# PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

by

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Table of Contents

List of Tables

List of Figures

# Introduction

Preparative High Performance Liquid Chromatography (HPLC) is an improved version of classical column chromatography. This improvement is the result of considerable development in theory and instrumentation of high performance liquid chromatography (1). Several recent reviews and editorials demonstrate the interest and the importance that is given to preparative HPLC (2-6).

Advantages of preparative HPLC over the classical method include better resolution, faster analysis time, and higher sample capacity (7, 8). Also, due to the higher efficiency of HPLC columns, separation of formerly unfeasible mixtures has become possible. The separation of the various intermediates in the synthesis of vitamin B-12 is a good example (9). An additional advantage is the possibility of continuous monitoring of the column effluent by detectors adapted for preparative work (2, 10). This continuous monitoring allows sharper fraction cuts to be made.

Best results in preparative HPLC are obtained when using totally porous particles of small diameter (5-10 μm). Particles of larger diameter also give good results and have the advantage of being cheaper and easier to pack (2). Several reviews on packing materials for HPLC exist (11-14).

The majority of analytical and preparative columns utilized in research laboratories are purchased from manufacturers. Most users do

not have the equipment or time required to pack efficient columns when micro-particles are used. Because of the high price of packed columns, there are no exhaustive studies that correlate the effect of column dimensions with column efficiency and its extension to preparative HPLC.

Wolf (15) presents one set of data for a 50 cm column at various internal diameters using a rather broad range (10-40 μm) of particle size. He did not study the effect of column length.

In preparative HPLC, the behavior of a column is usually given in terms of throughput (TPUT) which can be defined as the amount of a sample obtained from the column per unit time (16). It should be possible to evaluate different columns for potential preparative work with a single sample. Previous workers have evaluated different columns at constant resolution (16, 17). This is convenient because sample size must be varied for each column to reproduce a given resolution.

In this research, we studied a homemade slurry packing system that was built to obtain a set of packed columns with a wide range of lengths and internal diameters. These balanced density slurry packed columns were evaluated and the results correlated with column diameter and length.

Since resolution is an important factor in determining the purity of the collected sample, a new parameter that takes resolution into account was defined as Time Yield Factor (TYF). This factor should better express the preparative capability of different columns,

especially when these are operating at different resolutions.

The slurry packed column that presented, potentially, the highest preparative yield was used in the separation of three isomeric methoxy derivatives of 1,2,3,4,5-pentaphenylcyclo-2,4-pentadien-1-ol (18-21) obtained in our work. The collected fractions were then utilized for structure determination.

## Historical

Chromatography started as a preparative technique. In 1903 and 1906, Tswett (22, 23) described his classical experiment on the separation of colored pigments of leaves. He termed the procedure "chromatography," literally meaning "color writing." Although earlier experiments were performed by Runge (24) and Schoenbein (25), Tswett was the first to understand and define this separation method.

Tswett used a glass column packed with calcium carbonate. The petroleum ether extract of the colored pigments was introduced at the top of the packing. After continuous addition of solvent, separation into colored bands occurred. The packing was then carefully extruded and the sections containing the separated pigments were taken for further analysis. This was the first record of preparative chromatography.

The importance of Tswett's work was recognized by Kuhn in the 1930's (26) and chromatography since that time has expanded rapidly in its use and utility. A diversification of techniques and mobile phases has led to paper, thin layer, gel permeation, ion exchange, liquid-liquid, and gas chromatography.

Column chromatography is utilized almost exclusively as a preparative method. It has played an important role in the development of organic chemistry particularly in the area of natural substances.

Before the advent of chromatography, pure substances were isolated from mixtures chiefly by distillation, sublimation, and crystallization. However, when dealing with components having very similar structures, these techniques often fail to yield pure fractions.

From the original technique utilized by Tswett, improvements evolved so that components could be completely separated and the fractions eluted from the column. This eliminated the inconvenience of extruding the column packing and the extraction of the desired fraction from the column packing. The elution technique was extended to noncolored substances. The column eluent was collected in vials and the components present were detected by suitable methods.

The versatility of chromatography for handling so many different types of molecules can be explained as follows: by changing the natures of the stationary and mobile phases, different interactions between sample molecules and the stationary and mobile phases are established, resulting in the selective retardation of sample components. These selective interactions include partition, adsorption, ion exchange, and gel permeation.

Time and experience have made silica gel and alumina the two most popular adsorbents. Both can be obtained in a variety of particle sizes, pore diameters, surface areas, and activities. The excellent book by Snyder (27) on adsorption chromatography should be pointed out at this time. Using these two adsorbents and by varying the composition of the mobile phase, a wide variation in interactions is obtained suitable for many molecules up to molecular weights of 2000. These

adsorbents are also used as solid supports for liquid-liquid chromatography. Silica gel is used to obtain chemically bonded phases which when having ionic groups can be used for ion-exchange chromatography. Controlled porosity silica gel particles are used for gel permeation chromatography, although less active materials are preferred for Gel Permeation Chromatography (GPC).

Classical preparative liquid chromatography can be described as follows: a glass column with 10-25 mm diameter and 40-100 cm height is loaded with silica gel or alumina, usually as a slurry in the solvent to be later used as mobile phase. This slurrying process eliminates the air trapped between the particles and yields a more homogeneous column packing. There are two commonly used procedures to apply the sample to the column. The first consists of very slowly adding a concentrated solution of the sample to the top of the packing. This is frequently done with the help of a pipet which has a capillary tip. The second procedure consists of adding a solution of the sample to a small amount of packing, evaporating the solvent and placing this packing with sample on the top of the chromatographic column. In both cases the objective is to have a homogeneous and narrow band of sample on top of the column. The eluent is received in fraction collectors and the analyzed fractions combined accordingly. Pure components can be obtained by evaporating the mobile phase. The pure samples are now ready for the desired studies.

Although classical liquid chromatography is capable of yielding pure components from complex mixtures, it is often a very slow process.

Thin layer chromatography demonstrated that many separations were not obtained with classical liquid chromatography and that an increase in performance should be possible and had to be looked for.

Gas chromatographic theory pointed the way to higher column efficiencies: small particles had to be packed tightly into narrow diameter columns. Liquid Chromatographic column packings using small particles and showing higher performance were introduced. Because these required higher pressures, special pumps were constructed. Faster separations became possible because faster flow rates were available. Specially constructed inlet systems made possible the convenient introduction of samples by syringe or valve. Continuous monitoring of the effluent, by using specially designed detectors, simplified the measurement of the separation. Hence, this led to what today is known as High Performance Liquid Chromatography (HPLC). Other references (28-32) that account for some major work that led to HPLC are cited.

A typical instrumental set-up to perform preparative HPLC is shown in Figure 1. It consists of four different basic parts:

- Mobile Phase Supply
- Sample Injection System
- Column
- Detector (Monitor)

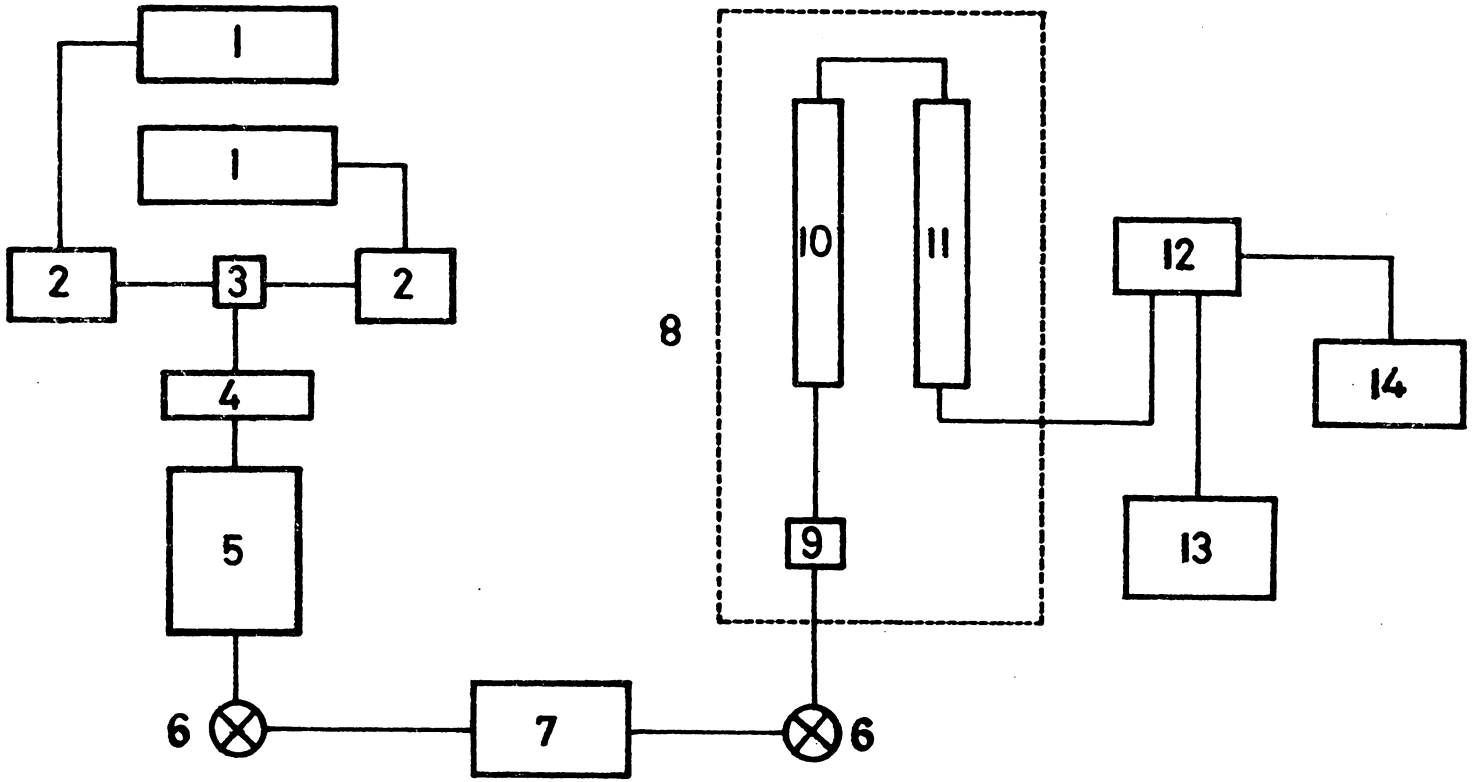



Figure 1. Typical preparative liquid chromatograph. (1) solvent reservoir, (2) degasser, (3) mixer, (4) filter, (5) pump, (6) shut-off valves, (7) pulse damper, (8) thermostat, (9) injection port, (10) pre-column, (11) column, (12) detector, (13) recorder, (14) fraction collector.

## Mobile Phase Supply

Basically the mobile phase supply consists of a solvent reservoir, a pump, and a filter to protect the pump and column from any particulates in the mobile phase. This section could also include a solvent degasser, a gradient forming device and a pulse dampener if a pulsating type of pump is being used. Degassing of the mobile phase has the advantage that it decreases the probability of gas bubbles forming in the detector and disturbing the generated signal.

Gradient elution might be desirable to save time and solvent volume when separating mixtures which have both early and late eluting peaks. Different types of gradient systems are available (33-35). High pressure gradient devices are placed behind the high-pressure pump and low pressure gradient devices in front of the high-pressure pump. The pump utilized in preparative HPLC should have a solvent delivery rate of 5-50 ml/min and a solvent reservoir larger than 1-2 liters. Pneumatic amplifier type pumps for which the flow rate is limited by column back pressure are very convenient for preparative work and can also be used for analytical purposes. Reciprocating pumps are usually more limited in their range of flow rate. Material of construction for pumps is preferably 316 stainless steel.

## Sample Injection System

This section consists of a sample injection device. In preparative

HPLC, sample valves are utilized almost exclusively. They are convenient in that reproducible volumes of samples are injected into a column by simply turning the valve. Also, by changing the volume of the sample loop, different volumes are injected with better reproducibility and convenience than with syringes. With syringe injection, there is a septum which often leaks or is contaminated, and the syringe needle often plugs up.

Considerable attention should be given to the sample introduction technique (36). It is usually accepted that the sample must be rapidly and uniformly swept from the sample loop into the top of the chromatographic column with a minimum dead volume. This is also true for preparative HPLC where usually larger diameter columns are used and a thin and homogeneous sample band on top of the column is desirable. The requirement for small dead volume in preparative HPLC is attained more easily than in analytical HPLC due to the larger samples that are used.

## Column

Stainless steel is the most frequently used material for constructing columns. Column inlets and outlets should be designed so that a laminar mobile phase flow pattern is obtained and the column exit is completely and uniformly swept.

The column packing procedure for HPLC is a very important factor. There are two basic packing procedures. The first is a "dry" packing

technique, the second is a "wet" or slurry packing technique. The "dry" technique is a simple manual method depending upon gravity. The packing is added slowly to the top of the column and the column tapped or vibrated (37). This is done in as reproducible a manner as possible to obtain columns with similar chromatographic behavior. Recently, Reeve Angel (Reeve Angel, Clifton, N.J.) has introduced a vibrator, Chrom-A-Tap IV, especially to make dry packings in 1/4 in. chromatographic columns. Using this vibrator, good results for packings of pellicular type and totally porous packings with particle diameters larger than 20 μm have been obtained (38).

For rigid solids with particle diameters below 20 μm, the best results are obtained by using a "wet" or balanced density slurry packing method (39-42). This method consists of preparing a 15-25% suspension of silica gel, dried at 200° for approximately 4 hr, in a solvent mixture of tetrabromoethane and tetracloroethane having the same density as the silica gel. The suspension is conveniently obtained by ultrasonic mixing of the packing with the balanced density solvent. If the particles remain in suspension after 10 min, the suspension is considered stable. Depending on whether the particles settle or float, more dense or less dense solvent is added until stability is obtained. A good starting point is 60 parts of tetrabromoethane and 40 parts of tetrachloroethane. Particles are dried to avoid agglomeration in hydrophobic solvents.

Cassidy (43) demonstrated that the ultrasonic bath is not necessary if the particles are dried at 210° for 4 hr. The stable suspension

is placed in a slurry reservoir (usually 50 to 100 ml volume); a layer of water is placed on top of the suspension; and the remainder of the reservoir is filled with n-hexane. A constant pressure pump, having n-hexane as the pumping liquid is pressurized to 5000 psig. The chromatographic column to be packed has previously been filled with the balanced density solvent and adapted with the appropriate end fittings. The valve between the pump and column is opened and the slurry rapidly transferred to the column at 5000 psig. After the first drops of water leave the column, the pump is turned off and the pressure in the column decreases slowly to zero.

This procedure should yield a uniform packing. It is recommended that the packing be activated by passing solvents through the column (44). The sequence should go in decreasing polarity (45): methanol, 2-propanol, acetone, diethyl ether, dichloromethane, and finally, n-hexane.

Tetrabromoethane is moderately toxic and not pleasant to handle. Several researchers have tried to work with suspensions that would not require the use of this solvent. Stable aqueous slurries of closely sized porous silica smaller than 10 μm have yielded efficient columns (46-48). Coq (49) packed liquid chromatographic columns under pressure using carbon tetrachloride as a dispersing agent. For aluminas such as Lichrosorb Alox T (5 μm particle diameter), the column efficiency was better with carbon tetrachloride than when using the balanced density method. However, with Lichrosorb SI-100 (silica of 5 μm particle diameter), the balanced density method gave better results.

## Detector (Monitor)

In preparative HPLC, solute concentrations are usually high and sensitive detectors are therefore not necessary. Many sensitive detectors can cause problems due to non linear response with the large sample concentrations common in preparative HPLC. It is recommended that the collected fractions be reinjected into an analytical column to check their purity. A sensitive detector is useful in this case to detect trace impurities in the mixture. The less sensitive refractive index (RI) detectors are well suited for preparative work. It is possible to use RI and UV detectors designed with larger diameter flow cells. Also, multiwave length UV/VIS detectors are convenient for the detection of high concentration effluents by choosing a wavelength where absorption is minimal (2). Transport detectors, like the flame ionization detector, are also useful in preparative work but rather expensive (50). A good combination is a sensitive UV detector for trace impurities in series with a RI detector for large concentrations.

In conclusion, instrument requirements for preparative HPLC are different from those for the best analytical performance. However, many liquid chromatographs are used with success for both analytical and preparative work. Waters Associates (8) has recently introduced a commercial unit specifically designed for preparative work having the capability of receiving several grams of sample at flow rates of up to 500 ml/min.

## Strategy of Preparative Separations

Baker and Larmann (51, 52) discussed preparative HPLC based on the amount of sample required. This will depend on the subsequent use of the material as shown in Table I. Based on these requirements, an estimate of the column dimensions can be made. Carr (4) presents the typical sample sizes for columns of different diameters under different conditions as shown in Table II. These are only guidelines and the actual sample size obtainable will vary greatly depending upon the column packing material, the sample, the mobile phase, and the selectivity of the system.

It is convenient at this point to discuss the requirements that should be used to describe a separation as of analytical, preparative, or intermediate scale. Some authors (53) prefer the designations and corresponding sample amounts as shown in Table III. However, the definition of preparative liquid chromatography varies with the individual worker. Many use the term preparative liquid chromatography irrespective of the amount of the isolated material. This latter approach considers the application of the separation, i.e. if the purpose is to collect and use pure fractions, this is preparative chromatography. We favor this latter definition.

It is recommended that before attempting the separation in a preparative column an analytical separation with the same packing material be developed. The conditions can then be more rapidly adapted to the preparative column. The main reason for this is economic, since

Table I: Sample Quantity Requirements for Preparative High Performance Liquid Chromatography

| Objective | Sample-Weight (mg) |
|---|---|
| Tentative Identification by Instrumental Methods | < 1 |
| Positive Identification by Instrumental Methods Including Nmr. Confirmation of Structure by Chemical Reaction | 1-100 |
| Positive Identification and Subsequent Use in Research or Synthesis Required | >100 |

Table II: Typical Sample Sizes for Various High Performance Liquid Chromatographic Columns

| Column I.D. | 2.1 mm | 6 mm | 23 mm |
|---|---|---|---|
| Difficult Separation | 0.1-1 mg | 1-10 mg | 10-100 mg |
| Easy Separation | 10-50 mg | 100-500 mg | 1-5 g |

Table III: Designation of HPLC Separation Based on Sample Amounts Injected for Different Types of Packing Materials

| Designation | Superfically Porous Supports | Totally Porous Supports | Exclusion Gels |
|---|---|---|---|
| Analytical | 0.1-0.2 mg | 1-2 mg | 10-20 mg |
| Semipreparative or Scale-Up | 10-20 mg | 100-200 mg | 1-2 g |
| Preparative Scale | -- | 0.5-1.0 g | 10 g |

in establishing the appropriate chromatographic conditions considerable amounts of solvents and time could be consumed. Preparative separations should preferably be performed by liquid solid chromatography because this mode provides the greatest flexibility and convenience in the separation of complex mixtures (3). Also, adsorbents cost relatively little. Many materials have been separated on similar adsorbents by TLC and a good preliminary indication of the mobile phase to be used can be obtained by a search of the TLC literature (54).

An important characteristic of the mobile phase for preparative liquid chromatography is volatility. While the solvent used for analytical liquid chromatography can be extended to preparative separations, it is important to be able to remove the solvent easily from the collected fractions. The successful use of the fraction depends upon the ability to remove the solvent and evaporation is the simplest way to perform this. Convenient solvents include hexane, dichloromethane, and even methanol. Less volatile solvents such as 2-propanol and water are not easily removed by evaporation and should be avoided if possible in preparative work.

It can be convenient to use TLC as an aid in finding the appropriate mobile phase even if no similar separations are reported in the literature. If the same adsorbent is used for TLC and for HPLC column, the mobile phase that is adequate for the TLC separation will be a good starting point for HPLC, but minor adjustments will be necessary.

Stahl has proposed a rapid screening procedure to determine the suitable solvent for LSC (55). Taking a standard 5 x 20 cm TLC plate,

he applied to one side several spots of sample, allowing the solvent to evaporate. He prepared a set of test solvents having increasing strength and applied dropwise one of the solvents to each spot. To check for the purity of the adsorbent on the TLC plate, he also applied the corresponding solvent to the left of the plate. The plates were dried and visualized by an appropriate method. The solvent composition that produced the most rings was the most appropriate mobile phase for that separation. Minor adjustments of solvent composition for HPLC separation were necessary. Figure 2 illustrates this procedure and shows that solvent D is the best solvent. This procedure saves time and cost for solvents that would be used in developing the analytical separation. Once the analytical separation is developed, the separation is taken to the larger column and similar conditions should yield a separation which now can be optimized for the preparative work.

It is convenient to make an estimate on some chromatographic parameters when scaling up a separation. Suppose that a separation on an analytical column having an internal diameter of 2.0 mm and a length of 100 cm is to be performed on a column having an internal diameter of 15.8 mm and 41 cm length (56). Typical chromatographic conditions are given in Table IV. The calculated scale-up values shown in Table IV are obtained by using the expressions shown in Table V.

It should be noticed that there is a considerable consumption of expensive mobile phase for the preparative column, justifying the preliminary work with TLC and analytical columns. If the separation cannot be obtained on the analytical column, it will probably not be

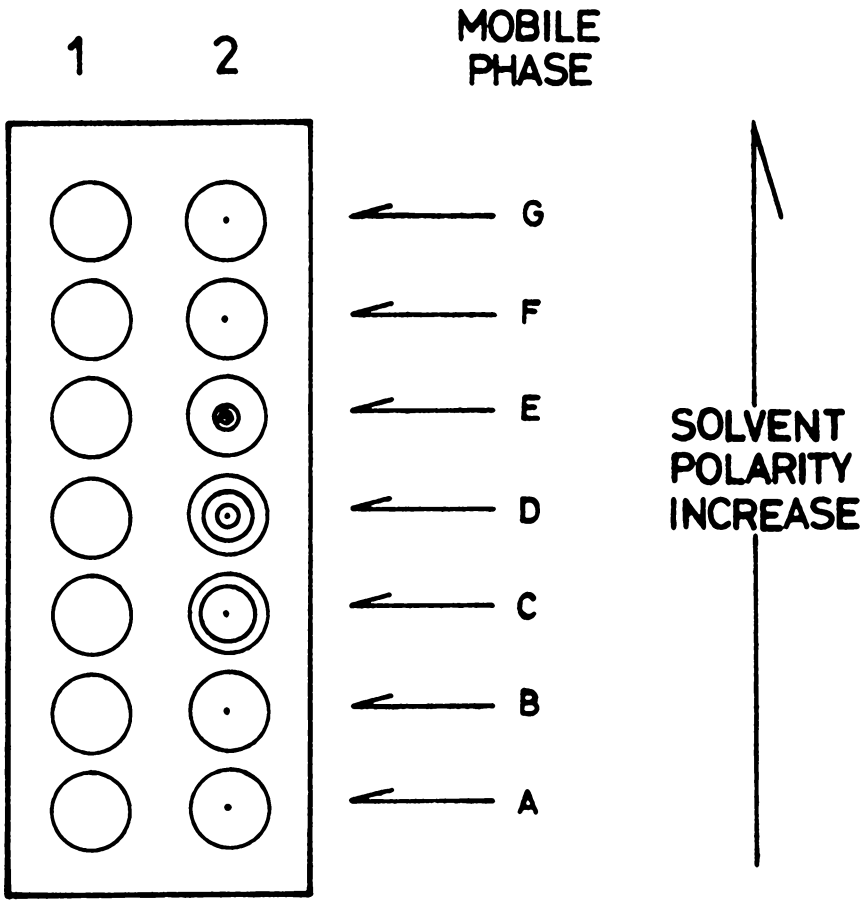



Figure 2. Rapid TLC screen for LSC mobile phase. (1) pure solvent; (2) solvent plus sample.

Table IV: Scale-Up Values Calculated for a Preparative HPLC Column

| Chromatographic Parameter | Analytical Column | Preparative Column |
|---|---|---|
| Column Length (cm) | 100 | 41 |
| Column I.D. (mm) | 2 | 15.8 |
| Column O.D. (inches) | 1/4 | 3/4 |
| Packing Weight (g) | 5.7 | 146* |
| Linear Velocity (cm/sec) | 1.30 | 0.31 |
| Flow Rate (ml/min) | 1 | 15* |
| $t_m$ (min) | 1.28 | 2.20 |
| $V_m$ (ml) | 1.28 | 33.0* |
| Analysis Time (min) | 16 | 28* |

*Calculated scale-up values

Table V: Equations to Determine the Theoretical Scale-Up for Preparative HPLC Columns

| Preparative Column | Formula |
|---|---|
| Theoretical Packing Weight | $\text{Packing wt. anal.} \times \left(\frac{\text{i.d. prep.}}{\text{i.d. anal.}}\right)^2 \times \frac{\text{L prep.}}{\text{L anal.}}$ |
| Theoretical Flow Rate | $\text{Flow rate anal.} \times \left(\frac{\text{i.d. prep.}}{\text{i.d. anal.}}\right)^2 \times \frac{\text{lin. vel. prep.}}{\text{lin. vel. anal.}}$ |
| Actual Dead Volume, $V_m$ | $t_m \times$ flow rate |
| Theoretical Dead Volume | $\text{Dead vol. anal.} \times \left(\frac{\text{i.d. prep.}}{\text{i.d. anal.}}\right)^2 \times \frac{\text{L prep.}}{\text{L anal.}}$ |
| Theoretical $t_r$ | $t_r \text{ anal.} \times \frac{t_m \text{ prep.}}{t_m \text{ anal.}}$ |

possible to perform the preparative work on the larger column.

Once the analytical separation has determined the type of packing material and the mobile phase, the same type of mobile phase is initially used to test the separation on the larger column with the same packing material. Because the preparative column contains more packing, a higher resolution is usually observed. This increased resolution is important in preparative work since it will allow for band broadening when the column is overloaded. With the analytical column, the interest is in separation at minimum time, at minimum resolution, with minimum amount of sample. In the preparative work, the interest lies in obtaining the maximum amount of sample in a minimum time with a resolution that will yield fractions with the desired purity. Flow rates of 10-20 ml/min would be necessary in the larger 8 mm i.d. columns to give linear solvent velocities comparable to those used in analytical HPLC. In practice, working at lower linear velocities, 3-6 ml/min, increased resolution will yield higher sample capacity (4). When components in the preparative column are excessively retained, a small adjustment of mobile phase polarity is made. Gradient elution, flow programming, and recycle chromatography (57, 58) are recommended in some specific separations.

After the resolution in the preparative column has been optimized, sample load is increased until a further increase in sample load would yield fractions with unacceptable purity. As the sample load is increased, proper adjustments on the detector may have to be made.

In conclusion, in order successfully to obtain a preparative

separation, the following sequence is recommended:

- Develop an initial separation on an analytical column using TLC as an orienting technique;
- Optimize the resolution on preparative column while adjusting solvent polarity;
- Gradually increase the sample load on the preparative column to the limit of fraction purity.

## Typical Preparative Problems

There are many different types of mixtures to which preparative HPLC can be applied. Frequently, it is possible to place a preparative problem in one of the categories illustrated in Figure 3. The efficient handling of these basically different problems requires that different strategies be applied.

In the case of one major component in a mixture, after the analytical separation is obtained, the resolution on the preparative column is optimized. The sample load is then increased until the minor and major component peak start to overlap. At this point, collection of the major component can be made. However, a higher yield is obtained if the column is further overloaded and only the central portion (heart cut) of the peak is collected. Purity of the collected portion can be

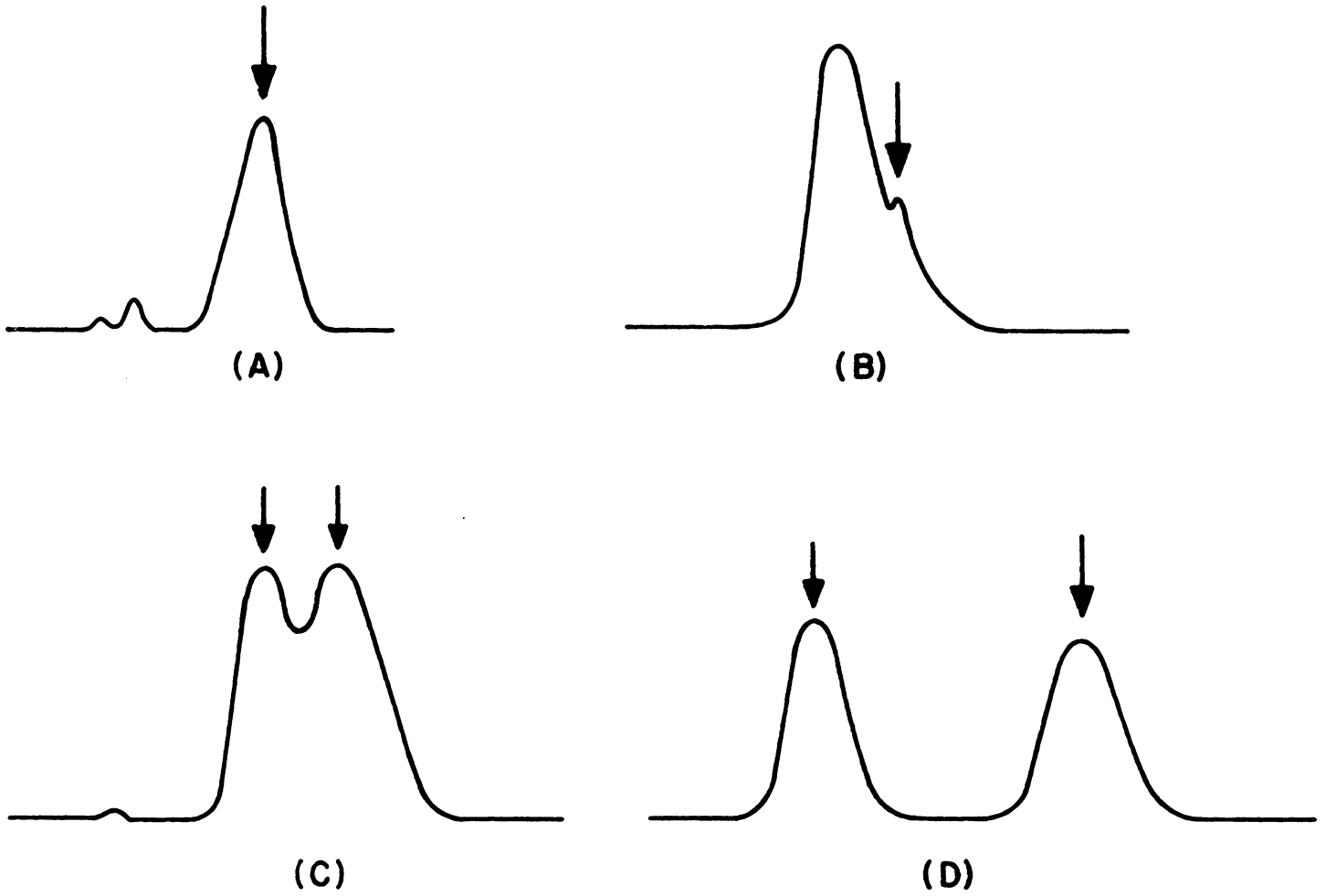



Figure 3. Typical preparative HPLC situations. (A) single major component is desired, (B) minor component is desired, (C) poorly resolved major components are desired, (D) well resolved major components are desired.

checked by injecting a sample into an analytical column.

If the component of interest is present as a minor part of the mixture, a different approach is taken. The first step is to overload the column and collect various fractions from the region where the sample of interest is present. The various fractions are analyzed and those richer in the minor component are combined. Now the mixture contains the compound of interest as a major component and is treated as discussed above.

The third possible situation happens frequently when isomeric compounds are separated with two or more major components being close together. If these two components are available in sufficient amount, the most efficient approach is to collect the leading edge of the least retained peak and the trailing edge of the last peak. These should consist of pure components. If the amount of sample available is small, the cross-contaminated portion can be reinjected, and again, the two pure fractions collected as previously described.

Finally, the last case is the most desirable but most seldom encountered situation: two major components are well separated and can be collected in high yields and high purity. The literature (9, 59-63) can be reviewed for examples of these various preparative circumstances.

# Experimental

## Column Packing

High pressure slurry packing apparatus. All columns were packed using the high pressure slurry packing apparatus shown schematically in Figure 4.

The Model DST-122 pneumatic amplifier pump used (Haskel Enginnering and Supply Co., Burbank, Calif) (B) was capable of delivering the solvent contained in the reservoir (A) at constant pressure up to 19000 psig. The pump head had a volume of 4 ml. The air pressure (D) applied to the pump inlet was amplified 122 times at the pump outlet. The balanced density slurry was fed into the 80 $cm^3$ slurry reservoir (G) through the valve (F) (Model SS-43S4, Whitey Research Tool Co., Emeryville, Calif) with the help of a special narrow bore glass funnel (E). Air escaped through the valve (C). The pressurized liquid forced the slurry through valve (H) into the chromatographic column (I) (Handy and Harman Tube Co., Norristown, Pa), fitted with a 2 μm stainless steel frit at the bottom (Mott Metalurgical Corp., Farmington, Conn). A cylinder (J) collected and measured the effluent from the column during the packing procedure. All the tubing and Swagelock® fittings connecting valve (F) to column (I) were specially drilled out to give a larger internal diameter to allow air to escape while the slurry was being loaded.

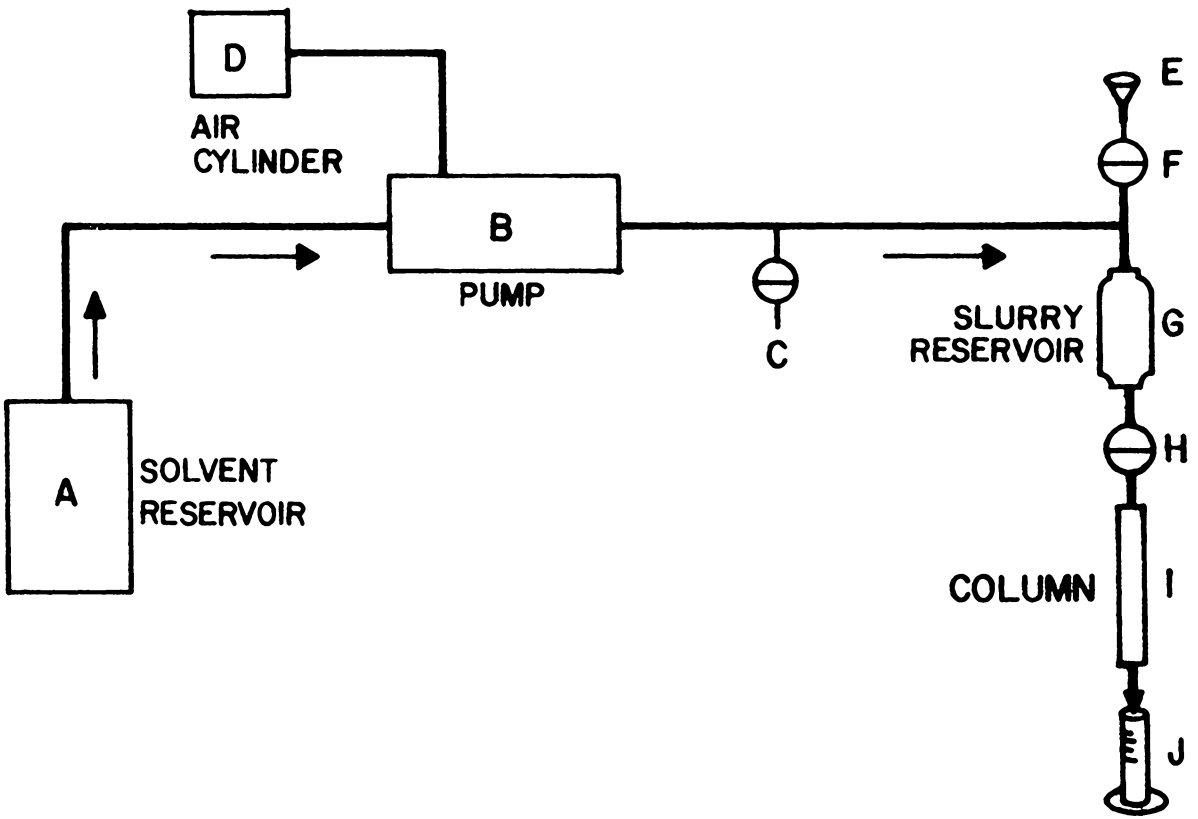



Figure 4. Slurry-packing apparatus.

Chemicals. Tetrabromoethane and tetrachloroethane were of reagent grade (Fisher Scientific Co., Fair Lawn, N.J.). Prior to use, the halogenated solvents were purified by passing them through silica gel, 60-200 mesh (Grace and Davidson Chemical, Baltimore, Md). A yellow impurity was removed by this treatment. The silica had been activated by heating for 4 hr at 200°.

Distilled water was used as an immiscible layer between the balanced density slurry and the pressurizing liquid, technical grade n-hexane (Fisher Scientific Co., Fair Lawn, N.J.). Reagent grade methanol, 2-propanol, and methylene chloride (Fisher Scientific Co., Fair Lawn, N.J.) were used as activation solvents. Solvents of lower polarity for further activation were not used because in these studies methylene chloride was the mobile phase.

Packing material. E. Merck Lichrosorb SI-100 (EM Laboratories, Elmsford, N.Y.), totally porous, nonspherical silica gel with an average particle diameter of 10 μm (particle size analysis: $d_{10}$=8 μm; $d_{50}$=10 μm; and $d_{90}$=13 μm, where $d_{90}$=13 μm means that 90% of the particles will pass through a 13 μm screen) was used.

Balanced density slurry packing procedure. For each column size, different volumes of balanced density solvent and weight of packing were used. For a 1/2 in. x 20 cm x 9.9 mm column, the procedure was as follows: into a 125 ml erlenmeyer flask was placed 8.3 g of silica, previously dried at 200° for 4 hr, and 70 ml of the balanced density solvent. This solvent was made up of 60% tetrabromoethane and 40% tetrachloroethane. Adjustment of the final density was performed by

trial and error whenever necessary. If the silica floated, tetrachloroethane (the less dense liquid) was added; if the silica had the tendency to precipitate, more of the denser tetrabromoethane was added. Experiments with these two solvents were carried out with good ventilation due to their moderate toxicity. If the silica was not adequately dried, the particles would stick together in the hydrophobic media. No ultrasonic degassing was utilized. Using a long stemmed funnel (E), the stable suspension was introduced to the slurry reservoir (G). Air escaped through valve (C). Column (I) had been previously filled with the balanced density solvent and valve (H) closed. At this point, 10 ml of water was carefully added to the top of the slurry, care being taken not to disturb the slurry since this would transfer silica to the water layer, and the volume was then completed with n-hexane. Valves (F) and (C) were then closed and the pump (B) was pressurized to 5000 psig with n-hexane from the solvent reservoir (A). Valve (H) was opened and the slurry rapidly transferred to column (I). When 65 ml of solvent had been collected in the graduated cylinder (J), the pump was shut off and the pressure allowed to decrease slowly to ambient pressure. Valve (H) was then closed, column (I) disassembled, and the top end fittings placed on the chromatographic column. The slurry reservoir was then rinsed with water and acetone.

Adsorbent activation. Activation of the silica packing was performed by pumping through the column 400 ml of methanol followed by 400 ml of methylene chloride with 1.5% 2-propanol added. This latter solvent was used as the mobile phase in these studies, thus, eliminating

the need of further activation with less polar solvents.

Columns. Sixteen columns with dimensions shown in Table VI were carefully prepared. Stainless steel tubing (Handy and Harman Tube Co., Norristown, Pa) were machine cut to obtain clean and flat ends. The columns were then washed with detergent, rinsed with acetone, and dried. The Swagelock® end fittings (Dibert Valve and Fitting Co., Inc., Richmond, Va) were machined out to a flat surface to allow for a "zero" dead volume with the flat tube end. The holes of the end fittings were drilled out to allow for the 1/16 in. inlet and outlet tubings to touch the surface of the 2 μm stainless steel frits (Mott Metallurgical Corp., Farmington, Conn) which were placed between the column ends and the end fittings.

## Column Evaluation

Equipment. The liquid chromatograph used was the MSI Model B-500 (Molecular Separations Incorporated, Champion, Pa) equipped with a pneumatic amplifier pump with a 230 ml capacity and 2000 psig maximum operating pressure, adapted with an injection valve Model HSPV (Spectra Physics, Santa Clara, Calif) and a dual cell ultraviolet detector operating at 254 nm and having a cell path of 10 mm.

Sample loops used were of 20 μl unless otherwise stated. Table VIII shows the volumes of the sample loops used when injecting sample volumes proportional to the volume of the columns.

Table VI: Dimensions of HPLC Columns Used

| Column Length in mm | Column Diameters | | | | |
|---|---|---|---|---|---|
| | O.D. 1/4" I.D. 2.1mm | O.D. 1/4" I.D. 3.9mm | O.D. 3/8" I.D. 7.0mm | O.D. 1/2" I.D. 9.9mm | |
| 200 | 0.693 | 2.39 | 7.70 | 15.4 | V |
| | 1.00 | 3.45 | 11.2 | 22.3 | NV |
| | 3.46 | 11.9 | 38.5 | 77.0 | A |
| | 1.00 | 3.45 | 11.2 | 22.3 | NA |
| 300 | 1.04 | 3.58 | 11.6 | 23.1 | V |
| | 1.51 | 5.19 | 16.7 | 33.5 | NV |
| | 3.46 | 11.9 | 38.5 | 77.0 | A |
| | 1.00 | 3.45 | 11.2 | 22.3 | NA |
| 450 | 1.56 | 5.38 | 17.3 | 34.6 | V |
| | 2.26 | 7.80 | 25.1 | 50.2 | NV |
| | 3.46 | 11.9 | 38.5 | 77.0 | A |
| | 1.00 | 3.45 | 11.2 | 22.3 | NA |
| 675 | 2.34 | 8.06 | 26.0 | 52.0 | V |
| | 3.38 | 11.7 | 37.7 | 75.3 | NV |
| | 3.46 | 11.9 | 38.5 | 77.0 | A |
| | 1.00 | 3.45 | 11.2 | 22.3 | NA |

NV = normalized volume
V = volume in $cm^3$

NA = normalized area
A = area in $mm^2$

Table VII: Designation of HPLC Columns Used

| L | Column Designation | | | |
|---|---|---|---|---|
| 20 | PC #1 | PC #5 | PC #9 | PC #13 |
| 30 | PC #2 | PC #6 | PC #10 | PC #14 |
| 45 | PC #3 | PC #7 | PC #11 | PC #15 |
| 67.5 | PC #4 | PC #8 | PC #12 | PC #16 |
| I.D. | 2.1 | 3.9 | 7.0 | 9.9 |

L = column length in cm
I.D. = column internal diameter in mm

Table VIII: Volumes of Sample Loops Proportional to Column Volumes

| Column | | Sample Loop |
|---|---|---|
| Designation | Volume ($cm^3$) | Volume ($\mu l$) |
| PC #1 | 0.69 | 20 |
| PC #2 | 1.04 | 30 |
| PC #3 | 1.56 | 45 |
| PC #4 | 2.33 | 67.5 |
| PC #5 | 2.39 | 69 |
| PC #6 | 3.58 | 104 |
| PC #7 | 5.38 | 156 |
| PC #8 | 8.06 | 234 |
| PC #9 | 7.70 | 224 |
| PC #10 | 11.6 | 334 |
| PC #11 | 17.3 | 502 |
| PC #12 | 26.0 | 754 |
| PC #13 | 15.4 | 446 |
| PC #14 | 23.1 | 670 |
| PC #15 | 34.6 | 1004 |
| PC #16 | 52.0 | 1506 |

A 10 mv Model 255 (Linear Instruments Corp., Irvine, Calif) recorder was used. A schematic representation of the instrumental set up is shown in Figure 5.

Chemicals. The methylene chloride and 2-propanol that were used were the same as described earlier for column activation. Dimethylphthalate (DMP) and acetanilide were of reagent grade (Eastman Kodak, Rochester, N.Y.) and diethyldiphenylurea was of technical grade (Story Chemical Corp., Muskegon, Mich).

All the samples were dissolved in methylene chloride and Table IX shows the concentration of the various samples utilized in this work.

Procedure. After each packed column had been activated as described above, samples having the different sample volumes and concentrations described below were injected.

Column efficiency. Twenty µl of sample number 1 (PS #1) with component concentrations as shown in Table IX was used to evaluate column behavior at constant sample load. Sample PS #1 was used with volumes proportional to the column volumes as shown in Table VIII to evaluate column behavior at proportional sample load.

Column throughput and time yield factor. Table X shows the concentrations of diethyldiphenylurea and acetanilide in the samples used to obtain the values for resolution (R), column throughput (TPUT), and time yield factor (TYF) for the 1/4 in. x 20 cm x 3.9 mm column (PC #5).

The results for TYF, TPUT, and R for column PC #9, PC #5, and PC #1, reported in Table VII, were obtained by using a 20 µl sample loop and sample concentrations as shown in Table IX. For this study only,

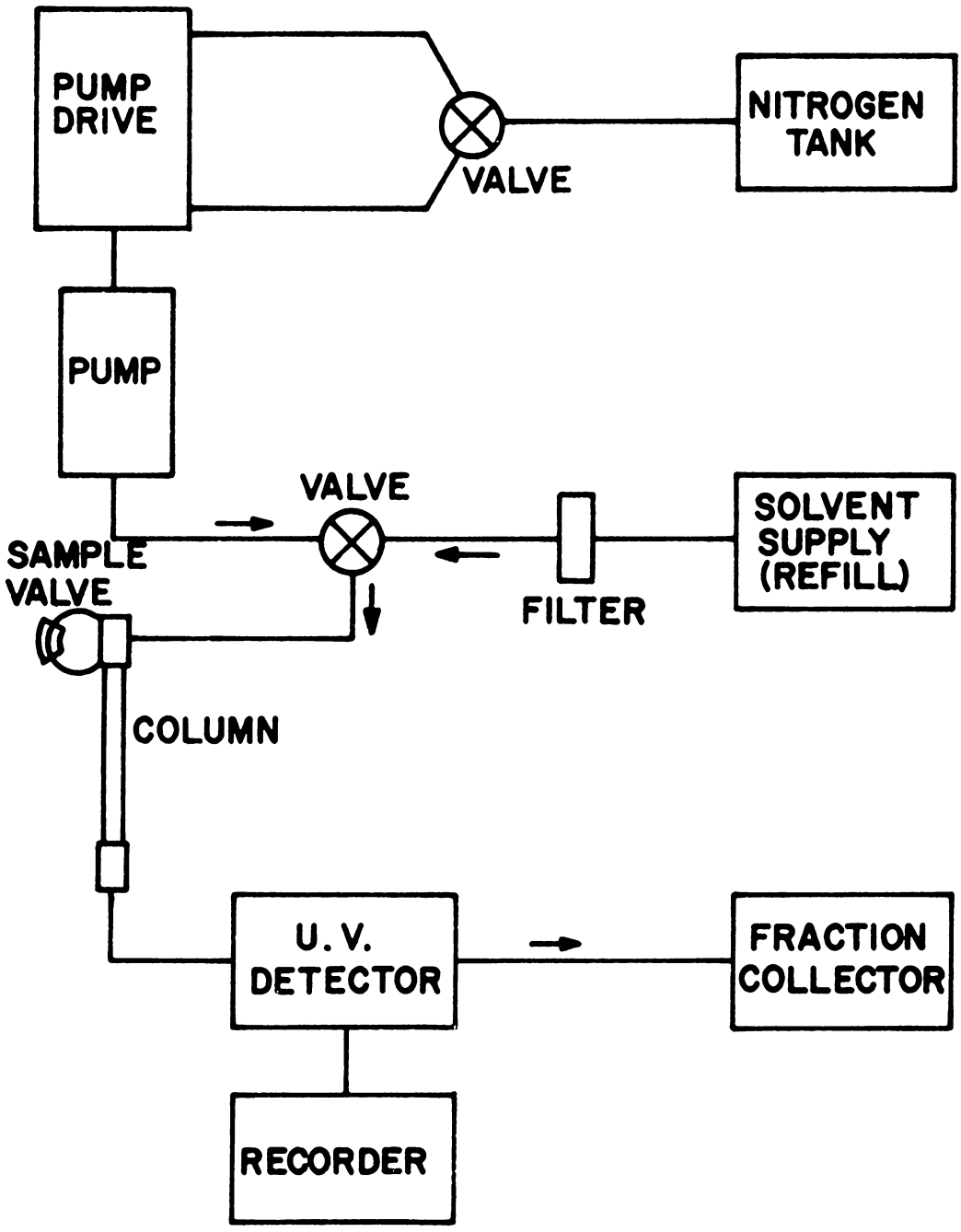



Figure 5. Schematic diagram of the preparative HPLC system used.

Table IX: Sample Concentrations for Dimethylphthalate (DMP), Diethyldiphenylurea (EPU), and Acetanilide in Methylene Chloride to Evaluate Columns PC #1 to PC #16

| Sample | Sample Concentration (g/l) $x10^2$ DMP, EPU, or Acetanilide |
|---|---|
| PS #1 | 4.000 |
| PS #2 | 6.000 |
| PS #3 | 9.000 |
| PS #4 | 13.50 |
| PS #5 | 32.00 |
| PS #6 | 64.00 |
| PS #7 | 90.00 |
| PS #8 | 115.0 |
| PS #9 | 148.0 |
| PS #10 | 180.0 |

Table X: Sample Amounts and Concentrations for Diethyldiphenylurea (EPU) and Acetanilide in Methylene Chloride to Obtain TPUT, TYF, and R Values for PC #5

| Sample | EPU or Acetanilide | |
|---|---|---|
| | Grams per Liter | Milligrams per 50 µl |
| S #1 | 5.88 | 0.29 |
| S #2 | 8.70 | 0.43 |
| S #3 | 12.50 | 0.63 |
| S #4 | 18.18 | 0.91 |
| S #5 | 25.00 | 1.25 |
| S #6 | 36.36 | 1.82 |
| S #7 | 50.00 | 2.50 |
| S #8 | 66.7 | 3.30 |
| S #9 | 80.00 | 4.00 |
| S #10 | 100.0 | 5.00 |

diethyldiphenylurea and acetanilide were considered. The same solutions as described in Table IX were used to obtain the values for R, TYF, and TPUT for PC #1.

Figure 6 illustrates how retention times and band widths (64) were determined to calculate various chromatographic parameters.

## Preparative Separations

Equipment. The liquid chromatograph used for the separation of the isomeric methoxy derivatives shown in Figure 7 were performed with the same instrumental set-up as described for the column evaluation.

The HPLC column for the analytical separation had the following characteristics: 1/4 in. x 20 cm x 3.9 mm, stainless steel, packed with E. Merck Lichrosorb SI-100, 10 μm totally porous particles (EM Laboratories, Elmsford, N.Y.). For the preparative separations, the column dimensions were 1/2 in. x 20 cm x 9.9 mm with the same packing material. The analytical samples were applied with a sample valve having a 334 μl loop. Other chromatographic conditions are shown in Figures 8 and 9.

Chemicals. The mobile phase for the analytical separations was 0.1% ethyl acetate in spectroquality n-hexane (Burdick and Jackson Laboratories, Inc., Muskegon, Mich). For the preparative separations, 0.08% ethyl acetate in n-hexane (by volume) was used.

Procedure. The separation developed for the analytical column

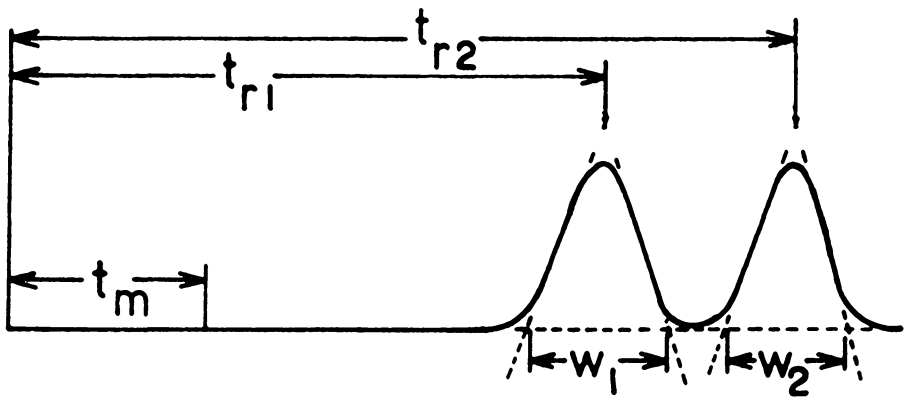



Figure 6. Chromatogram illustrating the definition of ($t_r$) retention time, ($t_m$) mobile phase retention time, and (w) band width.

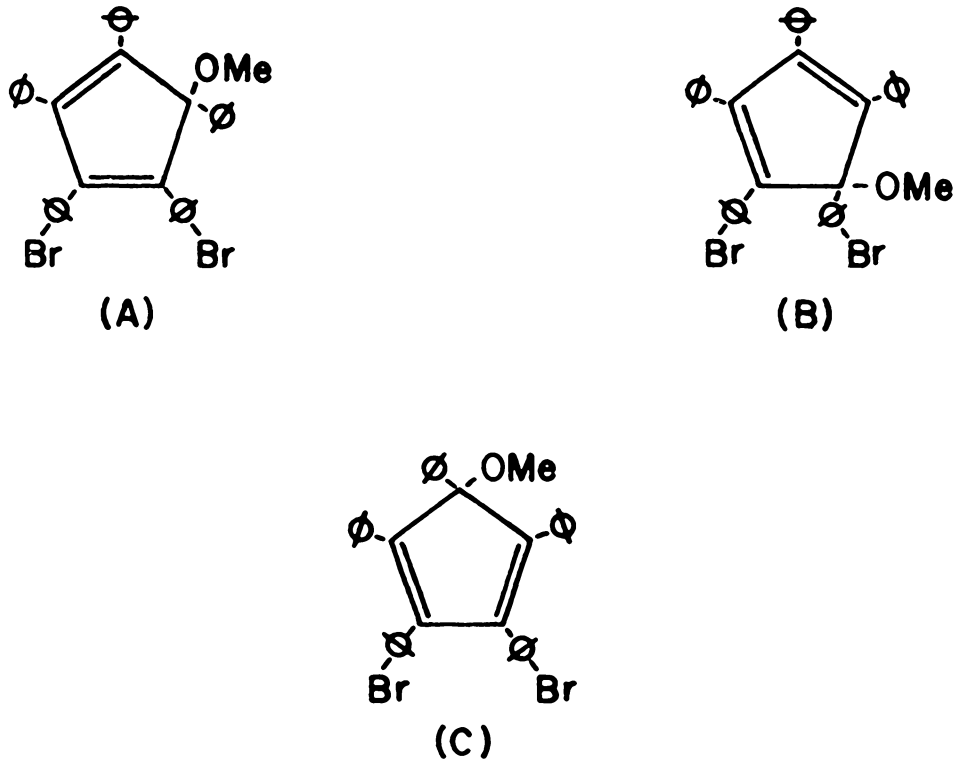



Figure 7. Structural formulas for (A) 2,3-di(p-bromophenyl)-1,4,5-triphenylcyclo-2,4-pentadien-1-methyl ether, (B) 1,2-di(p-bromophenyl)-3,4,5-triphenylcyclo-2,4-pentadien-1-methyl ether, and (C) 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-methyl ether.

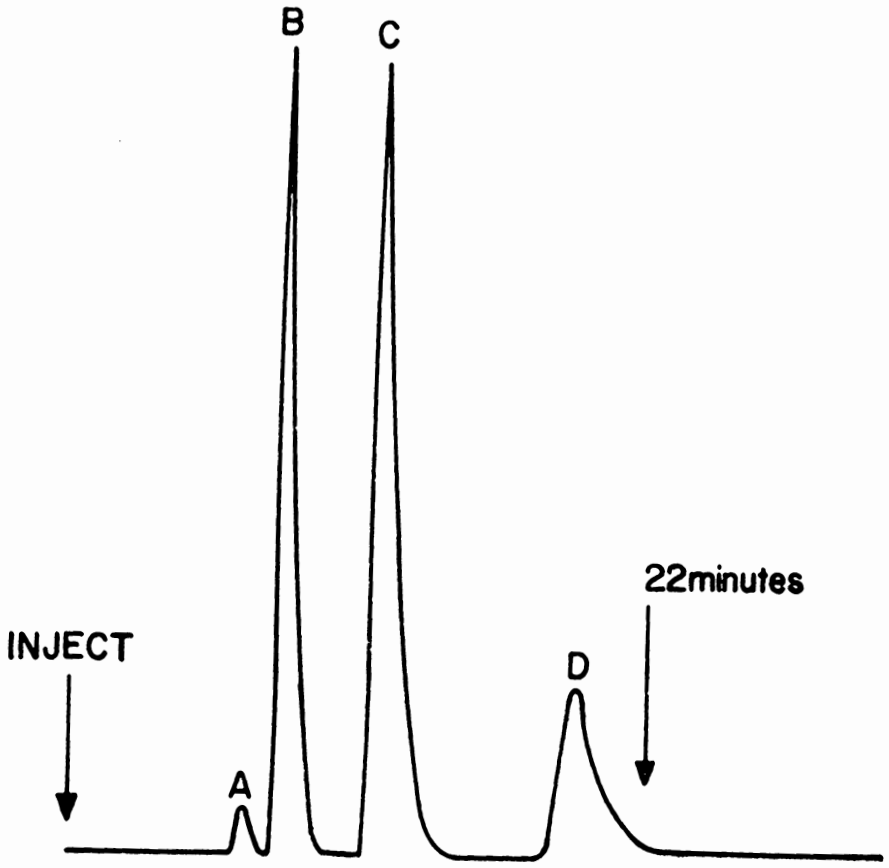



Figure 8. HPLC separation of isomeric methoxy derivatives (B), (C), (D). Column: 1/4 in. O.D. x 50 cm x 2.1 mm I.D.; packing: 10 μm Lichrosorb SI-100; mobile phase: n-hexane at 1.1 ml/min; detector: UV at 254 nm and 0.02 ABS sensitivity; sample size: 10 μl; A=starting product.

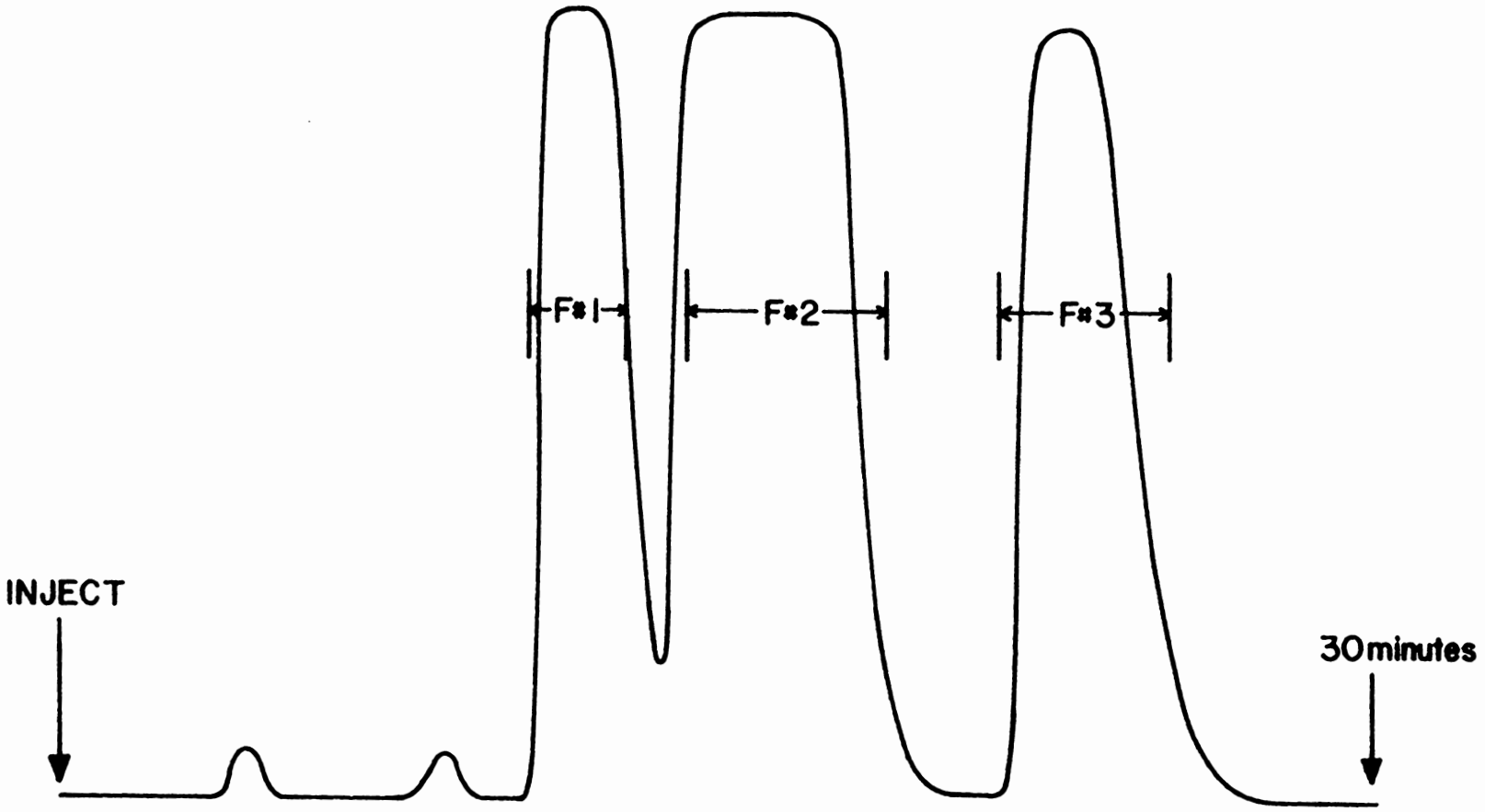



Figure 9. Preparative separation of isomeric methoxy derivatives. Column: 0.5 in. O.D. x 20 cm x 9.9 mm I.D.; packing: 10 µm Lichrosorb SI-100; mobile phase: 0.08% ethyl acetate in n-hexane at 4.9 ml/min; detector: UV at 254 nm and 0.64 ABS sensitivity; sample size: 1.5 mg in 334 µl.

indicated the initial mobile phase to be used in the preparative column. Resolution was then increased by decreasing the solvent strength by using 0.08% ethyl acetate in n-hexane. The sample size was increased by enlarging the sample loop volume and the sample concentration until the resolution reached the point where the purity of the collected fractions was unacceptable. Other chromatographic conditions are shown in Figure 8 and 9. The purity of the collected fractions was monitored by reinjections under analytical conditions.

Three fractions, as shown in Figure 9, were collected in ground glass stoppered glass bottles. The purity of each fraction is shown in the chromatograms reproduced in Figures 10 through 12. The fractions were concentrated by evaporation and transferred to 1 ml glass vials. By gently heating in a water bath and applying vacuum to the vials, solid precipitates were left behind. Each of the pure fractions was submitted for mass spectral and nmr analysis as discussed in the synthesis section.

The same approach to separating the three isomeric methoxy derivatives described above was used for the preparative separation of 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-methyl ether. Figure 15 shows the analytical HPLC separation of the impure alumina column fraction, while Figure 16 shows the developed preparative HPLC and Figure 17 the analytical HPLC analysis of the collected fractions from the preparative HPLC column. All conditions used are shown on these figures. Collected fractions were analyzed as discussed in the synthesis section.

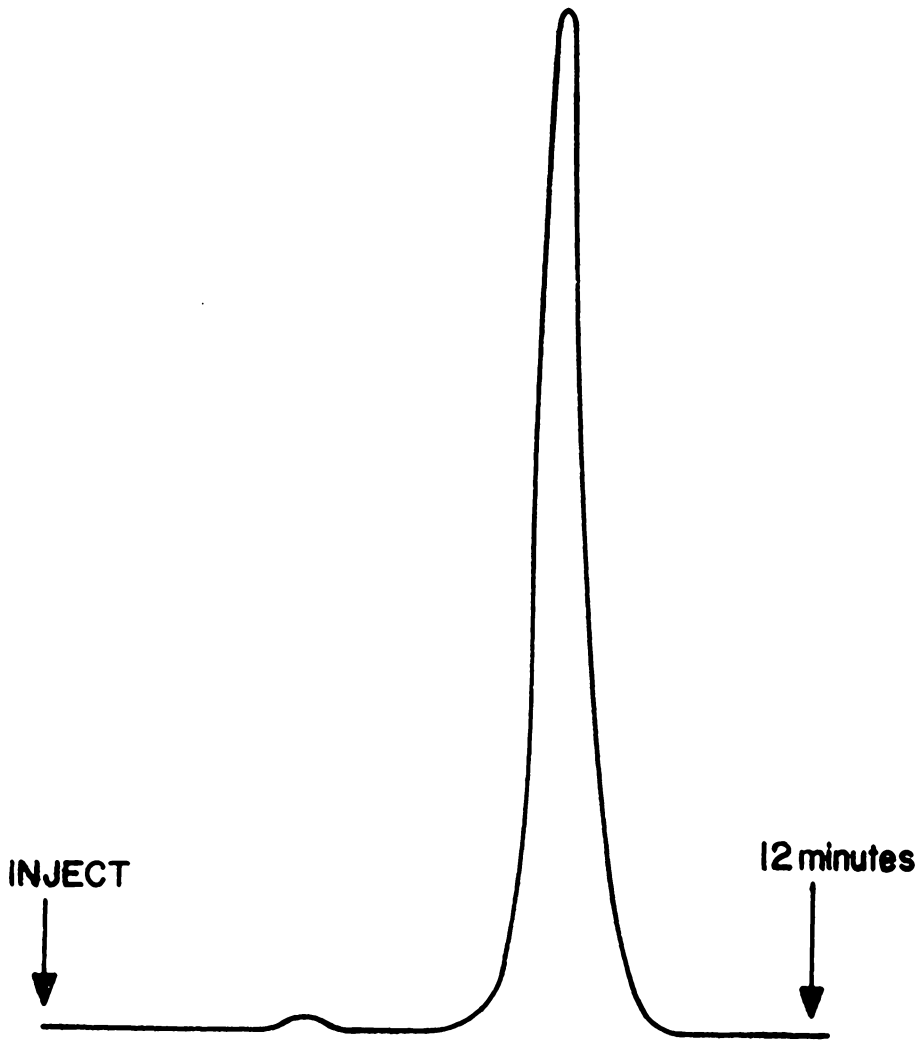



Figure 10. Analytical chromatogram of preparative fraction F #1 shown in Figure 9. Column: 1/4 in. O.D. x 20 cm x 3.9 mm I.D.; packing material: 10 μm Lichrosorb SI-100; mobile phase: 0.08% ethyl acetate in n-hexane at 1.5 ml/min; detector: UV at 254 nm and 0.01 ABS sensitivity; sample size: 20 μl.

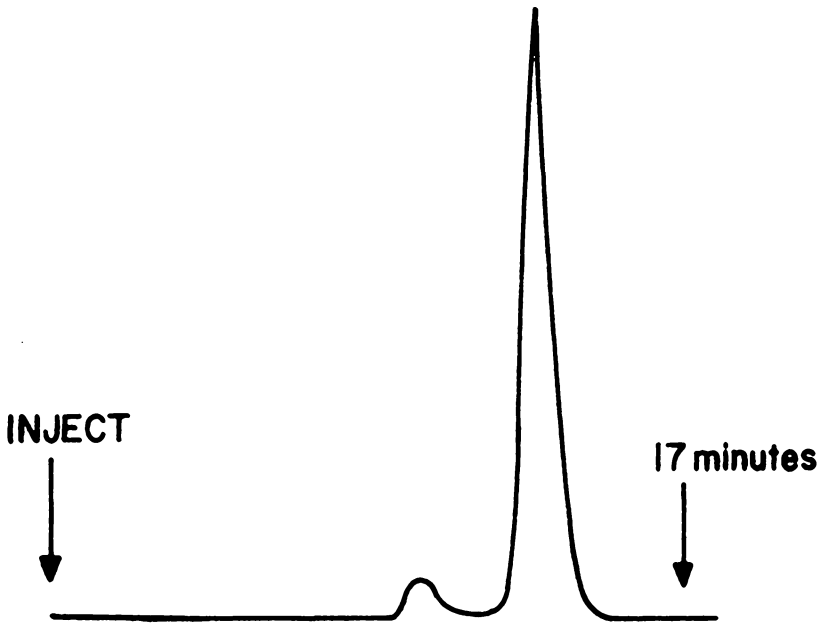



Figure 11. Analytical chromatogram of preparative fraction F #2 shown in Figure 9. Column: 1/4 in. O.D. x 20 cm x 3.9 mm I.D.; packing material: 10 μm Lichrosorb SI-100; mobile phase: 0.08% ethyl acetate in n-hexane at 1.5 ml/min; detector: UV at 254 nm and 0.02 ABS sensitivity; sample size: 20 μl.

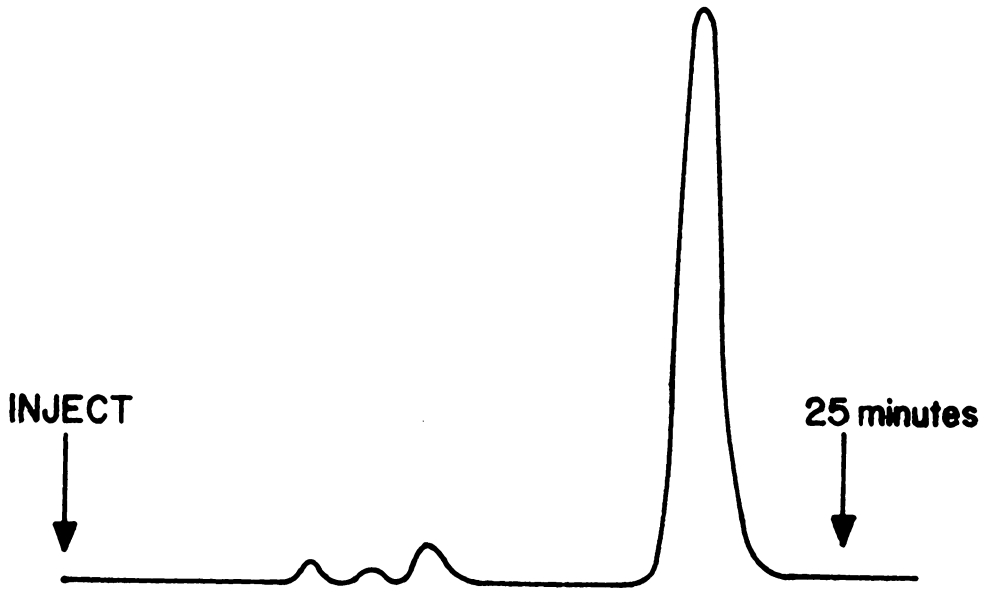



Figure 12. Analytical chromatogram of preparative fraction F #3 shown in Figure 9. Column: 1/4 in.O.D. x 20 cm x 3.9 mm I.D.; packing material: 10 μm Lichrosorb SI-100; mobile phase: 0.08% ethyl acetate in n-hexane at 1.5 ml/min; detector: UV at 254 nm and 0.01 ABS sensitivity; sample size: 20 μl.

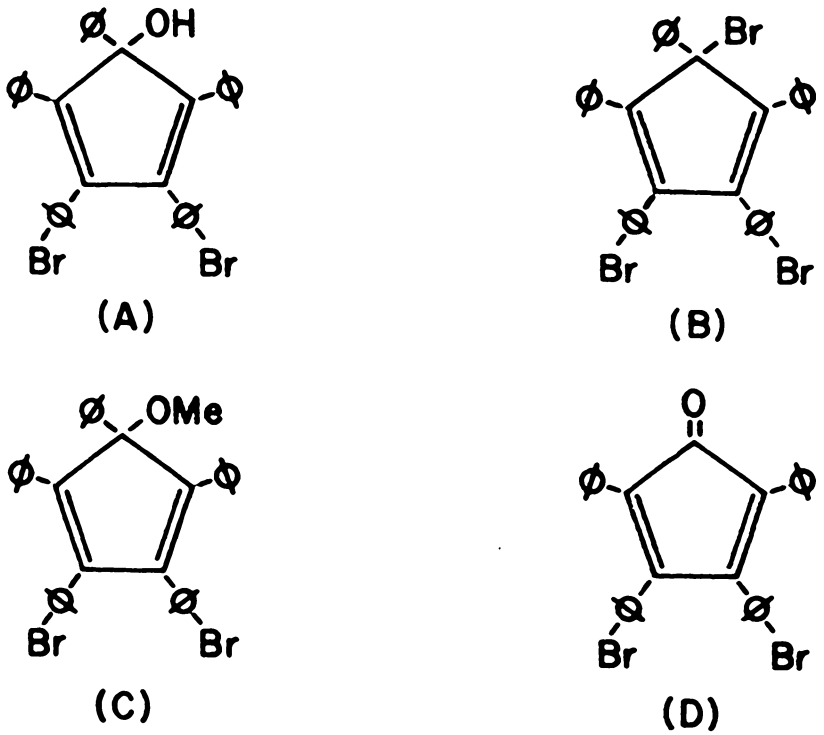



Figure 13. Structural formulas for (A) 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-ol, (B) 1-bromo-3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadiene, (C) 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-methyl ether, and (D) 3,4-di(p-bromophenyl)-2,5-diphenylcyclo-2,4-pentadien-1-one.

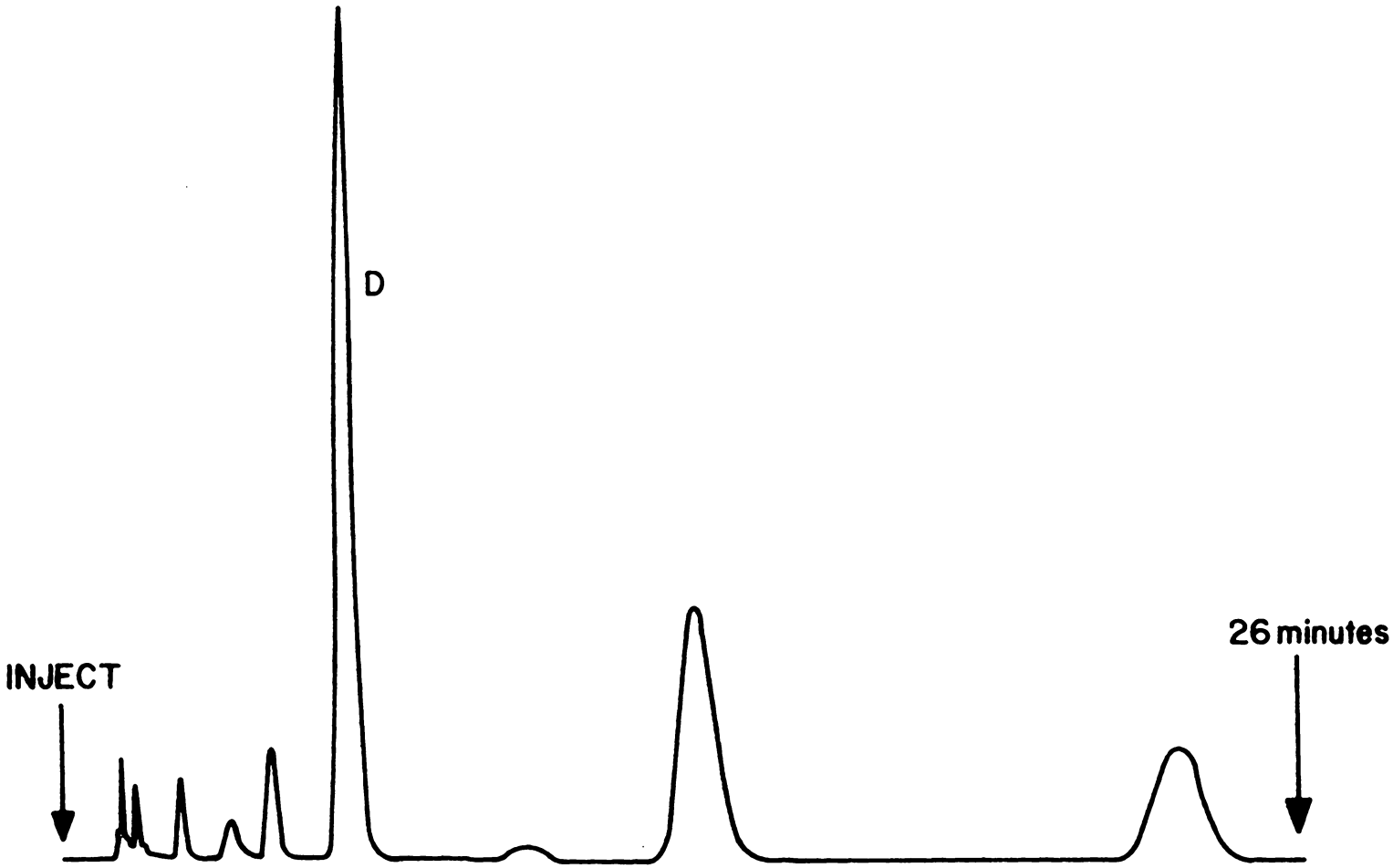



Figure 14. HPLC separation of reaction product (D) 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-methyl ether. Column: 1/4 in. O.D. x 20 cm x 3.9 mm I.D.; packing material: 10 μm Lichrosorb SI-100; mobile phase: 0.1% ethyl acetate in n-hexane at 2.7 ml/min; detector: UV at 254 nm and 0.02 ABS sensitivity; sample size: 20 μl.

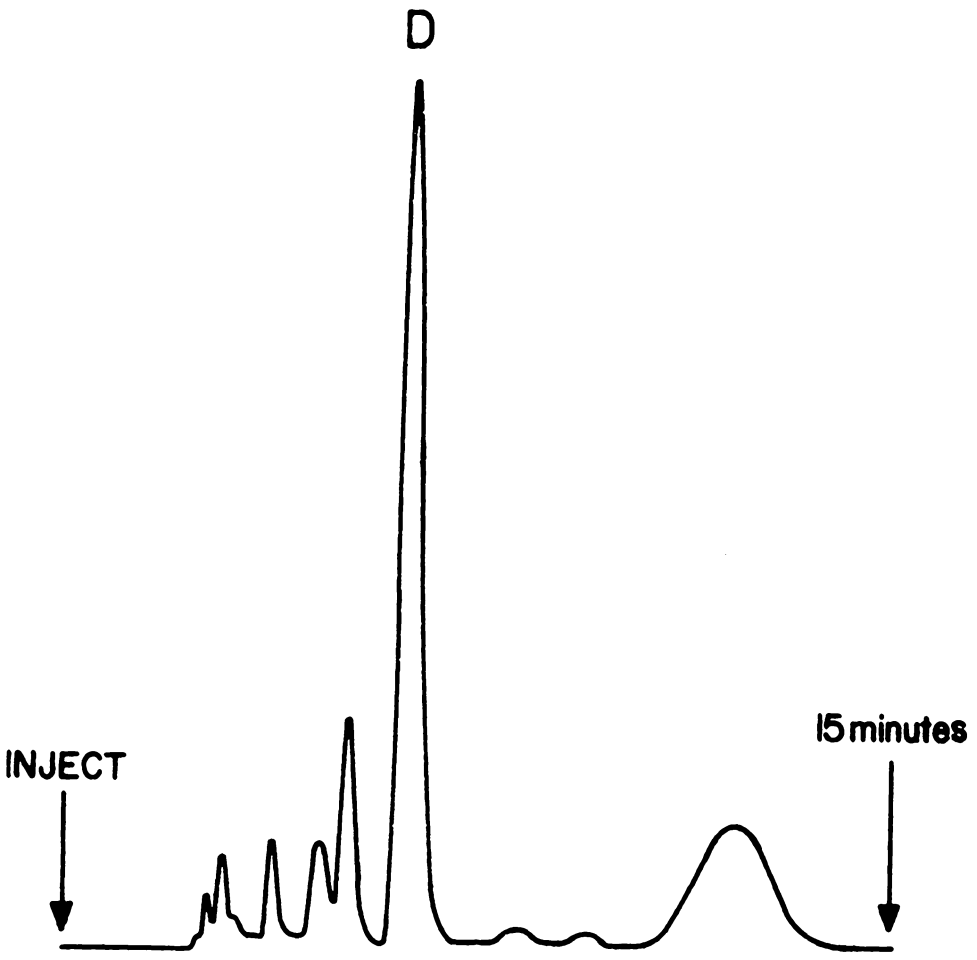



Figure 15. HPLC separation of alumina column fraction. (D) is 3,4-di-(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-methyl ether. Column: 1/4 in. O.D. x 20 cm x 3.9 mm I.D.; packing material: 10 µm Lichrosorb SI-100; mobile phase: 0.1% ethyl acetate in n-hexane at 2.8 ml/min; detector: UV at 254 nm and 0.04 ABS sensitivity; sample size: 20 µl.


Figure 16. Preparative HPLC separation of 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-pentadien-1-methyl ether (F #1). Column: 0.5 in. O.D. x 20 cm x 9.9 mm I.D.; packing material: 10 μm Lichrosorb SI-100; mobile phase: 0.08% ethyl acetate in n-hexane at 9.6 ml/min; detector: UV at 254 nm and 0.08 ABS attenuation; sample size: 502 μl.

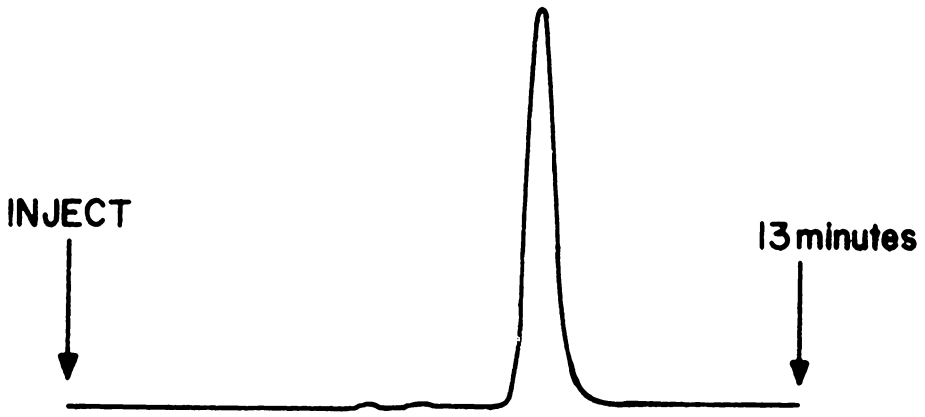



Figure 17. Analytical separation of collected fraction F #1 shown in Figure 16. Column: 1/4 in.O.D. x 20 cm x 3.9 mm I.D.; packing material: 10 μm Lichrosorb SI-100; mobile phase: 0.1% ethyl acetate in n-hexane at 1.5 ml/min; sample size: 20 μl.

## Synthesis and Identification

### Preparation of 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-ol (Figure 13A) (65)

Procedure. Into a 1-ml, three-necked, round-bottomed flask equipped with a reflux condenser, dropping funnel, nitrogen inlet, and magnetic stirrer, was placed 2.40 g (0.10 g-atom) of magnesium turnings and 15.70 g (0.10 mol) of bromobenzene in 125 ml of dry ether. After the Grignard reaction was completed, 13.55 g (0.025 mol) of 3,4-di(p-bromophenyl)-2,5-diphenylcyclo-2,4-pentadien-1-one (Figure 13D) in 125 ml of dry benzene was added slowly with stirring. After the addition was completed, the mixture was refluxed with stirring for 1.5 hr, then cooled to room temperature and hydrolyzed with 10% ammonium chloride solution. The organic layer was then separated, washed with water, dried over anhydous $MgSO_4$ and concentrated. The viscous brown oil was crystallized from 95% ethanol to give 12.9 g of product. The alcohol (Figure 13A) was purified by column chromatography as described below.

A portion of 3.50 g of the impure alcohol (Figure 13A) was dissolved in a minimum amount of carbon tetrachloride and placed on a glass column (60 cm length and 2.5 cm internal diameter) packed with Brockman activity I neutral alumina, 80-200 mesh. Gradient elution starting with pure carbon tetrachloride and ending with 50/50 $CCl_4/CHCl_3$ was utilized. The alcohol band was easily followed as a white fluorescence under ultraviolet radiation. The collected fractions were separated from the mobile

phase by evaporation. The solid dissolved in 95% ethanol and was concentrated until cloudiness appeared and the solution was left sitting overnight. The bright yellow crystals obtained, 2.45 g (0.004 mol, 70%), were washed with 95% ethanol and air dried. The melting temperature was 192-194°.

Equipment and analysis. Melting temperatures were determined on a Thomas-Hoover (Arthur H. Thomas Co., Philadelphia, Pa) melting temperature apparatus in open capillary tubes.

Infrared spectra were obtained on a Beckman IR-20A-X double beam spectometer (Beckman Instruments Inc., Fullerton, Calif).

Elemental analysis were obtained on a departmental Perkin-Elmer Model 240 carbon, hydrogen, and nitrogen analyzer (Perkin Elmer, Norwalk, Conn).

Nuclear magnetic resonance spectra were obtained on a departmental JEOL-PS-100 (Japan Electronic Optics Laboratories, Co. Ltd., Tokyo, Japan).

Mass spectra were obtained on a Varian Mat 112 (Bremen, Germany).

Benzene and ethyl ether were of reagent grade, dried over 50 pm molecular sieves. Carbon tetrachloride and chloroform were of reagent grade purified by passing through 200° activated, silica gel, 60-200 mesh. Ethanol and bromobenzene were of reagent grade. All chemicals mentioned were distributed by Fisher Scientific Co., Fair Lawn, N.J. The tetracyclone (m.t. 244°) and 3,4-di(p-bromophenyl)-2,5-diphenylcyclo-2,4-pentadien-1-one (Figure 13D) were kindly provided by Dr. M. A. Ogliaruso.

Preparation of 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-bromide (Figure 13B)

The synthetic procedure used was similar to the one reported by Youssef (66) and was followed by thin layer chromatography (TLC).

Into a 1000-ml, three-necked, round-bottomed flask equipped with a magnetic stirrer, a reflux condenser, and a gas dispersion tube, was placed 10 g (0.016 mol) of 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-ol in 100 ml of glacial acetic acid. Hydrogen bromide gas (Matheson Gas Products, East Rutherford, N.J.) was then passed through the solution for 45 min with gentle heating. The mixture was then heated under reflux and the course of reaction followed by observing the disappearance of the alcohol on a TLC plate. When the reaction was finished (2.15 hr), an orange precipitate was present. This precipitate was filtered and recrystallized from a 1:9 mixture by volume of benzene-petroleum ether (b.t. 30-60°) to give 9.3 g (0.014 mol, 85%) of an orange product with a melting temperature of 187-189°.

TLC conditions. Silica gel Q precoated 5 x 20 cm glass plates (Quantum Industries, Fairfield, N.J.) were used without further activation. The mobile phase was benzene. Spots were observed under ultraviolet radiation since only the alcohol showed fluorescence. Under visible radiation, the bromo derivative appeared as an orange spot and the alcohol as a bright yellow spot. The component of lower $R_f$ value corresponded to the alcohol.

Analysis. Calculated for $C_{35}H_{23}Br_3$:C, 61.49; H, 3.37; Br, 35.14.

Found: C, 61.63; H, 3.44.

Preparation of an isomeric methoxy mixture (Figure 7) containing 3,4-di (p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-methyl ether; 2,3-di(p-bromophenyl)-1,4,5-triphenylcyclo-2,4-pentadien-1-methyl ether; and 1,2-di(p-bromophenyl)-3,4,5-triphenylcyclo-2,4-pentadien-1-methyl ether

Into a 1-l., one-necked, round-bottomed flask equipped with a reflux condenser and a magnetic stirrer were put 7 g (0.01 mol) of 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-bromide, 210 ml of acetonitrile and 320 ml of methanol. The suspension was heated under reflux and nitrogen gas. There was a gradual dissolution of the bromide, which was complete after 9 hr. The solution turned brighter yellow as the reaction proceeded. Thin layer chromatography was used to follow the course of the reaction. Glass plates (5 x 20 cm), precoated with silica gel Q, were used without further activation. The mobile phase was 90% n-hexane/10% chloroform. Spots were observed under ultraviolet radiation where only the methoxy derivatives show fluorescence. The bromide derivative appeared as a dull spot which was orange under visible radiation as compared to bright and light yellow spots for the methoxy derivatives. With multiple development, the methoxy spot started to separate into 3 distinct spots showing similar yellow color and fluorescence. The spot of lower $R_f$ value corresponded to the methoxy derivatives. After 13 hr, the spot corresponding to the bromide was almost gone and the reaction was stopped.

The solution was filtered, heated, and distilled water added until cloudiness appeared. It was then cooled in an icebath and filtered. The precipitate, 5.6 g (0.009 mol, 90%) was filtered and washed with aqueous methanol and air dried overnight. The melting temperature was 165-185°.

Analysis. Calculated for $C_{36}H_{26}OBr_2$:C, 68.14; H, 4.10; Br, 25.14. Found: C, 67.87; H 4.41; Br, 25.43. The mass spectrum showed a molecular ion peak at m/e = 632; NMR($CCl_4$) $\delta$ 7.18(m, 23H) and 3.42(s, 3H).

## Preparation of 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-methyl ether (Figure 13C)

The procedure was adapted from the synthesis of the 1,2,3,4,5-pentaphenylcyclo-2,4-pentadien-1-methyl ether performed by Breslow (67).

Into a 1-l., three-necked, round-bottomed flask equipped with a magnetic stirrer, a dropping funnel, and a reflux condenser equipped with a drying tube to protect the reaction from moisture, was put 0.37 g (0.015 mol) of sodium hydride (Alfa Ventron, Beverly, Mass) in 250 ml of dry ether, and 1 g (0.0016 mol) 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-ol dissolved in 150 ml of dry benzene was slowly added from the dropping funnel with stirring. After the addition was completed, the mixture was refluxed with stirring for 2 hr until evolution of hydrogen ceased. Methyl iodide (2.13 g, 0.015 mol, dissolved in 250 ml of dry ether) was then slowly added with stirring. The mixture was then refluxed with stirring and the reaction followed by

TLC. Glass plates (5 x 20 cm), precoated with silica gel Q (Quantum Industries, Fairfield, N.J.), were used without further activation. The mobile phase was 90% n-hexane and 10% chloroform. Both spots showed fluorescence under the ultraviolet radiation but had distinct $R_f$ values as shown in Table XVI. The presence of a small fluorescent spot at the $R_f$ corresponding to the methoxy derivative occurred after 10 hr of reaction. After 170 hr of reaction, almost no alcohol was detected and the reaction was stopped. A sample separated by HPLC showed that there were, in addition to the peak corresponding to the 1-methoxy derivative, other major components. Water was added to the reaction mixture and the separated layer washed with distilled water, dried with anhydrous magnesium sulfate, filtered and concentrated. Purification of the 1-methoxy derivative was first attempted by classical column chromatography which yielded only a partially pure product as shown by the HPLC analysis (Figure 10). This fraction was then submitted to preparative HPLC as described in the section for preparative HPLC.

Partial purification of 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-methyl ether was performed to protect the preparative HPLC column from the more polar products as detected by TLC and analytical HPLC. The concentrated organic phase was applied to the top of the 80-200 mesh column of Brockman activity I alumina.

The column length was 40 cm and the internal diameter 15 mm. Elution was started with n-hexane and completed with 10% ethyl acetate in n-hexane. The compound of interest (together with several less polar impurities) left the column well separated from several more retained

nonfluorescent brownish-orange bands. The collected bands were followed down the column by their yellow color which showed a characteristic fluorescence under ultraviolet radiation.

The mass spectrum showed a molecular ion peak at m/e = 632.

## Preparation of 1-chloro-3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadiene

Procedure. Into a 250-ml, three-necked, round-bottomed flask equipped with a magnetic stirrer, a reflux condenser, a 50-ml addition funnel, and a nitrogen inlet was placed 4.0 g (0.0065 mol) of 3,4-di-(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-ol. Thionyl chloride (20 ml, 33.1 g, 0.269 mol), recently distilled from a mixture containing 30% by volume of cotton-seed oil, was slowly added from the addition funnel to the reaction mixture. The alcohol dissolved completely in the excess of thionyl chloride and the mixture was heated under reflux. The reaction course was followed by the evolution of $SO_2$ and HCl and stopped after 2.5 hr after these gasses could no further be detected. The excess thionyl chloride was distilled under vacuum and the precipitate was recrystallized from 60 ml of 1:9 by volume mixture of benzene and petroleum ether (b.t. 30-60°) to give 3.9 g (0.0062 mol, 95%) of a yellow-orange product with a melting temperature of 182-183°.

Analysis. Calculated for $C_{35}H_{23}ClBr_2$:C, 65.78; H, 3.60; Cl, 5.56; Br, 25.06. Found: C, 65.87; H, 3.60. Mass spectometric analysis

showed a peak at m/e = 636.

# Results and Discussion

## Column Packing and Evaluation

The wet packing procedure we developed is similar to the technique described by Kirkland (39) and Majors (40). However, we did not use ultrasonic degassing and found no problems in obtaining a good suspension when the silica packing was dried for 4 hr at 200°. Trying to obtain a stable suspension when the particles were dried at only 150° led to agglomeration of the hydrophilic silica gel particles. Similar results were reported by Cassidy (43). The water layer on top of the balanced density slurry was not allowed to pass through the column. However, no studies on the effect of the passage of water through the column impregnated with the hydrophobic solvent were made.

The concentration of the packing material in the balanced density solvent should affect the stability of the suspension. If this concentration exceeded 25-30% by weight, a very viscous suspension was obtained. It can be expected that the air contained inside the pores of the packing would become trapped by this viscous medium. The columns packed with high viscosity suspensions when taken off the packing apparatus showed a slow expansion of the bed out of the column. This is probably due to the expansion of the air trapped in the viscous medium. The hypothesis that this expansion is caused by the expansion of n-hexane in the column can not be valid since the pressure on the pump

outlet was decreased to zero and no expansion was observed when lower concentrations of suspensions were used. For these suspensions of higher viscosity, the use of ultrasonic degassing eliminated the trapping of air inside the pores of the particles and no expansion of the packed bed was observed. On the other extreme, when the concentration of the particles in the slurry was lower than 8-10% by weight, the volume of slurry that had to flow through the column bed was many times the volume of the column. This made the flow rate drop off rapidly, eliminating the advantages of a rapid transfer of the packing material into the column. Most of the time the column was not completely filled with packing and part of the packing would adhere to the sides of the slurry reservoir. As a result of these findings, the concentration of the suspension was maintained between 15% and 25% by weight. These data suggest that there should be an optimum slurry concentration and that for each column volume an appropriate slurry reservoir volume should be used.

Rapid addition of the water layer to the top of the slurry caused turbulence, and a portion of the hydrophilic packing together with trapped air was transferred into the water layer. Therefore, to avoid the possibility of this heterogeneous system, water was slowly added as a continuous film moving down the walls of the slurry reservoir.

One of the advantages of packing one's own columns is that the user can control the performance of the columns. After developing the packing technique, it is possible to compare the column behavior with previously determined standards. If necessary, the column can be

unpacked and repacked until it meets a certain standard. A second advantage of packing one's own columns is the lower cost. Figure 18 shows the prices of commercially available 25 cm columns of various internal diameters, packed with totally porous micro-particles and compares them with the cost of purchased materials used to pack one's own columns, as of 1975. The cost of the packing material used in the column is relatively low: $4 for a 25 cm x 3.9 mm i.d. column. Labor involved was not taken into account. The estimated total commercial value of the 16 packed columns described in Table VI is $5,000. Expenses incurred for making our columns were approximately $900 for the slurry apparatus, $320 for packing material, $80 for solvents, and $300 for tubing and fittings. A saving of approximately $3,400 was realized for 16 columns.

Waters Associates (68) guaranteed columns 25 cm x 4 mm packed with 10 μm totally porous μ Porasil for a minimum of 9000 plates per meter. Reeve Angel (69) reported obtaining the equivalent of 25000 plates per meter for a 25 cm x 4.6 mm column packed with totally porous 10 μm Partisil PXS packing. Table XI shows the results obtained by evaluating the 16 columns that were packed for this work. Results are presented in plates per meter for EPU and acetanilide (Table XI) using both 20 μl samples and sample volumes proportional to column volume as shown in Table VIII. The column efficiencies obtained compared favorably with the best published data available.

It is necessary to discuss the best way to express column efficiency. Workers in chromatography most often express column efficiency

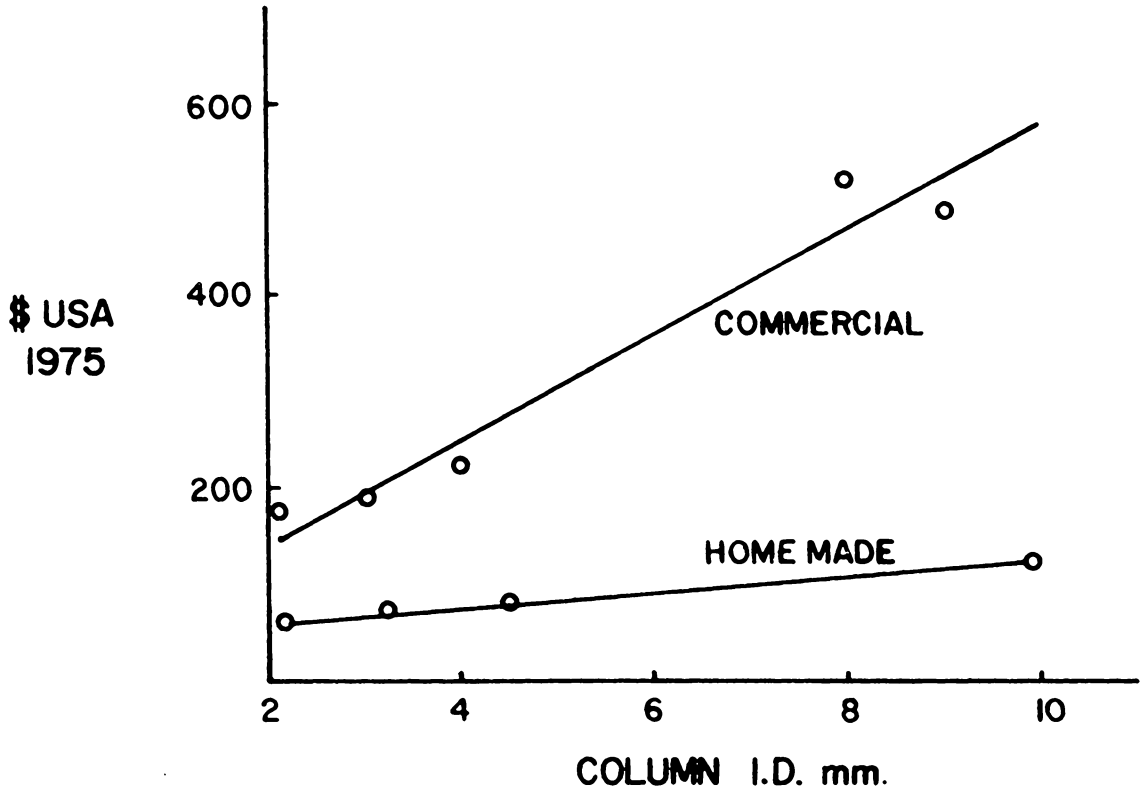



Figure 18. Comparison of prices for commercial and home made HPLC columns with 25 cm length and various internal diameters packed with microporous silica gel.

Table XI: Plates per Meter (N/m) Obtained for EPU and Acetanilide with PS #1

| Column | Plates per Meter | | | |
|---|---|---|---|---|
| | 20 µl Sample | | Proportional Sample Volumes | |
| | EPU | Acetanilide | EPU | Acetanilide |
| PC #1 | 8240 | 7080 | 8240 | 7080 |
| PC #2 | 7330 | 5910 | 7450 | 3190 |
| PC #3 | 2000 | 1250 | 3560 | 1400 |
| PC #4 | 2030 | 1660 | 5420 | 1300 |
| PC #5 | 18100 | 15400 | 20600 | 11300 |
| PC #6 | 13800 | 12000 | 15900 | 8400 |
| PC #7 | 9180 | 8432 | 12100 | 3910 |
| PC #8 | 6040 | 6110 | 7600 | 4700 |
| PC #9 | 24200 | 16500 | 28000 | 15000 |
| PC #10 | 17800 | 13000 | 20100 | 10100 |
| PC #11 | 13700 | 9270 | 13900 | 6290 |
| PC #12 | 9390 | 7160 | 9840 | 5040 |
| PC #13 | 32000 | 18700 | 43400 | 16700 |
| PC #14 | 20500 | 14800 | 27100 | 9880 |
| PC #15 | 17200 | 10900 | 21700 | 7880 |
| PC #16 | 11200 | 8560 | 13400 | 5520 |

Conditions: packing material: 10 µm Lichrosorb SI-100; mobile phase: 1.5% isopropyl alcohol in methylene chloride at $\bar{u}_o$=0.21 cm/sec; sample volumes proportional to column volume as shown in Table VIII.

in terms of plates (N) per meter (m). Therefore, a chromatographic column with a 20 cm length showing 5000 theoretical plates is said to have 25000 N/m. However, it is a known fact that if this column were 1 meter long, less than 25000 plates would be obtained. Height Equivalent to a Theoretical Plate (HETP or H) of a column is obtained by dividing the column length (L) by N (70). Therefore, H is an actual value for the column and seems a more universal expression for column efficiency. However, H values obtained for different column lengths will also vary. This fact makes it necessary always to mention the column dimensions when expressing column efficiency either in terms of H or N/m. Because of the limitations in expressing column efficiency, the more usual N/m representation will be used in this thesis.

Using the data for the 20 µl sample from Table XI, tridimensional plots of column efficiency versus column length and internal diameter were drawn. Figure 19 shows the data for EPU and Figure 20 shows the data for acetanilide. At constant column length, column efficiency increases as the internal diameter increases. Maintaining a constant internal column diameter, N/m decreases as column length increases.

Some researchers (71, 72) have suggested that this increase in column efficiency with increased diameter is related to wall effects. A centrally injected sample on top of the column does not reach the packing region close to the walls during the separation. This process is referred to as an "infinite-diameter column." Knox (71) presented the following equation to define an "infinite-diameter column":

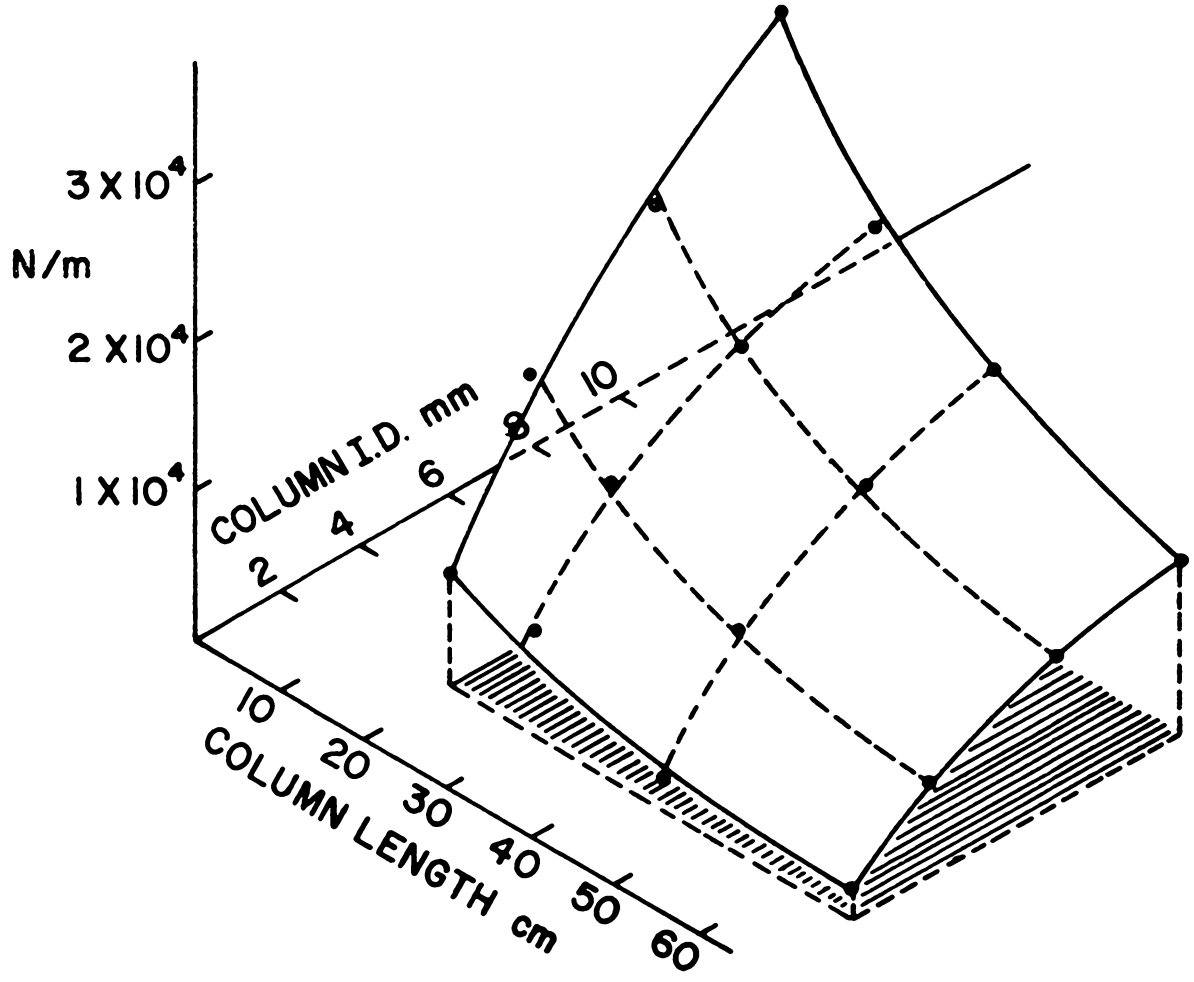



Figure 19. Plot of N/m versus column I.D. and column length for diethyldiphenylurea. Packing material: 10 μm Lichrosorb SI-100; mobile phase: 1.5% isopropyl alcohol in methylene chloride at $\bar{u}_o$=0.21 cm/sec; sample size: 20 μl.

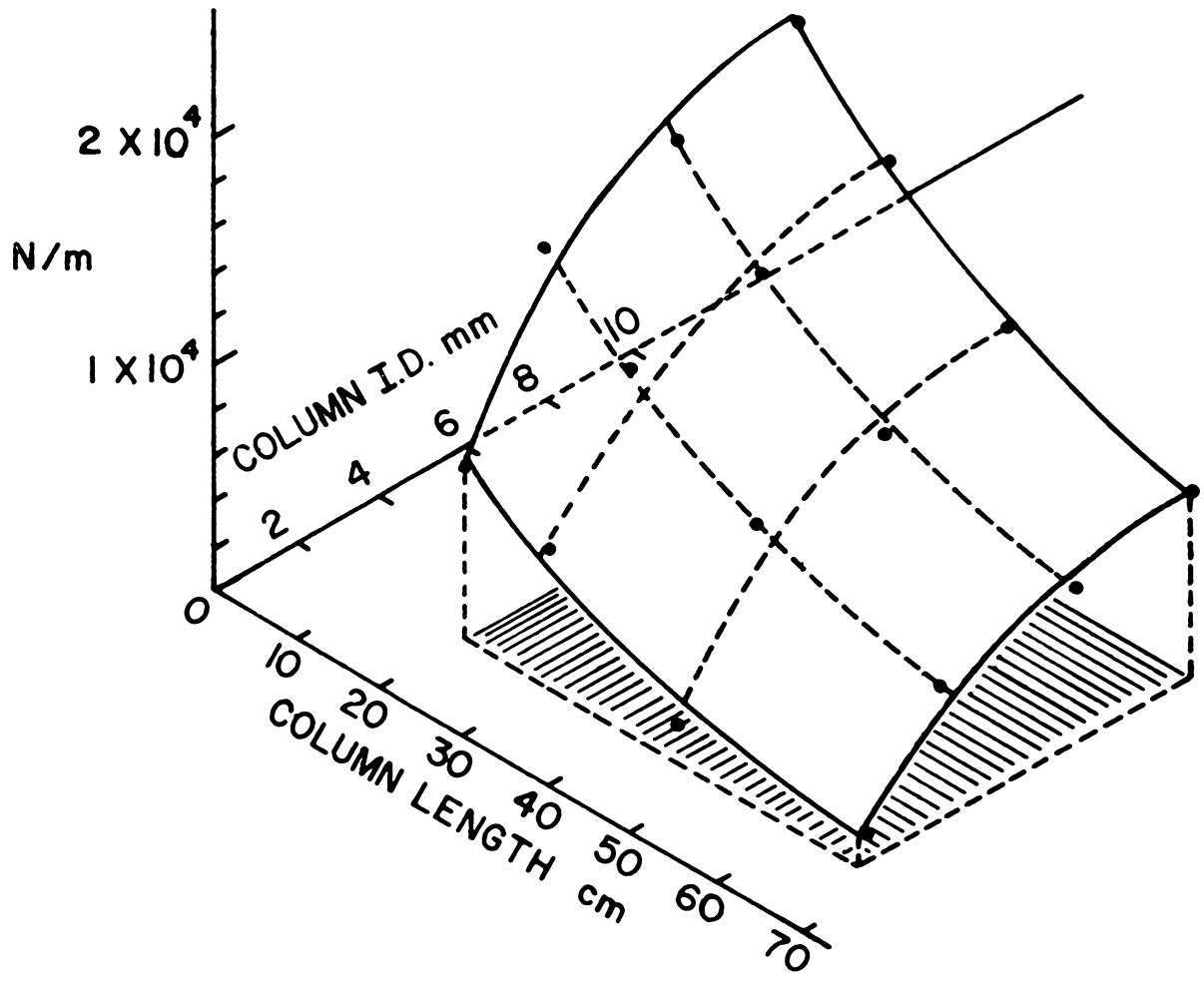



Figure 20. Plot of N/m versus column I.D. and column length for acetanilide. Packing material: 10 μm Lichrosorb SI-100; mobile phase: 1.5% isopropyl alcohol in methylene chloride at $\bar{u}_o$=0.21 cm/sec; sample size: 20 μl.

$$d_c \geq (2.4 d_p L)^{\frac{1}{2}}$$

where $d_c$ is the internal diameter of the column, $d_p$ is the adsorbent particle diameter, and L the column length. All dimensions must have the same units, usually cm. Any column satisfying this relationship is defined as an "infinite-diameter column." Photographic documentation (72) of a separation performed in a glass column shows that the sample never touched the walls while traveling through the column. However, Wolf (15) has pointed out that the "infinite-diameter" effect could not be used to explain the improved performance of his larger diameter columns. The column end fittings for his studies were designed so that no central injection of the sample was obtained. The sample was swept onto the entire cross section of the top of the column. Thus, his work shows that an "infinite-diameter" effect is not the only explanation for improved performance of larger diameter columns.

Comparison of column efficiencies for the 20 µl sample volume and for sample volumes proportional to column volume (Table XI) produces one unexpected result: for the EPU peak, column efficiencies are higher when a larger sample is injected. This is contrary to what is generally reported for the effect of sample weight on column efficiency (73). To investigate the possibility of a secondary sample effect, studies with various mixtures on a 20 cm x 3.9 mm column were performed. The mixtures used and the efficiencies obtained are shown in Table XII. It can be clearly seen that when EPU is injected in higher amounts as a mixture that also contains acetanilide, a distinctly higher column

Table XII: Secondary Sample Effect on Diethyldiphenylurea (EPU) Caused by the Presence of Acetanilide

| Sample | Plates per Meter | | | |
|---|---|---|---|---|
| | EPU | | Acetanilide | |
| | 20 µl | 69 µl | 20 µl | 69 µl |
| EPU | 13200 | 14500 | -- | -- |
| Acetanilide | -- | -- | 13900 | 6330 |
| EPU/DMP | 13400 | 14500 | -- | -- |
| EPU/ Acetanilide | 13500 | 19200 | 13600 | 6550 |
| DMP/Acetanilide | -- | -- | 13200 | 6620 |
| DMP/EPU/Acetanilide | 13100 | 19100 | 13600 | 6790 |

Conditions: column: 1/4 in. O.D. x 20 cm x 3.9 mm I.D.; packing material: 10 µm Lichrosorb SI-100; mobile phase: 1.5% isopropyl alcohol in methylene chloride at 1.6 ml/min; sample concentration: $4.00 \times 10^2$ g/l for each component.

efficiency is observed. A decrease in column efficiency, as sample size increases, is observed for acetanilide as would be expected. One explanation is that for the larger samples of both EPU and acetanilide, an increased amount of the more polar acetanilide is retained on the top of the column packing and this modified packing will strongly hydrogen bond with the EPU molecules. This results in a narrower band of more concentrated EPU molecules that will show less band spreading and give higher column efficiency. Apparently for the smaller samples, the EPU molecules see primarily the silica surface and are not appreciably affected by the presence of a smaller amount of acetanilide. Acetanilide is more strongly adsorbed and as sample size increases, no concentration effect occurs and column efficiency decreases.

The data from Table XI makes possible the verification of a relationship between HPLC column efficiency, column length, and internal diameter. The plot of column efficiency (expressed in N/m) for the 20 µl samples of EPU for the 16 different columns versus the ratio of the column internal diameter and column length is shown in Figure 21. It can be seen that using the same type of columns, what determines the column efficiency is the ratio of column internal diameter (I.D.) to column length (L). The column efficiency increases with the increase of this ratio.

This experimental observation has some important practical consequences:

- Existence of isoefficient columns

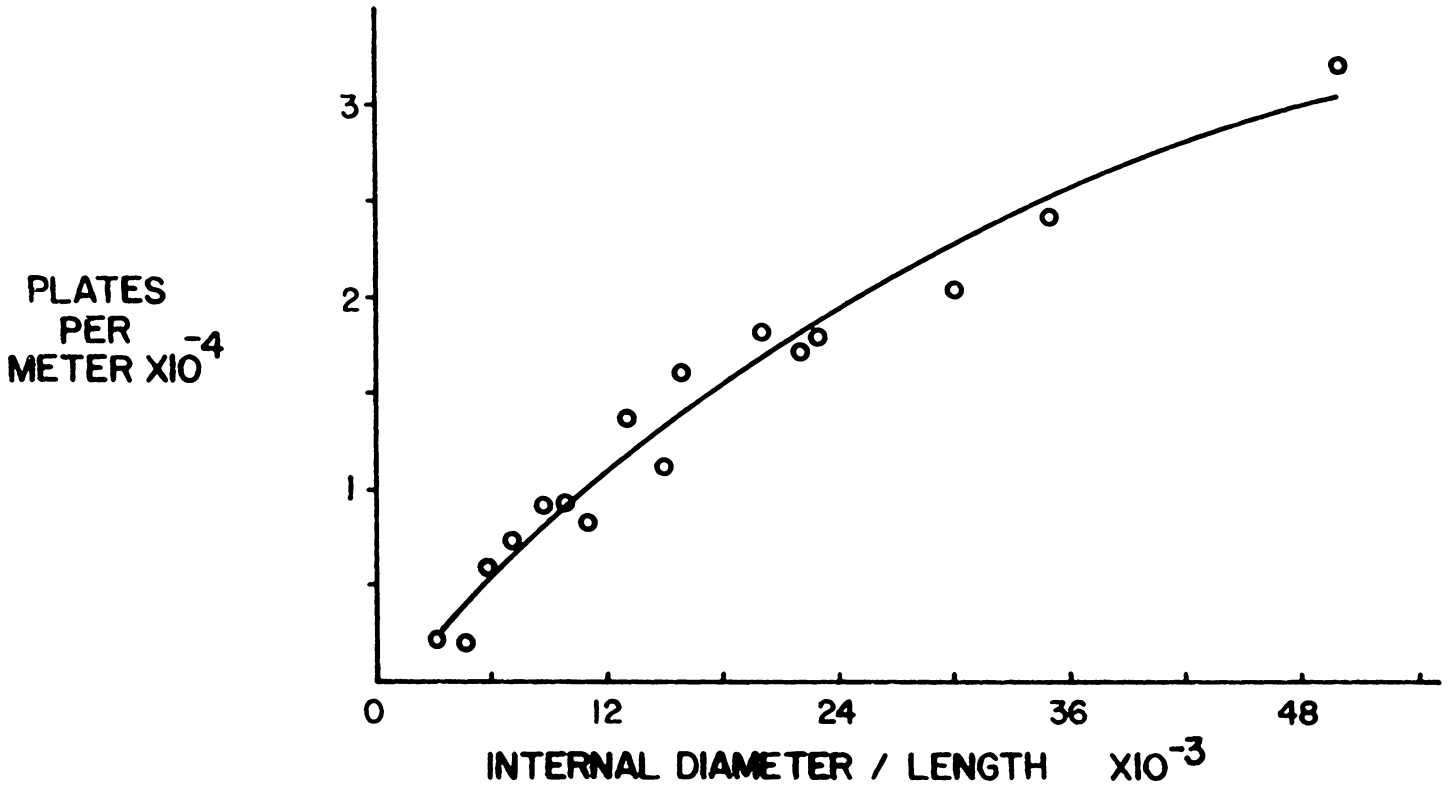



Figure 21. Plot of N/m versus column I.D./L for diethyldiphenylurea. Packing material: 10 μm Lichrosorb SI-100; mobile phase: 1.5% isopropyl alcohol in methylene chloride at $\bar{u}_o$=0.21 cm/sec; sample size: 20 μl.

- Prediction of column efficiency
- Minimum analysis time columns
- Preference for "short-fat" columns.

Since what determines the column efficiency is the ratio of column internal diameter to column length, by packing several different column lengths with the same (I.D.)/L ratio, chromatographic columns showing the same efficiency (isoefficient) should be obtained. The data in Table XIII shows that the experimental column efficiency values for EPU for the columns having a similar (I.D.)/L ratio is approximately the same. Thus, isoefficient columns were predicted and prepared.

Prediction of column efficiency is possible by calculating the (I.D.)/L of the column that is packed under the experimental conditions used to obtain the data for Figure 21.

For routine analytical work, speed of analysis can be very important. For this reason, the minimum column efficiency and minimum column length to perform the separation is desired. Minimum length is required because retention time is proportional to column volume.

The number of plates N for a required resolution R between two peaks is given by the expresssion

$$N_{req} = 16\ R^2\left(\frac{\alpha}{\alpha-1}\right)^2\left(\frac{k'_2+1}{k'_2}\right)^2$$

where $\alpha$ is the solvent efficiency and $k'_2$ is the capacity factor for peak 2 (74). Having calculated the N necessary, the desired column length and column efficiency (N/m) can now be fixed. Using the plot in

Table XIII: Column Efficiency (N/m) for EPU Related to Column I.D. and Length

| Column | I.D. (mm) | L (mm) | I.D./L | N/m |
|---|---|---|---|---|
| PC #4 | 2.1 | 675 | 0.0031 | 2030 |
| PC #3 | 2.1 | 450 | 0.0047 | 2000 |
| PC #8 | 3.9 | 675 | 0.0058 | 6040 |
| PC #2 | 2.1 | 300 | 0.0070 | 7330 |
| PC #7 | 3.9 | 450 | 0.0087 | 9180 |
| PC #12 | 7.0 | 675 | 0.010 | 9390 |
| PC #1 | 2.1 | 200 | 0.011 | 8240 |
| PC #6 | 3.9 | 300 | 0.013 | 13800 |
| PC #16 | 9.9 | 675 | 0.015 | 11200 |
| PC #11 | 7.0 | 450 | 0.016 | 13700 |
| PC #5 | 3.9 | 200 | 0.020 | 18100 |
| PC #15 | 9.9 | 450 | 0.022 | 17200 |
| PC #10 | 7.0 | 300 | 0.023 | 17800 |
| PC #14 | 9.9 | 300 | 0.030 | 20500 |
| PC #9 | 7.0 | 200 | 0.035 | 24200 |
| PC #13 | 9.9 | 200 | 0.050 | 32000 |

Conditions: packing material: 10 μm Lichrosorb SI-100; mobile phase: 1.5% isopropyl alcohol in methylene chloride at $\bar{u}_o$=0.21 cm/sec; sample size: 20 μl of PS #1.

Figure 21, the necessary column internal diameter to obtain the necessary N is determined. In case that the calculated column internal diameter is not available, the next larger diameter accessible should be used.

At the same linear velocity, peak retention time decreases with a decrease in column length. Therefore, shorter columns are to be preferred. However, to maintain the necessary number of plates (N) for a given separation, as the column length is decreased the column internal diameter has to increase. This leads to an important conclusion in this work: "short-fat" columns should be preferred for fast analytical separations.

This research also had the objective to define a better parameter than column throughput (TPUT) to express preparative efficiency. Column TPUT is defined as the amount of sample collected per unit time, and can be expressed as:

$$TPUT = g/t_r$$

where g is the amount of sample collected from the peak with a retention time $t_r$.

To compare the preparative efficiency of different columns in terms of TPUT, all columns would have to be operated under conditions which would generate the same resolution. Experimentally, this would be very inconvenient. Thus, column TPUT has the disadvantage of not taking into account differences in resolution of the components.

Taking R as a normalization parameter, a better expression for

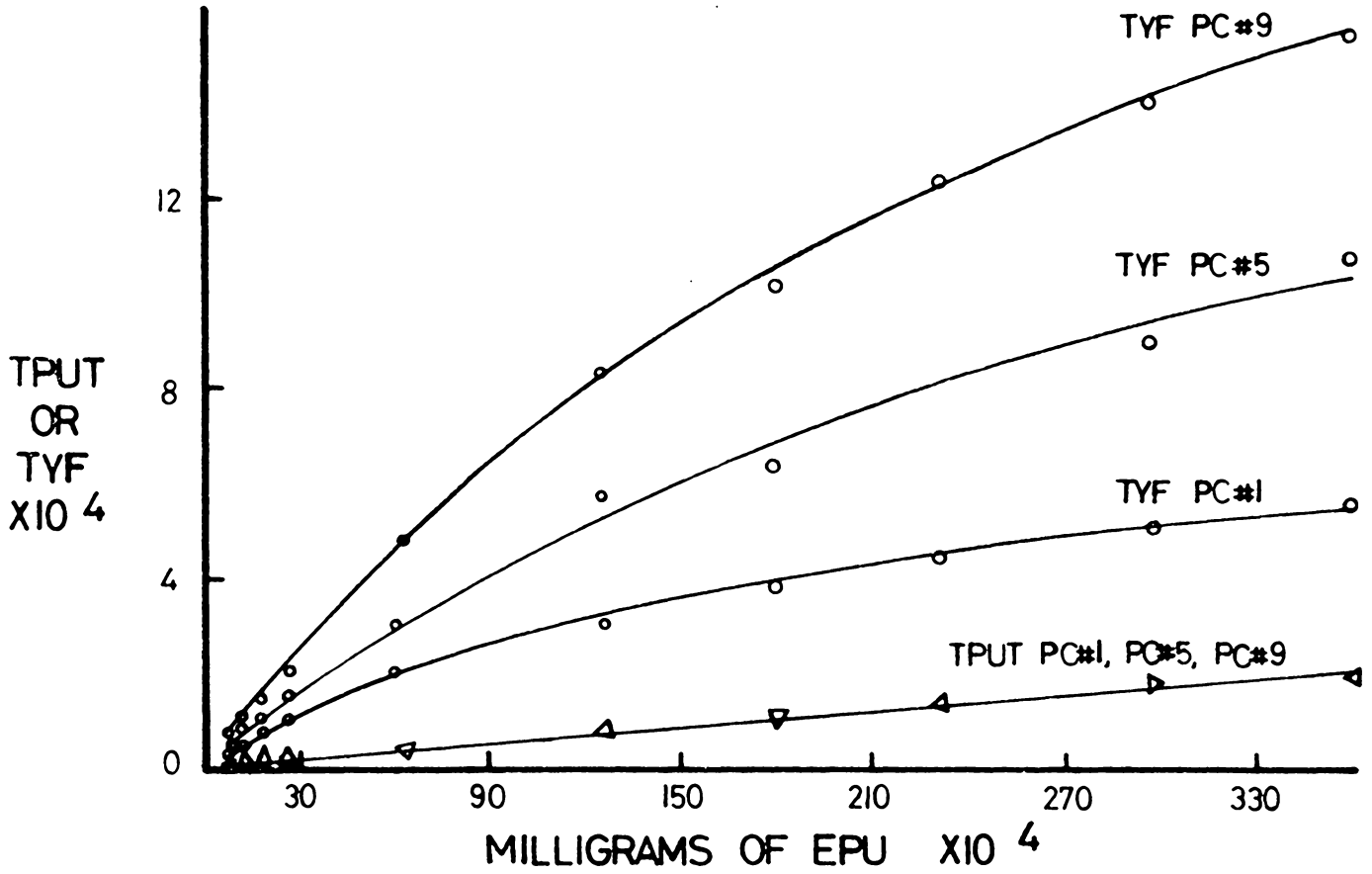



Figure 22. Plot of TPUT and TYF versus mg of diethyldiphenylurea separated from acetanilide. Column: (PC #1) 1/4 in. O.D. x 20 cm x 2.1 mm I.D.; (PC #5) 1/4 in. O.D. x 20 cm x 3.9 mm I.D.; (PC #9) 3/8 in. O.D. x 20 cm x 7.0 mm I.D.; packing material: 10 μm Lichrosorb SI-100; mobile phase: 1.5% isopropyl alcohol in methylene chloride at $\bar{u}_o$=0.21 cm/sec; sample size: 20 μl.

preparative efficiency is the Time Yield Factor (TYF) which can be defined as:

$$TYF = TPUT \times R$$

Different columns used for the same separation, under identical conditions, should give different resolutions. The column showing the highest R has the highest reserve to receive more sample and should potentially be the one that will give the largest preparative yield.

Three different columns, PC #1, PC #5, and PC #9, characterized in Table VII were used to separate 20 µl samples of EPU and acetanilide at various concentrations, but always with the same weight ratio of 1.0. TYF and TPUT for the columns were calculated and plotted versus mg of EPU injected as shown in Figure 22.

It can be seen that at any fixed sample load, all three 20 cm length columns show the same TPUT value, but column TYF increases as internal diameter increases. The highest TYF value is obtained for the column with the largest volume. Thus, TYF allows a rapid comparison of different size preparative scale columns without the need to adjust column conditions for equivalent resolution.

A more complete description of the chromatographic behavior of an HPLC column is obtained when plotting TYF, TPUT, and Resolution versus weight of sample injected. Figure 23 shows the results for the EPU and dimethylphthalate mixture. For the mixture of EPU and acetanilide, the chromatographic behavior is presented in Figure 24. For a certain amount of injected sample, the TYF values are always larger than the

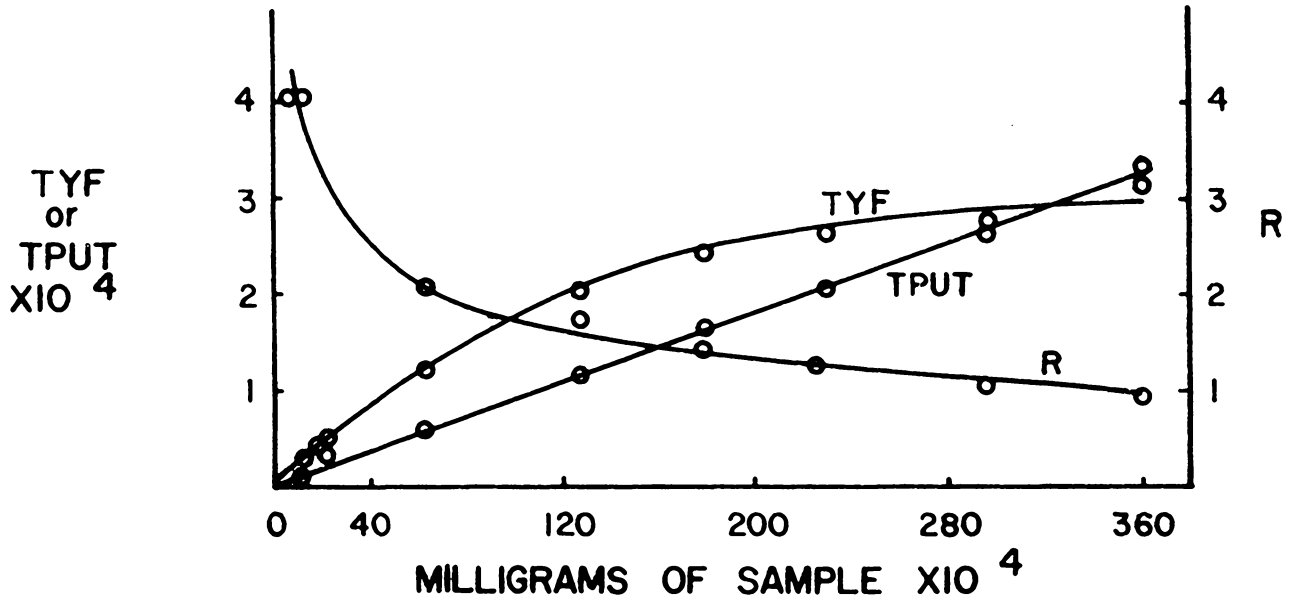



Figure 23. Plot of TYF, TPUT, and R versus mg of dimethylphthalate separated from diethyldiphenylurea. Column: 1/4 in. O.D. x 20 cm x 2.1 mm I.D.; packing material: 10 μm Lichrosorb SI-100; mobile phase: 1.5% isopropyl alcohol in methylene chloride at 0.4 ml/min; sample size: 20 μl.

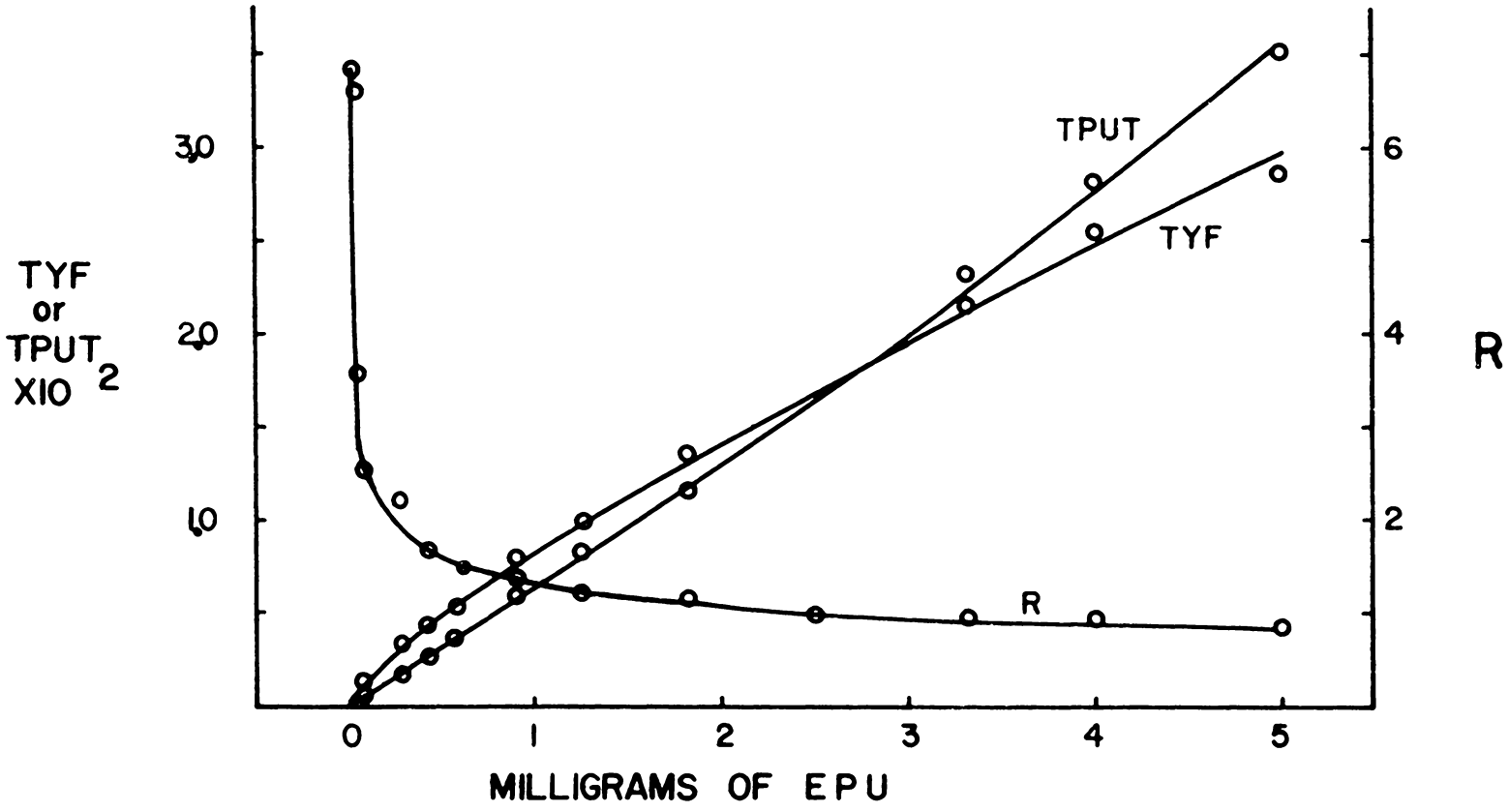



Figure 24. Plot of TYF, TPUT, and R versus mg of diethyldiphenylurea separated from acetanilide. Column: 1/4 in. O.D. x 20 cm x 3.9 mm I.D.; packing material: 10 μm Lichrosorb SI-100; mobile phase: 1.5% isopropyl alcohol in methylene chloride at $\bar{u}_o$=0.21 cm/sec; sample size: 50 μl.

corresponding TPUT values when R>1. By definition, TYF=TPUT at R=1. Values of TYF provide more meaningful data than TPUT and are easier to measure.

It would be useful to have each preparative column evaluated in terms of TYF and R over a wide range of sample sizes. This would give a better picture of column behavior and provide a better basis for predicting results with other samples.

The TPUT and TYF values obtained for EPU by injecting 20 µl of sample PS #1 (from Table IX) into the 16 columns used in these studies are given in Table XIV. To show the effect of column length and column internal diameter on TYF and TPUT, two plots are made. The results obtained for TPUT versus L and I.D. are shown in Figure 25. A similar plot for TYF is given in Figure 26.

Several conclusions can be made:

- At constant sample load, constant linear mobile phase velocity and constant column I.D., column TPUT and TYF increase with decrease in column length.
- At constant sample load, constant linear mobile phase velocity and constant column length, column TPUT is not affected by changes in column I.D., but column TYF increases with increases in column I.D.

From the 16 packed columns, PC #13 with L=20 cm and 9.9 mm I.D.

Table XIV: TYF and TPUT Values for the Sixteen Packed HPLC Columns

| Column | TYF $\times 10^5$ | TPUT $\times 10^6$ |
|---|---|---|
| PC #1 | 4.13 | 4.94 |
| PC #2 | 3.13 | 3.32 |
| PC #3 | 1.31 | 2.31 |
| PC #4 | 1.11 | 1.53 |
| PC #5 | 5.95 | 5.06 |
| PC #6 | 4.09 | 3.20 |
| PC #7 | 2.70 | 2.37 |
| PC #8 | 1.94 | 1.56 |
| PC #9 | 6.41 | 4.88 |
| PC #10 | 3.84 | 3.57 |
| PC #11 | 2.89 | 2.33 |
| PC #12 | 1.97 | 1.97 |
| PC #13 | 6.56 | 5.13 |
| PC #14 | 3.71 | 3.46 |
| PC #15 | 3.24 | 2.56 |
| PC #16 | 2.01 | 1.62 |

Conditions: packing material: 10 μm Lichrosorb SI-100; mobile phase: 1.5% isopropyl alcohol in methylene chloride at $\bar{u}_o$=0.21 cm/sec; sample size: 20 μl of PS #1.

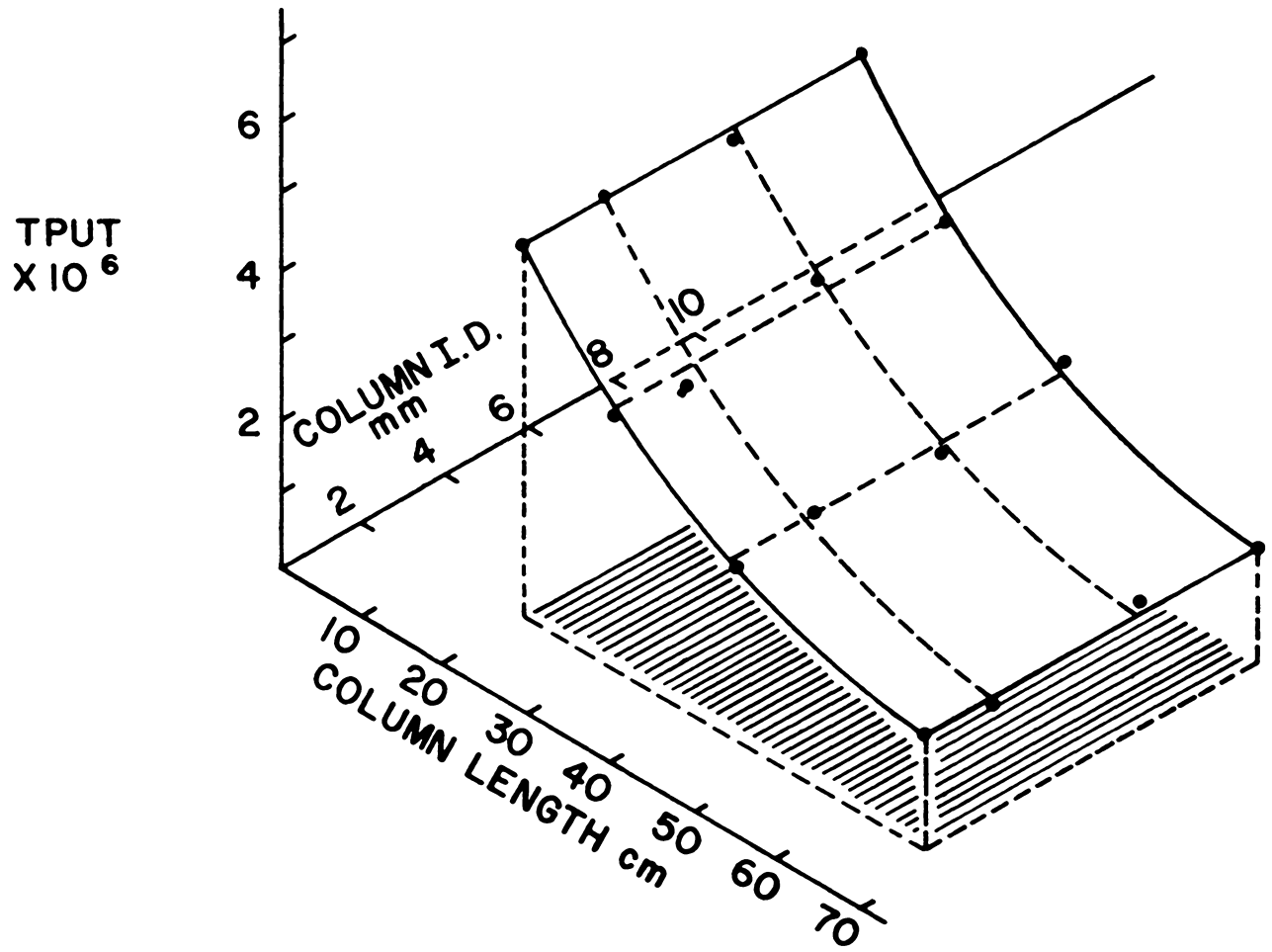



Figure 25. Plot of TPUT versus column I.D. and column length for diethyldiphenylurea. Packing material: 10 μm Lichrosorb SI-100; mobile phase: 1.5% isopropyl alcohol in methylene chloride at $\bar{u}_o$=0.21 cm/sec; sample size: 20 μl.

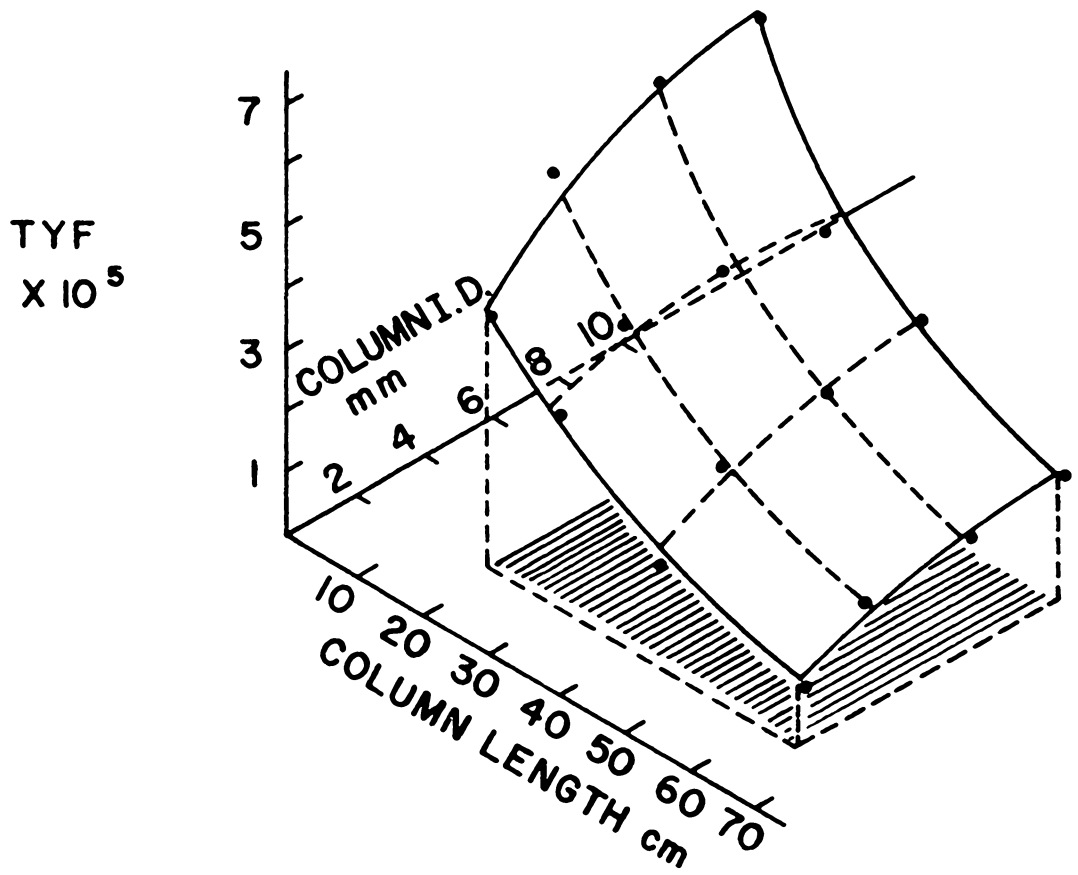



Figure 26. Plot of TYF versus column I.D. and column length for diethyldiphenylurea. Packing material: 10 μm Lichrosorb SI-100; mobile phase: 1.5% isopropyl alcohol in methylene chloride at $u_o$=0.21 cm/sec; sample size: 20 μl.

(highest I.D./L value) provides the highest preparative yield and will be used in the preparative separation of the mixtures described in the following section. As in the case of fast analytical separations, "fat -short" columns should be used to obtain the highest yield in preparative HPLC.

## Preparative Separations

### Preparative separations of 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-methyl ether; 2,3-di(p-bromophenyl)-1,4,5-triphenyl-cyclo-2,4-pentadien-1-methyl ether; and 1,2-di(p-bromophenyl)-3,4,5-triphenylcyclo-2,4-pentadien-1-methyl ether

The three positional methoxy isomers, shown in Figure 7, were obtained during the course of synthesizing a compound suitable for obtaining a chemically bonded phase for HPLC. Attempts to separate these three isomers by classical liquid chromatography failed. A preparative column, 20 cm x 9.9 mm I.D., packed with 10 μm Lichrosorb SI-100 was used. This column, as determined previously, should give the highest yield of separated material.

The strategy to establish the conditions for preparative separations was that described earlier.

The analytical separation, shown in Figure 8, was obtained by adjusting the polarity of the mobile phase (10% $CHCl_3$ in n-hexane) that

had been used with the TLC adsorbent. Similar conditions were used in scaling up the separation on the preparative column. A small percentage (0.08%) of ethyl acetate had to be maintained in the mobile phase (n-hexane) to maintain a constant activity of the packing material and to minimize tailing of the peaks. Separation was considered optimized with this mobile phase since a further decrease in ethyl acetate would decrease resolution and the peaks would tail badly.

Sample volume was gradually increased until the limit of 1.5 mg could be injected in the column. This is less material than typically expected from the data in Table II. This smaller sample size can be explained by the nature of the three components to be separated. We have three positional isomers of high molecular weight (634) in which the only differentiating feature is the position of a nonpolar methoxy group that is sterically hindered by bulky phenyl groups. It is known that high molecular weight solutes and steric hindrance are factors that decrease the selectivity of separation by adsorption chromatography. The yield of collected sample is further decreased by not collecting 100% of the peak as shown in Figure 9. Figure 27 shows the conditions used and the separation obtained during one intermediate step in the scaling up process. The purity of the collected fractions was determined by HPLC and the results are shown in Figures 10 through 12. The squared-off peaks obtained from the preparative separations result from the overload of the ultraviolet absorption detector.

An important aspect in preparative HPLC is the polarity of the sample solvent. Methylene chloride is a much better solvent for these

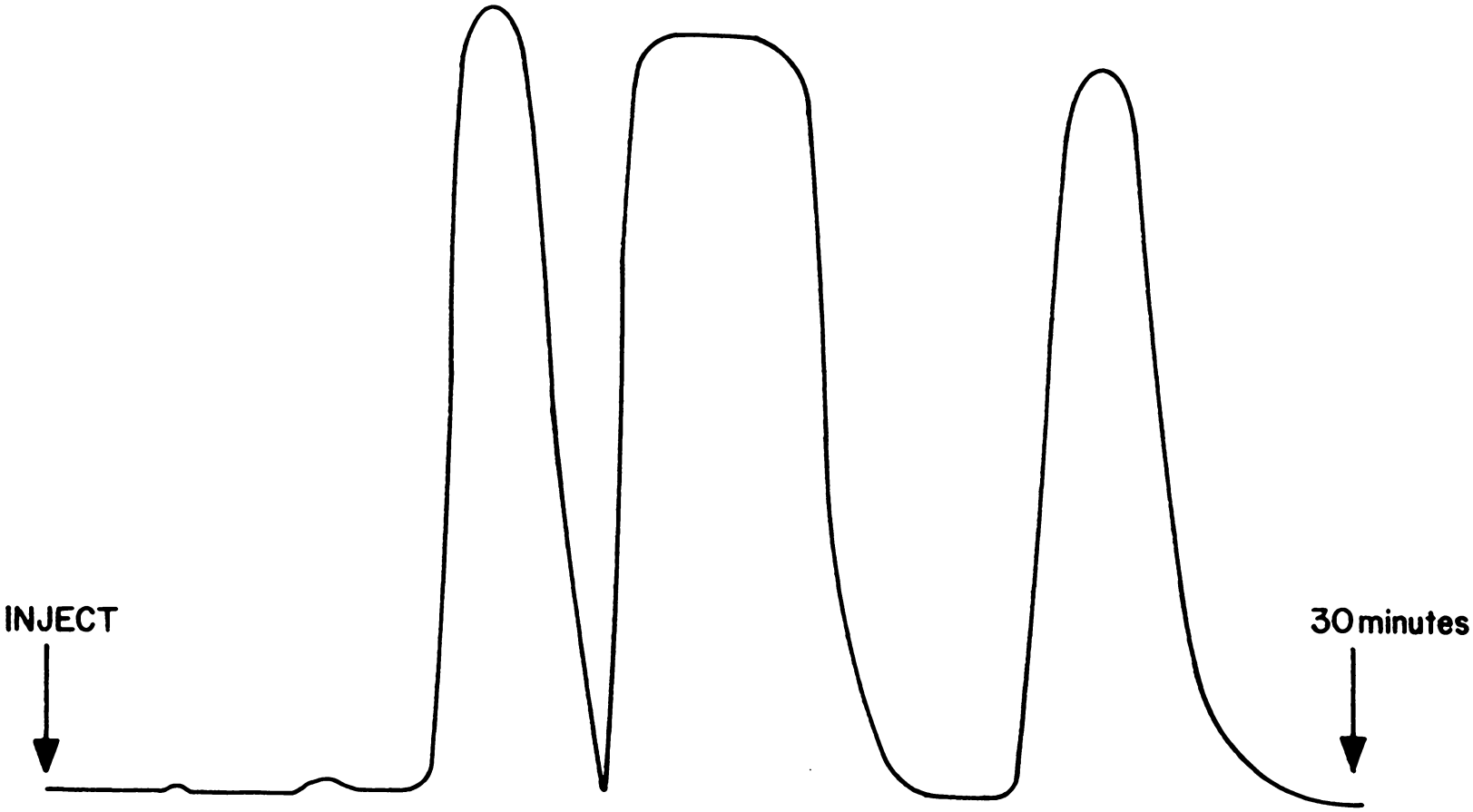



Figure 27. Preparative separation (intermediate sample size) for isomeric methoxy derivatives. Column: 0.5 in. O.D. x 20 cm x 9.9 mm I.D.; packing material: 10 μm Lichrosorb SI-100; mobile phase: 0.08% ethyl acetate in n-hexane at 4.9 ml/min; detector: UV at 254 nm and 0.64 ABS sensitivity; sample size 1.2 mg in 334 μl.

three methoxy isomers than n-hexane. However, when several hundred microliters of a more polar solvent (methylene chloride) were injected into the column having a less polar mobile phase (n-hexane) the separation process was disturbed momentarily, and as a result, the separation was diminished. When only a few microliters were injected, this effect was not seen. Recommended practice is to use the mobile phase to dissolve the sample. However, in this case, the sample was only sparingly soluble in the mobile phase.

It is usually believed that injecting the same amount of sample but using a larger volume of solvent will result in better resolution. The explanation given is that there is less localized column overload when using the less concentrated solutions (75). However, in this work, the opposite effect was observed. As can be seen in Figures 28 and 29, a better separation was obtained by injecting a smaller volume of the more concentrated sample. Polarity of the sample might have been a factor in establishing optimum sample volume and a more exhaustive study in this area should be made.

The collected fractions were treated and used for identification work as described in the synthesis and identification section.

## Preparative separation of 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-methyl ether

The separation of 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-

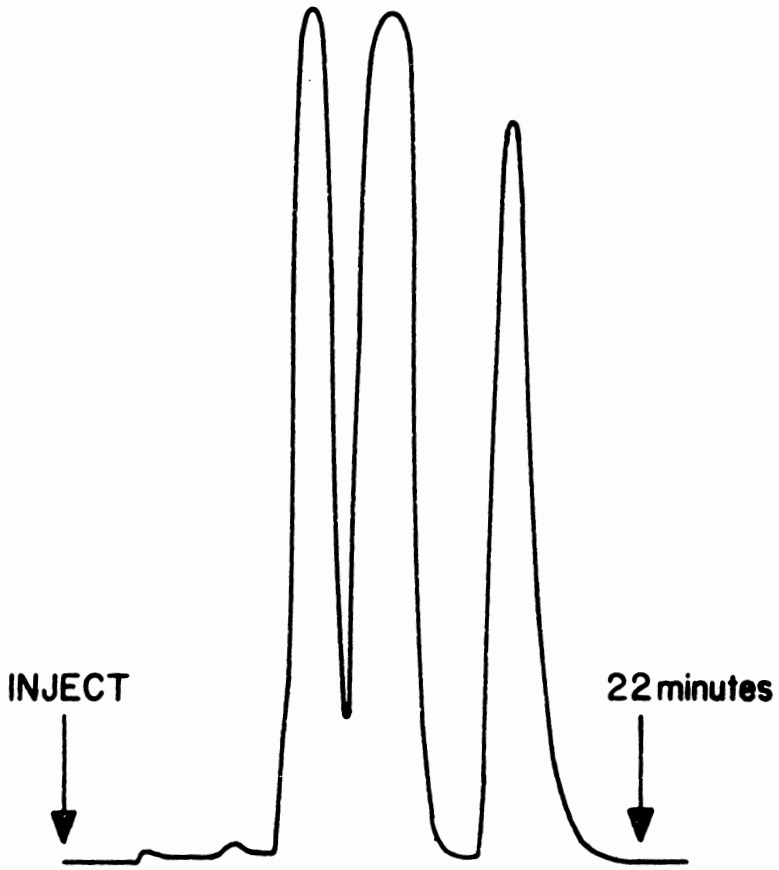



Figure 28. Sample volume effect in the separation of isomeric methoxy derivatives. Column: 0.5 in. O.D. x 20 cm x 9.9 mm I.D.; packing material: 10 μm Lichrosorb SI-100; mobile phase: 0.08% ethyl acetate in n-hexane at 9.0 ml/min; detector: UV at 254 nm and 0.08 ABS sensitivity; sample size: 1.2 mg in 1038 μl.

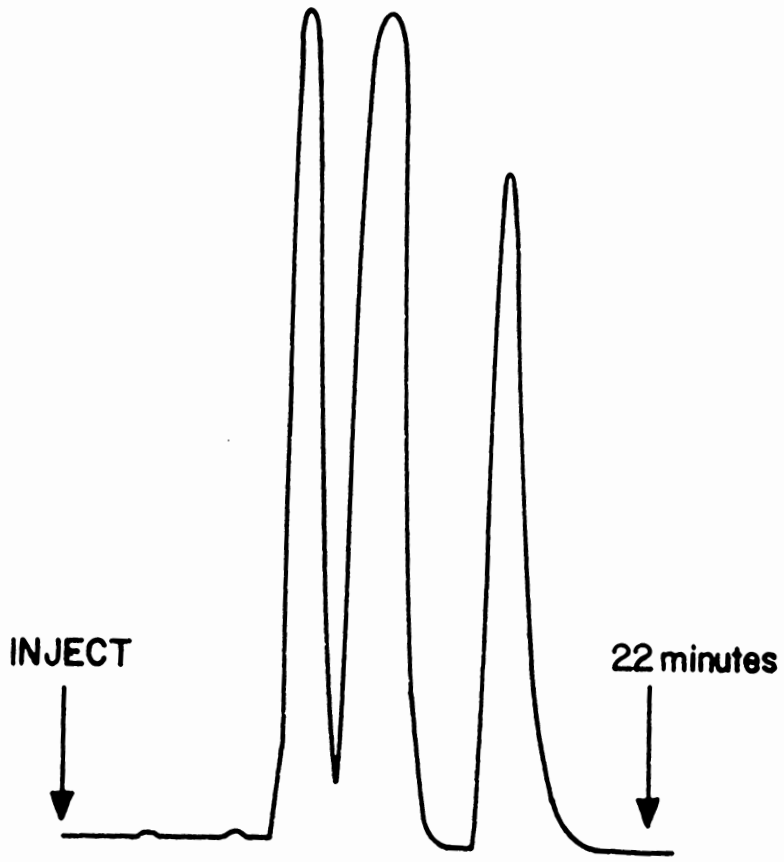



Figure 29. Sample volume effect in the separation of isomeric methoxy derivatives. Column: 0.5 in. O.D. x 20 cm x 9.9 mm I.D.; packing material: 10 μm Lichrosorb SI-100; mobile phase: 0.08% ethyl acetate in n-hexane at 90 ml/min; detector: UV at 254 nm and 0.08 ABS sensitivity; sample size: 1.2 mg in 234 μl.

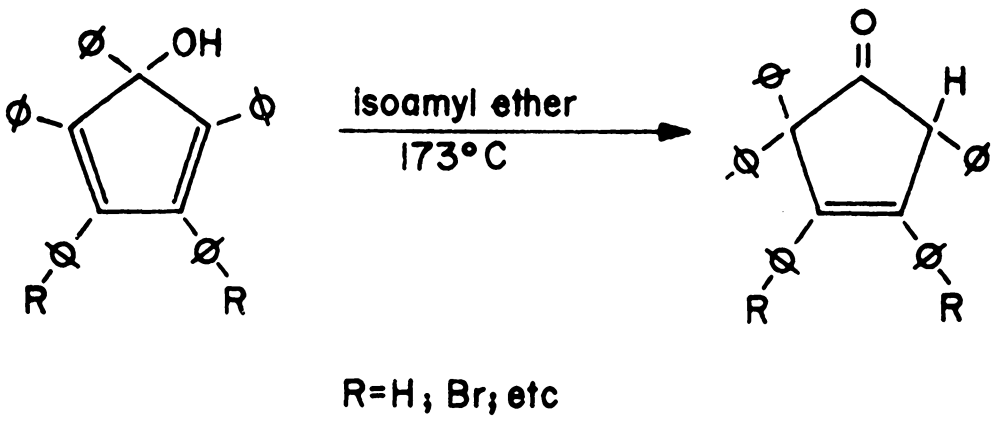
pentadien-1-methyl ether from the synthesis mixture by classical column chromatography yielded a fraction still containing several impurities as shown by Figure 15.

Preparative HPLC with the same conditions as in the previous separation was used. The fraction collected is shown in Figure 16 and the purity as analyzed by HPLC is shown in Figure 17. The collected fraction was treated and used for identification work as described in the synthesis and identification section.

These two preparative separations illustrate how the complexity of a sample mixture may decrease the yield of separated material. Simpler samples would allow larger yields to be obtained. For example, 10 mg of EPU and acetanilide could be easily handled on a smaller column (Figure 24).

## Synthesis and Identification

The sigmatropic rearrangement of the substituted 1,2,3,4,5-pentaphenylcyclo-2,3-pentadien-1-ol to the corresponding ketones (Figure 30) was reported by Youssef and Ogliaruso (47-50). Analysis by GC gave satisfactory results until the di(p-bromo) substituted derivative was studied. The GC conditions necessary for the separation of the alcohol and ketone caused the rearrangement of the alcohol to occur partially inside the gas chromatographic column as is shown in Figure 31. This is highly undesirable.




R=H; Br; etc

Figure 30. Thermal rearrangement of 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-ol to 3,4-di(p-bromophenyl)-2,4,4-triphenylcyclo-3-cyclopenten-1-one.

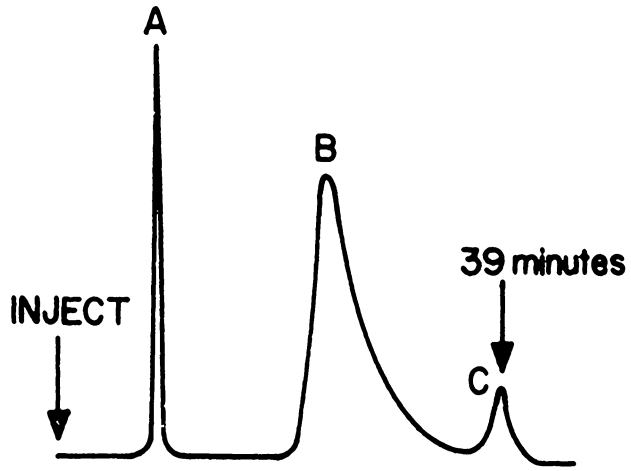



Figure 31. Typical results obtained by gas chromatography. (A) solvent peak, (B) 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-ol, and (C) 3,4-di(p-bromophenyl)-2,4,4-triphenylcyclo-3-cyclopenten-1-one.

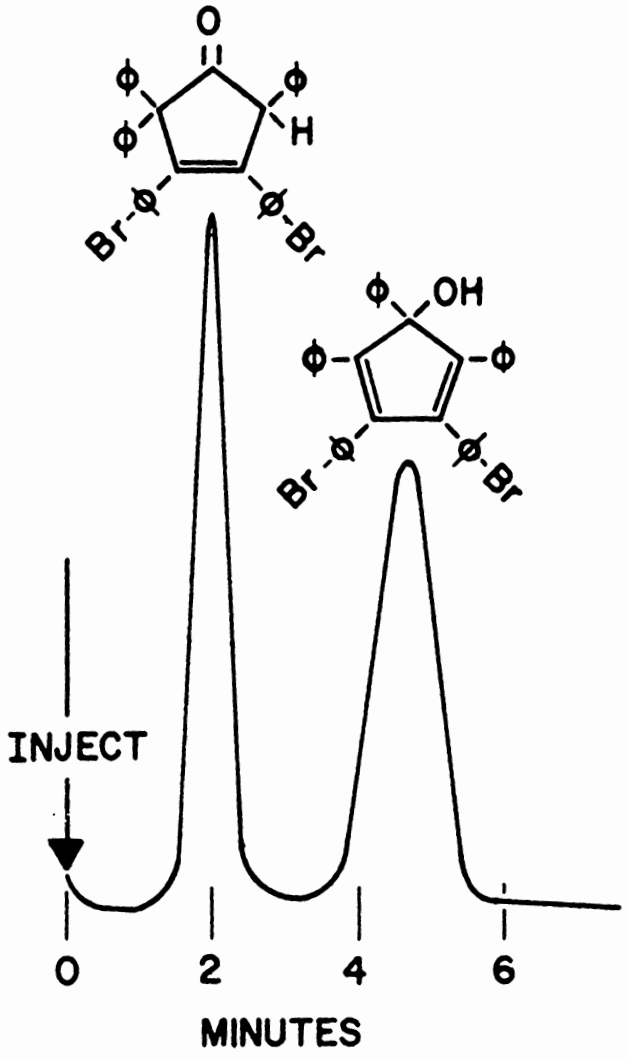



Figure 32. Typical results obtained by liquid chromatography. Column: 1/4 in. O.D. x 60 cm x 2.1 mm I.D.; packing material: 10 μm Lichrosorb SI-100; mobile phase: 1.5% chloroform in n-hexane at 1.45 ml/min; sample size: 20 μl.

HPLC separations usually occur at ambient temperature, thus avoiding this thermal rearrangement. Figure 32 shows the chromatogram and the necessary conditions to obtain the HPLC separation (76). The analysis time by HPLC is almost seven times shorter than by GC. This is an interesting example of the advantages of HPLC over GC in the separation of thermolabile compounds.

The 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-ol shows two bromo substituted phenyl groups. This compound should have a potential application as a stationary phase chemically bonded to silica gel. It should have two thermally and solvolytically stable Si-O-Si-C bonds via a Grignard coupling reaction with the chlorinated silica gel. The reaction that could occur is similar to what was obtained by Locke (77) using 1-bromonaphthalene.

The hydroxy group substituent would have to be tranformed into a methoxy group to avoid a Grignard reaction with the hydroxy group. The reactions predicted for this chemically bonded phase are shown in Figure 33.

The reaction to obtain 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-ol from dibromo substituted tetracyclone and bromobenzene is shown in Figure 34. This occurred without problems according to the procedure described by Allen (65).

To obtain the methoxy derivative of the above described alcohol, the sequence of reactions used by Youssef (66) to produce the corresponding unsubstituted methoxy compound was taken. The first reaction was the substitution of bromine for the hydroxy group by reacting the

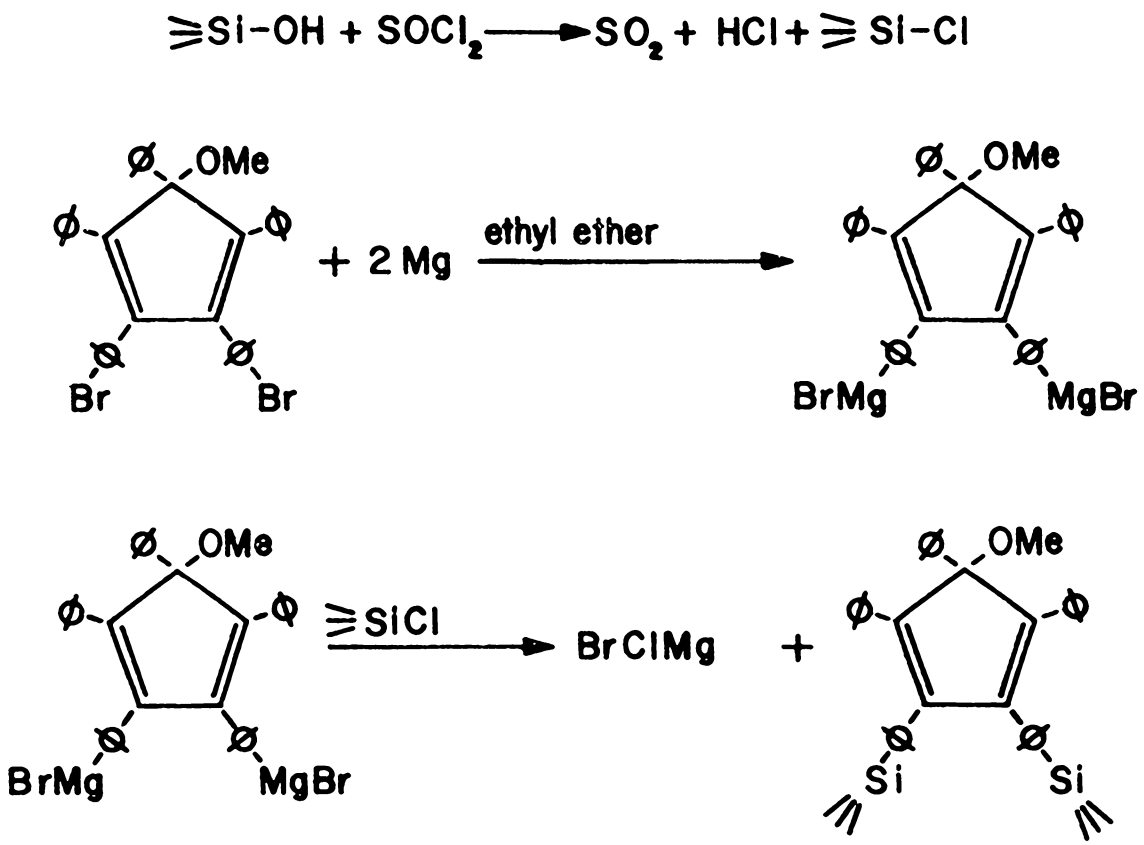



Figure 33. Reactions predicted for the production of a chemically bonded phase on silica gel.

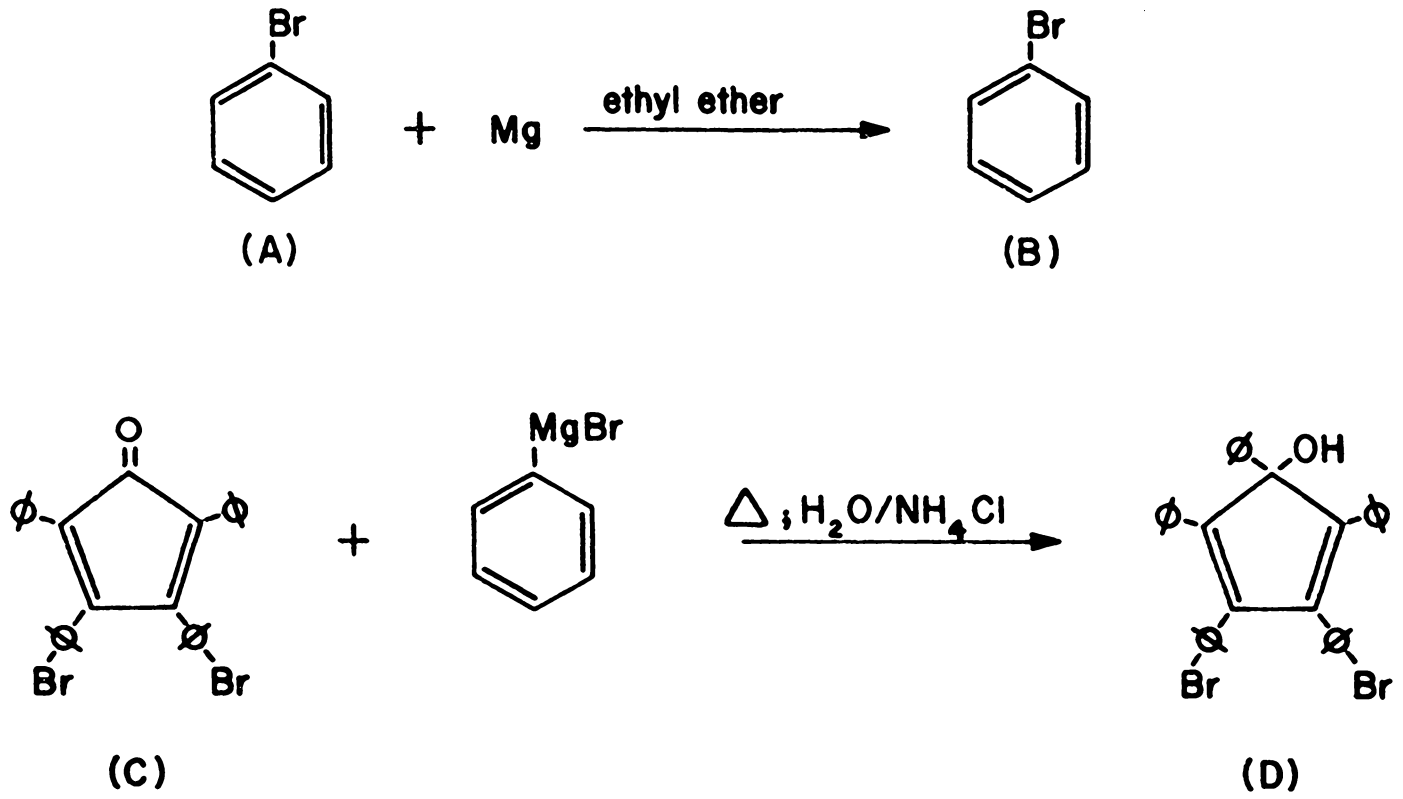



Figure 34. Reaction sequence to obtain 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-ol from 3,4-di(p-bromophenyl)-2,5-diphenylcyclo-pentadien-1-one.

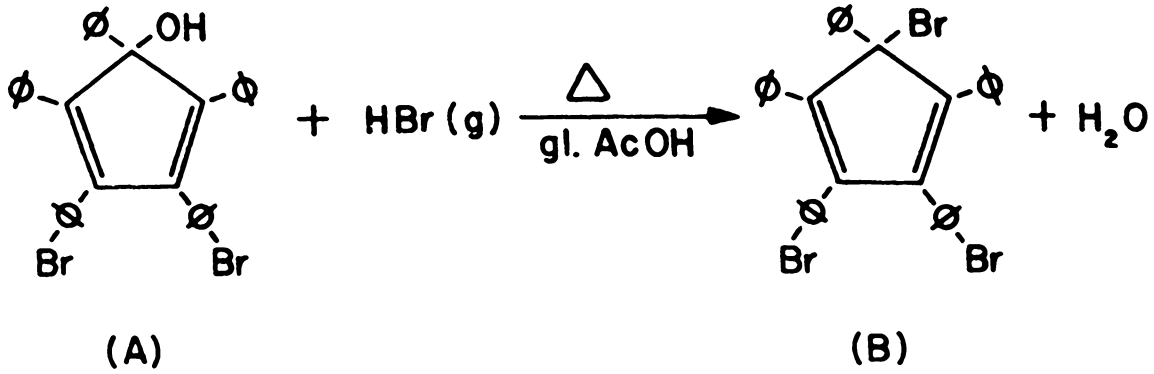



Figure 35. Reaction to obtain 1-bromo-3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadiene from the corresponding 1-hydroxy compound.

alcohol in glacial acetic acid with gaseous HBr, as shown by Figure 35. As the reaction approached completion, as determined by following the disappearance of the reactant and the appearance of the product by TLC, a precipitate formed. The results of the TLC analysis are shown in Table XV. An interesting observation is that by substituting the non-conjugated hydroxy group by bromine, the compound loses its property of fluorescing under ultraviolet radiation. The reaction product analysis given in the experimental part confirmed that the 1-bromo-3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-pentadiene was obtained. However, this reaction was completed only after 2.15 hr as compared to 1.15 hr for the unsubstituted system.

The reaction to substitute the 1-bromo group by the 1-methoxy group should occur by refluxing with anhydrous methanol, as shown in Figure 36. This reaction, followed by TLC was completed after 13 hr. Predicting even a longer reaction time if the corresponding 1-chloro compound (synthesis shown in Figure 37) would be used, no extensive studies for the production of the methoxy derivative were made. The TLC results for the 1-bromo compound are shown in Table XVI. The interesting observation is that substitution of the nonconjugated bromo group by methoxy transformed the nonfluorescent compound to one that fluoresced under ultraviolet radiation. Elemental analysis, nmr, ir, and m.s. satisfied the requirements for the desired methoxy derivative. However, the melting temperature (165-185$^{o}$) occurred over too wide a range for a pure compound. In addition, the fluorescent TLC spot corresponding to the methoxy compound showed a tendency of separating into several spots.

Table XV: Thin-Layer Chromatographic Conditions and Results for 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-ol and the Corresponding 1-bromo Compound.

| Compound | $R_f$ Value | Visualization | |
|---|---|---|---|
| | | UV | Visible |
| R-OH | 0.55 | F | Yellow |
| R-Br | 0.68 | NF | Orange |
| Stationary Phase | | Silica Gel Q | |
| Mobile Phase | | Benzene | |

R = 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadienyl
UV = under ultraviolet radiation
Visible = under visible radiation
F = fluorescent
NF = nonfluorescent

Table XVI: Thin-Layer Chromatographic Conditions and Results for 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-methyl ether and Corresponding 1-bromo Compound.

| Compound | $R_f$ Value | Visualization | |
|---|---|---|---|
| | | UV | Visible |
| R-Br | 0.30 | NF | Orange |
| R-OMe | 0.25 | F | Yellow |
| Stationary Phase | | Silica Gel Q | |
| Mobile Phase | | 90% n-hexane/10% chloroform | |

R = 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadienyl
UV = under ultraviolet radiation
Visible = under visible radiation
F = fluorescent
NF = nonfluorescent

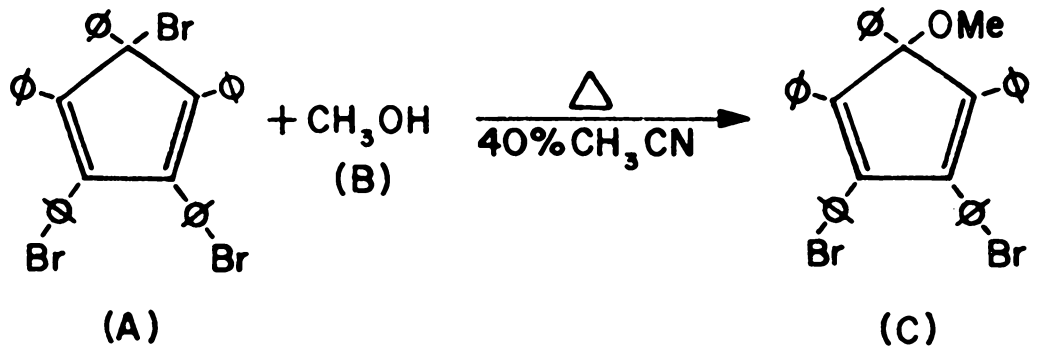



Figure 36. Predicted reaction for 1-bromo-3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadiene with anhydrous methanol.

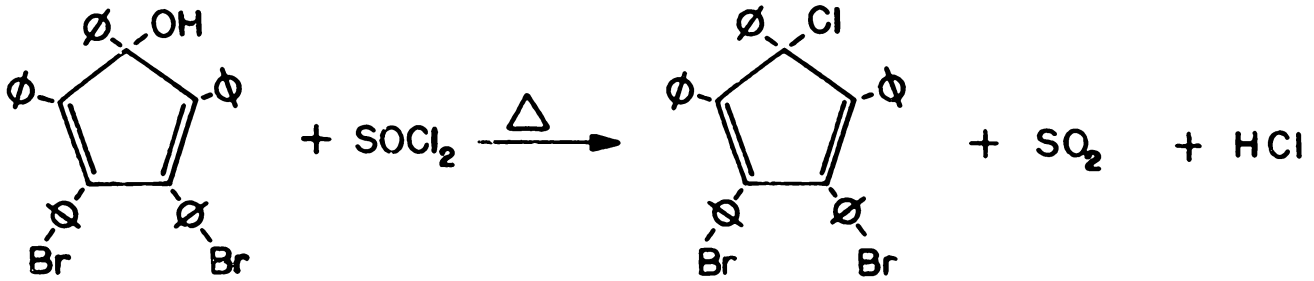



Figure 37. Reaction to obtain 1-chloro-3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadiene from the corresponding 1-hydroxy compound and thionyl chloride.

Multidevelopment produced 3 separated TLC spots with the same type of fluorescence under ultraviolet radiation. Finally, analysis by HPLC resulted in the chromatogram shown in Figure 8 in which 3 distinct peaks occurred. The explanation for obtaining three different components that satisfied the analysis for one compound is the production of three positional isomers.

The substitution reaction having bromide ion as a good leaving group and methyl alcohol as weaker nucleophile could go through an intermediate delocalized carbonium ion as shown in Figure 38. Breslow (67) studied some systems in which he was able to detect the unsubstituted pentaphenyl cyclopentadienyl cation in solutions with $BF_3$. The three different resonance structures can account for the production of three positional isomeric methoxy derivatives as shown in Figure 7.

The proof that the products obtained were the proposed isomers had to be obtained by submitting the separated components to analysis and attempting to synthesize the individual isomers and comparing their properties.

Preparative HPLC, as described in the previous section, yielded a few milligrams of each component. Analysis by mass spectrum and nmr spectrum were the same as what was obtained from the mixture, increasing the evidence for the presence of the three described isomers. Typical mass and nmr spectra are shown in Figures 39 and 40 respectively.

The preparation of 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclopentadien-1-methyl ether using a procedure similar to what was used by Breslow (67) to obtain a methoxy derivative of the unsubstituted

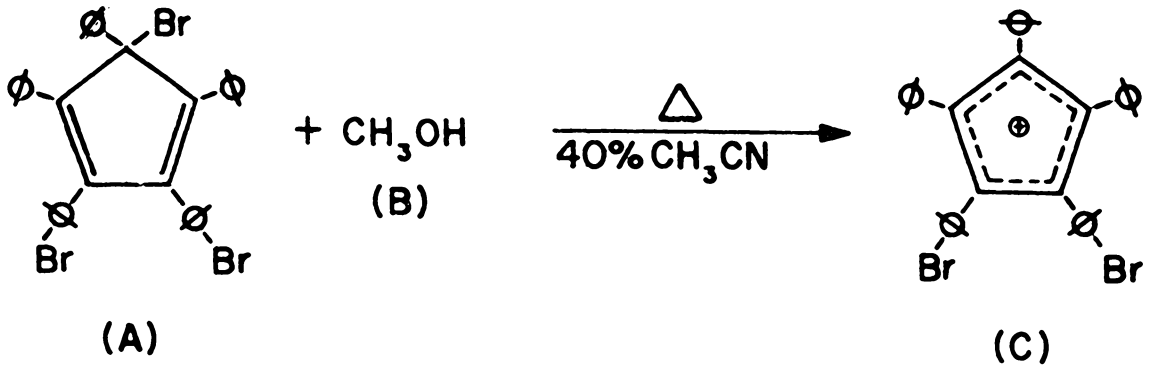



Figure 38. Equivalent representation (C) of three different intermediate carbonium ions in the reaction of (A) with (B).

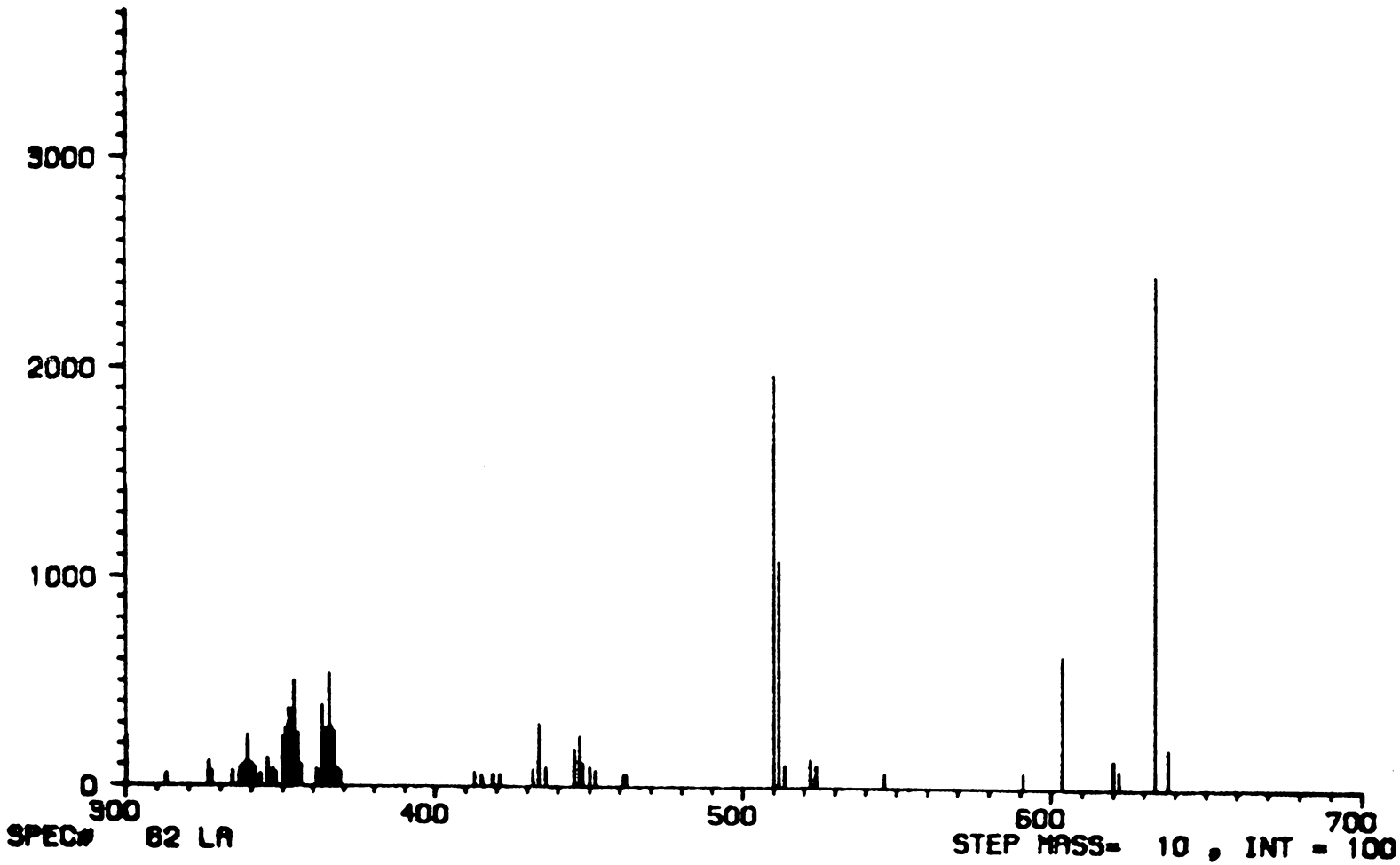



Figure 39. Mass spectrum for 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-methyl ether.

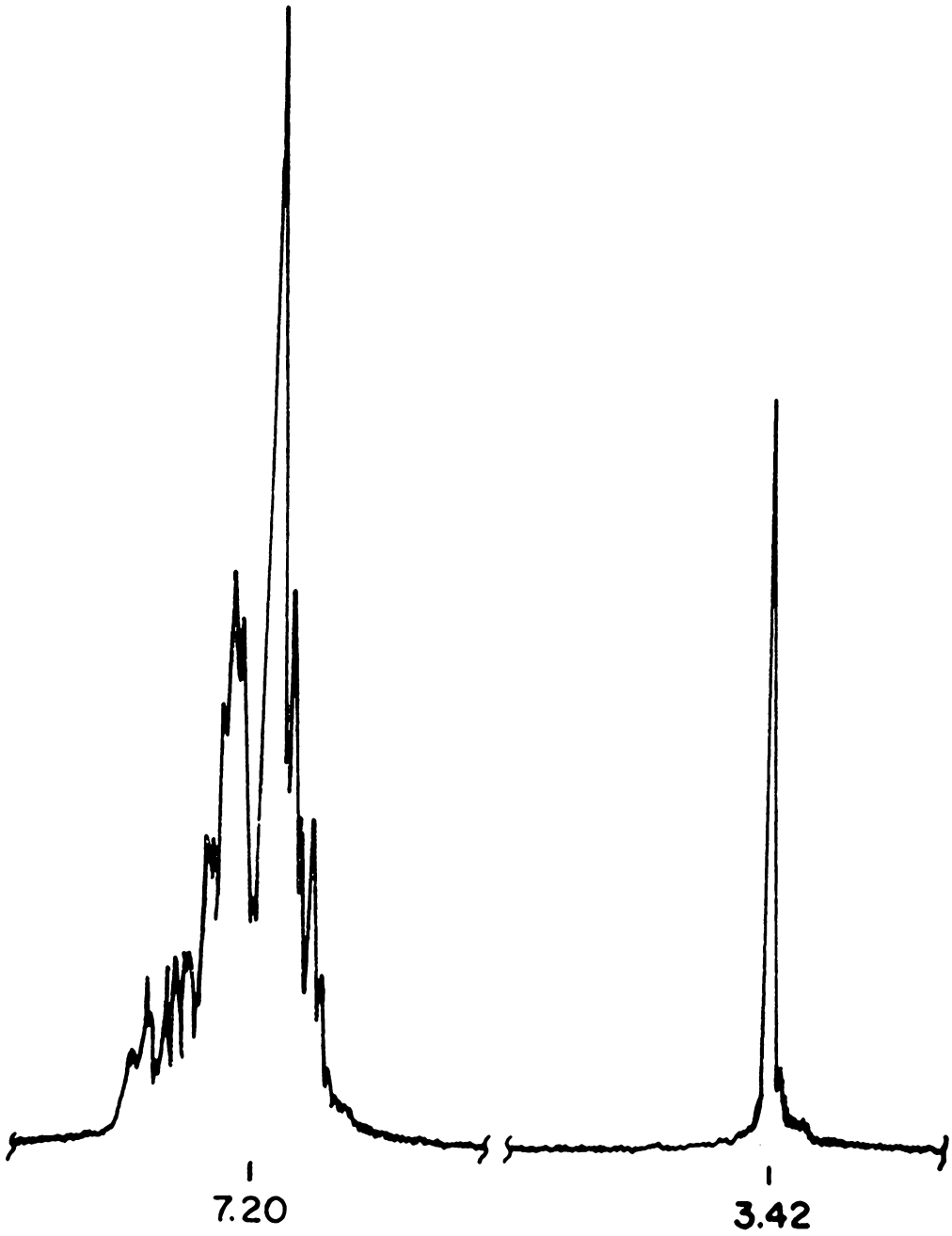



Figure 40. Nuclear magnetic resonance spectrum for isomeric methoxy derivatives. Values for $\delta$ are relative to TMS.

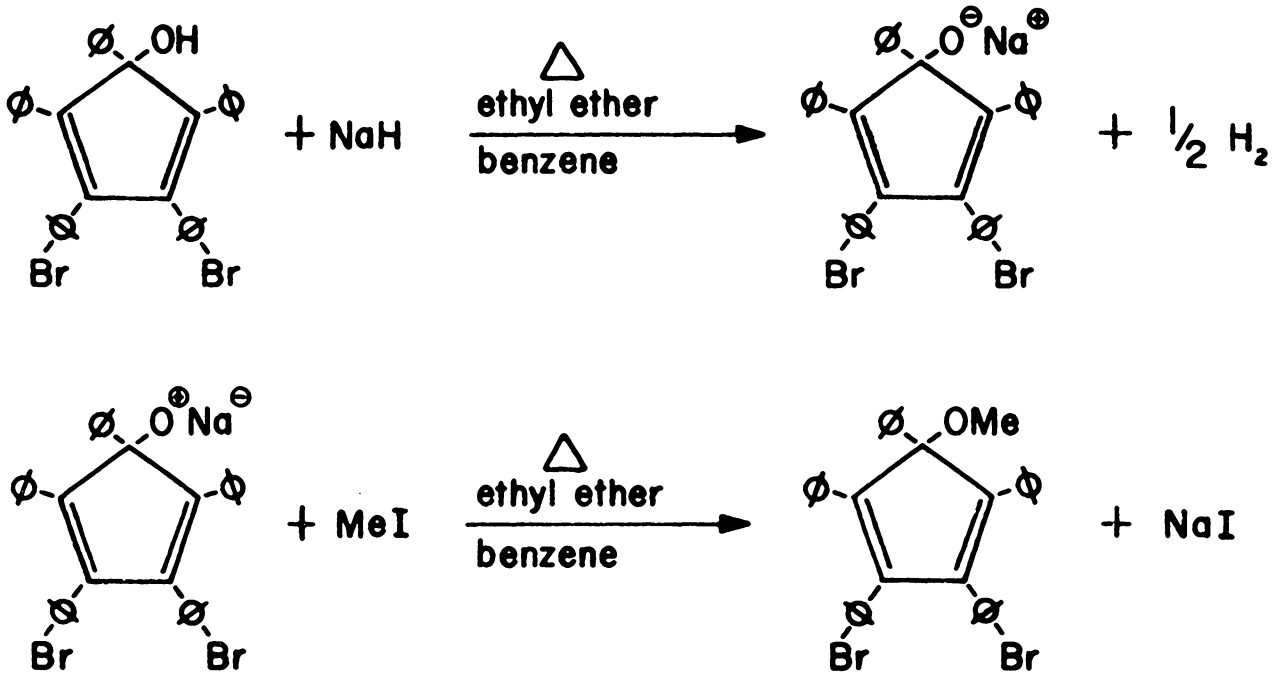



Figure 41. Reaction sequence to obtain 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-methyl ether from the corresponding 1-hydroxy compound.

pentaphenylcyclopentadienyl system was attempted. The reaction sequence is illustrated in Figure 41. The reaction was followed by TLC and HPLC analysis. A peak corresponding to component D in Figure 8 was formed during the reaction. The disappearance of all alcohol after 170 hr was used to terminate the reaction. The separation of the reaction mixture by classical column chromatography yielded the component of interest with many impurities, as shown by the HPLC analysis (Figure 15). A preparative HPLC separation, as discussed in the previous section, yielded the pure compound of interest for further analysis. The purity of collected fractions was determined by HPLC, as shown in Figure 17. Mass spectrometry gave the same results as those obtained for the methoxy compound obtained in the synthesis that yielded the three isomers. Chromatographic retention times were also the same. Also, when the mixture containing the three isomeric methoxy compounds was spiked with this collected fraction, the isomer showing the longest retention time showed an increase in peak height relative to the other two.

For future positive identification, all the isomeric methoxy compounds should be prepared in larger amounts by preparative HPLC so that elemental analysis and hydrogen and carbon-13 nmr could be done. Also, at least a second isomeric methoxy compound should be synthesized individually. Carbon-13 nmr analysis of the separated isomers, compared to the carbon-13 nmr spectra of some model compounds, could yield the answer to the question: which collected component corresponded to which positional isomers. Considering that about 50 mg of each component

would be necessary for a complete analysis and that only 1.5 mg of mixture could be injected each time into the 9.9 mm I.D. column; to make the preparative work practical, a much larger (at least 25 mm I.D.) preparative column should be used.

# Conclusions

A balanced density slurry-packing apparatus was developed and used to pack sixteen HPLC columns having different lengths and internal diameters with 10 μm Lichrosorb SI-100. The evaluation of these columns are as follows:

1) Columns of efficiency comparable to the better literature values were obtained. For the 20 cm length and 9.9 mm internal diameter column, 43400 plates per meter resulted for diethyldiphenylurea.

2) The column efficiency, expressed in plates per meter, is a function of the ratio of column internal diameter to column length. This relationship has never been published before.

3) A new definition for preparative efficiency, Time Yield Factor (TYF), is proposed. This factor differentiates among HPLC columns with different lengths and internal diameters using samples of the same volume and concentration.

4) The column that shows the highest TYF value was used for the preparative separation and identification of three positional isomers of high molecular weight (634): 3,4-

di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-methyl ether; 2,3-di(p-bromophenyl)-1,4,5-triphenylcyclo-2,4-pentadien-1-methyl ether; and 1,2-di(p-bromophenyl)-3,4,5-triphenylcyclo-2,4-pentadien-1-methyl ether, obtained in the synthesis work.

5) Samples of diethyldiphenylurea, in the presence of acetanilide, show higher column efficiency when injected in larger sample volumes as compared to when smaller sample volumes are injected. This is an unexpected secondary sample effect.

6) In the preparative separation of the three positional isomers, 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-methyl ether; 2,3-di(p-bromophenyl)-1,4,5-triphenylcyclo-2,4-pentadien-1-methyl ether; and 1,2-di(p-bromophenyl)-3,4,5-triphenylcyclo-2,4-pentadien-1-methyl ether, a better separation results when injecting the same amount of sample in a smaller volume as compared with a larger volume. This is opposite to what is commonly reported.

7) A carbonium ion intermediate in the synthesis of the methoxy derivative of 1-bromo-3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadiene is proposed. Three products with identical mass spectra support this proposal.

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# PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

by

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(ABSTRACT)

A balanced density slurry-packing apparatus was developed and used to pack sixteen HPLC columns having different lengths and internal diameters with 10 μm Lichrosorb SI-100. The evaluation of these columns led to several conclusions: the column efficiency, expressed in plates per meter, is a function of the ratio of column internal diameter to column length and increases as this ratio increases; a new definition for preparative efficiency, Time Yield Factor (TYF), is proposed. This factor differentiates among HPLC columns with different lengths and internal diameters using samples of the same volume and concentration; the column that showed the highest TYF value was used for the preparative separation and identification of three positional isomers of high molecular weight (634): 3,4-di(p-bromophenyl)-1,2,5-triphenylcyclo-2,4-pentadien-1-methyl ether; 2,3-di(p-bromophenyl)-1,4,5-triphenylcyclo-2,4-pentadien-1-methyl ether; and 1,2-di(p-bromophenyl)-3,4,5-triphenylcyclo-2,4-pentadien-1-methyl ether, obtained in the synthesis work.