errors in $q(k)/r$ can be absorbed with still good results.

The solution validation

The solution checks may be classified into two main groups: those which are common to any other input estimation technique, and those specific to the method. The obvious checks in the first group are: a) the solution must be non-negative; b) by processing $\hat{u}(k)$ through the system spreading function, the noise-free measured function should be recuperated; and c) the area under the corrected chromatogram must be equal to that of the measured curve. It should be emphasized that the check under b) is only a necessary (but not a sufficient) condition for good results; the reason being the algorithmic singularity of Eqns. (1) or (3). This implies that there are, in principle, infinite possible numerical solutions $\hat{u}(k)$ that can recover $y(k)$. With regards to the check under c), the area under the corrected curve will be smaller than that of the original, only when the ratio $q(k)/r$ is grossly undervalued. With overvalues or moderate undervalues of $q(k)/r$, then numerically meaningless discrepancies are observed.

The checks which are specific to the method are all based on the analysis of the innovations, that ideally should be zero-mean, Gaussian white sequences. Furthermore, the observed innovations should match the corresponding time-varying variance estimated through the filter:

$$\sigma^2_{\tilde{z}(k)} = \underline{h}^T(k)\ \Sigma(k/k-1)\ \underline{h}(k) + r \qquad (26)$$

Note that this last quantity depends again on the individual values of $q(k)$ and r. The filter results may be optimized by analyzing the innovations (and their estimated variances) under different adjustments. The innovations mean will, in general, be closer to zero with $\underline{f} = (0\ 0 \ldots 1)$ than with $\underline{f} = \underline{0}$. This may be

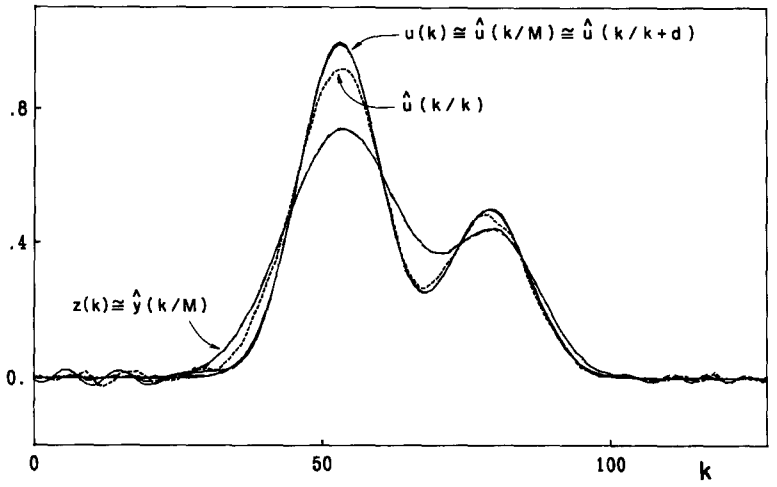

FIGURE 5 : Example 1; original curves and "best" solution with the correct constant ratio q/r = 100.

explained by the offset elimination effect that occurs when integration is incorporated into a closed loop.

EXAMPLES OF APPLICATION

Three applications of the technique will be considered. While the first two examples are synthetic, the third is based on real experimental data. All three examples were solved by means of a VAX 11/780 computer.

Example 1

By processing the curve $u(k)$ shown in Fig. 5 through a time-varying filter defined by the set of impulse responses of Fig. 1a, a noise-free chromatogram $y(k)$ is obtained. This curve was then corrupted by a Gaussian white noise of a relatively low variance (10^{-5}), to provide $z(k)$. Taking into account only the section of this series above the baseline, and defining the signal to noise ratio SNR as:

$$SNR = \frac{\sum_{k=1}^{M} z^2(k)}{(M-1)\,r} \tag{27}$$

one obtains, in this case, $SNR \cong 17400 \cong 132^2$. Throughout this Example $\underline{f} = (0\ 0 \ldots 1)$, and for simplicity a constant q will be adopted even though better results can, in principle, be obtained with a variable $q(k)$. Clearly, the best estimate for r is 10^{-5} . The best estimate of q may be obtained from:

$$q = \frac{1}{(M-1)} \sum_{k=1}^{M} [\Delta u(k)]^2 \tag{28}$$

Calculating this quantity for the $\Delta u(k)$ values above the baseline, 0.001 is obtained. Thus, the best q/r is 100. Note that if estimated through Eqn. (23), a value of q approximately 10-fold larger would have been obtained. The results of the filter-smoother when the best values for q and r are adopted are also shown in Fig. 5. While the filter estimate $\hat{u}(k/k)$ fails to reproduce the original curve, both the fixed-lag smoother output $\hat{u}(k/k+d)$ and the fixed-interval smoother output $\hat{u}(k/M)$ are practically overlapped with $u(k)$. The innovations corresponding to this case are represented in Fig. 6d, together with the estimated $\pm\sigma_{\tilde{z}(k)}$ limits. Ideally, the innovations should lie within these limits for approximately two thirds of the time, and this is roughly the case in Fig. 6d. The innovations sequence $\tilde{z}(k)$ is theoretically zero-mean Gaussian white, but its variance is time-varying and therefore $\tilde{z}(k)$ is non-stationary. In spite of this fact, it was found useful to calculate the sequence sample variance $\sigma_{\tilde{z}}^2$, the autocorrelation function and the power spectrum. Clearly, this approximation will not be valid when $\tilde{z}(k)$ is highly non-stationary. Figs. 6e and f illustrate the previously mentioned statistics. In the case of the power spectrum, the highest frequency shown corresponds to one half of the sampling frequency. Both the autocorrelation and the power spectrum show some low fre-

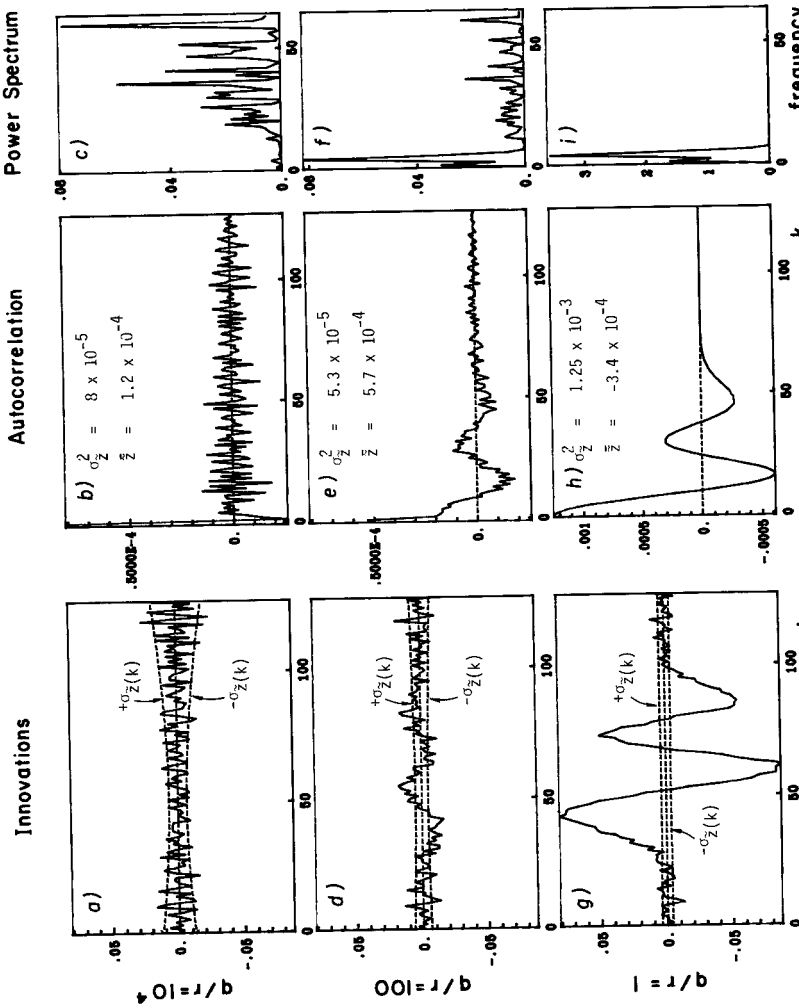

FIGURE 6 : Example 1; Innovations analysis corresponding to an overvalued q/r ratio (a,b,c), to the correct q/r ratio (d,e,f) and to an undervalued q/r ratio (g,h,i).

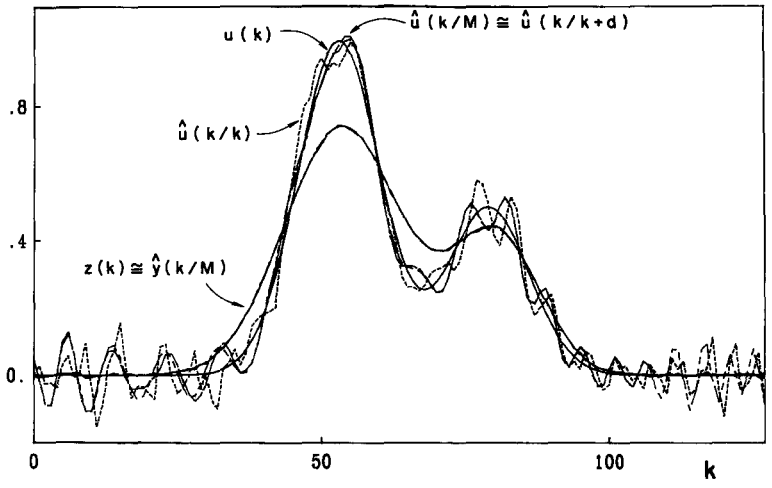

FIGURE 7 : Example 1; solution for $q/r = 10^4$.

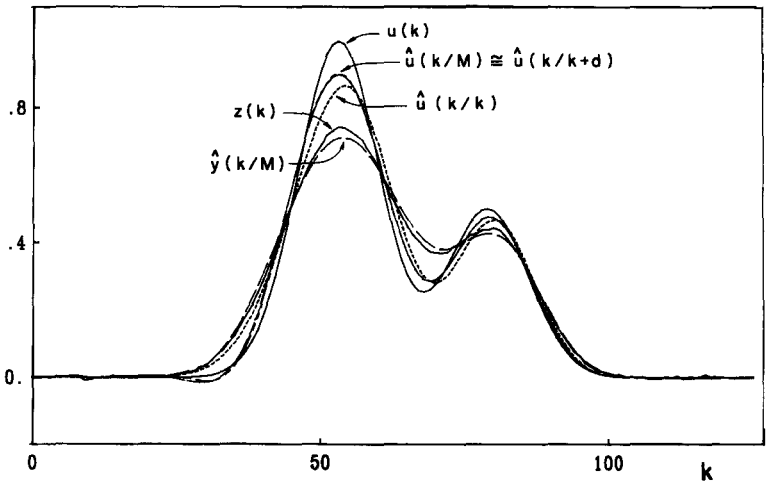

FIGURE 8 : Example 1; solution for $q/r = 1$.

quency oscillations. This, and the fact that the smoother estimate is satisfactory while the filter estimate is not indicate that some useful information is still remaining in the innovations sequence.

For illustrative purposes, the problem was solved again with the same value of r, but with erroneous estimates of q. In Figs. 7, 6a, 6b, and 6c, $q = 0.1$ (or $q/r = 10000$), while in Figs. 8, 6g, 6h, and 6i, $q = 10^{-5}$ (or $q/r = 1$). Note the following:

i) In both cases, the solutions are inadequate, but while $y(k/M)$ practically coincides with $z(k)$ when $q = 0.1$, this same function is crudely off those values if $q = 10^{-5}$.

ii) When q is overvalued, the innovations are less correlated at high lags than if the best value of q is employed. The opposite occurs at low lags however, and the overall variance of $\tilde{z}(k)$ is finally higher than before. If q is under valued, the situation is clearly worse at all lags.

iii) The standard deviation $\sigma_{\tilde{z}(k)}$ is overestimated if q is overvalued, and underestimated when undervalued.

iv) The high frequency components of the innovations are dominant if q is overestimated. Conversely, there is a low frequency components dominance when q is underestimated.

v) The percentages of variation of the areas under the corrected chromatograms with respect to those under the measured curves are -0.02%, -0.1% and -0.25% when $q = 0.1$, 0.001 and 10^{-5}, respectively.

Example 2

This Example was first suggested by Chang and Huang (6), and attempted later on by Hamielec and co-workers (12). The problem is illustrated in Fig. 9, which represents the following: $u(k)$, the uniform spreading function $g(k)$, the broadened curve $z(k)$ and the recuperated $u(k)$ by method 2 proposed in (12). Note that while $g(k)$ was generated from an analytical expression, and $z(k)$

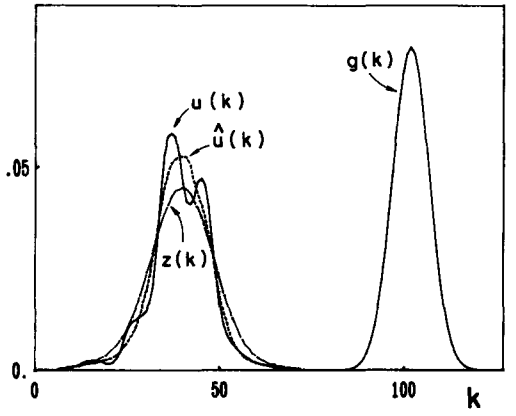

FIGURE 9 : Example 2; [after Hamielec and co-workers (12)].

was reproduced from a table of numbers given in (12), u(k) and u(k) were obtained by digitizing their graphical representations. For this reason, minor distorsions in these last two curves are to be expected. The solution $\hat{u}(k)$ shown in Fig 9 is practically coincident with that of (6), and with that of method 1 in (12). Clearly, these techniques are not able to appropriately recover the double-peaked input.

Consider now the solution via the present method. Assuming that the integer values of the table for z(k) are all accurate to the last digit, then one may interpret those numbers as contaminated by a noise v(k) of a uniform probability density function with limits at ±0.5. In this case, $\sigma_v^2 = 1/12$ and we adopt $r = 0.1$. The limits of the finite spreading function were taken at $-c = d = 20$. Beyond these values, the spreading function is below 10^{-3} .

As a first attempt, one could try to solve this Example through a constant q obtained by minimizing the variance of the innovations sequence. The solution $\hat{u}(k/M)$ is not shown here, but is very similar to that of Fig. 9, however. For better results, a variable q(k) must be adopted, and Fig. 10 illustrates this sit-

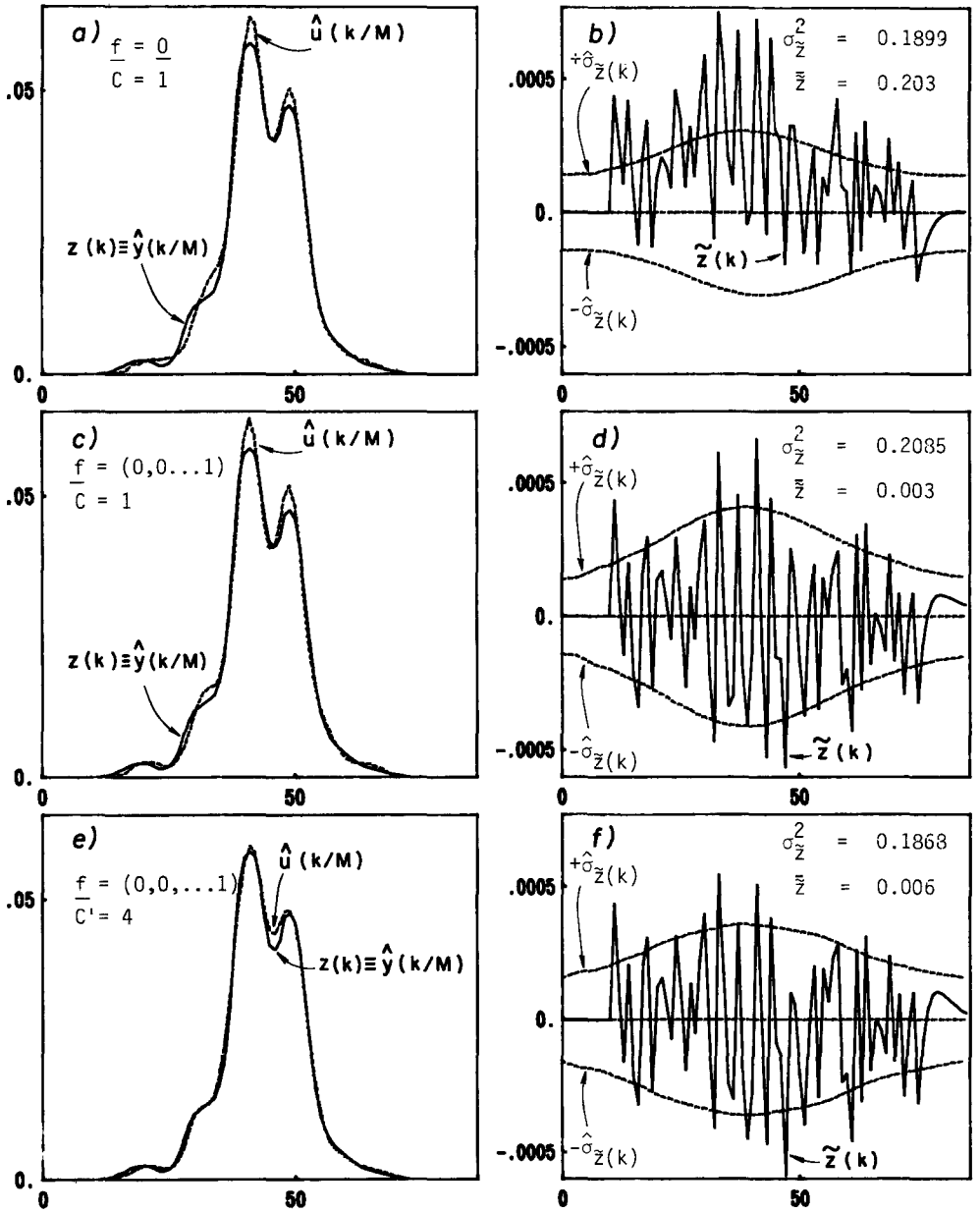

FIGURE 10 : Example 2; solutions considering u(k) white (a,b); considering u(k) a random walk sequence (c,d); and obtained through a two-step procedure (e,f).

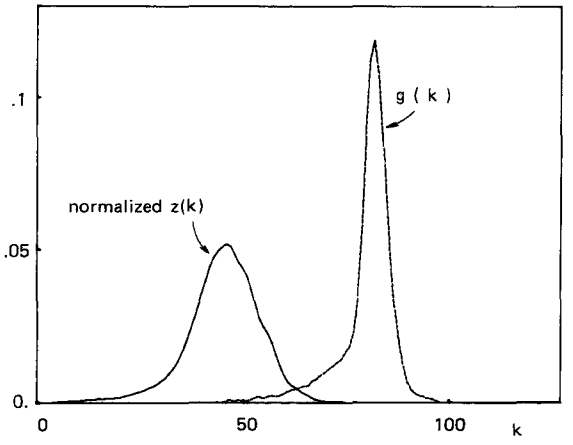

FIGURE 11 : Example 3; experimental chromatogram and spreading function.

uation. Note first that (even though not shown), all three solutions represented in Fig. 10, as well as that previously mentioned with a constant q, manage to recuperate z(k) without appreciable error.

The solution of Fig. 10a and 10b was obtained adopting $\underline{f} = \underline{0}$ and calculating q(k) through Eqn. (22) with C = 1. Clearly, the peaks of $\hat{u}(k/M)$ overpass those of u(k), and the innovations mean exhibits a certain bias. In Figs. 10c and 10d, Eqn. (22) is used again (with C = 1), in spite of the fact that in this case, $\underline{f}$ = (0 0...1). The result is similar to the previous, but now the innovations mean is very close to zero. Figs 10e and 10f were obtained through the following two-step procedure: i) based on the estimate of u(k) found in Fig. 10a, Eqn. (25) with C' = 4 was employed to estimate q(k); and ii) with this estimate, the smoother was run again with $\underline{f}$ = (0 0...1) to provide the shown results. Clearly, this solution is very acceptable. The corresponding innovations have a near zero mean, and the lowest sample variance of all three cases.

Example 3

Curve $z(k)$ in Fig. 11 represents the chromatogram of a PS standard of molecular weight $M_w = 525$, when fractionated through an A-802 Shodex column mounted on a Series 3-B Perkin Elmer liquid chromatograph. The chromatogram of pure benzene $g(k)$ is adopted as the uniform spreading function. The polymer sample is expected to be integrated by the first PS oligomers, with preponderance of the pentamer. Ideally therefore, delta functions ought to be recuperated, with the highest peak at a molecular weight of 520.

Three possible solutions to this problem are found in Fig. 12. In all three cases, the solutions accurately recuperate the measured chromatogram, r was estimated $5\text{x}10^{-5}$ and $q(k)$ was obtained through Eqn. (22). Figs. 12a and 12b show a quite acceptable solution, where all oligomers from dimer to hexamer are now clearly separated. The adjustments employed in this first solution are: $\underline{f} = \underline{0}$, and a C gain of 1.25 for Eqn. (22).

With C gains higher than 1.25, negative values in $\hat{u}(k/M)$ are produced. This situation is represented by Figs. 12c and d, where $\underline{f} = \underline{0}$ but $C = 75$. This value of C will clearly generate an overestimated $q(k)$. In this case, and in spite of the negative values in $\hat{u}(k/M)$, the low molecular weight peaks appear to be better separated, and two extra higher molecular weight components seem to be also detected. The innovations sample mean and variance indicated in Fig. 12d are lower than in Fig. 12b, but the estimated $\pm\sigma_{\tilde{z}(k)}$ limits confirm that this solution is not adequate.

Figs. 12e and f show a solution which is very similar to that of Figs. 12a and b; but in this case $\underline{f} = (0\ 0 \ldots 1)$ and $C = 2$ were adopted. Here again, by increasing C, negative values are also produced.

The estimates of Figs. 12a and 12e are the best obtained. The oligomers are clearly separated, and their retention times could be used for a more accurate column calibration. As expected, those retention times are, with good approximation, linearly re-

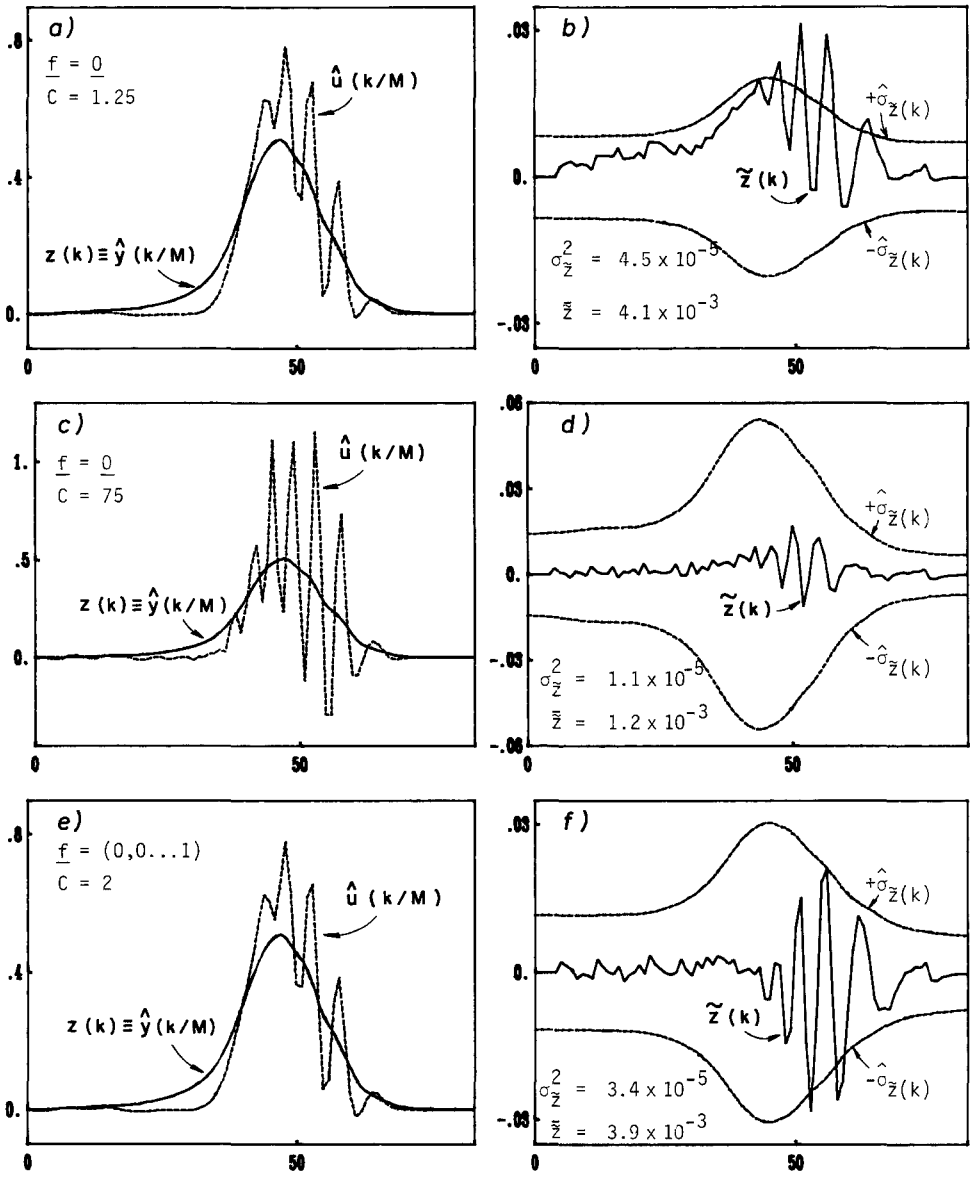

FIGURE 12 : Example 3; solutions considering u(k) white with the appropriate C gain (a,b); with C overvalued (c,d); and considering u(k) a random walk sequence with the appropriate C (e,f).

lated to the logarithm of their molecular weights.

The results of Fig. 12 seem to indicate that the lower molecular weight species are better separated than the higher. This bias is also reflected by the fact that in all cases, the innovations adequately match their $\pm\sigma_{\tilde{z}(k)}$ limits only on the right hand side of the chromatogram. A possible explanation to this effect is that the spreading function is, in reality, non-uniform. In this case, the given $g(k)$ is only accurate at the low molecular weight end. If as predicted by (21,22), the instrumental broadening increased towards intermediate retention volumes, then the correction would be more pronounced on the left hand side of the curve, and the bias would tend to be compensated.

CONCLUSIONS

The proposed technique has proved very powerful with both synthetic and real examples, and could be clearly extended to corrections in hydrodynamic chromatography (13,14). The results of Example 2, are better than those of other techniques. The computer program was written in FORTRAN 77 for a VAX 11/780 computer, and is available from the authors.

The main advantages of the method are: i) it is numerically very 'robust', thus allowing the solution of particularly ill-conditioned problems; ii) because a stochastic version of Eqn. (1) is employed, all 'a priori' information on the baseline noise may be conveniently employed; iii) under certain ideallized conditions, the solution is optimal from the standpoint of the estimation error variance; iv) the state-space representation of the system spreading function is obtained without calculations, thus involving no assumptions about the shapes of the calibration curves; v) the innovations analysis provides very powerful solution checks, and vi) the measurement noise is eliminated from the corrected chromatogram and the SNR of $y(k)$ is normally higher than that of $z(k)$.

The main drawback of the method deals with the relatively significant computational cost involved. With regards to memory, not only the system matrix H^* and the measured and calculated sequences must be stored, but (more important) the whole set of matrices $\Sigma(k/k)$. The computation time is also relatively high. For example, to solve an 80 point chromatogram with a calibration curve of 55 points (Example 3 above), approximately 12 minutes are required for the complete calculation, with half that time insumed in the fixed-interval smoother stage. The computation time increases with approximately the square of the system order n; and in the Kalman filter section, the main computational burden is related to the solution of the discrete Riccati equation. Note that if the same calibration $g(k,k_o)$ is to be repeatedly used, and the variances q and r are maintained constant, then this equation may be solved only once. Furthermore, when q and r are constant and the spreading function is uniform, then only the steady state solution of that equation is required.

In all cases considered, the results of the fixed-lag smoother when the lag was made equal to c or d were very similar to those of the fixed-interval smoother. Clearly, if the fixed-lag smoother results are adequate, not only the computation time is approximately halved, but also the storage of the covariance matrices set is no longer required.

In this work, the state variables [and consequently $u(k)$] were assumed white processes if $\underline{f} = \underline{0}$, and random walk processes when $\underline{f} = (0\ 0 \ldots 1)$. Both assumptions were seen to provide satisfactory results, but the innovations mean was in all cases smaller with $\underline{f} = (0\ 0 \ldots 1)$. As a counterpart in this last case, the estimation of $q(k)$ becomes more complex. Theoretically, the best results would require a specification of vector $\underline{f}$ that included all available information about $u(k)$. This was found not necessary in the processing of chromatograms, but in a different context, interesting efforts have been done in this direction although mainly dealing with time-invariant systems (23,24). In a

way, an appropriate estimation of $q(k)$ compensates a rather crude estimation of $\underline{f}$.

As explained above, the main advantage of the state-space time-varying model proposed is that no calculations for its development are required. The high order of the model so produced, makes the smoother computation a relatively arduous task, however. Alternatively, parameter estimation procedures could be employed to identify the system through lower order models. This identification stage could be implemented off-line, and then repeatedly used for a given calibration. Another potential advantage of this procedure is the elimination of the measurement noise from the set of curves $g(k,k_o)$; while the main disadvantage is that elaborate identification procedures for time-varying systems are not yet fully developed. The other possible modification to the proposed technique deals with the implementation of a variable gain scheme for an on-line estimation of $q(k)$ (25). Basically, the problem consists in choosing, along the calculation, the values of $q(k)$ which minimize the difference between the observed and the estimated innovations variance.

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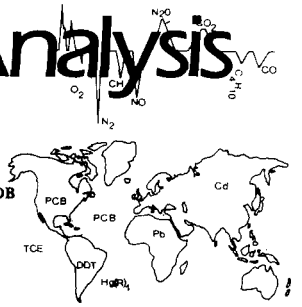

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(14), 2863-2873 (1984)

PREPARATIVE CHROMATOGRAPHY OF PROTEINS

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ABSTRACT

High performance liquid chromatographic methods that have been developed for microparticulate supports can be adapted to 30u supports, such as the SynChroprep series, for large scale purification. Loading capacity and resolution of the ion exchange and reversed phase macroparticulate supports are examined. One gram of protein may be loaded on the ion exchange support in a 250x10 mm I.D. column.

INTRODUCTION

Over the past five years, HPLC has been seen to be an effective way to analyze protein mixtures by a variety of mechanisms - steric-exclusion, ion-exchange, reversed-phase and hydrophobic-interaction chromatography (1). The use of rigid supports has allowed milligram amounts of proteins to be purified within minutes. As the use of this methodology became more widespread, the scale-up to columns of 1 cm I. D. was used to prepare hundreds of milligrams of proteins (2). Unfortunately, when pilot plant levels were desired, the extremely high cost of the HPLC grade silica was realized and possible alternatives were sought (3).

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One of the first problems in preparative HPLC is to define the goals of the analysis. A chromatographer wants to separate a mixture into the maximum number of components with the best resolution possible. The engineer in a pilot plant wants to separate his compound from its impurities and load it in the highest possible quantity. These differences in goals produce two definitions of "loading capacity." To a chromatographer, loading capacity is the amount of solute which can be applied to a column before resolution is decreased. To an engineer in a pilot plant, loading capacity means the absolute capacity of the support for the solute. These differences in definitions suggest that there may also be differences in support requirements.

This report examines the capacity and resolution of a series of 30u macroparticulate supports to see whether methods which have been developed on analytical columns can be adapted for preparative purposes.

EXPERIMENTAL

Chemicals

Tris(hydroxymethyl)aminomethane (TRIS) and sodium acetate were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Sodium chloride and Moni-Trol I.X were from American Scientific Products (McGaw Park, IL, U.S.A.). Propanol-2 was purchased from The Anspec Co., (Ann Arbor, MI, U.S.A.) and trifluoroacetic acid from Pierce Chemical Co. (Rockford, IL, U.S.A.). Lysozyme, ribonuclease A, α-chymotrypsin, chymotrypsinogen A, and bovine serum albumin (BSA) were all obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Apparatus

The 30u SynChroprep CM300, SynChroprep AX300, and SynChroprep RP-P, 250x10 mm I.D. and 6.5u SynChropak AX300, 250x4.1 mm I.D. columns were obtained from SynChrom, Inc.

(Linden, IN, U.S.A.). The 100x25 mm I.D. column was from HP Chemicals (St. Louis, MO. U.S.A.). A Varian Model 5000 gradient high-performance liquid chromatograph with a Valco Model CV-6-UHPA-N-60 injection valve (Varian Instrument, Walnut Creek, CA, U.S.A.) and Chem Research Model 2020 UV detector (Instrumentation Specialties Co., Lincoln, NE, U.S.A.) with a Linear Model 1200 recorder (Linear Instruments, Irvine, CA, U.S.A) were used for analyses.

METHODS

Because general guidelines have been established in previous analytical investigations, solvent selection for ion-exchange and reversed phase chromatography was not investigated (1,4,5). 0.02M TRIS buffers with sodium acetate or sodium chloride gradients from 0-0.5M were used for ion exchange, and propanol-2 gradients in .1% trifluoroacetic acid were used for reversed phase. The gradient times and flow rates used are given in the Tables and Figures.

RESULTS AND DISCUSSION

LOADING CAPACITY

Dynamic column capacity, which fits the needs of the chromatographer, may be described as the sample size that causes decreased retention time and loss of peak shape using normal gradient operating conditions (6). This column capacity was seen to vary according to the nature of the protein, its molecular weight, and its retention characteristics. The loss in peak shape with increased loading is evident in the analysis of bovine serum albumin in Fig. 1. Table I shows that the capacity of a weak anion-exchange support (SynChroprep AX 300) for BSA is approximately 6 times greater than that of a reversed-phase material (SynChroprep RP-P) for the same protein. When using a weak cation-exchanger (SynChroprep CM300), the capacity for a small protein, ribonuclease-A was about 5 times that of a protein twice its size, chymotrypsinogen-A.

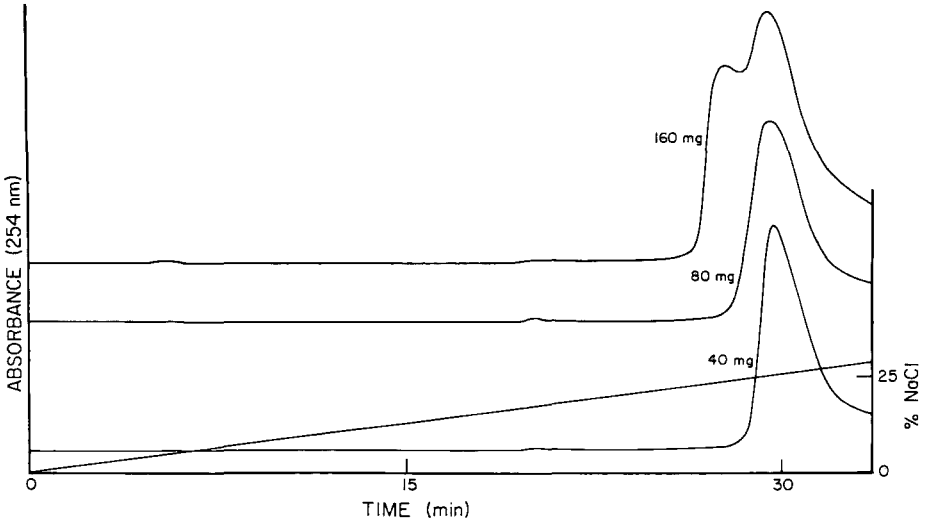

Fig. 1 Effect of sample loading on the resolution of a bovine serum albumin sample using a semi-preparative column of 30u support. Column: SynChroprep AX300, 250x10 mm I.D. Flow-rate: 1.5 ml/min. Buffer: 0.02M TRIS, pH7, 120 min. linear gradient from 0 to 0.5M CH_3CO_2Na.

TABLE I

Loading Capacity of 30u SynChroprep Supports

	Probe	Gradient Time (min)	Dynamic Capacity	Absolute Capacity
AX300	BSA	120	160 mg	1 g
RPP	BSA	40	25 mg	400 mg
CM300	α-chymotrypsin	120	80 mg	1 g

Mobile phase as in Methods. Flow-rate: 1.5 ml/m
Column: 250x10 mm I.D.

Such variance is probably due to the available surface area of the support.

Absolute column capacity may best be described as mass overload (7) utilizing the displacement mode (8) of chromatography. To utilize this technique, the column was equilibrated with a mobile phase that had a low affinity for the stationary phase. The sample was introduced and adsorbed on the column until it was completely loaded. Under these conditions, impurities with less retention eluted from the column during the sample application process. For example, the early eluting impurities of α-chymotrypsin were eluted with each injection on a SynChroprep CM300 column while α-chymotrypsin itself was retained. When absolute capacity was reached, the sample was released from the column by running a step gradient with a mobile phase having a stronger affinity for the stationary phase than the sample. By suitably choosing the mobile phase used in the "step", later eluting impurities could be retained on the column and dumped after the purification was finished. Again, different observations were made for various combinations of proteins and columns. While loading BSA on a SynChroprep RP-P column, a "breakthrough" impurity peak that had less column affinity than BSA was observed from almost the first injection. Sample was injected onto the column until a constant integrated area of the "breakthrough" peak was achieved. While loading α-chymotrypsin onto a SynChroprep CM300 support, an impurity "breakthrough" peak, that did not change in size was observed from the initial injection. αChymotrypsin did not "breakthrough" until absolute capacity was reached.

Table I compares the dynamic and absolute column capacities of three modes of chromatography supports - weak anion-exchange(AX300), weak cation-exchange(CM300) and reversed phase(RP-P), run in 250x10 mm I.D. columns. The dynamic capacity was calculated as the amount of sample that increased the width at peak half height by approximately 70%. If the resolution of the sample components is great, the actual dynamic capacity could be higher than these values (9). As would be expected, for fairly pure proteins,

TABLE II

BAND-SPREADING FOR 30u SUPPORTS

F_r (ml/min)	Gradient Time (min)	SynChroprep AX 300 Bovine Serum Albumin t_r (min)	SynChroprep AX 300 Bovine Serum Albumin t_r/t_w	SynChroprep CM 300 ∝Chymotrypsin t_r (min)	SynChroprep CM 300 ∝Chymotrypsin t_r/t_w
6.0	30			17.9	17.9
3.0	60	32	30.5	35.4	16.85
2.0	90	48	32	54.0	19.56
1.5	120	64	34.6	75.0	20.1
1.0	180	99	38.1	108.6	21.5
0.5	360			215	26.88

F_r (ml/min)	Gradient Time (min)	SynChroprep RP-P Bovine Serum Albumin t_r (min)	SynChroprep RP-P Bovine Serum Albumin t_r/t_w
3.0	20	13.5	38.5
2.0	30	20.4	42.7
1.5	40	27.1	43.3
1.0	60	40.5	40.5
0.5	120	82	45.5

Mobile phase as in Methods
Column: 250x10 mm I.D.

the absolute capacity was significantly greater than the dynamic capacity.

FLOWRATES

One easy way to increase resolution is to change the mobile-phase flowrate. Because proteins normally must be eluted with a gradient, ordinary plate height measurements cannot be used to determine the effect of flowrate velocity on band broadening. Therefore,the ratio of the retention time/peak width was used to indicate protein peak spreading.

In this method, larger numbers indicate better resolutiion. Flow-rates were varied from 0.5-3.0 ml/min while holding the gradient volume constant. The results are listed in Table II. In most cases, a decrease in flow-rate with a constant gradient volume resulted in both higher resolution and longer retention times. Due to the inaccuracy of some HPLC equipment at slow flowrates and low pressures, and the disadvantages of long retention times, flow-rates must be selected on these bases as well as that of resolution.

GRADIENT SHAPE

Solvent gradients can also be used to optimize separations with respect to both time and resolution. For example, on the SynChroprep CM300 column, a 90 minute linear gradient at 1.5 ml/min gave a very good separation of ribonuclease-A and lysozyme. However, it was necessary to use at least a 120 minute gradient at the same flowrate to separate chymotrypsinogen-A from the ribonuclease-A and lysozyme peaks as seen in Fig. 2. Similarly, on SynChroprep AX300, impurities in ovalbumin were not adequately separated until a 120 min. gradient at 1.5 ml/min. was used.

COLUMN DIAMETER

The scale-up of a chromatographic process from the analytical laboratory level to the pilot plant is accomplished by increasing the width of the column rather than the height. In this way, the parameters that have been worked out, such as the linear flow rates and gradient times would remain about the same. Because the capacity of a column roughly corresponds to the amount of packing material in it, the sample loading would be increased proportionately to the volume. Fig. 3 shows the same 3-component sample separation that was seen in Fig. 2 using a 2.5 cm I.D. column for 1.3 g of total protein instead of 75 mg.

RESOLUTION ON 30u VS. 6.5u

Once the operational parameters such as flow rate,

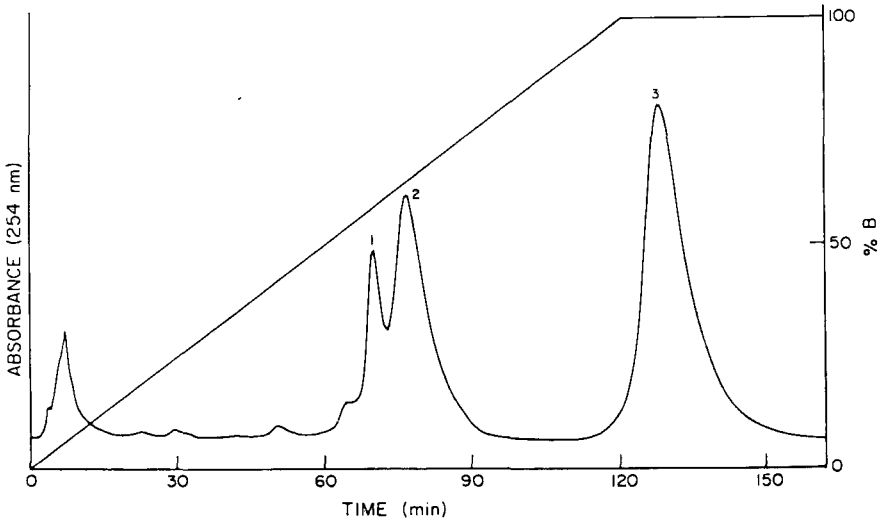

Fig. 2 Resolution of 45 mg ribonuclease-A (1), 15 mg chymotrypsingen-A (2), 15 mg lysozyme (3), using a semi-preparative column of 30u support. Column: SynChroprep CM300, 250x10 mm I.D. Flow-rate: 1.5 ml/min. Buffer: 0.02 M TRIS , pH7, 120 min linear gradient from 0 to 0.5M CH_3CO_2Na.

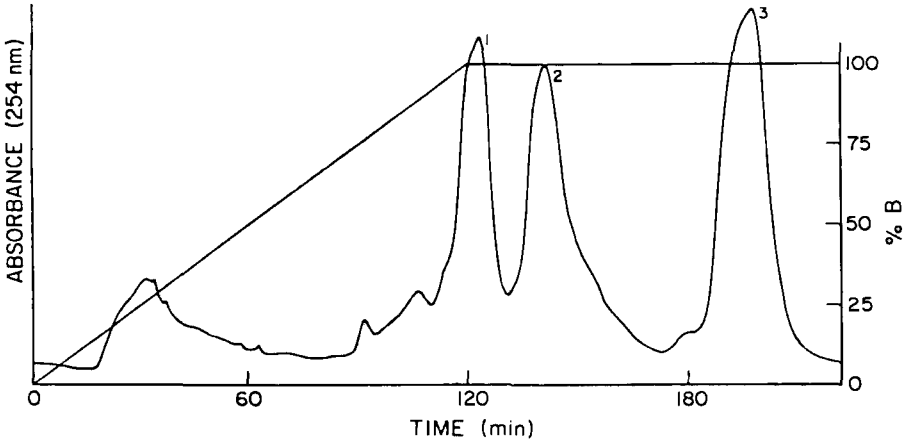

Fig. 3 Resolution of 750 mg ribonuclease-A (1), 130 mg chymotrypsinogen-A (2), and 150 mg lysozyme (3) using a preparative column (250x25 mm I.D.) of 30u support. Conditions as in Fig. 2.

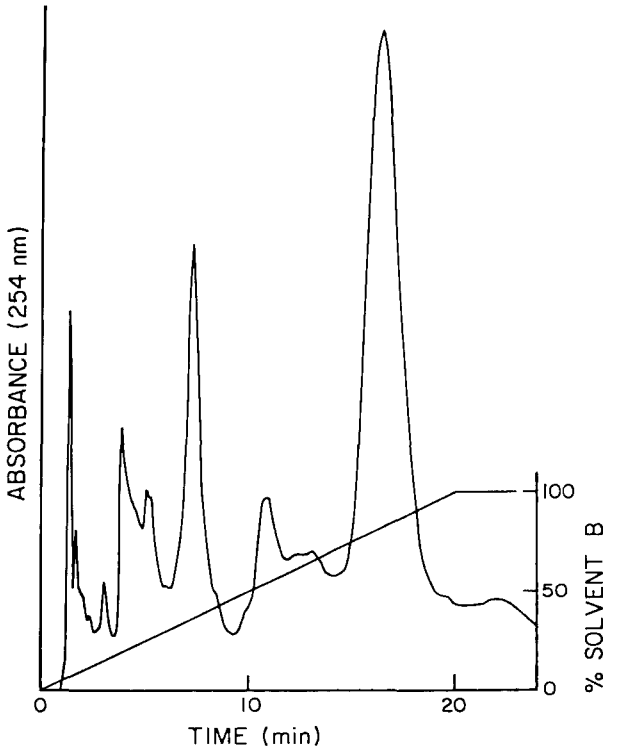

Fig. 4 Analysis of human adult serum using an analytical column of 6.5u support. Column: SynChropak AX300, 250x4.1 mm I.D. Flow-rate: 2 ml/min. Buffer: 0.02M TRIS, pH8. 20 min linear gradient from 0 to 0.5M CH_3CO_2Na.

gradient time, and component separation have been investigated for 30u supports, the chromatographer and engineer must know how the resolution of the 30u preparative material compares with that of the 6.5u supports used in the analytical columns. Fig. 4 and 5 compare the analysis of human adult serum on a 250x4.1 mm I.D. column packed with 6.5u SynChropak AX300 with the same analysis on a 100x25 mm I.D. column packed with SynChroprep AX300. There is a good

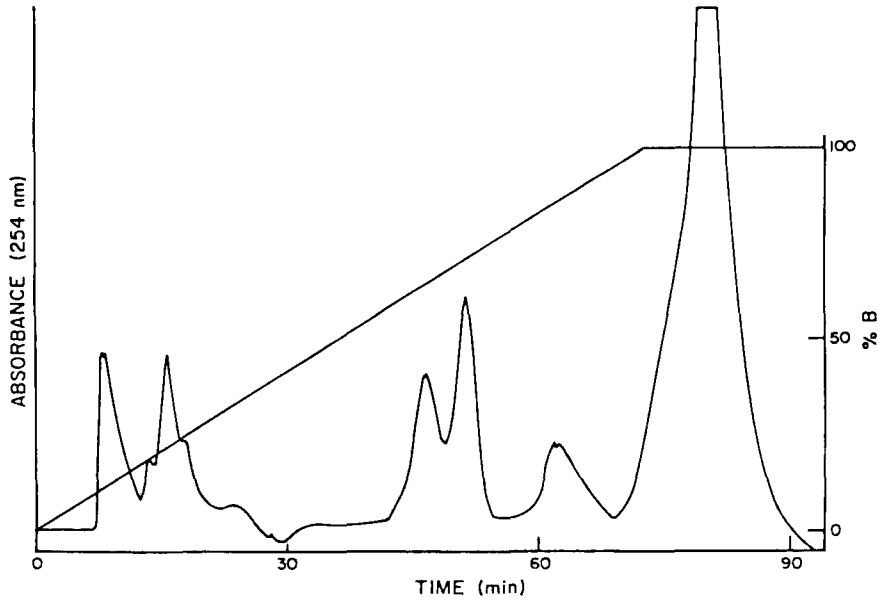

Fig. 5 Analysis of human adult serum using a preparative column of 30u support. Column: SynChroprep AX300, 100x25 mm I.D. Flow-rate: 3.2 ml/min. Buffer: 0.02M TRIS, pH7. 72 min. linear gradient from 0 to 0.5M NaCl.

correlation between the chromatograms, and the major components, and some minor ones, are sufficiently resolved on the 30u support to satisfy the engineers needs for purification.

CONCLUSIONS

This study shows that HPLC methods that have been developed on microparticulate supports can be adapted to 30u supports for large scale purification. Absolute loading of a gram of protein are possible on ion-exchange columns of 250x10 mm I.D. dimensions. Adequate resolution can be obtained by adjusting the flowrate and gradient shape of the analysis.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(14), 2875-2885 (1984)

GLYCOSYLATED HEMOGLOBIN DETERMINATIONS BY ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Glycosylated hemoglobins were separated isocratically by high-performance liquid chromatography (HPLC) on a cation-exchanger (CM-300). Both the stable and the labile fractions eluted together. The labile fraction was eliminated by incubating the red blood cells at 37°C for 20 min in a acidic buffer before injecting the sample on the column.

The column plate number was found to be dependent on the amount of sample injected. The capacity factor was dependent on the type of buffer, pH and ionic strength. Controls were preserved by preparing the hemolysates in 5% ethylene glycol. The method compared favorably with a commercial disposable minicolumn method.

INTRODUCTION

The aldehyde group of glucose reacts non-enzymatically and rapidly with the amino groups of hemoglobin (Hb) to form a reversible adduct (aldamine) which rearranges slowly to form a more stable ketoamine compound, HbA_{1c} (1). Other carbohydrates give similar reactions with Hb. These Hb derivatives are

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generally known as glycosylated Hb (2). Their level is greatly increased in diabetes. Measuring the level of glycosylated Hb is very helpful in monitoring the long-term therapy in the diabetic patient (3).

Several methods including high-performance liquid chromatography have been used for separating and determining the glycosylated Hb based on charge differences of the molecules. The weak cation-exchanger Bio-Rex 70 was used previously in HPLC methods for this determination (4,5). This ion-exchanger was not designed for HPLC work. Because the particles are large (>40 μm) the column did not yield a very high plate number. The Hb peak was therefore broad and overlapped with the non-glycosylated Hb peak (5). A buffer gradient in addition to double wavelength monitoring was necessary for the test. This complex instrumentation is not quite suitable for routine assays.

Recently, cation-exchangers designed specifically for protein separation by HPLC became available from different manufacturers. These ion-exchangers have small particle size with narrow particle distribution and wide pores yielding columns with high plate number. Here, we describe the isocratic separation of glycosylated Hb on such a column without the need for complex instrumentation. Factors which affect the separation, e.g., plate number, capacity factor, and temperature are studied. We also describe the preparation of stable controls.

MATERALS AND METHODS

Instrumentation:

The liquid chromatograph consisted of a Model 970A variable-wavelength detector (Tracor Instruments, Austin, TX, U.S.A.), a Constrametric I pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) and a 20 µl loop injector, Model 2120 (Rheodyne, Berkeley, CA U.S.A.). The detector was set at 405 nm and 0.010 A. The flow- rate was 1.0 ml/min.

A 250 mm x 4.6 (i.d.) column was packed with a CM-300 ion-exchanger, (Synchrom, Linden, IN) 6.5 µm average particle size, in the mobile phase by the slurry packing technique.

Reagents:

1. Mobile phase: Ammonium phosphate 28 mmol/l, pH 6.85. Initially the column may require higher concentration of buffer for elution.

2. Hemolysates: A 5 µl aliquot of blood collected in EDTA tubes was hemolyzed in 4 ml ammonium phosphate-buffer, pH 4.5, 14 mmol/l. The hemolysates were incubated for 20 min at 37°C before injecting on the column to eliminate the unstable fraction of the glycosylated Hb.

3. Controls: A large aliquot of a patient's hemolysate (twice concentrated) is mixed with ethylene glycol (20:1) and stored frozen in small aliquots. It is diluted 1:1 with hemolysate buffer before use.

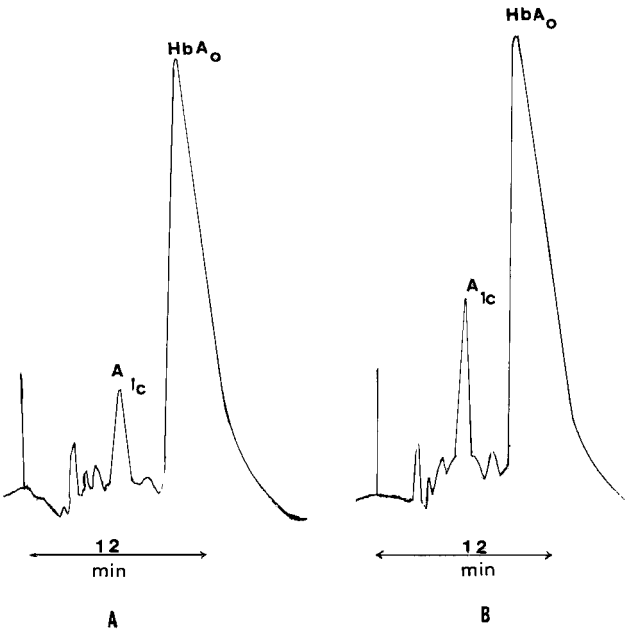

Figure 1:

Separation of the glycosylated Hb on the CM-300 at flow rate 0.7 ml/min: (A) without incubation of the red blood cells with glucose; (B) after glucose at (2000 mg/dl) is incubated with red blood cells for 1 hr. The hemolysates were injected immidiately on the column.

Procedure:

Inject 20 µl of the hemolysate on the column. Elute isocratically with the mobile phase.

Calculation:

The areas under the peaks were cut and weighed on a balance

and the ratio of the sum of all the glycosylates to the total hemoblobins were calculated.

RESULTS AND DISCUSSION

The isocratic separation of glycosylated Hb on the CM-300 column reveals several peaks, Fig. 1-A. This separation is better than the one obtained previously on the Bio-Rex 70 column with solvent gradient (5). Samples can be injected without the need for column equilibration. The need for double wavelength monitoring which is associated with the solvent gradient is eliminated.

When the red blood cells are incubated at 37°C with glucose even for short periods of time - e.g., 1 hr - a rapid increase in the height of several peaks on the chromatogram, especially HbA_{1c}, Fig. 1-B is noted. The degree of this increase depends on time of incubation, Fig. 2A. In contrast, if cell hemolysates are left for 20 min at 37°C instead of being injected directly on the column there is a gradual decline of the peaks, Fig. 2. These findings are consistent with the idea that glucose binds rapidly to hemoglobin altering the number of positive charges on the molecule; and this reaction is reversible in the acidic buffer used for hemolyzing the red blood cells (6). The fraction of the glycosylated Hb which reacts rapidly and reversibly with glucose represents the unstable fraction of aldamine. This fraction is not clinically useful. It arises from sample handling and from rapid fluctuations of glucose in the patient's

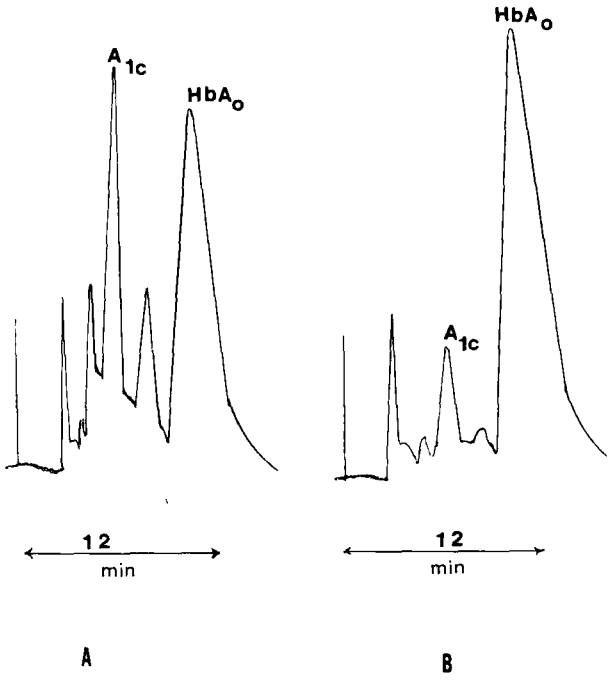

Figure 2:

A - Glycosylated Hb separation as in Fig. 1-B except the cells were incubated 24 hr. B - Same as in A except the hemolysates were kept for 30 min at 37°C before injection on the column.

blood. Unfortunately, the aldamine co-elutes with the stable fraction of the Hb (8). Thus it is necessary to incubate the patient's hemolysates in an acidic buffer for at least 20 min to eliminate this fraction (6).

The plate number (N), for the CM-300 column based on HbA_{1c} peak, is quite dependent on the amount of sample injected,

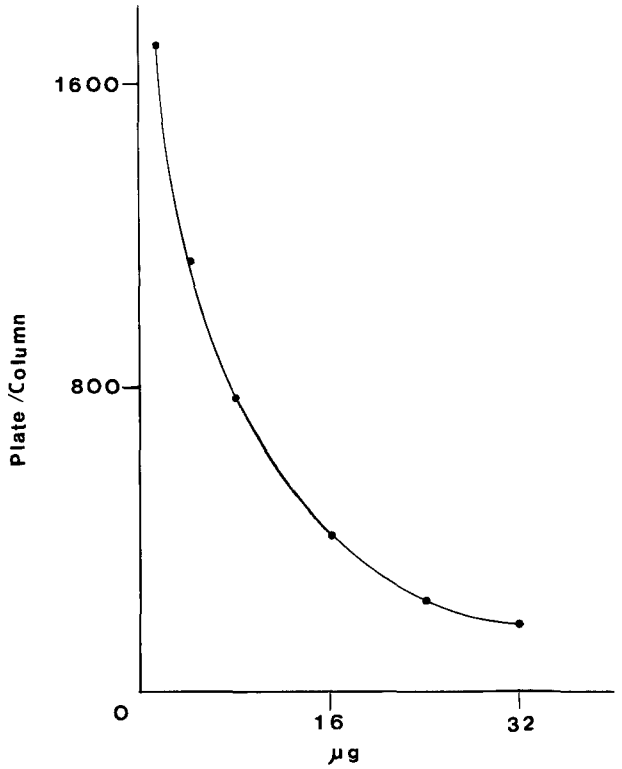

Figure 3:

Relationship of plate number of HbA_{1c} peak and the amount of Hb applied on the column as determined.

Fig. 3. Thus, in order to obtain a good separation it is necessary to inject a minimum amount of sample while operating the detector at maximum sensitivity. Injection of small amounts of sample increases the column lifetime. The present column yields about 10 times the plate number of the Bio-Rex column

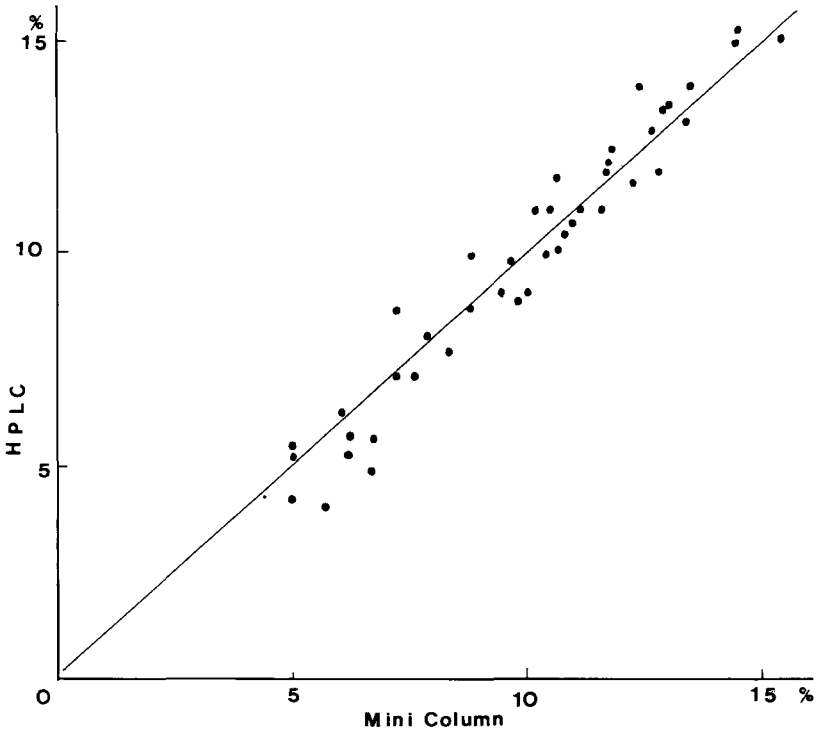

Figure 4:

The correlation of the results between CM-300 column by HPLC and the mini-column of Isolab for the glycosylated Hb. ($y = 1.26\ x + 2.8$; $r = 0.962$)

previously used (5). Flow-rates between 0.5 to 1.5 ml/min did not affect the plate number.

The within-run CV for 20 replicate determinations at a mean of 9.8% Hb is 3%. The correlation of the results of this method with that of the disposable commercial column (Isolab, Inc., Akron, OH) is 0.90, Fig. 4.

The capacity factor of HbA_{1c} decreased with increasing the ionic strength or increasing pH between 6 to 7.5. The potassium salts of the phosphate buffer gave the same capacity factor as the ammonium salts; however, the sodium salts decreased the capacity factor.

Column temperature between 25 and 40°C did not affect the percentage of glycosylated Hb but did affect the separation speed. For routine assays the separation was performed at room temperature.

Hemoglobin variants other than HbA elute at different capacity factors. The monozygotes will be recognized by the absence of HbA_o while the heterozygotes will have reduced HbA_o (about half of the concentration) relative to the other patients on the chromatograms.

Stable controls are very important in every assay. Hemoglobins are known to be unstable (8). They undergo continuous denaturation even when frozen. Several agents were added to the hemolysates in an attempt to preserve the glycosylated Hb as well as the total Hb. In the absence of any preservative, total Hb denatured and did not go back into solution giving low peak areas, (Fig. 5) and erratic values for the glycosylated Hb. However, we found that when ethylene glycol was added at 5% to the hemolysate, both the glycosylates and the total fractions were more stable. At this time, we have not studied the effect of different concentration of ethylene glycol or the effects of long-term storage.

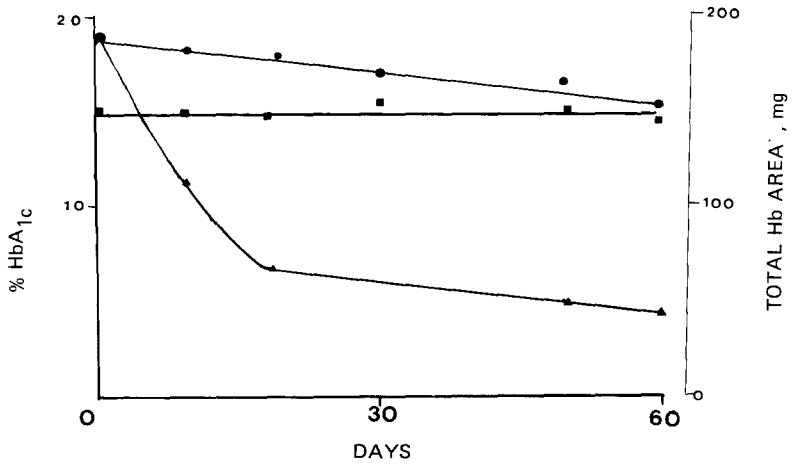

Figure 5:

The effect of ethylene glycol on % HbA_{1c} and total HbA_o after storage at -20°C:%HBA_{1c} (■——■) and total HBA_o (●——●) preserved with 5% ethylene glycol. Total HBA_o in absence of ethylene glycol (▲——▲).

Determination of glycosylated Hb on the CM-300 column is rapid (less than 10 min at flow-rates of 1.0 ml/min) and eliminates the need for solvent gradient and column equilibration after each run. In addition the column can be used for determination of other proteins.

ACKNOWLEDGMENT

We thank Dr. John W. Hartz and Dr. Michael L. O'Connor for reviewing this paper before submission.

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DETERMINATION OF TICARCILLIN IN SERUM BY REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Ticarcillin is a semi-synthetic penicillin useful against several Pseudomonas species. In order to easily quantitate this drug, a new procedure was developed whereby ticarcillin in serum is converted to its free acid form by the addition of citric acid and, subsequently, extracted into ethyl acetate. The organic extract which contains the nonionized form of ticarcillin is dried under nitrogen, the sample is reconstituted with mobile phase and analyzed by high performance liquid chromatography. Elution is completed in less than five minutes. The assay is linear from 1 mg/L through 100 mg/L. The correlation coefficient of ticarcillin concentration to peak area (r) was 0.999 over this concentration range. The small sample volume (100 µl) makes this assay particularly suitable for pediatric patients.

INTRODUCTION

Ticarcillin is a α-carboxypenicillin which is used predominately for the treatment of serious infections caused by gram-negative organisms, particularly Pseudamonas aeruginosa. Bacteriocidal efficacy is dependent on the maintenance of relatively high concentrations of the antibiotic during the dosing interval. It is frequently desirable, therefore, to be

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able to measure the concentration of ticarcillin in biological fluids during treatment to ensure adequate dosing.

During the past decade, a number of studies have employed bioassays of ticarcillin (1-5). However, the bioassays are subject to substantial random error, require a relatively long incubation time, and provide limited sensitivity. Furthermore, ticarcillin is invariably administered in combination with an aminoglycoside antibiotic which interfers with the bioassays, particularly at low ticarcillin concentrations (4).

The high performance liquid chromatographic method described here was designed to provide an accurate, sensitive, and rapid ticarcillin assay which can be performed in the presence of concurrently administered antibiotics. The small sample volume (100 µl) makes the assay readily applicable to pediatric patients.

MATERIALS AND METHODS

Reagents

Ticarcillin disodium (analytical grade) was a gift from Beecham Laboratories, Bristol, Tennessee, U.S.A. 8-chlorotheophylline was from ICN Pharmaceuticals, Plainview, New York, U.S.A. HPLC-grade acetonitrile, ethyl acetate, and methanol and reagent grade citric acid monohydrate, sodium acetate, and glacial acetic acid were obtained from Fisher Scientific Company (Fairlawn, New Jersey, U.S.A.).

Standards

A stock standard solution of ticarcillin, equivalent to 1 g/L in methanol, was prepared and stored in the freezer at $-20^{\circ}C$. Working standards were prepared daily in drug-free plasma to yield concentrations of 1 through 100 mg/L. The internal standard (8-chlorotheophylline) was added to the extraction solvent (ethyl acetate) to yield a concentration of 1 mg/L.

Instrumentation

Chromatography was performed on a Perkin-Elmer Series II HPLC system equipped with a LC 75 UV/VIS variable wavelength

detector interfaced to a Sigma-10 data system. A 4.6 mm x 12.5 cm HC ODS 5 μ-particle size reversed phase column (Perkin-Elmer Corporation, Norwalk, CT, U.S.A.) was used for the chromatography. The data system provided a readout of the digitally integrated area under the peaks, determined the retention times, calculated the response factors for ticarcillin and the internal standard and printed the results in the appropriate concentration units.

Chromatographic Conditions

The mobile phase consisted of 9% acetonitrile in 0.2 M sodium acetate containing 0.35% glacial acetic acid (pH = 2.7) which was filtered and thoroughly degassed prior to use. The flow rate was 1.5 ml/min, and the effluent was monitored at 220 nm. All analyses were performed at ambient temperature.

Procedure

One hundred μl of standard, control, or patient serum was placed in a 1.5 ml Eppendorf centrifuge tube. To each tube 200 μl of 1 M citric acid monohydrate (pH = 2.2, prepared fresh weekly and stored refrigerated) was added and mixed. This was followed by the addition of 1.0 ml of ethyl acetate containing the internal standard. The tubes were vortexed vigorously for at least 1 minute and then centrifuged for 3 minutes at 21,000 g in a Brinkman table-top microcentrifuge. The organic layer was then transferred to a 10 x 75 mm disposable glass tube and evaporated to a dryness at 40°C under a stream of nitrogen. The dried samples were reconstituted with 50 μl of mobile phase; 10 μl of which was injected onto the column.

RESULTS

Ticarcillin was extracted into ethyl acetate as its free acid after the addition of citric acid. Figure 1 shows typical chromatographs of (A) drug-free serum extracted with ethyl acetate containing the internal standard; (B) drug-free serum

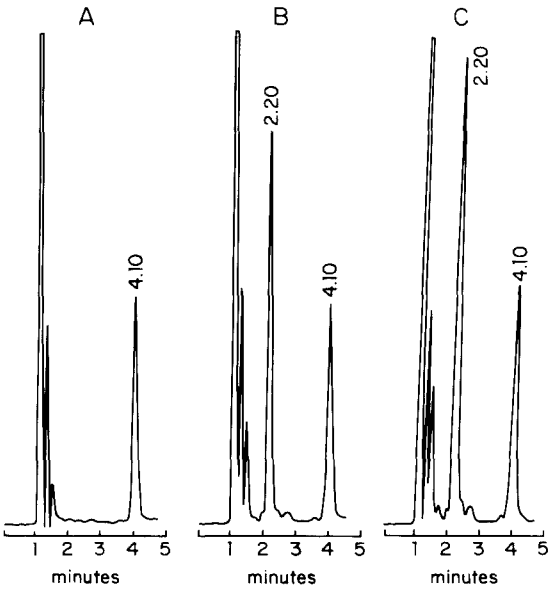

FIGURE 1. Chromatogram of blank serum, standard, and a patient serum. (See text for details.)

reconstituted with 100 mg/L of ticarcillin and the internal standard; and (C) a chromatogram of a patient serum extract containing 127 mg/L of ticarcillin. The retention times for internal standard and ticarcillin were 2.2 and 4.1 min., respectively.

The concentration of ticarcillin calculated from the integrated area under the peaks was linearily related to the internal standard area over a concentration range from 1.0 to 100 mg/L. The correlation coefficient (r) was 0.999 and the equation for the regression line was $y = 0.969\ x + 1.019$. Samples with concentrations greater than 100 mg/L were appropriately diluted with normal saline and re-analyzed. The mean recovery of ticarcillin from serum was 89%.

Table 1 shows the results of within-run and day-to-day precision studies conducted on spiked serum.

TABLE 1

Precision of Ticarcillin Assay

	Within Run	Day-to-Day
Concentration (Added) mg/L	50	20
Concentration (Obtained) mg/L		
Mean	49.7	21.4
SD	1.8	1.7
CV%	3.6	7.9
n	9	8

TABLE 2

Stability of Ticarcillin in Serum

Week	Value mg/L	Week	Value mg/L
1	46.9	5	51.9
2	50.4	6	50.7
3	46.3	7	49.6
4	52.9	8	49.7
Mean (mg/L)	50.1		
± SD	1.9		

Stability studies were conducted using a serum pool to which a known quantity of ticarcillin was added. Aliquots of this pool were frozen at -20°C and analyzed over a period of 8 weeks. Ticarcillin was stable for up to 8 weeks under these conditions (Table 2).

DISCUSSION

The ultraviolet absorption and solubility characteritistics of ticarcillin proved to be unusually problematic during the development of a satisfactory HPLC assay. The extinction coefficient of ticarcillin in the U.V. range is relatively low with an absorption maximum less than 200 nm. This region of the U.V. spectrum is

unsuitable for most analyses with U.V. detection because most solvents and many endogenous chromogens absorb below this wavelength, providing unacceptable background absorption. By monitoring the effluent at 220 nm we were able to reduce these background interferences while retaining satisfactory sensitivity in the detection of ticarcillin.

Because of less than optimal U.V. absorbance, it became necessary to extract ticarcillin from serum and concentrate it prior to chromatography in order to achieve satisfactory sensitivity. Ticarcillin is a highly water soluble dicarboxylic acid with a pKa of approximately 2.7, making it difficult to extract efficiently from an aqueous medium. We found that ticarcillin could be satisfactorily extracted into ethyl acetate as the free acid. The free acid was then concentrated by evaporating the ethyl acetate and reconstituting the drug with the acidic mobile phase.

Kwan, et al. (6) employed a back-extraction from ethyl acetate into 0.04 M pH 6.8 phosphate buffer to concentrate ticarcillin and separate it from interfering substances. However, we were unable to duplicate the recovery reported by Kwan and found the extra back extraction step to be cumbersome as well as unnecessary. In addition, we found the strong acidic mobile phase conditions used by Kwan to unacceptably shorten the functional life span of the C-18 column and to be unnecessary for optimal chromatography.

The method described here is specific and sensitive, may be carried out with a simple single extraction step and requires less than five minutes for elution of each sample. The sample size of 100 µl makes it an ideal assay for use in infants and young children where sample volume is frequently restricted.

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QUANTITATIVE DETERMINATION OF THE α-AMYLASE INHIBITOR FROM *PHASEOLUS VULGARIS* USING SIZE EXCLUSION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A quantitative determination of the α-amylase inhibitor from kidney beans, a glycoprotein (MW 49,000), by the use of a TSK-SW 3000 column is described. A correlation coefficient of 0.986 was obtained when the HPLC method was compared with a biological assay using Beckman Enzymatic Amylase-DS Reagent Kit. The within-day and between-day coefficients of variation were 4.8-8.9% and 3.7-11.6%, respectively. The results indicate this method is a reliable assay that has advantages over the biological assays presently available.

INTRODUCTION

High performance size exclusion chromatography has been used quite successfully in the separation of proteins (1-4). There have been, however, a limited number of reports in which proteins have been quantitatively assayed by HPLC (5,6). Size exclusion chromatography was chosen for the development of our quantitative chromatographic assay of the α-amylase inhibitor from kidney beans (*Phaseolus vulgaris*) because of the reportedly high degree of recovery of proteins in their active form (2,4) and the need to measure the biological activity of the inhibitor.

There have been numerous α-amylase inhibitors from plant and animal sources identified (7-10). This group of substances gained

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notoriety when they were introduced into the market as 'starch blockers' to be used as dietary aids in weight control, but they were subsequently removed from the market by the FDA. The efficacy of these products as starch blockers is controversial (11-14). Other clinical and agricultural applications of α-amylase inhibitors have also been investigated (15-17).

The α-amylase inhibitor from kidney beans is a glycoprotein with a molecular weight of 49,000 and characteristics of its inhibitory properties on α-amylase have been reported (7).

MATERIALS AND METHODS

The HPLC system was composed of a model 825 pump and model 226 UV (280 nm) detector (Instrument Specialties Company); model 210 injector and Spherogel TSK-SW 3000 size exclusion column, 300 x 7.5 mm (Beckman Instruments); and model 3390A integrator (Hewlett-Packard Company). The mobile phase flow rate was 1 ml/min and consisted of 85% of 0.15 M sodium chloride in 0.01 M phosphate buffer (pH 7.2) and 15% acetonitrile.

Partially purified samples of α-amylase inhibitor from kidney bean (Calbiochem-Behring) were used as the primary source of the α-amylase inhibitor. These samples were dissolved in water and used directly for HPLC and biological assays. Starch blockers which were previously available and marketed under the brand names of Phaseolamin (Vita Plus, Inc.), Carbo-Slim (Michigan Vitamin) and Starch Blocker (Advantage Supplements) were also used as a source of the inhibitor. Samples were prepared from 3 tablets (500 mg each) which were crushed, extracted with 100 ml of water, filtered to remove undissolved particles and assayed.

Biological activity of the inhibitor was determined by the use of Beckman Enzymatic Amylase-DS Reagent Kit (Beckman Instruments). This is an enzyme-coupled assay for α-amylase activity in which maltotetrose is the substrate and the rate of

production of NADH is followed by measuring the increase in absorbance at 340 nm. One unit of amylase activity was defined as the amount of enzyme that produced 1 μmole of NADH per minute under the conditions described below when no inhibitor was added. To assay the inhibitor, 1 ml of the Beckman reagent was preincubated at 37° C for 5 min in the spectrophotometer cuvette. At the same time another vial was preincubated and contained 750 μl of 0.05 M phosphate buffer (pH 5), 100 μl (approximately 0.03 units) of α-amylase (type I-A from porcine pancreas , Sigma Chemical Company), and inhibitor with HPLC mobile phase to give a final volume of 1100 μl. After preincubation the enzyme-inhibitor solution was transferred to the cuvette, incubated for 5 min at 37° C and the change in absorbance was measured during the next 3 min. All samples were assayed in duplicate. One unit of inhibitor was defined as the amount of inhibitor that inhibited 2 units of α-amylase by 50%.

The HPLC assay of the inhibitor was determined from a standard curve (peak height versus biological activity) which was prepared from a series of injections of the inhibitor. The equation for the standard curve was determined by linear regression analysis. Peak heights of all samples of inhibitor were calculated from the average of duplicate injections.

RESULTS AND DISCUSSION

Separation of α-amylase inhibitor from other constituents of partially purified inhibitor and starch blocker samples was accomplished by high performance size exclusion chromatography (Figure 1). Only samples collected under peak 4 were bioactive and re-injection of the bioactive fraction on the HPLC produced a single peak with the same retention time as peak 4. In addition, when the inhibitor (1.25 units/ml) was incubated at pH 5 with α-amylase (12.5 units/ml) for 40 min at 37° C and then

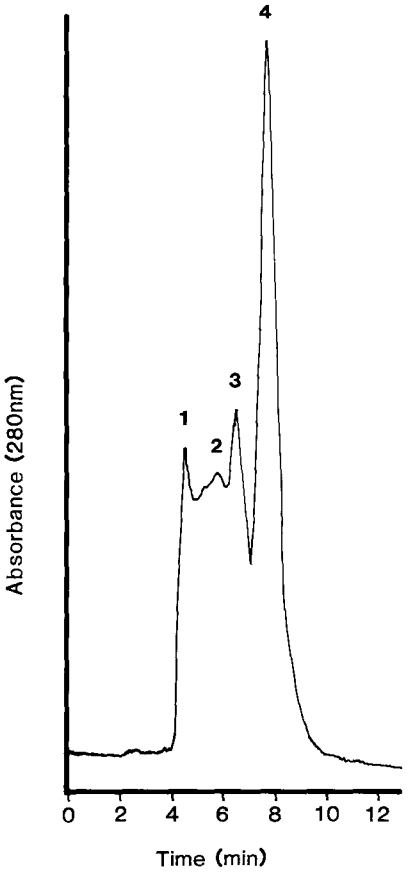

Figure 1. Chromatogram of partially purified α-amylase inhibitor using a Spherogel TSK-SW 3000 column at a flow of 1 ml/min. Injection volume was 20 µl (80 µg protein). Peak 4 contained all of the inhibitor activity.

chromatographed, a new peak appeared at 7.0 min whereas the inhibitor and α-amylase, when chromatographed separately under the same conditions, gave peaks at 7.3 and 8.8 min, respectively. The shorter retention time is indicative of a larger molecule as would be expected following the formation of an inhibitor-enzyme complex. The height of the peak at 7.0 min was also larger than either the inhibitor or amylase peaks alone.

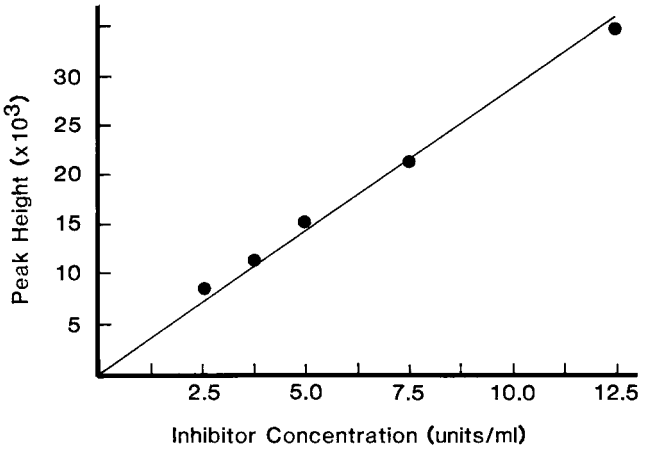

Figure 2. Standard curve for α-amylase inhibitor.

A typical standard curve for the α-amylase inhibitor is shown in Figure 2. Peak-height and units of biological activity were linearly related over this range (correlation coefficient of 0.997). The minimal detectable quantity (sensitivity at 95% confidence) of the inhibitor by the HPLC assay was 0.13 units/ml which is comparable to the minimal detection limit (0.11 units/ml) using Beckman Enzymatic Reagent Kit.

Loss of biological activity must correspondingly alter the chromatographic response if the HPLC assay is to be useful. Table 1 illustrates that the loss of biological activity of the inhibitor correlated well with the loss of HPLC response in a sample that was dissolved in water and stored either at room temperature or at 5° C. Boiling the sample for 10 min in pH 4 phosphate buffer eliminated both biological activity and HPLC response.

Within-day and between-day precision were estimated by multiple determinations of several inhibitor solutions (Table 2). The within-day and between-day coefficients of variation were 4.8-8.9% and 3.7-11.6%, respectively. Between-day precision was difficult to study over long time periods because of the instability of the inhibitor in water at 5° C.

TABLE 1

HPLC Response And Bioassay After Inactivation Of Inhibitor

Condition	% Activity Remaining HPLC	Bioassay
Boiling water bath, 10 min	0	0
5^{o} C for 7 days	64	66
Room temp for 2 days	70	58
Room temp for 14 days	0	0

TABLE 2

Precision Studies

	Mean (units/ml)	SD (units/ml)	CV (%)	n
Within-day	1.66	0.08	4.8	14
	2.46	0.16	6.5	15
	10.02	0.90	8.9	15
	15.06	1.03	6.8	15
Between-day	2.35	0.13	5.5	4
	2.88	0.15	5.2	4
	4.10	0.15	3.7	4
	8.48	0.54	6.4	5
	10.11	0.86	8.5	7
	16.89	1.96	11.6	7

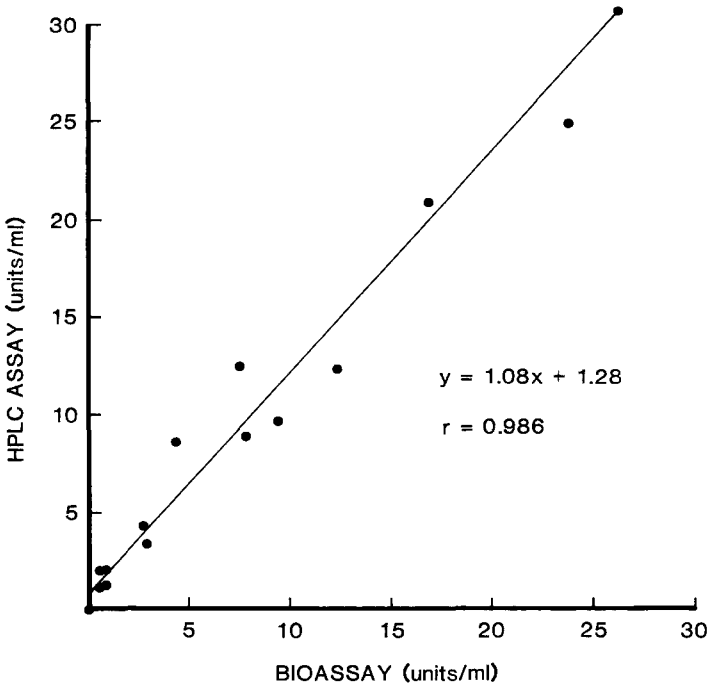

Figure 3. Comparison of HPLC assay with biological assay for α-amylase inhibitor.

A comparison of the HPLC assay and biological assay for the amylase inhibitor is shown in Figure 3. The correlation coefficient was 0.986, indicating a good agreement between the assay methods. These assays represent different samples of partially purified inhibitor and starch blockers and not merely dilutions of such samples.

In conclusion, this HPLC assay of α-amylase inhibitor from kidney bean is simple, rapid, sensitive and reproducible. Each assay can be completed within 10 min whereas biological assay methods for the inhibitor are more time consuming and cumbersome. The HPLC response correlates well with the bioactivity even following mild inactivation of the inhibitor during storage. The

HPLC assay, however, was not evaluated for proteins that may interfere with the assay although commercially produced starch blocker tablets and samples partially purified by the procedure of Marshall (7) that we assayed did not contain interfering compounds.

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SEMI-PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF CARBON-14 LABELED AVERMECTIN B_{1a} FROM A MIXTURE OF AVERMECTINS

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ABSTRACT

A semi-preparative high performance liquid chromatographic method has been developed to separate carbon-14 labeled avermectin B_{1a} from a fermentation mixture of carbon-14 labeled avermectins, i.e., avermectins A_{1a}, A_{1b}, A_{2a}, A_{2b}, B_{1a}, B_{1b}, B_{2a}, and B_{2b}. Two HPLC systems were employed for the separation: I. A Whatman M20, Partisil 10, normal phase column and a solvent system of 10% ethanol in isooctane (v/v), and II. A Whatman M20, Partisil 10, ODS-3, reverse phase column and a solvent system of acetonitrile/methanol/water (56:18:26, v/v/v); the flow rate was 18 ml/min. Avermectin separations were monitored using ultraviolet detection (254 nm). Further analyses of avermectin B_{1a} were done using analytical HPLC and TLC/radioassay to check compound purity and identity.

INTRODUCTION

Avermectin B_{1a} is one of the major avermectins (Figure 1) produced by the actinomycetes Streptomycetes avermetilis (1). It

*To whom inquiries should be directed.

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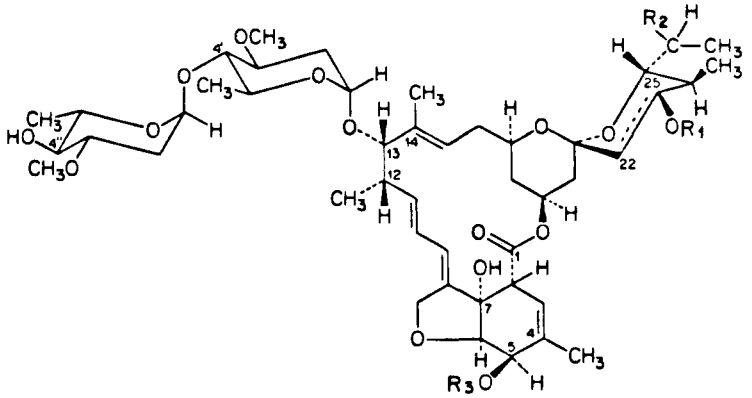


Figure 1. Structure of the Avermectins.

AVERMECTIN	R_1	R_2	R_3
A_1a		C_2H_5	CH_3
A_1b		CH_3	CH_3
A_2a	OH	C_2H_5	CH_3
A_2b	OH	CH_3	CH_3
B_1a		C_2H_5	H
B_1b		CH_3	H
B_2a	OH	C_2H_5	H
B_2b	OH	CH_3	H

Where R_1 is absent, the double bond (------) is present.
Both sugars are o-L-oleandrose.

is active at extremely low dosage against a wide variety of nematode and arthropod parasites, apparently by virtue of its action on the mediation of neurotransmission by Υ-aminobutyric acid (2). In addition, it exhibits excellent activity in controlling different phytophagus pests of field crops and citrus (3), and red imported fire ants (4). Pure, carbon-14 avermectin B_1a is needed for various metabolism studies and environmental chemistry studies. Miller et al. (5) separated avermectin major components, A_1, A_2, B_1, and B_2, by using a partition chromatography system: Sephadex LH-20.

EXPERIMENTAL

Material

Solvents used for the semi-preparative and analytical high-performance liquid chromatographic separations of avermectins were HPLC grade.

Crude mixture of carbon-14 labeled avermectins, used to obtain pure avermectin B_{1a} by semi-preparative HPLC, were isolated from the fermentation broth using the procedure reported by Ku et al. (6).

The TLC plates were E. Merck Sil GF, 0.25 mm thick plates. Autoradiography was achieved on Kodak ARO X-ray film. Both normal phase and reverse phase semi-preparative columns were purchased from Whatman.

Apparatus

The HPLC system consisted of two Altex model 110A pumps, a Rheodyne injector with a 4 ml sample loop, an Altex dual wavelength model 151 UV detector with a preparative flow cell (0.5 mm pathlength) and a recorder. The normal phase semi-preparative column (50 cm x 22 mm I.D.) was a Whatman M20, Partisil 10. Operating conditions were: mobile phase, ethanol-isooctane (10:90, v/v); flow rate, 18 ml/min; temperature, ambient; UV wavelength, 254 nm; chart speed, 12 cm/hr. The reverse phase semi-preparative column (50 cm x 22 mm I.D.) was a Whatman M20, Partisil 10, ODS-3. Operating conditions were: mobile phase, acetonitrile/methanol/water (56:18:26, v/v/v); flow rate, 18 ml/min; temperature, ambient; UV wavelength, 254 nm; chart speed, 12 cm/hr.

The analytical column (25 cm x 4.6 mm I.D.) was a Zorbax ODS (DuPont). Operating conditions were: mobile phase, methanol/water (85:15, v/v); flow rate, 1 ml/min; column temperature, ambient; UV wavelength, 245 nm; chart speed, 0.5 cm/min.

METHODS

Semi-Preparative HPLC Separations

A methanol solution of carbon-14 labeled avermectins was obtained from an isolate of the fermentation broth. One ml of this solution contained approximately 35 mg [^{14}C]avermectins with approximately 600 μCi of radioactivity. Three ml of the solution was injected per each run. A total of seven runs was made for the normal phase separation. The fractions containing avermectin B_{1a} were collected and concentrated using rotary evaporation. The separation of avermectin B_{1a} and B_{1b} was achieved by reverse phase HPLC. The pure avermectin B_{1a} was obtained by another normal phase separation to remove the trace contaminant, avermectin A_2. The fractions containing pure avermectin B_{1a} were collected and concentrated using rotary evaporation.

Analytical HPLC/Radioassay

The purified avermectin B_{1a} was analyzed for chemical purity by analytical HPLC. The effluent was collected in fractions for radioassay using standard liquid scintillation counting technique to determine the radiopurity.

TLC/Radioassay

The purified avermectin B_{1a} was also analyzed by TLC technique. The developing solvent was hexane/isopropyl alcohol (51:9, v/v). The avermectin B_{1a} spot on the developed plate was visualized by both autoradiography and UV light (254 nm). The radiopurity was determined by liquid scintillation counting of the TLC plate scrapings in a liquid scintillation counter.

RESULTS AND DISCUSSION

The chromatogram of the semi-preparative normal phase separation of avermectins A_1, A_2, B_1, and B_2 is shown in Figure 2. Through TLC with radioassay, the first peak with a retention time

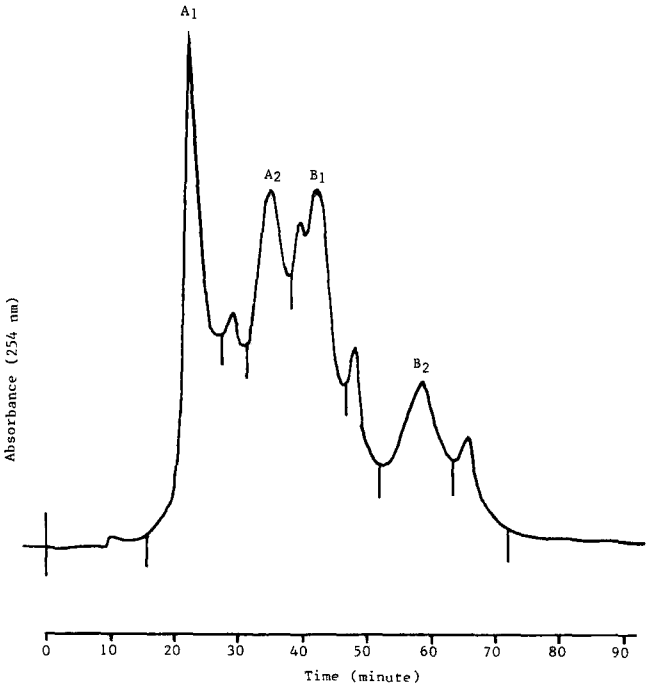


Figure 2. Semi-preparative Normal Phase HPLC Chromatogram of A_1, A_2, B_1, and B_2.

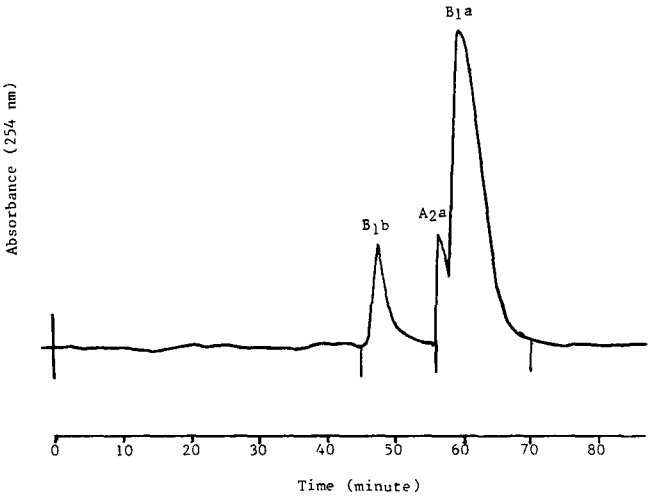


Figure 3. Semi-preparative Reverse Phase HPLC Chromatogram of Avermectins B_1a and B_1b.

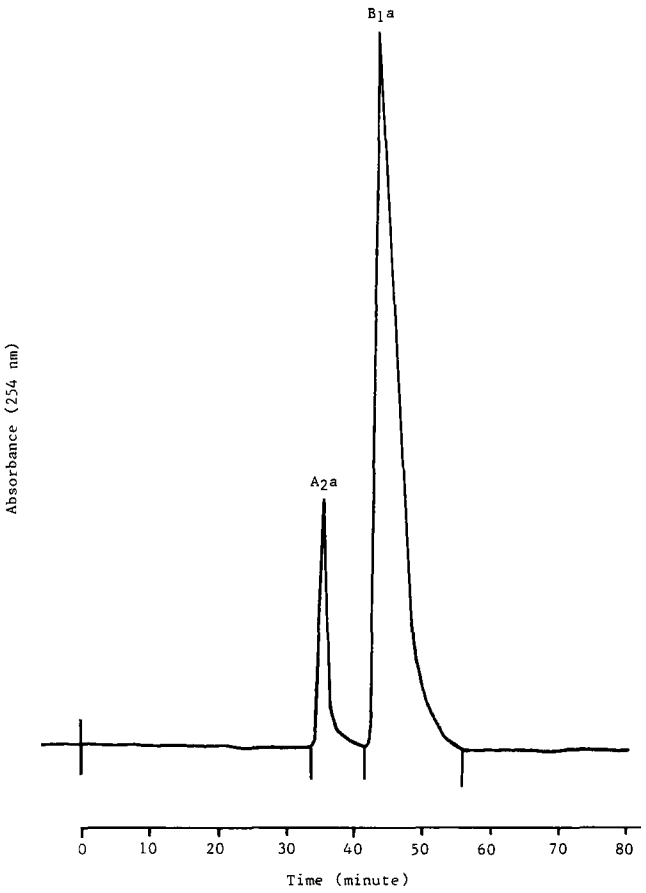
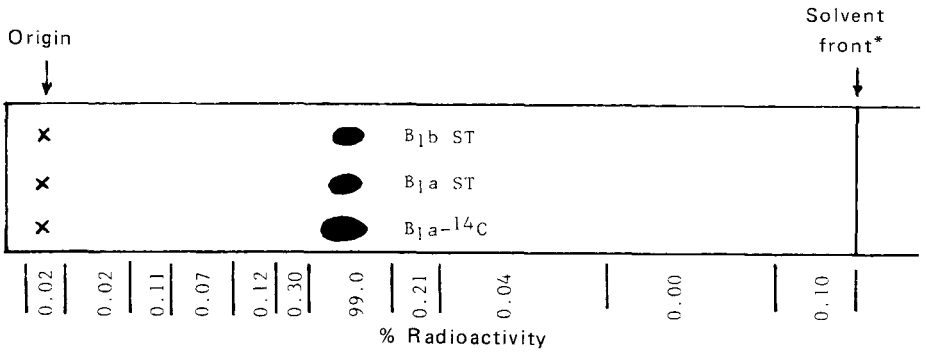


Figure 4. Semi-preparative Normal Phase HPLC Chromatogram of Avermectins B_1a and A_2a.

(R_T) of 22.5 min is identified as avermectin A_1, the peak with R_T = 35.5 min is avermectin A_2, the peak with R_T = 42.5 min is avermectin B_1a, and the peak with R_T = 59.0 min is B_2. In all four fractions, i.e., avermectin A_1, A_2, B_1, and B_2, the components a and b of the same avermectin e.g., A_1a and A_1b, were not separated



*Developed 3 times with hexane/isopropyl alcohol (51:9, v/v).

Figure 5. TLC Chromatogram of Avermectin B_1b (B_1b ST), Avermectin B_1a (B_1a ST) and Avermectin (B_1a-^{14}C) Collected from the Semi-preparative Separations. St, standards.

on normal phase HPLC. The absorption properties of silica gel for components a and b are nearly the same because the only difference between a and b structurally is the alkyl side chain at C-25, i.e., a is isobutyl and b is isopropyl (See Figure 1). However, when the fraction containing avermectin B_1 was concentrated and reinjected onto a semi-preparative reverse phase HPLC, a baseline separation of avermectin B_1a and B_1b was obtained. The chromatogram is shown in Figure 3. From the results of TLC and radioanalysis, the peak with R_T = 47.5 min is avermectin B_1b, the peak with 57.0 min is avermectin A_2a, and the peak with R_T = 59.5 min is avermectin B_1a.

Since avermectin B_1a contained some avermectin A_2a, these two components were collected together and concentrated, then reinjected onto the semi-preparative normal phase HPLC again to obtain a baseline separation of the two avermectins. The peaks again were analyzed by TLC and radioanalysis. The chromatogram is shown in Figure 4.

The fractions containing pure avermectin B_1a were collected and concentrated. In order to ascertain the chemical purity and radiopurity, and to check the identity of the purified avermectin B_1a, analytical HPLC and TLC/radioassay were employed. The results are presented in Figure 5 for the analyses by TLC and Figure 6 for the evaluation by analytical HPLC. The identity and

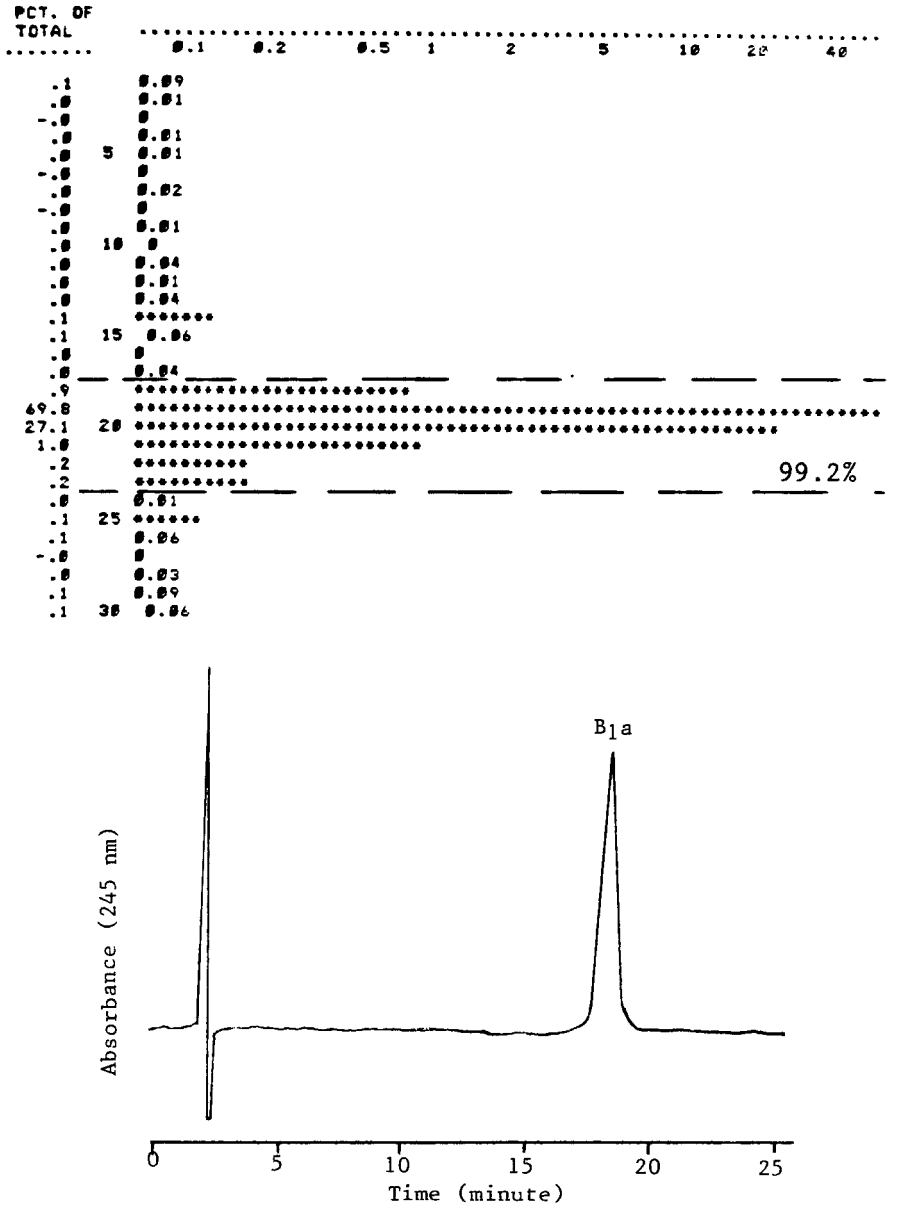


Figure 6. Analytical HPLC Chromatogram/Radio-histogram of Avermectin in B_1a.

Figure 7. UV Spectrum of Avermectin B_1a-^{14}C.

concentration of the purified avermectin B_1a were confirmed and measured by UV spectrometry (Figure 7).

This semi-preparative HPLC method offers a rapid means of obtaining [^{14}C]avermectin B_1a that are chemically and radiochemically pure (99+%) in sufficient quantity to do metabolism and environmental chemistry research.

ACKNOWLEDGEMENT

We are grateful to Drs. L. Kaplan, H. Mertel, and R. Ellsworth for valuable assistance in the biosynthesis of the [^{14}C]avermectins. We also thank Mr. H. Meriwether for technical assistance in radioassay and Dr. S. H. L. Chiu for valuable discussion.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(14), 2915-2920 (1984)

CONSTITUENTS OF HIGHER FUNGI. PART XVI.* IDENTIFICATION OF LACTARIUS SPECIES BY HPLC USING SESQUITERPENE MONOHYDROXYLACTONE CONTENTS AS CHARACTERISTIC CHEMOTAXONOMIC FEATURES.

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ABSTRACT

It has been shown that the sesquiterpene monohydroxylactone contents of several species of mushrooms of the Lactarius family are characteristic chemotaxonomic features of the species. The profile of such compounds can easily be determined by HPLC and the resulting chromatograms can serve for purposes of identification of the species.

INTRODUCTION

In our previous investigations on mushrooms (1-3) of the Lactarius family, we isolated and identified several monohydroxylactones with lactarane and secolactarane skeletons. During our investigations (1), it was found that the monohydroxylactone content in each species was a characteristic chemotaxonomic feature. The contents could be determined by HPLC using a simple procedure and the chromatograms could serve for identification of these species.

*Part XV, Daniewski, W. M. et al, Tetrahedron, in press

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Identification of mushrooms is sometimes very difficult. In 1972, Professor A. Nespiak in Poland suggested to us the use of standardised TLC chromatograms of mushroom extracts for their identification. The TLC method is not accurate as compared with HPLC. In our previous paper (1) we published HPLC chromatograms of the monohydroxylactone contents of the following mushrooms: Lactarius necator, L. quietus, L. helvus, L. torminosus, L. glyciosmus and L. subdulcis. Now we wish to report the chromatograms of the following mushrooms: L. necator (standard), L. turpis, L. controversus, L. vellereus, L. pergamenus, L. vietus L. spinosulus and L. blennius.

EXPERIMENTAL

Materials

All the mushrooms were collected in Poland. Solvents were purified by standard procedures which included distillation just before use.

Apparatus

HPLC chromatograms were obtained with a home assembled isocratic liquid chromatograph, which included Waters 6000 SDS, U6K universal injector, Varian RI detector, and Chemipan IC-01 integrator. The column had 4 mm i.d., 300 mm length and was packed with 10μ Si60 Lichrosorb using a high pressure slurry technique (9000 theoretical plates).

Preparation of Extracts

Fresh mushrooms (30 - 50 g) were ground with ethanol (200 ml) and stored for 24 hours at ambient temperature. The slurries of

mushrooms were filtered through a sintered glass funnel covered with a 1 cm layer of cellite filter aid. The ethanol extracts were evaporated to dryness and dissolved in a 1:1 water/ether mixture (100 ml). The water layer was extracted with ether (3 x 75 ml) and the extracts were combined and the ether evaporated. The residue was prepurified by passing through short columns filled with alumina (neutral) in a solute/adsorbent ratio 1:15. The columns were eluted with a benzene/ethanol (7:3) mixture until lactarorufin A (TLC, green spot, H_2SO_4) appeared in the eluate. The evaporated eluates were rechromatographed on TLC plates (Merck, benzene/acetone 8:2). The zones of spots containing monohydroxylactones (Rf, 0.3 - 0.4) were separated, extracted with ethyl acetate, the solvent evaporated and the residue weighed.

Analytical Procedure

Before the analysis, the column was conditioned by passing through 20 volumes of solvent (hexane/ethylacetate 85:15). The flow rate was 1 ml/min. Purified samples were injected (ca. 0.5 mg) through the Waters U6K injector. The duration of every analysis was ca. 90 min. Since the Lactarius necator is a very common mushroom in Poland, its extract was found to be very useful for standardisation of these chromatograms. The retention volume of each lactone was checked against the volumes obtained by injection of the mixture from Lactarius necator to which blennin A had been added [4]. Any doubts about the identity of a peak could be cleared up by collecting samples of the lactone and measuring its mass spectrum.

TABLE 1

Mushroom \ Peak No				1	2	3	4		5				7
L.necator	% time k'			33 2 42.5 8.44	7.3 45.0 9.0	15.8 47.1 9.5	28 2 51 5 10 4		7.2 55.8 11.4				14 4 69.2 14.4
L.controversus	% time k'			9.7	54.7	35.6							
L.vellereus	% time k'			6.6	9.9	45.6	15.8						22.1
L.turpis	% time k'				3.4	66.1	11.2						19.3
L.pergamenus	% time k'	9 9 37.8 7 5	6 0 41 4 8.2	20.5	18.2	29.7					15.6 62.1 12.34		
L.spinosulus	% time k'			4.7	11.2	15.9	4.2	42 3 54.2 10.75				21 8 68.1 13.5	
L.vietus	% time k'			3.2	11.4	9.9	22.7		14.9	18 9 59.9 11.9		19.1	
L.blennius	% time k'			15.33	33.49	10.61	6.60				28.30		5 67

RESULTS AND DISCUSSION

In figure 1 we can see the chromatograms of the monohydroxylactone contents of various species. The chromatogram of L necator has already been presented and serves as a standard (1). Table 1 summarizes the data obtained from the chromatograms. It was necessary to use a high capacity factor (k') in order to achieve separation. It was found again that the monohydroxylactone contents in the mushrooms investigated are characteristic features of every species. Extracts of Lactarius pergamenus, L. spinosulus and L. vietus contained new compounds in addition to the known monohydroxylactones. The compounds will be isolated in larger quantities and their structures will be investigated.

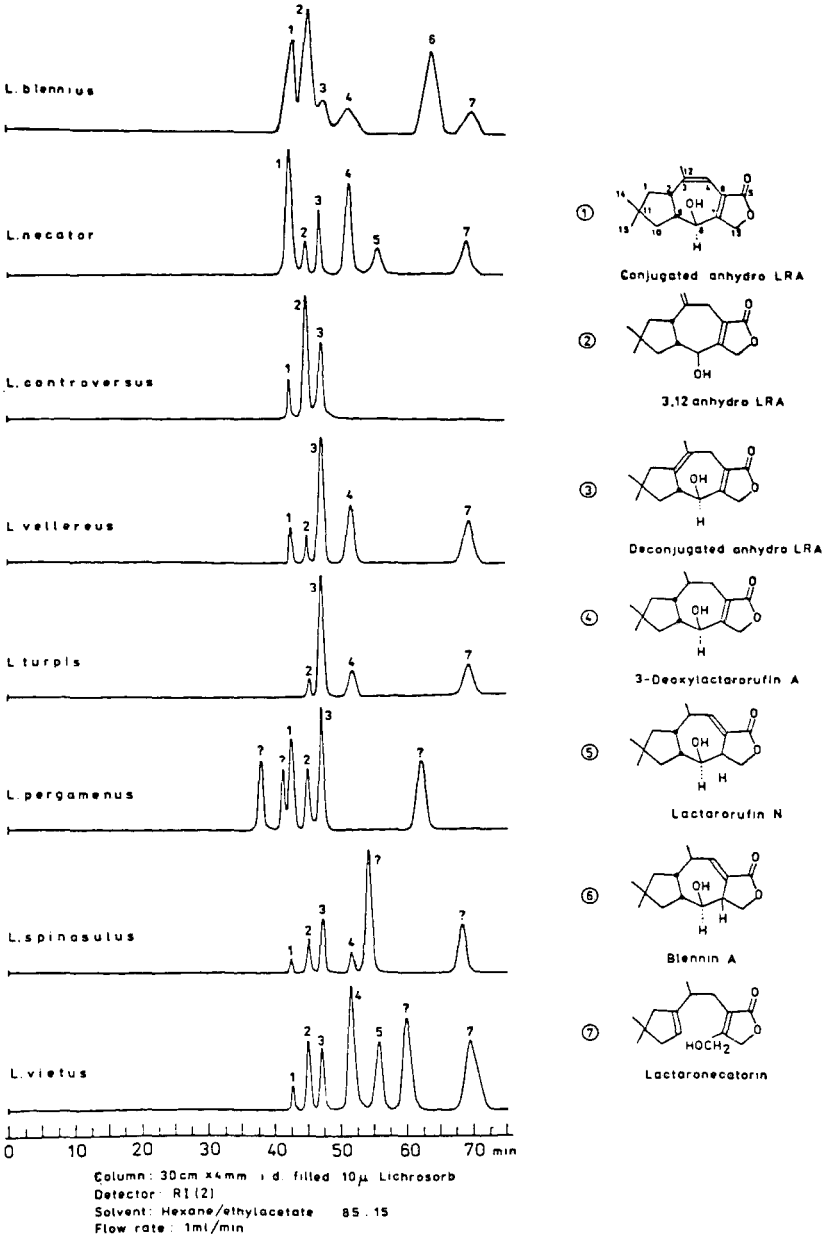

Figure 1. Chromatograms of the Monohydroxy-Lactone Contents of Various Species.

Lactarius controversus contained only three monohydroxylactones. Compound 3, the deconjugated anhydrolactarorufin A, was present in every species; thus it must be a very important intermediate in the biogenesis of these compounds. There are 70 species of Lactarius mushrooms in Poland and every one of them according to their supply will be investigated.

Monohydroxylactones play an important role in the life of these species as they have been found to be strong insect deterrents.(5)

ACKNOWLEDGEMENTS

We are grateful to Prof. Paola Vita-Finzi of the University of Pavia, Italy for providing us with a sample of blennin A, Mr. W. Ignacak for technical assistance, and The Polish Academy of Sciences for financial support under grant MR.I. 12. 1.5.3.2.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(14), 2921-2922 (1984)

BOOK REVIEWS

"STERIC EXCLUSION LIQUID CHROMATOGRAPHY OF POLYMERS", Josef Janca, ed., Marcel Dekker, Inc., New York and Basel, 1984, 329 pp., $55.00 (US).

This volume is another compilation of a type which has become popular in the last few years; that is, a series of chapters covers specific areas of a broader subject, each chapter written by a recognized authority in that area. The editor has done an excellent job of coordinating and assembling the material. Cross references among the various chapters are frequent and, as a result, the reader benefits from a review which is more coherent than many reviews of this type.

The book is divided into seven chapters as follows:

1. Principles of Steric Exclusion Chromatography.
2. Calibration of Separation Systems.
3. Correction of Axial Dispersion.
4. Effect of Experimental Conditions.
5. Use for Polymer Analysis.
6. Automatic Data Treatment.
7. Precision and Accuracy of Results.

As described by the contents above, this is not a "how to do it" book for the practitioner, but is a serious attempt to present to the reader an up-to-date review of the fundamental aspects of steric exclusion chromatography. As such, it fills a void in the published literature. The extensive reference list for each section should also be very useful.

For the most part, the various authors have done an excellent job of presenting to the reader the existing state of the method. The chapters on calibration, experimental conditions and polymer analysis are especially well written and informative.

If there is any criticism to be given, in several instances the authors present conflicting opinions or alternate explanations taken from the literature with no attempt to evaluate or express an opinion as to which may be the more correct. Perhaps this was done intentionally, but it is especially distracting in the chapter on "Correction for Axial Dispersion."

This book is especially recommended to polymer researchers and chromatographers who, in order to fully utilize the information available from the analysis, need to have a thorough understanding of the principles and mechanisms involved in size exclusion chromatography of polymers and oligomers.

Dale J. Harmon
B. F. Goodrich R & D Center
Brecksville, Ohio
August 9, 1984

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(14), 2923-2926 (1984)

LC CALENDAR

1984

DECEMBER 10-12: "TLC/HPTLC-84: Expanding Horizons in TLC," Sheraton-University City, Philadelphia, PA. Contact: J. C. Touchstone, University of Pennsylvania, Dept. OB-GYN, 3400 Spruce e Street, Philadelphia, PA.

DECEMBER 10-12: Fourth International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Baltimore, MD. Contact: Shirley E. Schlessinger, Symposium Manager, 400 East Randolph, Chicago, IL, USA.

DECEMBER 16-21: International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii, sponsored by the Chemical Inst. of Canada, Chemical Soc. of Japan, and the American Chem. Soc. Contact: PAC CHEM '84, International Activities Office, American Chem. Soc., 1155 Sixteenth St., NW, Washington, DC, 20036, USA.

1985

FEBRUARY 10-15: Symposium on the Interface Between Theory and Experiment, Canberra, Australia. Contact: Leo Radom, Research School of Chemistry, Australian National University, Canberra, ACT 2601, Australia.

FEBRUARY 11-14: Polymer 85, Int'l Symposium on Characterization and Analysis of Polymers, Monash University, Melbourne, Australia, sponsored by the Polymer Div., Royal Australian Chemical Inst. Contact: Polymer 85, RACI, 191 Royal Parade, Parkville Victoria 3052, Australia.

FEBRUARY 25 - MARCH 1: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, New Orleans, LA. Contact: Linda Briggs, 437 Donald Road, Pittsburg, PA, 15235, USA.

MARCH 23-24: Conference on Creativity & Science, Honolulu, Hawaii. Contact: D. DeLuca, Scientists and Humanities Conf., Winward Community College, University of Hawaii, 45-720 Keaahala Rd., Kaneohe, Hawaii, 96744, USA.

APrIL 8 - 11: 10th Annual AOAC Spring Training Worshop, Sheraton Hotel, Dallas, Texas. Contact: M. V. Gibson, USFDA, 332 Bryan, Dallas, TX, 75204, USA.

APRIL 15 - 17: Second International Symposium on Instrumental TLC, Wurzburg, West Germany. Contact: H. M. Stahr, 1636 College Veterinary Medicine, Iowa State University, Ames, IA, 50011, USA., or Prof. S. Ebel, Institute for Pharmacies, A. M. Hubland, D-8700 Wuerzburg, West Germany.

APRIL 15-18: Materials Research Society Spring Meeting, San Francisco, CA. Contact: Susan Kalso, Xerox Palo Alto Res. Center, 3333 Coyote Hill Road, Palo Alto, CA, 94304, USA.

APRIL 28 - MAY 3: 189th National ACS Meeting, Miami Beach. Contact: A. T. qinstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

APRIL 29 - MAY 2: Symposium on Analytical Metods in Forensic Chemistry & Toxicology, Miami, Florida; in conjunction with 189th ACS Nat'l Meeting. Contact: Dr. M. H. Ho, Dept. of Chem., University of Alabama, Birmingham, AL, 35294, USA.

MAY 13 - 15: Infant Formula Conference, Sheraton Hotel, Virginia Beach, VA. Contact: Dr. James Tanner, USFDA-Hff-266, 200 C Street, SW, Washington, DC, 20204, USA.

MAY 19: Middle Atlantic Regional ACS Meeting, Sponsored by ACS Monmouth County Section. Contact: M. Parker, Dept. of Chem., Monmouth College, West Long Branch, NJ, USA.

JUNE 9-15: ACHEMA 85, Frankfurt, West Germany. Contact: DECHEMA, Organization ACHEMA, P.O.Box 97 01 46, D-6000 Frankfurt, 97, West Germany.

JUNE 24 - 28: 59th Colloid & Surface Science Symposium, Clarkson College of Technology, Potsdam, NY. Contact: J. P. Kratohvil, Institute of Colloid & Surface Science, Clarkson College of Technology, Potsdam, NY, 13676, USA.

JULY 1-5: Ninth International Symposium on Column Liquid Chromatography, sponsored by the Chromatography Discussion Group and by the Royal Society of Chemistry's Chromatography & Electrophoresis Group, Edinburgh, Scotland. Contact: Prof. J. H. Knox, 9th ISCLC Secretariat, 26 Albany Street, Edinburgh, EH1 3QH,

Great Britain.

JULY 4: 4th International Flavor Conference, Greece. Contact: C. J. Mussinan, IFF R&D, 1515 Highway 36, Union Beach, NJ, 07735, USA.

SEPTEMBER 1-6: 6th International Symposium on Bioaffinity Chromatography & Related Techniques, Prague, Czechoslovakia. Contact: Dr. J. Turkova, Institute of Organic Chemistry & Biochemistry CSAV, Flemingovo n. 2, CS-166 10 Prague 6, Czechoslovakia.

SEPTEMBER 8-13: 190th National ACS Meeting, Chicago. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA

SEPTEMBER 29 - OCTOBER 4: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Marriott Hotel, Philadelphia, PA. Contact: T. Rains, NBS, Center for Analytical Chemistry, Chemistry B-222, Washington, DC, 20234, USA.

OCTOBER 27 - 30: 99th Annual AOAC Meeting, Shoreham Hotel, Washington, DC. Contact:Margaret Ridgell, AOAC, 1111 N. 19th Street, Suite 210, Arlington, VA, 22209, USA.

1986

APRIL 6-11: 191st National Am. Chem. Soc. Mtng., Atlantic City, NJ. Contact: A. T. Winstead, ACS, 1155 16th Streeet, NW, Washington, DC, 20036, USA.

SEPTEMBER 7-12: 192nd National Am. Chem. Soc. Mtng., Anaheim, Calif. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA

SEPTEMBER 21-26: XVIth International Symposium on Chromatography, Paris, France. Contact: G.A.M.S., 88,Boulevard Malesherbes, F-75008 Paris, France.

OCTOBER 12 - 16: 100th Annual AOAC International Meeting, Shoreham Hotel, Washington, DC. Contact: Margaret Ridgell, AOAC, 1111 N. 19th Street, Suite 210, Arlington, VA, 22209, USA.

1987

APRIL 5-10: 193rd National Am. Chem. Soc. Mtng., Denver, Colo. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

AUGUST 30 - SEPTEMBER 4: 194th National Am. Chem. Soc. Mtng., New Orleans, LA. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

1988

JUNE 5 - 11: 3rd Chemical Congress of the North American Continent, Toronto, Ont., Canada. Contact: A. T. Winstead, ACS, 1155 Sixteenth St, NW, Washington, DC, 20036, USA.

SEPTEMBER 25 - 30: 196th ACS National Meeting, Los Angeles, CA. Contact: A. T. Winstead, ACS, 1155 Sixteenth Street, NW, Washington, DC, 20036, USA.

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

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