JOURNAL OF LIQUID CHROMATOGRAPHY

VOLUME 7 NUMBER 3 1984

Editor: DR. JACK CAZES
Associate Editor: DR. HALEEM J. ISSAQ

CODEN: JLCHD8 7(3) i-x, 441-646 (1984)
ISSN: 0148-3919

ASI HPLC PACKINGS

GUARANTEED* REPLACEMENTS FOR ALL WATERS' uBONDAPAK™ PACKINGS

ASI offers a complete line of 10um, fully porous packings to compete with the Waters' uBondapak™ series. We believe we have duplicated or, in some cases improved the chemistry that has made uBondapak™ the most popular packings in the world.

ASI-packed columns are more stable and last longer than those packed by our competitors – we guarantee it! If you are not delighted with any ASI product you may return it for a prompt refund.

Why 10 micron, irregular particles?

SELECTIVITY

Enhanced selectivity is possible because irregular particles have more surface area than spherical particles. Selectivity is more important than efficiency. Selectivity is an inherent property of a particle; efficiency is not. Efficiency can change quickly and separations based on efficiency alone can be lost suddenly and without warning. Loss of selectivity happens gradually and predictably allowing for planned column replacement.

COLUMN LIFE

ASI believes that HPLC columns should last until the coating is used up or wears off. You cannot produce a stable packed bed using irregularly shaped particles of less than 10um. Excessive operating pressures cause premature column failures providing a poor value for your column dollar.

ASI PACKING	DIRECT REPLACEMENT FOR	PREPACKED		REPACKED	
		PART #	PRICE	PART #	PRICE
ASI C18					
3.9 x 15	uBondapak™ C18	101	$195.	RP-101	$150.
3.9 x 30		102	245.	RP-102	195.
7.8 x 30		103	550.	RP-103	450.
ASI PHENYL					
3.9 x 15	uBondapak™ Phenyl	201	$195.	RP-201	$150.
3.9 x 30	&	202	245.	RP-202	195.
7.8 x 30	Fatty Acid Analysis	203	550.	RP-203	450.
ASI SILICA					
3.9 x 15	uPorasil™	301	$180.	RP-301	$135.
3.9 x 30		302	220.	RP-302	170.
7.8 x 30		303	495.	RP-303	395.
ASI NH_2					
3.9 x 15	uBondapak™ NH_2	401	$195.	RP-401	$150.
3.9 x 30	&	402	245.	RP-402	195.
7.8 x 30	Energy Analysis	403	550.	RP-403	450.
ASI CYANO					
3.9 x 15	uBondapak™ CN	501	$195.	RP-501	$150.
3.9 x 30		502	245.	RP-502	195.
7.8 x 30		503	550.	RP-503	450.
ASI CARBOHYDRATE					
3.9 x 15	Carbohydrate	601	$195.	RP-601	$150.
3.9 x 30	Analysis	602	245.	RP-602	195.
7.8 x 30	Column	603	550.	RP-603	450.

asi ANALYTICAL SCIENCES INCORPORATED
SUITE B-24, AIRPORT PARK • 1400 COLEMAN AVENUE • SANTA CLARA, CALIF. 95050 • (408) 779-0131
TELEX 176646

ASI

Circle Reader Service Card No. 101

JOURNAL OF LIQUID CHROMATOGRAPHY

JOURNAL OF LIQUID CHROMATOGRAPHY

JOURNAL OF LIQUID CHROMATOGRAPHY

March 1984

Aims and Scope. The journal publishes papers involving the application of liquid chromatography to the solution of problems in all areas of science and technology, both analytical and preparative, as well as papers that deal specifically with liquid chromatography as a science within itself. Included will be thin-layer chromatography and all modes of liquid chromatography.

Indexing and Abstracting Services. Articles published in *Journal of Liquid Chromatography* are selectively indexed or abstracted in:

• Analytical Abstracts • ASCA • BioSciences Information Service of Biological Abstracts (BIOSIS) • Chemical Abstracts • Current Awareness in Biological Sciences • Current Contents/Life Sciences • Current Contents/Physical and Chemical Sciences • Engineering Index • Excerpta Medica • Journal of Abstracts of the All-Union Institute of Scientific and Technical Information of the USSR • Physikalische Berichte • Science Citation Index

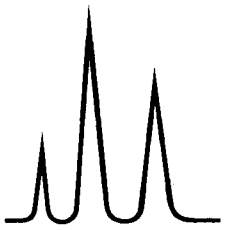
Manuscript Preparation and Submission. See the last page of this issue.

Subscription Information. *Journal of Liquid Chromatography* is published in fourteen numbers and two supplements in January, February, March (2 numbers), April, May (2 numbers), June, July (2 numbers), August, September, October (2 numbers), November, and December by Marcel Dekker, Inc., 270 Madison Avenue, New York, New York 10016. The subscription rate for Volume 7 (1984), containing fourteen numbers and two supplements, is $350.00 per volume (prepaid). The special discounted rate for individual professionals and students is $175.00* per volume. To secure this special rate, your order must be prepaid by personal check or may be charged to MasterCard or VISA. Add $40.00 for surface postage outside the United States. For airmail to Europe, add $72.32; to Asia, add $91.52.

Mailing Address. *Please mail payment with order to:* Marcel Dekker Journals, P. O. Box 11305, Church Street Station, New York, New York 10249.

Contributions to this journal are published free of charge. Application to mail at second-class postage rates is pending at New York City, New York and additional mailing offices.

***THIS REFLECTS A 50% DISCOUNT GIVEN TO INDIVIDUAL SUBSCRIBERS.**



Fourth International Symposium on HPLC of Proteins, Peptides and Polynucleotides

December 10-12, 1984, Baltimore, Maryland

The Fourth International Symposium on HPLC of Proteins, Peptides, and Polynucleotides is being organized to bring together researchers involved in this rapidly growing field. The Symposium will be held at the Hyatt Regency Hotel in Baltimore, MD. This hotel is located in Baltimore's scenic Inner Harbor close to a variety of attractions, including the National Aquarium and Baltimore Science Center. The three-day program will include oral presentations and poster sessions covering the following topics:

- Column Technology and Support Materials
- Micropreparative Techniques and Recovery of Biological Activity
- HPLC of Polynucleotides
- Peptide Mapping by HPLC
- Membrane Proteins
- HPLC Analysis of Amino Acids
- Analytical Applications of HPLC
- Scale-up and Process-Level Preparative Chromatography
- Multistep and Multidimensional Chromatographic Techniques
- Special Topics, including Clinical Applications

Call for Papers

You are invited to submit an abstract describing original research in any of the above areas by August 1, 1984. The chairmen of the Symposium, Dr. Fred E. Regnier of Purdue University, Dr. Klaus Unger of Johannes Gutenberg-Universität, Dr. Milton T.W. Hearn of St. Vincent's School of Medical Research, Dr. C. Timothy Wehr of Varian Associates, Inc., and Dr. Jan-Christer Janson of Pharmacia Fine Chemicals AB, welcome your suggestions for additional categories. Abstract forms and registration information may be obtained from:

Shirley E. Schlessinger, Symposium Manager
Fourth International Symposium on HPLC
of Proteins, Peptides, and Polynucleotides
400 East Randolph
Chicago, IL 60601; telephone (312) 527-2011

Circle Reader Service Card No. 117

INS-3021/22

SHOWA DENKO

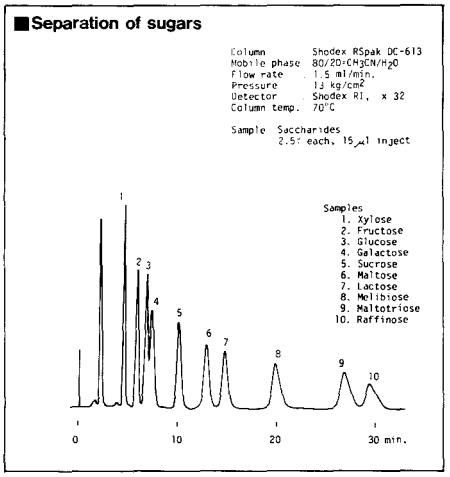
Shodex RSpak D Series

Columns packed with Porous Polymer Gels for High-Performance Partition/Adsorption Liquid Chromatography

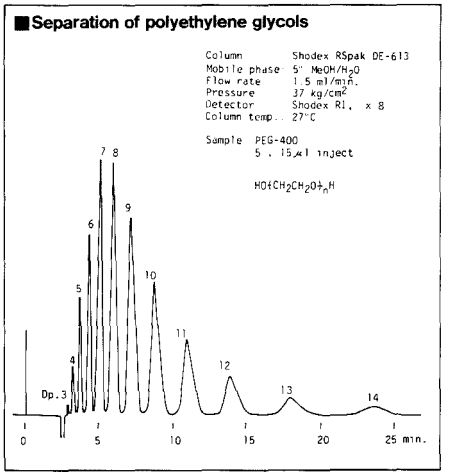
Features

1. **Effective over pH range of 1 to 13**
   In contrast to columns packed with chemically bonded silica gels, there are practically no pH value limitations on the mobile phase they use.
2. **Usable for wide variety of mobile phases**
   The variety of mobile phases that can be used is greater than in the case of columns packed with conventional porous polymer gels.
3. **High performance with 40,000 theoretical plates per meter**
   The bands formed in the chromatogram are narrower than those formed by columns packed with conventional porous polymer gels.
4. **Packings in different polarities**
   Since the packings come in four different polarities, samples ranging in polarity from high to low can be readily separated.
5. **Versatility**
   The columns of this series are also suitable for ion-pair chromatography.

■Separation of sugars



■Separation of polyethylene glycols



Nomenclature	Theoretical plates	Size	In-column eluent	Packed gel
DS-613	5,000	6mmφ×150mm	$CH_3N/THF/H_2O$ 40/30/30	Hydrophobic polystyrene gel
DE-613	5,000	〃	H_2O	Polymethacrylate gel
DM-614	4,000	〃	0.005M H_2PO_4	Hydrophilic polyester gel
DC-613	5,000	〃	CH_3N/H_2O 70/30	Hydrophilic polystyrene ionexchange gel

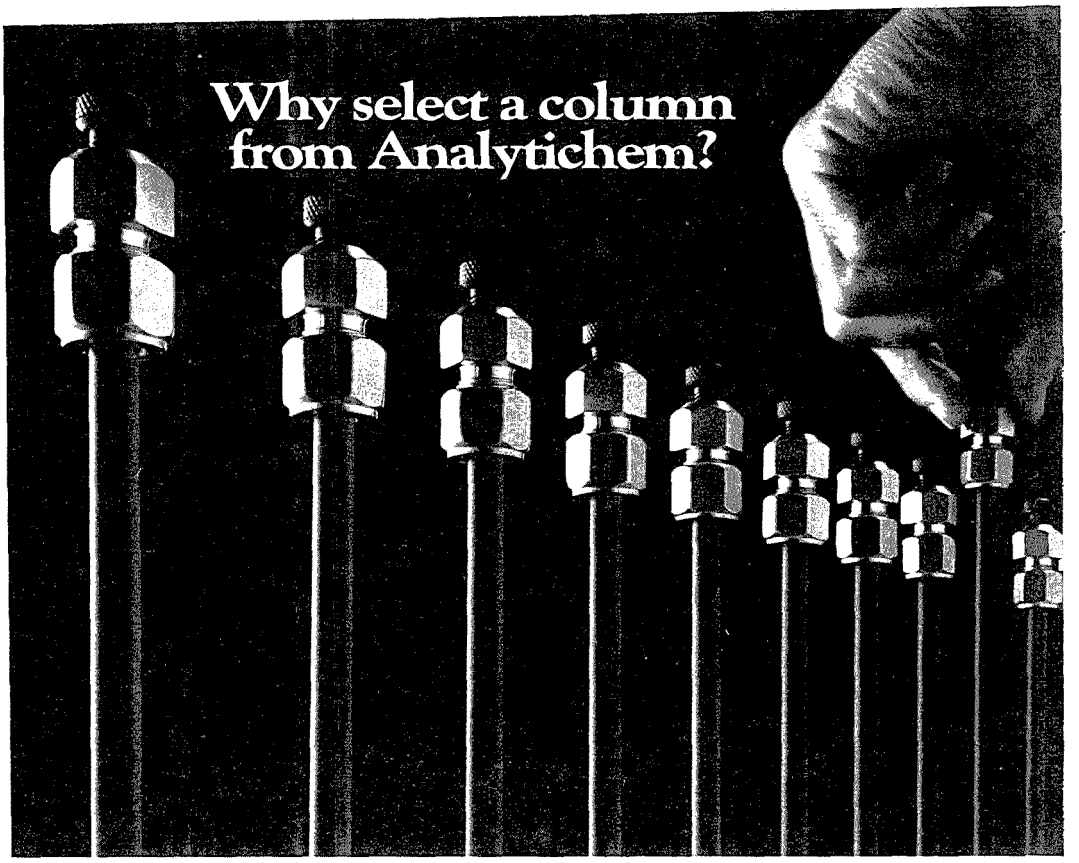
Contact us for further information.

SHOWA DENKO K.K.
Instrument Products Division
13-9, Shiba Daimon 1-chome, Minato-ku, Tokyo 105, Japan
Telephone: (03) 432-5111 Telex: J26232
Cable: SECIC TOKYO

SHOWA DENKO AMERICA, INC.
280 Park Avenue
West Building, 27th Floor
New York, N.Y. 10017, U.S.A.
Telephone 212-687-0773
Telex 23423898

SHOWA DENKO (EUROPE) GmbH
4000 Dusseldorf, Charlottenstr 51
Federal Republic of Germany
Telephone (0211) 362037
Telex 8587649 SDK D

Circle Reader Service Card No. 109



Because the greater the selection the greater the selectivity.

The new line of HPLC columns from Analytichem provides selectivity unequalled by anyone. The reason is simple. Analytichem offers a wider selection of phases than anyone.* Now you can choose the phase that is precisely suited to your particular application. These new columns, packed with our unique Sepralyte™ 5μm spherical media, set an unprecedented standard of chromatographic efficiency... regardless of the phase you select.

The performance of each new Analytichem column is fully guaranteed and backed by the industry's strongest customer service and technical support teams. Our technical advisors have the training and hands-on experience to assist you in solving virtually any separation problem. Next time you're considering HPLC columns, be selective. Call Analytichem. You'll find the columns you need and the service you deserve.

Analytichem International
24201 Frampton Ave., Harbor City, CA 90710. USA. (800) 421-2825. In California (213) 539-6490
TELEX 664832 ANACHEM HRBO

JOURNAL OF LIQUID CHROMATOGRAPHY

Volume 7, Number 3, 1984

CONTENTS

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(3), 441-461 (1984)

SIZE EXCLUSION CHROMATOGRAPHY OF POLY (VINYLPYRROLIDONE): I. THE CHROMATOGRAPHIC METHOD

E. G. Malawer, J. K. DeVasto,
S. P. Frankoski, and A. J. Montana*
GAF Corporation
1361 Alps Road
Wayne, NJ 07470

*Present Address: Diamond Shamrock Corporation
350 Mt. Kemble Avenue
Morristown, New Jersey 07960

ABSTRACT

The optimum size exclusion chromatographic (SEC) method for poly-(vinylpyrrolidone) (PVP) was found to be based upon a stationary phase of diol derivatized silica of pore sizes 3000, 500, and 75Å and a mobile phase of 50:50 (v/v) $MeOH/H_2O$ containing 0.1M-$LiNO_3$. Sample recovery under identical conditions varied for the commercial packings investigated and was found to be inversely related to molecular weight. The latter phenomenon was rationalized on the basis of a limited number of active substrate sites available for binding. Methanol was found to be a more effective mobile phase modifier than either dimethyl formamide or acetonitrile apparently due to its ability to function as a proton donor in hydrogen bonding with PVP. Chromatographic evidence for the existence of semipolyampholyte character in PVP is presented. A procedure for the construction of a column set log-linear in calibration and of extended dynamic range is described and is based upon hydrodynamic volume theory.

INTRODUCTION

Traditionally, the molecular weight of PVP has been characterized indirectly by means of the Fikentscher K-value, K,

 0148-3919/84/0703-0441$3.50/0

(1,3) which is defined in terms of the relative viscosity, η_{rel}, and the concentration c (in g/dl), of a given solution:

$$\frac{\log \eta_{rel}}{C} = \frac{75K_o^2}{1 + 1.5\ K_o C} + K_o$$

$$\text{and } K = 1000\ K_o$$

At present, PVP is produced in four basic K-value grades in the United States; namely, 15, 30, 60, and 90 which correspond to nominal viscosity-average molecular weights of 7,700, 38,000, 216,000, and 630,000 amu, respectively. These amounts are based upon a Mark-Houwink coefficient of 1.4×10^{-4} dl/g and an exponent of 0.7 for water at 25°C as described by Scholtan and coworkers (4,5).

Knowledge of the absolute molecular weight distribution of a polymeric material allows one to predict end-use properties which are not dependent upon and thus cannot be predicted by viscosity parameters such as K-value. The production of a molecular weight distribution by gel permeation chromatography (generally known as size exclusion chromatography) is inherently a two-part process: the development of a suitable chromatographic method and the establishment of an absolute means of calibration. The present paper will concern itself with the former aspect only for the case of PVP.

Chromatographic supports of crosslinked agarose (6) and dextran gels (7,9) have both been reported to successfully elute poly (vinylpyrrolidone). As a result of the poor mechanical strength of these gels, they cannot tolerate the normal flow rates (1-2 ml/min.) desirable for high performance liquid chromatography and, consequently, run times are long. They are also prone to irreversible swelling and deswelling phenomena which are tantamount to loss of resolution and efficiency. To overcome the difficulties associated with soft gels researches have resorted to the use of semi-rigid gels such as crosslinked poly(styrene/ divinyl benzene) in conjunction with a mobile phase

of N,N-dimethyl formamide (DMF) (10,11) or N-methyl pyrrolidone (NMP) (12) with or without the addition of 0.1M - LiBr.

This method has been found to be impractical by this laboratory from two standpoints. First, column efficiencies were observed to drop by more than 75% over several months of continuous use. This was attributed to irreversible swelling in either DMF or NMP as has been cited by one manufacturer of such gels, Waters Associates (Milford, MA). Second, as differential refractometry is the detection system most commonly employed and as PVP is particularly hygroscopic, a large negatively-oriented peak at the total permeation volume (V_t) is observed due to water. Because PVP is so similar in structure and thus in refractive index to either NMP or DMF, the residual water has a much higher response factor than the polymer in these solvents. These large water peaks have been noted to obliterate the low molecular weight tails of low K-value grades of PVP molecular weight distributions making quantitation extremely difficult.

Rigid packings based upon porous silica are not subject to the irreversible swelling/deswelling phenomena exhibited by both the soft and semi-rigid gels. A method for PVP involving a hydrophobically modified silica was investigated by this laboratory. This material (produced by E. I. DuPont de Nemours & Co., Inc., and consisting of porous silica deactivated by chlorotrimethylsilane) was utilized with a mobile phase of DMF containing 0.1M - LiBr. While this method was found to provide reproducible chromatograms, high resolution, and high sample recovery, it also suffered from the interference of residual water peaks. In addition, it did not offer resolution of material greater than 1.5 million amu due to the inadequacy of the largest pore size used, 1000 Å.

Recently, several groups of workers have reported on a variety of SEC methods applicable to PVP based upon an aqueous mobile phase. A novel hydrophilic semi-rigid polymeric gel containing the group $\{CH_2CHOHCH_2O\}$ was introduced under the name TSK-GEL type-PW (Toya Soda Co., Japan). The fractionation of PVP

using an aqueous mobile phase in conjunction with this packing material has been reported (13). While the water peak was eliminated here, column efficiency appeared to decline markedly over several months of use (14).

Engelhardt and Mathes described a bonded (protic) amide stationary phase and a mobile phase of 0.1M - TrisHCl buffer (pH = 8.0) with 10% ^{v}/v) ethylene glycol adjusted to an ionic strength of 0.5 with $Li_2 SO_4$(15). The purpose of the ethylene glycol was to eliminate partition due to a hydrophobic interaction between the stationary phase and PVP. This packing material is not commercially available. An interesting material which was found to be incapable of eluting PVP was PVP-coated silica (16). While the mobile phase was not specified, the adsorption of PVP was said to be a result of the dipolar interaction between the two PVP phases. As PVP is an aprotic amide its failure as a stationary phase is at odds with the work of Engelhardt and Mathes.

The primary purpose of this work was to describe a successful method for the aqueous size exclusion chromatography of PVP based upon a diol-bonded silica packing (also referred to as possessing glyceryl, glycol or carbohydrate functionality). This work was produced independently with respect to that of Herman, Field and Abbott who report a similar separation (17). The two methods are contrasted in the body of this report.

EXPERIMENTAL

The chromatograph employed in this study was a Waters Model 150C GPC operated at ambient temperature. Data collection was achieved with a Perkin-Elmer Sigma 10 data station. SEC stationary phases investigated included commercial diol columns (250 x 4.6 mm ID) obtained from E. Merck (LiChrospher-DIOL) and Synchrom, Inc. (Syn Chropak GPC) as well as bulk packing from Electro-Nucleonics, Inc., (Glyceryl-CPG). The former materials are based upon nominal 10μm diameter spherical silica while

the latter consists of crushed irregular glass particles in the 37-74μm range. The Glyceryl-CPG material was dry-packed into Waters 300 x 7.8 mm ID S.S. columns by the "tap-fill" method of Snyder and Kirkland (18).

The poly(vinylpyrrolidone) samples studied represent typical batches of the four major grades produced by GAF Corporation. The narrow polystyrene calibration standards were obtained from Pressure Chemical Co. The methanol, water, tetrahydrofuran, and acetonitrile used were distilled-in-glass solvents obtained from Burdick & Jackson, Inc. LiBr, $LiNO_3$, and KH_2PO_4 were reagent grade materials obtained from J.T. Baker Co. The Glyceryl-CPG column bank was operated at a flow rate of 2.0 ml/min. which corresponds to a nominal analysis time of 30 minutes. Unless indicated otherwise in the figure captions, the injections consisted of 160μl volumes of 0.25% ($^{w}/v$) solutions for all molecular weight grades. (K-90 grade PVP was studied at a 0.1% $^{w}/v$ concentration as well. No peak shift ascribable to viscous fingering was detected.) The development of the mobile phase composition is presented in the Results/Discussion section.

RESULTS AND DISCUSSION

A. Glyceryl-CPG Column Bank Design

Apart from increasing resolution and minimizing adsorptive effects, there are two main considerations in constructing a well-designed SEC column set. These are the maximization of dynamic range (selective permeation) to include all molecular weight components of interest and the optimization of the shape of the calibration curve to be log-linear in character over a broad range with respect to the dependence of molecular weight upon elution volume (V_e). The latter allows the abscissa of a molecular weight distribution plot to be divided into equal increments corresponding to decades of molecular weight and facilitates the use of polydisperse (broad) standard calibration. A non-log-linear column bank can cause a symmetric, Gaussian peak shape to

appear skewed and resolution to be a function of elution volume in the selective permeation range.

In this study the column bank was required to possess a selective permeation range extending from several hundred to ten million amu (or five decades). It has been demonstrated that a column set constructed from only two pore sizes can span a linear dynamic range of four but not five decades and that the pore volumes of each size should be equal for highest linearity (19). The latter stems from the dependence of calibration slope upon pore volume in the range covered by a particular pore size. Therefore to span five decades, at least three pore sizes are required.

The Glyceryl-CPG material pore sizes chosen were nominally 75, 500, and 3000 Å. The 75Å material is the smallest commercially available pore size and is capable of resolving components in the several hundred amu range. The remaining sizes were chosen so that their individual selection permeation ranges barely overlapped. The spacing between the three pore sizes to insure maximum linearity was dictated by the following consideration.

The diameter of an individual pore can be thought of as equivalent to twice the Stoke's radius of the largest random coiled polymer molecule (Gaussian chain) which can penetrate it. (The SEC mechanism operates upon the molecular hydrodynamic volume.) According to the Flory-Fox equation the hydrodynamic volume, $[\eta]$ M, of such a molecule can be expressed by

$$[\eta] M = \Phi \langle r^2 \rangle^{3/2}$$

where, $[\eta]$ = the intrinsic viscosity

Φ = a universal constant

$\langle r^2 \rangle$ = the mean square end-to-end distance (equal to six times the mean square radius of gyration)

Utilizing the Mark-Houwink equation which empirically relates [η] to M by

$$[\eta] = K'M^{a}$$

where K' and a are the Mark-Houwink coefficient and exponent respectively, one may substitute for [η] in the Flory-Fox equation to yield

$$K'M^{1+a} = \Phi\langle r^2\rangle^{3/2}$$

Assuming that K', a, and Φ are essentially independent of molecular weight and taking the logarithm of both sides of this equation, the following corollary is obtained, viz..

$$\log M \propto \log r$$

or any other linear dimension of such a Gaussian chain.

Thus, the difference in the logarithms of the three pore sizes must be equal in order for the log M values corresponding to their respective exclusion limits to be equally spaced yielding a log-linear calibration curve. In this case,

$$\log 3000 - \log 500 = 0.778$$
$$\log 500 - \log 75 = 0.824$$

i.e., the differences are nearly equivalent, indicating proper selection.

Because the pore volume of the 75Å material was reported to be approximately half that of the 500 and 3000 Å materials by their manufacturer, the bed volume of the former was doubled. The Glyceryl-CPG column set consisted of two columns of the 75Å and one each of the 500Å and 3000Å materials. The calibration curve corresponding to this column set as defined by a narrow

polystyrene standards in a THF mobile phase is depicted in Figure 1. A correlation coefficient of 0.9981 was achieved, indicative of high linearity (n=13).

B. Mobile Phase Development

The first mobile phase attempted in conjunction with the Glyceryl-CPG column set consisted of 5% DMF ($^{V}/v$), 95% H_2O ($^{V}/v$) and 0.1 M -LiBr. The purpose of the salt was to counteract any residual ionic electrolyte character of the PVP as well as to screen any remaining anionic $-SiO^-$ sites on the support. DMF was included to interact with the remaining silanol sites via hydrogen bonding in order to eliminate PVP absorption. While commercial K-90, -60, -30, and -15 samples were found to elute under these conditions in correct SEC order, the K-90 and K-60 peak shapes were highly skewed to low molecular weight. This apparent adsorptive effect could not be overcome by either increasing the DMF concentration or by substitution of the DMF by NMP.

In order to overcome this effect, a different approach was taken. Methanol was substituted for the DMF. The former was expected to interact via hydrogen-bonding with the carbonyl groups of PVP leaving the unreactive methanol methyl group exposed to the support surface. What was not known a priori was what concentration of methanol was required for optimum efficiency in shifting the equilibrium between methanol and water to the left:

$$\backslash\!\!/C = O \cdots HOCH_3 + HOH \quad \rightleftharpoons \quad \backslash\!\!/C = O \cdots HOH + HOCH_3$$

A series of aqueous 0.1M-LiBr mobile phases were prepared containing 10, 25, 40, and 50% ($^{V}/v$) of methanol (MeOH) in water. (The pH of these mixtures was normally in the range of 6 and was not adjusted.) The 10% composition resulted in total retention of all PVP samples injected. The chromatography observed for the compositions 25, 40 and 50% ($^{V}/v$) MeOH is depicted in Figure 2 and the peak crest times are reported in

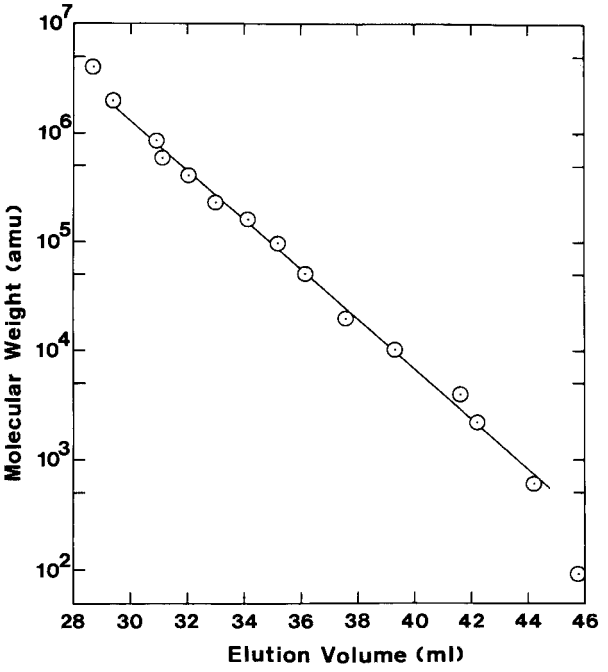


FIGURE 1. Calibration curve for the Glyceryl-CPG column set consisting of 2x75Å, 500Å, and 3000Å, (300 x 7.8mm) columns using narrow polystyrene standards in THF. Conditions: flow rate of 2.0 ml/min., injection volume of 80 µl, concentration of 0.1% W/v.

Table 1. The peak symmetry of the K-90 and -60 materials was found to improve as the methanol concentration was increased while the peak crest retention times monotonically decreased (although minimally from 40 to 50%). As adsorption appears to have been minimized at the 50% methanol level, this composition was subsequently maintained. At this point in the development of the method, $LiNO_3$ was substituted for LiBr because of the potential of corrosion of stainless steel tubing in the prolonged presence of bromide ions. Equimolar replacement was found to have no effect upon the observed chromatography as shown in Figure 3. The 2000 Å column utilized initially to perform the chromatography

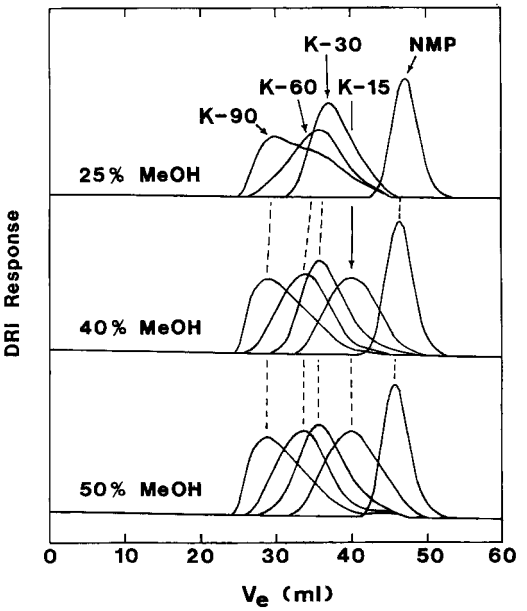


FIGURE 2. The effect of methanol concentration in the mobile phase of PVP peak shape and position. Conditions: MeOH/H_2O mobile phases containing 0.1 M-LiBr, column bank of Glyceryl-CPG 2x75Å, 500Å, and 2000Å (300 x 7.8mm), flow rate of 2.0 ml/min., injection volume of 80 µl, concentration of 0.25% $^{w}/_{v}$.

TABLE 1

Peak Crest Times (in min.) of Various Grades of PVP as Observed on a Glyceryl-CPG Column Bank

Sample	% Methanol ($^{v}/_{v}$) in Mobile Phase		
	25%	40%	50%
K-90	14.6	14.2	14.1
K-60	17.5	16.7	16.4
K-30	18.4	17.7	17.4
K-15	-	19.7	19.7
NMP	23.4	22.7	22.5

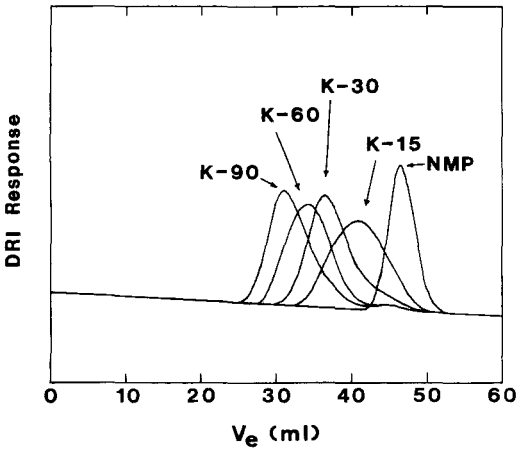


FIGURE 3. Size exclusion chromatography of PVP under the final chromatographic conditions: a mobile phase of 50:50 v/v MeOH/H_2O containing 0.1M-$LiNO_3$, a column bank of Glyceryl-CPG 2x75Å, 500Å, and 3000Å (300 x 7.8mm), flow rate of 2.0 ml/min., injection volume of 160 µl, concentration of 0.25% w/v.

depicted in Figure 2 was replaced by a 3000Å column in Figure 3 as per part A of this Discussion.

Column efficiency was measured using an analogous totally permeating species, NMP, and performing the test at normal operating conditions (i.e., 2.0 ml/min flow rate, all four columns, 50% MeOH/50% H_2O containing 0.1M-$LiNO_3$, ambient temperature). Using the 5σ method (peak width measured at 4.4% of peak height from baseline) the column bank efficiency was found to be 720 plates. This result is not surprising considering the particle size and particle irregularity of the packing material.

C. Comparison to Other Systems

Herman, Field and Abbott have demonstrated the use of a mobile phase consisting of 40% CH_3CN / 60% H_2O in 0.01 M-KH_2PO_4 adjusted to pH = 2.5 in conjunction with a 100Å diol column to successfully perform SEC on a K-15 grade PVP(17). Sample recovery was reported to be 100%. The function of the

acidity (low pH) was to suppress the ionization of PVP which was said to be an acidic polyelectrolyte. Our laboratory has to date found no viscometric evidence to support the existence of polyelectrolyte character in unmodified PVP as would be revealed by the nonlinear dependence of inherent or reduced viscosity upon concentration in water (3). A minimal electrophoretic mobility has been observed for PVP and ascribed to carboxyl end groups (20).

The effect of removing salt from a 50:50 $^{v}/_{v}$ MeOH/H_2O mobile phase was investigated for K-90, K-30, K-60, K-15, and NMP on Glyceryl-CPG columns as depicted in Figure 4. These chromatograms should be compared to the 50% MeOH chromatograms of Figure 2. The distribution of the K-90 material exhibits a distinct shoulder whereas the K-60, K-30, and K-15 grade samples possess bimodal distributions: one peak at the total exclusion volume and one corresponding to the expected SEC peak. This phenomenon is consistent with a polyelectrolyte effect despite the lack of supporting viscometric evidence. However, PVP has been reported to be a semi-polyampholyte on the basis of conductometric titration (21,22) and ^{13}C-NMR data (23). A hydrolysis/ring-opening reaction results in a Zwitterionic (amino acid) form whose positive charges on the nitrogens are screened by concomitant negatively charged carboxyl groups which are furthest from the backbone. Mutual repulsion between such negative charges and with respect to residual $-SiO^-$ groups on the silica substrate would result in increased hydrodynamic volume in the absence of free cationic counterions and is consistent with a portion of the PVP eluting at the total exclusion volume. The bimodal nature of these chromatograms is not completely understood, however, and is under further investigation. The frequency of the Zwitterionic form has been reported to increase dramatically at both high and low pH values (21).

Thus, the adjustment of the mobile phase to low pH is unwarranted and can be as detrimental to the stability of the

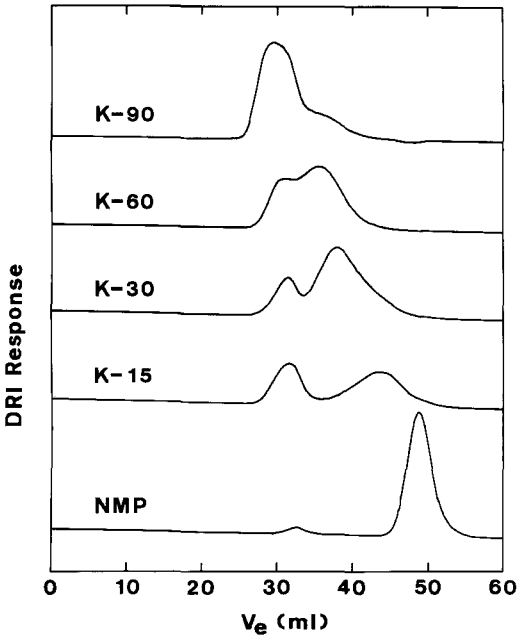


FIGURE 4. The effect of the removal of salt from the mobile phase upon PVP peak shape and position. Conditions: a mobile phase of 50:50 $^{v}/_{v}$ MeOH/H_2O, column bank of Glyceryl-CPG 2x75Å, 500Å, and 3000Å (300 x 7.8 mm), flow rate of 2.0 ml/min., injection volume of 160 µl, concentration of 0.25% $^{w}/v$.

bonded phase as high pH is to the silica substrate (24). Residual ionic effects exhibited by either the polymer or chromatographic support are better counteracted by the addition of an adequate quantity of a neutral salt. The acetonitrile incorporated into the mobile phase of Herman et. al. can participate in hydrogen-bonding although, unlike methanol, strictly as a proton acceptor.

To assess the relative efficacy of the two mobile phases, a direct comparison of sample recovery was performed using the same diol column bank, that of the Glyceryl-CPG. Peak areas of 160µl injections made on these columns were compared to 10µl injections made on a flow restrictor coil (in the absence of the columns)

with compensation for differences in injection volume, chart speed, flow rate, and detector sensitivity. The detector response for the case of the flow restrictor alone is taken to represent 100% recovery. The results of this study are summarized in Table 2. In both cases a generally monotonic increase is observed for % recovery as molecular weight is decreased. At every molecular weight studied, the methanol based mobile phase resulted in a substantially higher recovery than that based upon acetonitrile. This is particularly evident for the K-90 grade material. The greater efficacy of the former mobile phase modifier is evidence for the role of proton donation in overcoming adsorption in this system.

The inverse dependence of percent recovery upon molecular weight is not surprising. An individual mer segment on a low molecular weight PVP molecule has an equal probability of interacting with an active substrate site as does a mer segment on a high molecular weight molecule. However, once such an interaction occurs, the mass of PVP retained is greater for the high molecular weight case. (This argument presumes a limited number of active substrate sites. In the event that the number of sites is very high, as in unmodified silica, all PVP molecules are retained eliminating the molecular weight dependence.) This molecular weight dependence is contrary to what would be expected from end-group effects alone. The high recovery of K-15 material in the study of Herman et. al. is consistent with the selection of a low molecular weight test sample and a lesser amount of packing material than in the present study.

An effort was made to investigate the characteristics of commercially available 10μm particle size diol columns such as SynChropak-GPC and LiChrospher-DIOL in order to improve efficiency while retaining the good features of the Glyceryl-CPG columns: log-linear calibration and high sample recovery. A 250 x 4.6 mm ID column containing 500 Å pore size SynChrom material was compared chromatographically to a similar column of the LiChrospher material. These comparative chromatograms appear in

TABLE 2

% PVP Sample Recovery as Affected by Mobile Phase Composition

Sample	Mobile Phase of Present Study[1]	Mobile Phase of Herman et al[2]
K-90	87.1	37.3
K-60	93.3	60.7
K-30	92.8	59.7
K-15	98.1	75.1
NMP	100	62.2

(1) 50% MeOH/50% H_2O (v/v) containing 0.1M-$LiNO_3$ (pH 6).

(2) 40% CH_3CN/60% H_2O (v/v) containing 0.01M-KH_2PO_4, pH = 2.1.

Figure 5. While recovery of K-30 and K-15 materials appeared to be adequate and comparable for the two columns, K-90 eluted well from the LiChrospher column but was almost completely retained by the Synchrom column. This result indicates that the diol derivatization of the Glyceryl-CPG and LiChrospher materials is more complete than that of the SynChrom material. Sample recovery of K-90, -60, -30, -15 and NMP was determined to be 100% for a LiChrospher-DIOL column bank consisting of one 100 Å, one 500 Å, and one 4000 Å column. The efficiency of this column bank was determined to be 2060 theoretical plates by the 5σ method at a 0.8 ml/min flow rate.

The 100 Å packing represents the smallest, commercially available pore size LiChrospher-DIOL. According to the pore size selection scheme developed in part A of this section, the correct

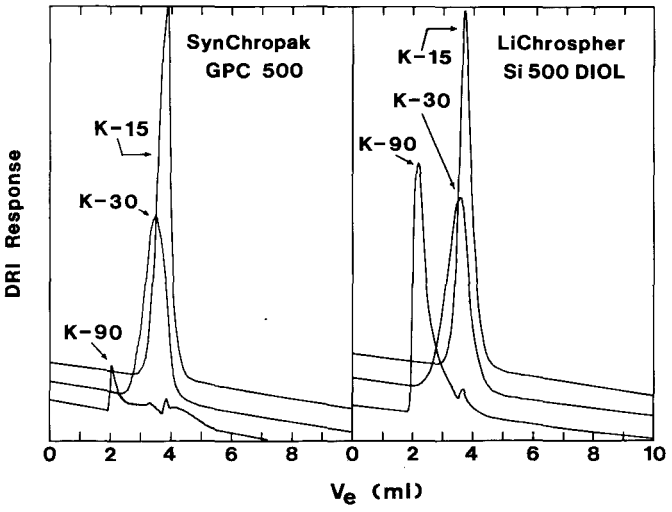


FIGURE 5. Comparison of two commercially-available 10 µm particle size 500Å (250 x 4.6mm) diol columns with regard to PVP recovery. Conditions: a mobile phase of 40:60 $^{v}/_{v}$ MeOH/H_2O containing 0.1M-$LiNO_3$, flow rate of 1.0 ml/min., injection volume of 20 µl, concentration of 0.25% $^{w}/_{v}$.

pore size to mate with 4000 Å and 500 Å pore sizes would be 60 Å (assuming equal pore volumes.) A calibration curve for the LiChrospher column set used was constructed from narrow polystyrene standards in a THF mobile phase and is depicted in Figure 6. The non-linearity of this calibration curve (it appears to be comprised of three distinct linear segments of different slope) is indicative of both poorly matched pore volumes and the absence of a sufficiently small pore size. That a 60 Å size would be a clear improvement is seen by the lack of selective permeation below 500 amu.

Chromatograms of all four grades of PVP as well as NMP obtained for the LiChrospher column bank recovery study are presented in Figure 7. The highly skewed appearance and sharp high molecular weight cutoff of the K-60 and K-90 peaks appeared unusual in light of the peak shapes observed for these materials

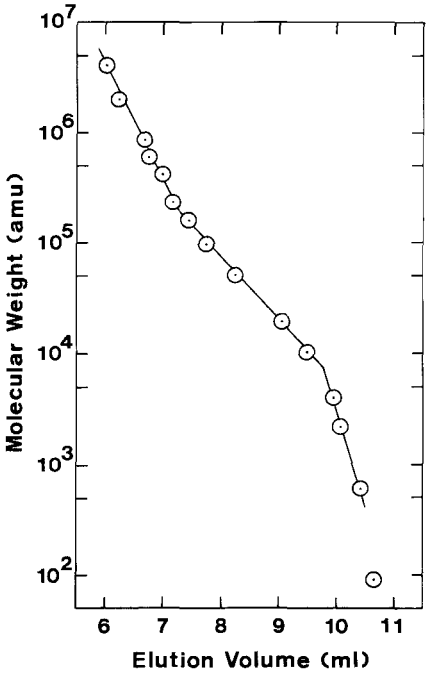


FIGURE 6. Calibration curve for the LiChrospher DIOL column set consisting of 100Å, 500Å, and 4000Å, (250 x 4.6mm) columns using narrow polystyrene standards in THF. Conditions: flow rate of 1.0 ml/min., injection volume of 20 µl, concentration of 0.1% $^{w}/_{v}$.

in conjunction with the Glyceryl-CPG column. In addition, a 4000 Å column would be expected to show good selectivity for the high molecular weight tail of PVP K-90 whose weight average molecular weight has been reported to be in the range of 1.5 million amu (25). According to the polystyrene calibration curve given in Figure 6, total exclusion appears to occur prior to 4.1 million amu (6.03 ml). The peak crest retention volume of 7.1 ml (and leading edge retention volume of 6.4 ml) for the K-90 peak representing 20µl of a 0.25% solution was significantly later than the estimated lower limit of the total exclusion volume. For a 200µl injection volume of a 0.15% solution of the same material

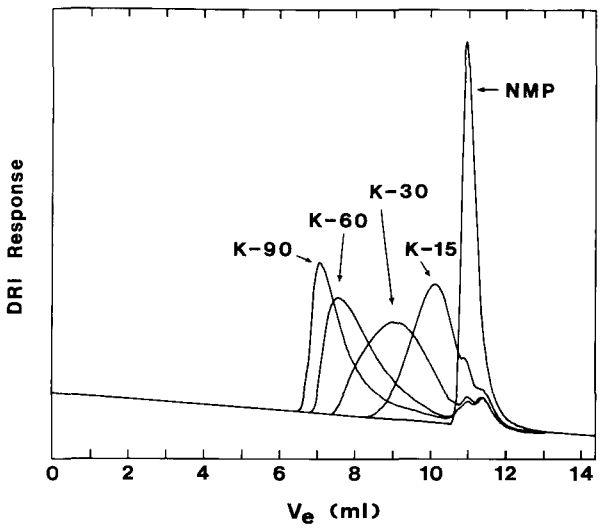


FIGURE 7. Size exclusion chromatography of PVP using the LiChrospher column bank of 100Å, and 500Å, and 4000Å (250 x 4.6mm). Conditions: a mobile phase of 50:50 v/v MeOH/H_2O containing 0.1M-$LiNO_3$, flow rate of 0.8 ml/min., injection volume of 20 µl, concentration of 0.25% w/v.

the peak crest retention volume of 7.6 ml (and leading edge retention volume of 7.0 ml) reflects even further retardation and a dependence upon concentration. These facts imply an undesirable mixed mode separation of SEC and partition or adsorption of PVP for the LiChrospher material.

By comparison the polystyrene calibration curve shown in Figure 1 for the Glyceryl-CPG column bank indicates that total exclusion is not expected before 28.7 ml (corresponding to the 4.1 million amu standard). The K-90 peak crest retention volume was 31.2 ml but the leading edge retention volume was 25.1 ml or less than the estimated total exclusion volume. This result confirms the presence of a very high molecular weight component in K-90 grade PVP (and indicates axial dispersion in this column set) which is consistent with a predominantly SEC mechanism.

CONCLUSIONS

The optimum size exclusion chromatographic method for poly(vinylpyrrolidone) was found to be one based upon a stationary phase of diol derivatized silica and a mobile phase of 50:50 ($^{v}/_{v}$) MeOH/H_2O with 0.1M-$LiNO_3$. Sample recovery under identical conditions varied for the commercial packings investigated but was found to be acceptable for two. This variation underscores the requirement of complete surface deactivation of the silica substrate. The percent recovery was found to be inversely related to molecular weight. This phenomenon was rationalized on the basis of a limited number of active substrate sites available for binding. Methanol was found to be a more effective mobile phase modifier than either DMF or acetonitrile which appears due to its ability to act as a proton donor in hydrogen bonding with PVP. Chromatographic evidence for the existence of semipolyampholyte character in PVP has been reported. A procedure for the construction of a log-linear (in calibration) column set of extended dynamic range has been described and is based upon hydrodynamic volume theory.

The primary drawback to the method described lies in the relatively low column efficiency exhibited by 37-74 m packing material. Unfortunately, the commercially available, prepacked 10 μm (high efficiency) column materials were found to be inferior to the above with regard to PVP recovery, mixed-mode separation character, mismatched pore volumes and lack of availability of a crucial pore size (60 Å).

ACKNOWLEDGEMENT

The authors are grateful to GAF Corporation for granting permission to publish this work and to C. P. Talley for helpful comments.

REFERENCES

1. H. Fikentscher, Cellulose - Chem., 13, 58 (1932).

2. G. M. Kline, Mod. Plast., 22, 157 (1945).

3. E. G. Malawer, J. K. DeVasto, and S. P. Frankoski, Pharmacopeial Forum, 9 (1), 2651 (1983).

4. W. Scholtan, Makromol. Chem., 7, 209 (1951).

5. J. Hengstenberg and E. Schuch, Makromol. Chem., 7, 236 (1951).

6. G. E. Fleig and F. Rodriguez, Chem. Eng. Commun., 13, 219 (1982).

7. H. Nogami, T. Nagai, and A. Kondo, Chem. Pharm. Bull., 18, (11), 2290 (1982).

8. B. G. Belenkii, L. Z. Vilenchik, V. V. Nesterov, V. J. Kolegov, and S. Ya. Frenkel, J. Chromatogr., 109, 233 (1975).

9. Yu, E. Kirsh, T. M. Karaputadze, V. I. Svergun, T. A. Sus', O. I. Sutkevich, I. I. Iverdokhlebova, and V. P. Panov, Khim. - Farm. Zh., 14 (7), 107 (1980).

10. F. Comelli, R. Francesconi, and E. Lanzi, Polym. Eng. & Sci., 20 (5), 370 (1980).

11. M. Ezrin, private communication (Springborn Laboratories, Inc., Enfield, CT).

12. H. N. Cheng, T. E. Smith, D. M. Chiou, and E. Malawer, unpublished data. (A GAF method involved the use of NMP at 80°C in conjunction with a bank of Waters μ-Styragel columns.)

13. T. Hashimoto, H. Sasaki, M. Aiura, and Y. Kato, J. Polym. Sci., Polym. Phys. Ed., 16, 1789 (1978).

14. P. L. Dubin, Polym. Prepr., 22, (1), 132 (1981).

15. H. Engelhardt and D. Mathes, J. Chromatogr., 185, 305 (1979).

16. L. Letot, J. Lesec, and C. Quivoron, J. Liq. Chromatogr., 4, (8), 1311 (1981).

17. D. P. Herman, L. R. Field, and S. Abbott, J. Chromatogr. Sci., 19, 470 (1981).

18. L. R. Snyder and J. J. Kirkland, "Introduction to Modern Liquid Chromatography," 2nd ed., (1979), Wiley-Interscience, p. 206-207.

19. W. W. Yau, J. J. Kirkland, and D. D. Bly, "Modern Size-Exclusion Liquid Chromatography," (1979), Wiley-Interscience, p. 265 ff.

20. L. May, M. Hines, L. Weintraub, J. Scudder, and S. Graff, Surg., 35, 191 (1954).

21. H. P. Frank, J. Polym. Sci., 12, 565 (1954).

22. A. Conix and G. Smets, J. Polym. Sci., 15, 221 (1955).

23. H. N. Cheng, D. M. Vitus, and T. E. Smith, unpublished data (GAF Corporation).

24. N. H. C. Cooke and K. Olsen, Am. Lab., 11, (8), 45 (1979).

25. BASF product bulletin B382e, "Kollidon Grades: Polyvinylpyrrolidone for the Pharmaceutical Industry," (May, 1982).

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(3), 463-473 (1984)

REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF FENTANYL HOMOLOGUES AND ANALOGUES

I. AN OPTIMIZED ISOCRATIC CHROMATOGRAPHIC SYSTEM UTILIZING ABSORBANCE RATIOING*

Ira S. Lurie and Andrew C. Allen
Special Testing and Research Laboratory
Drug Enforcement Administration
McLean, Virginia 22102

Haleem J. Issaq
Chemical Synthesis and Analysis Laboratory
NCI/Frederick Cancer Research Facility
Frederick, Maryland 21701

ABSTRACT

An optimized isocratic chromatographic system was developed using overlapping resolution mapping for the reversed-phase separation of 26 fentanyl homologues and analogues. The system consisted of a Partisil 10-ODS-3 column with a quaternary mobile phase consisting of phosphate buffer, methanol, acetonitrile and tetrahydrofuran. All 26 compounds were distinguished when UV detection at 215nm was employed in series with UV detection at 230nm.

INTRODUCTION

In the course of our work it became desirable to develop a reversed-phase high-performance liquid chromatographic separation of 26 homologues and analogues of fentanyl, a powerful narcotic analgesic. The goal was to develop an isocratic system which dis-

*Presented in part at 1983 Pittsburgh Conference, Atlantic City, N. J. March 7-11.

 0148-3919/84/0703-0463$3.50/0

tinguished among the compounds of interest in a reasonable run time, i.e., less than twenty minutes. Various close-ended methods are available for developing an optimum mobile phase. Some are based on treating retention behavior as a function of mobile phase composition or temperature (1-2), while others are based on statistical or sequential search techniques (3-6). The method utilized in this study is based on the work of Snee and employs computer generated overlapping resolution mapping (6,7).

EXPERIMENTAL

The liquid chromatograph employed consisted of the following components: Model 8800 4-solvent gradient system with oven (DuPont); Model LC85 variable UV detector set at 215nm or 254nm containing a 2.5µl flow cell, either alone or in series with a second Model LC85 variable UV detector set at 230nm and containing a 1.5µl flow cell (Perkin-Elmer); IS-100 autosampler (Perkin-Elmer); Sigma 15 Data System interfaced with a Model 3600 Data Station (Perkin-Elmer); a prepacked, 4.6mm x 25cm stainless steel column, with 10µm C18 packing material (Partisil 10-ODS-3, Whatman). Temperature was maintained at 40°C.

Materials

The following solvents were used: acetonitrile, methanol and tetrahydrofuran (Burdick and Jackson). Other chemicals used to prepare mobile phases were reagent grade. The fentanyl compounds were synthesized at the Special Testing and Research Laboratory as the hydrochloride salts, except for the 2-methyl homologue which was obtained from Dr. Thomas Riley of the University of Mississippi.

For experiments where binary or ternary solvent systems were employed solvent 1 consisted of water; solvent 2 consisted of a concentrated phosphate buffer comprised of 16 parts water, 3 parts 2N sodium hydroxide and 1 part phosphoric acid; and solvents 3 and 4 consisted of organic solvent. Solvent 2 was kept constant at 20%. For the quaternary mobile phases employed solvent 1 consisted of phosphate buffer and solvents 2-4 were either pure organic components or pre-mixed 50:50 with solvent 1. The overall phosphate concentration was kept constant for all mobile phases examined.

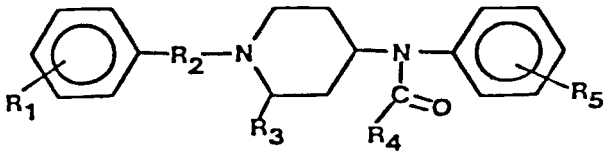
RESULTS AND DISCUSSION

The structures of the 26 homologues and analogues of fentanyl studied are presented in Table 1. In order to establish the composition of the optimum mobile phase we were required to run 7 experiments, as depicted in Figure 1, using different combinations of three organic solvents based on Snyder's selectivity triangle (8). The seven experiments were chosen to estimate the coefficients of a cubic equation which described the surface of the relationship between resolution and mobile phase composition. The composition of each of the first three mobile phases (which contained a single organic modifier) were adjusted to give k' values for fentanyl of approximately 3.5. For all seven mobile phases the approximate k' range for all compounds was between 1 and 10. Retention data for the 7 experimental runs are presented in Table 2.

A resolution map was created for all pairs of adjacent compounds in each of the seven experiments with a computer program

TABLE 1

STRUCTURE OF FENTANYL HOMOLOGUES AND ANALOGUES



	R^1	R^2	R^3	R^4	R^5
1.	. . .	CH_2	. . .	CH_3	. . .
2.	. . .	CH_2	. . .	CH_2CH_3	. . .
3.*	. . .	CH_2CH_2	. . .	CH_2CH_3	. . .
4.	. . .	CH_2CH_2	. . .	CH_3	. . .
5.	. . .	$CH_2CH_2CH_2$	. . .	CH_3	. . .
6.	. . .	$CH_2CH_2CH_2$	. . .	CH_2CH_3	. . .
7.	. . .	$CH_2CH\ CH_3$	. . .	CH_2CH_3	. . .
8.	. . .	CH_2CH_2	. . .	CH_3	o-CH_3
9.	. . .	CH_2CH_2	. . .	CH_3	m-CH_3
10.	. . .	CH_2CH_2	. . .	CH_3	p-CH_3
11.	. . .	CH_2CH_2	. . .	CH_2CH_3	o-CH_3
12.	. . .	CH_2CH_2	. . .	CH_2CH_3	m-CH_3
13.	. . .	CH_2CH_2	. . .	CH_2CH_3	p-CH_3
14.	o-CH_3	CH_2CH_2	. . .	CH_3	. . .
15.	m-CH_3	CH_2CH_2	. . .	CH_3	. . .
16.	p-CH_3	CH_2CH_2	. . .	CH_3	. . .
17.	o-CH_3	CH_2CH_2	. . .	CH_2CH_3	. . .
18.	m-CH_3	CH_2CH_2	. . .	CH_2CH_3	. . .
19.	p-CH_3	CH_2CH_2	. . .	CH_2CH_3	. . .
20.	. . .	CH_2CH_2	CH_3	CH_2CH_3	. . .
21.	. . .	$CH_3CH\ CH_2$	. . .	CH_3	. . .
22.	. . .	$CH_3CH\ CH_2$	. . .	CH_2CH_3	. . .
23.	. . .	CH_2CH_2	. . .	CH_3	o-F
24.	. . .	CH_2CH_2	. . .	CH_3	m-F
25.	. . .	CH_2CH_2	. . .	CH_2CH_3	m-F
26.	. . .	CH_2CH_2	. . .	CH_2CH_3	p-F

*Fentanyl

Buff = Buffer
MeOH = Methanol
ACN = Acetonitrile
THF = Tetrahydrofuran

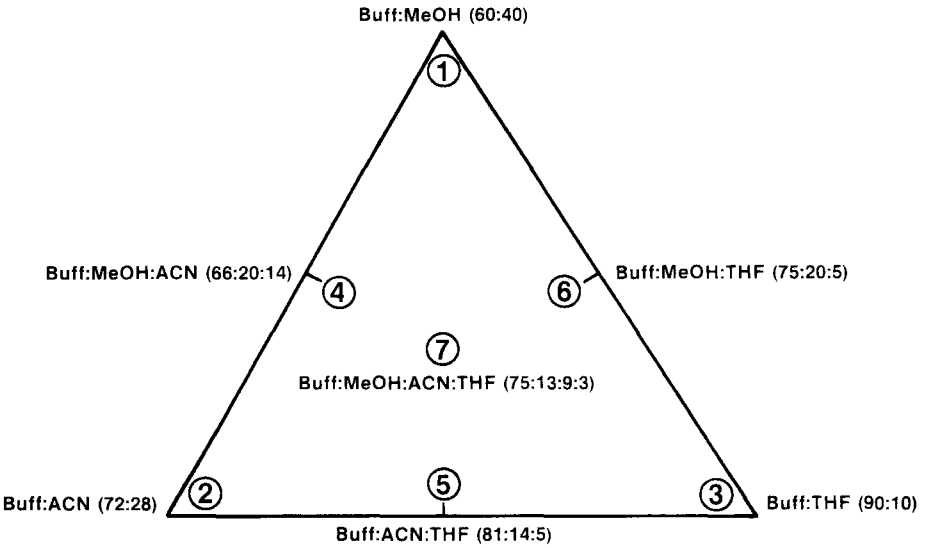


Figure 1 - Simplex experimental for mobile phase optimization utilizing experimental runs 1-7.

that corrected for peak crossover. A union of these plots, which represents overlapping resolution mapping, portrays the region where the maximum number of pairs of peaks are resolved above a preselected resolution level. Resolution can be defined by either of the following equations.

$$Rs = \tfrac{1}{4}(\alpha-1)\ (N)^{\frac{1}{2}}\ \ (k' / (k' + 1) \qquad 1)$$

$$Rs = (Rt2 - Rt1) / \tfrac{1}{2}\ (W1 + W2) \qquad 2)$$

Where α, N and k' are the selectivity factor, column efficiency and capacity factor respectively; and RT and W are retention times

TABLE 2

RETENTION TIME DATA FOR SEVEN EXPERIMENTAL RUNS

Compound No.	Retention time (minutes) Run #1	Run #2	Run #3	Run #4	Run #5	Run #6	Run #7
1	3.71	3.29	3.16	4.20	3.80	3.47	4.32
2	5.61	4.59	5.04	6.53	5.94	5.39	7.02
3	6.28	5.87	6.47	8.03	7.77	6.51	8.80
4	4.17	4.14	4.03	5.19	4.95	4.21	5.47
5	5.61	5.51	6.27	7.37	7.34	6.14	8.22
6	8.80	8.15	10.78	11.74	12.08	10.01	13.84
7	7.04	7.24	7.46	9.63	9.54	7.37	10.46
8	5.42	5.20	5.37	6.91	6.57	5.51	7.43
9	6.21	5.78	6.57	8.03	7.77	6.43	8.80
10	6.52	5.97	6.88	8.36	7.30	6.86	9.39
11	8.93	7.89	9.60	11.59	11.29	9.34	13.29
12	10.37	8.88	11.56	13.53	13.44	11.15	15.55
13	10.99	9.29	12.63	14.34	14.29	12.03	16.75
14	5.54	5.49	5.90	7.30	7.14	5.90	7.94
15	6.47	5.87	6.93	8.29	8.29	6.92	9.33
16	6.52	6.06	7.11	8.49	8.48	7.02	9.51
17	8.59	8.11	9.91	11.68	11.68	9.35	13.22
18	10.22	8.88	11.86	13.41	13.35	11.20	15.51
19	10.41	9.06	12.27	13.69	13.78	11.40	15.93
20	7.57	7.19	7.81	10.13	9.81	7.73	11.03
21	5.66	5.18	5.38	7.02	6.51	5.62	7.50
22	8.90	7.65	9.24	11.31	10.72	9.11	12.57
23	4.58	5.41	4.83	5.74	5.66	4.82	6.19
24	4.31	4.63	4.88	5.61	5.77	4.73	6.18
25	6.52	6.92	8.38	9.01	9.56	7.67	10.45
26	6.47	6.68	8.80	8.90	9.58	7.87	10.48

and peak widths of peaks designated 1 and 2. In this work, resolution was approximated by Rt2 - Rt1 because we were not interested in simultaneously separating 26 compounds, but in distinguishing the various fentanyl analogues and homologues. In this vein it is important that the retention time difference between two adjacent peaks be greater than the experimental variation in retention

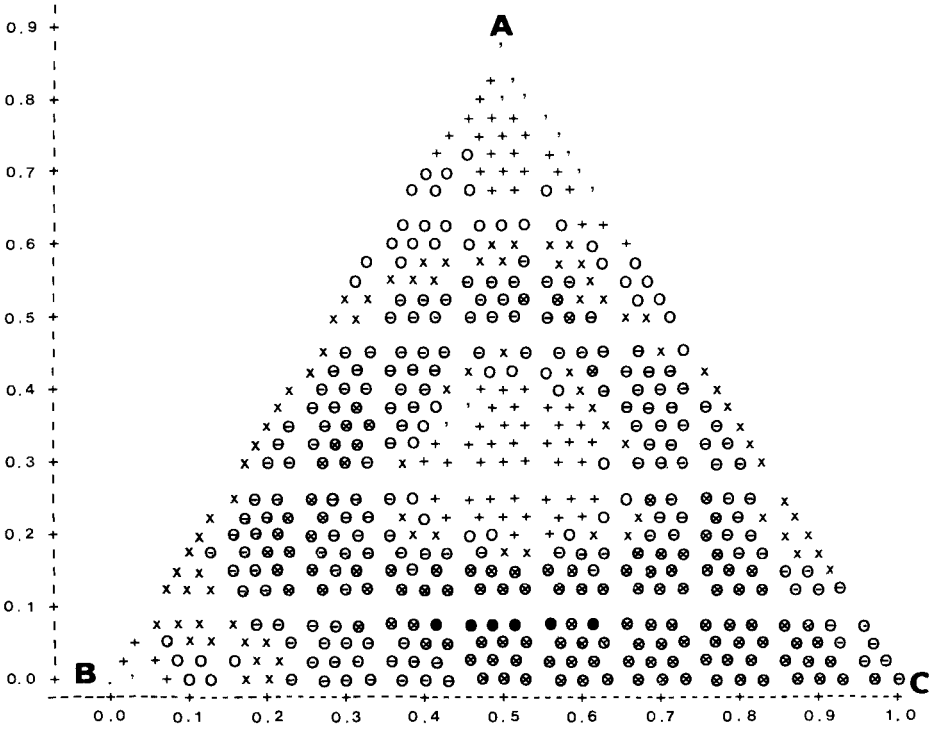


Figure 2 - Contour plot showing number of pairs of peaks where retention time difference between each pair is greater than 0.5 minutes versus mobile phase composition. Points A, B, and C refer to mobile phases depicted in the apexes of the triangle in Figure 1.

time for either peak. A retention time difference of 0.5 minutes was found to be suitable for this purpose. As illustrated in Figure 2, overlapping resolution mapping predicted that a mobile phase consisting of 11%A, 37%B and 52%C was optimum. A, B, and C refers respectively to the concentration of organic modifier which was employed in the first three experiments. Several mobile phases close to the latter solvent system were tested and were found to

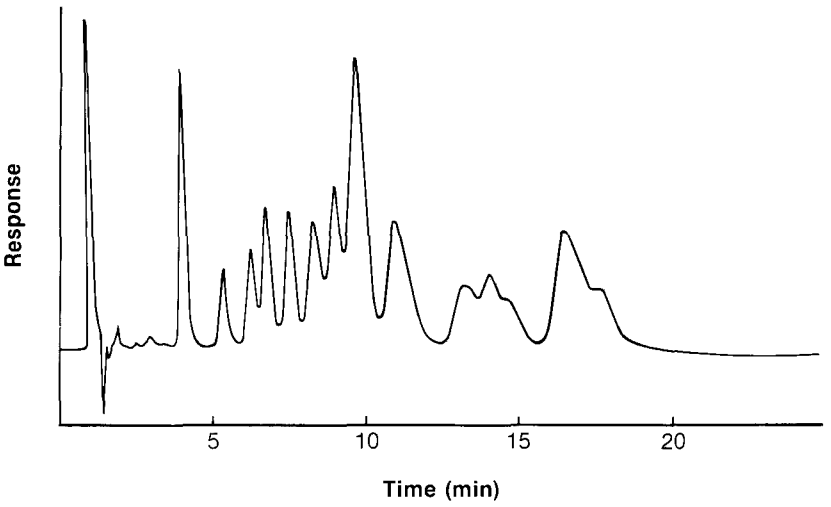


Figure 3 - Chromatogram of a mixture of 26 homologues and analogues of fentanyl utilizing optimum mobile phase predicted by overlapping resolution mapping. Mobile phase consists of 81% phosphate buffer (99 parts water, 3 parts 2 N sodium hydroxide and 1 part phosphoric acid), 4% methanol, 10% acetonitrile and 5% tetrahydrofuran.

Symbol	# of Peaks	Symbol	# of Peaks	Symbol	# of Peaks
.	274-276	@	300-305	Θ	295-300
+	281-286	,	276-281	Φ	305-310
X	290-295	O	286-290		

give no improvement in the separation of the compounds studied. An examination of the chromatographic run, which is depicted in Figure 3, using the predicted optimum conditions showed peak overlap for several pairs of peaks. In fact, 13 pairs of peaks were not separated by at least 0.5 minutes.

The discriminating power of our chromatographic system was tremendously improved by employing absorbance ratios obtained by using two UV detectors in series. This technique has been successfully employed for the analysis of drugs (9-11). As depicted

TABLE 3

Short Term and Long Term Relative Retention Times (RRT's) and Absorbance Ratios for Compounds in Table 1

Chromatographic Conditions Described in Figure 1
RRT's Calculated Relative to Fentanyl

CPD	RRT	215/230 Un-corrected	215/230 Corrected	RRT*	215/230* Un-corrected	215/230* Corrected
1	0.451	0.765	1.19	0.466	0.851	1.16
4	0.594	0.684	1.04	0.614	0.770	1.05
23	0.688	0.743	1.15	0.702	0.831	1.14
24	0.692	0.578	0.904	0.707	0.600	0.838
2	0.754	0.714	1.11	0.759	0.801	1.10
8	0.836	1.22	1.88	0.842	1.37	1.87
21	0.840	0.685	1.06	0.855	0.756	1.04
14	0.915	0.915	1.40	0.923	1.00	1.37
5	0.943	0.738	1.12	0.948	0.820	1.13
15	1.07	0.976	1.51	1.07	1.03	1.41
3	1.00	0.643	1.00	1.00	0.726	1.00
9	1.03	0.852	1.33	1.01	0.947	1.30
16	1.07	0.938	1.46	1.09	1.04	1.44
10	1.09	0.632	0.975	1.08	0.670	0.920
7	1.22	0.703	1.08	1.22	0.763	1.06
25	1.22	0.485	0.753	1.22	0.556	0.760
26	1.24	0.914	1.42	1.23	1.04	1.44
20	1.27	0.632	0.989	1.26	0.716	0.993
22	1.45	0.648	1.01	1.43	0.723	0.992
11	1.51	1.01	1.58	1.50	1.12	1.54
17	1.57	0.864	1.35	1.54	0.944	1.28
6	1.64	0.693	1.09	1.62	0.772	1.07
12	1.83	0.798	1.25	1.81	0.880	1.20
18	1.83	0.909	1.42	1.81	0.953	1.32
19	1.90	0.979	1.54	1.85	1.00	1.38
13	1.96	0.594	0.925	1.93	0.635	0.871

*Re data obtained after 7 weeks

in Table 3, when we employed UV detection at 215nm in series with UV detection at 230nm, the corrected absorbance ratios coupled with relative retention time distinguished all 26 compounds. The corrected absorbance ratios obtained by peak height for the individual drugs were found by dividing the absorbance ratio for

a compound by the absorbance ratio of an internal standard (fentanyl). Due to variations in wavelength accuracy and wavelength repeatability large differences in uncorrected absorbance ratios could be expected. Since the various compounds we studied, including our internal standard, have similar slopes in their UV spectrum at the wavelengths used, the corrected absorbance ratios would be expected to be considerably more reproducible. This was verified experimentally. The average long term reproducibility measured after seven weeks was 1.6% relative standard deviation for corrected ratios versus 4.7% for uncorrected ratios. However, as expected, the short term reproducibility of the corrected versus the uncorrected absorbance ratio was almost identical, 0.50% versus 0.45%. The long term average percent difference in relative retention time was 0.65% while the short term average percent difference in relative retention time was 0.10%.

Absorbance ratios, both short time and long time, are presented in Table 3.

CONCLUSION

A reversed-phase high-performance liquid chromatographic system has been developed which can distinguish between 26 analogues and homologues of fentanyl with run times less than twenty minutes.

ACKNOWLEDGEMENT

We would like to thank Mrs. Helen Paquette for her excellent secretarial assistance, and Ms. Karen McNitt of FCRF for running the ORM program.

REFERENCES

1. Gant, J. R., Dolan, J. W., and Snyder, L. R., J. Chromatogr., 185, 153, (1979).

2. Toon, T. and Rowland, M., J. Chromatogr., 208, 391, (1981).

3. Svoboda, V., J. Chromatogr., 201, 241, (1980).

4. Berridge, J. C., J. Chromatogr., 244, 1, (1982).

5. Glajch, J. L., Kirkland, J. J., Squire, K. M., and Minor, J. M., J. Chromatogr., 199, 57, (1980).

6. Issaq, H. J., Klose, J. R., McNitt, K. L., Haky, J. E., and Muschik, G. M., J. Liquid Chromatogr., 4, 2091, (1981).

7. Snee, R. D., Chem. Tech. 9, 702, (1979)

8. Snyder, L. R., J. Chromatogr. Sci., 16, 223, (1978).

9. Baker, J. K., Skelton, R. E., Ma, C. Y., J. Chromatogr., 168, 417, (1979).

10. White, P. C., J. Chromatogr., 200, 271, (1980).

11. Lurie, I. S., Sottolano, S. M., and Blasof, S., J. Forensic Sci., 27, 519, (1982).

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(3), 475-482 (1984)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATIONS USING SHORT COLUMNS PACKED WITH SPHERICAL ODS PARTICLES - II EFFECT OF MOBILE PHASE COMPOSITION ON RESOLUTION

Haleem J. Issaq
NCI-Frederick Cancer Research Facility
Frederick,MD 21701

ABSTRACT

The effect of instrumental parameters, mobile phase composition, and flow rate on high performance liquid chromatography separations using 3 cm, 5 cm, and 10 cm columns packed with 3μ spherical ODS materials is discussed. The results indicate that when instrumental parameters were optimized, the 10 cm column gave better separation than the shorter columns under the same experimental conditions. However, when the mobile phase composition was adjusted so that the solute residence time was comparable in the three columns, short and long columns gave comparable results.

INTRODUCTION

In a previous study (1) a comparison was made of the separation of a mixture on 5 cm and 10 cm columns packed with 3μ and 5μ spherisorb ODS spherical particles using standard high performance liquid chromatography (HPLC) equipment without any modification. The results indicated that

This work was supported by Contract NO. NO1-CO-23910, with the National Cancer Institute, NIH, Bethesda, MD 20205.

0148-3919/84/0703-0475$3.50/0

the separations achieved on the 10 cm column were not significantly better than those on 5 cm columns packed with supports of the same size and physical properties.

In the present study, which is an extension of the previous one, modifications were made on the instrument to accommodate the requirements of 3 cm, 5 cm, and 10 cm columns packed with 3μ ODS spherical particles. The results indicate that the 10 cm columns would give much better resolution than the 5 cm and 3 cm columns under the same experimental conditions, i.e., mobile phase compositions and flow rate. The results also show that the 5 cm and 3 cm columns can be made to give resolutions comparable to those of the 10 cm column if the composition of the mobile phase is adjusted to meet the requirements of solute residence time in the shorter columns.

EXPERIMENTAL

Materials: Perylene, benz(a)anthracene, and coronene were received from the Chemical Carcinogenesis Reference Standard Repository, function of the Division of Cancer Cause and Prevention, NCI/NIH, Bethesda, MD 20205. Acetonitrile (ACN) was glass distilled (Burdick and Jackson).

Apparatus: A modular HPLC system consisting of Laboratory Data Control (LDC) constametric I and II pumps attached to an LDC Gradient Master, a Chromatronix dual-channel UV absorbance detector (254 and 280 nm), a Rheodyne injector, and a strip-chart recorder operated at 0.2 in/min was used.

Three sets of columns were used (30 mm x 4 mm, 50 mm x 4 mm, and 100 mm x 4 mm) and were each packed with 3μ Spherisorb ODS packings obtained from Phase Separations, Inc. (see reference 1 for packing physical properties).

The experiments were run at room temperature using a mobile phase of acetonitrile/water (ACN/H_2O). One microliter of sample solution was injected. The mobile phase was degassed before use. The modifications made on the

instrument to accommodate the requirements of the short columns were as follows: a 10µl solvent loop was used in place of the 100µl loop. The column was attached as closely as possible to the detector, and stainless steel tubing of the narrowest bore possible was used.

Column Packing: Supports were slurry packed into columns with acetone: ACN (1:1) at 8000 psi by the upward technique using a Haskel pneumatic pump.

RESULTS AND DISCUSSION

The effect of column length using 5µ and 3µ ODS spherical and irregular ODS materials on resolution was studied (1) using an unmodified HPLC system. Using 10 cm and 5 cm columns, the results indicated that it was possible to achieve the separation of a mixture without an appreciable loss of resolution. This was attributed to the fact that the instrument's parameters (such as sample loop, detector cell volume, internal diameter of tubing, distance between column and detector) that are acceptable when 25 to 30 cm columns are used are inadequate when used with the short columns. As a result, short and long columns did not give appreciably different results. In the present study, instrument parameters were optimized and the sample injected was scaled down from 10µl to 1µl. The results (Fig 1) show that the longer the column the better the resolution, in this case the 10 cm column is superior to the 3 cm column. If the flow rate was changed from 2 ml/min to 1 ml/min (Fig 2) and to 0.5 ml/min (Fig 3) resolution improved. However, the 3 cm column did not give baseline separation, which means that increasing solute residence time in the column by decreasing the flow rate is not the best possible approach, as will be discussed later.

Based on a previous study (2) in which the mobile phase was adjusted to meet the hydrophobic properties of the columns, and our knowledge of reversed phase HPLC, we decided to increase the residence time of the solute mixture in the column by changing the composition of the mobile

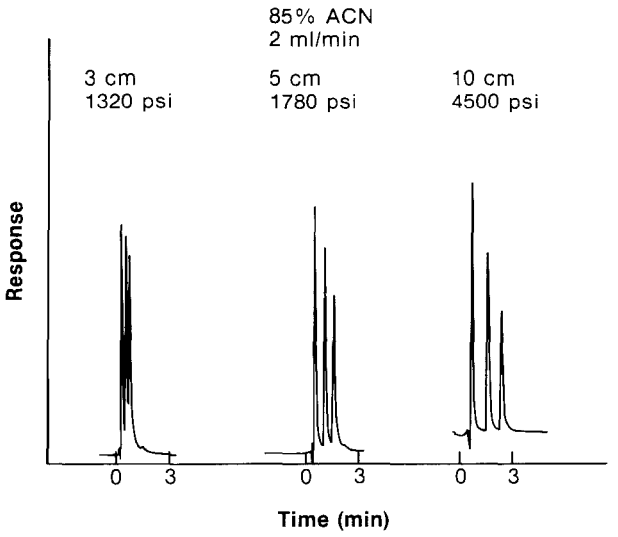


Figure 1. Separation of a test mixture on 3 cm, 5 cm, and 10 cm long columns packed with 3μ Spherisorb ODS material using a mobile phase of 85% acetonitrile/water at a flow rate of 2 ml/min.

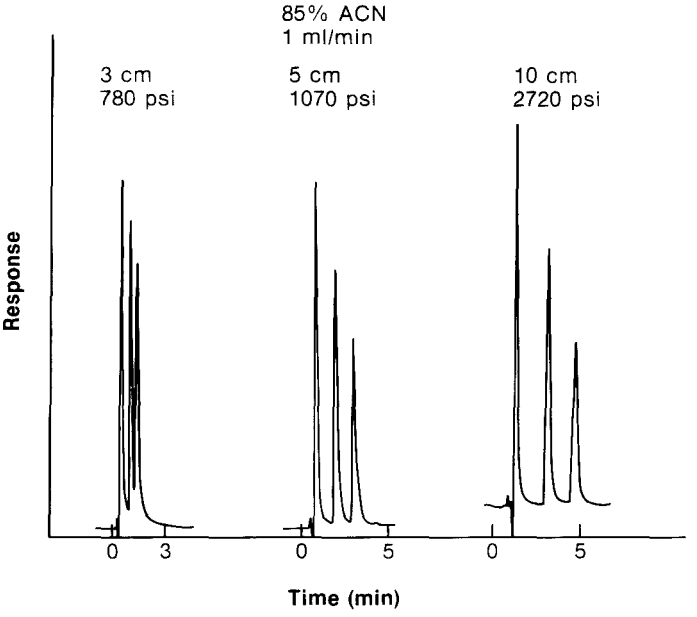


Figure 2. Same as Figure 1, except a flow rate of 1 ml/min.

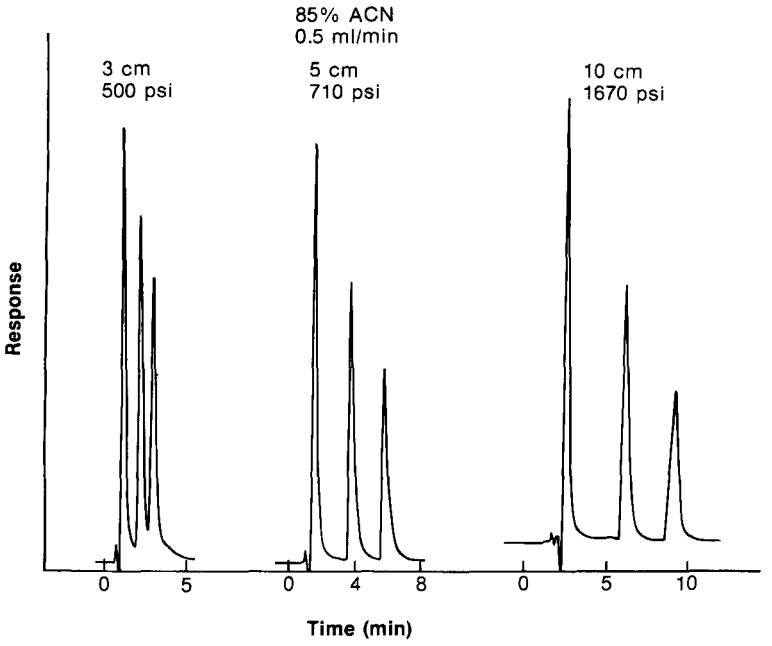


Figure 3. Same as Figure 1, except a flow rate of 0.5 ml/min.

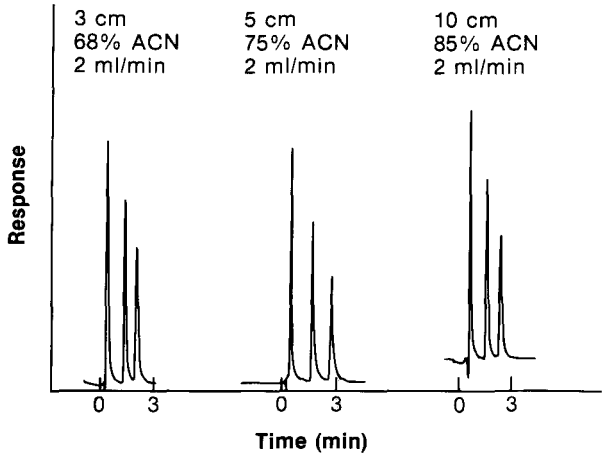


Figure 4. Separation of the test mixture on 3 cm, 5 cm, and 10 cm columns packed with 3μ Spherisorb ODS material using different ratios of acetonitrile/water at a flow rate of 2 ml/min.

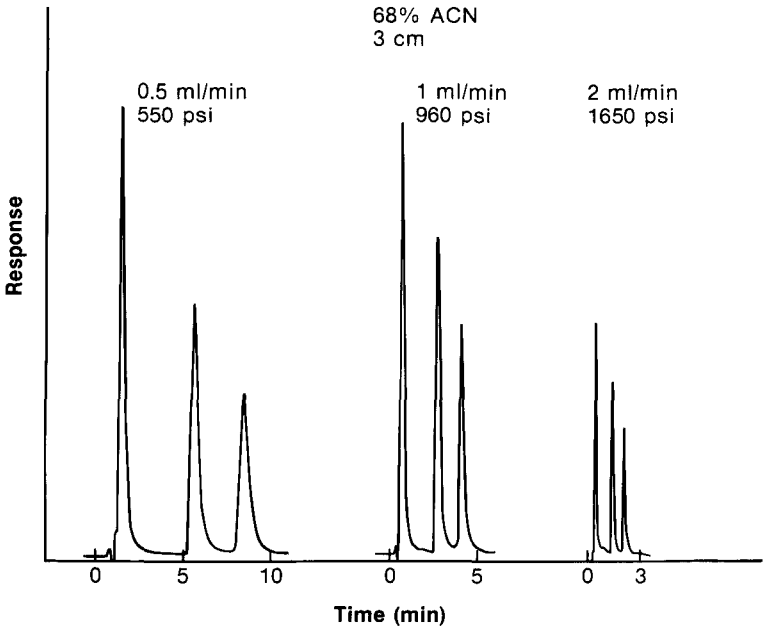


Figure 5. Separation of the test mixture on 3 cm column using a mobile phase of 68% acetonitrile/water and a mobile phase flow rate of 0.5, 1.0, and 2.0 ml/min.

phase. This was achieved by making the binary mobile phase richer in water as the column length decreased. Fig 4 shows that the same resolution of the test mixture was obtained on columns of the three different lengths when the mobile phase composition was changed. The 3 cm column and a 68% ACN/H_2O mobile phase gave the same separation as the 5 cm column with 75% ACN/H_2O and the 10 cm column with 85% ACN/H_2O at a flow rate of 2 ml/min. The separation was achieved in each case in less than 3 minutes. Fig 5 shows that when an optimum mobile phase is found, decreasing the flow rate will give better separation.

Mobile phases of the same composition, e.g., ACN/H_2O, methanol/water, etc., that contain a higher percentage of water result in higher back

TABLE 1

Effect of Column Length on Back Pressure

at a Mobile Phase Flow Rate of 2 ml/min

Column Length (cm)	Mobile Phase (% ACN)	Back Pressure (psi)
3	68	1650
5	75	1960
10	85	4500

pressure. Since the mobile phase had to be adjusted so that it contained a higher percentage of water as the column length decreased, it was necessary to compare the back pressure resulting from the use of each of the columns under optimum mobile phase conditions. Table 1 shows that the 3 cm column with a mobile phase of 68% ACN/H_2O gave a much lower back pressure than the 10 cm column with a mobile phase of 85% ACN/ H_2O and the 5 cm column with 75% ACN/H_2O.

CONCLUSION

This study shows that improving the instrumental parameters will lead to better results when columns of different lengths packed with the same material are used: the longer the column, the better the resolution. A 3 cm column can be made to give the same results as a 10 cm column, packed with the same material, if the residence times of the solutes in the column are comparable. This is achieved by decreasing the percentage of organic modifiers as the column length decreases. Although slowing the flow rate from 2 ml/min to 0.5 ml/min improves the resolution, it may not result in baseline separation.

ACKNOWLEDGEMENT

By acceptance of this article, the publisher or recipient acknowledges the right of the U.S. Government to retain a nonexclusive, royalty-free license in and to any copyright covering the article.

REFERENCES

1. Issaq, H.J. and Gourley, R.E., J. Liquid Chromatogr. 6, 1375 (1983).

2. Issaq, H.J., J. Liquid Chromatogr. 4, 1917 (1981).

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(3), 483-492 (1984)

EFFICIENCY OF GLASS CGC COLUMNS IN REVERSED-PHASE HPLC

Pavel Špaček, Stanislav Vozka[+], Jiří Čoupek[+],
Miroslav Kubín, Jaroslav Voslář, and Bedřich Porsch

Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, 162 06 Prague 6, Czechoslovakia

[+]Laboratory Instruments Works, 160 00 Prague 6, Czechoslovakia

ABSTRACT

The design of metal-jacketed glass columns of CGC type is described, and the dependence of their efficiency on the flow rate is given, both for a single column and for two columns combined in series. The results have been treated in terms of reduced quantities; the optimal values of the reduced plate height $h \sim 2.5$ suggest a good quality of the sorbent packing. It is shown that the described combination of columns in series does not reduce the total efficiency of the system.

INTRODUCTION

Column is the heart of each chromatographic apparatus: its parameters govern the efficiency, resolving power and working throughput of each chromatograph. For all these reasons, manufacturing of columns for high-performance

 0148-3919/84/0703-0483$3.50/0

liquid chromatography is an exacting process, which of course is also reflected in the price of the column. If the latter loses its utility properties, the purchase of a new one means a comparatively high additional expense.

Many manufacturers try to solve this problem by introducing jacketed columns in which the jacket assumes several functions of the column. The latter may then be composed of a comparatively simple tube with closures, placed in the jacket (the so-called "cartridge" type). Advantages of such system cannot be denied; they not only reduce the price of the column itself, but also make possible a simple joining of several columns, or a combination precolumn - - column, without capillary connections and with a small loss of efficiency.

At present, there are several systems offered on the market which make use of the principle just mentioned. The columns are made of stainless steel; their jackets are always made of a combination of stainless steel and light metals. With respect to some disadvantages of metal tubes and to our own long-time experience with high-pressure glass columns for HPCL [1], a system of metal-jacketed glass columns has been developed by the Laboratory Instruments Works in cooperation with the Institute of Macromolecular Chemistry of the Czechoslovak Academy of Sciences [2]. This system, while maintaining advantages of the cartridge columns, has stainless steel parts replaced with glass which in all respects is more advantageous. At present, the columns are manufactured by the Laboratory Instruments Works,

Prague. Their design and some of their properties are dealt with in this paper.

Design of the column

Fig.1 shows the design diagram of the glass column. The CGC (compact glass column) consists of a glass tube (1), inner diameter 3.3 mm, 150 mm long. The ends of the tube are ground, smoothed by melting, and the whole tube is chemically reinforced so as to withstand average pressures of 80 MPa. At the ends, the glass tube is provided with cemented guide rings (2) which serve to fix the closing and sealing elements and to guide the column in the metal jacket. The guide rings contain centering collars (3) with a fine gauze which retains the column packing. A sealing collar (5) containing a pressed-in sealing ring (6) is placed on the centering collar. The centering and sealing collars are made from PTFE, the sealing ring is made from high-quality stainless steel. Under pressure, the sealing collars are pressed into the thread of the guide rings, thus preventing their loosening. The system makes possible an easy fixation of the column packing and an additional sealing of the column in the jacket by applying a relatively low axial pressure.

The column is placed in a jacket which consists of a metal tube (8) provided with threads for cap nuts (9) at its ends, and with end caps (10) screwed into the threads which serve for additional sealing of the column in the jacket and for joining the column with the chromatographic

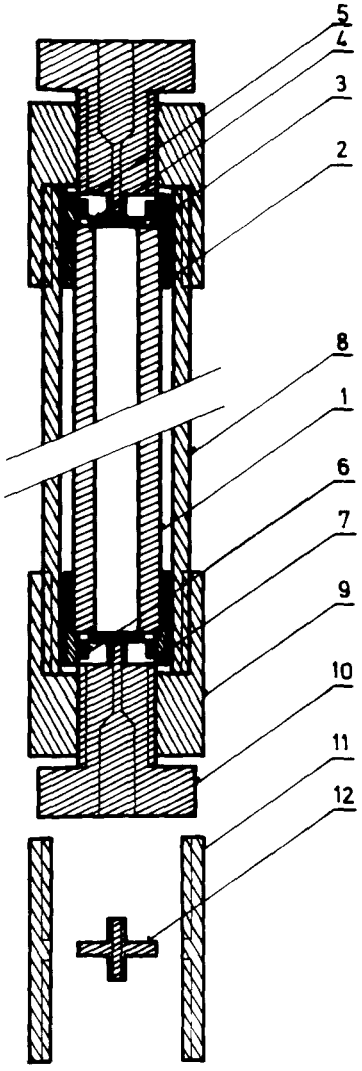


Fig.1 Schematic view of glass column of the CGC system (description cf. text)

apparatus. The jacket and nut caps are made from light metals, the end caps are made from stainless steel. The faces of the end caps are provided with grooves; after the column has been inserted into the jacket and the end caps have been screwed into the nut caps, the whole system is fixed by additional tightening of the nuts. This produces axial pressure which is needed to ensure stability of the system centering collar - sealing collar with the ring-grooved face of the end cap up to high pressures (50 MPa).

The columns are packed at 40-50 MPa by the slurry technique used for stainless steel columns. Only sorbents with particle size 5 μm are used as column packing; along with the design which minimizes dead volumes and the ideally smooth glass surface, they allow high chromatographic efficiencies to be achieved. The column dimensions have been chosen bearing in mind the use of 5 μm particles, and are optimized for limiting the wall effects according to[3].

The suggested system makes possible a simple joining of the columns in series and the use of protective precolumns. By using the cap nut (11) and the insert (12) (Fig.1), a column of double length can be obtained from the standard system using an additional jacket tube. The use of a short jacket allows a precolumn to be attached. Precolumns for the CGC system are 30 mm long.

EXPERIMENTAL PART

The liquid chromatograph used consisted of a syringe-type, positive displacement pump VLD 30[4,5] (Development

Workshop of the Czechoslovak Academy of Sciences, Prague), a home-made "stop-flow" sample injector [5], differential refractometer R 401 (Waters Ass., Milford, USA), and a potentiometric recorder (Servogor 220, Goerz, Austria).

The columns were packed with spherical silica [6], surface-modified with covalently bonded octadecyl groups [7] (SEPARON SIX C 18 , manufactured by Laboratory Instruments Works, Prague). The average size of sorbent particles was d_p=5µm. An acetonitrile-water mixture was the mobile phase (8:2 vol.), n-octyl alcohol (OcOH) and n-octyl acetate (OcAc) injected in a volume of 1 µl were used as the testing compounds.

Two columns (denoted as A and B) were examined with respect to their efficiency in the range of the mobile phase flowrate 0.1 - 1.2 ml/min, first each column separately and than both columns combined in series. The flowrate was first gradually increased from the minimal to the maximal and then decreased again. In this manner, each column was tested five times (to check the stability of the packing and to reduce the experimental error); the average from all measurements was taken for further treatment. A similar procedure was employed when both columns were connected in series. The results are summarized in Table I, together with the measured values of the capacity factor k' and with the corresponding values of pressure drop ΔP across the column or system of columns. The linear flow velocity u was calculated directly from the chromatogram using the retention time of an unretained peak

TABLE I

Plate number N, capacity factor k', and pressure drop, ΔP, at different volume flowrates, V, and linear velocities, u

	V ml/min	ΔP MPa	u cm/min	OcOH k'	OcOH N	OcAc k'	OcAc N
Column A	0.1	0.75	2.01	1.36	8050	2.82	8400
	0.2	1.50	3.98	1.32	10000	2.75	10000
	0.4	3.25	7.76	1.29	11150	2.68	11250
	0.6	4.50	11.32	1.27	11300	2.66	11100
	0.8	6.25	15.00	1.28	10100	2.64	10250
	1.0	7.50	18.26	1.26	9200	2.63	9650
	1.2	9.25	23.50	1.27	8350	2.63	8900
Column B	0.1	0.50	1.74	1.35	8700	2.81	8800
	0.2	1.25	3.42	1.32	10500	2.74	10500
	0.4	2.75	8.11	1.28	11250	2.68	11000
	0.6	4.25	12.62	1.27	10600	2.66	10350
	0.8	5.50	16.36	1.26	9650	2.64	9750
	1.0	6.50	19.35	1.27	8650	2.64	9100
	1.2	7.75	22.98	1.26	8000	2.63	8500
Column A+B in series	0.1	1.25	2.00	1.37	21150	2.83	19350
	0.2	2.75	4.22	1.33	23200	2.75	24700
	0.4	5.50	7.96	1.30	25150	2.69	26500
	0.6	8.25	12.00	1.28	24650	2.65	25000
	0.8	11.25	16.36	1.26	21800	2.63	21500
	1.0	13.75	20.22	1.27	19850	2.63	19500
	1.2	16.00	23.29	1.27	18650	2.63	18450

and column length. The number of theoretical plates, N, was calculated from the peak width at half height.

RESULTS AND DISCUSSION

Table I demonstrates the good reproducibility of the capacity factor k', although its values seem to increase

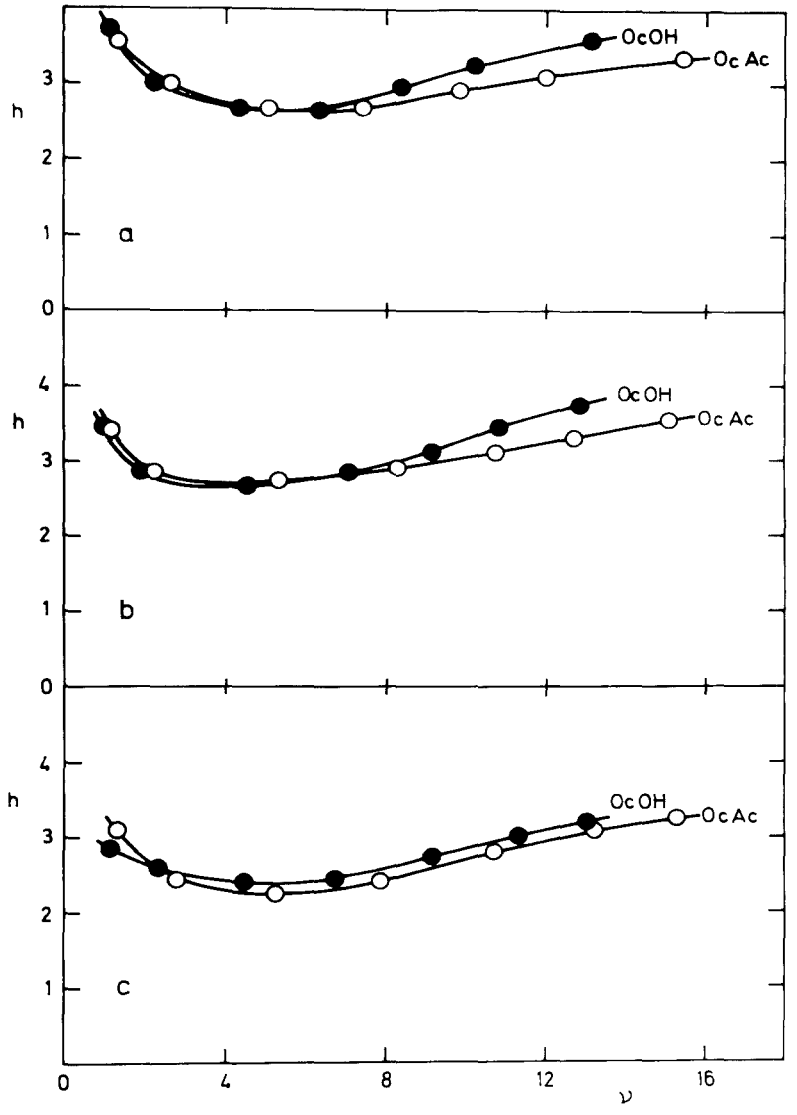


Fig.2 Dependence of reduced plate height,h, on reduced velocity, ν , for a) column A, b) column B, and c) columns A+B connected in series

somewhat at the minimal linear velocities of the mobile phase. The observed plate numbers are relatively high, considering that a column only 150 mm long packed with an octadecyl phase is involved; their dependence on the flow rate has a flat maximum near u = 10 cm/min.

It is worth mentioning that the sum of plate numbers of columns A and B is lower by 2-3 thousands on the average than that directly measured with both columns in series. This suggests that the mode of directly connecting the columns obviously does not impair essentially the efficiency of the whole system. It should be borne in mind, however, that with two columns connected in series, the extracolumn spreading in the refractometer cell is operative only once, while in the case of the individual columns A and B this negative effect is included twice in the sum of the plate numbers.

The experimental values were further evaluated in terms of reduced quantities; the required diffusion coefficients of the separated compounds in the mobile phase, D_m, were estimated from the approximative Wilke-Chang formula[8]. The reduced plate height $h = H/d_p$ (where H is the actual plate height and d_p is the particle diameter) is plotted against the reduced velocity $\nu = u \cdot d_p/D_m$ for the individual columns (A,B) and for their combination in series (A+B) in Fig.2. The curves show a minimum of about $h \simeq 2.5$ at $\nu \simeq 5$; this comparatively low value of the reduced plate height indicates a good quality of the sorbent packing and its narrow particle size distribution.

CONCLUSION

The results of this study show that columns of the CGC system can be used in high-performance liquid chromatography within a broad range of flow rates. The column efficiency (with Separon SIX C 18 used as sorbent) does not depend to any considerable degree on the retention of the compound in the column. By connecting two CGC columns directly in series the obtained plate number is virtually doubled.

REFERENCES

1. Vozka S., Porsch B., Špaček P., Kubín M., Čs.AO 183468.
2. Špaček P., Vozka S., Čoupek J., Kubín M., Voslář J., Porsch B., Čs.Patent Application, Čs.Patent PV 4635-81.
3. J.H.Knox, in "Practical High Performance Liquid Chromatography" (C.F.Simson,Ed.), Heyden and Son Ltd. 1978, pp. 19-46.
4. Porsch B., Voslář J., Vozka S., Špaček P., Čs.AO 181895.
5. Vozka S., Porsch B., Voslář J., Špaček P., Kubín M., Čs.AO 187774.
6. Vozka S., Špaček P., Kubín M., Porsch B., Čs.AO 200997.
7. Vozka S., Špaček P., Kubín M., Porsch B., Holler P., Čs.AO 207861.
8. Wilke C.R., Chang P.: Am.Inst.Chem.Eng., 1, 264 (1955).

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(3), 493-508 (1984)

APPLICATIONS OF A TECHNIQUE FOR THE HPLC ANALYSIS OF LIQUID CARBON DIOXIDE SOLUTIONS*

Christopher S. Nieass

The Commonwealth Industrial Gases Limited
138 Bourke Road, Alexandria, N.S.W. Australia 2015

and

Mark S. Wainwright and Rodney P. Chaplin

School of Chemical Engineering and Industrial Chemistry
The University of New South Wales
P.O. Box 1, Kensington, N.S.W.
Australia. 2033

ABSTRACT

A novel technique has been developed whereby substrate and solvent quantitation can be effected by means of reversed-phase high performance liquid chromatography. Carbon dioxide is detected by a differential refractometer.

Applications of this technique include the analysis of liquefied carbon dioxide-based aerosol mixtures, solubility measurements and liquid carbon dioxide extraction studies. Preliminary experiments suggest that this technique may also find application to the direct analysis of supercritical carbon dioxide extraction systems.

INTRODUCTION

Although the phenomenon of solubility of organic compounds in compressed and liquefied gases has been explored by numerous

*Based on a paper presented at the International Conference on Detectors and Chromatography, University of Melbourne, Melbourne, Australia. May 30 to June 3, 1983.

 0148-3919/84/0703-0493$3.50/0

researchers during the past century, it is only in the last decade or so that this subject has been exploited to advantage. Some advances such as tertiary enhanced oil recovery using nitrogen and carbon dioxide miscible flooding (1,2) have fostered much basic research of vapour-liquid equilibria for selected organic-compressed gas systems (3-11). Compressed and liquefied gases have found application as extraction media and thus replaced commonly used solvents such as methylene chloride and dichloroethylene (12). Many natural products such as chrysanthemum flowers, hops, soya beans, fruit, coffee, sunflower and rapeseeds and fats and oils have yielded useful extracts when treated with liquid carbon dioxide (13-22). Other uses of liquid and supercritical carbon dioxide include the stripping of organic contaminants from activated carbon used in the treatment of wastewater (23) and the recovery of neutral oils from coal tar (24).

A patent granted in 1978 (25) demonstrated that two-phase carbon dioxide could be used to dispense as aerosols a variety of insecticides and odour absorbers, thus replacing the traditional hydrocarbon and chlorofluorocarbon solvent-propellants. As a direct result of the invention of this liquid carbon dioxide based system by the Commonwealth Industrial Gases Limited (CIG), we have been engaged in the development of suitable analytical procedures whereby condensed gas systems can be directly analyzed for their organic content. Initially aimed at facilitating the study of the physical behaviour of products based upon this technology, the developed procedures (26-28) permit the direct analysis by modern liquid chromatography of liquefied carbon dioxide solutions for quality control and fundamental solubility measurements.

The purpose of this paper is to present details of some analyses of carbon dioxide-based systems which have been performed with these procedures. Although the range of systems investigated so far is limited, it is hoped that many of the foregoing applications may benefit from the development of this novel analytical technique.

EXPERIMENTAL

Analyses were performed by means of a Waters Associates Liquid Chromatograph assembled from separate components, viz, an M6000A solvent delivery system, a model 440 absorbance detector operating at a wavelength of 254 nm and an R401 differential refractometer. Chromatograms were recorded with either a Linear Instruments dual-channel strip chart recorder or two Hewlett-Packard 3390A reporting integrators. The latter devices were modified to permit simultaneous remote start and stop and facilitated calibration and quantitation procedures.

Liquid and supercritical gas mixtures were introduced to the chromatograph by means of Valco sample injection valves of both external and internal loop configurations; various delivery volumes were used and the procedures followed for liquefied gas injection have been described elsewhere (26-28).

Separations were effected on either a Brownlee Labs RP-8 25 cm x 4.6 mm ID column or a Waters Associates μ-Bondapak C_{18} 30 cm x 3.9 mm ID column. Eluents were prepared from HPLC grade acetonitrile, freshly distilled methanol and water. All mobile phases were thoroughly degassed by vacuum filtration to 0.45 μm.

RESULTS AND DISCUSSION

Analysis of Envirosols

Envirosols is the term used in reference to the liquid carbon dioxide-based aerosol mixtures produced by CIG. There are three products in the current range: Pestigas which comprises 0.4% w/w natural pyrethrins synergized with piperonyl butoxide in liquid carbon dioxide; Insectigas, 5% w/w dichlorvos in carbon dioxide, and Deodourgas, a complex mixture of esters dissolved in liquid carbon dioxide at a concentration of 1% w/w. The most abundant components of the presently-used concentrate are triethyl citrate, isopropyl myristate and methylated resin acid

esters. This latter group of compounds comprises at least 35 components, not all of which have been identified.

Liquid samples for quality control procedures are taken directly from industrial cylinders into small volume double-ended sample cylinders and analyzed by reversed phase liquid chromatography. Sample handling is confined to pressurization with nitrogen or other inert gas (26). Figure 1 presents a chromatogram obtained from a methanolic solution of the Pestigas concentrate and one obtained from the direct injection of the liquid carbon dioxide solution. The two traces are qualitatively similar with no spurious baseline perturbations arising from the introduction of the liquefied gas. Eluted with a mobile phase comprising 85 volume percent methanol/15 volume percent water at a flowrate of 3.0 $cm^3 min^{-1}$, an analysis takes 6 minutes; temperature programmed gas chromatographic assays of the active ingredient typically require 35 minutes.

Employing the same isocratic chromatographic conditions, Insectigas is analyzed in approximately two minutes. Comparative chromatograms for this product are presented in Figure 2. The leading peak in each case is due to a product of the partial hydrolysis of the organophosphate insecticide which may occur during storage of the pure material.

The multiplicity of components in Deodourgas is illustrated in Figure 3. Although this chromatogram was obtained with elution by a 90 volume percent methanol/10 volume percent 0.01 M $(NH_4)_2HPO_4$, no significant change in the separation was observed when the buffer was omitted from the mobile phase.

The technique which permits the direct sampling and analysis of liquid carbon dioxide-based aerosol mixtures has one major advantage over other methods of quality assurance in that the need to evaporate the solvent and to then dissolve the recovered substrate in an appropriate solvent is obviated. A less apparent advantage is that solvent evaporation may result in the loss of the more volatile compounds from a complex substrate which comprises

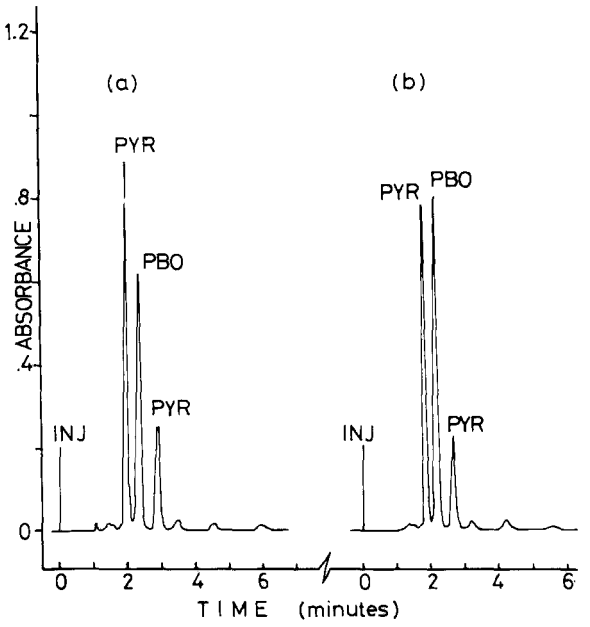


FIGURE 1. Comparative chromatograms of Pestigas Organic Solute (a) Methanolic Solution, (b) Direct CO_2 Solution Injection. HPLC conditions: sample volume - 10 mm^3; eluent - 85 v% methanol/15 v% water; flowrate - 3.0 $cm^3 min^{-1}$; column - Brownlee Labs RP8; detector sensitivity - 2.0 AUFS; chart speed - 10 mm min^{-1}.

components with widely differing boiling points. Thus direct sampling and analysis eliminates the potential loss of substrate.

Solubility Measurements

The development of a technique which would permit the determination of the limits of solubility of organic substrates in liquefied carbon dioxide (28) was an extension of the basic analytical technique (26). The technique has been tested using the naphthalene-carbon dioxide system (28) and the results obtained compare favourably with published data (29).

Although carbon dioxide does not yield a response on an ultraviolet absorbance detector, it can be detected with a differential refractometer (27). However, as illustrated in Figure 4,

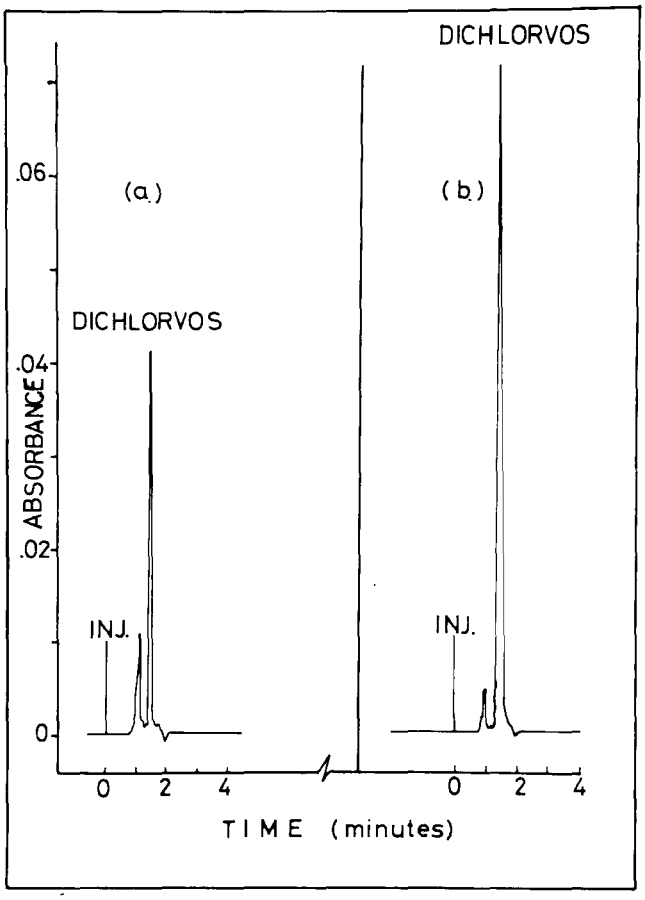


FIGURE 2. Comparative Chromatograms of Insectigas Solute (a) Methanolic Solution, (b) Direct CO_2 Solution Injection. HPLC conditions - as Figure 1 except detector sensitivity - 0.1 AUFS.

the carbon dioxide peak is of negative polarity. This arises because the refractive index of carbon dioxide is much lower than most organic compounds used as mobile phases in liquid chromatography. Consequently, the integrator must be capable of peak polarity inversion if both substrate and solvent are to be detected by means of a differential refractometer. Alternatively, two

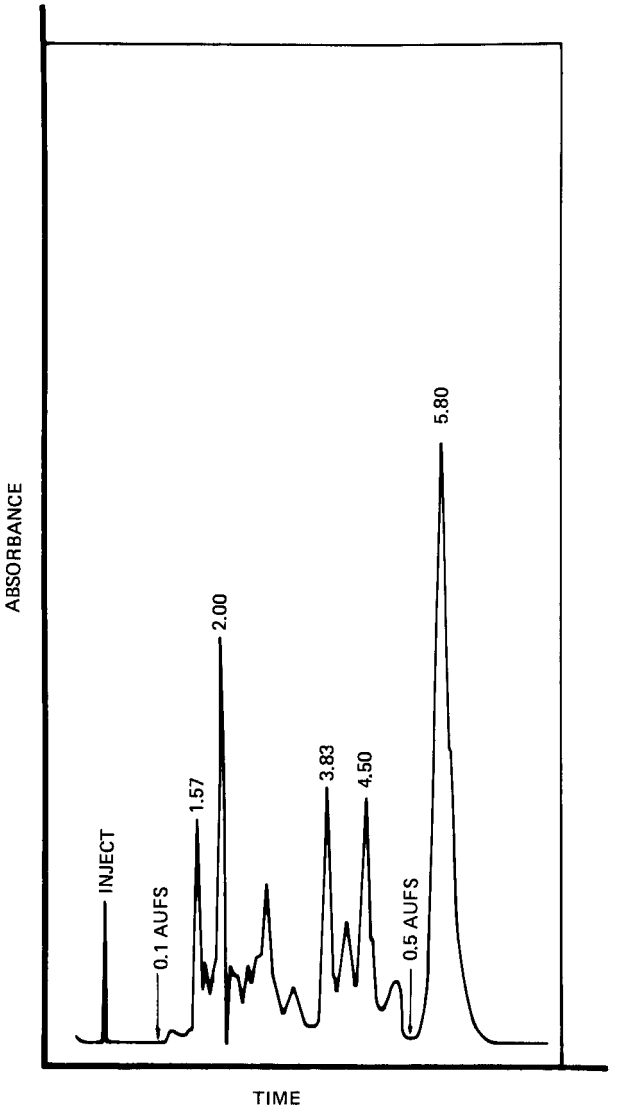


FIGURE 3. Chromatogram of Deodourgas Solute by Direct CO_2 Injection. HPLC conditions: sample volume - 10 mm^3; eluent - 90 v% methanol/10 v% 0.01 M $(NH_4)_2 HPO_4$; flowrate - 3.0 $cm^3 min^{-1}$; column - Brownlee Labs RP-8; detector sensitivity - 0.1 AUFS, 0.5 AUFS; chart speed - 10 mm min^{-1}.

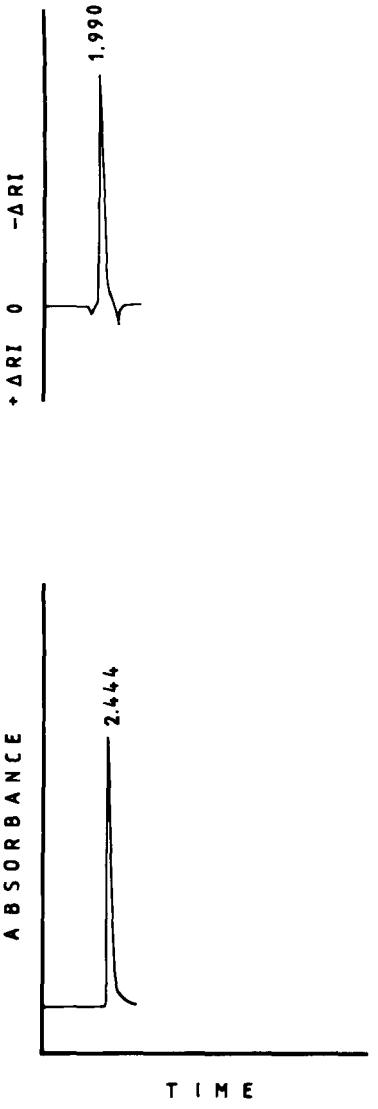


FIGURE 4. Naphthalene and Carbon Dioxide Chromatograms. HPLC conditions: sampl- volume - 2 mm^3 (nominal); eluent - 80 v% acetonitrile/20 v% water; flowrate - 2.0 $cm^3 min^{-1}$; column - Waters Associates C-18; detector sensitivity, RI-4X; UV - 2.0 AUFS; chart speed - 5 mm min^{-1}.

integrators may be interfaced to the one detector. Quantitation of carbon dioxide involves calibration of response versus absolute sample volume. It is important, therefore, that the solvent peak be separated from the solute peak if such is detected by the differential refractometer. It is fortuitous in this regard that carbon dioxide demonstrates anomalous retention behaviour in the aqueous methanol-C_{18} chromatographic system (27): the capacity factor of this compound increases very slowly with decreasing organic content in the mobile phase. Thus carbon dioxide should be readily separable from the substrate. This statement requires some qualification, however, as alkaline media are not satisfactory for carbon dioxide analyses due to reaction of CO_2 with the mobile phase (27). Alkaline substrates, such as aniline, may also cause difficulties due to adsorption on the stationary phase and the concomitant change in eluent pH in the region of the stationary phase-mobile phase boundary. In cases such as this, paired-ion chromatography in acid-buffered media may overcome this problem.

Solvent Extraction

The liquid carbon dioxide extraction from natural products of potentially useful constituents is receiving considerable attention. In order to assess the utility of the developed procedures to solvent extraction, we conducted a simple experiment involving the contacting of liquid carbon dioxide with macerated chrysanthemum cinerariaefolium flowerheads. The system was pressurized with nitrogen and the liquid analyzed by direct injection. Figure 5 illustrates both the ultraviolet absorbance and differential refractometer detector responses from an analysis of the liquid sample. A comparison of the organic substrate analysis from liquid CO_2 and commercial refined pyrethrins extract appears as Figure 6. The extract composition is similar in all respects to the commercial material with the exception of the first major peak. This component is present in the liquid carbon dioxide extract but not in the commercial product and may be a waxy compound removed in the refining process.

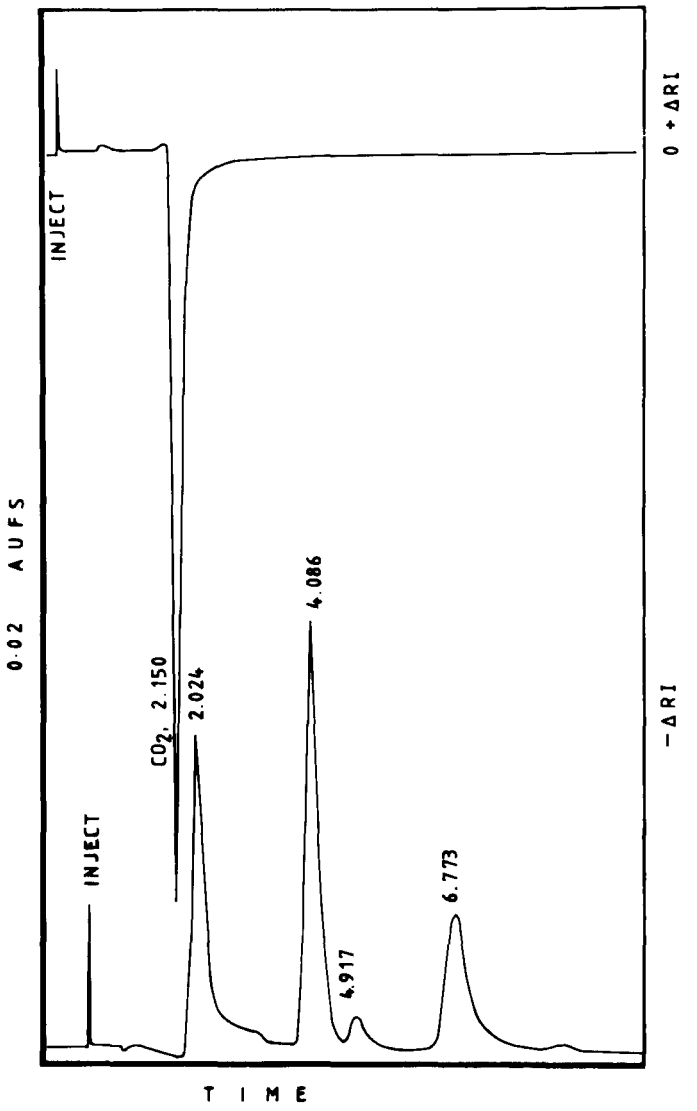


FIGURE 5. Chromatogram of CO_2-soluble Pyrethrum Extract. HPLC conditions: sample volume - 2 mm^3 (nominal); eluent 85 v% methanol/15 v% water; flowrate - 2.0 $cm^3 min^{-1}$; column - Waters Associates C-18; detector sensitivity, RI-32X; UV - 0.02 AUFS; chart speed - 10 mm min^{-1}.

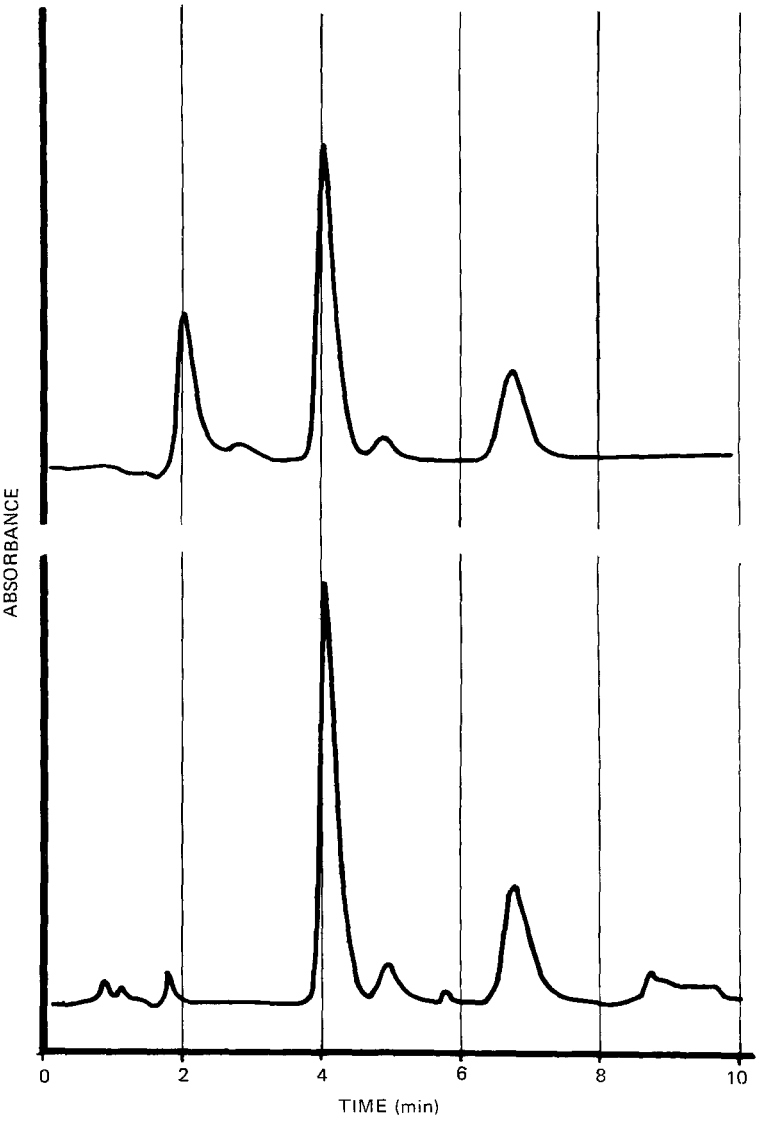


FIGURE 6. Comparison of LCO_2 and Commercial Pyrethrum Extracts. Upper - LCO_2 extract. Lower - Commercial extract. HPLC conditions - see Figure 5.

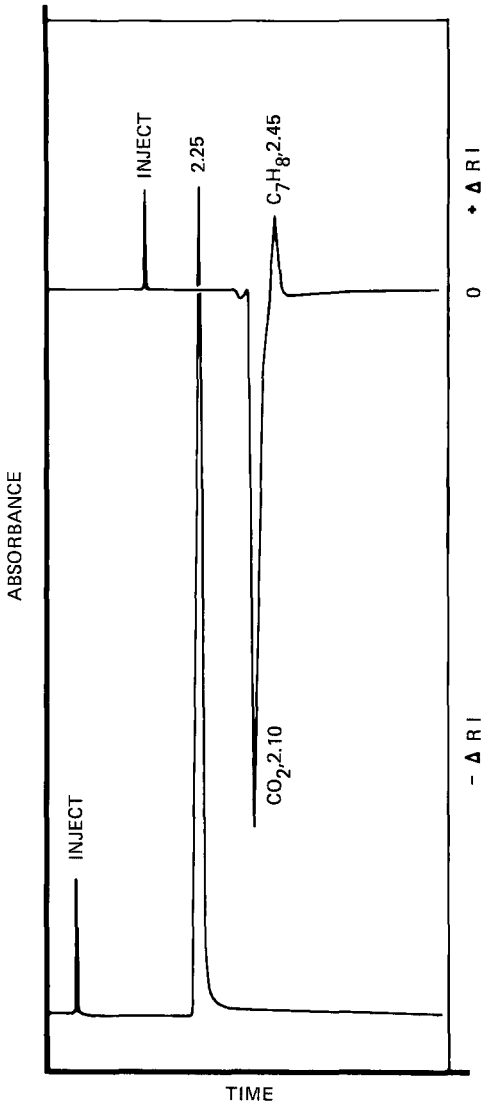


FIGURE 7. Supercritical CO_2 - Toluene Analysis. HPLC conditions: sample volume - 0.2 mm^3; eluent - 90 v% methanol/10% water; flowrate - 2.0 cm^3min^{-1}; column - Waters Associates C-18; detector sensitivity, RI-32X, UV - 1.0 AUFS; chart speed - 10 mm min^{-1}.

These chromatograms indicate that carbon dioxide has a selectivity similar to that of the conventional organic solvent used in the commercial extraction of the insecticide and demonstrate that, with appropriate apparatus, the developed techniques could be of benefit to the study of liquefied gas extraction.

Supercritical Carbon Dioxide Solutions

In a previously reported study (27) it was found that liquid carbon dioxide dissolved in aqueous methanol eluents at the point of injection. It was of interest to find out whether or not supercritical carbon dioxide behaved similarly. Thus a dilute solution of toluene in liquid carbon dioxide was rendered supercritical by heating to 40°C and samples of the homogeneous fluid introduced to the chromatographic system. Sample pressure was maintained by means of a pressurized mercury reservoir (28). A chromatogram similar to that presented in Figure 7 was obtained. The carbon dioxide response is consistent with that obtained from liquid injections which demonstrates that the supercritical solution may be analyzed with reversed phase liquid chromatography.

In principle, therefore, a conventional high performance liquid chromatograph can be used for the analysis of suitable supercritical systems. Quantitative analysis relies on the availability of accurate pressure-density-temperature data for carbon dioxide.

CONCLUSIONS

We have attempted to show that a technique for the sampling and analysis of liquefied carbon dioxide solutions may be applied to activities such as quality control, solubility measurements and liquid and supercritical extraction studies. Although the technique has limitations which are primarily the suitability of the analytical method to the substrate of interest, we feel that such a technique will find many applications, not necessarily restricted to the carbon dioxide system. Indeed, the principle could be used for the analysis of many liquefied gases such as hydrocarbons.

ACKNOWLEDGEMENT

Permission from The Commonwealth Industrial Gases Limited to publish this paper is gratefully acknowledged.

REFERENCES

1. Wilson, K.B., Nitrogen Use in EOR Requires Attention to Potential Hazards, OGJ, Oct 18, 1982, p.105-110.

2. Schendel, R.L., EOR + CO_2 = A Gas-Processing Challenge, ibid, Oct. 25, 1982, p.158-166.

3. Besserer, G.J., and Robinson, D.B., A High Pressure Auto Collimating Refractometer for Determining Coexisting Liquid and Vapour Phase Densities, Can. J. Chem. Eng., 49, 651, 1971.

4. Besserer, G.J., and Robinson, D.B., Refractive Indices of Ethane, Carbon Dioxide and Isobutane, J. Chem. Eng. Data, 18, 137, 1973.

5. Besserer, G.J., and Robinson, D.B., Equilibrium-phase Properties of i-Butane-Carbon Dioxide System, ibid, 18, 298, 1973

6. Besserer, G.J., and Robinson, D.B., Equilibrium-phase Properties of n-Pentane-Carbon Dioxide System, ibid, 18, 416, 1973.

7. Besserer, G.J., and Robinson, D.B., Equilibrium-phase Properties of Isopentane-Carbon Dioxide System, ibid, 20, 93 1975.

8. Kalra, H., Kubota, H., Robinson, D.B., and Ng, H-J., Equilibrium-phase Properties of the Carbon Dioxide-n-Heptane System, ibid, 23, 317, 1978.

9. Ng, H-J., and Robinson, D.B., Equilibrium-phase Properties of the Toluene-Carbon Dioxide System, ibid, 23, 925, 1978.

10. Ng, H-J., Huang, S., and Robinson, D.B., Equilibrium-phase Properties of Selected m-Xylene Binary Systems. m-Xylene-Methane and m-Xylene-Carbon Dioxide, ibid, 27, 119, 1982.

11. Sebastian, H.M., Simnick, J.J., Lin, H-M., and Chao, K-C., Gas-Liquid Equilibrium in Mixtures of Carbon Dioxide + m-Xylene, ibid, 25, 246, 1980.

12. Sims, M., New Interest in CO_2 Extraction of Spices, 'Naturals' - Offers Process/Quality Advantages, Food Processing, January 1980, p.92.

13. Stahl, E., Schutz, E., and Mangold, H.K., Extraction of Seed Oils with Liquid and Supercritical Carbon Dioxide, J. Agric. Food Chem., 28, 1153, 1980.

14. Stahl, E., and Schutz, E., Extraction of Natural Compounds with Supercritical Gases. 3. Comm.: Pyrethrum Extracts With Liquefied and Supercritical Carbon Dioxide, Planta Medica, 40, 12, 1980.

15. Sims, M., Process Uses Liquid CO_2 for Botanical Extractions, Chem. Eng. (N.Y.), 89, 50, 1982.

16. Schultz, W.G., and Randall, J.M., Liquid Carbon Dioxide for Selective Aroma Extraction, Food Technol., 24, 1282, 1970.

17. Schultz, W.G., Schultz, T.H., Carlson, R.A., and Hudson, J.S., Pilot Plant Extraction with Liquid CO_2, ibid, 28, 32, 1974.

18. Schultz, T.H., Flath, R.A., Black, D.R., Guadagni, D.G., Schultz, W.G., and Teranishi, R., Volatiles from Delicious Apple Essence - Extraction Methods, J. Food Sci., 32, 279, 1967.

19. Laws, D.R., Bath, N.A., and Pickett, J.A., Production of Solvent-free Isomerized Extracts, J. Am. Soc. Brew. Chem., 35, 187, 1977.

20. Laws, D.R.J., Bath, N.A., Pickett, J.A., Ennis, C.S., and Wheldon, A.G., Preparation of Hop Extracts Without Using Organic Solvents, J. Inst. Brew., 83, 39, 1977.

21. Nestle's Products Ltd., U.K. Pat., 1,106,468 (1968).

22. Caragay, A.B., and Krukonis, V., "Supercritical Fluid Extraction for Purification and Fractionation of Fats and Oils". Presented at 72nd Annual Meeting of American Oil Chemists Society, New Orleans, LA., May 18, 1981.

23. Worthy, W., Hazardous Waste: Treatment Technology Grows, Chem. Eng. News, 60, 10 (1982).

24. Valteris, R.L., Vapour Phase Extraction, Ph.D. Thesis, Dept. Chem. Eng., University of Birmingham (1966).

25. Shervington, E.A., Ryan, R.F., and Catchpoole, D.J., Austr. Pat. 494,198 (1978).

26. Nieass, C.S., Wainwright, M.S., and Chaplin, R.P., Sampling and High Performance Liquid Chromatographic Analysis of Organic Compounds in Liquefied Carbon Dioxide, J. Chromatogr., 194, 335, 1980.

27. Nieass, C.S., Wainwright, M.S., and Chaplin, R.P., Reversed-phase High Performance Liquid Chromatography of Carbon Dioxide, J. Liq. Chromatogr., 5, 2179, 1982.

28. Nieass, C.S., Chaplin, R.P., and Wainwright, M.S., Quantitation of Carbon Dioxide by High Performance Liquid Chromatography: the Key to Solubility Measurements in Liquefied Gas Solutions, ibid, 5, 2193, 1982.

29. Quinn, E.L., The Internal Pressure of Liquid Carbon Dioxide from Solubility Measurements, J. Am. Chem. Soc., 50, 672, 1928.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(3), 509-538 (1984)

THE DETERMINATION OF CATECHOLAMINES, INDOLEAMINES, METABOLITES, AND RELATED ENZYMATIC ACTIVITIES USING THREE MICRON LIQUID CHROMATOGRAPHY COLUMNS

P. Y. T. Lin, M. C. Bulawa, P. Wong, L. Lin,
J. Scott, and C. L. Blank
Department of Chemistry
University of Oklahoma
Norman, OK 73019

ABSTRACT

Liquid chromatography with electrochemical detection has become an established technique for the determination of catecholamines, indoleamines, precursors, metabolites, and related enzymatic activities in tissues and fluids. Previously available instrumentation, however, has limited the number of individual species readily and simultaneously accessible with reasonable throughput to only a few. Determinations of other species required either extended amounts of time per individual chromatogram or the use of an entirely separate chromatographic setup employing different columns and eluting solvents. Using reversed-phase columns packed with 3 micron particles, we have been able to produce the separation of 16 different catecholamine and indoleamine related species and two different internal standard compounds in 5 or 7 minutes. Samples may be analyzed directly after only homogenization, centrifugation, and clarification by filtration. No further purification steps are required. The enzymatic activities of 6 separate enzymes may be determined using the same chromatographic apparatus and simply monitoring selected metabolites following appropriate incubation of pretreatment. The metabolites and transmitters currently accessible with this apparatus include norepinephrine, dopamine, epinephrine, serotonin, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylalanine, normetanephrine, metanephrine, 3-methoxytyramine, 3,4-dihydroxyphenylethyleneglycol, vanillylmandelic acid, homovanillic acid, 5-hydroxytryptophan, 5-hydroxyindoleacetic acid, 5-hydroxytryptophol, and N-acetyl-5-hydroxytryptamine. The enzymatic activities include tyrosine hydroxylase, tryptophan hydroxylase, dopa decarboxylase, 5-hydroxytryptophan decarboxylase, monoamine oxidase, and catechol-O-methyltransferase.

 0148-3919/84/0703-0509$3.50/0

INTRODUCTION

The initial utilization of liquid chromatography combined with electrochemical detection for the determination of catecholamines by Refshauge et al. (1) offered a unique and highly selective technique for this purpose. Likewise, the same basic technique was found applicable to the determination of serotonin (2). This was quickly followed by announcements of procedures to determine catecholamine (3-8) and indoleamine (9-11) metabolites. Other workers (2, 12-19) reported the determination of enzymatic activities by coupling liquid chromatography with electrochemical detection to previously established pretreatments or incubation procedures.

The desire for greater amounts of information per sample motivated some to attempt to increase the number of individually determined species in single chromatographic runs. Such efforts (2, 3, 20-27) have been shown capable of separating as many as 10-12 endogenous species, although individual chromatograms may have required as much as 30-60 minutes to accomplish this task. This is clearly too long for most routine applications. On the other hand, the determination of only 3 or 4 major species, which may be obtained on a more rapid basis, leaves the investigator with the feeling that s/he would like to know a little more about the sample.

The recent advent of reversed-phase columns containing 3 μ packing materials has offered a substantial improvement in this situation along with some notable difficulties (28-32). The difficulties primarily arise from two major factors. First, the extremely small peak widths of the early eluting components require much greater attention to dead volumes and detector response times. Secondly, the large number of conceivably desirable tyrosine and tryptophan metabolites, which may be as great as 50, means we still cannot measure all the metabolites in a reasonable period of time. Nonetheless, the judicious choice of some 10-14 commonly measured metabolites will allow their rapid and routine determination in 4-7 minutes. This is exactly

what we have done in the current work. While our selection of the particular species to be included is unquestionably a biased one, we feel that the individual components and enzymatic activities to which the separation applies will be generally useful to a number of other investigations.

MATERIALS

Liquid Chromatograph

The apparatus employed for this report was constructed from readily available components. Two separate systems are described, since both have been shown independently capable of performing all of the described determinations. System A employed a Beckman Ultrasphere ODS reversed-phase (75 x 4.6 mm) column, while system B employed a Perkin-Elmer HC-18 reversed-phase (100 x 4.6 mm) column. The average particle diameter for both columns was 3 microns. Besides the obvious differences in columns and eluting solvents (vide infra), the two systems further differed only in respect to flow rates (system A - 2.2 ml/min; system B - 1.85 ml/min) and mean operational back pressures (system A - 3350 p.s.i.; system B - 3525 p.s.i.). The pump was a Milton Roy reciprocating Minipump. This was connected to a Mark III pulse dampener from Alltech Associates for mechanical dampening and a 5000 p.s.i. pressure gauge. In succession, this was connected to an injection port, the column, the electrochemical flow cell, the reference electrode compartment, and the waste container. The entire system was not thermostatted (room temp. = 21°C), since it was felt that most potential users would not have access to this capability. However, even further improvements in the currently reported separations could feasibly be obtained through judicious use of temperature control (28). The Rheodyne model 7010 injection port was modified to contain a 5 µl injection loop by replacing the standard loop with a short piece of 0.006 inch (i.d.) stainless steel tubing (1/16 inch o.d.). The same, narrow bore tubing was employed to connect the injection port

to the column inlet (6 cm length) and to connect the column outlet to the flow cell (6 cm length). The flow cell was similar to a BioAnalytical Systems model TL-3 with the following exceptions. The tubing forming the inlet to the flow cell was press fitted into the upper Kel-F block such that it penetrated completely to the level of the cell gasket. The carbon paste working electrode was located in the upper Kel-F block between the inlet and outlet openings. The bottom block was constructed entirely of stainless steel and served as the auxiliary electrode. The reference electrode, a Ag/AgCl (3 N NaCl) unit obtained from BioAnalytical Systems, was located downstream in the reference electrode holder unit. A model LC-4B dual electrochemical potential controller, also from BioAnalytical Systems, was employed to maintain a constant potential of 0.85 to 0.90 volts vs. Ag/AgCl. Only one channel of output is displayed in this report. We also would advise using a potential setting of only 0.65 to 0.70 volts for the in vitro tryptophan hydroxylase determination; the large amounts of L-tryptophan used as substrate in this assay will otherwise cause the noticeable appearance of this component at an elution time of approximately 15 min when using the higher potential. The lower potential could also be used to gain a higher signal to noise ratio in many of the other determinations described. However, it does provide a considerable lowering of signal for the methoxylated catecholamines and, thus, should not be employed when these are of primary concern.

The time constant afforded by the RC damping of the LC-4B detector was modified to a value of 0.25 sec. This was accomplished by altering the capacitor in the primary amplification stage. While such modification is essential to avoid instrumental broadening of the early eluting components, it also allows considerable amounts of pump pulsation, with a period of ca. 0.5 sec, to feed through to the detector as noise. The use of more extensive mechanical damping, a 6-or 8-pole Butterworth filter, a higher frequency pump, curve fitting by subtracting the underlying pump 'signal,' or all of these may, thus, be very appro-

priate to obtain the maximum signal to noise ratio for systems employing 3 μ columns.

Eluting Solvents

The eluting solvents for the two systems were quite similar and are presented in Table 1. The mobile phase is typically prepared in 4.0 liter batches, which is described as follows for System B. Approximately 0.5 to 1.0 g of NaOH and 74.45 mg $Na_2EDTA \cdot 2H_2O$ are added with stirring to 3700 ml of distilled/deionized (Milli-Q) water. The dissolution of the EDTA is aided here by the initially basic nature of the solution. After dissolution, citric acid monohydrate (84.04 g), diethylamine (3.4 ml), and sodium octyl sulfate (236.9 mg) are added with stirring. The mixture is filtered through an 0.22 μ Millipore vacuum filtering unit and combined with 300 ml of previously filtered acetonitrile. The final mixture is then titrated to a measured pH value of 2.45; no further filtration is effected in order to preserve the acetonitrile content. The diethylamine, only necessary when using the Perkin-Elmer column, is added to eliminate peak tailing problems which were particularly observed with the amine-containing species.

Fine tuning of the solvent conditions appears to be essential to obtain the desired separation for each new column received as well as, occasionally, throughout the lifetime of individual columns. We are currently preparing a paper which will discuss this problem in more detail. Briefly, however, useful adjustments have been determined to be afforded by the pH (in 0.02 to 0.05 unit increments), the concentration of SOS (in 0.05 to 0.10 mM increments), and the concentration of acetonitrile (in 0.5% increments). The latter parameter simply effects contraction (upon increased CH_3CN concentration) or expansion (upon decreased CH_3CN concentration) of the chromatogram. The former two parameters, however, alter the relative retention times of individual species. Acetonitrile and pH effects typically require only a few hours, at normal flow rates, to become apparent. Effects

TABLE 1

Eluting Solvents

Parameter	Concentration or Value*	
	Beckman Ultrasphere (System A)	Perkin-Elmer HC-18 (System B)
Citric Acid	0.1 M	0.1 M
EDTA	0.05 mM	0.05 mM
CH_3CN (vol:vol)	7.5%	7.5%
pH (final measured value)	2.50	2.45
Sodium Octyl Sulfate (SOS)	0.175 mM	0.255 mM
Diethylamine (wt./vol.)	0	0.06%

*Final concentrations assume the aqueous and nonaqueous portions of the solvent are completely additive.

due to increasing (decreasing) SOS concentrations, represented by stronger (weaker) relative retention of the amines and amino acids, may require as long as 48 hours.

Chemicals

All chemicals used were obtained from commercial sources at the highest available purity.

Animals

All mice used were adult males of the ARS-HA/ICR strain (Sprague-Dawley, Madison, WI) weighing 20-30 g at the time of sacrifice. The rats were of the Sprague-Dawley strain and typically weighed 300-325 g at the time of sacrifice. These animals were maintained on a 12 hr. light/12 hr. dark cycle and allowed access to food and water ad libitum. No animal was used until at

least one week after arrival. Also, the animals were typically sacrificed at 3-4 hr into the light cycle.

METHODS

Tissue and Fluid Determination of Endogenous Components

A sample of tissue (0.5 g) or bodily fluid (0.5 ml) is precisely measured and added to 750 µl of homogenizing solution. Appropriate adjustments are made in these quantities for different weights or volumes, although a lower limit of 250 µl of homogenizing solution is employed for practical manipulative purposes. The homogenizing solution contains 0.5 M acetic acid, 0.5 M sodium acetate, 0.4 M sodium perchlorate, and has a pH of 4.8. It is degassed for ca. 15 min before use with O_2-free N_2 (1). A 100 µl aliquot of internal standard, containing approximately 300 ng 3,4-dihydroxybenzylamine and 250 ng N_ω-methyl-5-hydroxytryptamine (for whole mouse brain) then added to each sample and the resultant mixture is subjected to ground glass homogenization. Standard samples are prepared by replacing the tissue or fluid with a roughly equivalent volume of a stock external standard solution containing the species to be quantitated at levels appropriate to the unknown samples. Thus, for a whole mouse brain (ca. 0.5 g), 0.5 ml of a stock external standard containing ca. 200 ng norepinephrine, 450 ng dopamine, etc. would be employed. In general, we recommend that the concentration of substances to be measured not be lower than 100 ng/ml in the external standard to minimize the attendant problems associated with extracting the signal from the noise. Standard samples also receive a 50 µl aliquot of 1 mg/ml ascorbic acid, prepared fresh on the day of analysis.

Following homogenization, the sample is centrifuged at 50,000 x g for 45 min or 40,000 x g for 60 min to separate the macromolecules and cellular debris from the supernate. An aliquot of the supernate is then transferred to an MF1 Microsample Filtering unit (BioAnalytical Systems) and clarified by centri-

fugation/filtration at 13,000 x g for 15 min. The pore size of the filter is 0.22 μ. The temperature during both centrifugal operations is 4°C. A 5 μl aliquot of the filtrate is then injected into the liquid chromatograph for quantitation.

Two internal standards are employed in this determination; one (3,4-dihydroxybenzylamine) is more suited to the catechols, while the other (N_ω-methyl-5-hydroxytryptamine) is more suited to the indoles. This use, which might be labelled extravagant by some, is really quite appropriate since neither of these substances overlaps any of 26 tested catecholamine and indoleamine derivatives. However, the N_ω-methyl-5-hydroxytryptamine could be eliminated to obtain faster throughput, since it elutes last at the current time.

in vitro Tyrosine Hydroxylase Activity

The in vitro tyrosine hydroxylase determination described is for whole mouse brains, although it has been used for brain parts and should be applicable to other tissues.

The tissue is removed and weighed. A tissue homogenate is prepared by adding ca. 0.5 g tissue to 2.00 ml H_2O and 200 μl of an internal standard solution containing 8 x 10^{-5} M 3,4-dihydroxybenzylamine in 1 x 10^{-3} M ascorbic acid. Homogenization is effected with a glass/Teflon mechanical unit. Standard 'homogenates' are prepared by adding 500 μl of a solution containing 9.84 μg L-3,4-dihydroxyphenylalanine (L-DOPA) in 10 ml of 1 x 10^{-3} M ascorbic acid to 2.00 ml H_2O and 200 μl of the above internal standard solution.

The incubation mixture is prepared by mixing the following components to obtain a final volume of 500 μl: 100 μl of 2.0 M acetate buffer having a pH of 6.40; 50 μl of a solution which contains 20.0 mM 6-methyltetrahydropterin, 0.50 M 2-mercaptoethanol, and 0.4 mM NSD-1015 (3-hydroxybenzylhydrazine); 50 μl of 1.0 mM ferrous sulfate; 50 μl of 2.5 mM L-tyrosine in 0.01 M HCl; and, 250 μl of brain (or standard) homogenate. All the solutions except the L-tyrosine and the homogenate are preincu-

bated for 30 min at 37°C after mixing. The L-tyrosine and homogenate are then added and the incubation is carried out, with shaking, at 37°C for 30 min.

Since tyrosine solutions contain L-DOPA, the reaction product, as an impurity and L-DOPA is also produced by non-enzymatic routes, it is important to run blanks with these determinations. A blank 'homogenate' is prepared by mixing 500 µl of 1×10^{-3} M ascorbic acid, 2.00 ml H_2O, and 200 µl of the above described internal standard solution. Blanks then receive the same treatment as the tissue samples except that 250 µl of this 'homogenate' replaces the 250 µl of tissue homogenate in the above sample preparation.

Following incubation, the reaction is terminated by the addition of 200 µl of 1.0 M $HClO_4$. 50 µl of a solution containing 0.30 M $NaHSO_3$ and 0.030 M EDTA is also added to prevent oxidation of the catechols. The samples are centrifuged at 16,000 x g and 4°C for 15 min. 500 µl of the supernatant fraction is transferred to a small plastic vial and subjected to the alumina isolation procedure (1). This is the only determination of all being described which requires such a special isolation step. However, it must be used here to prevent substantial interference between the 6-methyltetrahydropterin and L-DOPA in the succeeding chromatography. The final eluent from the Al_2O_3 is subjected to centrifugation/filtration for sample clarification. 5 µl of the filtrate is injected into the liquid chromatograph for quantitation.

in vivo Tyrosine Hydroxylase and Tryptophan Hydroxylase Activities

The simultaneous determination of the *in vivo* hydroxylase activities are surprisingly simple. The method is patterned after that presented by Carlsson *et al*. (33, 34). Mice are injected with 200 mg/kg (i.p. or i.v.) NSD-1015 (N-3-hydroxybenzylhydrazine) 2-30 minutes prior to sacrifice. A dose of 150 mg/kg is completely adequate for rats. The longer the time between injection and sacrifice, the greater the resultant signal to noise

ratio obtained. Following sacrifice, the desired tissue is removed, weighed, and subjected to the procedure described above for "Tissue and Fluid Determinations." Of course, the standard 'homogenates' in these determinations should contain appropriate concentrations of L-DOPA and L-5-hydroxytryptophan, the tyrosine and tryptophan hydroxylase products, respectively. The NSD-1015 blocks metabolism of these products by DOPA decarboxylase and 5-hydroxytryptophan decarboxylase. Thus, their buildup following blockade is a direct measure of the in vivo activity of their respective hydroxylase source activities. While endogenous concentrations of these two products are typically very small (ca. 2 ng/g and 15 ng/g), blanks may be appropriate for very short times between treatment and sacrifice, i.e., 2-3 minutes. The blanks would simply be saline injected controls.

in vitro DOPA Decarboxylase Activity

DOPA decarboxylase and 5-hydroxytryptophan decarboxylase may well be a single enzyme, or at least the same entity with two active sites (35). Alternatively, they may represent two distinct, but very similar, entities (36). We have proceeded as if the two substrates act differently toward decarboxylation and, thus, have optimized assays for each. The procedures of Sims et al. (36) were used to obtain workable conditions, and the determinations were then optimized for whole mouse brain tissue. However, we feel the results should be readily applicable to other tissues and fluids as well.

For the assessment of DOPA decarboxylase activity, a 10% tissue homogenate is formed by adding 500 mg of tissue to 500 µl of 2.0×10^{-3} M 3,4-dihydroxybenzylamine or epinine, either being a satisfactory internal standard, and 4.00 ml of H_2O. The water content is actually adjusted for each sample to assure a 10% homogenate. A standard 'homogenate' is prepared by adding 500 µl of 2.0×10^{-3} M dopamine to 500 µl of the same internal standard solution, 50 µl of ascorbic acid (75 mg/ml), and 3.95 ml of an 0.10 M acetate buffer of pH 4.80. Homogenization is effected by

ultrasonication or with a ground glass apparatus. For each sample, the following components are combined in a small incubation tube: 100 l of a 1:1 mixture of 1.0×10^{-3} M pargyline and 1.0×10^{-3} M pyridoxal-5'-phosphate; 100 µl of 0.75 M phosphate buffer of pH 6.60 (this is replaced with 100 µl of the pH 4.80 acetate buffer mentioned above for standards); and 100 µl of 1.0×10^{-2} M L-DOPA as substrate. The samples are briefly mixed by vortexing and then incubated at 37°C for 30 min. Inactivation of the enzyme is accomplished by heating the sample at 100°C, by immersion in boiling water, for 45 seconds. 100 µl of a solution containing 1.5 M HCl and 0.10 M Na_2EDTA is added to each incubation tube. The samples are then clarified by centrifugation/filtration through 0.22 µ MFl Microsample Filters (BioAnalytical Systems) at 13,000 x g and 4°C for 15 min. A 5 µl aliquot of the filtrate is finally injected into the liquid chromatograph for quantitation of the product, dopamine.

in vitro 5-Hydroxytryptophan Decarboxylase Activity

As with dopa decarboxylase, this determination initially employed the procedural conditions outlined by Sims et al. (36). These were then modified in our laboratory to provide optimal results for whole mouse brains.

The tissue homogenate is prepared by adding 500 mg of tissue to 500 µl of 8.0×10^{-4} M N_ω-methyl-5-hydroxytryptamine, the internal standard, and 4.00 ml of water. Again, the actual amount of water is adjusted for each sample according to the weight of tissue to yield a constant 10% homogenate. A standard 'homogenate' employs 500 µl of 8.0×10^{-4} M serotonin, the product of the reaction, 500 µl of the same 8.0×10^{-4} M internal standard solution, 50 µl of 75 mg/ml ascorbic acid, and 3.95 mls of an 0.1 M acetate buffer of pH 4.80. Homogenization is accomplished with either ultrasonication or a ground glass apparatus. For incubation, the following solutions are added to the individual tubes: 100 µl of a 1:1 mixture of 1.6×10^{-3} M pargyline and 5.0×10^{-3} M pyridoxal-5'-phosphate; 100 µl of 0.75 M phosphate buffer of

pH 8.40; 100 µl of 4.0 x 10^{-3} M L-5-hydroxytryptophan as substrate; and 100 µl of the homogenate. For standards, the phosphate buffer is replaced with 100 µl of 0.1 M acetate buffer of pH 4.80. After incubation at 37°C for 30 min, the reaction is quenched by immersion of the tube in boiling water for 45 sec. A 100 µl aliquot containing 1.5 M HCl and 0.1 M Na_2EDTA is added to prevent subsequent loss of the product, 5-hydroxytryptamine, through oxidation. The samples are then clarified by centrifugation/filtration, as described above for DOPA decarboxylase, and the activity quantitated by injection of a 5 µl aliquot into the liquid chromatograph.

in vitro Tryptophan Hydroxylase

This procedure was also optimized in our laboratory for whole mouse brains, although it has been shown applicable to other species, other tissues, and brain regions. Tissue homogenates are prepared by homogenizing the following mixture with a ground glass apparatus: 500 mg of tissue; 50 µl of a solution containing 12.2 µg of N_ω-methyl-5-hydroxytryptamine (internal standard) in 1 x 10^{-3} M HCl; and 3.00 mls of H_2O. The volumes, of course, should be adjusted appropriately for other tissue sizes. The incubation mixture is prepared by adding the following components to each sample: 100 µl of a 2.0 M acetate buffer of pH 8.0; 50 µl of a solution containing 5.0 mM 6-methyltetrahydropterin, 0.20 M 2-mercaptoethanol, 0.10 mM NSD-1015, and 52.5 units of catalase per µl; 50 µl of 100 mM Ca^{2+}; 50 µl of 4.0 mM L-tryptophan, the substrate; 200 µl of homogenate; and, 50 µl of 1.0 mM ascorbic acid. For standards, 50 µl of a working standard solution containing 3 µg/ml of L-5-hydroxytryptophan in 1 x 10^{-3} M ascorbic acid replaces the 50 µl of ascorbic acid in the above incubation mixture. Blanks are prepared exactly as described above for tissue samples. Both blanks and standards, however, are additionally treated with 50 µl of 60% $HClO_4$ prior to incubation. For all samples, incubation is carried out at 37°C for 25 min. For tissue samples, the reaction is terminated by the addi-

tion of 50 µl of 60% $HClO_4$. After an initial centrifugation at 27,750 x g and 4°C for 20 min, the sample is clarified by centrifugation/filtration at 13,000 x g and 4°C for 15 min. The quantitative results are obtained by injection of 5 µl aliquots into the liquid chromatograph.

in vitro Monoamine Oxidase Activity

Based upon previous work by others (37, 38), we decided to design a determination of monoamine oxidase which coupled this enzyme to aldehyde dehydrogenase. Thus, the final product would be the corresponding acid rather than the more difficult to analyze aldehyde. However, due to the existence of isozymes of monoamine oxidase (39), we also decided to optimize the determination utilizing two different substrates: dopamine and serotonin. The conditions used by Lovenberg et al. (40) were initially employed, although the final procedure obtained was optimized for whole mouse brains in our laboratory. The following description applies only to the determination employing 5-hydroxytryptamine as substrate.

Tissue homogenates are prepared by adding 500 mg of tissue to 4.00 mls of an isotonic, 0.050 M phosphate buffer of pH 10.0 and sonicating until homogeneous. Kidney homogenates, used as a source of aldehyde dehydrogenase, are prepared by adding an appropriate amount of isotonic, 0.35 M sucrose to whole female mouse kidneys to obtain 0.20 g/ml; the mixture is homogenized in a ground glass apparatus, centrifuged at 39,900 x g and 4°C for 20 min, and the resultant supernatant fraction used as the kidney homogenate.

The preincubation mixture is composed of the following components: 500 µl of 0.50 M phosphate buffer of pH 8.10; 100 µl of 17.5 mM NAD^+; 100 µl of H_2O; 100 µl of 0.578 M 2-mercaptoethanol; 100 µl of 1.33 mM 5-hydroxyindole-3-carboxylic acid, the internal standard; 250 µl of the kidney homogenate; and, 500 µl of the tissue homogenate. Following preincubation at 44°C for 20 min, the substrate is added as a 100 µl aliquot

containing 17.0 mM serotonin and the resultant mixture incubated at 44°C for 20 min. The reaction is terminated by placing a 250 µl aliquot of the incubation mixture into 750 µl of a solution containing 0.5 M acetic acid, 0.5 M sodium acetate, and 0.4 M $NaClO_4$. The resultant solution is centrifuged at 40,000 x g and 4°C for 1 hour. Sample clarification is obtained by centrifugation/filtration at 13,000 x g and 4°C for 15 min. Quantitation is afforded by injection of 5 µl aliquots of the filtrates into the liquid chromatograph. Standard samples are prepared by replacing the 500 µl of brain homogenate with 500 µl of isotonic, 0.050 M phosphate buffer (pH 10.0), replacing the 100 µl of H_2O with 100 µl of 5.58×10^{-4} M serotonin, and replacing 250 µl of the kidney homogenate with 250 µl of isotonic sucrose. Blanks are prepared by only replacing the 500 µl of brain homogenate with 500 µl of isotonic, 0.050 M phosphate buffer (pH 10.0).

in vitro Catechol-O-Methyltransferase Activity

The determination of catechol-O-methyltransferase activity is virtually identical to that described by Shoup *et al.* (41). Dopamine is used as the substrate, while 3,4-dihydroxybenzylamine serves as the internal standard. After terminating the incubation, the solution is clarified by centrifugation/filtration at 13,000 x g and 4°C for 15 min. 5 µl aliquots of the filtrate are used for quantitation by liquid chromatography.

Calculations

The calculation of a single endogenous species in a routine determination of tissue or fluid components is given as:

$$\text{nmol/g} = \frac{R_{\text{sample}}}{R_{\text{std}}} \times \frac{\text{nmol in std}}{\text{g tissue}}$$

where

R = ratio of peak height (area) of desired component to that of internal standard. The subscripts 'sample' and

'std' refer to the final, injected sample or standard mixtures, respectively.

nmol in std = the number of nmol of the desired substance contained in the final standard mixture, an aliquot of which was injected to obtain the above R_{std}.

g tissue = number of grams of tissue of the sample initially employed.

Of course the final result could be expressed in pmol/ml, or any other suitable unit, by altering the appropriate expressions in this formulation. Expressions of desired components in weight (e.g., ng/g), however, should be careful to use the amount in the standard as the free base.

The calculation of an enzymatic activity is given as:

$$\text{nmol/g/hr} = \frac{R_{sample}-R_{blank}}{R_{std}} \times \frac{\text{nmol in std}}{(\text{g tissue})(\text{time,hr.})}$$

The same definitions and comments apply here as to the simpler calculation above. Additionally,

R_{blank} = ratio of peak height (or area) of desired component to that of internal standard for a blank.

time,hr. = the time of incubation, for an in vitro determination, or the time between pretreatment and sacrifice, for an in vivo determinations.

The R_{std} expression in the denominator of this calculation should be replaced with $R_{std}-R_{blank}$ for the in vitro tryptophan hydroxylase determination.

All results in the current report are expressed as mean $\pm$ S.E.M. for at least 4 separate determinations.

RESULTS AND DISCUSSION

The determinations described may all be performed with a single liquid chromatographic apparatus, as represented by either System A or System B. The individual components, along with their common abbreviations are presented in Table 2 to aid the reader in deciphering the figures.

TABLE 2

Catecholamine and Indoleamine Related Species and Their Abbreviations

Compound (In order of elution)	Abbreviation
3,4-Dihydroxyphenylglycol	DOPEG
3-Hydroxy-4-methoxyphenylglycol	MHPG
Vanillylmandelic acid (coelutes with MHPG)	VMA
Norepinephrine	NE
3,4-Dihydroxyphenylalanine	DOPA
Epinephrine	EPI
3,4-Dihydroxybenzylamine	DHBA
3,4-Dihydroxyphenylacetic acid	DOPAC
Normetanephrine	NM
Dopamine	DA
5-Hydroxytryptophol	5-HTOL
Metanaphrine (coelutes with EPIN)	MET
Epinine	EPIN
5-Hydroxyindole-3-acetic acid	5-HIAA
5-Hydroxytryptophan	5-HTP
N-Acetyl-5-hydroxytryptamine	N-Ac-5-HT
Homovanillic acid	HVA
3-Methoxytyramine	3-MT
5-Hydroxytryptamine	5-HT
N_{ω}-Methyl-5-hydroxytryptamine	N-MET

A computer drawn chromatogram of a synthetic mixture of 18 catecholamine and indoleamine related compounds is shown in Fig. 1. As can be seen, all components are readily resolved in 4.3 and 7.0 minutes on Systems A and B, respectively.

A typical analysis of a whole rat brain yielded the resultant chromatogram shown in Fig. 2, while the results for a typical analysis of whole mouse brain are presented in Table 3. The values obtained are quite comparable to the many previous reports for such determinations. It is, however, interesting to note the

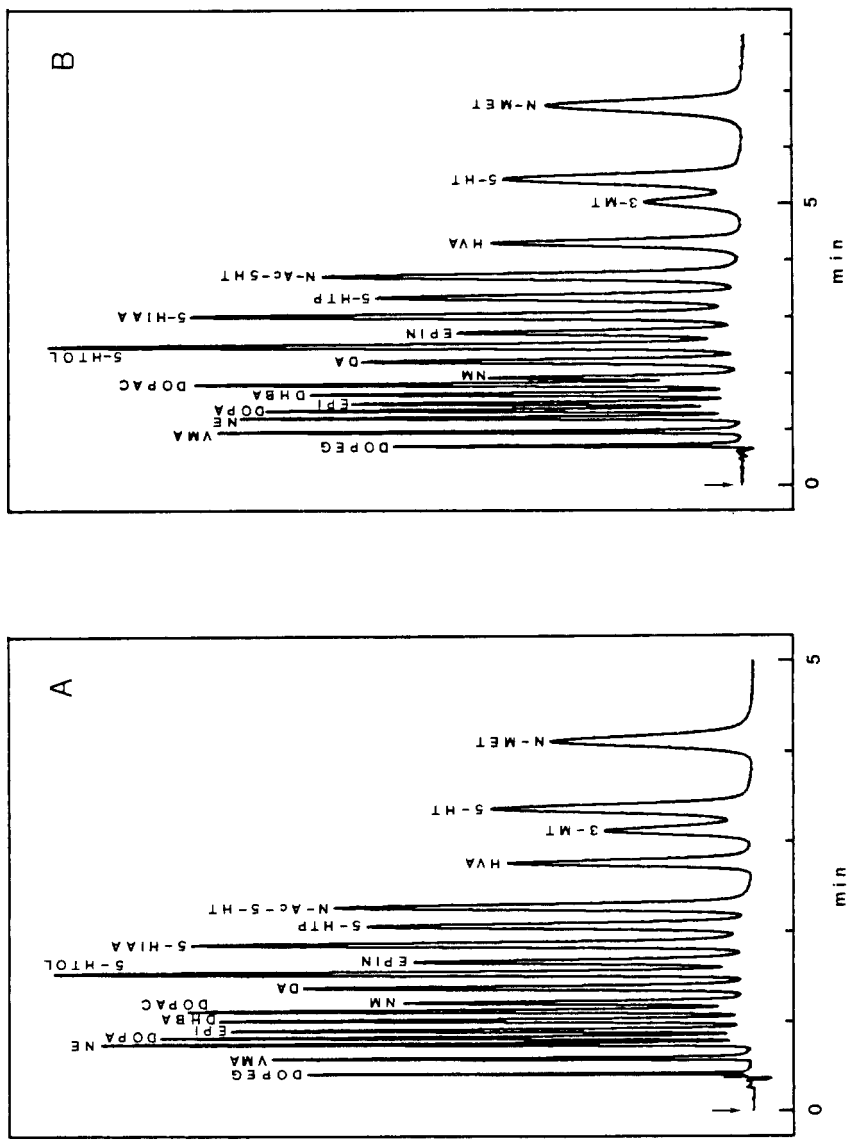


FIGURE 1. Separation of Eighteen Different Catecholamines and Indoleamine Related Compounds. The 5 µl injection contains each compound at a concentration of ca. 1 µM. System used (A or B) is indicated in the upper right of each chromatogram.

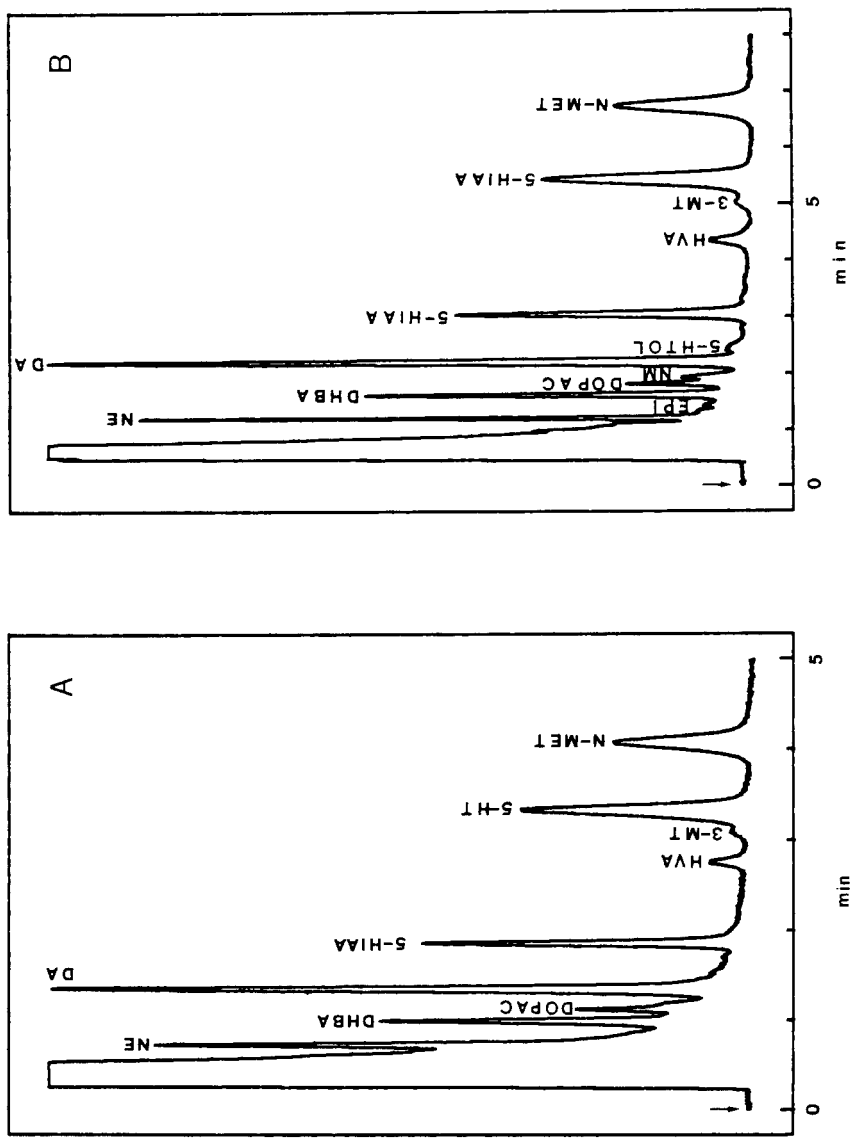


FIGURE 2. Whole Rat Brain Catecholamines, Indoleamines, and Related Metabolites.

TABLE 3

Whole Mouse Brain Content of Various Compounds*

Compound	Content (ng/g)
NE	392 ± 18
EPI	12 ± 14
DOPAC	116 ± 32
DA	1042 ± 83
5-HTOL	28 ± 6
5-HIAA	414 ± 31
HVA	263 ± 46
5-HT	745 ± 57
3-MT	238 ± 64

*Results from system B.

differences observed between the two chromatographic systems. In particular, the early eluting compounds are much more distinctly recognizable in System B. Thus, determinations of these components, especially norepinephrine, epinephrine and DOPA would appear to be better approached with System B. However, the resolution of System A could be enhanced by decreasing the acetonitrile content of its eluting solvent and, simultaneously, increasing its required time per analysis. Alternatively, one could employ an alumina separation prior to injection to greatly decrease the relative size of the solvent front in relationship to these components for either system.

Both systems cannot, unfortunately, be used with the currently described isolation procedure for the determinations of 3,4-dihydroxyphenylglycol, 3-hydroxy-4-methoxyphenylglycol, or vanillylmandelic acid. Further purification of the sample prior to injection would be necessary to avoid their obliteration by the solvent front.

The outlined procedures for the determination of enzymatic activities all rely on the same fundamental bases. The product

of the enzyme of interest is quantitated following blockade of its normal metabolism. For the *in vitro* determinations, the procedures also require incubation under proper conditions with appropriate cofactors to enhance production.

The simultaneous determination of both tyrosine and tryptophan hydroxylase *in vivo* incorporates simple blockade of both DOPA decarboxylase and 5-hydroxytryptophan decarboxylase with NSD-1015 at a specified time prior to sacrifice. As seen in Fig. 3, the resultant buildup of L-DOPA and L-5-hydroxytryptophan may then be directly used to assess the *in vivo* activities of these two enzymes. A typical determination of whole mouse brain yields values of 4.82 $\pm$ 0.56 nmol/g/hr and 3.37 $\pm$ 0.41 nmol/g/hr for tyrosine and tryptophan hydroxylase, respectively. The pretreatment time for Fig. 3, it should be noted, was only 5 minutes. Thus, the peaks observed are only *ca.* 1/6 of that which would be obtained at 30 minutes. At this short time, we again observe the difficulty in determining L-DOPA with system A. However, the remedies described above for component analyses of tissue and fluid samples would also apply to this situation.

The *in vitro* determination of tyrosine hydroxylase, not shown, yields a typical result of 108 $\pm$ 4 nmol/g/hr for whole rat brain with a signal to noise (tissue to blank) ratio of 34. This compares favorably to the radiometric results of Waymine *et al.* (120 $\pm$ 4 nmol/g/hr and 36). The *in vitro* tryptophan hydroxylase assay produced a typical value of 80.4 $\pm$ 0.7 nmol/g/hr for whole mouse brain. No comparable report of this tissue's activity by others could be found in the literature. The detection limit for this procedure, assuming a signal to noise ratio of two, is currently 1.0 pmol of 5-HTP. This is compared to the limits of 100 pmol reported for the fluorometric assay of Gal and Patterson (43) and 2.5 pmol for the radiometric assay of Kizer *et al.* (44).

The chromatographic results of an *in vitro* DOPA decarboxylase and a 5-hydroxytryptophan decarboxylase determinations are shown, respectively, in Figs. 4 and 5. For DOPA decarboxylase,

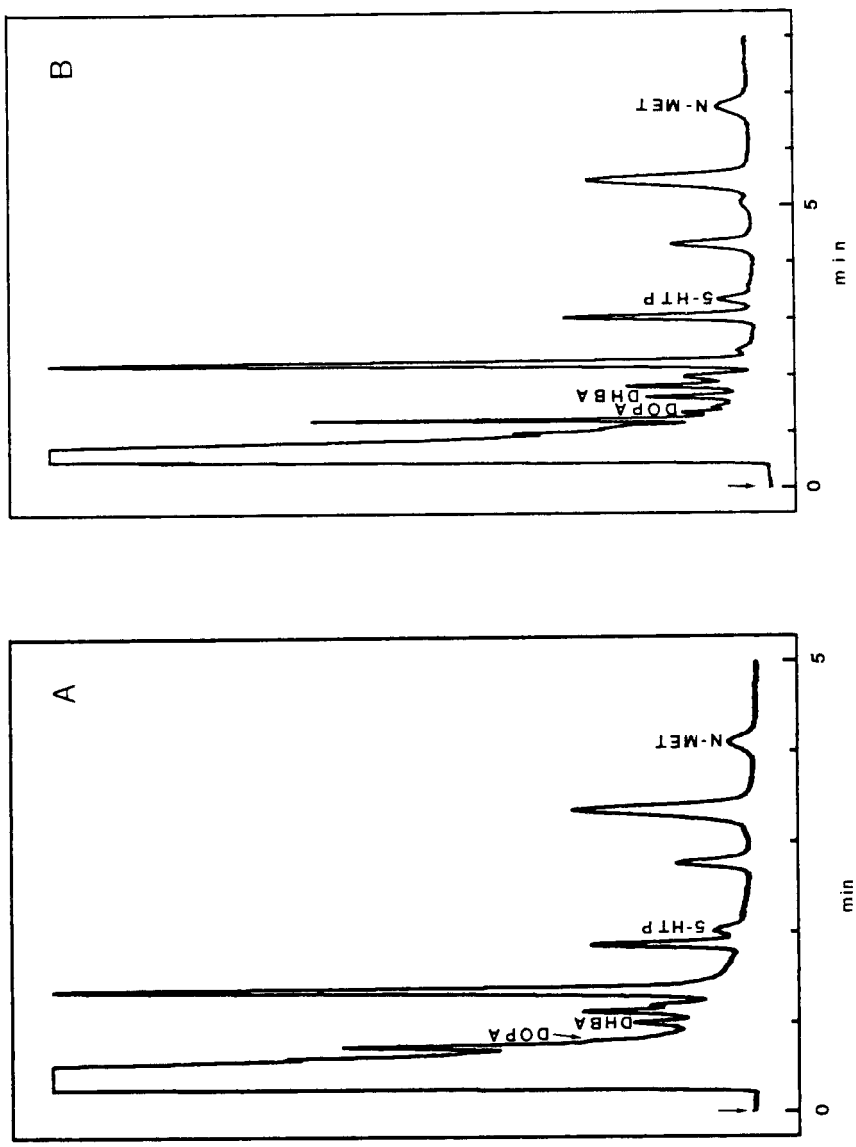


FIGURE 3. Simultaneous Determination of in vivo Tyrosine Hydroxylase and in vivo Tryptophan Hydroxylase in Whole Mouse Brain.

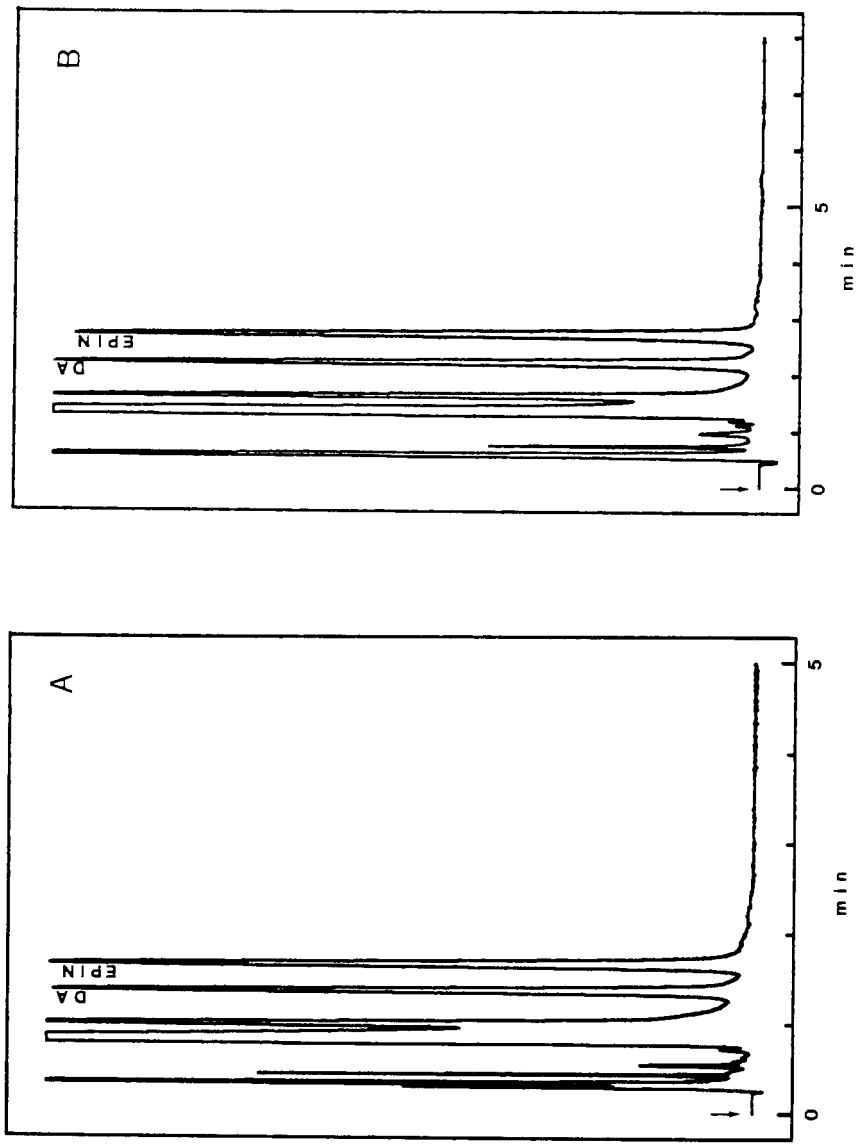


FIGURE 4. Whole Mouse Brain Determination of in vitro DOPA Decarboxylase.

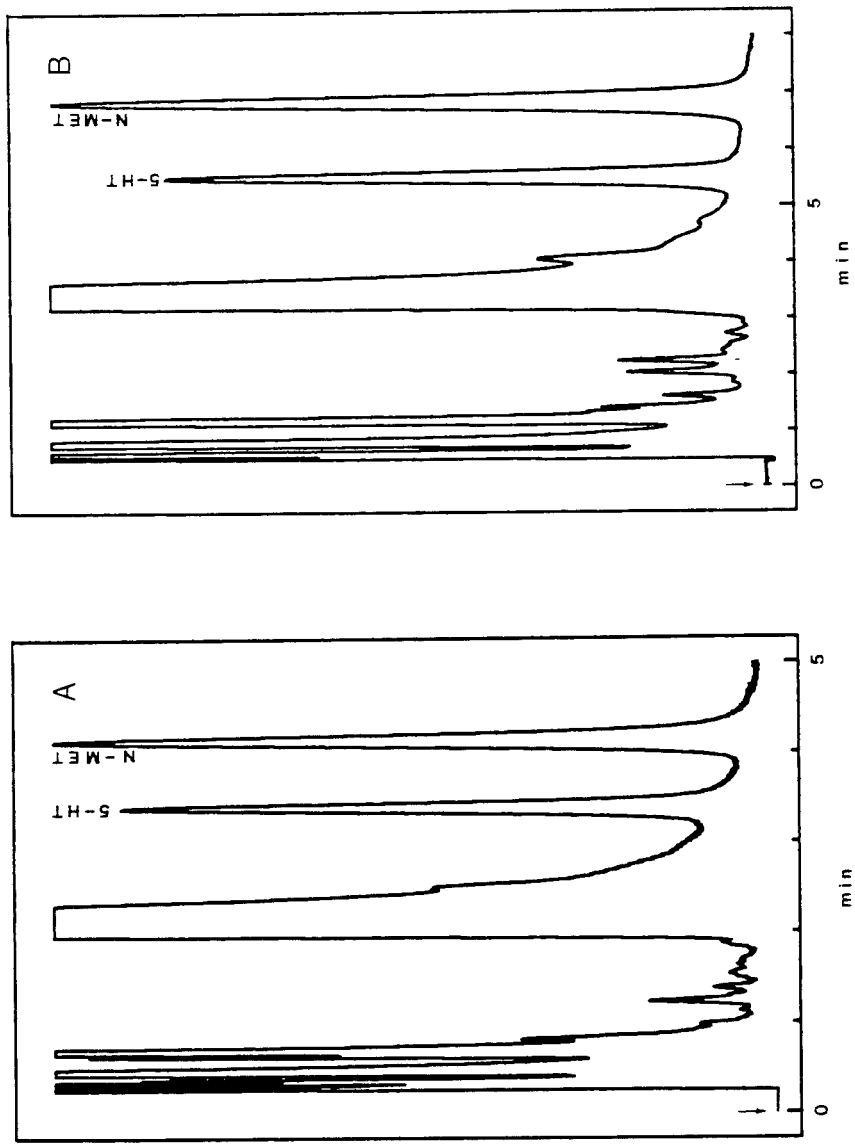


FIGURE 5. Whole Mouse Brain Determination of 5-Hydroxytryptophan Decarboxylase.

epinine has been employed as the internal standard. 3,4-Dihydroxybenzylamine, which can be seen but is not labeled, could also be employed as the internal standard. However, its peak is somewhat crowded by that for L-DOPA, the substrate. Thus, we would recommend the use of epinine. The very large amount of product formed in the DOPA decarboxylase assay, approximately 1000 times the endogenous value for dopamine, provides two advantages. First, blanks are not needed for these determinations. Secondly, the effective time required per individual sample is decreased to 1.8 minutes for System A and 3.0 minutes for System B. This considerably increases the potential throughput for these determinations. Typical results for whole mouse brain are 7.22 $\pm$ 0.58 μmol/g/hr and 0.88 $\pm$ 0.07 μmol/g/hr for DOPA decarboxylase and 5-hydroxytryptophan decarboxylase, respectively.

A typical chromatogram obtained for a sample taken from a determination of mouse whole brain monoamine oxidase activity is shown in Fig. 6. The essentially complete conversion of the intermediate, 5-hydroxyindoleacetaldehyde, to 5-hydroxyindole-3-acetic acid by excess aldehyde dehydrogenase is virtually assured by the lack of any observed broad, underlying background elevation in the chromatography (45). Whole mouse brain values obtained from a typical determination are 76.3 $\pm$ 6.4 pmol/mg/min, while regional values from seven major mouse brain regions varied between 53.8 $\pm$ 4.7 and 151.2 $\pm$ 16.6 pmol/mg/min.

A typical determination of catechol-O-methyltransferase activity in a selected portion of rat liver yielded a value of 462 $\pm$ 7 nmol/g/min for 3-methoxytyramine produced and a value of 135 $\pm$ 2 nmol/g/min for 4-methoxytyramine produced. A representative chromatogram is shown in Fig. 7.

These 3 μ systems do, indeed, exhibit a broad range of applicability to catecholamines, indoleamines, metabolites, and related biosynthetic and degradative enzymes. In addition to the determinations presented here, we feel both of these systems should be readily adaptable to the reported determinations of

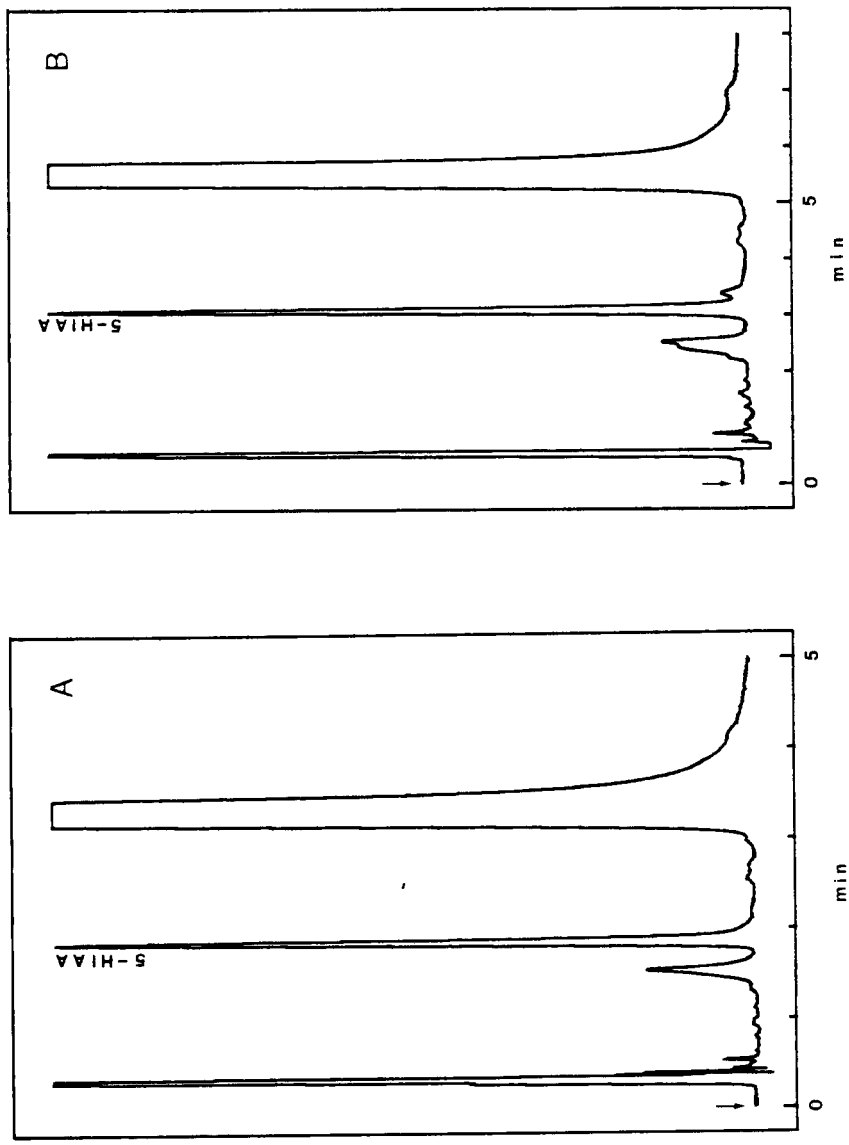


FIGURE 6. Determination of Monoamine Oxidase Activity in Whole Mouse Brain Using 5-Hydroxytryptamine as Substrate.

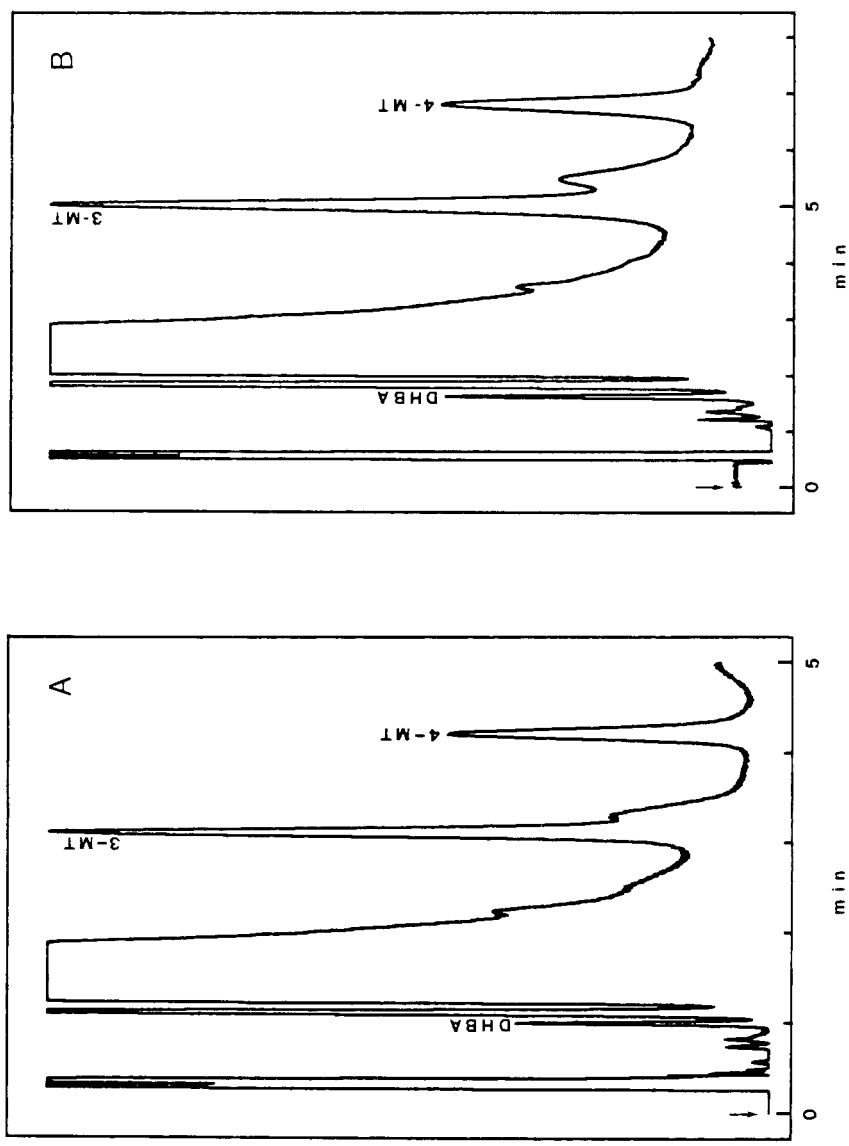


FIGURE 7. Determination of Catechol-O-methyltransferase Activity in a Selected Portion of Rat Liver.

in vitro dopamine-β-hydroxylase (14) and phenethanolamine-N-methyltransferase (15). But, there are cases where the current systems would be advantageously replaced with the older 5μ or 10μ packing materials. In particular, when the concentration of the species to be determined in the final mixture to be injected is extremely low, larger volumes must be injected to allow quantitation. The current systems will allow injection volumes of only 5 μl for early eluting components, while 20 or even 50 μl could be used for the later eluting components. But, injection volumes of 100 or 200 μl would almost certainly require the larger (5 or 10μ) packing materials. This problem could be circumvented by the use of additional purification or preconcentration steps in the procedure; the overall analysis time for the individual determination will need to be examined to obtain optimal throughput.

Three micron systems are also somewhat more 'touchy' than the 5 or 10μ systems. While this problem should diminish as the user's experience increases, more attention to details such as connecting tubing lengths, dead volumes, and damping time constants are essential at the outset.

ACKNOWLEDGMENTS

We gratefully acknowledge support of this research through grant no. NS 16887-03 from NINCDS/PHS as well as locally from the University of Oklahoma Research Council. One of us (P.Y.T.L.) recognizes the help provided through a Conoco/Apex Fellowship from the Chemistry Department at Oklahoma University, and another (L.L.) recognizes the support provided through a Graduate Professional Opportunities Program Fellowship from DHEW/PHS. The financial support provided to C.L.B. during his current sabbatical leave at the Pharmacology Department, University of Texas Health Sciences Center at San Antonio, TX, is also appreciated.

REFERENCES

1. Refshauge, C., Kissinger, P. T., Dreiling, R., Blank, L., Freeman, R., and Adams, R. N., Life Sci., 14, 311 (1974).

2. Sasa, S. and Blank, C. L., Anal. Chem., 49, 354 (1977).

3. Kissinger, P. T., Bruntlett, L. S., Davis, G. C., Felice, L. J., Riggin, R. M., and Shoup, R. E., Clin. Chem., 23, 1449 (1977).

4. Freed, C. R. and Asmus, P. A., J. Neurochem., 32, 163 (1979).

5. Mefford, I. N., J. Neurosci. Methods, 3, 207 (1981).

6. Felice, L. J. and Kissinger, P. T., Anal. Chem., 48, 794 (1976).

7. Shoup, R. E. and Kissinger, P. T., Clin. Chem., 23, 1268 (1977).

8. Wightman, R. M., Plotsky, P. M., Strope, E., Delcore, R. J., and Adams, R. N., Brain Res., 131, 345 (1977).

9. Koch, D. D. and Kissinger, P. T., J. Chromatogr. Biomed. Appl., 164, 441 (1979).

10. Mefford, I. N. and Barchas, J. D., J. Chromatogr. Biomed. Appl., 181, 187 (1980).

11. Ponzio, F. and Jonsson, G., Dev. Neurosci., 1, 80 (1979).

12. Blank, C. L. and Pike, R., Life Sci., 18, 859 (1976).

13. Nagatsu, T., Oka, K., and Kato, T., J. Chromatogr., 163, 247 (1979).

14. Kissinger, P. T., Bruntlett, C. S., Davis, G. L., Felice, L. J., Riggin, R. M., and Shoup, R. E., Clin. Chem., 23, 1449 (1977).

15. Borchardt, R. T., Vincek, W. C., and Grunewald, G. L., Anal. Biochem., 82, 149 (1977).

16. Borchardt, R. T., Hegazi, M. F., and Schowen, R. L., J. Chromatogr., 152, 255 (1978).

17. Shoup, R. E., Davis, G. C., and Kissinger, P. T., Anal. Chem., 52, 483 (1980).

18. Christensen, H. D. and Blank, C. L. in Biological/Biomedical Applications of Liquid Chromatography, ed. G. L. Hawks, Marcel Dekker, 1979, p. 163.

19. Rahman, M. K., Nagatsu, T., and Kato, T., Life Sci., 28, 485 (1981).

20. Sasa, S. and Blank, C. L., Anal. Chim. Acta, 104, 29 (1979).

21. Loullis, C. C., Felton, D. A., and Shea, P. A., Pharmacol. Biochem. Behav., 11, 89 (1979).

22. Kempf, E. and Mandel, P., Anal. Biochem., 112, 223 (1981).

23. Kilts, C. D., Breese, G. R., and Mailman, R. B., J. Chromatogr., 225, 347 (1981).

24. Ishikawa, K. and McGaugh, J. L., J. Chromatogr., 229, 35 (1982).

25. Co, C., Smith, J. E., and Lane, J. D., Pharm. Biochem. Behav., 16, 641 (1982).

26. Wagner, J., Vitali, P., Palfreyman, M. G., Zraika, M., and Huot, S., J. Neurochem., 38, 1241 (1982).

27. Mefford, I. N., Foutz, A., Noyce, N., Jurick, S. M., Handen, C., Dement, W. C., and Barchas, J. D., Brain Res., 236, 339 (1982).

28. Cooke, N. H. C., Archer, B. G., Olsen, K., and Berick, A., Anal. Chem., 54, 2277 (1982).

29. Lin, P. Y. T. and Blank, C. L., Current Separations, 5, 3 (1983).

30. Kirkland, J. J., Yau, W. W., Stoklosa, H. J., and Dilks, C. H., Jr., J. Chromatogr. Sci., 15, 303 (1977).

31. Unger, K. K., Messer, W., and Krebs, K. F., J. Chromatogr., 149 1 (1978).

32. Krull, I. S., Bratin, K., Shoup, R. E., Kissinger, P. T., and Blank, C. L., American Laboratory, 15, 57 (1983).

33. Carlsson, A. and Lindqvist, J. Neural Trans., 34, 79 (1973).

34. Carlsson, A., Davis, J. N., Kehr, W., Lindqvist, M., and Atack, C. V., Naunyn-Schmiedeberg's Arch. Pharmacol., 275, 153 (1972).

35. Christenson, J. G., Dairman, W., and Udenfriend, S., Arch. Biochem. Biophys., 141, 356 (1970).

36. Sims, K. L., Davis, G. A., and Bloom, F. E., J. Neurochem., 20, 449 (1973).

37. Erwin, V. G. and Dietrich, R. A., J. Biol. Chem., 244, 3533 (1966).

38. Weetman, D. F. and Sweetman, A. J., Anal. Biochem., 41, 517 (1971).

39. Monoamine Oxidase: Structure, Function, and Altered States, Singer, T. P., Von Kroff, R. W., and Murphy, D. L., eds., Academic Press, New York, 1979.

40. Lovenberg, W., Levine, R. J., and Sjoerdsma, A., J. Pharmacol. Exp. Ther., 135, 7 (1962).

41. Shoup, R. E., Davis, G. C., and Kissinger, P. T., Anal. Chem., 52, 483 (1980).

42. Waymire, J. C., Bjur, R., and Weiner, N., Anal. Biochem., 43, 588 (1971).

43. Gal, E. M. and Patterson, K., Anal. Biochem., 52, 625 (1973).

44. Kizer, J. S., Ziven, J. A., Saavedra, J. M., and Brownstein, M. J., J. Neurochem., 24, 779 (1975).

45. Bulawa, M. C., Ph.D. Thesis, University of Oklahoma, 1981.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(3), 539-558 (1984)

DANSYL AMINO ACIDS BEHAVIOR ON A RADIAL PAK C_{18} COLUMN. DERIVATIZATION OF GRAPE WINE MUSTS, WINES AND WINE VINEGARS.

Paloma Martín*, Carmen Polo*, M. Dolores Cabezudo* and Manuel V. Dabrio**

*Instituto de Fermentaciones Industriales, **Instituto de Química Orgánica General. (C.S.I.C.) Juan de la Cierva, 3. Madrid-6. Spain.

ABSTRACT

Investigation was carried out into the retention behaviour of dansyl amino acids with the concentration of the organic modifier, the pH and the ionic strenght of the eluting buffer. The quantitative result precision obtained using gradients of polarity and the stability of dansyl amino acids have been calculated. Finally, the dansylation conditions of grape wine musts, wines and wine vinegars are fixed.

INTRODUCTION

Dansylation has been largely used as a method for determining free amino acids, as well as protein hydrolyzing amino acids and terminal amino acids from proteins and peptides (1-4). Dansyl amino acids (Dns-aa) are highly fluorescent compounds, which can easily be obtained; thus they are very adequate for the analysis of these nitrogenized compounds.

Dns-aa have been separated using electrophoresis (5) and thin layer chromatography (1, 6, 7) but during recent years High Performance Liquid Chromatography (HPLC) has been used to separate and detect these

 0148-3919/84/0703-0539$3.50/0

compounds, with either silica as a stationary phase (8, 9) or phases linked with silica (10, 11, 12).

The reaction of amino acids with dansyl chloride in conventional pH and temperature conditions produces Dns-aa and HCl (I). At the same time a hydrolysis of the reagent is produced and dansic acid is obtained (II). For some amino acids, dansyl chloride excess reacts in turn with the Dns-aa and the reaction (III) takes place.

$$AA\text{-}NH_2 + Dns\text{-}Cl \longrightarrow AA\text{-}NH\text{-}Dns + HCl \quad (I)$$

$$H_2O + Dns\text{-}Cl \longrightarrow DNS\text{-}OH + HCl \quad (II)$$

$$AA\text{-}NH\text{-}Dns + Dns\text{-}Cl \longrightarrow Dns\text{-}NH_2 + \text{other products} \quad (III)$$

Neadle and Pollit (13) show that dansyl amide formation is an unavoidable limitation of the method, the quantity formed depending upon the amino acid concerned and the dansyl chloride excess. Therefore some reaction conditions should be found for each kind of sample, conditions in which the reaction (I) is favoured (which implies a dansyl chloride excess) and in which the reaction (III) is minimized. This excess should not be very high.

In this survey Dns-aa behaviour with regard to the buffer molarity, pH and concentration of the organic modifier has been investigated. To do that a Radial Pak C_{18} column was used.

The variability of the results obtained with this method of analysis and the stability of these derivatives have also been calculated.

In addition, the dansylation conditions of grape wine must, wine and wine vinegar amino acids have been determined so that the reagent quantity should be sufficient but not excessive.

In fact, it is interesting to know the amino acid contents for these kind of samples, because amino acids

are yeast and bacteria nutrients and due to they are flavour precursors as well.

EXPERIMENTAL

Instrumentation

All separations were performed on a Waters Associates instrument with two 6000 A pumps, a 660 solvent flow programmer, radial compression module RCM-100 and a U6K injector. Fluorescence was detected using a fluorometer 420AC with standard flow-cell and standard filters: excitation filter 340 $\pm$ 6 nm; emission filter 425 nm (long pass). Reversed phase column (10 cm x 8 mm ID), Radial Pak C_{18} (10µm). Bondapak C-18/Corasil (37-50 µm) guardcolumn.

Chemicals and Buffers

Methanol was HPLC grade from Scharlau. All buffers were prepared from analytical grade chemicals and Milli-Q (Millipore Corp. Bedford, MA) water. Before use, all buffers were filtered using a Millipore Type HA filter with a pore diameter of 0.45 µm, and degassed.

Dns- amino acid standards and amino acids were obtained from Sigma (St. Louis, Mo. USA) and Dns-Cl from Fluka.

Methods

Dns-derivatization was carried out under conditions similar to those used by Tapuhi et al (14). The reactant solution consisted of Dns-Cl disolved in acetone (1.5 mg/ml, 5.56 mM). Dansyl derivatization of a standard mixture of amino acids was carried out with Dns-Cl 5-10 fold higher in concentration than amino acids and 40 mM lithium carbonate buffer, pH 9.5, during 1 hour in the dark, at room temperature.

RESULTS AND DISCUSSION

Methanol Concentration Influence upon Retention

In order to study the influence of the methanol concentration on retention and therefore the Dns-aa separation, a series of tests has been carried out using respectively 20%, 40% and 60% (w/w) of methanol. Phosphate buffer concentration (0.03 M) and pH 6.9 were constant.

In most cases of separations in HPLC reversed phase, the capacity factor (k') logarithm of solutes decreases linearly when the organic modifier percentage increases in the mobile phase. Karger et al. (15) have verified this linear relationships of log. k' with methanol percentage for two solutes n-hexane and octanol. However we have observed that this not happen in the case of Dns-aa, at least in the conditions we have used (Fig. 1).

This fact was observed by Hearn and Grego (16) for polypeptides and acetonitrile as organic modifier. Bij et al. (17) who observed it for very polar compounds, interpreted this as something due to interaction between the solute and silanol groups which are not blocked during the union between the stationary phase and the support.

As Fig. 1 shows, a total Dns-aa separation in isocratic conditions cannot be carried out as low methanol concentration should be used. This would imply very long time analysis. For instance, using 20% methanol, proline would be eluted after 50 minutes. The remaining amino acids of which polarities are lower than proline (among which there are leucine and isoleucine) would be retained in the column and their elution could even last several days. On the other

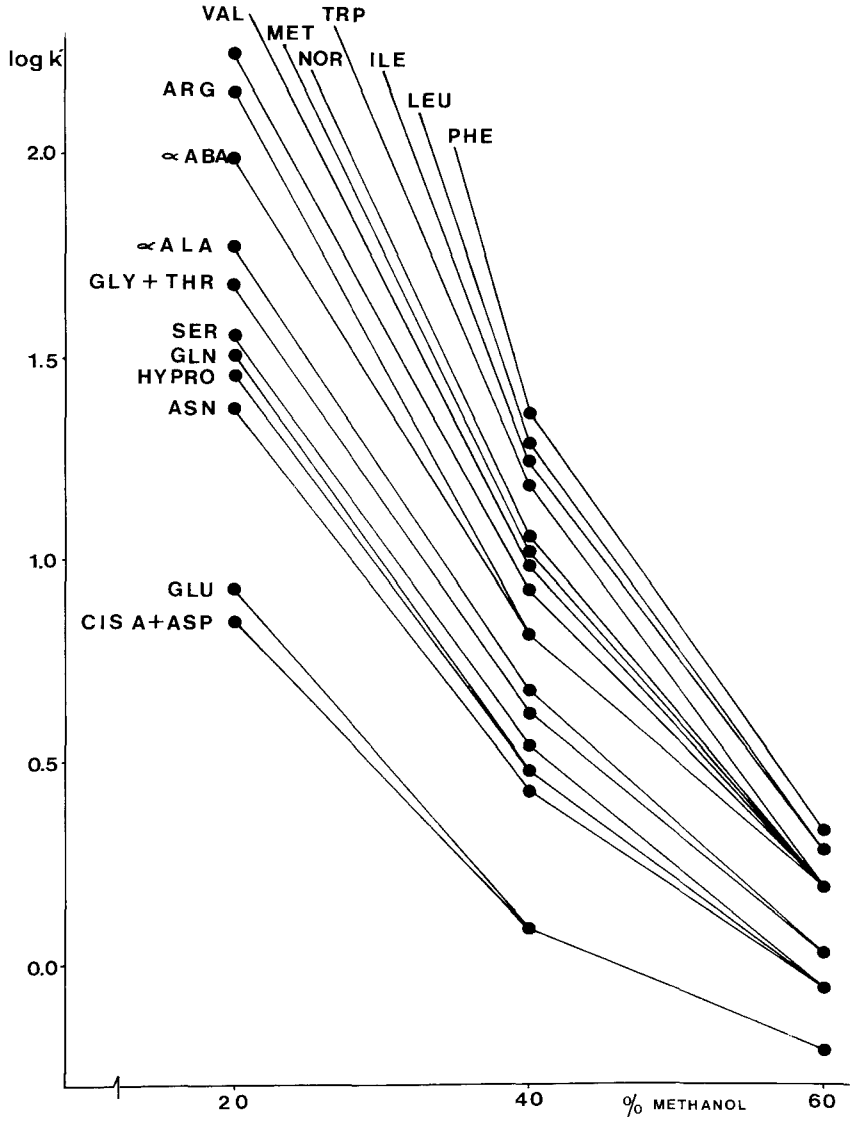


FIGURE 1. Plots of the log k' of dansyl amino acids againts concentration of methanol. Isocratic elution, mobile phase: 0.03 M sodium phosphate buffer, pH 6.9.

hand, some amino acids are not resolved at concentration higher than 20%. However, if 40% methanol concentration is used the most of the amino acids can be eluted in a reasonable time and with a fear good separation. For subsequent tests.

Molarity and pH Influence upon Retention

The effect of the ionic eluent concentration is interesting as related to the ionic exclusion phenomenon due to the carboxilate group. An interaction exists between the carboxilate groups of the dansyl aminoacids and the sodium ions presents, the concentration of which increases as the phosphate molarity does.

In order to prove this point, several tests were carried out with constant methanol (40%) and pH (6.9) values; phosphate concentration being changed: 0.01, 0.03 and 0.05M. These low molarities have been chosen in order to avoid precipitations in the analytical system.

Fig.2 shows the increase in the retention of the most of the Dns-aa used when the phosphate buffer concentration increases. Dns-arginine is an exception, probably due to the existence of a highly polar guanidinic group in its lateral chain. As much as phosphate buffer concentration increases, more sodium ions are present, being predominant the guandidinic group effect.Therefore, the retention decreases because of the polarity increases as it was suggested by Hill et al. (18) on the basis of amino acid ortho--phthaldialdehide/ethanethiol derivatives. The guanidinic group polarity is inversed to the carboxilate one and in certain conditions, these opposed polarities could be compensated up to a certain point.

The pH effect in the separation was studied for a 5.9 - 7.9 interval. Within these limits, the most of

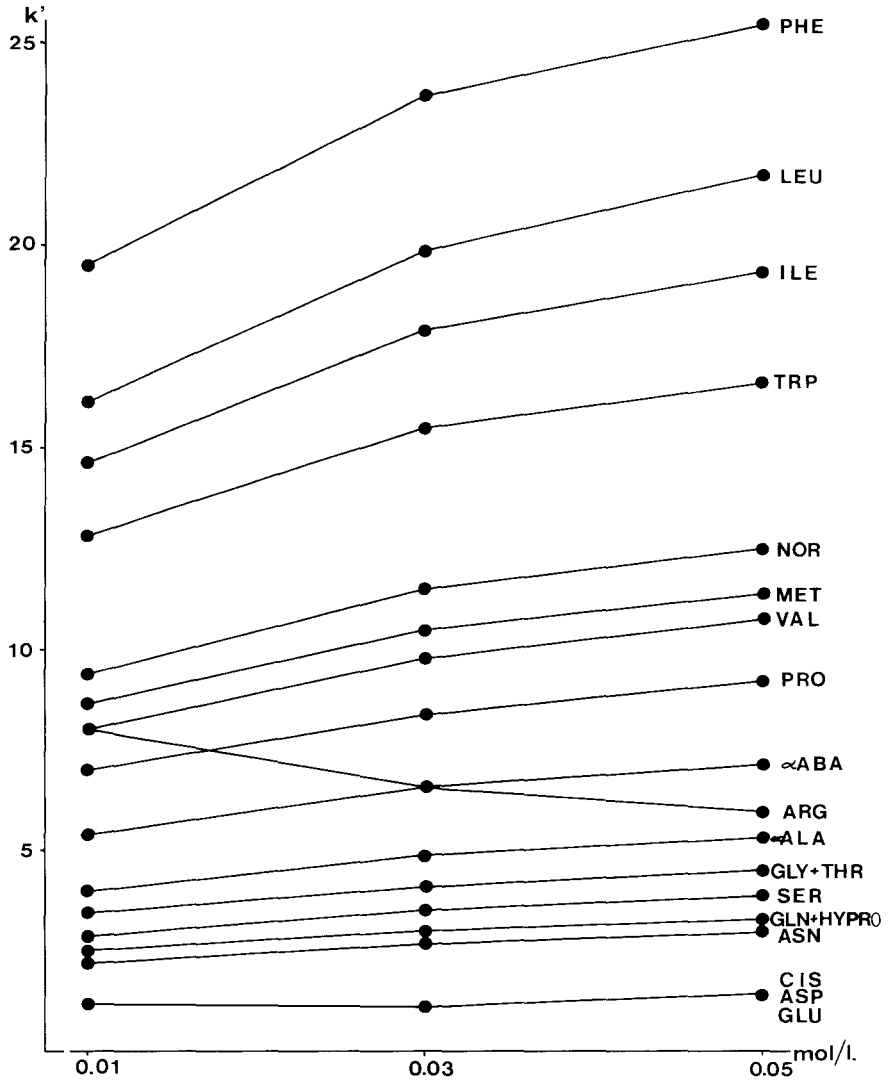


FIGURE 2. Plots of the log k' of dansyl amino acids againts phosphate buffer concentration. I Isocratic elution: methanol - sodium phosphate buffer pH 6.9 (40-60 w/w).

the carboxilic groups of the Dns-aa are ionized. The Dns-arginine presents the nitrogen of the guanidinic group protoned and a zwitterion is formed; this is why its net charge is null in these tests.

Fig. 3 shows a retention increase as pH decreases. The k' increase when pH decreases may be due to an ionic suppression phenomenon. According to Lindroth and Mopper (19), when pH decrease Dns-aa carboxilate protonization is favoured and its hydrophobicity increases; therefore retention is favoured in the apolar stationary phase.

Fig. 4 shows the chromatogram obtained in the conditions of the central point of these tests: Methanol 40%, phosphate buffer 0.03 M, pH 6.9. Although several double peaks appear most of the amino acids are resolved.

Quantitative Dansyl Amino Acids Analysis. Derivatives Stability

A synthetic solution of the dansyl amino acids in which they are in similar proportions to those in musts, wines and vinegars (20 - 22) has been chromatographied.

Owing to the fact that Dns-aa cannot be separated in isocratic conditions, from now on, gradient of polarities described by Martin et al. (23) have been used.

Eluent A: 15% (w/w) methanol/phosphate buffer 0.01 M, pH 6.3.

Eluent B: 35% (w/w) methanol/phosphate buffer 0.01 M, pH 6.3.

Linear gradient from 0 to 100 B in 30 minutes.

Flow: 2 ml/min.

Fig. 5 shows a standard solution chromatogram obtained in these conditions.

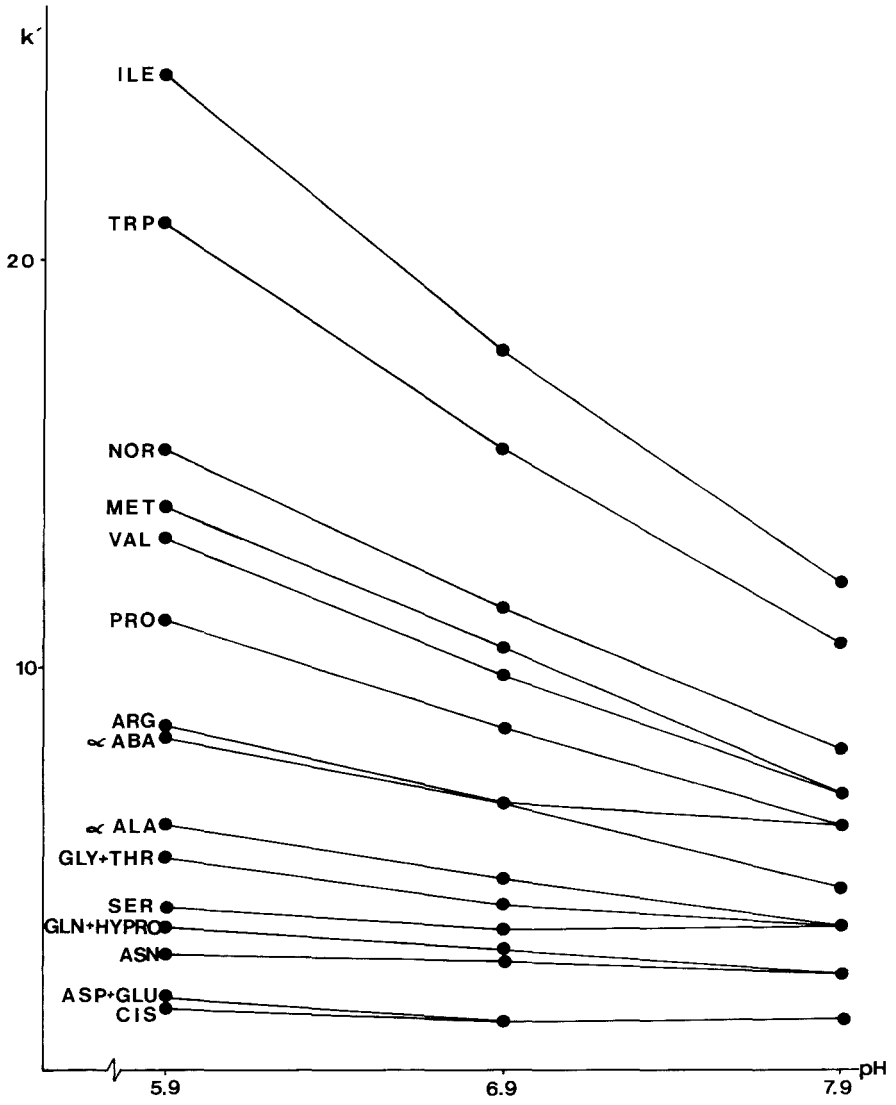


FIGURE 3. Plots of the log k' of dansyl amino acids againts pH. Isocratic elution: methanol - 0.03 M sodium phosphate buffer.

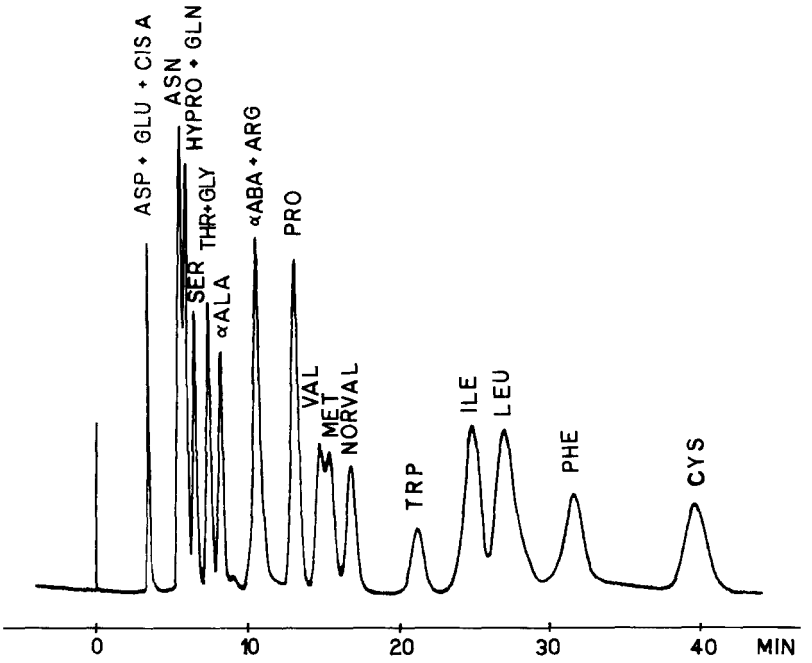


FIGURE 4. Chromatogram of a synthetic solution of Dns-amino acids. Isocratic elution, mobile phase: methanol-0.03 M sodium phosphate buffer pH 6.9 (40-60 w/w).

Table 1 sums up the results obtained from eight replicated analysis of the standard solution. Variation coefficients are from 0.9% for threonine + glycine to 4.5% for aspartic acid. It is observed that the highest values (4.5, 4.1 and 4.0) correspond to the amino acids in lower proportions (aspartic acid, serine and valine). In addition, the valine and the aspartic acid are peaks which are not chromatographically well resolved.

De Jong et al. (24) do not observe loss in the Dns- amino acid when the samples are analyzed within a reasonable time (1 day) after Dns-derivatization while remaining somewhat protected from direct light. We have

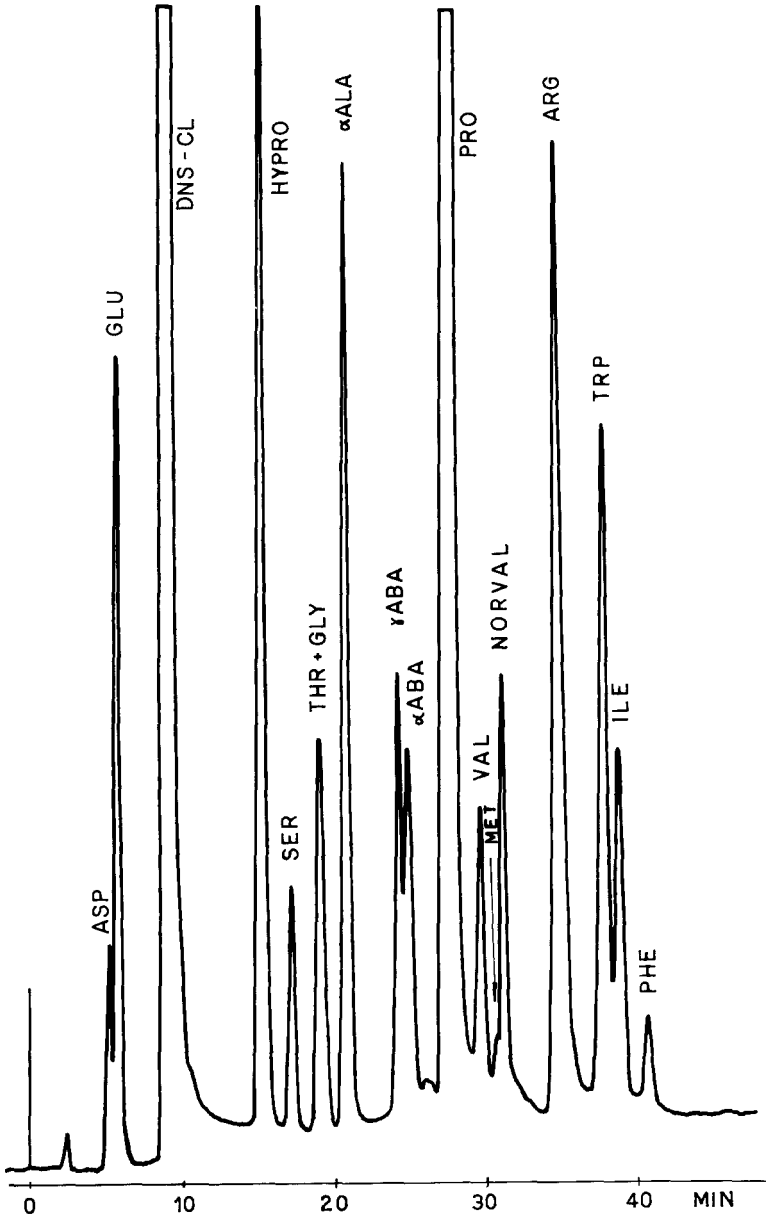


FIGURE 5. Chromatogram of a synthetic solution of Dns-amino acids. Amino acids concentration as stated in Table 1. Gradient elution, mobile phase: A = Methanol-0.01 M sodium phosphate buffer pH 6.3 (15-85 w/w); B = methanol- 0.01 M sodium phosphate buffer pH 6.3 (35-65 w/w), 30 min., flow rate 2 ml/min.

TABLE 1

Variability of the Data Obtained from 8 Replicates of a Test Solution. (Chromatographic Conditions as in Fig.5)

Amino acid	ng	$\overline{Ai/As}$	$s_{Ai/As}$	v
Aspartic acid	400	0.460	0.021	4.5
Glutamic acid	950	0.948	0.019	2.0
Hydroxyproline	300	0.336	0.008	2.4
Serine	174	0.243	0.010	4.1
Threonine+Glycine	301	0.756	0.007	0.9
alfa alanine	592	1.809	0.035	1.9
gamma aminobutyric acid	252	0.959	0.014	1.5
alfa aminobutyric acid	274	0.777	0.014	1.8
Valine	93	0.224	0.009	4.0
Norvaline (IS)	960	1	-	-
Arginine	1210	2.403	0.027	1.1
Tryptophane	400	0.425	0.010	2.4
Isoleucine	50	0.130	0.004	3.2
Phenylalanine	199	0.459	0.013	2.8

	$\overline{As}$	s_{AS}	v
Norvaline	72729380	2849162	3.9

$\overline{Ai/As}$ = mean of peak area / internal standard peak area distribution.

$s_{Ai/As}$ = standard deviation of the Ai/As distribution.

v = coefficient of variation.

$\overline{As}$ = mean of the internal standard peak areas (counts).

s_{As} = standard deviation of the As.

observed that Dns-aa are still stable for at least seven days if the are kept at -4ºC., as may be deduced from Table 2, where the dispersion of the results is within the values obtained for the variability coefficients of the analysis method, as shown in Table 1

TABLE 2

Dansyl Amino Acids Stability in Course of Time

Amino Acid	$\bar{X}$ (ng)	s	v
Aspartic acid	454	11	2.4
Glutamic acid	430	8	1.9
Serine	483	17	3.5
Threonine+Glycine	568	5	0.9
alfa Alanine	345	6	1.7
Proline	365	13	3.6
Valine	420	12	2.9
Methionine	640	34	5.3
Arginine	628	5	0.8
Phenylalanine	740	11	1.5

$\bar{X}$ = mean of the values obtained from the chromatograms of a standard solution stored up 7 days. Each x_i is the mean of two replicates.

s = standard deviation.

v = coefficients of variation.

Must, Wines and Wine Vinegars Dansylation.

For a fixed quantity of sample, dansyl chloride amount has been progressively increased until has been checked that it did not imply an increase in the area of the corresponding peaks. Results show that the amount of dansyl chloride required is at least eight times higher than the amino nitrogen content of the sample. Considering that there is no reliable analytical method for determining amino nitrogen in musts, wines and vinegars (in the usual methods, proline nitrogen is not evaluated), an approach has been taken into account.

There is a quite satisfactory correlation between total nitrogen and amino nitrogen in must as well as in wine samples. These correlation exists for many grape varieties and it is independent of the maturity grade of the grapes (25). Thus, it is possible to estimate the

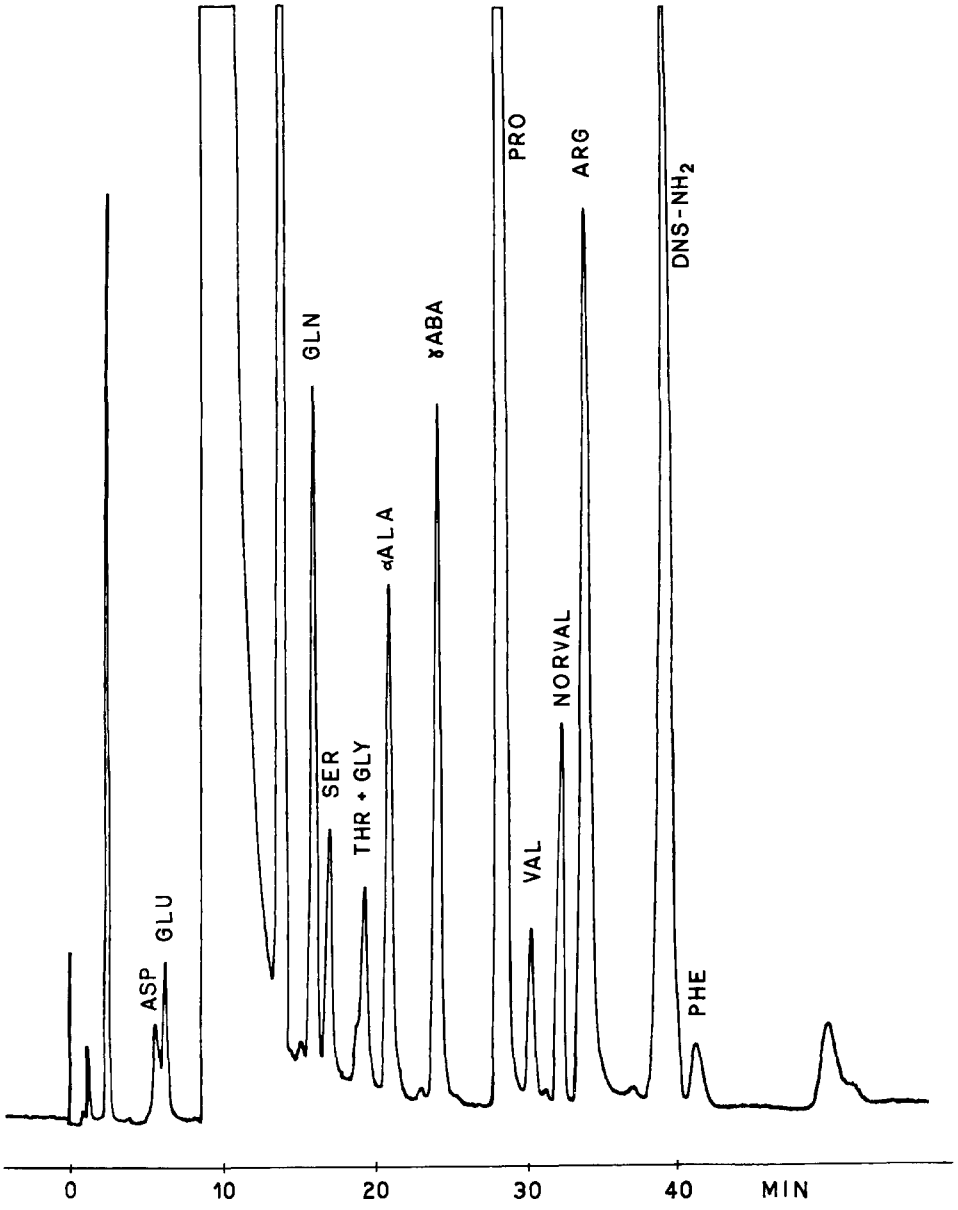


FIGURE 6. Grape must chromatogram after Dns-derivatization. Separation procedure as stated in Fig. 5.

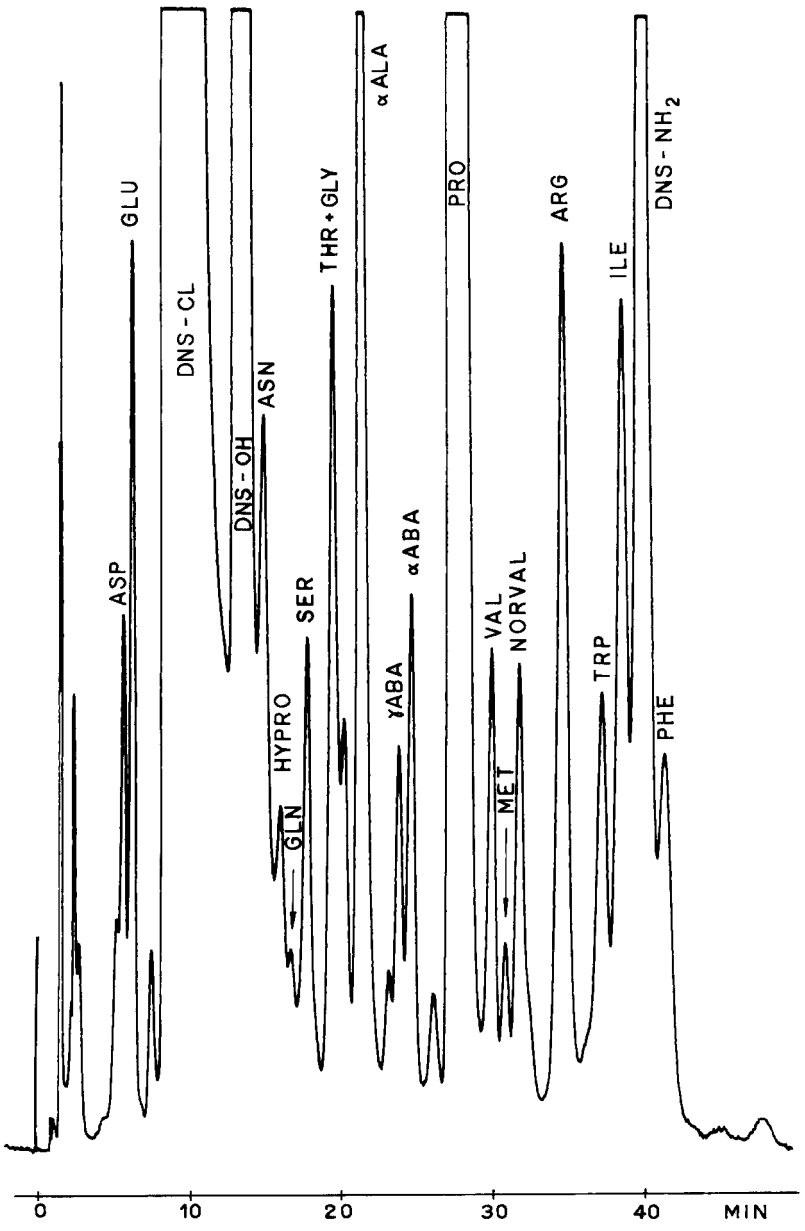


FIGURE 7. Wine chromatogram after Dns-derivatization. Separation as stated in Fig. 5.

amino nitrogen amount of unknown samples on the basis of such a correlation.

The optimun dansylation conditions deduced from our own experience are summed up as follows.

For must samples: The must is raised to a pH of around 9 and a aliquot containing approximately 25 µg total nitrogen is taken. 1 ml dansyl chloride 5.56 mM (1.5 mg in 10 ml acetone without water) and 1 ml lithium carbonate buffer 40 mM, pH 9.5 are added. The reaction is allowed to take place for 1 hour in the dark at room temperature and the internal standard (25 µ litres Dns-norvaline 0.1 mg/ml) is added. It is concentred to dryness at room temperature and is dissolved again in 0.3 ml methanol. It is filtered through a Millipore Type FH Filter with a pore diameter of 0.50 µm before injection.

For wine and vinegars samples: It is raised to a pH of around 9, an aliquot containing approximately 60 µg tctal nitrogen is taken and 2.7 ml dansyl chloride and 2.7 ml buffer are added. It is continued as in the case of musts.

The injected quantities correspond to approximately 4 µg total nitrogen for musts and 10 µg total nitrogen for wines and vinegars (fig. 6 and 7).

CONCLUSIONS

The decrease of Dns-amino acids k' log with the increase of the solvent polarity is not linear, as is the case of most solutes and solvents. When buffer molarity increases, Dns-amino acids retention increases except for arginine, wich does not behave in the same way as the remaining Dns-amino acids. On the other hand, pH increase within a 5.9 - 7.9 interval implies a higher Dns-amino acid retention.

The quantitative results obtained and the Dns-amino acids stability are satisfactory.

Dansyl derivatives of the amino acids from musts, wines and vinegars are suitable ones for the analysis of these samples.

The amount of dansyl chloride required is at least eight times higher than the amino nitrogen content of the sample. In practice, 1 ml dansyl chloride 5.56 mM should be added to the must sample amount equivalent to 25 µg total nitrogen. To carry out wine and vinegar analysis, the use of a higher initial sample amount is suggested.

AKNOWLEDGEMENT

This research was supported (in part) by a grant from the Spanish Vinegar Industries Research Association. We are grateful to Waters Associates by his technical support.

REFERENCES

1. Gross, C. and Labousse, B., Study of the dansylation reaction of amino acids peptides and proteins, Eur. J. Biochem., 7, 463, 1969.

2. Seiler, N., Methods of Biochemical Analysis, Vol. 18, D. Glick, ed., John Wiley & Sons, New York, 1970, p. 259.

3. Zanetta, J.P., Vincendon, G., Mandel, P., and Gombos, G., The utilisation of 1-dimethylaminonaphtalene 5-sulphonyl chloride for quantitative determination of free amino acids and partial analysis of primary structure of proteins, J. Chromatogr., 51, 441, 1970.

4. Weiner, S. and Tishbee, A., Separation of Dns-amino acids using reversed-phase high-performance liquid chromatography: a sensitive method for determining N-termini of peptides and proteins, J. Chromatogr. 213, 501, 1981.

5. Gray, W.R., Methods in Enzymology Vol. 11, C.H.W. Hirs and S.N. Timasheff, eds., Academic Press, New York and London 1967, p. 139.

6. Spivak, V.A., Scherbukhin, V.V., Orlov, V.M. and Varshavsky, JA.M., Quantitative ultramicroanalysis of amino acids in the form of their Dns-derivatives. II. On the use of the dansylation reaction for quantitative estimation of amino acids, Anal. Biochem., 39, 271, 1971.

7. Biou,D., Queyrel, M., Visseaux, M.N., Collignon, I. and Pays, M., Separation and identification of dansylated human serum and urinary amino acids by two-dimensional thin-layer chromatography, Aplication to aminoacidpathies, J. Chromatogr., 226, 477, 1981.

8. Bayer, E., Grom, E., Kalteneggar, B. and Uhmann, R., Separation of amino acids by high performance liquid chromatography, Anal. Chem., 48, 1106, 1976.

9. Hsu, K. and Currie, B.L., High performance liquid chromatography of Dns-amino acids and application to peptide hydrolysates, J. Chromatogr., 166, 555, 1978.

10. Wilkinson, J.M., The separation of dansyl amino acids by reversed phase high performance liquid chromatography, J. Chromatogr., Sci., 16, 547, 1978.

11. Hogan, D.L., Kraemer, K.L. and Isenberg, J.I., The use of high-performance liquid chromatography for quantitation of plasma amino acids in man, Anal. Biochem., 127, 17, 1982.

12. Wiedmeier, V.T., Porterfield, S.P. and Hendrich, C.E., Quantitation of Dns-amino acids from body tissues and fluids using high-performance liquid chromatography, J.Chromatogr., 231, 410, 1982.

13. Neadle, D.J. and Pollit, R.J., The formation of 1-dimethylaminonaphtalene-5-sulphonamide during the preparation of 1-dimethylamino naphtalene-5-sulphonyl amino acids, Biochem., J., 97, 607, 1965.

14. Tapuhi, Y., Schimidt, D.E., Lindner, W. and Karger, B.L., Dansilation of amino acids for high performance liquid chromatography analysis, Anal. Biochem., 115, 123, 1981.

15. Karger, B.L., Gant, J.R., Hartkoff, A. and Weiner, P.H., Hidrophobic effects in reversed-phase liquid chromatography, J. Chromatogr., 128, 65, 1976.

16. Hearn, M.T.W. and Grego, B., High-performance liquid chromatography of amino acids, peptides and

proteins. XL. Further studies on the role of the organic modifier in the revesed-phase high-performance liquid chromatography of polypeptides. Implications for gradient optimisation, J. Chromatogr., 255, 125, 1983.

17. Bij, K.E., Horvath, Cs., Melander, W.R. and Nahum, A., Surface silanols in silica-bonded hydrocarbonaceaus stationary phases. II Irregular retention behaviour and effect of silanol masking. J. Chromatogr., 203, 65, 1981.

18. Hill, D.W., Walter, F.H., Wilson, T.D. and Stuart, J.D., High performance liquid chromatographic determination of amino acids in the picomole range, Anal. Chem., 51, 1338, 1979.

19. Lindroth, P. and Mopper, K., High performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with o.phthaldialdehyde, Anal Chem. 51, 1667, 1979.

20. Polo, C. and Llaguno, C., Evolution des acides aminés libres dans le moût de raisin sous l'action des levures de fleur. I.- Etúde qualitative et quantitative des acides aminés libres des moûts de raisin, Conn. Vigne Vin, 8, 81, 1974.

21. Polo, C. and Llaguno, C., Evolution des acides aminés libres au cours de la fermentation alcoolique des jus de raisin par les levures de fleur. II.- Composition des vins et des cellules de levures, Conn. Vigne et Vin, 8, 321, 1974.

22. Polo, C., Suárez, M.A. and Llaguno, C., Aportación al estudio de los vinagres españoles. I.- Contenido en aminoácidos libres y nitrógeno total, Rev. Agroquim. Tecnol. Aliment., 16, 257, 1976.

23. Martin, P., Suárez, A., Polo, C., Cabezudo, D. and Dabrio, M.V., Análisis de 19 aminoácidos por HPLC, Anal. Bromatol., 32, 289, 1980.

24. De Jong, C., Hudghes, G.J., van Wieringen, E. and Wison, K.J., Amino acid analysis by high-performance liquid chromatography. An evaluation of the usefulness of pre-column Dns derivatization, J. Chromatogr. 241, 345, 1982.

25. Polo, C., Herraiz, M. and Cabezudo, M.D., A study on nitrogen fertilization and fruit maturity as an

approach for obtaining the analytical profiles of wines and wine grapes. 3th. International Flavour Conference. Corfu. Greece. July 27 - 30, 1983.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(3), 559-568 (1984)

THE USE OF LARGE VOLUME INJECTIONS FOR THE ISOCRATIC SEPARATION OF PHENYLTHIOHYDANTOIN AMINO ACIDS BY MICROBORE LIQUID CHROMATOGRAPHY

M.R. Silver, T.D. Trosper, M.R. Gould, J.E. Dickinson and G.A. Desotelle

EM SCIENCE
A Division of EM Industries, Inc.
480 Democrat Road
Gibbstown, New Jersey 08027

ABSTRACT

The use of microbore columns in high performance liquid chromatography (HPLC) has remained limited primarily due to difficulties in adapting conventional HPLC analysis to the smaller volumes and lower flow rates of microbore chromatography while maintaining high quality chromatographic results.

Although conventional HPLC techniques are the method of choice for PTH-amino acid analysis, these techniques are rapidly approaching their useful limits in the field of microsequencing. A suitable method for the isocratic room temperature separation of PTH-amino acids employing microbore liquid chromatography is discussed herein. As described, the system employed for this type of analysis makes it readily adaptable to the field of protein sequencing. A comparison of a large volume mobile phase injection and a large volume "non-eluting" solvent injection with microbore columns is also presented.

INTRODUCTION

The general applicability of microbore columns in liquid chromatography is by no means unique and has, in fact, been discussed quite extensively be several investigators (1-5). More specifically, the use of stainless steel microbore columns for the separation and identification of phenylthiohydantoin (PTH) amino

 0148-3919/84/0703-0559$3.50/0

acids has been discussed to some extent as early as 1980 (5). However, in the last several years, numerous technological advances related to microbore HPLC allow for refinements of this specific application.

The advantages of microbore columns, relative to their classical analytical counterparts, include extremely high efficiencies, low solvent consumption under normal operating conditions, and the capability of obtaining high linear velocities at very low flow rates. Moreover, the most distinct advantage of microbore columns is the increase in relative mass sensitivity which can be achieved when sample concentrations are limited. This increase in relative mass sensitivity has been examined and discussed in detail by Scott and Kucera (3).

In the past, the use of microbore columns for routine analysis was somewhat limited. This was due to the small sample injection volumes required (0.5 µl to 5.0 µl) and a lack of readily available instrumentation which did not require expensive modification. This latter point has been gradually rectified with the introduction of commercially available microbore chromatographic systems.

As with any attempt to develop an analytical methodology which has a direct application for routine use in the laboratory, careful consideration must be given to both the advantages and disadvantages of the system employed. This becomes quite apparent when attempting to adapt an analysis system from conventional high performance liquid chromatography to microbore liquid chromatography.

Although modern HPLC techniques have met with considerable success for the analysis of PTH-amino acids, These conventional methods are rapidly approaching their useful limits as research trends advance towards sequencing "microquantities" of protein, i.e. less than 0.5 nanomoles. However, the use of microbore columns with their inherent increase in relative mass sensitivity will extend the usefulness of liquid chromatography into the field of microsequencing; provided that relatively simple analysis systems can be devised.

The basic procedure for the identification of PTH-amino acids is predicated on the comparison of the retention times of the sample PTH-amino acid and the same PTH-amino acid in a standard mixture. Therefore, any microbore chromatographic procedure employed for this type of analysis must provide sufficient resolution of all standard components to make sample identification reliable. Furthermore, analysis time must

be of sufficient length (i.e. thirty minutes or less) in order for the identification process to handle the volume of samples generated by automated sequencing equipment.

Finally, sample working volumes must be large enough (i.e. 50-100 microliters) to obtain complete and reproducible dissolution of the sequencing fractions. This final condition dictates the use of larger injection volumes than are typically used with microbore columns in order to obtain an accurate representation of the sample PTH-amino acid at a reasonable detector attenuation. Thus, the size of the injection volume can be a difficult problem to overcome.

The use of large volume injections with microbore columns for the isocratic separation of twenty PTH-amino acids at room temperature is reported here. A comparison is presented between the use of a 20 µl mobile phase injection and a 20 µl sample volume injection employing a "non-eluting" solvent technique similar to that described by Broquaire and Guinebault (6).

EXPERIMENTAL

Equipment, Solvents and Standards

The microbore chromatographic system used for this analysis was composed of a MACS^{tm} 100 single piston pump, a MACS 700 variable wavelength U.V. detector equipped with 0.5 µl flow cells (1.0 mm pathlength), obtained from EM SCIENCE a Division of EM Industries, Inc., A Kipp and Zonen model BD 40 chart recorder, A Hewlitt Packard 3390A integrator, a Rheodyne model 7125 manual injection valve (volume of sample loop 20 µl).

The microbore column used in this study was 500 mm in length with a 1.0 mm internal diameter. This particular column was packed in the laboratory with LiChrosorb RP-18, 7 micron sorbent (E. Merck, Darmstadt, West Germany). Column efficiency, under optimal conditions determined as plates per meter (P/M) was calculated from the following formula;

$$N = 5.54 \left(\frac{t_r}{t_{w0.5h}} \right)^2$$

where N plates per column, t_r is the retention time in seconds of ethylbenzene (k' = 1.52), and $t_{w0.5h}$ is the width at half height of

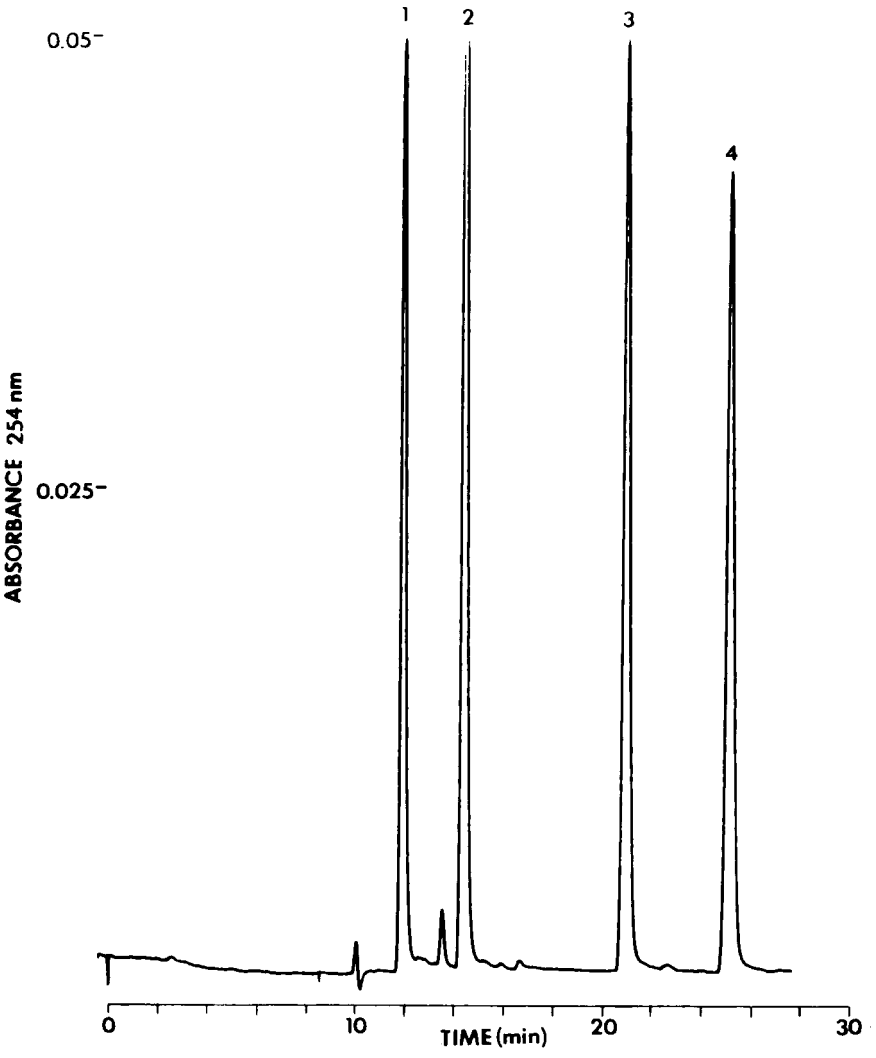


FIGURE 1 Separation of a standard mixture of phenol, ethylphenol, toluene and ethylbenzene.

ethylbenzene expressed in seconds. All values are based on the separation of a standard mixture (Figure 1) of phenol (1.0 g/l, peak #1) ehtylphenol (2.0 g/l, peak #2) toluene (4.0 g/l, peak #3) and ethylbenzene (4.0 g/l, peak #4) using a methanol/water (80:20) solvent system, flow rate 30 µl/minute, 0.5 µl sample injection volume, U.V. detection at 254 nm, at a sensitivity of 0.5 Absorbance Units Full Scale (AUFS), and a chart speed of 5 mm/minute.

Low-absorbance grade sodium acetate (AcONa), OmniSolv® HPLC grade acetonitrile and OmniSolv water used for the mobile phase were obtained from EM SCIENCE, a Division of EM Industries, Inc. (Gibbstown, New Jersey). All PTH-amino acid standards were purchased from Pierce Chemical Co. (Rockford, Illinois) with the exception of PTH-glutamic acid methyl ester (EoMe) and PTH-aspartic acid methyl ester (DoMe) which were synthesized in the laboratory.

Chromatographic Conditions

The chromatographic conditions employed for the analysis of the PTH-amino acid standards were as follows:

Sample: 1.0 nanomole of each of 20 PTH-amino acid standards
Injection Volume: 20 µl
Mobile phase: Acetonitrile/AcONa 0/01M pH 4.5 (42/58 v/v)
Flow rate: 75 µl/min. (2200 psi)
U.V. detection: 269 nm
Detector sensitivity: 0.2 AUFS
Chart speed: 1.0cm/min.
Temperature: Ambient
The "non-eluting" solvent used for sample injections was composed of 25% mobile phase and 75% water.

RESULTS AND DISCUSSION

The separation of the standard mixture used for the calculation of column efficiency is shown in Figure 1. The value of N as calculated from the ethylbenzene peak (#4) was 37,250 plates (74,500 P/M).

The chromatogram shown in Figure 2 demonstrates the effects of a 20 µl mobile phase injection containing 1.0 nanomole of each standard PTH-amino acid. The standard single letter abbreviations are used for peak identification. The lack of resolution and peak tailing observed in this chromatogram is ultimately attributable to elution of the sample before the injection is complete.

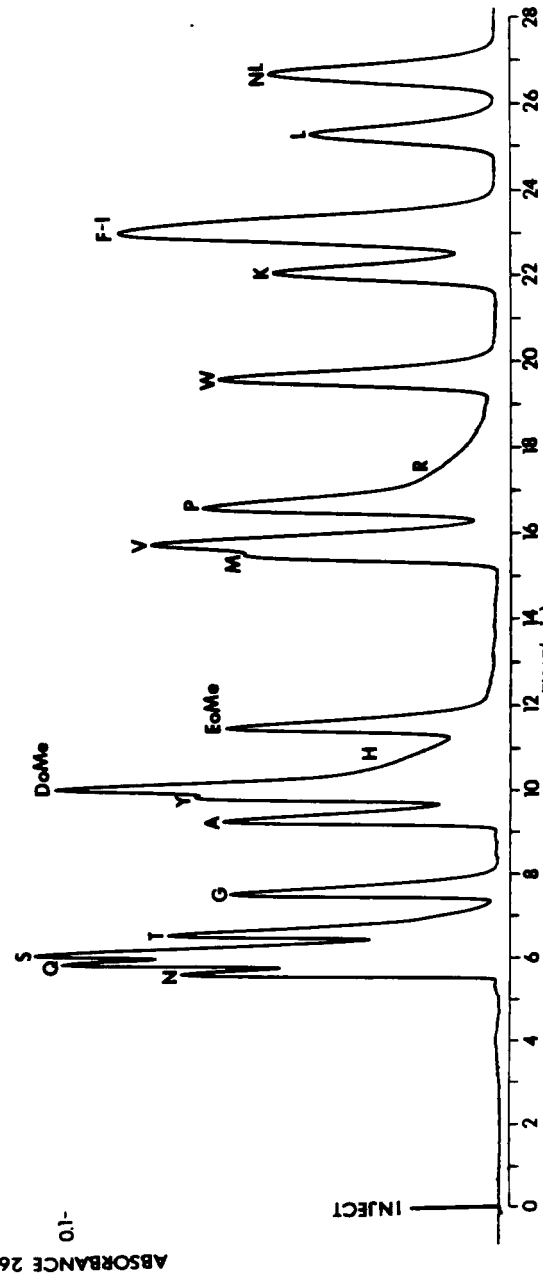


FIGURE 2 Separation of twently PTH-amino acids using a 20 µl mobile phase injection.

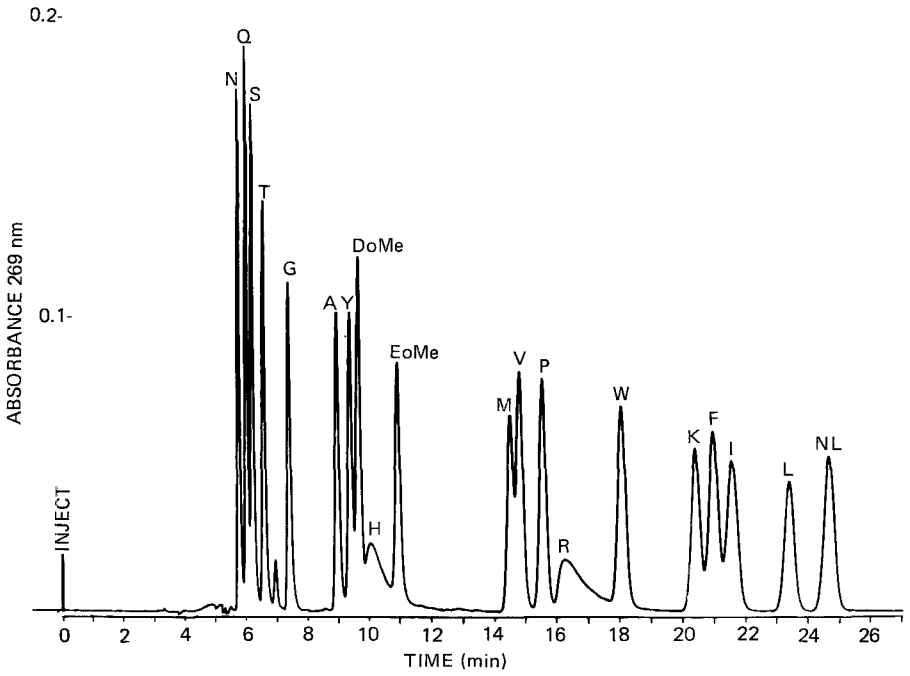


FIGURE 3 Separation of twenty PTH-amino acids using a 20 µl "non-eluting" solvent injection.

In contrast, the chromatogram shown in Figure 3 demonstrates the effects of a 20 µl "non-eluting" solvent injection containing an identical quantity of each standard. In this chromatogram, resolution of all components is greatly increased over the mobile phase injection making identification of the individual standards reliable. This dramatic increase in resolution is due to a concentrating effect whereby the sample remains at the head of the column until the injection is complete and thus maintaining column efficiency.

A comparison of the two injection techniques reveals not only a loss of efficiency with the mobile phase injection but also an increase in overall analysis time resulting from a shift in retention times of the individual components. Furthermore, There is a measurable decrease in sensitivity of the early eluting peaks due primarily to peak broadening. These results are expected in accordance with the data reported for large bore columns by Broquaire and Guinebault (6).

TABLE I

Reproducibility Of The "Non-eluting" Injection Technique

PTH-Amino Acid	t_r (minutes)	Mean t_r	Standard Deviation	PTH-Amino Acid	t_r (minutes)	Mean t_r	Standard Deviation
N	5.88	5.87	0.017	M	14.67	14.67	0.025
Q	6.12	6.12	0.021	V	14.93	14.94	0.029
S	6.36	6.36	0.021	P	15.85	15.86	0.013
T	6.75	6.74	0.022	R	16.24	16.22	0.026
G	7.69	7.67	0.024	W	18.25	18.23	0.026
A	9.32	9.30	0.027	K	20.68	20.68	0.057
Y	9.70	9.73	0.025	F	21.11	21.10	0.069
DoMe	9.90	9.88	0.026	I	21.87	21.86	0.074
H	10.17	10.15	0.027	L	23.71	23.70	0.082
EoMe	11.06	11.04	0.026	NL	24.88	24.87	0.086

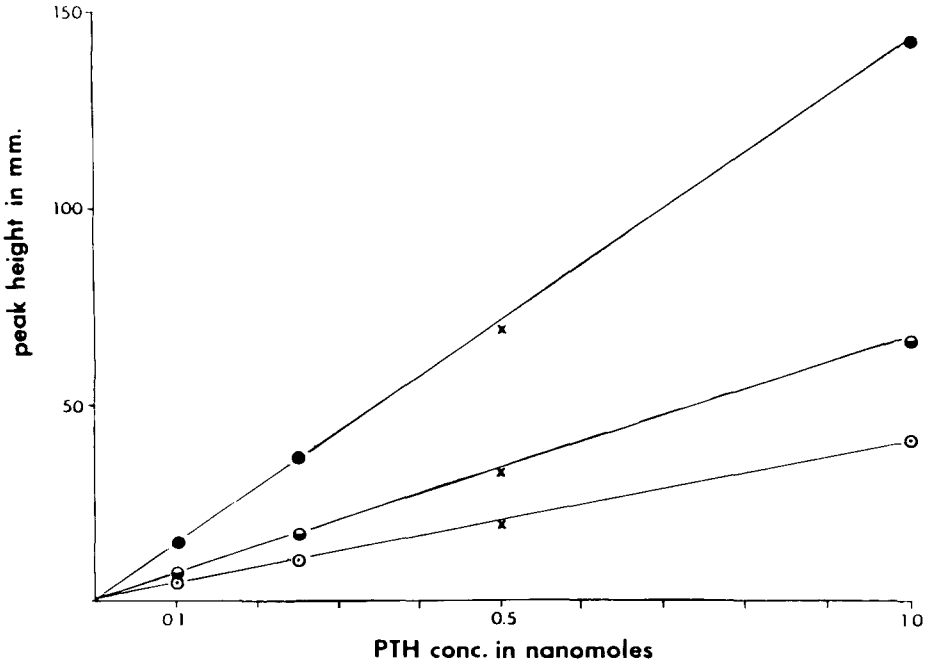


FIGURE 4 Linearity of the isocratic microbore system represented by Asparagine (●), Proline (◒), and Leucine (⊙). Peak heights obtained for a 0.5 nanomole sample are represented by x.

Table I demonstrates the reproducibility of the "non-eluting" injection technique in conjunction with the isocratic system. The mean retention times were calculated from four replicates of 1.0 nanomole injections. The maximum time between replicates was 5.0 minutes. Figure 4 demonstrates the linearity of the system based on peak height in millimeters. Three representative PTH-amino acids were chosen from the beginning (N), middle (P), and end (L) of chromatograms generated from 0.1, 0.25 and 1.0 nanomole samples. The peak heights obtained for the same three PTH-amino acids from a 0.5 nanomole sample were found to be in good agreement with the values determined graphically.

The techniques described herein demonstrate that the use of large volume injections in conjunction with microbore liquid chromatography is a viable alternative to conventional HPLC. The system employed for the analysis shown in Figure 3 provides a rapid and relatively simple technique for the separation of PTH-amino acids which meets the criteria necessary for direct application to protein sequence analysis. Furthermore, serious consideration should be given to the advantage of increased mass sensitivity which will be addressed in a future study.

ACKNOWLEDGEMENTS

MACS and OMNISOLV are trademarks of EM Industries, Inc.

LiChrosorb is a registered trademark of E. Merck, Darmstadt, West Germany.

REFERENCES

1. Scott, R.P.W. and Kucera, P., Mode of Operation and performance characteristics of microbore columns for use in liquid chromatography. J. Chromatogr. 169, 51, 1979.

2. Scott, R.P.W., Kucera, P. and Munroe, M., Use of microbore columns for rapid liquid chromatographic separations. J. Chromatogr. 186, 475, 1979.

3. Scott, R.P.W. and Kucera, P., Use of microbore columns for the separation of substances of biological origin. J. Chromatogr. 185, 27, 1979.

4. Ishii, D., Asai, K., Hibi, K., Jonokuchi, T. and Nagaya, M., A study of micro high performance liquid chromatography. 1 Development of techniques for the miniaturization of high performance liquid chromatography. J. Chromatogr. 144, 157, 1977.

5. Godtfredsen, S.E. and Oliver, R.W.A., On the analysis of phenylthiohydantoin amino acids by high performance liquid chromatography. Carlsberg Res. Commun. 45, 35, 1980.

6. Broquaire, M. and Guinebault, P.R., Large volume injection of biological samples dissolved in a non-eluting solvent: a way to increase sensitivity and a means of automatic drug determination using HPLC. J. Liq. Chromatogr. 4, 2039, 1981.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(3), 569-580 (1984)

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF ALBENDAZOLE AND ITS SULFOXIDE METABOLITE IN HUMAN ORGANS AND FLUIDS DURING HYDATATIDOSIS.

MEULEMANS A. Département de Byophysique, U.E.R. Xavier Bichat
Université Paris VII, 75018 PARIS.
GIOVANANGELI M.D. Institut Médecine et Epidémiologie Tropicale
I.M.E.T., 75019 PARIS.
MOHLER J. Département de Byophysique, U.E.R. Xavier Bichat
Université Paris VII, 75018 PARIS.
VULPILLAT M. Département de Byophysique, U.E.R. Xavier Bichat
Université Paris VII, 75018 PARIS.
HAY J.M. Clinique Chirurgicale, Hôpital Louis Mourier, Colombes.
SAIMOT A.G. Institut Médecine et Epidémiologie Tropicale
I.M.E.T., 75019 PARIS.

ABSTRACT

Albendazole and albendazole sulfoxide were quantified in serum, bile, liver, lungs and hydatid cyst fluid and walls of man receiving albendazole before surgical procedure during therapy of hydatidosis. The frozen tissue specimens were crushed and suspended in phosphate buffer, then the suspension was extracted with ethylacetate as for liquid specimens. The parent drug and its metabolite were extracted at the same time. Albendazole was quantified on a bonded hydrophobic stationnary phase and albendazole sulfoxide on a silica column at 254 nm. This assay was designed for monitoring albendazole and albendazole sulfoxide in serum of patients on long course treatment. The limit of sensitivity in serum was 10 $ng.ml^{-1}$ for the parent drug and its metabolite. An accumulation of these two drugs was shown in organs and bile, sometimes in the liquid and membrane of the cyst.

INTRODUCTION

Albendazole (methyl (5-propylthio) - 1 H - benzimidazol 2-yl) carbamate is a broad spectrum anthelmintic agent generaly used in animals. Recently benzimidazole anthelmin tics were used in man to test their efficacy in E. Granulosus

 0148-3919/84/0703-0569$3.50/0

human echinococcosis (1) (2) (3) ; however the results of clinical trials required reevaluation as well as studies on the pharmacokinetics of these drugs in man. The determination of albendazole and its metabolite in man could serve to standardize the use of this drug in therapy of hydatidosis.

Nine metabolites of albendazole were identified in urine of different species (cattle, sheep, rats and mice) using radiolabeled product (4) ; the synthetic pathway to obtain metabolites and the thin-layer chomatographic technique corresponding to their identification were given by the authors (4). Despite this fact only albendazole and albendazole sulfoxyde were present in human samples tested in these thin-larger chromatographic systems.

Benzimidazoles were also investigated by high performance liquid chromatography : mebendazole and its metabolites in human plasma (5) (6), thiabendazole for industrial hygiene measurement (7), and all different commercial benzimidazoles in sheep plasma (8) ; but in this later paper the limit of sensitivity was approximately 80 $ng.ml^{-1}$ for albendazole on one ml of plasma ; albendazole was not detectable in any plasma of sheep ; all separations in these papers were obtained after an organic extraction on a reverse phase system, but none indicated metabolites of parent drug.

In this study a bonded phase system was used for quantitation of albendazole and a silica column was used for quantitation of a albendazole sulfoxyde ; but for the two products the same extraction procedure was performed.

A first group of patients had liver hydatidosis and lung hydatidosis. A surgical operation was scheduled for all patients from this group. There were volunteers to receive albendazole prior to surgery. This group was designed to evaluate the drug concentration in the target organs for hydatid cysts and finally in the cysts themselves as well as the monitoring of the blood drug levels during the course of a potential therapeutic treatment.

A second group of patients received the test drug therapeutically and/or prophylactically following cyst rup-

tures during surgical procedures or for residual or recurrent single or multiple cysts.

MATERIAL AND METHODS

a) Chromatographic equipment

Analyses were performed with a Waters Associates liquid Chromatograph (Waters Assoc., Paris, France) equipped with a model 440 absorbance detector, a model 6,000 A pump, a Wisp 710 B injector and 10 mv recorder.

b) Solvents and standards

Freshly distilled deionized water was used throughout the procedure. Methanol, acetonitril and ethylacetate were analytical grade (Merck, Darmstadt, Germany). Albendazole and albendazole sulfoxide were kindly given by Smith, Kline French (France).

c) Chromatographic eluent

For the determination of albendazole sulfoxide the mobile phase consisted of a mixture of acetonitrile : methanol : water (250/12/5). For the determination of albendazole it consisted of a mixture of methanol : water (65 :35) Each mobile phase was filtered through a 0,6 μm filter (Millipore corp., Paris, France) and degassed using ultrasonics.

d) In vitro samples

Plasma Albendazole and albendazole sulfoxide were directly prepared in pooled human plasma and congealed in aliquots at - 80°C. Each day of analysis an aliquot of each point of the standard curve was decongealed for standardization.

Bile Albendazole and albendazole sulfoxide were prepared in pooled human bile and congealed in aliquots at - 80°C

Organs Albendazole and albendazole sulfoxide were prepared in phosphate buffer saline (pH 7.4) and congealed in aliquots at - 80°C.

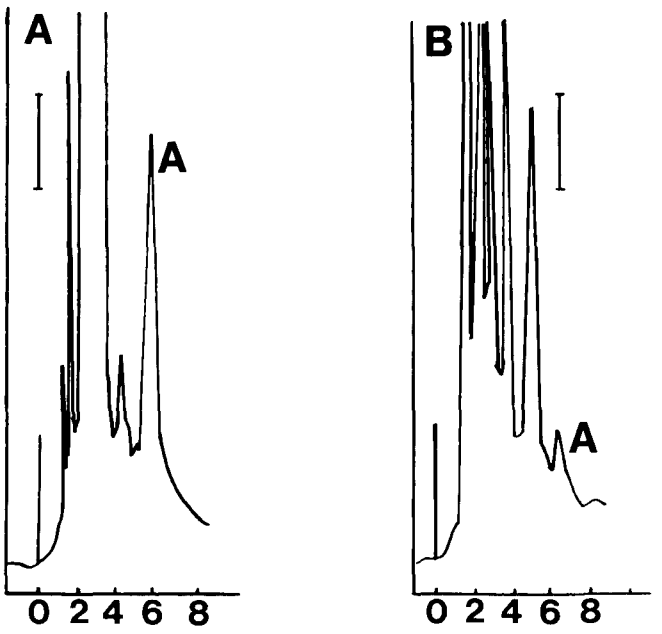


FIGURE 1

Chromatographic separation of albendazole in extracts of bile (A) and serum (B). Albendazole's peak is indicated by the letter A.
The vertical lines correspond to 0.002 absorbance units.

e) In vivo samples

Organs of patients (liver, lungs and cyst) or liquid (serum, bile or hydatic liquid) were sampled during surgery. They were immediately congealed at - 80°C.

f) Chemical assay

Extraction One ml of phosphate buffer (pH 7.4) and one ml of ethylacetate was added to one ml of serum, bile or hydatic liquid. The extraction was performed during 15 minutes on a rotor type agitator.

Organs (a few milligrammes depending on surgery) were weighted after decongealation, put in a few milliliters of

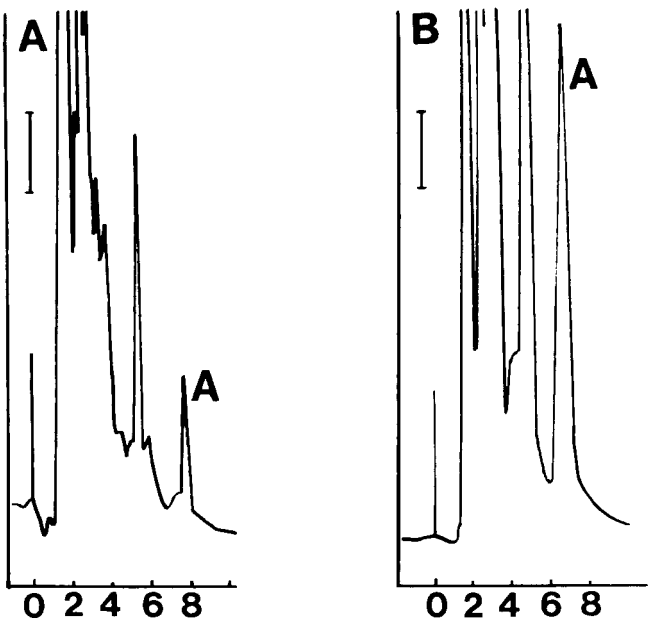


FIGURE 2

Chromatographic separation of albendazole in extracts of lungs (A) and liver (B). Albendazole's peak is indicated by the letter A. The vertical lines correspond to 0.002 absorbance units.

phosphate buffer (pH 7.4) and crushed with an ultrathurax apparatus. On ml of the supernatant was added to one ml of ethyl acetate and the extraction was performed during 15 minutes on a rotor type agitation. Standards were treated in the same manner. In each case 700 µl of the organic phase was evaporated and 100 µl of the mobile phase was added to the residue and passed through 0.22µm filters and 35 µl of this liquid injected into the chromatograph.

Chromatography A reverse phase system was chosen to quantitate albendazole and a normal phase system for albendazole sulfoxide. In each cas 35 µl of the extract was injected onto the columns : a µBondapack C18 (Waters) for

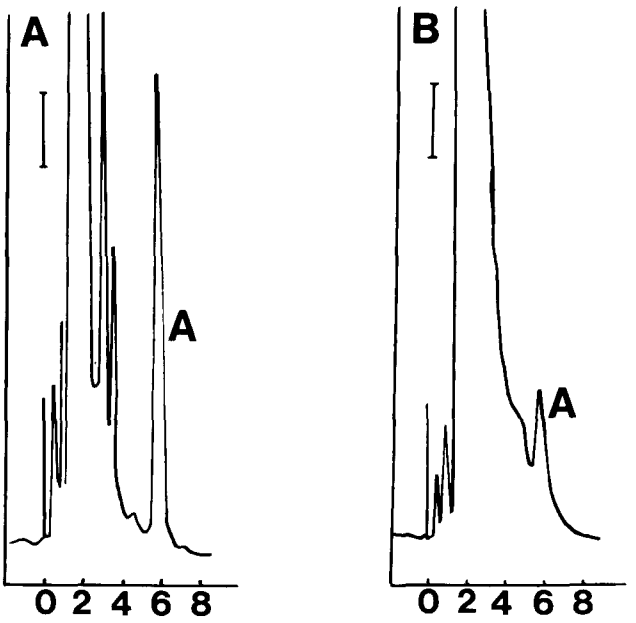


FIGURE 3

Chromatographic separation of albendazole sufoxide in extracts of serum (A) and bile (B). The peak of albendazole sufoxide is indicated by the letter A. The vertical lines correspond to 0.002 absorbance units.

albendazole and a micro-Porasil (Waters) for albendazole sulfoxide. Eluent pumped through at 1.5 ml/min. The absorbance detector was set at 254 nm at a sensitivity of 0.005 absorbance units full scale. Quantitation was based on recorded peak heights. Three standard curves were used : one for bile and another for organs.

RESULTS

Chromatographic separation

The retention time of albendazole was 6.6. minutes in the described conditions and 6 minutes for albendazole sufoxide ; They were constant in serum, bile or supernatant of organs. In the system of albendazole, albendazole sulfoxide

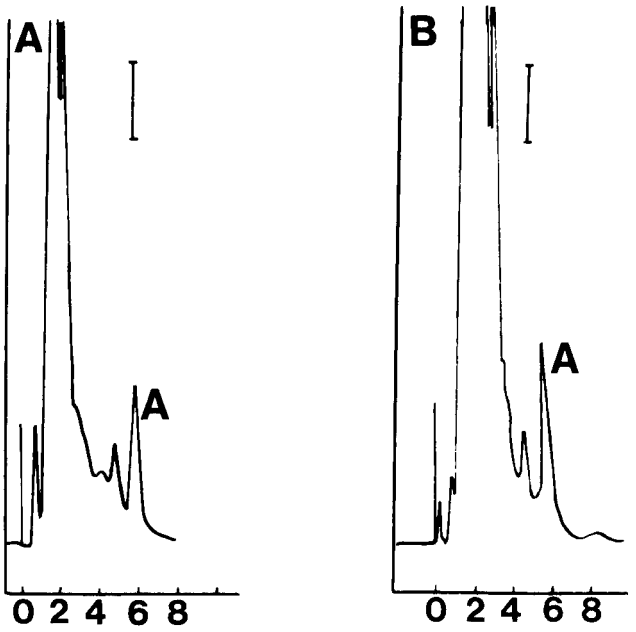


FIGURE 4

Chromatographic separation of albendazole sulfoxide in extracts of cyst wall (A) and cyst liquid (B). The peak of albendazole sulfoxide is indicated by the letter A. The vertical lines correspond to 0.002 absorbance units.

is undetectable : its time of retention is too near the dead volume and it is the same for albendazole in the system of albendazole sulfoxide. The chromatograms were shown in Figures (1-2) for albendazole and Figures (3-4-5) for albendazole sulfoxide in the different extracts.

Recovery

The three standard curves showed good linearity in the range studied : 10 - 100 $ng.ml^{-1}$ for albendazole and 10 - 800 $ng.ml^{-1}$ for albendazole sulfoxide . The recovery of these assays was tested using the dilution test and by adding known amounts of drug to known extracts. For albendazole the range of the recovery was between 94 % to 102 %

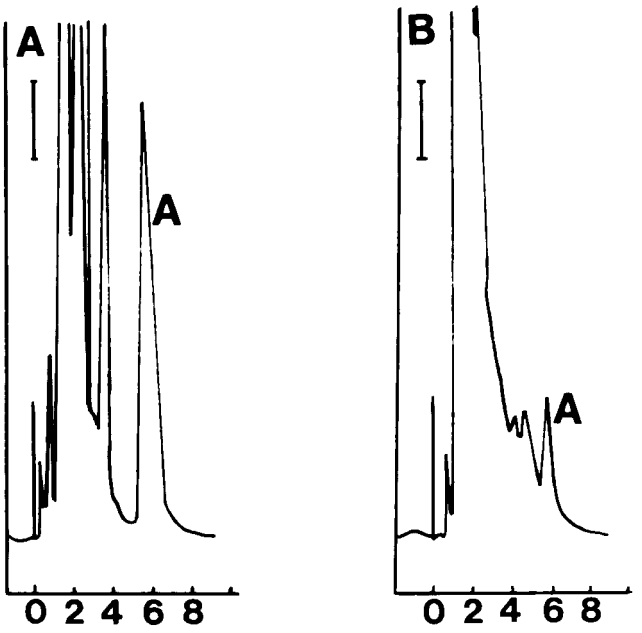


FIGURE 5

Chromatographic separation of albendazole sulfoxide in extracts of lung (A) and liver (B). The peak ol albendazole sulfoxide is indicated by the letter A. The vertical lines correspond to 0.002 absorbance units.

and for albendazole sulfoxide from 93 % to 101 % in the series of two tests.

Sensitivity

The limit of sensitivity of the method was determined with pooled serum, bile or phosphate buffer and by dilution of known human samples. The limit of detection could be evaluated to 10 $ng.ml^{-1}$ for albendazole and 10 $ng.ml^{-1}$ for albendazole sulfoxide in the described conditions. This limit is largely sufficient for clinical monitoring.

Precision

. Intra-assay variation was studied on three sera and biles which were determined tenfold on the same day with

TABLE I

PATIENTS	Serum ng.ml^{-1}		Bile ng.ml^{-1}		Cyst wall ng.g^{-1} wet tissue		Cyst liquid ng.ml^{-1}		Liver ng.g-1 wet tissue		Lungs ng.g-1 wet tissue	
	*A	*B	A	B	A	B	A	B	A	B	A	B
1	6	133	5	350	< 4	83	< 1	180		1 730		
2	< 5	130	< 5	248	< 5	138	8	504	121	272		
3	< 5	480			< 5	53	< 10	94			< 5	712
4	< 5	350			< 5	68	< 5	98			< 5	718
5	< 5	180			< 5	92	< 5	158			< 5	816
6	< 5	50									10	720
7	24	65	200	90							406	554
8	38	90	60	350							139	97
9	10	160			< 10	< 10	< 5	< 10	70	732		
10	45	350	30	570	15	83	8	180	40	1 210		

*A Albendazole *B Albendazole sulfoxide

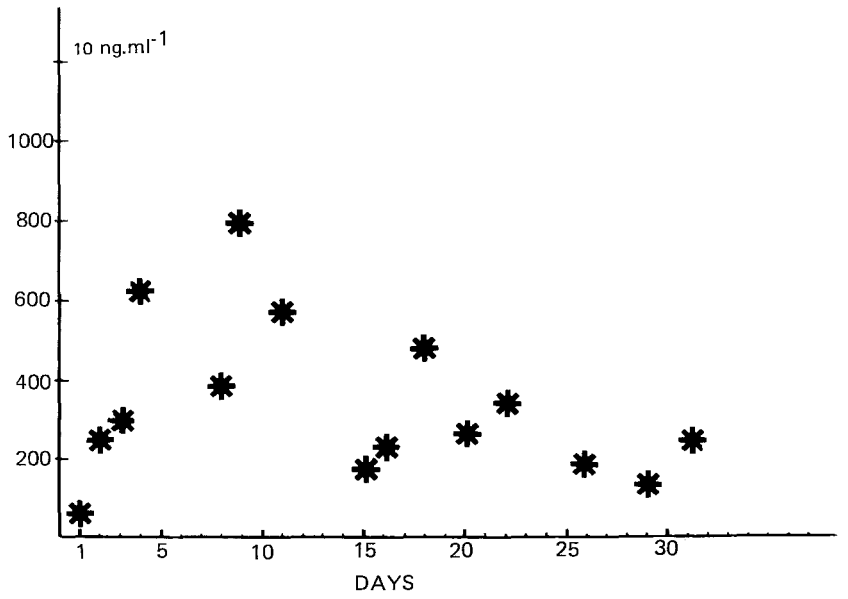


FIGURE 6

Concentration of albendazole sulfoxide in serum for a patient of the second group plotted against days of treatment.

the two techniques. For albendazole sulfoxide, the maximum variation was 8 % and 7.5 % for albendazole on the range of the standard curve.

. Inter-assay variation was calculated on the same sera and biles which were stored at - 80°C and assayed ten times on different days ; the variation was 10 % for albendazole sulfoxide and 9.5 % for albendazole (the coefficient of variation was defined with one standard deviation).

. Patient values

The first group received 5 to 7 mg. Kg^{-1} of albendazole for two 39 consecutive days. The last dose of albendazole was administred 10 to 12 hours before surgical operation. The drug was administered morning and evening before meals as 200 mg tables (see table I).

The second group received 5 to 7 $mg.Kg^{-1}$ of albendazole for 30 consecutive days the treatment being renewed after an interval of two weeks. The observed values were shown in Figure 6 for one patient ; the same type of curve was obtained for other patients. The mean value for albendazole was below 10 $ng.ml^{-1}$ and most of time albendazole was undectectable in patient's serum. Albendazole sulfoxide had very high values 275 $ng.ml^{-1} \pm 210\ ng.ml^{-1}$.

DISCUSSION

The procedure described above permits one to monitor chemotherapy of human hydatidosis with albendazole . Albendazole and albendazole sulfoxide needed to be determined in serum ; albendazole had a too low concentration in serum, except in a few patients (cirrhosis). The two different ways to quantitate albendazole and its metabolite were needed because of the difficulty of having an isocratic separation on a column in extracts. Tissue penetration was better for the metabolite than for the parent drug. Albendazole was undetectable in the cyst and cystic liquid. It is clear that the determination of albendazole sulfoxide in serum could serve to monitor albendazole therapy and permit controlled clinical trials. This HPLC analysis of albendazole sulfoxide is a simple, sensitive and reliable method for such studies.

REFERENCES

1. Heath D.D., Christie M.J., Chevis R.A.R., Parasitology, 70, 273-285 (1975).
2. Braithwaite P.A., Aust. NZ J. Surg., 51 : 23-27 (1981)
3. Saimot A.G., Meulemans A., Hay J.M., Mohler J., Manuel C. Coulaud J.P., Nouv. Pr Med., 38, 3121-3126 (1981)
4. Gyurik R.J., Show A.W., Zaber B., Brunner E.L., Miller J.A., Willani A.J., Petka L.A., Parish R.C., Drug Metab. Disp., 9, 6, 503-508 (1981).

5. Alton K.B., Patrick J.E., Mc Guire J.L., J. of Pharm.Sci 68, 7, 880-882 (1979).
6. Allan R.J., Goodman H.T. Watson T.R., J. of Chrom., 1983 311-319 (1980).
7. Belinky B.R., J. of Chrom., 238, 506-508 (1982).
8. Bogan J.A., Marriner S., J. of Pharm. Sci, 69, 4, 421-423 (1980).

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(3), 581-590 (1984)

SEPARATION AND COLUMN PERFORMANCE OF CERTAIN RADIOTRACERS USING A CHROMATOGRAPHIC COLUMN OF CELITE LOADED WITH ADOGEN-381 AND SELECTED ELUENTS

M.Raieh, S.M.Khalifa, M.El-Dessouky and H.F.Aly
Nuclear Chemistry Department, Nuclear Research Centre,
Atomic Energy Establishment, Cairo, Egypt.

ABSTRACT

The radiotracers Mo(VI), Sc(III), Ce(III), Eu(III), Tm(III), Fe(III), Hg(II), Cd(II), Zn(II), Cu(II) and Co(II) could be separated from each others using a hydrophobic celite column coated with Adogen-381 together with the mobile phases hydrochloric acid, nitric acid, potassium thiocyanate, ammonium acetate or water. The elution behaviour was satisfactorilly interpreted. The effect of the flow rate (50-300 ul/min) and temperature (26-70°C) on the column performance of Co(II) was also studied.

INTRODUCTION

Amines and quaternary ammonium salts found many applications in column chromatography (1-6). Different classes of amines were utilized for the separation of many elements using a variety of supporting agents and different mobile phases. In this contribution, the

 0148-3919/84/0703-0581$3.50/0

system comprising the commercially available tertiary amine Adogen-381 together with stationary phase, hydrophobic celite and selected mobile phases of HCl, HNO_3, KSCN, CH_3COONH_4 or H_2O was employed to isolate eleven radiotracers from each others. Considering the importance of the flow rate and temperature on the column performance (7, 8) these parameters were also studied.

EXPERIMENTAL

Chemicals and radiotracers

Adogen-381 of average molecular weight 360 was delivered from Archer-Daniels Midland Company, USA. Commercial diatomaceous earth celite No. 560 was supplied from John Manville, USA. The radiotracers : Sc-46, Ce-141, Eu(152+154), Tm-170, Fe-59, Co-60, Cu-64, Zn-65, Cd-115, Hg-203, and Mo-99 were prepared by neutron activation, and their radiochemical purity exceeded 99%. All other chemicals and reagents were of A.R. grade and obtained from B.D.H. or Merck.

Chromatographic Procedure

The stationary phase, celite, was dried at 105°C over night and made hydrophobic by the addition of 50 cm^3 of 5% silane-ethanol with thorough mixing. The mixture was left in a dessicator under slight vacuum to remove excess silane and ethanol. The hydrophobic celite was washed with distilled water to remove any chloride ions followed by oven drying at 105°C. The Adogen-381 was loaded on the hydrophobic celite by mixing 5.0 g with 100 cm^3 5% Adogen solution in acetone. Excess acetone was removed by evaporation with interrupted stirring at 80°C. A 0.5 cm i.d. x 10 cm

borosilicate glass column with platinum calibrated tip to produce 25 ul drop size was used. This column was loaded with 1.6 celite coated with Adogen-381 according to the procedure previously reported (9), to give a bed height of 9 cm. In all cases, the column was preconditioned with 5.0 cm^3 of the first eluent eomployed and the radiotracers were loaded on the column from a 50 ul syring . Unless otherwise stated, the eluent solutions were allowed to percolate with a flow rate of 75 ul/min. Known volume fractions of the eluent were collected for radiometric assay using an ORTEC-Scintillation counter.

The effects of the flow rate and temperature on column performance were studied using Co-60 and 2M HCl solution as eluent.

From the elution curve, the distribution coefficient, D, was determined using the relation (10) ;

$$D = (V_{max} - v) / w$$

where V_{max} is the eluent volume to the elution maximum, v is the free column and w is the weight of the stationary phase. The number of theoretical plates, N, was calculated by means of the equation (11) :

$$N = 8 (V_{max} / \beta)^2$$

where β is the band width of the elution peak at 1/e of the maximum elution concentrati n. Dividing the height of the column by N, the height equivalent to theoretical plate, HETP, was obtained.

RESULTS AND DISCUSSION

The radiochemical separation procedures developed in this work are based on the expected extraction behaviour of Adogen-381 and the types of the species of the different radiotracers in the different solutions used

as eluents. Thus, a- the tertiary amine Adogen-381 is expected to extract both neutral and anionic species(12), b- the lanthanide elements are hardly complexed from hydrochloric or nitric acid solutions up to 8 molar (13), c- in aqueous HCl solution of concentrations higher than 8M, iron(III) is present in neutral or anionic chloride forms (14) and at lower HCl molarities, cationic iron chloride complexes are predominating, d- molybdate is extracted by tertiary amines from all hydrochloric acid concentrations and unextracted from all nitric acid concentrations (15), e- in HCl solutions, copper is present as the chloride complex, while from thiocyanate solutions it is poorly extracted by the tertiary amines due to the competition of SCN^- anion to the amine solution (15,16), f- zinc forms strong chloride and thiocyanate complexes which are extracted by tertiary amines, When zinc is slightly complexed from hydrochloric acid solution, water was found quite suitable to bring it to the free cationic state and thus be eluted. In presence of HCl and KSCN, water was found not enough to elute zinc in a suitable volume. Therefore, 1M NH_4CH_3COO eluted zinc, most probably as the strong cationic ammino complex (16), $[Zn(NH_3)_n]^{2+}$, g-cadmium is highly complexed by chloride ions and extracted by tertiary amine from HCl solution of different concentrations. Cadmium nitrate complexes are hardly present in nitric acid medium and the elution of Cd(II) using nitric acid was found convenient, h- mercury forms chloride and thiocyanate complexes, therefore, its uptake from these solutions is expected (16). Elution from high SCN^- solution is mainly related to the replacement of the SCN^- anion with the mercury complex.

In figures 1, and 2, the elution profiles of the eight radiochemical separation procedures developed are

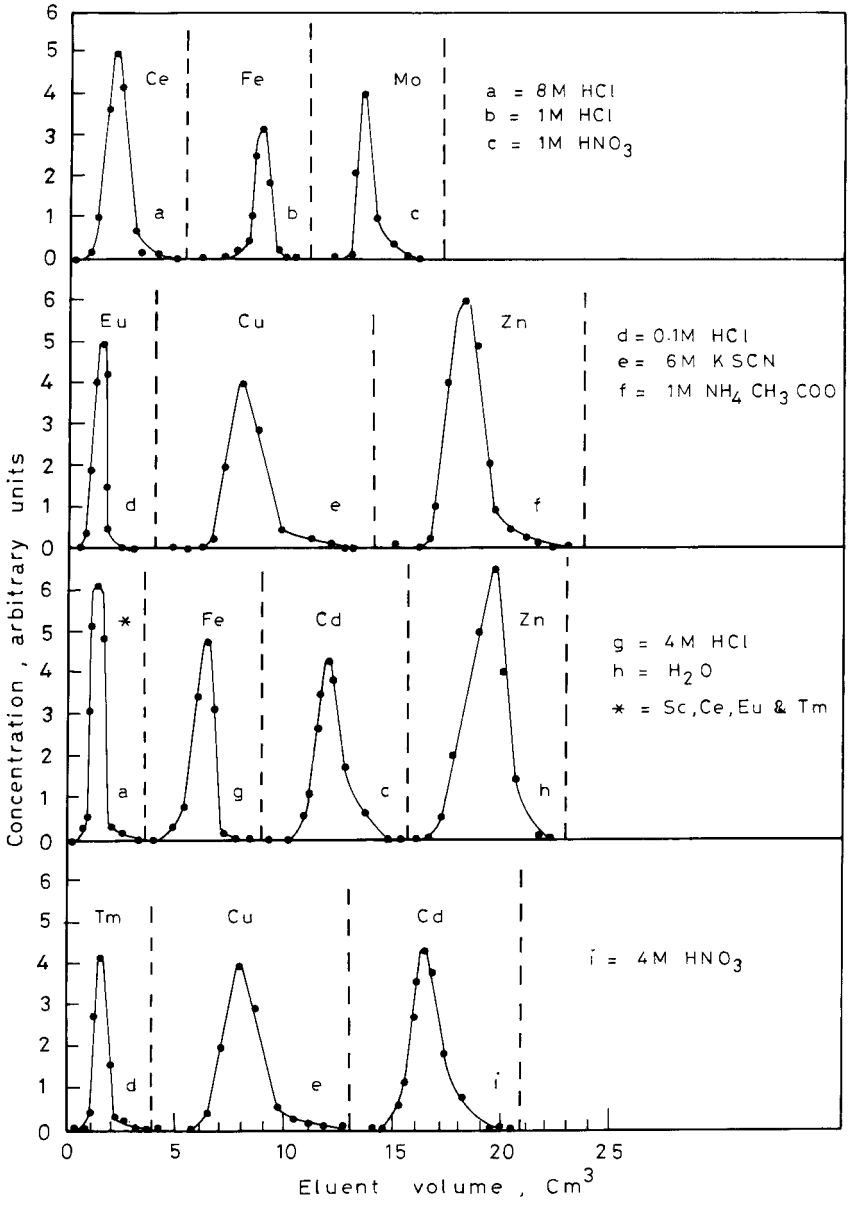


Figure 1 Elution profile : Ce(III), Fe(III), Mo(VI), Eu(III), Cu(II), Zn(II),(Sc(III), Ce(III), Eu(III) & Tm(III)), Tm(III) and Cd(II).

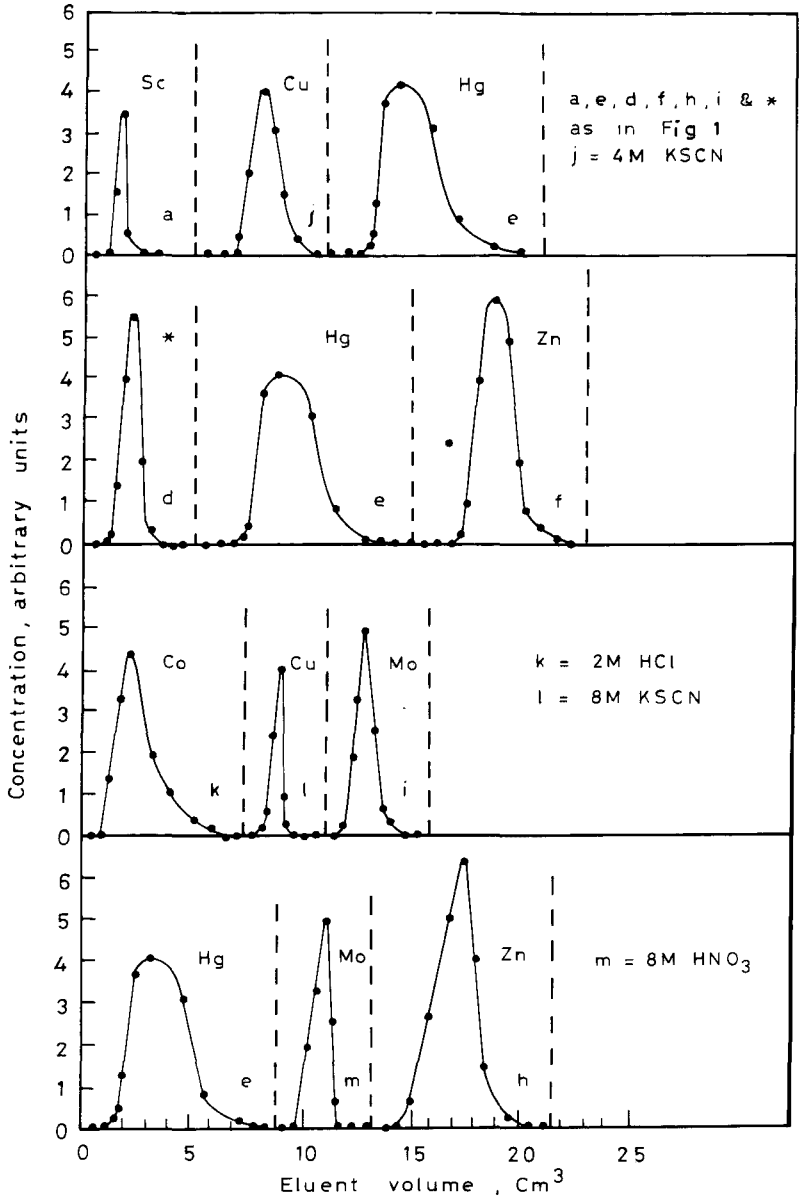


Figure 2 Elution profile : Sc(III), Cu(II), Hg(II), (Sc(III), Ce(III), Eu(III) & Tm(III)), Zn(II), Co(II) and Mo(VI).

TABLE 1

Effect of Flow Rate on the Elution Profile(Co(II)

Flow rate ul/min.	Distribution coefficient.	Band width cm^3	Number of theoretical plates.	Plate height (HETP) mm
50	0.42	0.58	60.50	1.49
75	0.42	0.89	26.60	3.38
150	0.42	1.00	20.50	4.39
225	0.42	1.30	12.51	7.19
300	0.42	1.45	9.70	9.28

given. In all cases, the investigations were carried out at 26±1°C. The total concentration of the radiotracers did not exceeded 10^{-4}M (equimolar amounts of each tracer). The radiochemical purity of each separated fraction was more than 98%.

In order to evaluate the column performance utilized, the effects of eluent flow rate and temperature on the elution profile were investigated using Co(II). It was found that, the distribution coefficient does not change with the flow rate (50-300 ul/min) whereby the band width of the eluted cobalt increased by increasing the flow rate (Table 1).

When the theoretical plate height was calculated and plotted against the flow rate, a linear relationship was obtained (Fig. 3). The effect of temperature on the distribution coefficient, elution profile and theoretical plate height of Co(II) is given in Table 2.

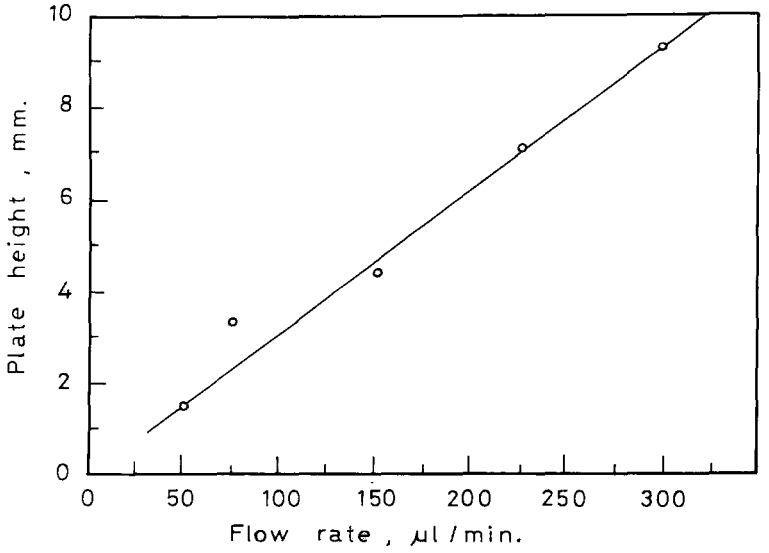


Figure 3 Effect of flow rate on the plate height for the elution of Co(II) by 2M HCl at 26±1°C.

TABLE 2

Effect of Temperature at 75 ul/min.

Temperature °C	Distribution coefficient.	Band width cm^3	Number of theoretical plate.	Plate height (HETP), mm
26	0.42	0.89	26.6	3.38
30	0.42	0.87	28.2	3.19
44	0.42	0.75	37.7	2.39
55	0.42	0.62	55.6	1.62
70	0.42	0.37	155.3	0.58

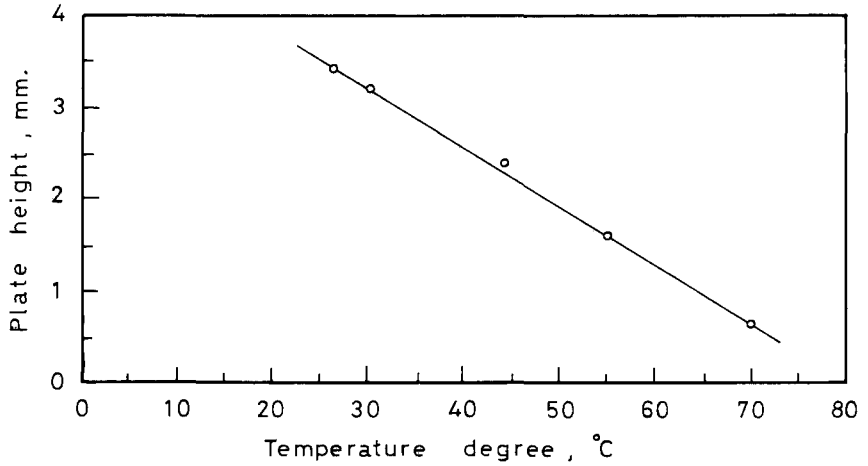


Figure 4 Effect of temperature on the plate height for the elution of Co(II) by 2M HCl at 75 ul/min.

It is clear that within the temperature range investigated (26-70°C), the D value does not change, however, the band width decreased by increasing temperature. Subsequently, the number of theoretical plates of the column increased. This is illustrated in Fig. 4, where the theoretical plate height decreased linearly by increasing temperature.

REFERENCES

1. Mikulski, J. and Stronski, I., J. Chromatoger., 17, 197, 1965.
2. Akaza, I. and Inamura,J., J. Radioanal. Chem., 54, 27, 1979.
3. Horwitz,E.P., Orlandini,K.A. and Bloomquist,C.A.A., J. Inorg. Nucl. Chem. Letters, 2, 87, 1966.

4. Shimizu,T. and Ikeda,K., J. Chromatoger., 85, 123, 1973.
5. Aly,H.F. and Abdel-Rassoul,A.A., Z. Anorg. Allg. Chem., 387, 252, 1972.
6. Murray,R.W. and Passarelli,R.J., J. Anal. Chem., 39, 282, 1967.
7. Horwitz,E.P. and Bloomquist,C.A.A., J. Inorg. Nucl. Chem., 34, 3851, 1972.
8. Cerrai,E., Testa,C. and Triulizi,C., Energia, Nucleare, 9, 377, 1962.
9. Abdel-Rassoul, A.A., Aly,H.F. and Zakareia,N., Z. Anal. Chem., 27, 272; 1974.
10. Tompkins,E.R. and Mayer, S.W., J. Amer. Chem. Soc., 69, 2866, 1974.
11. Glueckauf,E., Trans. Faraday, Soc., 51,34,1955.
12. McKay,H.A.C., Healy,T.B. Jenkins,I.L. and Naylor,A., Solvent Extraction Chemistry of Metals, MacMillan, London, 1966.
13. Moeller,T., The Chemistry of Lanthanides, Pergamon Press, 1973.
14. Nicholls,D., The Chemistry of Iron, Cobalt and Nickel, Pergamon Press, 1973.
15. Sekine, T. and Hasegawa,Y., Solvent Extraction Chemistry, Marcel Dekker, Inc. 1977.
16. Sillen,L.G. and Marrell, A.E., Stability Constants of Metal-Ion Complexes, The Chemical Society, Special Publication No. 25, 1971.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(3), 591-598 (1984)

ESTIMATION OF AZADIRACHTIN CONTENT IN NEEM EXTRACTS AND FORMULATIONS

J. D. Warthen, Jr., J. B. Stokes, and M. Jacobson
Biologically Active Natural Products Laboratory
Agricultural Environmental Quality Institute
Agricultural Research Service
U.S. Department of Agriculture
Beltsville, Maryland 20705
and
M. F. Kozempel
Eastern Regional Research Center
Agricultural Research Service
U.S. Department of Agriculture
Philadelphia, Pennsylvania 19118

ABSTRACT

A high performance liquid chromatographic reversed-phase procedure has been developed whereby azadirachtin content can be estimated in crude extracts of neem and in dust formulations of neem. An estimation of the azadirachtin content is achieved through the use of an external azadirachtin standard and valley-to-valley integration. Since azadirachtin seems to be the most potent insect feeding deterrent in these extracts and formulations, its content is a measurement of potency and represents an attempt at standardization.

INTRODUCTION

Azadirachtin ($C_{35}H_{44}O_{16}$) is a tetranortriterpenoid (1,2) (Fig. 1) present in neem kernels [Azadirachta indica A. Juss. (Melia azadirachta L., M. indica Brandis., Margosa tree or Indian lilac)] and the chinaberry tree (M. azedarach L., Persian lilac). It is a highly active feeding deterrent and growth regulator for insects (3).

 0148-3919/84/0703-0591$3.50/0

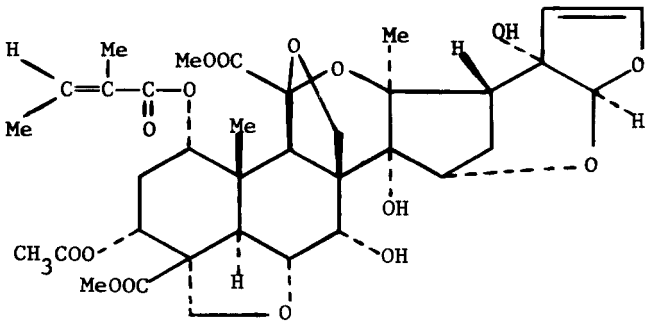


FIGURE 1 Structure of azadirachtin ($C_{35}H_{44}O_{16}$) as proposed by Zanno et al. (2).

Azadirachtin has not yet become a generally used insect feeding deterrent for several reasons: 1) it is difficult to isolate and the yield is low (4), 2) formulations have not yet been stabilized (5), and 3) its effectiveness on any one particular insect pest in the field has not been totally studied, etc. (6-10).

In order to study the effectiveness of crude extracts or formulations containing neem, we developed a high performance liquid chromatographic (HPLC) technique for estimating the azadirachtin content of these samples. The technique is partially based on some of our prior work dealing with the preparative isolation of azadirachtin (4) and the effects of sunlight on azadirachtin (5).

MATERIALS AND METHODS

Apparatus

A Waters Associates Model ALC-100 Liquid Chromatograph equipped with a Model 720 System Controller, a Model 730 Data Module, two Model 6000A Pumps, a U6K Injector, and a Model 440 Absorbance Detector with an Extended Wavelength Module at 214 nm was used for all HPLC. The column for HPLC, in a Z-Module™ Radial

Compression Separation System, was a 10 μ Radial-Pak™ μBondapak® C_{18}.

A Comitrol Model MG, Urschel Cutter, with a 0.15 cm cutting head; a Hamilton Kettle, style A 227 L, double motion Teflon scraper agitator; a Sparkler Filter with E-5 filter paper; a Precision Scientific, 3 L (D-1) laboratory, vacuum, glass evaporator; and a Stokes Vacuum Dryer were used for the large scale extraction of neem seed kernels.

A Brinkmann Centrifugal Grinding Mill ZM-1 was used to decrease the particle size of hexane extracted neem kernel powder needed for kaolin (hydrated aluminum silicate) formulations.

Ethanol (95%) was obtained from Publicker Industries, Inc. Solvents (methanol, methylene chloride, acetone, and ether) were HPLC grade and obtained from Fisher Scientific Co. Hexane (laboratory grade) was also obtained from Fisher Scientific Co. Water was distilled. Kaolin (USP) was obtained from Mallinckrodt Inc.

Plant Material and Formulations

1) Neem extracts on a small scale were prepared by grinding 50 g of neem kernels in a Waring Blendor with 100 ml 95% ethanol or an appropriate solvent for 30 sec. The mixture was then filtered through Whatman #1 filter paper. When neem was extracted with methylene chloride or ether, the solvent was removed in vacuo and replaced with 100 mL 95% ethanol for HPLC analysis.

2) Neem extract on a large scale (Fig. 2) was prepared by first grinding 114 kg of neem seed kernels into a coarse powder with a Comitrol Model MG, Urschel cutter. The first 23 kg was extracted with 95 L 95% ethanol for 2 hr at room temperature in a Hamilton Kettle. The mixture was allowed to settle and the supernatant was clarified by filtration with a Sparkler Filter. The filtrate was concentrated at a temperature generally below 40°C in a Precision Scientific Evaporator. The yield of extract was 4 L. The extraction was repeated twice with yields of 2.5 L and 0.25 L, respectively. The neem seed kernel marc was dried in

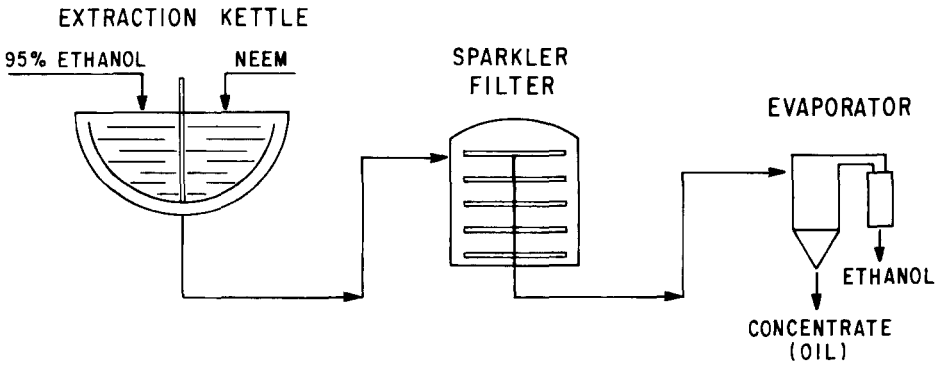


FIGURE 2 Pilot plant extraction process.

a Stokes Vacuum Dryer to 2.2% volatiles. The remaining 91 kg of ground neem seed kernels was extracted with 76 L 95% ethanol for 2 hrs, allowed to settle overnight, and filtered. Eight similar extractions of this large batch gave a total yield of 19.5 L after concentration. A 1 g sample from each of the extracts was prepared for analysis by adding 10 mL 95% ethanol.

3) Neem-kaolin formulations were prepared by first grinding 454 g of neem seed kernels with 1 L hexane in a Waring Blendor for 60 sec. The mixture was filtered through Whatman #1 filter paper and the procedure repeated. The hexane-extracted powder was air dried and then ground in the Brinkman Mill with a screen of 0.5 mm pore size. The powder was then mixed with kaolin on a weight to weight basis. The neem-kaolin formulations (1 g) and 10 mL 95% ethanol were mixed for the analytical estimation of azadirachtin content.

Sample Preparation

The neem-kaolin formulations (1.0 g) were mixed with 2-10 mL 95% ethanol and sonicated for 10 min. The mixtures were then centrifuged at low speed for 5 min and the supernatant was used for the analytical estimation of azadirachtin content.

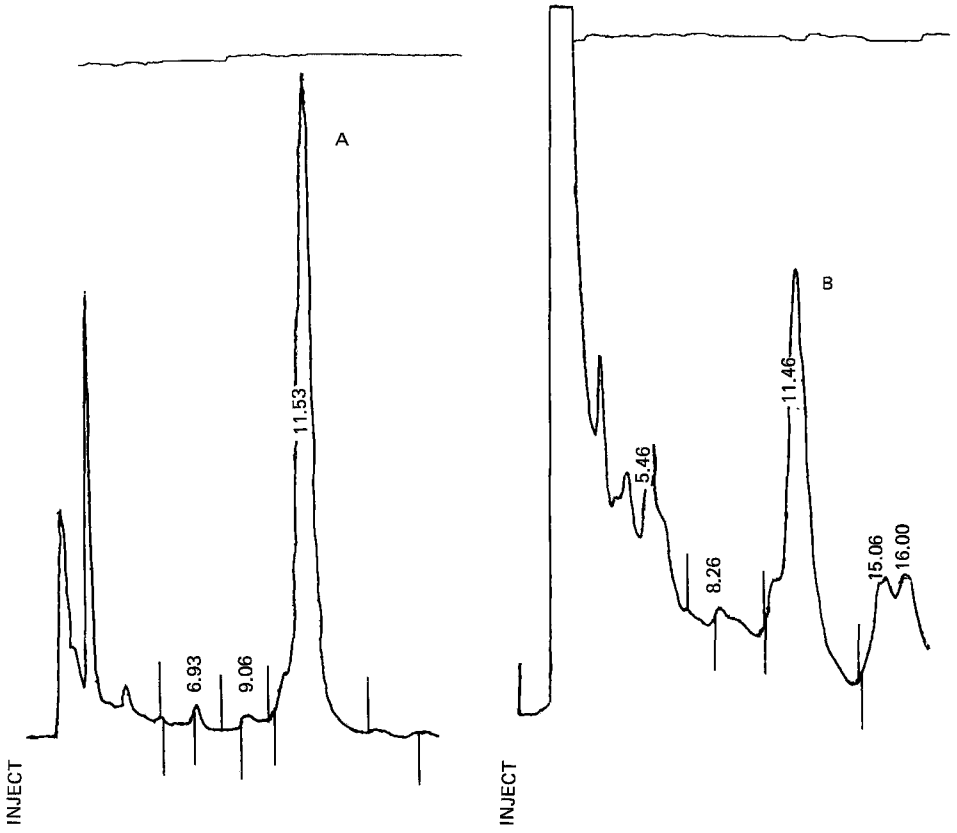


FIGURE 3 A typical chromatogram of (A) the standard, azadirachtin, and (B) a crude neem sample.

Chromatographic Procedure

A standard azadirachtin sample (1.0 µg/10 µL) was injected into a 10 µ Radial-Pak µBondapak C_{18} in a Z-Module Radical Compression Separation System with a flow rate of 2 mL/min (50/50 methanol/water) and detection at 214 nm.

The Model 730 Data Module was set with a peak width of 40, noise rejection of 6, and valley-to-valley integration. At 214 nm and 0.05 attenuation, 1.0 µg of azadirachtin gave a 1/2 scale peak

which was entered in the calibration table as an external standard.

Solutions prepared from crude neem extracts or neem-kaolin samples were injected and the peak corresponding to the azadirachtin standard was analyzed by the Data Module at the same attenuations. A typical chromatogram of the standard, azadirachtin, and a crude neem sample is shown in Fig. 3.

When crude neem extracts were analyzed, it was necessary to flush the column after each run with a 10 min flush of 100% methanol. This was accomplished by the System Controller with a 10 min linear program from 50/50 methanol/water to 100% methanol, a hold for 10 min at 100% methanol, and then another 10 min linear program from 100% methanol to 50/50 methanol/water. This procedure was not necessary with the analyses of the neem-kaolin samples because the neem kernels in these formulations had been pre-extracted with hexane.

RESULTS AND DISCUSSION

1) The efficiency of azadirachtin extraction from neem kernels was determined by a series of small scale extractions. Of the solvents tried, 95% ethanol was the most effective for the removal of azadirachtin from the kernels (Table 1).

2) The utilization of this estimation technique for monitoring the completeness of azadirachtin removal by extracting neem kernels in 95% ethanol has proven to be very useful. The amount of azadirachtin/g of extract for the 3 successive extracts from the 23 kg batch revealed that azadirachtin was effectively removed (1 - 1.36 µg/g, 2 - 2.72 µg/g, 3 - 0.22 µg/g). The amount of azadirachtin/g of extract for each of the 8 successive extracts from the 91 kg batch also revealed the completeness of azadirachtin extraction.

3) The stability of the kaolin-neem powder formulations was demonstrated by this estimation technique also. It was found that the 1:4 neem:kaolin formulation contained 474 µg azadirachtin/g of extract and 2 months later contained 465 µg/g. Other neem-kaolin

TABLE 1
Extraction of Neem Kernels

Solvent	µg Azadirachtin/10 µL Solvent
95% Ethanol	2.80
Methanol:Water 85:15	2.60
Methanol	2.19
Methylene Chloride	1.73
Ether	1.28
Acetone	0.74

formulations of 10%, 5%, 2%, and 1% were also analyzed and found to be stable with time and room temperature.

This high performance liquid chromatographic technique has been extremely valuable in estimating azadirachtin content. Other applications of this technique could include the determination of azadirachtin yield from different batches of neem kernels; determination of azadirachtin degradation in other neem extracts; determination of azadirachtin content in extracts of other Melia species; and possibly the determination of azadirachtin content in the environment (insects, food, clothing, animals, soil, etc.).

ACKNOWLEDGMENT

We thank Robin L. Trexler, Physical Science Technician, for running the HPLC analyses.

Trade names are used in this publication solely for the purpose of providing specific information. Mention of a commercial or a proprietary product does not constitute a recommendation, guarantee, or warranty of the product by the U.S. Department of Agriculture or an endorsement by the Department over the products mentioned.

REFERENCES

1. Butterworth, J. H., Morgan, E. D. and Percy, G. R., The structure of azadirachtin; the functional groups, J. Chem. Soc. Perkin Trans., 1, 2445, 1972.

2. Zanno, P. R., Miura, I., Nakanishi, K. and Elder, D. L., Structure of the insect phagorepellent azadirachtin. Application of PRFT/CWD carbon-13 nuclear magnetic resonance, J. Am. Chem. Soc., 97, 1975, 1975.

3. Warthen, Jr., J. D., Azadirachta indica: a source of insect feeding inhibitors and growth regulators, USDA, Sci. & Educ. Adm., Agric. Reviews and Manuals, Northeastern Ser. 4, 21 p., 1979, Beltsville, MD.

4. Uebel, E. C., Warthen, Jr., J. D. and Jacobson, M., Preparative reversed-phase liquid chromatographic isolation of azadirachtin from neem kernels, J. Liquid Chromatog., 2, 875, 1979.

5. Stokes, J. B. and Redfern, R. E., Effect of sunlight on azadirachtin: antifeeding potency, J. Environ. Sci. Health, Part A, 17, 57, 1982.

6. Ladd, Jr., T. L., Jacobson, M. and Buriff, C. R., Japanese beetles: extracts from neem tree seeds as feeding deterrents, J. Econ. Entomol., 71, 810, 1978.

7. Meisner, J., Ascher, K. R. S., Aly, R. and Warthen, Jr., J. D., Response of Spodoptera littoralis (Boisd.) and Earias insulana (Boisd.) larvae to azadirachtin and salannin, Phytoparasitica 9, 27, 1981.

8. Reed, D. K., Jacobson, M., Warthen, Jr., J. D., Uebel, E. C., Tromley, N. J., Jurd, L. and Freedman, B., Cucumber beetle antifeedants: laboratory screening of natural products, USDA, Sci. & Educ. Adm., Technical Bulletin No. 1841, 13 p, 1981.

9. Reed, D. K., Warthen, Jr., J. D., Uebel, E. C. and Reed, G. L., Effects of two triterpenoids from neem on feeding by cucumber beetles (Coleoptera: Chrysomelidae), J. Econ. Entomol., 75, 1109, 1982.

10. Webb, R. E., Hinebaugh, M. A., Lindquist, R. K. and Jacobson, M., Evaluation of aqueous solution of neem seed extract against Liriomyza sativae and L. trifolii (Diptera: Agromyzidae), J. Econ. Entomol., 76, 357, 1983.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(3), 599-614 (1984)

SOLUTE RETENTION IN COLUMN LIQUID CHROMATOGRAPHY. I. BINARY NON-ELECTROLYTE MOBILE-PHASE ADDITIVES AT HIGH DILUTION WITH SILICA SORBENT

A.-J. Hsu, R. J. Laub*, and S. J. Madden

Department of Chemistry
San Diego State University
San Diego, CA 92182

ABSTRACT

The two principal models purporting to describe elution behavior are presented, and it is argued that they cannot be distinguished solely on the basis of chromatographic data. A recent modification (so as to describe all known isotherm types) of one of these is fitted in this work to the retentions of five test solutes with silica sorbent and mobile phases comprised of hexane + the additive pairs: THF/MC; ACN/MC; IPA/THF; IPA/MC; ACN/THF; and ACN/IPA over the compositional range 0-0.12% v/v, for which advantages in routine analysis are said to obtain. The fits describe without exception all systems studied to within experimental error, although there is no immediately-apparent correlation of the resultant parameters with the bulk properties either of the solutes or solvents. Nevertheless, the ability to forecast retentions with blended mobile phases from data pertaining to at most four or five measurements is claimed to represent a considerable advance over all other models in use in liquid chromatography at the present time.

INTRODUCTION

There can be little doubt that considerable interest is currently focussed on "high-performance" liquid chromatography. Indeed, the technique has already found extensive application in organic and

*Author to whom correspondence may be addressed.

 0148-3919/84/0703-0599$3.50/0

biochemistry, and is rapidly becoming a major analytical tool in biological and medicinal studies. However, the number of system parameters which may influence lc separations is greater than that e.g., in gc, due primarily to the presence of condensed-state mobile phase. Moreover, and as is true in gc, the sorption process (be this solution or adsorption) remains only poorly understood. For example, Locke (1) re-stated in simplified terms a model of sorption originally developed by Everett (2), and concluded that differences in retentions can be attributed, at least in part, to solute solubility in the mobile phase. It was then argued that log(retention) should be inversely proportional to -log(solute solubility) for homologous series of compounds on the assumption that the support is covered with a monolayer or so of adsorbed solvent, and that solutes desorbed from the mobile phase compete with solvent for adsorption sites during the elution process. However, no account was taken of the undoubted differences between bulk and adsorbed solvent and so, only modest agreement was found when solute bulk solubilities were contrasted with those deduced from lc experiments.

Since the original report by Locke, there have evolved three principal models of retentions in liquid chromatography. The first is that by Snyder (3), whose assumptions parallel those of Locke. He arrived ultimately at the expression:

$$\log K^{o} = \log V_{a} + \alpha(S^{o} - A_{(1)}\epsilon^{o}) \qquad (1)$$

where K^{o} is taken as the solute sorption isotherm ($cm^{3}\ g^{-1}$), defined as the ratio of moles of adsorbed solute per gram adsorbent to moles of solute per cm^{3} of mobile phase; V_{a} is the volume of adsorbed mobile phase per gram adsorbent; α is a function of the adsorbent surface activity; $A_{(1)}$ is the surface area occupied by adsorbed solute; and S^{o} and ϵ^{o} are said to represent energies of adsorption of solute and solvent per unit area of adsorbent at some "standard" activity. Thus, V_{a} and α are properties of the adsorbent; S^{o} and $A_{(1)}$ pertain to the solute; while ϵ^{o} relates to the solvent.

Extension of eqn. 1 to include binary mobile phases requires expansion only of the ϵ^{o} term. The result, for solvents A and S, is given by:

$$\epsilon^{o}_{(M)} = \epsilon^{o}_{(S)} + \frac{\log\left[x_A \; 10^{\alpha A_{(A)}\left(\epsilon^{o}_{(A)} - \epsilon^{o}_{(S)}\right)} + 1 - x_A\right]}{\alpha A} \qquad (2)$$

where as before $A_{(i)}$ refers to a molecular size and x_i (i = A or S) is the mole fraction of the ith component in the mobile phase. Substitution of this relation for ϵ^{o} into eqn. 1 then yields an approximate description of the variation of K^{o} with mobile-phase composition.

It is fair to say that despite the considerable assumptions utilized in simplifying the development of this model, and aside from its limited success in practice, it represents nevertheless the first attempt at a comprehensive description of retentions as a function of mixed solvents in liquid chromatography.

Snyder's model is essentially thermodynamic in nature in that it describes the partition coefficient as a function of the exponent of the partial molar excess free energy associated with transfer of solute from the mobile phase to the stationary phase. However, this approach is limited in that the (differential) thermodynamic properties as formulated pertain only to the **net** interactive forces acting upon the solute when it is distributed between phases. The alternative is description of individual interactions occurring in each phase, which leads to the model proposed initially by Scott and Kucera (4):

$$K^{o}_{R(M)} = \frac{\left[\sum_{i=1}^{n} \Theta_i \; F_i \; P_i \; f(T)\right]_S}{\left[\sum_{i=1}^{n} \Theta_i \; F_i \; P_i \; f(T)\right]_M} \qquad (3)$$

where Θ is a constant which incorporates the probability of contact with stationary S or mobile M phases, F is the magnitude of the force between the solute and the respective phase, P is the probability of molecular interaction, and f(T) is the thermal energy of the molecule at the moment of contact. So long as the stationary phase remains at some state of

constant activity, this arising as a result of sorbed mobile-phase component, the numerator of eqn. 3 will be fixed and so,

$$\frac{1}{K^{o}_{R(M)}} = A + B\,C_A \tag{4a}$$

This can also be cast in terms of volume fractions, viz.,

$$\frac{1}{K^{o}_{R(M)}} = \frac{\phi_A}{K^{o}_{R(A)}} + \frac{\phi_S}{K^{o}_{R(S)}} \tag{4b}$$

where A and B are constants. Eqn. 4 thus is the liquid-chromatographic equivalent of the diachoric solutions hypothesis of Laub and Purnell (5,6):

$$K^{o}_{R(M)} = \phi_A\,K^{o}_{R(A)} + \phi_S\,K^{o}_{R(S)} \tag{5}$$

Eqn. 4 is found to hold for very many systems so long as the concentration of "polar" component of the mobile phase is greater than about 5-10%, i.e., such that the surface of the adsorbent is completely deactivated. In addition, eqns. 3 and 4 are equivalent to eqn. 1 if the latter is cast in terms of fractional surface coverage of the adsorbent by the mobile phase, as brought out clearly in the work of Jandera and Churacek (7), Soczewinski and Golkiewicz (8), Jaroniec and his coworkers (9), and Slaats and colleagues (10). The three models represented by eqns. 1-5 cannot generally be distinguished, therefore, solely on the basis of retention data.

As a result of considerable testing of the above retention relations, very many solute-sorbent systems have now been documented in the literature. All bear strong resemblance to the shapes of (curved) BET adsorption isotherms and, consequently, McCann, Purnell, and Wellington (11), followed by Madden, McCann, Purnell, and Wellington (12), proposed a modification of eqn. 4 with which all six isotherm shapes could be represented. Their result, applicable strictly only to nonionic systems at this time, is given by:

$$\frac{1}{K^{o}_{R(M)}} = \phi_A \left\{ \frac{1}{K^{o}_{R(A)}} + \frac{b\,\phi_S}{1 + b'\phi_S} \right\} + \frac{\phi_S}{K^{o}_{R(S)}} \tag{6}$$

where values of b and b' are derived from an analysis of the experimental data. In every case tested thus far the fits have been excellent (13). However, it must be pointed out that the fitted parameters have yet to be rationalized either from one solute to the next or from one solvent system to another. Nevertheless, the ability to represent generally (hence predict) the variation of solute retentions as a well-defined function of mobile-phase composition represents a very considerable advance.

From the standpoint of analysis, the most useful carrier compositions self-evidently are those which provide the "best" separation in the fastest time. However, the "best" separation frequently is defined in terms of local criteria established by the analyst and which may or may not correspond to this or that general principle. For example, it is often the case that a particular solute elution order is mandated in order to retrieve quantitative data for trace components whose retentions have been adjusted to fall well removed from some major analyte constituent. As a result, the mobile-phase compositions of overriding interest in chemical analysis comprise those with which solute retentions and retention order can most readily be altered. Experience with silica sorbent has shown that these amount in practice to high (ca. 0-0.1%) dilution of this or that **individual** mobile-phase additive such that the surface of the stationary phase is far from completely covered. As a result, it would seem that adjustment of the concentrations of **binary, ternary, ...** additives over this limited range could provide an even more powerful means of control of elution behavior. We have therefore carried out in this work a study of the utility of ternary mobile phases, where two of the components were varied only over the range 0-0.12% v/v. The opportunity was taken as well to explore the limits of application of eqn. 6 with such systems.

EXPERIMENTAL

Solvent Purification. Reagent-grade hexane (wet; a blend of several hexane isomers) was found to contain numerous aromatic impurities which were removed as follows. Concentrated sulfuric acid was added (1:10 v/v) to the hexane and the mixture stirred overnight. The organic layer was then drawn off and treated again if the resultant acid layer was yellow in

color. A solution of 5% sodium bicarbonate was next added (Caution!) to the separated organic layer to neutralize any residual acid and the hexane was then washed to neutrality with water distilled from dichromate solution. Fresh Drierite was added next to remove most of the water, following which dried nitrogen (molecular sieves; calcium chloride or magnesium perchlorate) was passed through the solvent to which had been added metallic sodium and a small amount of benzophenone. (The latter acts in this instance as an indicator and changes from light yellow to dark blue upon consumption of the last traces of moisture). Finally, the organic solvent was refluxed for several hours under nitrogen until it turned dark blue, following which it was distilled and stored over molecular sieves.

Mobile-Phase Preparation. Solutions of hexane as treated above were mixed with the six distilled and pre-dried (molecular sieves) additive pairs: tetrahydrofuran (THF)/methylene chloride (MC), acetonitrile (ACN)/MC, iso-propyl alcohol (IPA)/THF, IPA/MC, ACN/THF, and ACN/IPA over the volume-percent range of each of 0-0.12% in steps of 0.024%.

Test Solutes. The test samples were polycyclic aromatic hydrocarbons (PAH) dissolved in hexane and comprised naphthalene (1), anthracene (2), fluoranthene (3), pyrene (4), and benzo(a)anthracene (5). Injection of these individually or in admixture confirmed that retention times were independent of the manner of sample introduction.

Instrumentation. The liquid chromatograph used throughout this work was a Varian Model 5000. The detector was an LDC UV Monitor (254 nm) or a Varian Vari-Chrom variable wavelength UV monitor set at 254 nm, very little difference being found between the two units. The column was a Varian Micro-Pak Si-5 (5 um silica), and was 30 cm by 4 mm id. It was thermostated at constant temperature (25°C) with a water-jacket. The injector was a Valco valve with a 10-ul external loop.

Procedure. The column was allowed to equilibrate with each new mobile phase (3 cm^3 min^{-1} flow rate) until retention times were constant. Dead volumes were then measured by injection of 10-ul amounts of pentane or of iso-octane, a small blip being obtained in the recorder trace in each case.

TABLE 1

Equilibration Times/min for Listed Additive (A + B) Systems[a]

		% v/v B					
A	B	0.0	0.024	0.048	0.072	0.096	0.120
THF	MC	15	30	30	30	30	60
ACN	MC	30	30	30	50	60	60
IPA	MC	15	30	30	50	50	70
ACN	THF	15	20	20	30	45	95
IPA	THF	10	20	20	30	45	95
ACN	IPA	50	55	45	45	40	35

[a]tetrahydrofuran (THF), methylene chloride (MC), acetonitrile (ACN), and iso-propyl alcohol (IPA)

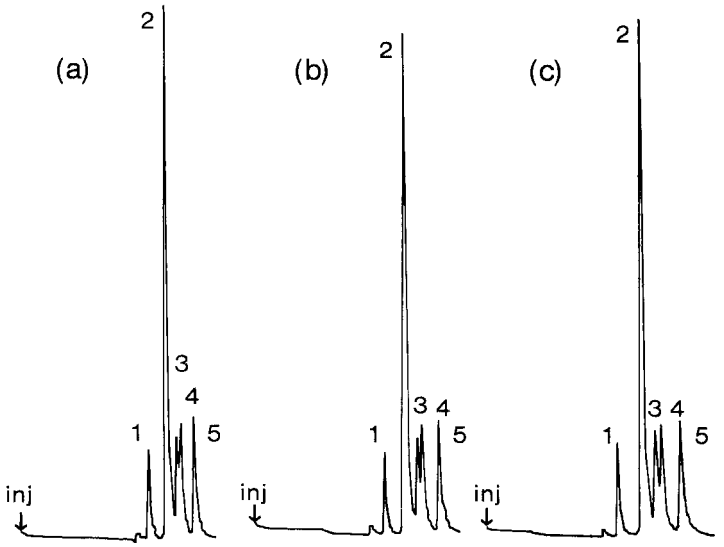


FIGURE 1

Illustration of the variation of solute retention times (ca. 5 min for the last-eluting compound) prior to equilibration of stationary phase with new mobile phase (here, 0.024% v/v IPA + 0.096% THF).

RESULTS AND DISCUSSION

Equilibration Time. Our first concern was with the time required to establish equilibrium surface coverage of the silica sorbent with each combination of mobile phases, this being of particular importance in obtaining reproducible results in gradient elution. The consequences of slow kinetics of displacement of one component by another have been studied and commented upon at length by Scott and Kucera (4), and our results establish that their findings hold equally true in the instance of high dilution of mobile-phase additives. Table 1 presents the equilibration times for all binary-additive/hexane mixtures used in this work. The first five sets of these required increasing times in accordance with solvent "strength" while, in contrast, the pair ACN/IPA gave the opposite result (where the equilibration time decreased from 50 min to 35 min on passing

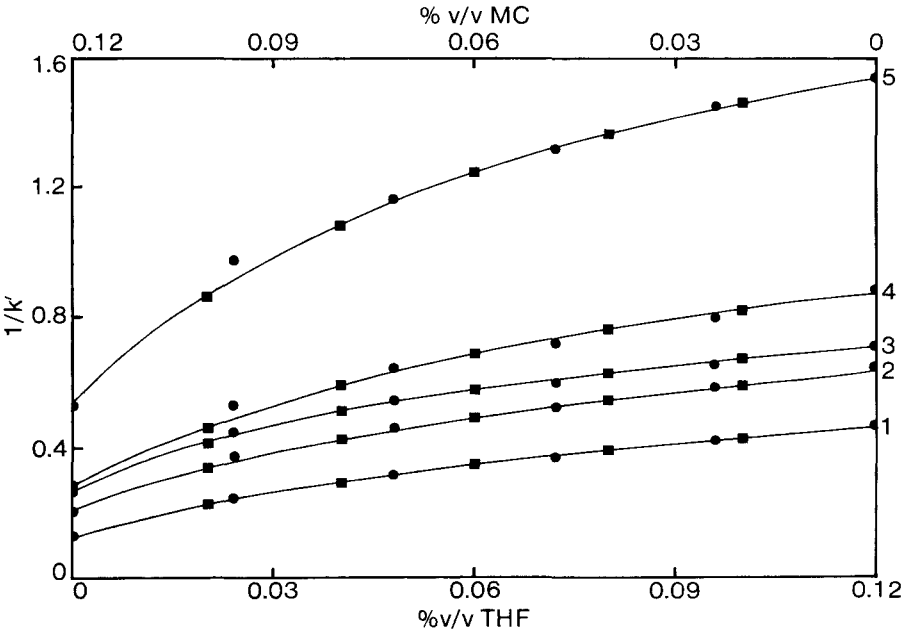


FIGURE 2

Plots of inverse capacity factor against composition (volume percent) of the indicated mobile-phase additive pairs for the solutes: (1) naphthalene, (2) anthracene, (3) pyrene, (4) fluoranthene, and (5) benzo(a)pyrene. Circles: experimental data; squares and solid curves: eqn. 6.

from 0.12% ACN to 0.12% IPA). The situation is illustrated further in Figure 1, where the chromatograms resulting from three successive injections of the test mixture prior to the establishment of column equilibrium are shown (0.024% IPA + 0.096% THF). There is a noticeable improvement in the separation of solutes 3 and 4 on passing from (a) to (c), while the absolute retention time for the last-eluting compound increased roughly by 10%. We also observed that the equilibration times in Table 1 were independent of whether or not the solute test mixture was injected during the course of a particular determination, which would seem to lend weight to a model (4) of solute interaction with sorbed mobile-phase constituent as opposed to one of displacement (3).

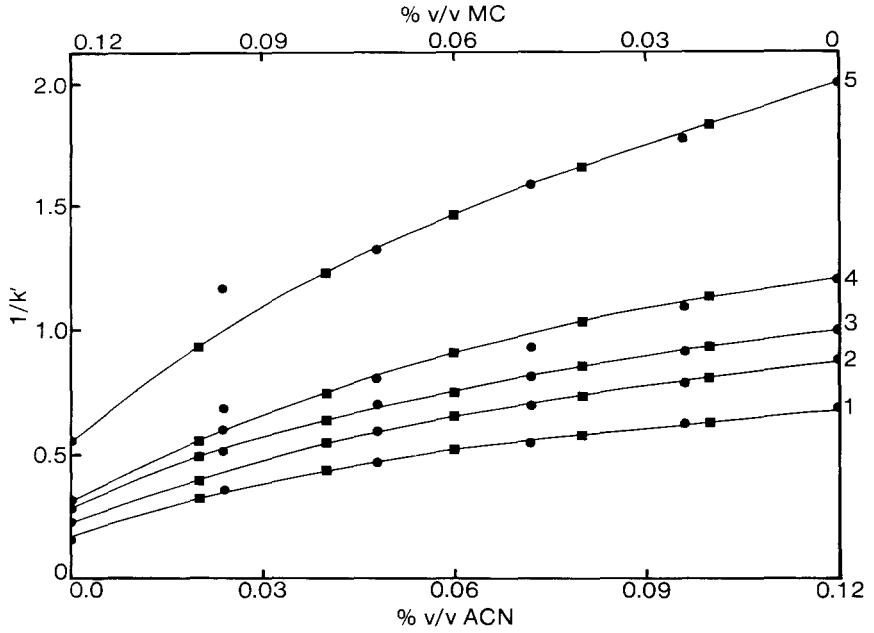


FIGURE 3. As in Figure 2.

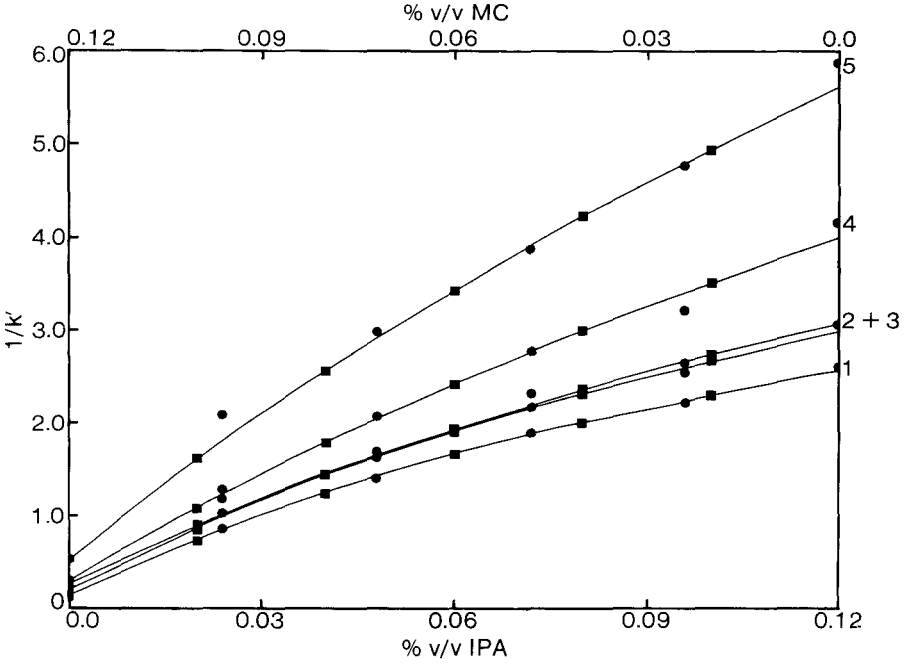


FIGURE 4. As in Figure 2.

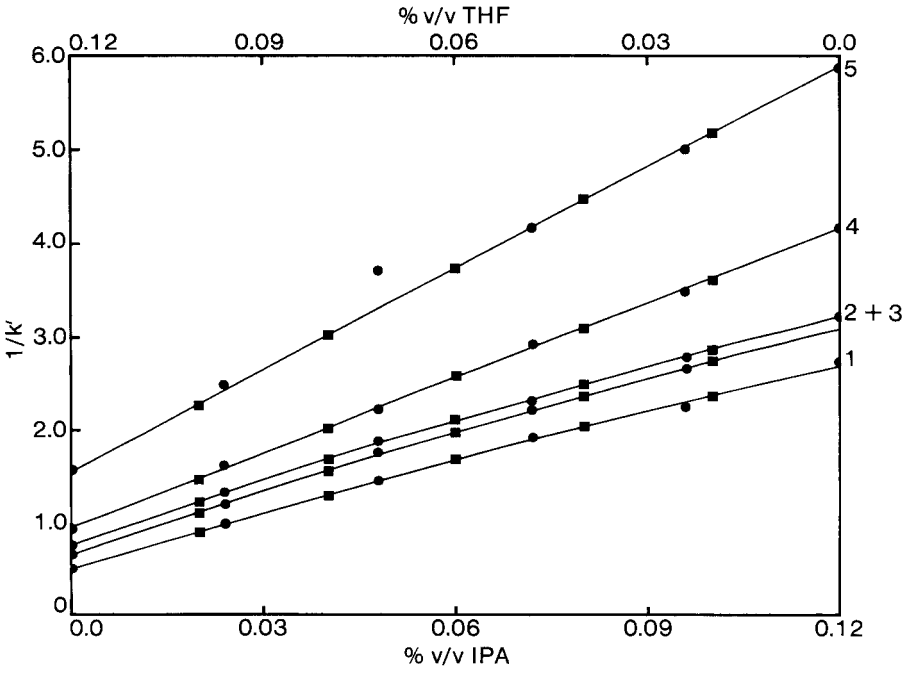


FIGURE 5. As in Figure 2.

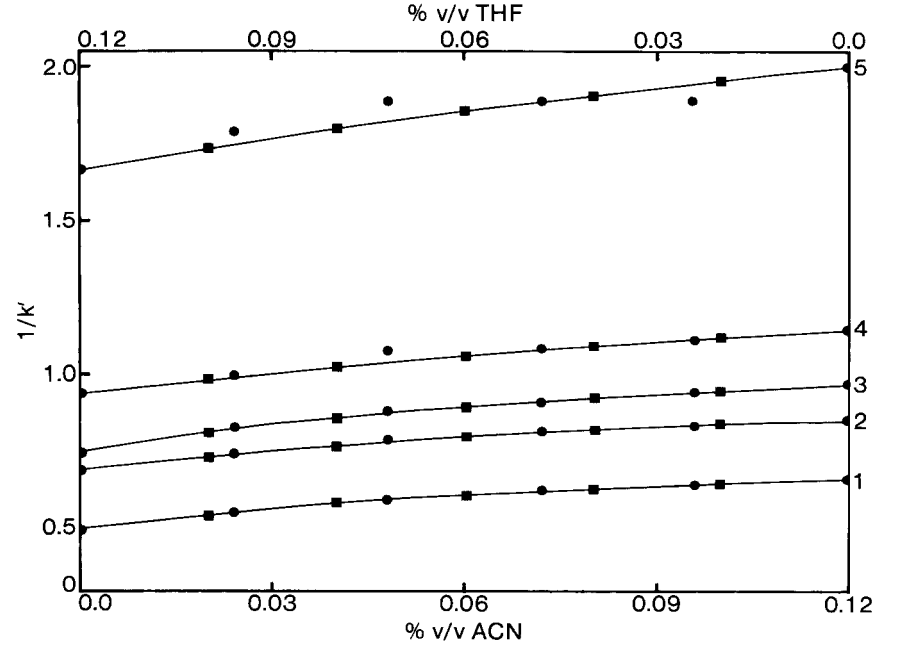


FIGURE 6. As in Figure 2.

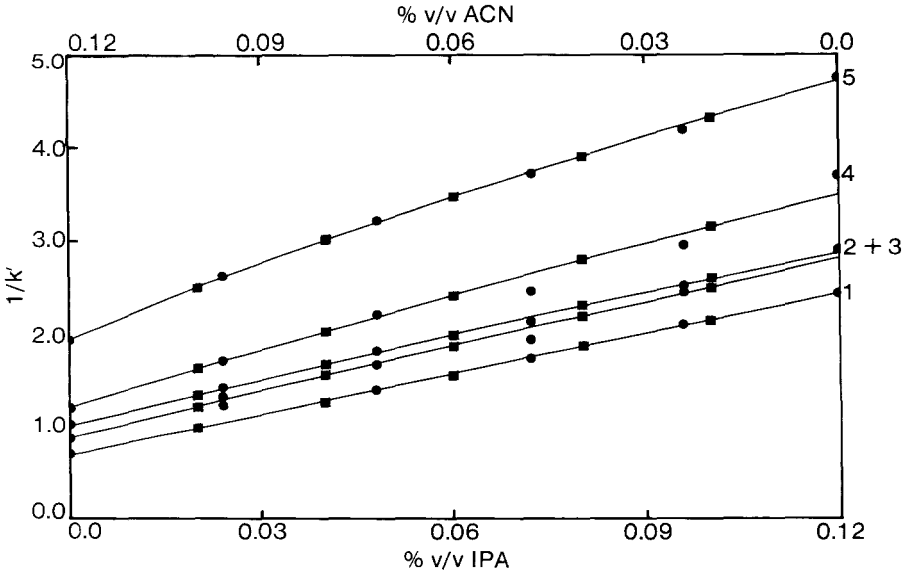


FIGURE 7. As in Figure 2.

Graphical Presentation and Interpretation of Retention Data. Figures 2-7 provide plots of reciprocal solute capacity factor against ϕ for the indicated mobile-phase additive pairs over the range of each of 0-0.12%. The filled circles represent the experimental data ($\pm$ ca. 3%) while the squares were generated from best-fits of eqn. 6 with which were constructed also the solid curves.

The plots in Figures 2-4 exhibit substantial curvature, while those in Figures 5-7 are very nearly linear. Moreover, there is in some instances a considerable improvement in the resolution of pairs of solutes upon alteration of the binary-additive composition, as found for example in Figures 2 and 3. In contrast, the separation of solutes 2 and 3 is consistently poor with IPA/MC (Figure 4), improved somewhat with IPA/THF (Figures 1 and 5), and decreases from left to right on passing from ACN to IPA modifier (Figure 7). Thus, retentions are indeed sensitive to minute changes in the type and make-up of mobile-phase additive even at levels of the latter of 0.1%.

Table 2

Best-Fit Parameters of Eqn. 6

Solvent Pair		Solute				
		Naphthalene	Anthracene	Pyrene	Fluoranthene	Benzo(a)pyrene
THF/MC	b	2103.	1087.	720.2	700.5	526.8
	b'	1247.	969.0	940.3	810.4	770.2
ACN/MC	b	2176.	1500.	1190.	1057.	928.0
	b'	696.0	800.0	828.0	791.0	984.0
IPA/MC	b	5741.	4237.	3329.	3758.	3449.
	b'	297.2	302.6	337.3	516.7	586.3
ACN/THF	b	406.1	252.3	364.8	278.5	295.1
	b'	352.6	366.0	823.9	854.3	914.9
IPA/THF	b	3754.	2731.	2510.	2397.	2121.
	b'	30.26	17.39	177.2	147.5	129.5
IPA/ACN	b	3020.	2222.	1738.	1767.	0.
	b'	251.9	123.2	104.1	80.40	0.

We note in passing that the capacity factors (hence times of analysis) are substantially altered in each figure. For example (Figure 2), k' for benzo(a)anthracene (no. 5) is 1.9 with 0.12% MC yet is reduced to 0.65 with 0.12% THF, an improvement of a factor of 3. On the other hand, this change induces a higher number of theoretical plates required to effect a separation (14), the increase demanded amounting also to a factor nearly of 3. The gain achieved in analysis time may well then be lost if the system efficiency is incapable of the higher required resolving power. The use of highly-dilute binary mobile-phase additives thus permits optimization in terms both of resolution and analysis time even in the case of the very simple example presented here (15). (The optimum turns out in fact to be 0.12% ACN.)

Fitted Parameters of Eqn. 6. We present in Table 2 the derived values of b and b' for all systems studied. We note at the outset that these are moderately insensitive on a relative basis, that is, if for a particular system b is increased by 10% b' can then be decreased by the same amount without affecting to any great extent the goodness of fit of the experimental data. Even so, we have thus far been unable to formulate a correlation of the values in terms either of solute or solvent bulk properties. Nevertheless, there is no question that eqn. 6 provides an accurate fit of all retention data with each of the six binary-additive pairs, hence the means of prediction of separations, which we intend taking up further in subsequent reports.

ACKNOWLEDGMENT

We gratefully acknowledge support received for this work from the Department of Energy.

REFERENCES

1. Locke, D. C., **J. Chromatogr. Sci., 12,** 433, 1974.

2. Everett, D. H., **Trans. Faraday Soc., 61,** 2478, 1965.

3. Snyder, L. R., **Principles of Adsorption Chromatography,** Marcel Dekker, New York, 1968.

4. Scott, R. P. W., and Kucera, P., **Anal. Chem., 45**, 749, 1973; **J. Chromatogr. Sci., 12**, 473, 1974; **13**, 337, 1975; **J. Chromatogr., 112**, 425, 1975; **122**, 35, 1976; **149**, 93, 1978; **171**, 37, 1979.

5. Laub, R. J., and Purnell, J. H., **J. Am. Chem. Soc., 98**, 30,35, 1976; Purnell, J. H., and Vargas de Andrade, J. M., **J. Am. Chem. Soc., 97**, 3585,3590, 1975.

6. Of special note is the peculiar notion put forth by Snyder and Poppe (**J. Chromatogr., 184**, 363, 1980) that eqn. 5 (hence, 3 and 4) violates the Gibbs-Duhem relation and that it cannot therefore be considered to be rigorously valid under any circumstances. Self-evidently, this argument is specious on several grounds, as pointed out by Laub (in **Physical Methods in Modern Chemical Analysis**, Vol. **3**, T. Kuwana, Ed., Academic Press, New York, 1983, Ch. 4). First of course, since the Gibbs-Duhem relation is formulated in terms of mixed **solvents**, it offers no information regarding the variation of infinite-dilution activity or partition coefficients of probe **solutes** as a function of blended-phase composition. Secondly, since in the diachoric solutions formulation the solvent components are assumed to be immiscible the activity coefficients of each must be taken to be unity over all compositions. The Gibbs-Duhem relation is thereby obeyed exactly although, since d ln γ_i = 0, little further insight is gained. The point thus emphasizes that for the purposes of eqns. 4 and 5, there is in fact no difference between completely ideal mixtures and those whose (diachoric) components are completely demixed.

7. Jandera, P., and Churacek, J., **J. Chromatogr., 91**, 207,223, 1974; **93**, 17, 1974; **104**, 257, 1975; Chandera, P., Churacek, J., and Janderova, M., **J. Chromatogr., 115**, 9, 1975.

8. Soczewinski, E., **Anal. Chem., 41**, 179, 1969; Soczewinski, E., and Golkiewicz, W., **Chromatographia, 6**, 269, 1973.

9. Jaroniec, M., Narkiewicz, J., and Borowko, M., **Chromatographia, 11**, 581, 1978; Narkiewicz, J., Jaroniec, M., Borowko, M., and Patyrkiejew, A., **J. Chromatogr., 157**, 1, 1978.

10. Slaats, W. M., Kraak, J. C., Brugman, W. J., and Poppe, H., **J. Chromatogr., 149**, 255, 1978.

11. McCann, M., Purnell, J. H., and Wellington, C. A., in **Chromatography, Equilibria, and Kinetics**, Faraday Society Symposium No. 15, D. A. Young, Ed., The Royal Society of Chemistry, London, 1980, p. 82.

12. Madden, S. J., McCann, M., Purnell, J. H., and Wellington, C. A., paper presented at the 184th National Meeting of the American Chemical Society, Kansas City, Missouri, 1982; Madden, S. J., Ph.D. Thesis, University College of Swansea, Swansea, Wales, 1983.

13. It is important to note that in the studies cited the abscissa covered the entire compositional range, pure S to pure A. It is also significant

that in all but two of the six types (V and VI) linearity of the plots extended over a considerable range; any theoretical attempt to derive eqn. 6 hence must take this into account.

14. Purnell, J. H., **J. Chem. Soc.**, 1268, 1960.

15. There is the additional practical advantage that since the modifiers are used at such low concentration the column efficiency and pressure drop remain nearly invariant.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(3), 615-637 (1984)

SOLUTE RETENTION IN COLUMN LIQUID CHROMATOGRAPHY. II. OPTIMIZATION OF MOBILE-PHASE COMPOSITIONS

A.-J. Hsu, R. J. Laub*, and S. J. Madden

Department of Chemistry
San Diego State University
San Diego, CA 92182

ABSTRACT

Retentions are reported for nine phenol solutes with a reverse-phase column packing and acidified aqueous mobile phases containing tetrahydrofuran, acetonitrile, and methanol additives. Plots of $\log(k'_{(M)})$ against additive volume fraction ϕ exhibit both positive and negative deviations from linearity. In contrast, plots of inverse capacity factor against ϕ are in every instance convex to the abscissa. The latter, as well as several systems comprising ionic solutes and/or solvents, are fitted exactly by a semi-empirical relation; however, only one of the fitted parameters appears to exhibit identifiable trends and then only very approximately so. It is deduced by simple inspection of the plots and without resorting to higher-component carriers, then verified experimentally, that the test mixture is completely separable with 30% methanol. Window diagrams of α, S_f, and R_s ordinates are used to identify alternative mobile phases and compositions in the instance that one of the solutes is present in large excess. The three methods of data reduction prove to be entirely equivalent since the solute capacity factors approach 10. When this is not the case, window diagrams constructed with the separation-factor parameter are said to be favored.

INTRODUCTION

The analytical utility of mixed phases is firmly established at the present time both in gas chromatography (gc) and in variants of liquid

*Author to whom correspondence may be addressed.

 0148-3919/84/0703-0615$3.50/0

chromatography (lc). Moreover, much of the sucess of resultant systems optimization strategies (1) is due directly to the formulation of functions which describe quantitatively, that is, within reasonable limits of experiment, the variation of solute retentions with composition of the respective phase. For example, it has been argued on various occasions (see preceding paper) that inverse retentions vary in a well-defined fashion with concentration (in whatever units) of one or another of the mobile-phase components in lc, while many have shown that with few exceptions, solute specific retention volumes, capacity factors, and partitition coefficients are additive in stationary-phase weight- or volume-fraction compositional units in gc. Indeed, novel interpretations inter alia of solutions thermodynamics notwithstanding, the preponderance of reputable experimental evidence derived to date points strongly in the direction of relations appropriate for lc which are in fact intimately related to (if not directly based upon) those pertinent to gc (2).

Upon selection of the appropriate description of partitioning, the only valid criterion for which self-evidently being faithful representation of the experimental data, optimization of the relevant system parameter(s) in terms of maximal separation in minimal time of the solutes at hand is straight-forward. Since the initial report of the data-reduction procedure in 1975 by Laub and Purnell (christened by them the window-diagram strategy) (3), a number of variants of the optimization technique has been detailed. For example, Deming and Turoff (4) utilized the methodology for optimization of mobile-phase pH for the lc separation of benzoic acids, while Sachok, Kong, and Deming (5) later extended its use to include multiple and interdependent system variables. The strategy has also been employed in mobile-phase gradient elution (6,7), the most promising approach being that taken by Edwards and Wellington (8).

Recently, Glajch, Lehrer, and colleagues (9) and Issaq and coworkers (10) have shown that substitution of a triangular coordinate system for one of three-dimensional Cartesian coordinates also provides the means of optimization (window diagrams) of ternary- (or higher-) component lc mobile phases. However, since the results from one coordinate system self-evidently must be identical to those derived from the other, there

appears to be little from which to choose with regard to each other than on the basis of convenience of presentation.

The separation factor S_f derived by Jones and Wellington (11) appears to offer considerable advantage when used as the (dependent) ordinate parameter for representation of separations in lc, where $S_f = 2R_s/N^{1/2} = (t_{R2} - t_{R1})/(t_{R2} + t_{R1})$. Thus, measurement is no longer required of column void volumes, which frequently proves to be troublesome (12), nor of peak baseline-widths, as required for utilization of R_s.

Nevertheless, there remains some controversy with regard to each of these aspects of liquid chromatography. We have therefore examined in this work, first, appropriate relations for graphical representation of retentions in terms of mobile-phase compositions and, secondly, the accuracy of the resultant window diagrams constructed with values of relative retentions α, separation factors S_f, and resolution R_s.

The analysis of phenols with reverse-phase packings was chosen as the test system, in part because of the report by Lehrer (9) where it was claimed that a quaternary-component solvent was required for resolution of a few simple phenolic compounds, and in part because the separation of these species continues to be of considerable interest in a variety of fields. For example, phenols are known to be present in hardwood smoke used in curing meat and fish products which incorporate the compounds directly. Thus, heavily-smoked bacon has been shown to contain up to 250 ppm of phenols (13). It has also been reported that they can inhibit (14,15) or accelerate (16-18) the formation of carcinogenic nitrosamines depending upon the reaction conditions.

Chlorinated phenols are of course also of intense interest at the present time, these having been used extensively over the years as insecticides, fungicides, antiseptics, and disinfectants. Moreover, effluents from pulp and paper industries are known (19) to contain derivatives of guaiacol, catechol, and trihydroxybenzes (20-22). However, difficulties are often encountered in the separation of these and related materials due to the variety of positional isomers. We therefore intend in this work to provide also at least a basis for choice of systems and conditions pertinent to their analysis.

THEORY

Retention and selectivity with "reverse-phase" systems are taken generally to be more complex than with "normal" (i.e., underivatized) sorbents. In point of fact in either case, the actual retention mechanism(s) remain largely undefined. Nevertheless, there is today agreement, broadly speaking, that retention and selectivity are related in the former mode of lc to carbon chain length (23-27), surface coverage (carbon content) (25-31), eluent composition and type (23,26,27,31-38), solute "polarity" (31,39), and column temperature (32-34,40). In particular, equations of the general form:

$$\log k'_{(M)} = \log k'_{(S)} - S\,\phi_A \qquad (1)$$

have been observed by a number of workers (31,36,39-51) to describe retentions with specifically-defined reverse-phase systems comprising aqueous mobile phases with organic additives, where $k'_{(S)}$ is the solute capacity factor with pure water eluent, ϕ_A is the volume fraction of organic modifier, and S is an empirically-derived constant for the system at hand. For a given column and modifier, S appears to be approximately constant for solutes of varying molecular size and structure. However, it is found to vary somewhat from one additive to the next as well as with the type of reverse-phase sorbent (e.g., different chain length of surface-attached organic species). Schoenmaker and his coworkers (41) have also noted that eqn. 1 is not strictly obeyed by the systems studied by them, where their plots of $\log k'_{(M)}$ against ϕ_A were slightly concave to the abscissa. Further work by others has since verified the approximate nature of the relation (52).

A model purporting to account for interactions between the analyte and each phase has alternatively been proposed wherein the solute inverse capacity factor is said to be related to mobile-phase composition in the manner (30,53-56; see also preceding paper):

$$\frac{1}{k'_{(M)}} = \frac{\phi_A}{k'_{(A)}} + \frac{\phi_S}{k'_{(S)}} \qquad (2)$$

where the subscripts A and S pertain to a pure mobile phase. Thus, eqn. 2 predicts that $1/k'_{(M)}$ will vary linearly with ϕ_A (= $1 - \phi_S$), which is

frequently borne out in practice. However, the relation is by no means universally applicable. As a result of study of very many systems that in fact give plots of $1/k'_{(M)}$ which are curved, Madden, McCann, Purnell, and Wellington (57,58) have recently proposed a modification of eqn. 2 that applies also to the "normal" mode of lc:

$$\frac{1}{k'_{(M)}} = \phi_A \left\{ \frac{1}{k'_{(A)}} + \frac{b\ \phi_S}{1 + b'\ \phi_S} \right\} + \frac{\phi_S}{k'_{(S)}} \qquad (3)$$

The equation has in all cases studied thus far been shown to account for the (at-times sharp) curvature found with many systems at either end of the mobile-phase volume-fraction range as well as those which show curvature over the entire compositional span. However, the values of b and b' must at the present time be deduced from experimental data. Moreover, there has yet to be provided a rationalization of these in terms of properties either of the solutes or solvents. Thus, this work is intended as well not only to explore the applicability of eqns. 2 and 3 but in addition, the derivation and interpretation in particular of values of b and b' in terms of compound structure, elution order, and composition and type of mobile-phase additive.

EXPERIMENTAL

Solvents. Spectrophotometric-grade methanol (MeOH) and acetonitrile (ACN) mobile-phase components (A) were employed as received, while reagent-grade tetrahydrofuran (THF) (A) was distilled prior to use. Demineralized and doubly-distilled water + reagent-grade acetic acid comprised the solvent component (S).

Mobile-Phase Preparation. Acetic acid at the level of 1% v/v was added to the water component of the mobile phase in order to diminish any interactions between residual unreacted or accessible silanol groups of the reverse-phase packing as well of course to supress dissociation of the phenols, and did indeed sharply reduce peak asymmetry. Solutions of the aqueous blend with organic modifier were then prepared by volume over the additive concentration range of 25-45% (MeOH) or 20-35% (ACN and THF) at intervals in each case of 5%.

TABLE 1

Test-Solute Properties[a]

No.	Name	M/daltons	m.p./°C	b.p./°C	pK_a (25°C)
1	Phenol	94.11	41.8	182.	9.99
2	p-Nitrophenol	139.11	114.	-	7.16
3	2,4-Dinitro-phenol	184.11	114.	-	4.09
4	o-Chlorophenol	128.56	7.	175.6	8.48
5	o-Nitrophenol	139.11	45.	216.	7.21
6	2,4-Dimethyl-phenol	122.17	27.8	211.5	10.45
7	4,6-Dinitro-o-cresol	198.06	86.5	-	-
8	4-Chloro-m-cresol	142.52	66.	235.	-
9	2,4-Dichloro-phenol	163.00	45.	210.	7.51

[a]**CRC Handbook of Tables for Organic Compound Identification,** Third Ed., Rappoport, Z., Ed., CRC Press, Boca Raton, Florida, 1979.

Solutes. Solutions of phenols in methanol comprised the test samples, these being phenol (1), p-nitrophenol (2), 2,4-dinitrophenol (3), o-chlorophenol (4), o-nitrophenol (5), 2,4-dimethylphenol (6), 2,4-dinitro-o-cresol (7), 4-chloro-m-cresol (8), and 2,4-dichlorophenol (9). Their properties are provided below in Table 1.

Instrumentation. The liquid chromatograph employed in this work was a Varian Model 5000 with an LDC UV Monitor or Vari-Chrom variable-wavelength UV detector, each being utilized at 254 nm. The injector was a Valco valve fitted with a 10-ul external loop, while the column was 25 cm by 4.5-mm id of 8 um Zorbax C_8. Both the injector and column were immersed in a water-bath, the temperature of which was maintained constant at 50°C. The mobile phase was also brought to this temperature prior to entering the column.

Procedures. The column was allowed to equilibrate with each new mobile phase under constant flow until the solute retention times remained

constant. Dead volumes, which were found to vary with mobile-phase composition (see below), were then calculated from the flow rate and the retention time of the methanol solvent peak.

RESULTS AND DISCUSSION

Column Dead Volume. The values of V_A found in the present work are provided in Table 2, the smallest being that with 40:60 (v/v) THF and the largest with 25:75 MeOH. In general, depending upon the presumed-unretained marker compound employed, the apparent void volume of the column is expected to vary as a function of surface coverage of the mobile-phase additive, as pointed out inter alia by Boehm and Martire (2) and by Billiet, Van Dalen, Schoenmaker, and De Galan (59). That is, a void volume calculated from chromatographic measurements may well be larger than the true value, the excess comprising the sum of contributions arising from interactions with sorbed mobile-phase additive as well as those with underivatized surface silanol groups and the chemically-bound alkyl groups. Nor, clearly, would isotope marking, as in the work of McCormick and Karger (60), speciate the individual contributions (61).

Presumably, retentions for methanol would be largest in those instances, first, where the surface is least covered by additive, i.e., available for sorption and, secondly, when interactions with sorbed additive are most favored. In general, surface coverage of hydrocarbon reverse-phase packings would be expected to be smallest with MeOH, increased with ACN, and highest with THF. The trends followed in Table 2 appear to bear out this notion; thus, at 25% mobile-phase composition, V_A is largest for MeOH (smallest surface coverage; most-favorable interaction with injected MeOH), decreases with ACN, and is lowest for THF (highest surface coverage; least-favored interaction with injected MeOH). In addition, and without exception, V_A decreases upon increasing concentration (surface coverage) of additive irrespective of its identity.

From the standpoint of analysis, the spread on V_A in Table 2 (ca. 8% of the average of the extremes, 2.82 cm^3) emphasizes the utility of separation factors rather than values of alpha in calculating window diagrams, since the dead volume need not be determined with the former.

TABLE 2

Apparent[a] Column Dead Volumes V_A/cm^3

Composition, % v/v	Organic Additive		
	MeOH	ACN	THF
20		2.94	2.91
25	3.03	2.88	2.76
30	2.94	2.84	2.68
35	2.91	2.78	2.64
40	2.91	2.72	2.61
45	2.91		

[a]MeOH solvent peak; flow rate 3 cm^3 min^{-1}

The same holds true also in calculating the resolution, where $R_s = (t_{R2} - t_{R1})/w_m$ and where w_m is the peak mean baseline width. That is, the apparent dead volume (or time) does not enter into the calculations. Nor is the matter a trivial one since the apparent capacity factors of the phenol solutes (see later) are in many instances less than 2.

Graphical Representation of Retention Data. Plots of $1/k'_{(M)}$ against % v/v mobile-phase additive are shown for all systems studied in Figures 1-3. All are clearly convex to the abscissa to varying degrees, and eqn. 2 must therefore be said not to hold in these instances. We also show plots of $\log(k'_{(M)})$ against ϕ_A for the phenol solutes with THF additive in Figure 4. Both convex and concave curves as well as straight lines were found and, in addition, solute no. 5 appeared to pass through a point of inflection. The log plots for all solutes with MeOH and ACN gave, respectively, concave and convex curves. Hence, eqn. 1, too, is not obeyed for the systems studied here. Nor can the curves be ascribed to inaccuracies in the dead-volume determinations, since the apparent capacity factors e.g. for solutes 7-9 were often in excess of 20. In order to force conformity of the data either to eqns. 1 or 2, we would therefore have to assume that the dead volume pertinent to these solutes varied with mobile-phase composition from 0 to in excess of 10 cm^3. This seems unlikely, since the total volume of the empty column tube was only 3.9 cm^3 (61).

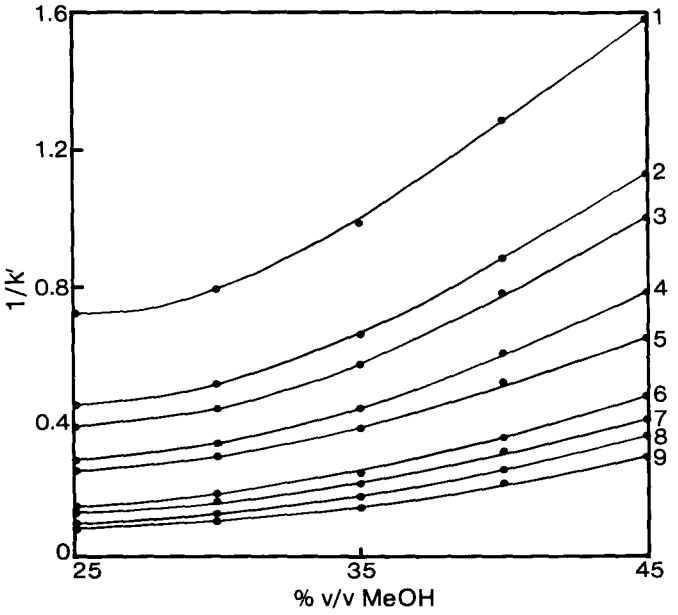


FIGURE 1

Plots of inverse capacity factors of solutes of Table 1 against volume percent MeOH additive. Curves constructed with fitted parameters of eqn. 3 (cf. Table 3).

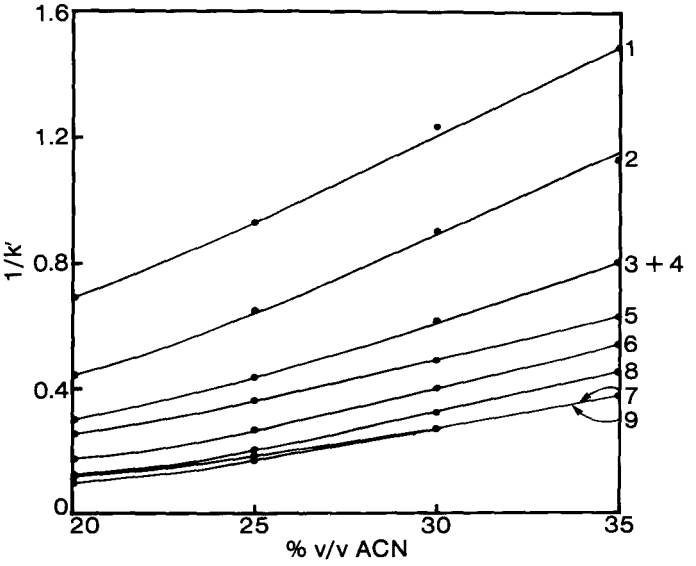


FIGURE 2. As in Figure 1; acetonitrile additive.

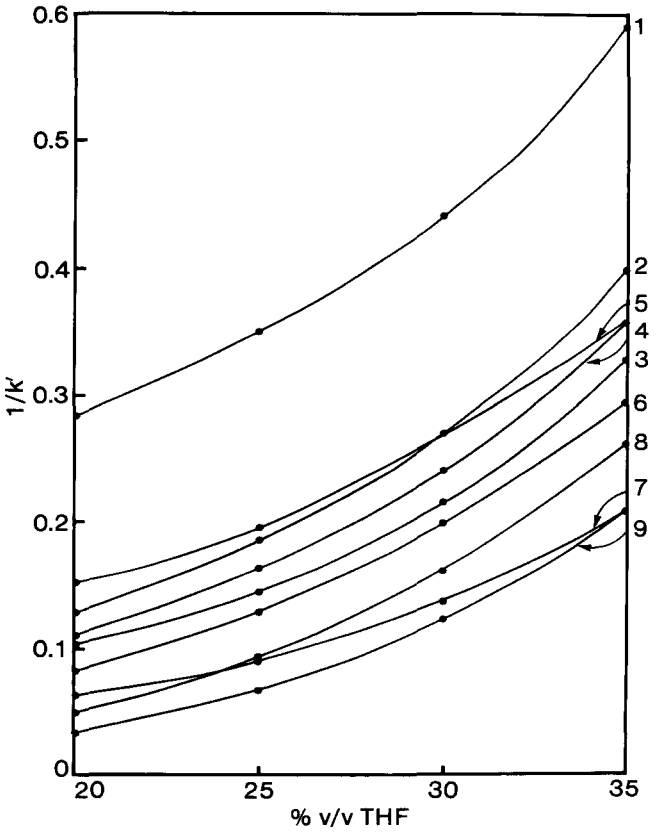


FIGURE 3. As in Figure 1; tetrahydrofuran additive.

The data of Figures 1-3 (solid curves in each) were found to be fitted exactly by the semi-empirical relation eqn. 3, the values of b and b' being presented in Table 3. However, there is no immediately-apparent correlation of these either with solute molecular weight or with pK_a (cf. Table 1). Nevertheless, and while b' appears to vary randomly, the values of b, excluding phenol solute and in contrast to those presented in the preceding paper are, very roughly, constant from one solvent system to the next. In addition, the **averaged** values are nearly identical with MeOH and ACN (ca. -0.3), but are diminished by a factor of 3 with THF (ca. -0.1). This seems

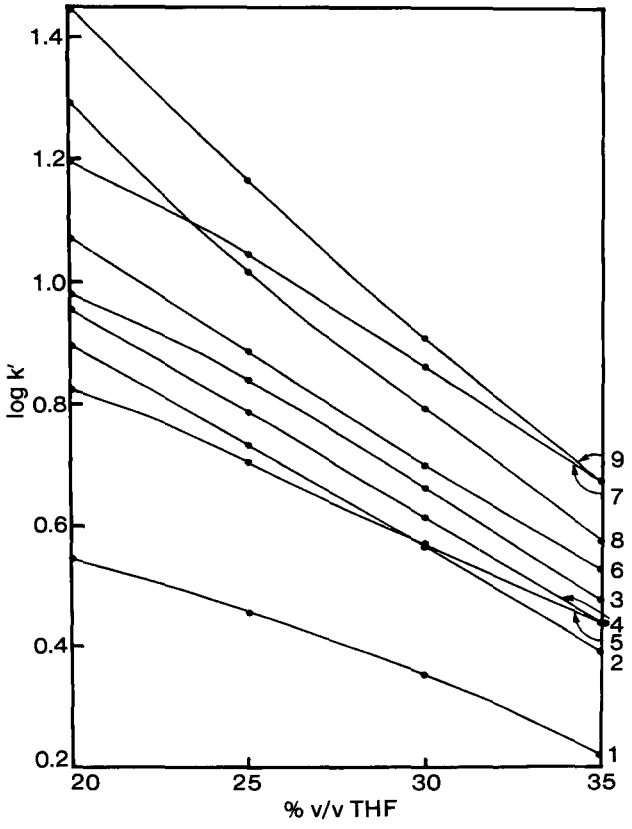


FIGURE 4

Plots of $\log(k'_{(M)})$ against volume percent THF additive for solutes of Table 1.

to suggest that b reflects, at least to some extent, the degree to which solutes interact with additive sorbed on the surface of the stationary phase (62). For example, the values of pK_a for p- and o-nitrophenol are virtually identical yet the latter is eluted much more quickly than the former with mobile phases containing either MeOH or ACN. On the other hand, the solutes nearly coelute with THF additive. Presumably, intramolecular hydrogen bonding with the latter solute diminishes its interactions with sorbed MeOH and ACN but, since hydrogen bonding with THF is small to

TABLE 3

Fitted Parameters of Eqn. 3

	Organic Additive					
	MeOH		ACN		THF	
Solute No.	-b	b'	-b	b'	-b	b'
1	1.23	2.06	0.325	6.27	0.135	-0.644
2	0.650	0.410	0.338	4.33	0.124	-0.522
3	0.554	0.395	0.229	1.89	0.142	-0.338
4	0.410	0.123	0.229	1.89	0.117	-0.426
5	0.298	0.215	0.376	11.6	0.122	-0.379
6	0.275	0.268	0.395	6.18	0.098	-0.239
7	0.219	0.115	0.109	1.50	0.092	-0.241
8	0.197	-0.156	0.275	3.22	0.113	-0.235
9	0.184	-0.146	0.345	8.19	0.099	-0.354

begin with, the compounds are eluted together with this carrier modifier. However, we can infer little else from the data at this time, and the situation thus remains as one which invites further and comprehensive study.

Separation of the Test Mixture. The retention orders of the phenols are altered in several instances on passing from one organic modifier to another. For example, solutes 3 and 4 are well separated with MeOH additive (Figure 1), overlap completely with ACN (Figure 2), and elute with order reversal with THF (Figure 3). However, **by inspection**, and after having examined only three solvent systems (utilizing only four or five compositions of each), we see immediately that the **binary** MeOH system offers the best separation in the fastest time. With the flow rate fixed at 3 cm^3 min^{-1} (in order to compare the results with those of ref. 9), and utilizing Purnell's relation (63) for the number of plates N_{req} requisite for a particular separation at a given a and capacity factor, we deduce further that a methanol concentration of 30% should provide baseline resolution of all solutes with the order of elution being compounds 1 through 9 in numerical sequence. The **first-time** chromatogram is shown in Figure 5 and corresponds very nearly exactly to that predicted (see also ref. 10).

Alternative Mobile-Phase Combinations. Selection of a mobile phase for separation of simple mixtures of solutes, such as the test sample

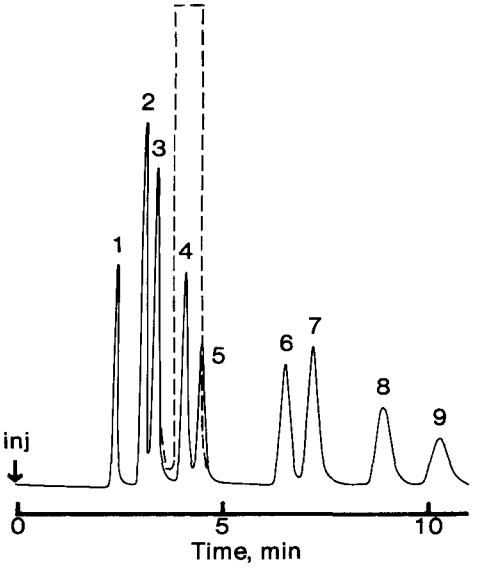


FIGURE 5

First-time chromatogram of the solute test mixture with mobile phase containing 30% MeOH additive. Dashed curve corresponds to hypothetical large amount of solute no. 4.

utilized above, generally presents no real difficulties. More often than not an analyst has simply to plot the retentions in accordance with some or other preferred method of data reduction (e.g., eqns. 1-3) and deduce the optimal mobile-phase composition by inspection. However, only rarely are such mixture types encountered in practice. Those confronted most frequently generally comprise some solutes (often of unknown identity) that are present in overwhelming quantity (hence masking the compounds of interest); a natural-products matrix (or matrices) superimposed on the chromatogram; peak distortion due to this or that column/mobile-phase/stationary-phase effect(s) or artifact(s); and so on. Thus, for purposes of quantitation, preparative collection, spectrometric identification, etc., it is often found in practice that a particular separation must be adjusted such that the compound(s) of interest is(are) eluted well away from others. For these reasons, it may well be that the

optimum **practical** mobile-phase composition is much different from that with which all solutes could be baseline-resolved when present in equal amounts.

For example, suppose that solute no. 4 (dashed curve) were in great excess over no. 5 in Figure 5, and that quantitative data were required for the latter. Because of the overlap, quantitation would be rendered less accurate than if the two compounds were present, as in the solid curves, in nearly identical amount. Other mobile phases with which the solute elution order can be changed must therefore be considered.

Reference back to Figure 2 shows, as mentioned earlier, that solutes 3 and 4 coelute at all compositions of ACN considered and so, this mobile-phase additive is not useful in terms of the analysis desired. In contrast, Figure 3 shows that, with small amounts of THF additive (left-hand ordinate), solute no. 5 will elute second (after phenol), followed by 2 and then by 4. Moreover, solutes 4 and 3 approach coelution at 20% THF; 5 and 2 overlap at ca. 30%; while 2 and 4 are not resolved at 35%. Thus, there are substantial shifts in elution order with this mobile-phase modifier, and it is therefore ideally suited for adjustment of the separation in the desired manner.

The resultant window diagrams formulated in terms of α, S_f, and R_s were superimposable in shape and are shown in Figure 6 composited with the relevant ordinate values. The most favorable window is that occurring at 22% THF, with the solute pair 4/3 being the most difficult to resolve at this mobile-phase composition. The most-difficult alpha is 1.095 which, since the capacity factors are ca. 10, requires 4800 theoretical plates for complete separation. Further, the order of solute elution is predicted from Figure 3 to be 1, 5, 2, 4, 3, 6, 7, 8, and 9.

The **first-time** chromatogram is shown in Figure 7, where resolution has been achieved precisely as predicted. In addition, separation of the major component, solute 4 (dashed curve), is more than adequate for quantitation of it as well as its nearest neighbor, no. 3. Solute 5 (that of main interest from the standpoint of this example) elutes well away from 1 and is also baseline-resolved from 2. Thus, the overall optimum **practical** mobile-phase additive and concentration turn out to be much different from those utilized to produce Figure 5.

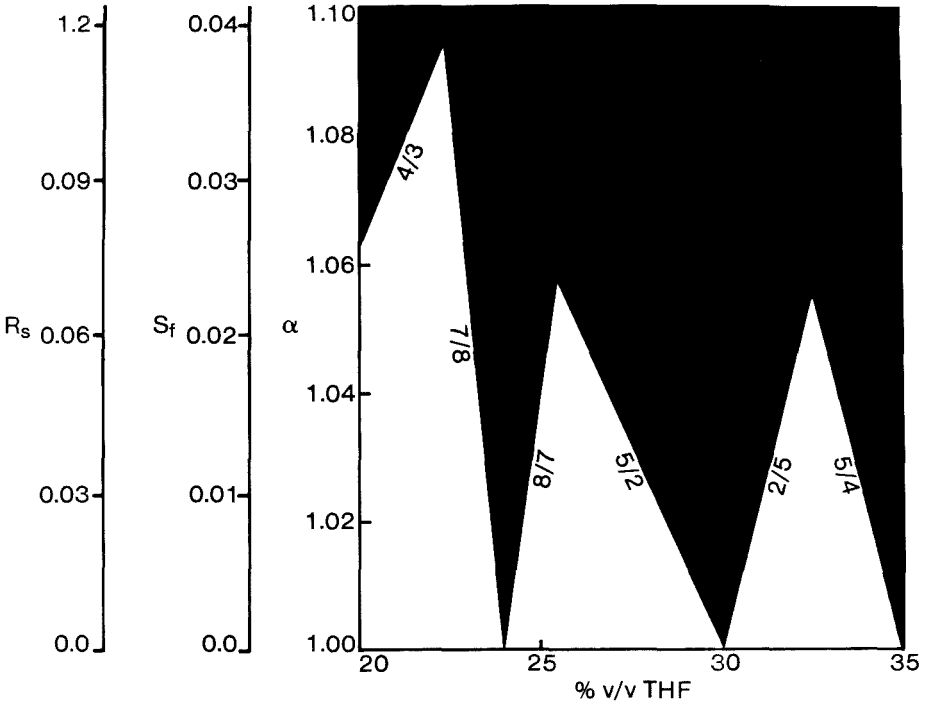


FIGURE 6

Window diagram (1) for test solutes with mobile phase containing THF additive. Ordinates: resolution R_s; separation factor S_f; and relative retention α. Optimum concentration of THF is predicted to be 22% v/v.

Window-Diagram Ordinate Parameters. The equivalence of the three versions of window diagram appears to confirm that the values taken for the system dead volume were not seriously in error since, if that were the case, the alpha plot would have been substantially different from that utilizing S_f. In fact, the various forms of window diagrams must approach coincidence when the solute capacity factors are on the order of 10 or more. However, when that is not the case, there may arise substantial differences in the optimum mobile-phase compositions deduced from each. The separation-factor parameter S_f should then be employed. Plots utilizing an ordinate of R_s would also be correct in such instances, although

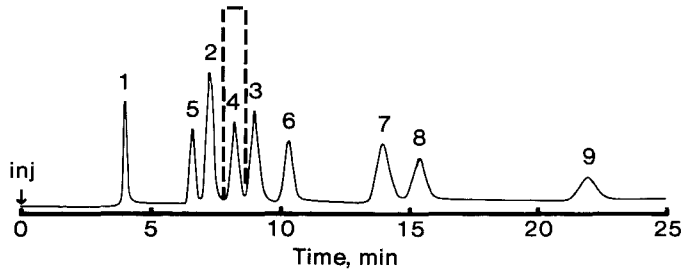


FIGURE 7

First-time chromatogram of the the solute test mixture with 22% THF additive, the optimum mobile-phase composition deduced from Figure 6.

there is some inconvenience in having to measure peak baseline widths in addition to retention times with this method. (There may also be introduced some uncertainty if the pure components or some other suitable reference materials are not available for separate evaluation of w_m.) In any event in this case, where the capacity factors of the most-difficult solutes approach 10, the three versions of window diagrams prove to be entirely equivalent.

Generality of Eqn. 3. It has been established at this point that eqn. 3 applies to very many solvent systems in liquid chromatography (57,58; this work; preceding paper). In addition, and now that a generalized function is in place with which retentions can be represented quantitatively (hence predicted after measurement of only a few datum points), the window-diagram optimization strategy can be employed directly and on a routine basis even for the analysis of mixtures of initially-unknown content and complexity (64,65). Moreover, it would seem that the methodology detailed here could easily be automated: to do so would require only a simple algorithm with which to acquire, thence fit, retention data to the relation, followed by application of a second program to provide plots (window diagrams) of S_f against the system parameter(s) to be optimized (66-68). We take this matter up in the following paper; however, before doing so, we note that all systems shown to date to be described by eqn. 3 have comprised non-ionic solutes and solvents. We therefore enquire at this point whether the relation does in fact apply to ionic systems.

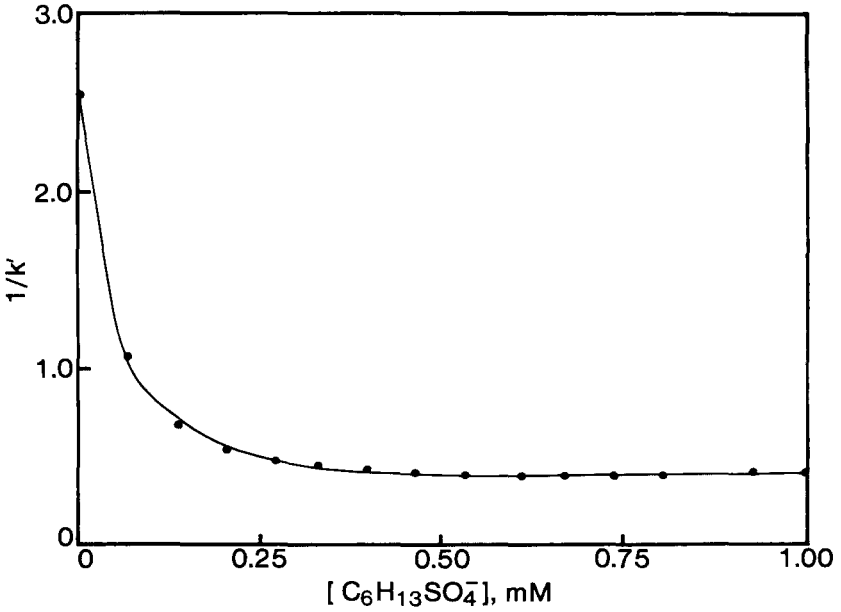


FIGURE 8

Plot of inverse capacity factor for epinephrine solute against mobile-phase surfactant concentration. Data from ref. 68; solid curve constructed with eqn. 3.

Figure 8 presents as an example a plot of the inverse capacity factor data exhibited by epinephrine solute with millimolar concentrations (0 to 1 mM) of hexyl sulfate additive in water:MeOH (80:20; 2.55 pH) mobile phase (69). The circles represent the experimental data, while the solid curve was constructed from eqn. 3 with the fitted values: b = -56.88 and b' = 24.15. The inverse retentions fall off, as shown, very rapidly upon the introduction of some surfactant, and pass through a very shallow minimum at ca. 0.65 mM. Even so, eqn. 3 clearly yields an exact fit over the entire range of additive concentration considered in the work.

The capacity factors $k'_{(S)}$ and $k'_{(A)}$ were taken, for purposes of data reduction, to be those at zero and at unit millimolar amounts of additive; i.e., as if these concentrations corresponded to hypothetical

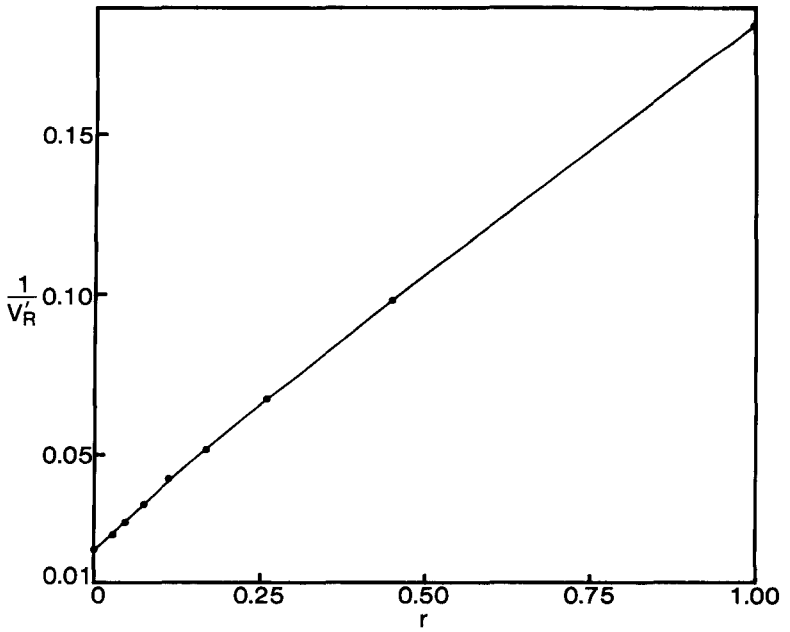


FIGURE 9

Plot of inverse adjusted retention volume for 4-hydroxybenzoic acid against anion/acid ratio for mobile phase containing acetic acid, sodium hydroxide, and sodium chloride. Data from ref. 4; solid curve constructed with eqn. 3.

values of "ϕ" of 0 and 1. Thus, the **actual** units on the abscissa were disregarded; self-evidently, this can be done with data of any form (e.g., pH, ionic strength, concentration of additive, etc.). For example, we show in Figure 9 a plot of the inverse corrected retention volumes of 4-hydroxybenzoic acid solute against the anion/acid concentration ratio, where the mobile phase consisted of various amounts of acetic acid, sodium hydroxide, and sodium chloride so as to maintain a constant ionic strength (4). The experimental data (slightly concave to the abscissa) are again represented by circles, while the solid curve was constructed with b = 0.0361 and b' = 2.504. Figure 10 offers further illustration of the matter, where the inverse capacity factors of tyrosinamide solute are plotted against

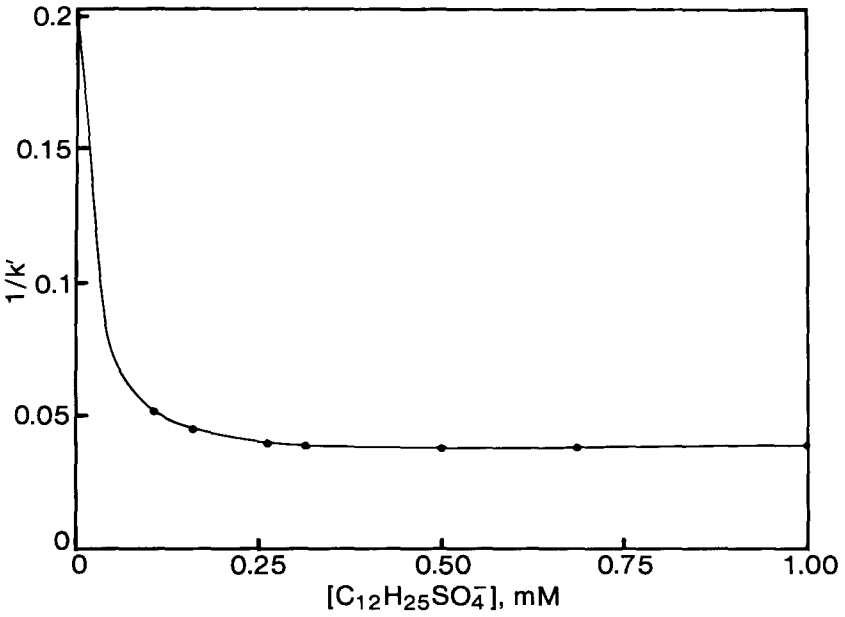


FIGURE 10

Plot of inverse capacity factor for tyrosinamide solute against mobile-phase surfactant concentration. Data from ref. 69; solid curve constructed with eqn. 3.

millimolar concentration of dodecyl sulfate anion in water:MeOH mobile phase (80:20; buffered at pH 6 with 0.018 M KH_2PO_4 and 0.002 M $NaHPO_4$) (70). The values of b and b' were in this instance -10.45 and 61.9.

It therefore appears that eqn. 3 can be fitted also to systems wherein the solutes and/or solvents are ionic. In fact, we have thus far been unable to identify a single case to which it does not apply. Nevertheless, this does not preclude the generality of its form. Indeed, given its complete success up to this time, the relation invites at the very least consideration of models of partitioning from which it may be derived, as well as compel reinterpretation of those already extant.

ACKNOWLEDGMENT

We gratefully acknowledge support of this work received from the Department of Energy.

REFERENCES

1. Laub, R. J., **Am. Lab., 13**(3), 47, 1981; in **Physical Methods in Modern Chemical Analysis**, Vol. **3**, T. Kuwana, Ed., Academic Press, New York, 1983, Ch. 4.

2. Laub, R. J., **Anal. Chem.**, submitted for review. See also: Stranahan, J. J., and Deming, S. N., **Anal. Chem., 54**, 2251, 1982; **55**, 425, 1983. Ngoc, L. H., Ungvarai, J., and Kovats, E. sz., **Anal. Chem., 54**, 2410, 1982. Boehm, R. E., and Martire, D. E., **J. Phys. Chem., 84**, 3620, 1980; Martire, D. E., and Boehm, R. E., **J. Phys. Chem., 87**, 1045, 1983.

3. Laub, R. J., and Purnell, J. H., **J. Chromatogr., 112**, 71, 1975.

4. Deming, S. N., and Turoff, M. L. H., **Anal. Chem., 50**, 546, 1978.

5. Sachok, B., Kong, R. C., and Deming, S. N., **J. Chromatogr., 199**, 317, 1980. See also: Issaq, H. J., Muschik, G. M., and Janini, G. M., **J. Liq. Chromatogr., 6**, 259, 1983 for an example of the use of window diagrams with reverse-phase systems.

6. Jandera, P., and Churacek, J., **J. Chromatogr., 91**, 207, 1975; **93**, 17, 1975; **104**, 9, 1975.

7. Hartwick, R. A., Grill, C. M., and Brown, P. R., **Anal. Chem., 51**, 34, 1979.

8. Edwards, W. G., and Wellington, C. A., **J. Chromatogr., 135**, 463, 1977.

9. Glajch, J. L., Kirkland, J. J., and Squire, K. M., **J. Chromatogr., 199**, 57, 1980; Lehrer, R., **Am. Lab., 13**(10), 113, 1981.

10. Issaq, H. J., Klose, J. R., McNitt, K. L., Haky, J. E., and Muschik, G. M., **J. Liq. Chromatogr., 4**, 2091, 1981; Issaq, H. J., Muschik, G., and Janini, G. M., **J. Liq. Chromatogr., 6**, 259, 1983.

11. Jones, P., and Wellington, C. A., **J. Chromatogr., 213**, 357, 1981.

12. Al-Thamir, W. K., Laub, R. J., and Purnell, J. H., **J. Chromatogr., 173**, 388, 1979.

13. Lustre, A. O., and Issenberg, P. J., **J. Agr. Food Chem., 18**, 1056, 1970.

14. Challis, B. C., and Lawson, A. J., **J. Chem. Soc.**, 770, 1970.

15. Challis, B. C., **Nature (London), 244**, 466, 1973.

16. Challis, B. C., and Bartlett, C. D., **Nature (London), 254**, 532, 1975.

17. Walker, E. A., Pignatelli, B., and Castegnaro, M., **Nature (London), 258**, 176, 1975.

18. Walker, E. A., Pignatelli, B., and Castegnaro, M., **J. Agr. Food Chem., 27**, 389, 1979.

19. Ander, P., Ericksson, E.-E., Kolars, M.-C., Krigstad, K., Rannug, V., and Ramel, C., **Sv. Papperstidn., 80**, 454, 1977.

20. Bjørseth, A., Carlberg, G. E., and Moller, M., **Sci. Total Environ., 11**, 197, 1979.

21. Rogers, J. H., **Pulp Pap. Mag. Can., 74**, 111, 1973.

22. Lindstrom, K., and Nordin, J., **J. Chromatogr., 128**, 13, 1976.

23. Hemetsburger, H., Mansfield, W., and Ricken, H., **Chromatographia, 9**, 303, 1976.

24. Karch, K., Sebastian, I., and Halasz, I., **J. Chromatogr., 122**, 3, 1976.

25. Roumeliotis, P., and Unger, K. K., **J. Chromatogr., 149**, 211, 1978.

26. Cooke, N. H. C., and Olsen, K., **J. Chromatogr. Sci., 18**, 512, 1980.

27. Horvath, C., and Melander, W., **J. Chromatogr. Sci., 15**, 393, 1977.

28. Grushka, E., and Kikta, Jr., E. J., **Anal. Chem., 46**, 1370, 1974.

29. Grushka, E., and Kikta, Jr., E. J., **Anal. Chem., 48**, 1098, 1976.

30. Scott, R. P. W., and Kucera, P., **J. Chromatogr., 142**, 213, 1977.

31. Tanaka, N., Goodwell, M., and Karger, B. L., **J. Chromatogr., 158**, 233, 1978.

32. Schmit, J. A., Henry, R. A., Williams, R. C., and Dieckman, J. F., **J. Chromatogr. Sci., 9**, 645, 1971.

33. Gant, J. R., Dolan, J. W., and Snyder, L. R., **J. Chromatogr., 185**, 153, 1979; **165**, 3, 1979.

34. Melander, W., Chen, B. K., and Horvath, C., **J. Chromatogr., 185**, 99, 1979.

35. Gant, J. R., Dolan, J. W., and Snyder, L. R., **J. Chromatogr., 165**, 31, 1979.

36. Horvath, C., Melander, W., and Molnar, L., **J. Chromatogr., 125**, 129, 1976.

37. Karch, K., Sebastian, L., and Halasz, L., **J. Chromatogr., 122**, 171, 1976.

38. Locke, D. C., **J. Chromatogr. Sci., 12**, 433, 1974.

39. Bakalyar, S. R., McIlwrick, R., and Roggendorf, E., **J. Chromatogr., 142,** 353, 1977.

40. Laub, R. J., and Purnell, J. H., **J. Chromatogr., 161,** 49, 1978.

41. Schoenmaker, P. J., Billiet, H. A. H., Tijssen, R., and De Galan, L., **J. Chromatogr., 149,** 519, 1978.

42. Snyder, L. R., and Kirkland, J. J., **Introduction to Modern Liquid Chromatography,** Wiley-Interscience, New York, 1974, Chs. 2,3.

43. Erni, F., and Frei, R. W., **J. Chromatogr., 130,** 169, 1977.

44. Karger, B. L., Karger, J. R., Hartkopf, A., and Weiner, P. H., **J. Chromatogr., 128,** 65, 1976.

45. Hemets, H., Maasfeld, W., and Richer, H., **Chromatographia, 9,** 303, 1976.

46. Lafosse, M., Keravis, G., and Durand, M. H., **J. Chromatogr., 118,** 283, 1976.

47. Abbott, S. R., Berg, J. R., Achener, P., and Stevenson, R. L., **J. Chromatogr., 126,** 421, 1976.

48. Mulshoff, A. H., and Perrin, J. M., **J. Chromatogr., 129,** 263, 1976.

49. Grafeo, A. P., and Karger, B. L., **Clin. Chem., 22,** 184, 1976.

50. Tanaka, N., and Thornton, E. R., **J. Am. Chem. Soc., 99,** 7300, 1979.

51. Colin, H., and Guiochon, G., **J. Chromatogr., 141,** 289, 1978.

52. Westerlund, D., Theodorsen, A., and Carlquist, J., paper presented at the Third International Symposium on Column Liquid Chromatography, Salzburg, September 1978.

53. Oscik, J., **Prsem. Chem., 44,** 129, 1965; Oscik, J., and Chojnacka, **J. Chromatogr., 93,** 167, 1974.

54. Jandera, P., and Churacek, J., **J. Chromatogr., 93,** 207, 1974.

55. Soczewinski, E., and Golkiewicz, W., **Chromatographia, 4,** 501, 1971; **6,** 269, 1973; Soczewinski, E., **J. Chromatogr., 130,** 23, 1977.

56. Jaroniec, M., Rozylo, J. K., and Oscik-Mendyk, B., **J. Chromatogr., 179,** 237, 1979; Jaroniec, M., Klepacka, B., and Narkiewicz, J., **J. Chromatogr., 170,** 299, 1979; Jaroniec, M., Rozylo, J. K., and Golkiewicz, W., **J. Chromatogr., 178,** 27, 1979.

57. McCann, M., Purnell, J. H., and Wellington, C. A., in **Chromatography, Equilibria, and Kinetics,** Faraday Society Symposium No. 15, D. A. Young, Ed., The Royal Society of Chemistry, London, 1980, p. 82.

58. Madden, S. J., McCann, M., Purnell, J. H., and Wellington, C. A., paper presented at the 184th National Meeting of the American Chemical Society, Kansas City, Missouri, 1982; Madden, S. J., Ph.D. Thesis, University College of Swansea, Swansea, Wales, 1983.

59. Billiet, H. A. H., Van Dalen, J. P. J., Schoenmaker, P. J, and De Galan, L., **Anal. Chem., 55,** 847, 1983. We thank D. E. Martire for calling this work to our attention.

60. McCormick, R. M., and Karger, B. L., **Anal. Chem., 52,** 2249, 1980.

61. Even in the absence of interactions of marker species with the stationary phase there remains open the question of the relevance of such data in terms of solutes (here phenols) of differing molecular volume, for which the method (12) of determining an **effective** V_A with an homologous series of compounds closely related to the analytes of interest appears to have merit.

62. The injected amounts of each (effectively at infinite dilution) are completely soluble in the mobile-phase combinations employed here and in fact, are soluble even in pure water. For example, the weight-based aqueous solubility of phenol at 50°C is 33%.

63. Purnell, J. H., **J. Chem. Soc.,** 1268, 1960.

64. Laub, R. J., and Purnell, J. H., **Anal. Chem., 48,** 1720, 1976.

65. Laub, R. J., and Purnell, J. H., **J. Chromatogr., 161**; 59, 1978.

66. Laub, R. J., Purnell, J. H., and Williams, P. S., **Anal. Chim. Acta, 95,** 135, 1977.

67. Our experience has been that commercially-available systems heretofore purported capable of automated data acquisition and reduction fail when there occur reversals in solute elution order. Yet such reversals are precisely what is desired in optimizing separations.

68. An nth-order polynomial (or some other function) could obviously be substituted for the relation in order to achieve the same end. However, eqn. 3 is overwhelmingly appealing as a result of the close proximity of its form to what would be derived from fundamental considerations of the partitioning process; see preceding paper.

68. Horvath, C., Melander, W., Molnar, L, and Molnar, P., **Anal. Chem., 49,** 2295, 1977.

69. Knox, J. H., and Hartwick, R. A., **J. Chromatogr., 204,** 3, 1981.

JOURNAL OF LIQUID CHORMATOGRAPHY, 7(3), 639-640 (1984)

LETTER TO THE EDITOR

Sir,

In a recent paper in your Journal (1) Vemulapalli and Gnanasambandan present an equation that relates chromatographic retention with the interfacial tensions of the solute and the solvent each in combination with the stationary phase. This equation is quite remarkable, as it suggests that

a) the interaction between the solute and the solvent plays no part in the retention mechanism and
b) the interfacial adsorption mechanism leads to a linear adsorption isotherm and hence symmetrical peaks, at least for dilute solutions.

These remarkable features caused me to take a closer look at the derivation of the retention equation. Besides the possible errors introduced by substituting an approximate equation for the surface tension of binary mixtures for the interfacial tension and extrapolation of the magnitude of the constants in this equation, the derivation seems to be reasonably sound until after eq. (10) is obtained.

In going from eq. (10) to eq. (12) a factor $(1/X^s_2)$ is ignored. This is explained from eq. (11), which reads

$$(1/X^s_2) \quad (1-X^s_2) = \ln (X^s_1).$$

Since the authors state (correctly) that X^s_1 is nearly equal to unity and since $X^s_2 + X^s_1 = 1$, this equation seems rather odd. E.g., if we taken X^s_2 to be 0.01, it reads

 0148-3919/84/0703-0639$3.50/0

$$100 \quad 1 = -0.01 \ .$$

This, of course, is an absurd statement. Moreover, its function in the derivation is unclear. However, it is used to omit the factor $(1/X^{s}_{2})$, which is both large and variable from the final equation.

The evidence supplied for the final result is minimal. Four datapoints from a literature source merely indicate that retention increases with surface tension of the pure solute (no further information about the chromatographic system given !) and an inaccurate and incomplete reference to the observed variation of retention with stationary phase chainlength is of hardly any value.

Therefore, I must conclude that the cited paper is erroneous, and, moreover, that it should not have been published.

Nov. 1, 1983

Peter J. Schoenmakers
Philips Research
Eindhoven, The Netherlands

(1) G.K. Vemulapalli and T. Gnanasambandan,
Journal of Liquid Chromatography 6 (10) (1983) 1777.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(3), 641-642 (1984)

LC NEWS

GPC+, FIRMWARE-BASED PROGRAM, calculates analytical results in gel permeation chromatography. It resides on a 8K chip enables the calculation of calibration curves via point-to-point, quadratic, or cubic fits of the data. Statistical data are presented to aid in selecting the best fit. Spectra-Physics Corp., JLC/84/3, 3333 North First Street, San Jose, CA, 95134, USA.

GPC/SEC SCOUT COLUMN is available in a highly cross-linked 10 micron divinylbenzene gel, which permits the use of many organic solvents, including DMF. It can be used for molecular weight distributions of polymers from 500 to 4 million. IBM Instruments, Inc., JLC/84/3, P. O. Box 332, Danbury, CT, 06810, USA.

CUSTOM HPLC PHASES AND PACKINGS SERVICE is available. One can specify particle size, shape, and pore diameter as well as structure of the desired stationary phase. ES Industries, Inc., JLC/84/3, 8 S. Maple Avenue, Marlton, NJ, 08053, USA.

ELECTROCHEMICAL DETECTOR FOR HPLC combines a large electrode surface area with low internal volume (1.5 microliter) for enhanced sensitivity and compatibility with microbore requirements. The solid state reference electrode is an inytegral part of the flow cell and requires no regular maintenance. LKB Instruments, Inc., JLC/84/3, 9319 Gaither Rd., Gaithersburg, MD, 20877, USA.

INTELLIGENT VALVE POSITIONER consists of a valve driver assembly including valve, synchronous motor and encoder, plus a controller unit which uses a standard RS232 interface. A valve may be moved to any position, at any time, in any direction. Hamilton Co., JLC/84/3, P. O. 10030, Reno, NV, 89520, USA.

GLASS CARTRIDGE COLUMNS FOR HPLC withstand up to 200 bars pressure. Advantages offered include low solvent consumption, inertness, visibility of the packed bed, and smoothness of the internal wall. Chromapack Nederland BV, JLC/84/3, P. O. Box 3, NL4330 AA Middelburg, The Netherlands.

CONTINUOUSLY VARIABLE HPLC DETECTOR digital setting of wavelengths from 190 to 600 nm in 1 nm steps with a wide range of cells from micro to prep. For automated systems, all features can be externally controlled by computers and other devices. An optional

microprocessor spectrocontroller can change wavelength and range at preset times and provides baseline correction. Sonntek, Inc., JLC/84/3, P. O. Box 8731, Woodcliff Lake, NJ, 07675, USA.

CHROMATOGRAPHY INTEGRATOR FOR THE APPLE II COMPUTER is dual channel and includes hardware, software, and 128K additional RAM. Data from two independent chromatographs may be acquired, displayed, and analyzed at up to 40 points/sec. A finished report includes graphical presentation of data. Anadata, Inc., JLC/84/3, 516 N. Main Street, Glen Ellyn, IL, 60137, USA.

REVERSED-PHASE ODS COLUMNS are constructed of stainless steel with a highly polished interior surface. They are packed with Spherisorb ODS-II, a spherical silica of very narrow particle size distribution. It is coated with a C18 function and is fully end capped. Columns are guaranteed to deliver 50,000 to 80,000 plates per meter. HPLC Specialties, JLC/84/3, P. O. Box 484, Edmond, OK, 73083, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(3), 643-646 (1984)

LC CALENDAR

1984

MARCH: "Basic GC School," a 3-day course (date to be announced), sponsored by the Chicago Chromatography Discussion Group. Contact: N. Armstrong, LC Company, P. O. Box 72125, Roselle, IL, 60172, USA.

MARCH 20: "New Developments in HPLC of Water Soluble Macromolecules," sponsored by the Chicago Chromatography Discussion Group. Contact: N. Armstrong, LC Company, P. O. Box 72125, Roselle, IL, 60172, USA.

APRIL: "HPLC of Water Soluble Polymers," a 2-day course (date to be announced) sponsored by the Chicago Chromatography Discussion Group. Contact: N. Armstrong, LC Company, P. O. Box 72125, Roselle, IL, 60172, USA.

APRIL 8-13: National ACS Meeting, St. Louis, MO. Contact: Meetings, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

APRIL 15-17: Short Courses: "LCEC" taught by Dr. R. Shoup, BAS, Inc.; "Derivatization & Sample Preparation" taught by Dr. C. Poole, Wayne State University - sponsored by the Minnesota Chromatography Forum; held at the Minneapolis Audirorium & Convention Hall. Contact: Meeting Management, 1421 E. Wayzata Blvd., Wayzata, MN, 55391, USA.

APRIL 17: "New Developments in TLC," sponsored by the Chicago Chromatography Discussion Group. Contact: N. Armstrong, LC Company, P. O. Box 72125, Roselle, IL, 60172, USA.

APRIL 29 - MAY 3: "Analytical Applic. of Supercritical Fluids - Supercritical Fluid Technol. Symposium" at the meeting of the AOCS, Dallas, TX. Contact: Dr. J. W. King, CPC Int'l., Moffett Tech Center, Argo, IL, 60501, USA.

MAY 17: Symposium on Therapeutic Drug Monitoring & Toxicology for the 80's: Clinical & Instrumental Perspectives, Farmington, CT,

sponsored by the UConn School of Medicine & AAAC Connecticut Valley Chapter. Contact: Dr. Steven H. Wong, Dept. of Lab. Med., UConn School of Medicine, Farmington, CT, 06032, USA.

MAY 20 - 26: 8th Intl. Symposium on Column Liquid Chromatography, New York Statler Hotel, New York City. Contact: Prof. Cs. Horvath, Yale University, Dept. of Chem. Eng., P. O. Box 2159, Yale Stn., New Haven, CT, 06520, USA.

JUNE 3-5: International Symposium on LCEC and Voltammetry, Indianapolis Hyatt Regency Hotel, Indianapolis, IN. Contact: The 1984 LCEC Symposium, P. O. Box 2206, West Lafayette, IN, 47906, USA.

JUNE 10-14: 14th Northeast Regional ACS Meeting, sponsored by the Western Connecticut and New Haven Sections, at Fairfield University, Fairfield, CT. Contact:D. L. Swanson, American Cyanamid Co., Stamford, CT, USA.

JUNE 18-20: Second International Conference on Chromatography & Mass Spectrometry in Biomedical Sciences, sponsored by the Italian Group for Mass Spectrometry in Biochemistry & Medicine, Milan, Italy. Contact: Dr. A. Frigerio, via Eustachi 36, I-20129 Milan, Italy, or Dr. H. Milon, P. O. Box 88, CH-1814 La Tour-de-Peilz, Switzerland.

JUNE 18-21: Symposium on Liquid Chromatography in the Biological Sciences, Ronneby, Sweden, sponsored by The Swedish Academy of Pharmaceutical Sciences. Contact: Swedish Academy of Pharmaceutical Sciences, P. O. Box 1136, S-111 81 Stockholm, Sweden.

AUGUST 26-31: National ACS Meeting, Philadelphia, PA. Contact: Meetings, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

SEPTEMBER 10-14: Advances in Liquid Chromatography, including the 4th Annual American-Eastern European Symposium on LC and the Int'l Symposium on TLC with Special Emphasis on Overpressured Layer Chromatography, sponsored by the Hungarian Academy of Sciences' Chromatography Committee & Biological Research Center and the Hungarian Chemical Society, in Szeged, Hungary. Contact: Dr. H. Kalasz, Dept. of Pharmacology, Semmelweis University of Medicine, P.O.Box 370, H-1445 Budapest, Hungary, or Dr. E. Tyihak, Research Inst. for Plant Protection, P.O.Box 102, H-1525 Budapest, Hungary.

SEPTEMBER 20 - 22: Labcon Central 83, 3rd Annual Lab Instrument & Equipment Conference & Exhibition, O'Hare Exposition Center, Rosemont, IL. Contact: Tower Conference Mgmt. Co., 143 N. Hale Street, Wheaton, IL, 60187, USA.

OCTOBER 1-5: 15th Int'l. Sympos. on Chromatography, Nurenberg, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, D-6000 Frankfurt Main, West Germany.

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 Street, Philadelphia, PA.

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 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.

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

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

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.

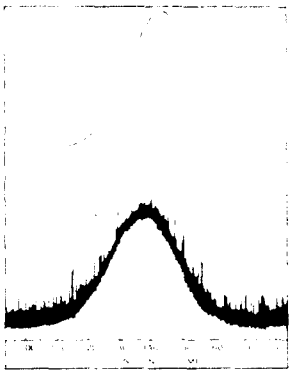
AUGUST 30 - SEPTEMBER 4: 194th National Am. Chem. Soc. Mtng., New Orleans, LA. Contact: A. T. Winstead, ACS, 1155 16th Street, NW, Washington, DC, 20036, USA.

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.

Providing state-of-the-art understanding of . . .

Steric Exclusion Liquid Chromatography of Polymers

(Chromatographic Science Series, Volume 25)



edited by
JOSEF JANČA
Institute of Analytical Chemistry
Czechoslovak Academy of Sciences
Brno, Czechoslovakia

January, 1984
352 pages, illustrated
$55.00
(Price is 20% higher outside the U.S. and Canada)

". . . Dr. Janča has succeeded in bringing together several of the most innovative giants in this field. Each has contributed significantly. In this monograph, we are able to look over their shoulders to see where future advances will lie."
—**Dr. JACK CAZES**
Fairfield, Connecticut *(from the Foreword)*

WITH ITS UNSURPASSED capacity to determine a polymer's molecular weight distribution and other properties, it is easy to recognize the vast importance of steric exclusion chromatography—*AND the value of this unparalleled single-source reference!*

Certainly, no other work can match *Steric Exclusion Liquid Chromatography of Polymers* for its up-to-date, fully detailed coverage—provided by leading international experts—with features that include

- in-depth examination of separation mechanisms and secondary interactions and their correlation with the structure and behavior of macromolecules
- refinements in calibration and data evaluation techniques
- complete information on solvent and column packing, correction of zone dispersion, and experimental variables
- practical applications involving molecular weight distribution, polymer branching, chemical composition, chain growth and degradation mechanisms, and others

Complete with illustrative experimental data, this state-of-the-art volume leads the way to future innovations for a wide range of scientists. Furthermore, this volume serves as an excellent reference for a number of graduate-level chemistry courses.

Readership: Liquid Chromatographers; Analytical, Polymer, Organic, and Industrial Chemists; Physicists; Polymer Technologists; Polymer Processors; and Graduate Chemistry Students.

CONTENTS

ISBN: 0-8247-7065-X

MARCEL DEKKER, INC.
270 MADISON AVENUE, NEW YORK, N.Y. 10016
(212) 696-9000

INSTRUCTIONS FOR PREPARATION OF MANUSCRIPTS FOR DIRECT REPRODUCTION

Journal of Liquid Chromatography is a bimonthly publication in the English language for the rapid communication of liquid chromatographic research.

Directions for Submission

One typewritten manuscript suitable for direct reproduction, carefully inserted in a folder, and two (2) copies of the manuscript must be submitted. Since all contributions are reproduced by direct photography of the manuscripts, the typing and format instructions must be strictly adhered to. Noncompliance will result in return of the manuscript to the authors and delay its publication. To avoid creasing, manuscripts should be placed between heavy cardboards and securely bound before mailing.

Manuscripts should be mailed to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography
P. O. Box 1440-SMS
Fairfield, Connecticut 06430

Reprints

Owing to the short production time for articles in this journal, it is essential to indicate the number of reprints required upon notification of acceptance of the manuscript. Reprints are available in quantities of 100 and multiples thereof. For orders of 100 or more reprints, twenty (20) free copies are provided. A reprint order form and price list will be sent to the author with the notification of acceptance of the manuscript.

Format of Manuscript

1. The general format of the manuscript should be as follows: title of article; names and addresses of authors; abstract; and text discussion.

2. Title and Authors: The entire title should be in capital letters and centered on the width of the typing area at least 2 inches (5.1 cm) from the top of the page. This should be followed by three lines of space and then by the names and addresses of the authors in the following way (also centered):

A SEMI-AUTOMATIC TECHNIQUE FOR THE SEPARATION AND DETERMINATION OF BARIUM AND STRONTIUM IN SURFACE WATERS BY ION EXCHANGE CHROMATOGRAPHY AND ATOMIC EMISSION SPECTROMETRY

F. D. Pierce and H. R. Brown
Utah Biomedical Test Laboratory
520 Wakra Way
Salt Lake City, Utah 84108

3. Abstract: Three lines below the addresses, the title ABSTRACT should be typed (capitalized and centered on the page). This should be followed by a single-spaced, concise, abstract comprising less than 10% of the length of the text of the article. Allow three lines of space below the abstract before beginning the article itself.

4. Text Discussion: Whenever possible, the text discussion should be divided into such major sections as INTRODUCTION, MATERIALS, METHODS, RESULTS, DISCUSSION, ACKNOWLEDGMENTS, and REFERENCES. These major headings should be separated from the text by two lines of space above and one line of space below. Each heading should be in capital letters, centered, and underlined. Secondary headings, if any, should be flush with the left margin, underscored, and have the first letter of all main words capitalized. Leave two lines of space above and one line of space below secondary headings.

5. Paragraphs should be indented five (5) typewriter spaces.

6. Acknowledgment of collaboration, sources of research funds, and address changes for an author should be listed in a separate section at the end of the paper.

7. References (including footnotes) in the text will be numbered consecutively by numbers in parentheses. All references (and footnotes) should then be aggregated in sequence at the end of the communication. No footnotes should be shown at the bottom of pages. The reference list follows immediately after the text. The word REFERENCES should be capitalized and centered above the reference list. It should be noted that all reference lists should contain initials and names of all authors; *et al.* will not be used in reference lists. Abbreviations of journal titles and styles of reference lists will follow the American Chemical Society's Chemical Abstracts List of Periodicals. References should be typed single-spaced with one line space between each reference.

8. Each page of manuscript should be numbered lightly at the bottom of the sheet with a light blue pencil.

9. Only standard symbols and nomenclature approved by the International Union of Pure and Applied Chemistry should be used.

10. Any material that cannot be typed, such as Greek letters, script letters, and structural formulae, should be drawn carefully in black India ink (do not use blue ink).

Typing Instructions

1. The manuscript must be typewritten on good quality white bond paper measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). Do not use Corrasible bond or its equivalent. The typing area of the article opening page, including the title, should be 5½ inches wide by 7 inches deep (14 cm x 18 cm). The typing area of all other pages should be no more than 5½ inches wide by 8½ inches deep (14 cm x 21.6 cm).

2. In general, the chapter title and the abstract, as well as the tables and references, are typed single-spaced. All other text discussion should be typed 1½-line spaced, if available, or double-spaced. Prestige elite characters (12 per inch) are recommended, if available.

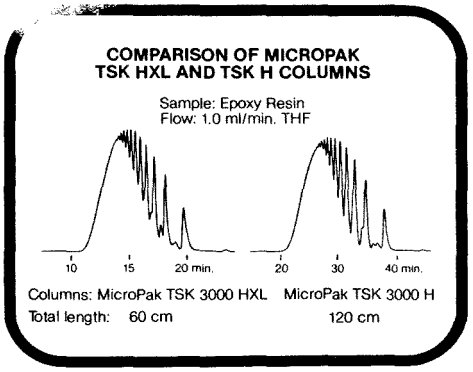
3. It is essential to use black typewriter ribbon (carbon film is preferred) in good condition so that a clean, clear impression of the letters is obtained. Erasure marks, smudges, creases, etc., may result in return of the manuscript to the authors for retyping.

4. Tables should be typed as part of the text but in such a way as to separate them from the text by a three-line space at both top and bottom of each table. Tables should be inserted in the text as close to the point of reference as possible, but authors must make sure that one table does not run over to the next page, that is, no table may exceed one page. The word TABLE (capitalized and followed by an Arabic number) should precede the table and be centered on the page. The table title should have the first letters of all main words in capitals. Titles should be typed single-spaced. Use the full width of the type page for the table title.

5. Drawings, graphs, and other numbered figures should be professionally drawn in black India ink (do not use blue ink) on separate sheets of white paper and placed at the end of text. Figures should not be placed within the body of the text. They should be sized to fit within the width and/or height of the type page, including any legend, label, or number associated with them. Photographs should be glossy prints. A typewriter or lettering set should be used for all labels on the figures or photographs; they may not be hand drawn. Captions for the pictures should be typed single-spaced on a separate sheet, along the full width of the type page, and preceded by the word FIGURE and a number in arabic numerals. All figures and lettering must be of a size to remain legible after a 20% reduction from original size. Figure numbers, name of senior author, and arrow indicating "top" should be written in light blue pencil on the back or typed on a gummed label, which should be attached to the back of the illustration. Indicate approximate placement of the illustrations in the text by a marginal note in light blue pencil.

6. The reference list should be typed single-spaced although separated from one another by an extra line of space. Use Chemical Abstract abbreviations for journal titles. References to journal articles should include (1) the last name of all author(s) to any one paper, followed by their initials, (2) article title, (3) journal, (4) volume number (underlined), (5) first page, and (6) year, in that order. Books should be cited similarly and include (1) author, surname, first and middle initials, (2) title of book, (3) editor of book (if applicable), (4) edition of book (if any), (5) publisher, (6) city of publication, (7) year of publication, and (8) page reference (if applicable). E.g., Journals: Craig, L. C. and Konigsber, W., Use of Catechol Oxygenase and Determination of Catechol, Chromatogr., 10, 421, 1963. Books: Albertsson, P. A., Partition of Cell Particles and Macromolecules, Wiley, New York, 1960. Article in a Book: Walter, H., Proceedings of the Protides of Biological Fluids, XVth Colloquim, Pteeters., H., eds., Elsevier, Amsterdam, 1968, p. 367.

Increase GPC resolution and speed with new MicroPak TSK HXL Columns



Varian announces a new family of high-resolution GPC columns, manufactured by Toyo Soda, the leader in microparticulate gel technology. Featuring new 5-μm spherical particles, these HXL Gels provide outstanding performance characteristics for GPC analysis.

- **Unexcelled performance for GPC polymer characterization**—Excellent column stability provides highly reproducible calibration for precision of molecular weight distribution calculations.
- **Extremely high resolving power for GPC separation of polymer additives and other small molecules**—Column efficiency specification of 50,000 plates/meter provide twice the efficiency of conventional GPC columns.
- **Increased separation speed**—Higher separation efficiency means equivalent resolving power can be obtained with half the column length of conventional GPC columns. Note the equivalency in separation efficiency with only half the length of the HXL columns as shown in the separation. Result: Faster Separations.

Varian MicroPak TSK HXL Columns are in stock and available now.

MICROPAK TSK TYPE HXL COLUMNS FOR GPC

Description	Exclusion Limit (MW of PS)	Order Number
MicroPak TSK 1000HXL	1×10^3	00-997149-74
MicroPak TSK 2000HXL	1×10^4	00-997150-74
MicroPak TSK 2500HXL	2×10^4	00-997151-74
MicroPak TSK 3000HXL	6×10^4	00-997152-74
MicroPak TSK 4000HXL	4×10^5	00-997153-74
MicroPak TSK 5000HXL	4×10^6	00-997154-74
MicroPak TSK 6000HXL	4×10^7*	00-997155-74
MicroPak TSK 7000HXL	4×10^8**	00-997156-74
MicroPak TSK GMHXL**	4×10^8**	00-997157-74

* Estimated
** GMHXL = mixed bed packing.
Columns are available packed in ODCB for use in high temperature GPC.

To order call: 800-538-1735 *or in California 800-672-1405 or write: Varian Instrument Group, 220 Humboldt Court, Sunnyvale, CA 94089—ATTN: Order Desk. For further information circle the Reader Service Number.*

varian

Intelligent chromatography . . . from Varian

For assistance contact: • Florham Park, NJ (201) 822-3700 • Park Ridge, IL (312) 825 7772 • Sugar Land, TX (713) 491-7330
In Europe: Steinhauserstrasse, CH-6300 Zug, Switzerland

Circle Reader Service Card No. 120