INDIRECT CONDUCTIVITY DETECTION
IN SIZE EXCLUSION CHROMATOGRAPHY OF SMALL MOLECULES

by

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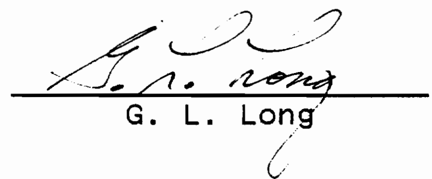
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(ABSTRACT)

Size Exclusion Chromatography (SEC) is a liquid chromarographic technique used for the characterization of polymers and polymer related materials and also for the separation of small molecules. The major drawback of SEC is the lack of availability of a universal and true mass detector for providing a homogeneous response for all samples. Indirect detection methods have demonstrated to supply an alternative way to obtain a universal, sensitive and mass response in liquid chromatography.

This research evaluated the indirect conductivity detection method for the size exclusion chromatographic separation of small molecules. Several studies were developed in order to understand the performance of this novel indirect detection mode. The evaluation of the initial experimental conditions showed a dependence on the response, efficiency and elution time with the concentration of the conductivity probe added to the mobile phase. The SEC calibration curves developed for a series of standards indicated that selected conditions do not affect the separation by size. Response factors revealed a slight increase with

molecular weight when they are expressed in volume or mass. Limits of detection were in the order of 20 nanoliters for small molecules. The effect of different conductivity probes on the separation and response was also studied. A modification of the original detector cell design resulted in improvement in the signal to noise ratio.

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CHAPTER I

INTRODUCTION AND THEORY

Chromatographic processes, in general, are defined according to the IUPAC [1] as:

'A method used primarily for the separation of components of a sample, in which the components are distributed between two phases, one of which is stationary while the other moves. The stationary phase may be a solid, or a liquid supported on a solid, or a gel. The stationary phase may be packed in a column, spread as a layer, or distributed as a film. In these definitions, "chromatographic bed" is used as a general term to denote any of the different forms in which the stationary phase may be used. The mobile phase may be gaseous or liquid'.

From the IUPAC definition the essential parameters are the two phases on which the process takes place, one of which is stationary and the other is mobile. However, this definition does not make reference to the nature of the sample. Both factors, mobile phase and stationary phase, contribute to the classification and sub-division of the different chromatographic techniques: Gas Chromatography {GC}; Liquid Chromatophy {LC} and Thin-Layer Chromatography {TLC}. Normally, the range of operation modes is much greater in LC than in GC because both phases, stationary and mobile, affect the separation and because a wide range of both phases can be used in LC.

Size Exclusion Chromatography (SEC) is one of the versions of liquid chromatography on which a liquid mobile phase is used to drive the

sample through a stationary phase that has sieving properties. In other words, size exclusion chromatography is a liquid chromatographic technique which separates molecules according to their size and shape.

The origin of SEC is attributed to the development of Porath and Foldin [2] in the size separation of water-soluble biopolymers on a highly cross-linked polydextran. Later, in 1964 Moore [3] from Dow Chemical Company revealed the use of "gels" or cross-linked polystyrene polymers for separating synthetic polymers soluble in organic solvents.

This commonly used separation technique has been divided into two categories according to the type of sample analyzed. The first technique is operated to separate and identify water soluble macromolecules of biochemical origin using aqueous mobile phases and hydrophylic packing materials (dextran gels). This form of SEC is known as Gel Filtration Chromatography (GFC). The other SEC technique, normally employs organic mobile phases and lipophylic (hydrophobic) packing materials and is termed Gel Permeation Chromatography (GPC). Although, there is a difference in the type of samples and chromatographic conditions, the analytical goal remains the same for both techniques, i.e., the separation of sample molecules by their molecular size.

SEC has been considered as the preferred technique for determining the molecular weight distribution of polymers, but it also has become in recent years a valuable tool for the separation of small molecules [4].

SEC is the simplest of the liquid chromatographic methods to understand and use and one of the most predictable. There are three factors which indicate SEC as the first choice in solving separation

problems of wide variety of sample types. First, SEC is the most convenient method for separating high molecular weight compounds (Mw>1500); this applies to separations based on molecular weight. As a consequence, not only is the separaton accomplished, but also the molecular weight distribution of the sample is obtained. Second, SEC is the easiest method for separation of simple mixtures when the components differ in molecular size. Third, this technique can be used as a preliminary and exploratory separation technique for unknown samples from which a separation profile of sample complexity is generated with a minimum of method development. Frequently, an initial SEC separation of a complex mixture is a convenient step to decide what liquid chromatographic methods to follow. SEC is relatively simple to use. Sample components in solution are separated according to size by means of a column packed with porous particles. Retention and separation are determined by the molecular hydrodynamic volume of the solutes in solution, where the solutes retention is expressed as elution time or volume and related to the logarithm of the solutes' molecular weight.

The basic mechanism for size exclusion chromatographic separation can be described as follows. A sample dissolved in the eluent and containing analytes with a variety of sizes is introduced to the column; the column is packed with a stationary phase that is inert to the analytes but contains pores of about the same size as the solvated analytes (hydrodynamic volume). The smaller solute molecules are able to penetrate a larger effective volume within the particle pores relative to the larger solute molecules. As a consequence, in the same time period,

larger molecules move further through the column than smaller ones which spend more time diffusing into the pores. Sample molecules that are too large to enter the pores are excluded from the internal pore volume, moving rapidly through the interparticle space and eluting first from the column. Since solvent molecules typically are smallest, they appear last and the entire sample elutes prior to this solvent dead-time peak.

The basic chromatographic equation is:

$$Vr = Vm + k.Vs \qquad (1)$$

where, Vr= retention volume of the peak

Vm= volume of mobile phase within the column but outside the particle, i.e., the interparticle volume

Vs= volume of the pores in the particles (packing)

k= partition coefficient

By rearrangement of equation (1) it can be obtained:

$$k = (Vr - Vm)/Vs \qquad (2)$$

from which the following observations can be established:

- for a solute that is totally excluded from the column (very high molecular size) its Vr will be the same as Vm, and a $k = 0$ is obtained.
- on the other hand, for a solute that permeates all the pores of the particles in the column (very small molecular size) its Vr will be (Vm + Vs) and a $k = 1$ will be obtained.

Thus, the partition coefficient k ($0<k<1$) measures the extent to which

a given analyte has penetrated the pores and a separation is produced.

It is important to emphasize that in SEC solutes are partially excluded from the pores of the packed particles and elute ahead of the solvent peak. In other liquid chromatography methods, sample components are retained (absorption/adsorption) by the column packing and elute after the unretained solvent peak. This difference in elution behavior in liquid chromatographic methods makes SEC a very predictable separation technique. All solutes should elute between k values of 0 to 1.0.

In the size separation mechanism, as a solute band moves along the column and around the packing particles, the solute molecules repeatedly permeate or diffuse in and out of the pores of the packing. The driving force for this process is the concentration gradient established between the two phases (external/solvent and internal/solvent).

From the thermodynamic point of view, solute distribution in SEC is governed mainly by changes in entropy between phases [5]. The solute mobility becomes more limited inside the pores of the packing than in the bulk mobile phase and as a consequence solute permeation in SEC is associated with a decrease in entropy. This occurs largely since solute-stationary phase interactions are minimal in SEC.

In developing a SEC analysis, two chromatographic parameters are normally obtained: (a) retention time or retention volume of the separated components and (b) area or amount of each component. Considering the first parameter and in the case of individual single components, the retention time is useful and essential for qualitative analysis. On the other hand, when dealing with polymer samples, where a

broad peak is usually obtained, the retention time or volume is commonly converted to a molecular weight for calculation of the molecular weight distribution of the polymer. This is accomplished by correlation with retention behavior of known molecular weight standards. The second parameter, the area of the molecular weight profile of the polymer sample, is a result of the detector response, which is a direct function of the physical property measured. In the case of single components analysis, a calibration curve (Area vs. Concentration) can be established and a reliable quantitative analysis performed. But, when analyzing polymers, the area or detector response is the parameter that offers major limitations in the accuracy of the SEC analysis, since it is the result of the physical property measured by the detector and this can vary widely with the molecular weight.

In analyzing these two factors, it is convenient to remark that the retention time is determined by major variables such as the precision of the flow rate (given by the precision of the pump), compressibility of the solvent, column temperature and column packing stability. These variables are easy to control and understand with currently well developed instrumentation. But, the detector response is still a great source of uncertainty in generating a highly accurate molecular weight distribution of a polymer.

In spite of all the advances achieved in the last two decades in liquid chromatographic instrumentation, SEC still has one major drawback which is also applicable to general liquid chromatography: to date, there is no reliable and simple to use detector. The introduction of

flowthrough detectors represented a major breakthrough from which modern liquid chromatography could evolve. However, the major problem concerning the performance of a reliable SEC analysis is the availability of a true mass detector. Ideally, detectors in SEC are required to provide an identical response for all components as well as a proportional response in terms of mass or volume of the separated analytes.

The selection of a detector for SEC analysis requires consideration of various performance criteria. Two types of detectors are commonly available: (1) bulk-property detectors that measures a change in some overall physical property of the eluent due to the presence of the analyte; and (2) solute-property or selective detectors that are sensitive only to some sample components. Either of these detector types generates an output signal that is related to the concentration of solute in the column effluent. While no liquid chromatography detector is "universal" in applicability and response, bulk-property detectors represents an obvious alternative for SEC as compared to selective detectors, since they can "see" all the sample components.

Major important detector characteristics include sensitivity, noise level, detectivity and linearity and these factors have to be evaluated in conjunction with the sample type when selecting a detector for SEC analysis.

Refractive index detectors have been used most frequently in SEC due to their "almost universal" response. But, on the other hand, this detector posses limited sensitivity as a consequence of its dependence on

temperature, mobile phase purity, dissolved gases, pressure and flow rate. Several recent refractive index detectors have incorporated improved design features that have made them more sensitive and stable [6]. The refractive index response of most polymers depends on the mobile phase refractive index and is approximately constant above 1000 daltons in molecular weight. For complex matrices however, this detector may offer differences in response for the solutes being separated as a function of their molecular sizes. For some samples a combined positive and negative response can occur with certain solvents. Thus, quantitative SEC results can be significantly affected when using a refractive index detector.

There are some other bulk-property detectors that can be used for SEC analysis. The evaporative or light scattering detector, the Laser Low Angle Light Scattering detector (LLAS), the LC-Flame Ionization detector (LC-FID) and the viscometer are examples of some other useful universal detectors in SEC. These detection systems however, are more dependent on operational variables than the refractive index detector and results can be subjected to difficult interpretation.

Selective detectors, such as UV and fluorescence, are normally used in SEC as secondary detectors to monitor specific sample components of interest and, due to their selectivity, they do not offer advantages when used alone.

Thus, the development of improved universal detection techniques in SEC constitutes an area of new research in detection modes and LC detector designs.

A detailed comparison of detectors for SEC of heavy oils related

samples was reported by Coulombe [7]. In this study, a differential refractive index detector, a flame ionization detector and an evaporative detector were evaluated in terms of: linearity, response factors and detection limits. For the type of samples studied, it was difficult to select one detector as the best in terms of accuracy of the molecular size profile.

A relatively new and technicaly simple alternative for detection in liquid chromatography is the "Indirect Detection Method". This detection technique is derived from a comprehensive development of the concept that some detectors may be used to monitor "transparent" species commonly thought not to be amenable to the type of detector operated.

One simple indirect detection mechanism can be defined in the following way: One way to use a liquid chromatography detector for analytes that do not respond to that detector is to introduce a probe or visualization agent in the mobile phase that interacts with that detector; when the non-interacting solutes, separated by the column, enter the detector cell they cause a decrease (dilution) in signal or baseline, thus a negative or vacancy in detection is generated and the solute is observed indirectly.

The response is therefore an indirect one and is the result of the "absence" of the mobile phase additive rather than the "presence" of the analyte. This is the key feature of indirect detection methods.

The classification and description of some other indirect detection mechanisms have been well documented by Ishii and Takeuchi [8], where either positive and negative peaks can be generated.

However, those detection mechanisms work favorably in partition liquid chromatography, like reversed-phase liquid chromatography, and do not apply in SEC.

A simple scheme of how an indirect detection experiment can be developed is shown in figure 1. The horizontal scale is the sequence stages. The ordinate is the detector response. In the stage A, the mobile phase that is used for the separation is equilibrated with the column and detector. The mobile phase has no interaction with the LC detector and a low background is produced. A substance that interacts with the detector is intentionally added, at a given concentration, in the mobile phase and this 'new' mobile phase is equilibrated with the column and detector. This additive is inert and ideally has no effect on the separation. Due to the presence of this agent a raise in baseline ocurrs (stage B). The new baseline or background can be set at a convenient level, by adjusting the baseline as shown on stage C, where the injection of a sample that is transparent to the detector is made. Since the elution of a component in the sample is accompanied by a displacement of the additive from the mobile phase, a transitory decrease in the background level is thereby produced, and a negative peak is obtained (Stage D). This is the basic mechanism of the indirect detection. Finally, as shown on step E, by simple polarity reversing of the detector output terminals, positive peaks are obtained for proper recording and integration.

The primary advantage of this simple indirect detection approach is that it affords the detection and quantitation of species which

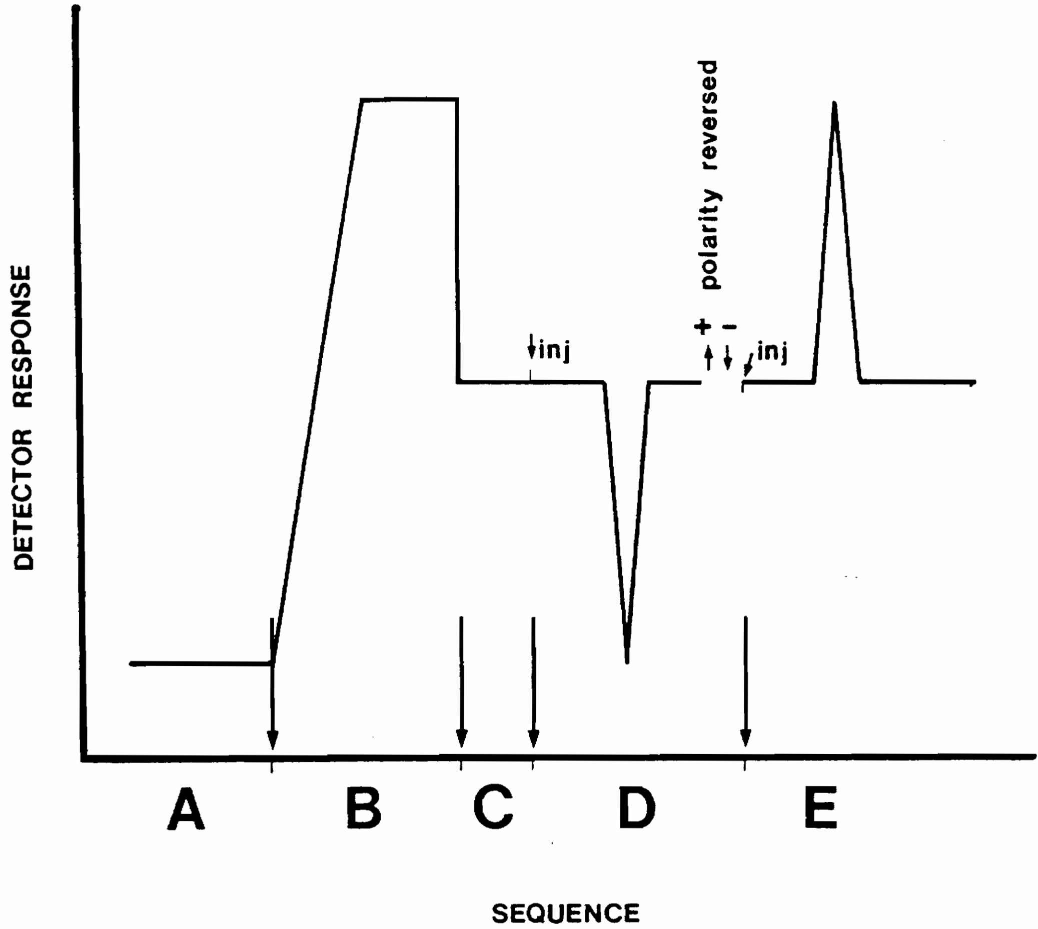


Figure 1. Stages in Indirect Detection Experiments.

themselves do not possess a functional group for direct monitoring by the usual detection modes.

There are good reasons to develop indirect detection modes. First, indirect detection is universal, i.e., there is little requirement as to the exact nature of the analyte. It is obvious that it has to be different from the mobile phase or added component in terms of the response at the detector. Generally, the more unique the mobile-phase component (more selective response) the more broadly applicable the indirect detector becomes. Second, some of the high sensitivity detectors are also very selective. Thus, by proper design it is possible to take advantage of the available sensitivity. Also, by implementing indirect detection the applicability of these detectors is expanded. Third, quantitation is easier with indirect detection. The appropiate and unique mobile phase component can be selected to fit the detector, where the response of most analytes should be fairly uniform. Fourth, indirect detection is nondestructive and the analyte can be collected for further analysis. The problem in this case is the contamination from the mobile phase additive.

Recently, different indirect detection schemes in high performance liquid chromatography have been successfully practiced. This detection mode initiated by Small and Miller [9] with indirect ultraviolet detection was succesfully applied in ion chromatography. In this work, indirect photomeric chromatography was developed using a single column ion analysis, a mobile phase containing UV absorbing ions and a UV photometer as the detector. Thus, sample ions that are transparent to the UV radiation were amenable to detection in this way.

The notable advantages of this technique were its single column simplicity, its applicability to a wide range of ionic species and a high sensitivity.

Some others applications of indirect UV absorbance has been reported [10-14]. In each method, a careful selection of experimental parameters was essential in order to develop the desired separation and detection performance. UV absorption photometers are still the most popular detectors in liquid chromatography and one of the most utilizied in indirect detection. The performance of some commercial UV detectors for indirect photometric chromatography has been evaluated [15].

Polarimetry, which usually is designed for highly selective detection of optically active molecules, has also proven to be a useful indirect detection mode [16-17].

Fuorescence is a much more selective process than absorption. In this way, indirect fluorescence is more broadly applicable. Several works on indirect fluorescence has been reported including applications in ion chromatography [18-19] and micro-column chromatography [20].

In the same area of selective detectors, an electrochemical detection system for quantitation of non-electroactive species separated by reversed-phase liquid chromatography was also developed with simple instrumentation [21].

In a different approach, ionic and non-ionic organic species were separated and detected with a conductivity detector operated in the indirect mode [22]. In this later case, positive peaks were obtained for the ionic species and negative peaks for the non-ionic organics.

So, these recent contributions in indirect detection clearly demonstrate that this is an area of active research.

Although indirect detection in liquid chromatography is expected to be both universal and sensitive, as discussed before, this detection mode has not been explored as an alternative method for Size Exclusion Chromatography. The first reported use was a presentation by this author at the 1989 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy [23].

Thus, it is of interest to investigate the characteristics and applicability of Indirect Conductivity Detection in Size Exclusion Chromatography. The reasons for selecting this detector system are: (1) the conductivity detector is a very sensitive (ng-pg range) and stable detector; and (2) most polymers are non-conductive materials. Another reason is based on the anticipated sensitive and universal response and, consequently, a more accurate molecular weight distribution determination of the sample should be possible. In applying this new tecnique, experimental conditions which maintain the size separation mechanism and simultaneously obtain optimal sensitivity need to be developed. From the standpoint of sensitivity, a study of the response factors of model compounds and limits of detection estimation will be essential in the evaluation of this novel detection system.

CHAPTER II

EXPERIMENTAL.

Liquid Chromatograph Description.

The liquid chromatograph used was assembled with different parts of various manufacturers. The description of each component is discussed separately.

Solvent Delivery System.

The pump was a HITACHI Model 655A-11 high speed liquid chromatograph (HITACHI, ltd., Tokyo, Japan). This is a dual-piston reciprocating pump capable of operating in a range of flow rate of 0.1 to 9.9 mL/min. and at a pressure up to 400 Kg/cm^2. The flow rate setting can be controlled in 0.1 mL/min. increments by a digital switch. The accuracy of the flow rate setting is ± 0.03 mL/min. The flow rate stability is ± 1% in the range of 0.6 to 5.0 mL/min. which is essential for size exclusion chromatography. Reproducibility of flow rate is required in SEC in order to employ the retention time in component identification. The pump includes a built-in mechanism for automatic compensation of solvent compressibility. All the materials of parts in contact with liquid are stainless steel, ruby, sapphire, carbon-impregnated Teflon and Teflon. The design of this serially connected double headed piston pump with only two check valves, guarantees a virtually pulse free and compressibility-corrected flow.

The pump was fed with a Teflon line (1/8" outer diameter) from a glass solvent reservoir of 1.0 liter. The teflon line had a stainless steel

porous frit of 2 microns to prevent any particulate matter from entering the pump and the rest of the system.

Sample Injection Valve.

The injection valve was a Rheodyne Model 7010 (Rheodyne Inc., Cotati, CA, USA) equipped with a 10 microliter sample loop. The model 7010 is a conventional six-port injector for complete loop filling. By means of this valve the sample is transferred at atmospheric pressure from the syringe to the sample loop. The loop is then connected by valving action to the high pressure mobile phase stream, which carries the sample into the column. The volume injected was the loop volume (10 uL) and an excess of sample was used to completely fill the loop to insure a representative sample. This sample injection method produces exceptional volumetric precision, especially when loops exceed 5 uL, where better than 0.1% (RSD) is typical. Different sample loop volumes can be adapted to this valve in order to vary the amount of sample injected into the column. This is the simplest form of sample injector, yet the complete filling method gives the highest precission. All the parts in contact with solvent are made of stainless steel.

Chromatographic Columns.

Two sets of high performance liquid chromatography columns were employed. Table 1 shows the characteristics of these columns. Each individual column was evaluated according to the manufacturer's specifications to insure optimum chromatographic performance. Before and after use, the columns were washed with 10 to 15 column volumes

Table 1: Characteristics of Chromatographic Columns.

Column	Dimensions (mm) (length x i.d.)	Packing Material	Particle Size (um)	Pore Size (Å)
1	250 x 4.0	Lichrospher (Silica Gel)	3	60
2	2 x (150 x 3.0)	Lichrosorb-DIOL (Silica Gel based)	5	60

All columns manufactured by E.M.Science (Cherry Hill, NJ, USA)

of methanol. These columns are of the removable cartridge type. All the connections of the columns with the injection valve and the detector were made of high quality stainless steel tubing 1/16" outer diameter and 0.007" internal diameter to maintain minimum dead volume and insure high performance during the transfer of solutes between components.

Liquid Chromatography Detector.

The detector used was a Perkin-Elmer Tri-Det (Perkin-Elmer Corp., Norwalk, CT, USA). This detector has a trifunctional flowcell configuration where it is possible to simultaneously monitor three physical properties of a sample: Ultraviolet absorption [UV], Fluorescence emission [FL] and Conductivity [CD].

For the conductivity measurement, a simple voltage divider network, driven by a 5 KHz oscillator, is made from a 1 meg resistor in series with the equivalent resistance of the sample passing through the flowcell. The voltage across the flowcell, which is proportional to its resistance, is then amplified and filtered in the conductivity amplifier section. The output of this stage is then passed to the recorder terminal and LED driver.

The detector flowcell housing is a molded acetal block. Figure 2 illustrates the basic components of the cell. The mobile phase input and output tubes, made from stainless steel hypodermic tube, are silver soldered into a stainless steel doughnut. These input and output tube/disc assemblies serve as the conductivity detector connectors

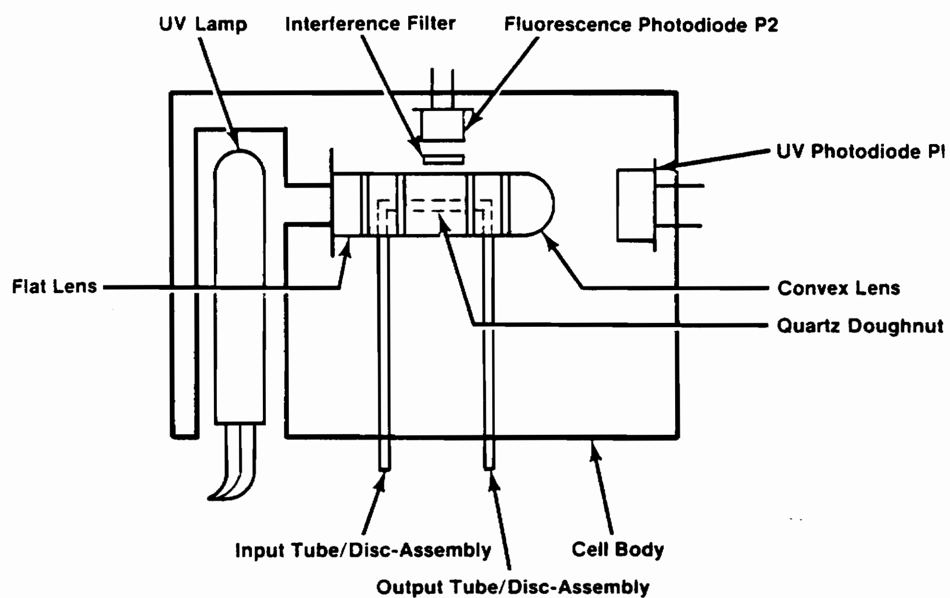


Figure 2. TriDet Detector Cell Schematic.

(electrodes) since they are electrically isolated from one another.

According to manufacturer specifications, using sodium chloride as the analyte and water as mobile phase, three orders of magnitude linearity and a sensitivity of 50 nanograms/mL can be achieved. To minimize extra-column band broadening the Tri-Det has a 2.4 uL volume flowcell.

Detector Cell Modification.

As a part of the sensitivity improvement study, a flowcell modification was carried out. According to figure 2, the quartz window spacer was removed from the cell reducing the separation between the two electrodes. The same seals were used to maintain the cell leaks free. In this case, the separation distance between the two electrodes was reduced from 4 mm to approximately 2 mm. After the removal of the quartz spacer the cell was assembled again and inspected for leaks.

Reporting Integrator.

A reporting integrator Hewlett-Packard Model 3390A (Hewlett-Packard, Palo Alto, CA, USA) was connected to the recorder output of the conductivity terminal function of the Tri-Det detector. The integration parameters such as peak width, threshold, attenuation and area rejection were set and optimized according to the chromatographic peak characteristics. Table 2 shows typical integration parameters values.

Table 2: Integration Parameters.

ATTENUATION	0
PEAK WIDTH	0.041
THRESHOLD	0
CHART SPEED	1.0 and 0.5 cm/min.
AREA REJECTION	1000

Mobile Phase Preparation.

Solutions of different tetraalkylammonium salts were prepared in methanol and used as the mobile phase. The necessary amount of salt was weighed in an analytical balance (± 0.001 g.), transferred to a 1.0 liter solvent reservoir and dissolved in the appropriate amount of methanol to obtain the desired salt concentration. The solutions were filtered through a 0.5 microns Teflon membrane and degassed by bubbling helium gas for 3 minutes.

Reagents and solvents.

High performance liquid chromatography grade methanol from Fisher (Fisher Co., Fair Lawn, NJ, USA) was used throughout this work without further treatment. The tetraalkylammoniun chloride salts were received from Aldrich (Aldrich, Milwakee, WI, USA) at a 97-98% purity and a recrystallization step was necessary prior their use. The tetramethylammonium chloride was recrystalized from ethanol. The tetraethylammonium chloride was recrystalized from ethyl acetate. After recrystallization, these salts were maintained in a dessicator under vacuum. The tetrabutylammonium chloride was used as received (+98%) since it was impossible to find a convenient solvent for recrystallization.

Standard Analytes.

Different groups of standard compounds were used in this work for the characterization of the detector response. A series of n-alkanes and aromatic hydrocarbons, alcohols and alkylphthalates were obtained from

Aldrich at a +99% purity. Solutions of these analytes were prepared in methanol in 10.00 mL volumetric flasks at the needed concentration. Polypropylene glycol standards of known molecular weight (4000, 2000, 1200 and 800 daltons) were obtained from Waters (Millipore, Waters Chromatography Division, Milford, MA, USA). Specifications on purity levels are not available for these series of standards. Solutions of each molecular weight standard were prepared in methanol in 10.00 and 20.00 mL volumetric flasks at the required concentration.

CHAPTER III

RESULTS AND DISCUSSION

The thesis statment of this work was the study of the characteristics and applicability of indirect conductivity detection as an alternative detection mode for size exclusion chromatography. From this goal a series of criteria and analytical procedures were defined and developed to acquire the data necessary for the evaluation. The results of each criterion will be described separately.

Optimization of Conditions.

The fundamental consideration in developing an indirect detection method is based on the dynamic equilibrium that needs to be established between the mobile phase, the added probe and the stationary phase. As mentioned in the introduction, ideally in SEC there is no interaction between the analyte and the stationary phase; the packing chemistry and the solvent type are carefuly selected to eliminate and avoid any possible interaction. In the case of this research work, the chromatographic conditions were adjusted to match the analytical goal: "SEC and indirect conductivity detection mode".

Selection of the mobile phase system and chromatographic column.

The mobile phase system is the combination of the solvent, which is necessary to dissolve the sample and transport it through the stationary phase, and the added probe that is responsible for generating the desired

background signal at the detector. In SEC the selection of the solvent is mainly limited to the solubility of the sample, its compatibility with both the column packing material and the detector used. There are a few solvents that are routinely used in SEC. Among them, toluene, chloroform, methylene chloride, tetrahydrofuran, o-dichlorobenzene, trichlorobenzene and methanol are routinely used for organic polymers. The primary consideration in this work was the selection of a solvent that could dissolve the samples and allow ionization of the added probe to produce the necessary conductivity background. From the solvents mentioned, methanol was the best mobile phase in terms of solvation and ionization for probes.

For conductivity probes, tetraalkylammonium chloride salts were found to be most suitable since they are soluble in methanol and produce a high conductivity background. These salts are unreactive with most polymers and also with common chromatographic packing materials like silica gels and polymeric packings. Different salts were studied with distinct alkyl groups (-methyl, -ethyl and -butyl). The effect of these salts on separation and detection will be discussed later as a part of this work.

In the initial stages of this work, additional experiments were conducted with tetrahydrofuran and methanol as solvents and nitric and sulfuric acids as probes for the conductivity signal, but these combinations gave rise to undesirable positive and negative peaks. Thus, the mobile phase system throughout this work consisted of a tetraalkylammonium salt solution in methanol.

In order to optimize the mobile phase system with chromatographic variables, different salt concentrations were used and three parameters evaluated: area (AR) or response for a 10 uL of methanol injected; number of theoretical plates (N) for the methanol peak; and the retention time (tr). The results shown in Table 3 indicate an increase in the methanol peak area with increases in salt concentration up to approximately 5 mM with a maximum between 2.5 and 10.0 mM. The area decreases from 10 to 20 mM. The peak width, expressed as AR/HT shows a decrease with increased salt concentration. The number of plates and retention time both increases when salt concentration increases. These trends are better illustrated in figures 3 and 4. From the overall data it can be noted that there is a compromise between response (maximum signal) and efficiency (maximum number of plates). A reasonable working range was determined to be between 10 - 12 mM salt concentration for a high signal and efficiency.

The increase of signal with salt concentration can be attributed to the increase in the conductivity background level and upon the injection of the nonconductive methanol a greater decrease in conductivity is obtained. This has been observed before in other modes of indirect detection [24]. The fact that the response decreases after some concentration level of salt is explained by the decrease in dissociation of the salt at elevated concentration (more than 12 mM) and also that the detector is saturated.

Surprisingly there was a shift in retention time for the methanol peak with the salt concentration. This result can be attributed to the

Table 3: Effect of salt concentration on response and efficiency in indirect conductivity detection.

Conc.(mM)	Area	AR/HT	N(plates)	tr(min.)
1.44	11,700	0.058	1,480	0.89
2.00	12,200	0.055	1,950	0.97
5.25	13,220	0.046	3,270	1.05
10.50	12,500	0.042	4,080	1.07
19.20	10,340	0.041	4,280	1.07

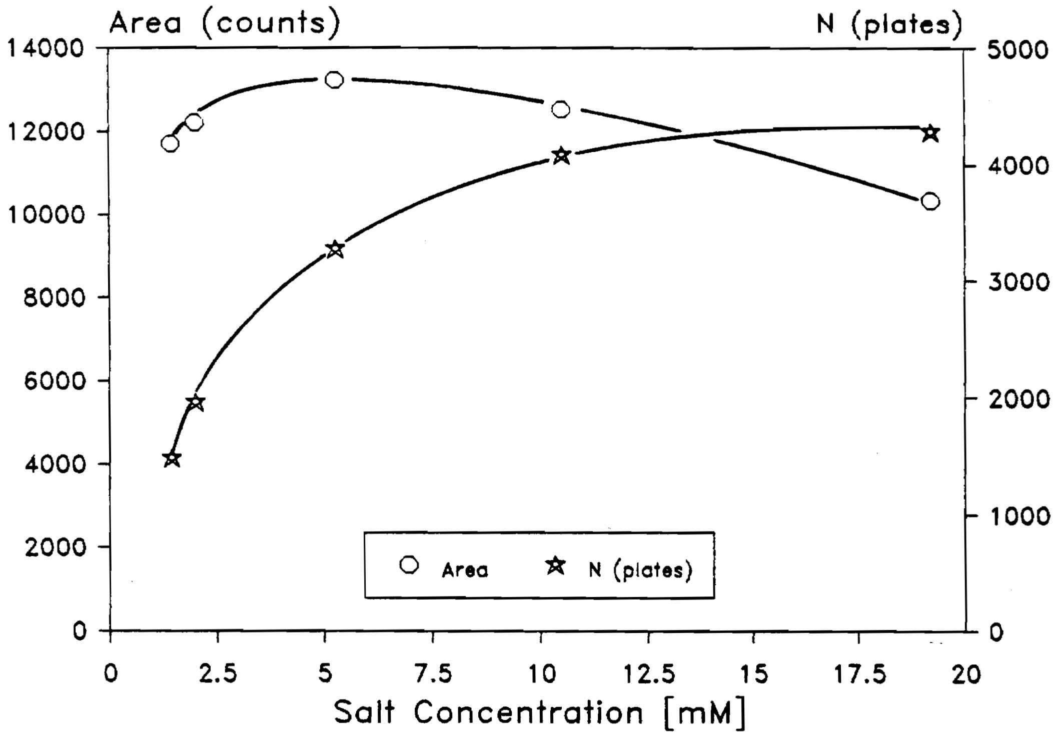


Figure 3. Area and Number of Plates (N) as afunction of Salt Concentration.

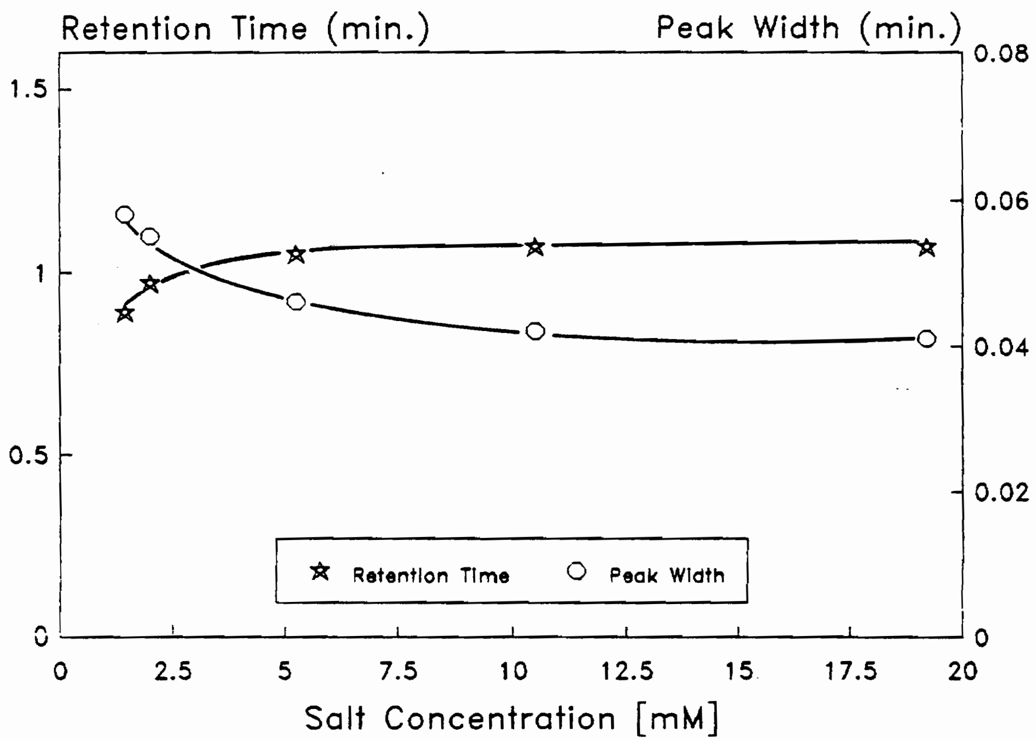


Figure 4. Retention Time and Peak Width as a Function of Salt Concentration.

"slow mass transfer" of the methanol into the pores that are equilibrated with the salt; the higher the salt concentration the slower the equilibration rate for the permeation process and the longer the retention time. In SEC, the driving force of permeation is controlled by the concentration gradient of the solute between the bulk mobile phase and the mobile phase inside the pores.

To further evaluate this mechanism, the retention time of the methanol was monitored as a function of the column temperature for a mobile phase containing 10 mM of the tetramethylammonium salt. Table 4 shows that increasing the column temperature decreases the retention time to a value equivalent to a 2 mM mobile phase at room temperature. In this case, the methanol permeation is faster at higher temperatures, which explains the shift to longer retention times observed at higher salt concentration. A slight shift toward smaller retention volumes with increasing temperature has also been observed for polystyrene polymer standards chromatographed on bioglass columns [25]. In addition, there is a reduction in solvent viscosity that favors the permeation process of the solute.

Methanol was chosen as a reference solute for the evaluation of the chromatographic variables since this molecule is very small and will elute last. One disadvantage of this solvent is that not many polymers are soluble in methanol and only silica gels and hydrophylic gels can be used as column packing materials. The highly efficient SEC columns packed with polystyrene divinylbenzene based gels are incompatible with methanol.

Table 4: Temperature effect on retention time of methanol.

Temperature (°C)	tr (min.)
23 ± 1	1.07
30 ± 1	1.01
40 ± 1	0.97

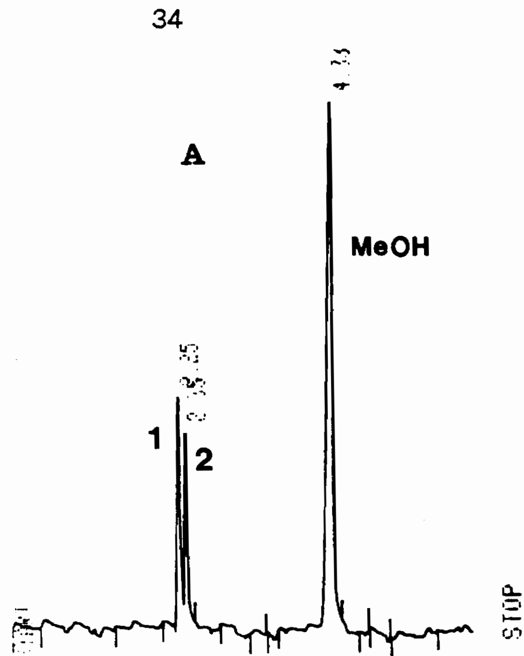
Silica gel represented the best option from the available chromatographic packings in high performance SEC. Hydrophylic gels such as polydextrans are also used with polar solvents like methanol, THF and aqueous mobile phases, but these packings are less well characterized and less efficient than silica gels. Silica gel offers superior advantages in contrast to most other packings. They are rigid solids which can support the high pressures (6000 psi) of high performance SEC. Silica gels are commercially available in a wide range of particle sizes (3, 5, 7, 10 microns) and pore sizes (60 Å to 4000 Å) with very narrow size and pore distributions.

Mobile phase Flow Rate.

The mobile phase velocity can have a significant influence on the efficiency and the resolution in SEC depending on the molecular size of the solute analyzed. For high molecular weight solutes the increase in flow rate has a dramatic effect on decreasing the efficiency due to the very slow equilibration of the permeation process [26]. In contrast for the low molecular weight solutes, there is a relatively small decrease in efficiency with increased mobile phase velocity, which is characteristic of the rapid solute equilibration associated with the permeation process. Linear velocities of 0.15 cm/sec. are normally found optimun for suitable efficiencies. The working linear velocity was between 0.15 and 0.23 cm/sec. which can be considered satisfactory since the column effiency is in the order of 27,00 plates/meter.

The observed changes in resolution were very small for the low

molecular weight solutes. By halving the flow rate from 1.00 mL/min. to 0.5 mL/min. an average 10% increase in resolution was found for low molecular weight solutes. As illustrated in figure 5 the separation of n-hexane from p-xylene at 1.0 mL/min. and 0.5 mL/min. does not show a significant improvement in resolution at lower flow rates; n-hexane elutes first and p-xylene second because of the larger molar volume of n-hexane (130.77 mL/mol) as compared to p-xylene (122.60 mL/mol).



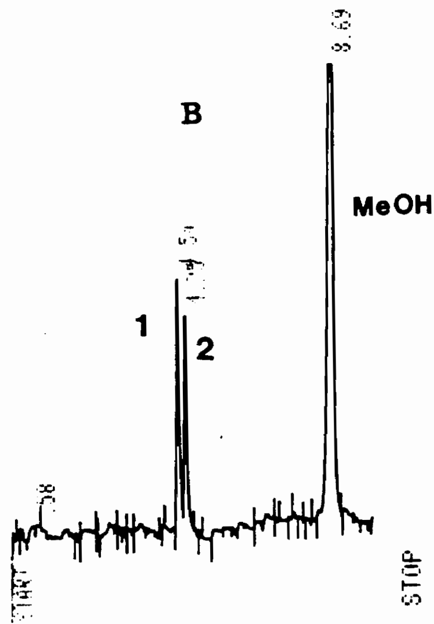


Figure 5. Effect of Flow Rate on SEC Separation of n-Hexane (1) and p-Xylene (2).
A: Flow Rate: 1.00 mL/min. and Chart Speed: 1.0 cm/min.
B: Flow Rate: 0.50 mL/min. and Chart Speed: 0.5 cm/min.

Separation Characteristics.

To evaluate the effect of the selected conditions on the separation characteristics of the system, a series of molecular weight calibration curves were constructed using different standards. Since the logarithm of the molecular weight is linearly related to the retention time or volume, these calibration curves can serve as the best indication if the ideal SEC mechanism is operating under the selected conditions.

Analytes of different chemical nature were selected to cover the variety of functional groups commonly found in polymers. Figure 6 shows the chromatograms obtained for the different groups of standards and figure 7 shows the calibration curves (Log MW vs. Retention Time) for a series of low molecular weight n-alkanes, alcohols, aromatics and phthalates. Although different slopes were obtained for each functional group, a linear relationship is observed indicating that a 'size separation mechanism' is operating in the system. The slope of the different curves can be attributed to several factors. First, each calibration standard has a particular hydrodynamic volume since each compound has a characteristics geometry and molecular configuration and the molecular shape and size (not the molecular weight) is the physical parameter that controls the separation. A second factor could be the slight adsorption of the polar groups in the polar silica gel surface; in this case the shift of the phthalates calibration curve to the right (longer retention time) as compared to the alkanes could be explained by adsorption of this polar group. Also, the position of the alcohols and aromatics curves as compared to that of the n-alkanes could be explained by the slight

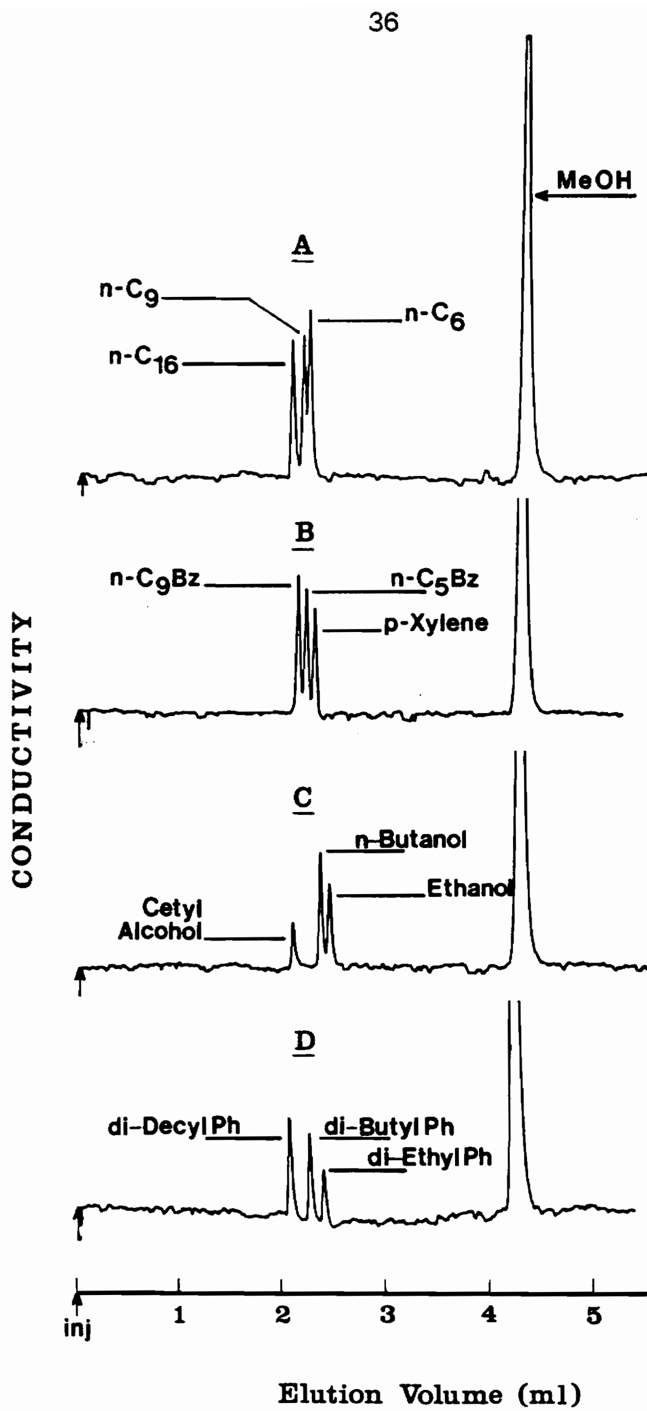


Figure 6. Size Exclusion Chromatograms of Different Functional Groups.

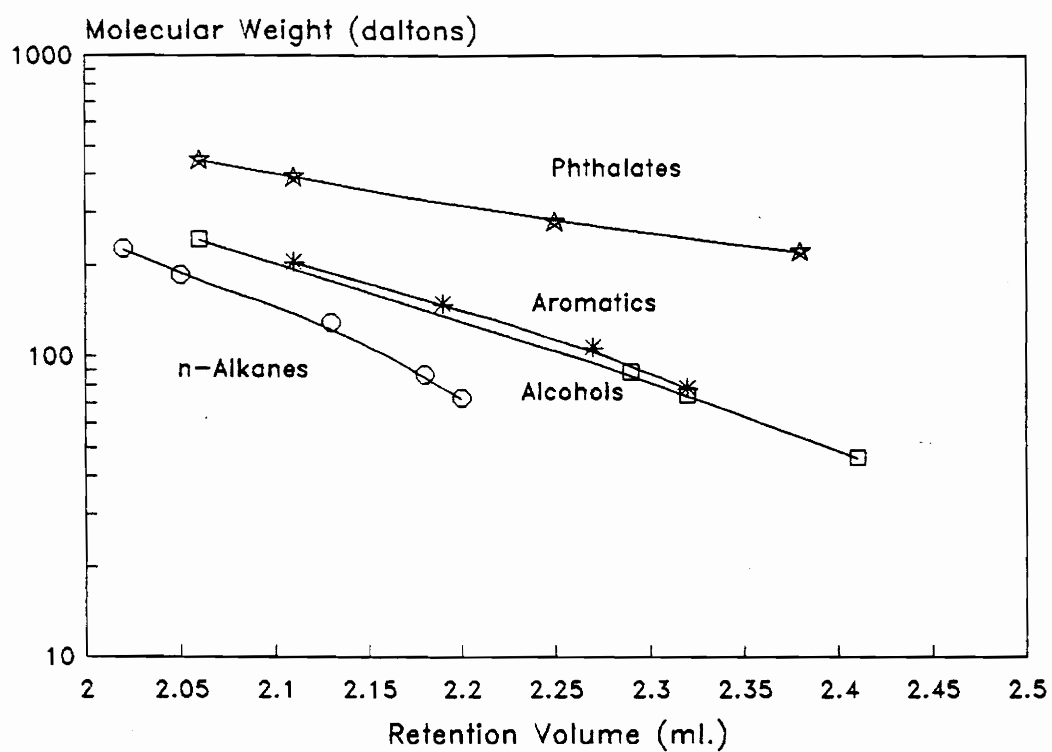


Figure 7. SEC Calibration Curves of Different Functional Groups.

adsorption of these compounds in the silica gel surface. However, considering the highly polar mobile phase (organic salt + methanol) interacting primarily with the polar silica gel surface, the adsorption mechanism can be considered as secondary when compared to the hydrodynamic volume differences. A third possible explanation of the different slopes could be a distinct solvation effect for each group.

Response Factors Study.

The response factor study was developed with the same series of standards used for the SEC calibration curves. In chromatographic analysis the response factor of a solute represents the sensitivity of the detector, expressed as output (mV or area) per concentration unit of the solute. This is usually determined for more than one mass of analyte, resulting in linear graphs of detector signal (response) as a function of analyte mass.

In the determination of response factors, a four point calibration curve was constructed for each compound; each point representing the average of 4 to 5 injections.

Response factors can be expressed in different units. Normally, the detector signal is represented as counts (area unit) given by the reporting integrator and the amount of analyte as microliters, miligrams or milimols. Table 5 summarizes the response factors obtained for the n-alkanes, alcohols, aromatics and phthalates. These response factors (Rf) are expressed in Area/uL since solutions of the analytes were prepared on a volume concentration basis. It can be noted that Rf increases with

Table 5: Indirect Conductivity Detection Response Factors of Different Functional Groups.

Compounds	Response Factor (Area/uL.)
n-Alkanes	
n-Pentane	200
n-Hexane	210
n-Nonane	220
n-Undecane	240
n-Hexadecane	245
Aromatics	
Benzene	132
Toluene	134
p-Xylene	140
p-di-iso-propylbenzene	180
n-Amylbenzene	165
1-Heptylbenzene	188
1-Nonylbenzene	190
Alcohols	
2-Propanol	170
n-Butanol	182
n-Octanol	214
Benzyl alcohol	100
Phthalates	
di-Butylphthalate	97
di-iso-Octylphthalate	145
di-Decylphthalate	184

increasing molecular weight for all the functional groups. This trend can be explained as follows: compounds with larger molecular weight are more effective in inhibiting the mobility of the solvated salt species and consequently they show a greater decrease in conductance. This is explained by the higher viscosity of the larger molecules that affects in a large degree the mobile phase conductance (Stokes law). The conductivity of ions decreases with increasing solvent viscosity.

These Rf's can also be expressed as Area/mg and Area/mmol and figures 8, 9 and 10 show the response factors of n-alkanes, alcohols and aromatics in units of Area/uL, Area/mg and Area/mmol respectively and as a function of the molecular weight. The molar Rf's (Area/mmol) show a more pronounced increase with increasing molecular weight . On the other hand, Rf's expressed as Area/mg show an almost flat plot for the n-alkanes indicating that the response (as mass) of this group is practically independent of the molecular weight.

To further evaluate these results, the response factors of larger molecules were studied. Polypropylene glycol standards of known molecular weight (4000, 2000, 1200 and 800) were used for this purpose. Calibration curves of Area vs. mg of each standard were generated. Figure 11 illustrates the curves obtained for each standard. Apparently all the slopes appear to be the same, but only the 1200 and 800 standards have statistically the same value (based on the t-test @ 95% confidence limit). Table 6 shows the Rf's and their statistical comparison. The Rf's of the 4000, 1200 and 800 standards seem to be similar even only the 1200 and 800 are statistically the same.

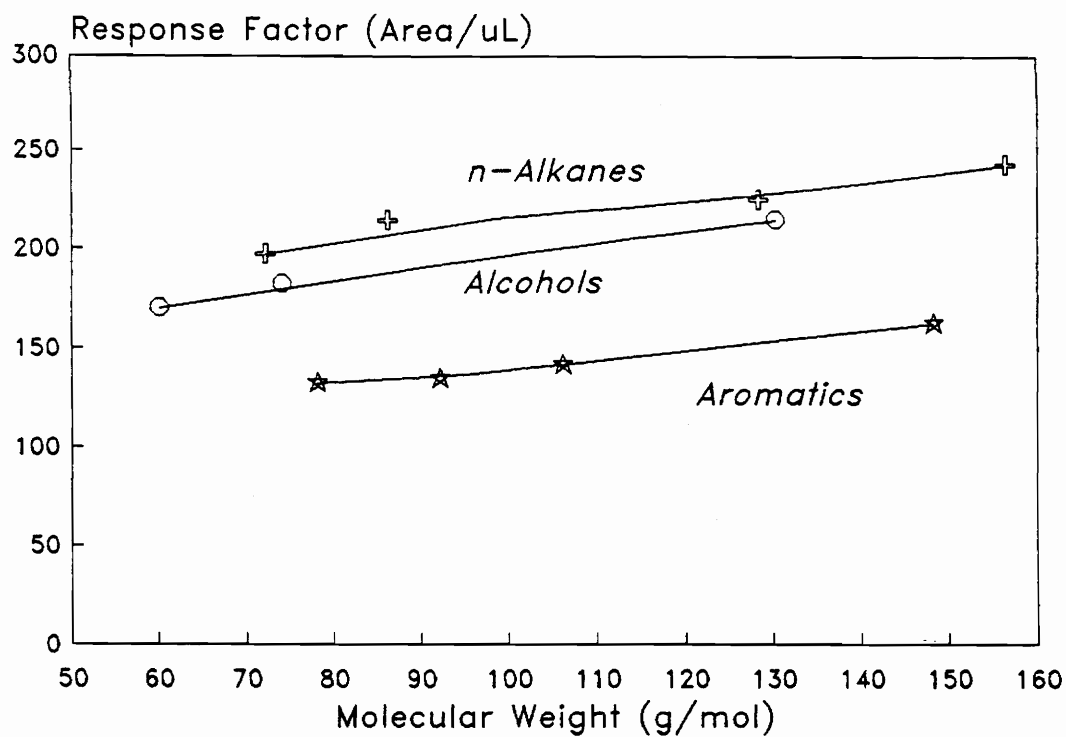


Figure 8. Response Factors (Area/uL) for Different Functional Groups.

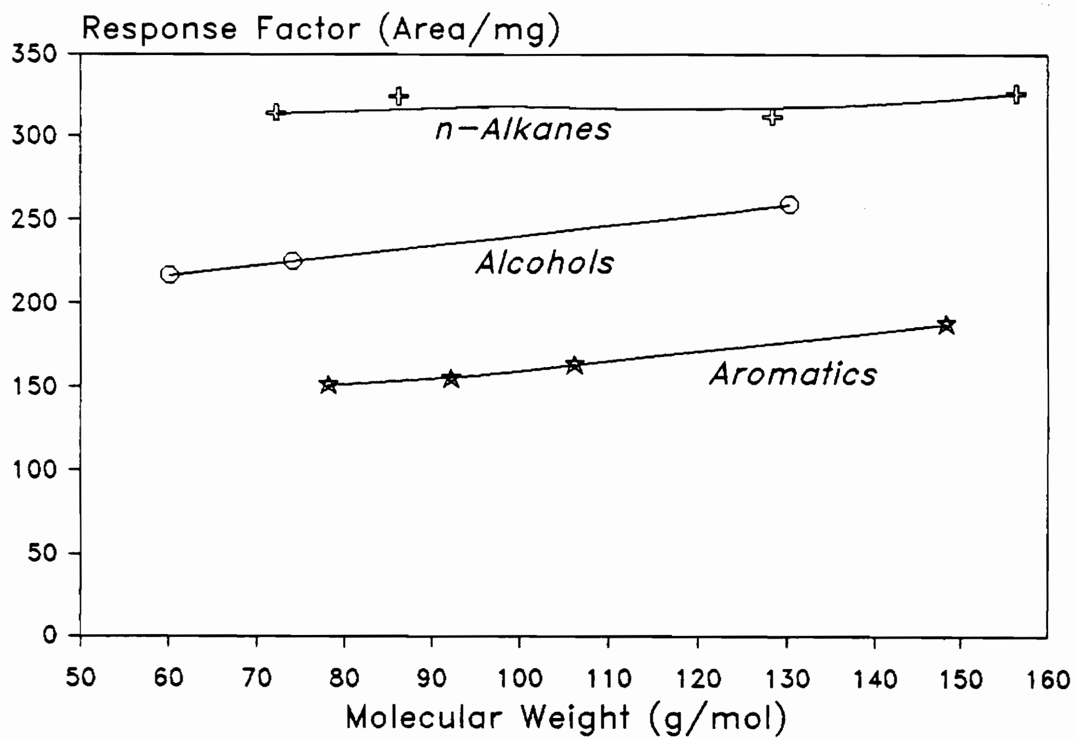


Figure 9. Response Factors (Area/mg) for Different Functional Groups.

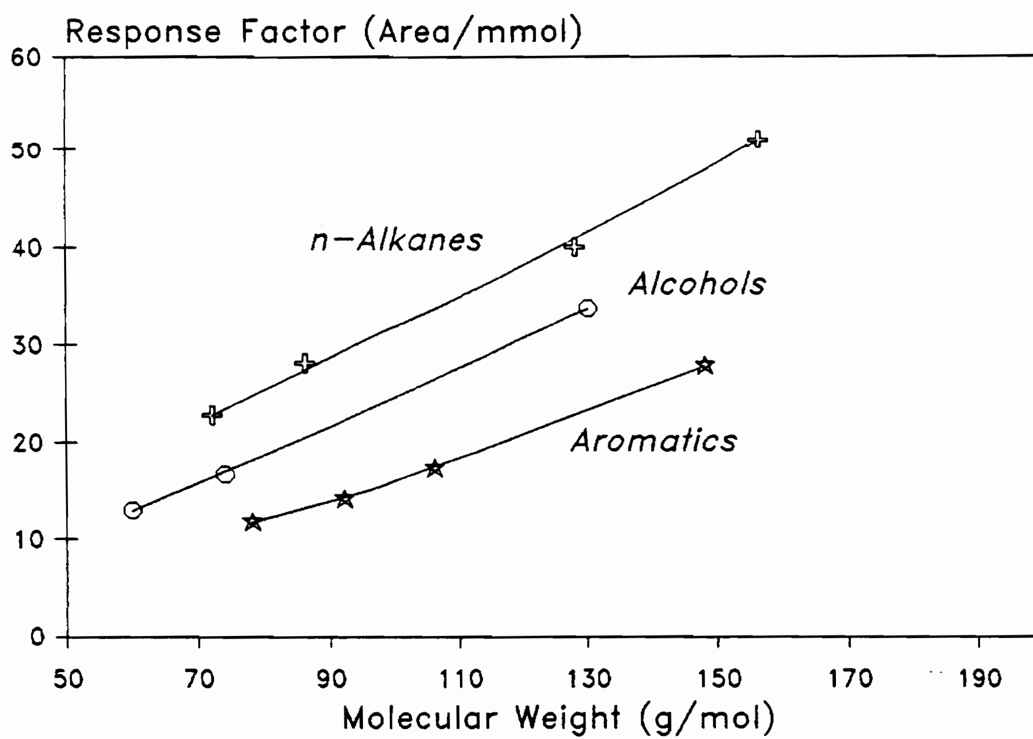


Figure 10. Response Factors (Area/mmol) for Different Functional Groups.

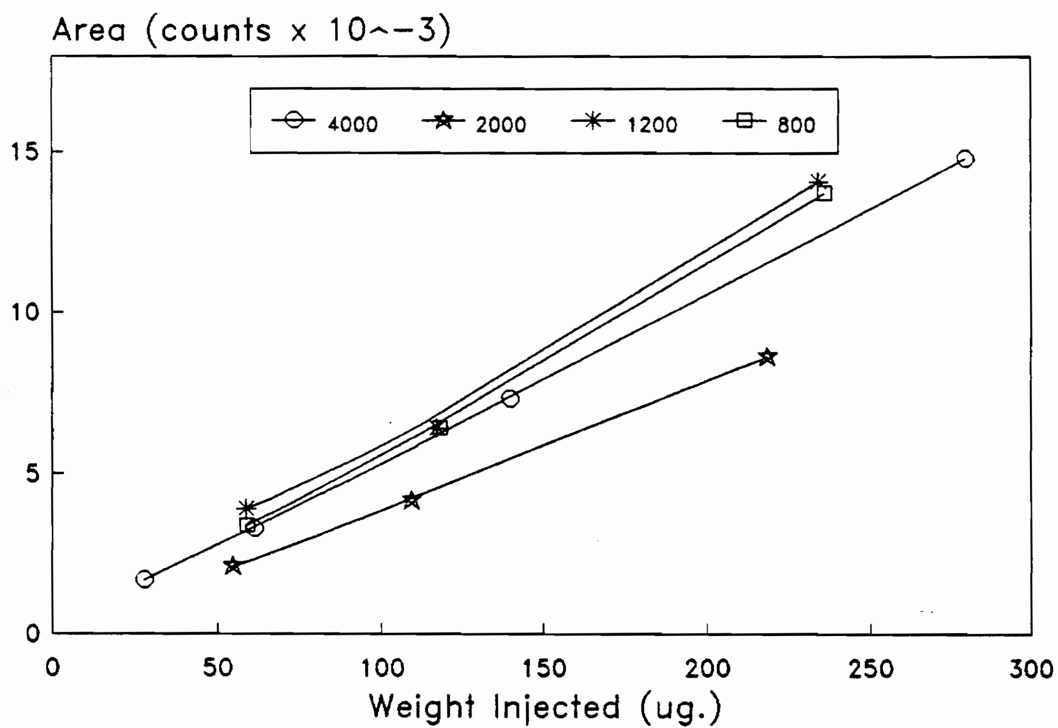


Figure 11. Response Factors of Polypropylene Glycol Standards.

Table 6:

Response Factors of PPG* Standards.

M.W.	Rf (Area/ug.)
4000	52 ± 1
2000	40 ± 1
1200	60 ± 6
800	59 ± 3

*Polypropylene glycol

Statistical Comparison of PPG Response Factors**.

Compared Standards	t (calc.)
4000 vs 2000	11.31
4000 vs 1200	2.63
4000 vs 800	4.43
1200 vs 800	0.30

** Based on the t-test; t (table value) @ 95% C.L. = 2.45

The difference observed in the 2000 standard could be justified by impurities (water or glycol) contained in this standard. Also, the differences in molecular weight distribution between all the standards can be a factor to consider.

Limits of Detection.

The procedure described by Miller [27] was used for limits of detection calculation. Calibration curves for four different functional groups were developed; n-hexane, o-xylene, n-butanol and cyclohexyl-acetate were used as standards. The statistical standard deviation (Sy/x) of each point point was estimated from the calibration plots. The limit of detection (Xcl) was then calculated by using three times the statistical standards deviation (Sy/x) divided by the analytical sensitivity (b) (slope of the line):

$$Xcl = 3 \cdot \frac{Sy/x}{b} \qquad (3)$$

Figures 12, 13, 14 and 15 show the linear calibration plots developed for these calculations. A linear regression coefficient better than 0.9998 was obtained in all cases. Table 7 contains the limits of detection expressed as mass and volume. Similar values were obtained among the functional groups. These limits of detection are comparable with conventional refractive index detectors. However, limits of detection are solvent dependent in the case of the refractive index detector and only a relative comparison can be established.

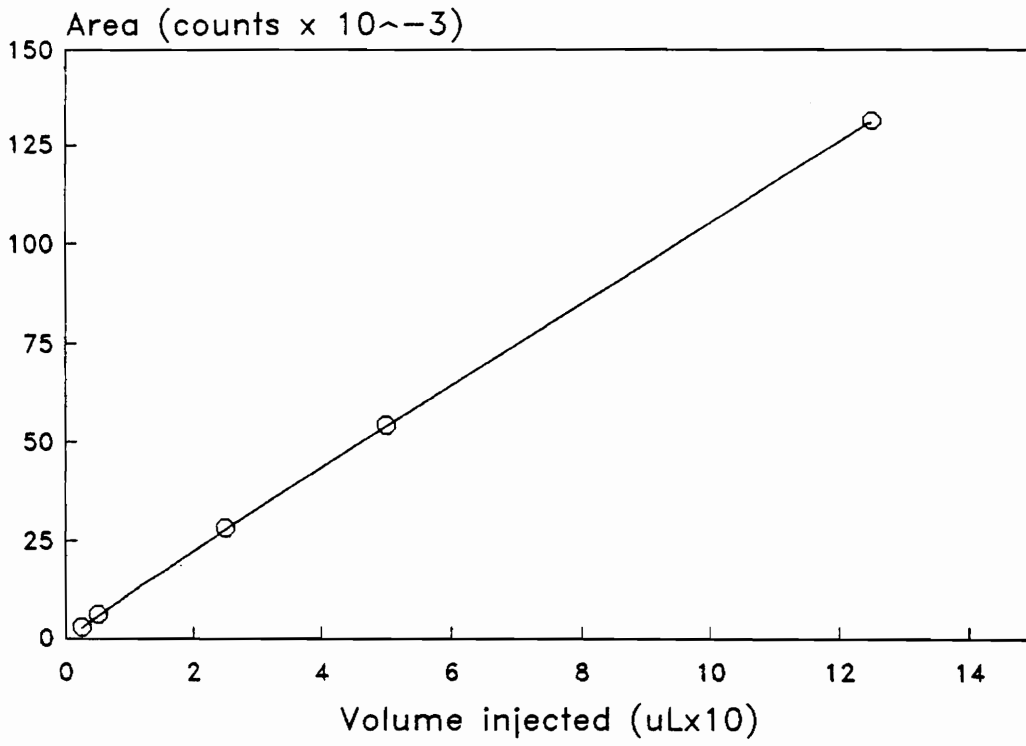


Figure 12. Calibration Curve of n-Hexane for Limit of detection Calculations.

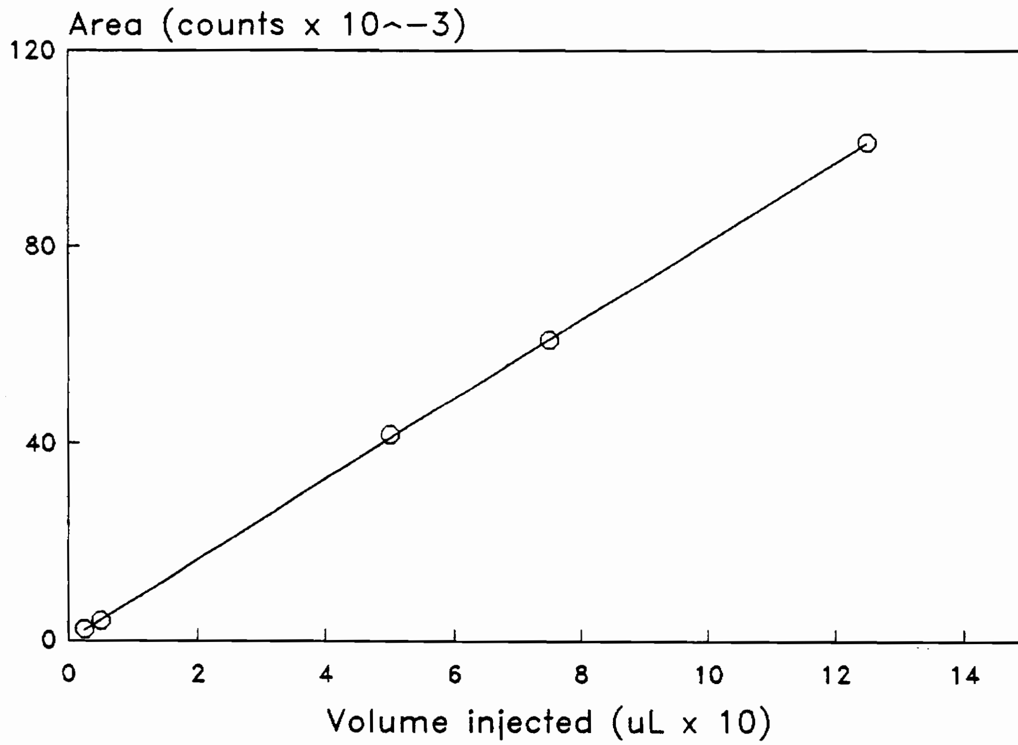


Figure 13. Calibration Curve of o-Xylene for Limit of Detection Calculations.

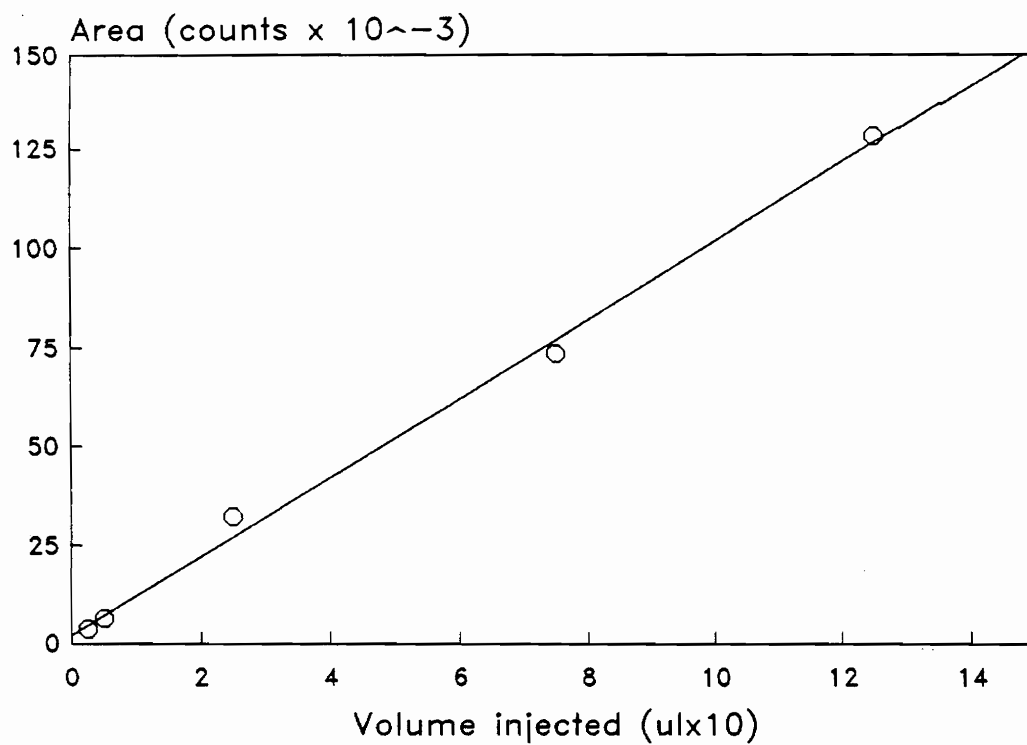


Figure 14. Calibration Curve of n-Butanol for Limit of Detection Calculations.

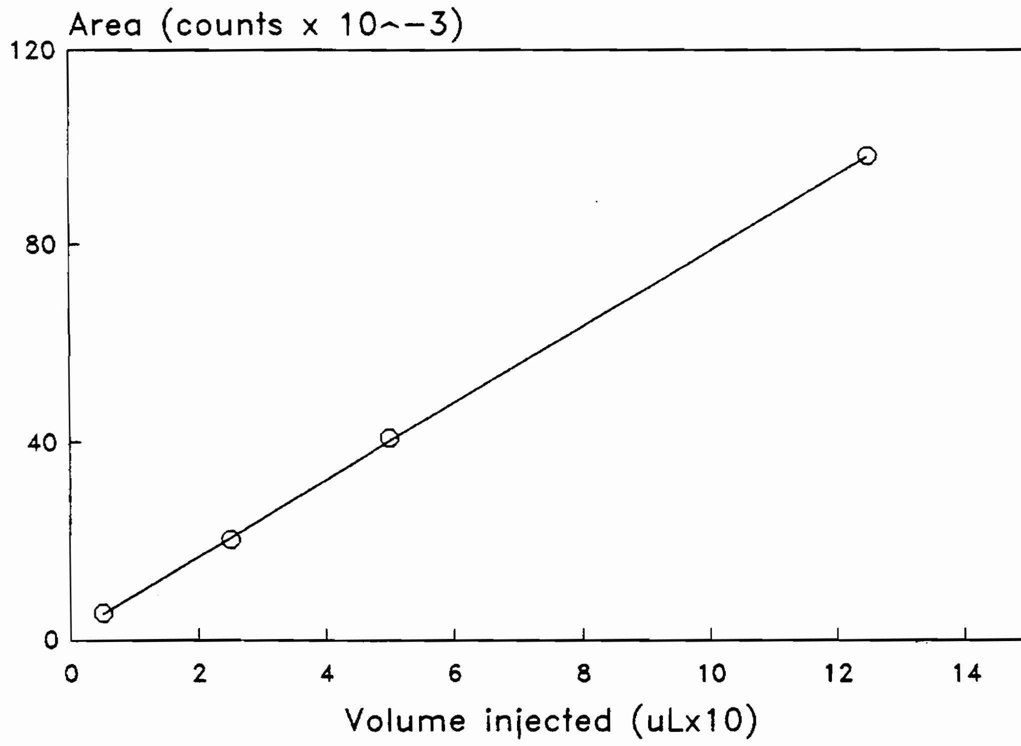


Figure 15. Calibration Curve of Cyclohexylacetate for Limit of Detection Calculations.

Table 7: Limits of Detection (Xcl) of Different Functional Groups.

Compound	Xcl*	
	Volume (nL)	Mass (ug)
n-Hexane	20	10
o-Xylene	20	15
n-Butanol	30	20
Cyclohexylacetate	20	20

* Based on k=3

Effect of the Salt Type on Detector Response and Separation.

In addition to the tetramethylammonium chloride salt used during this work, tetraethyl- and tetrabutylammonium chlorides were also employed to evaluate the detector response. Solutions of similar salts concentration (10 mM) were prepared and equilibrated with the column. Methanol was used as evaluation solute as in previous studies. Table 8 shows the results on the effect of these salts on retention time, peak width (AR/HT), number of plates (N) and Area. The reduction in efficiency (N) with increasing the length of the alkyl chain in the probe is due to the decrease in retention time at a constant peak width (AR/HT). The decrease in retention time of methanol with increasing the alkyl chain of the salt can be explained with the help of the illustration in the figure 16. The surface and the pores of the silica gel are equilibrated and covered with the solvated salt molecules. The structure of the silica gel can be considered as formed of macropores, which are used for the sieving process, and micropores. When equilibrating the silica gel phase with the probes, the larger and bulky tetrabutyl salt will cover the micropores more extensively than the smaller tetramethyl and tetraethyl salts. Upon injection of the small molecule methanol, with the bulky tetrabutyl probe the micropores are blocked and unavailable for the methanol to permeate. Thus, the elution time is reduced with the bulkier probe. In the opposite case, with the smaller probes, the methanol can permeate in some extent the micropores and a larger effective retention time is observed. To further verify this observation, different polypropylene glycol standards were analyzed with these three

Table 8: Effect of the Salt Type on Retention Time and Response.

Sal Type*	tr(min.)	AR/HT	N(plates)	Area
TMACl	1.07	0.042	4080	12380
TEACl	0.99	0.042	3490	13250
TBACl	0.91	0.042	2950	14170

* Salt Concentration : 10.0 mM

TMACl: tetramethylammonium Chloride
TEACl: tetraethylammonium Chloride
TBACl: tetrabutylammonium Chloride

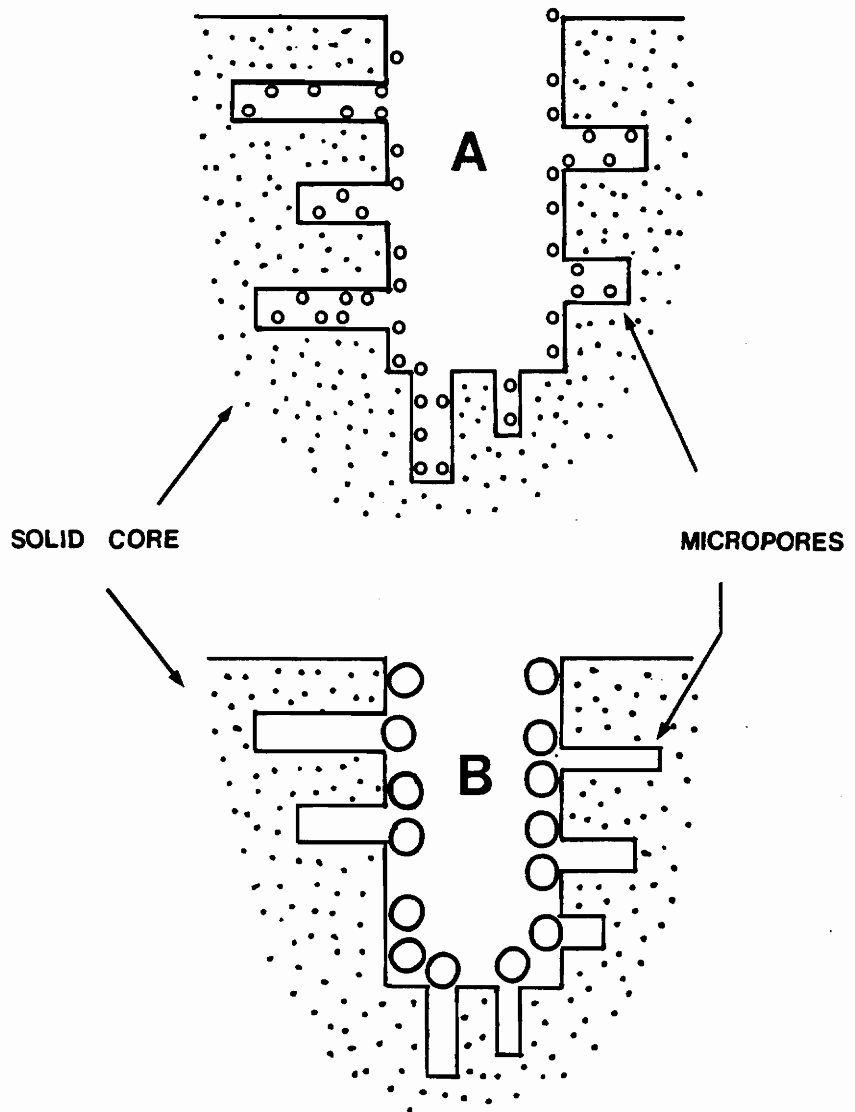


Figure 16. Schematic of the Silica Gel Pore Structure and the Salt Effect.
A: o tetramethylammonium chloride

B: O tetrabutylammonium chloride

probes. Figures 17, 18 and 19 show the chromatograms of the separation of the PPG standards 4000, 1200 and 800 from methanol. In all cases there is a reduction in the retention time of methanol when increasing the size of the conductivity probe. The retention time of the PPG standards remains the same. In figure 19 the 800 PPG standard and methanol co-elute and there is no separation. The coverage of the silica gel micropores with the probes is affecting the elution of the methanol but not of the larger polymers which can not permeate in the small pores. Figure 20 shows the SEC calibration curves with the different probes where the only effect is the variation in retention time of methanol.

The surprising result was the increase in Area (response) with the size of the probe. The larger the probe the smaller the conductivity background should be and also the signal, since the conductivity is a function of the ion mobility and this is a function of the ion size. This result may be explained by the fact that the tetrabutylammonium salt was used without purification (97% purity). The small amount of impurities present in this salt could contribute to increase the conductivity background.

Detector Cell Modification.

The construction design of the TriDet detector cell permitted a modification. According to the schematic in figure 21, the quartz spacer that separates the two electrodes was removed and the cell was reconstructed again. With this new configuration the separation of the

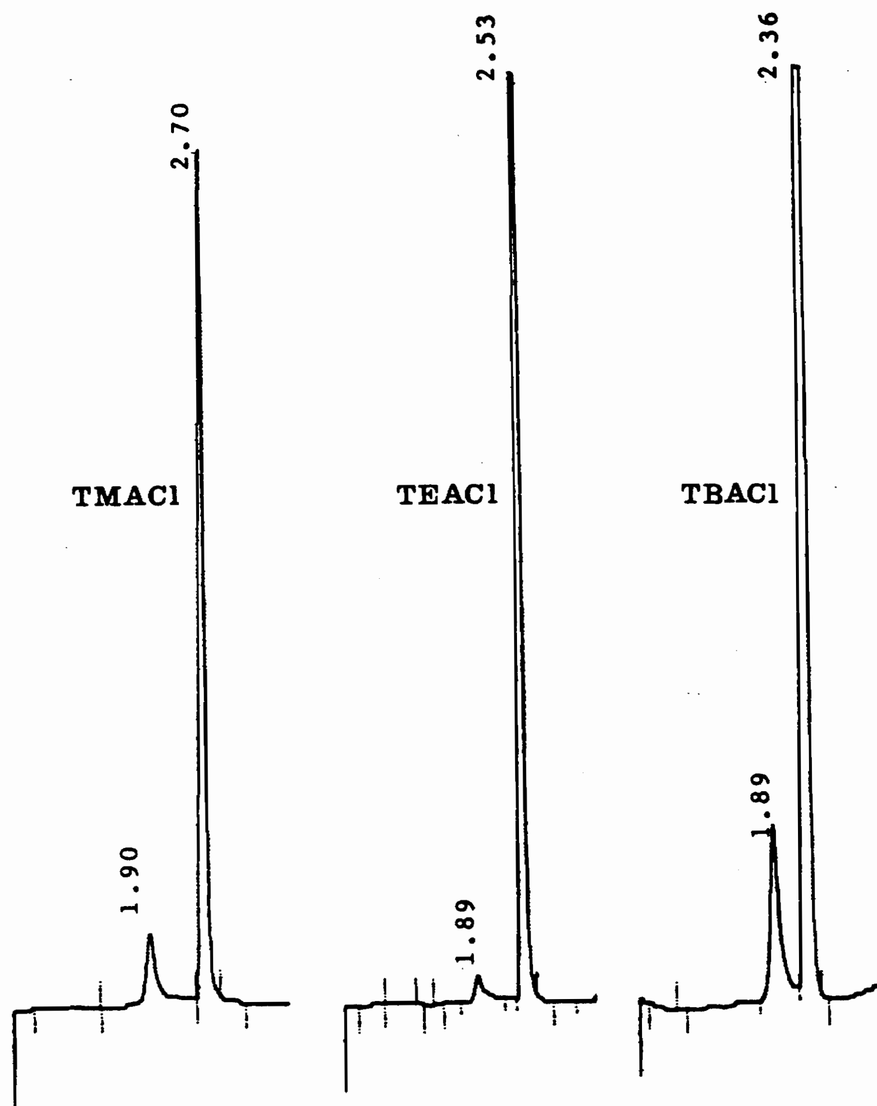


Figure 17. Separation of 4000 PPG Standard and Methanol with Different Salts.
TMACl: tetramethylammonium chloride
TEACl: tetraethylammonium chloride
TBACl: tetrabutylammonium chloride

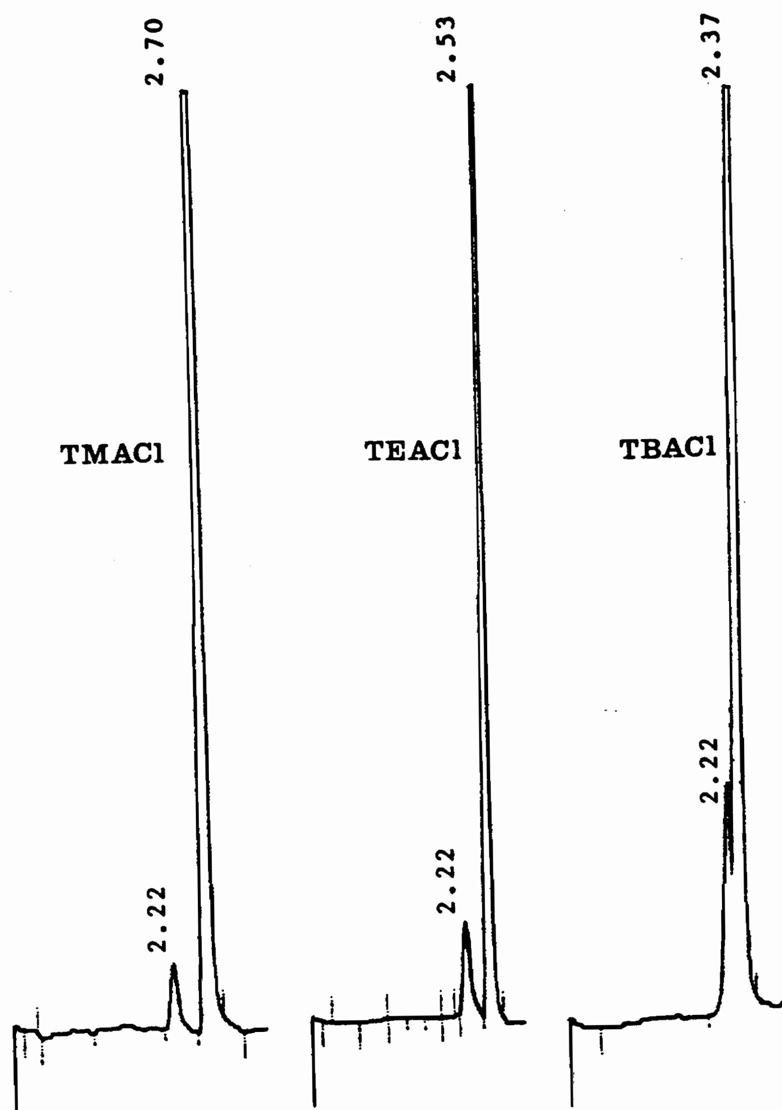


Figure 18. Separation of 1200 PPG Standard and Methanol with Different Salts.
TMACl: tetramethylammonium chloride
TEACl: tetraethylammonium chloride
TBACl: tetrabutylammonium chloride

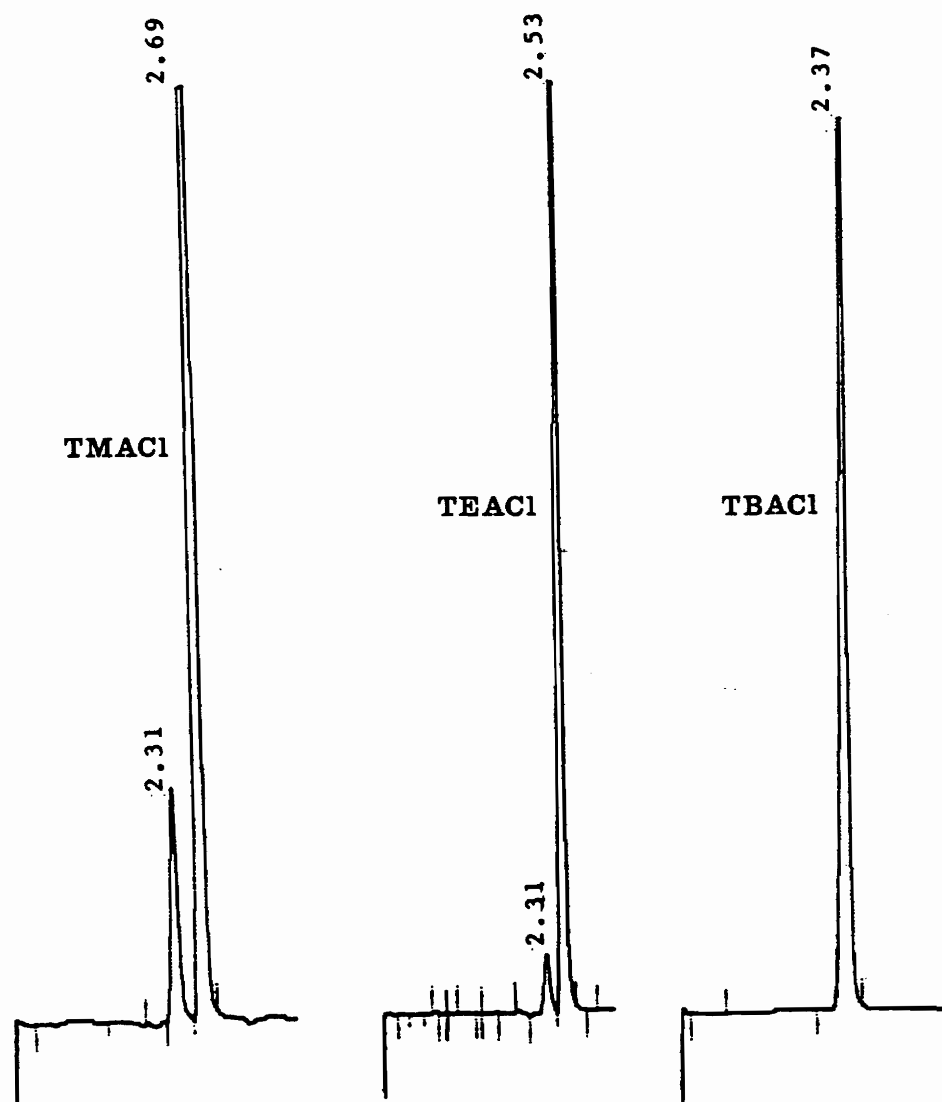


Figure 19. Separation of 800 PPG Standard and Methanol with Different Salts.
TMACl: tetramethylammonium chloride
TEACl: tetraethylammonium chloride
TBACl: tetrabutylammonium chloride

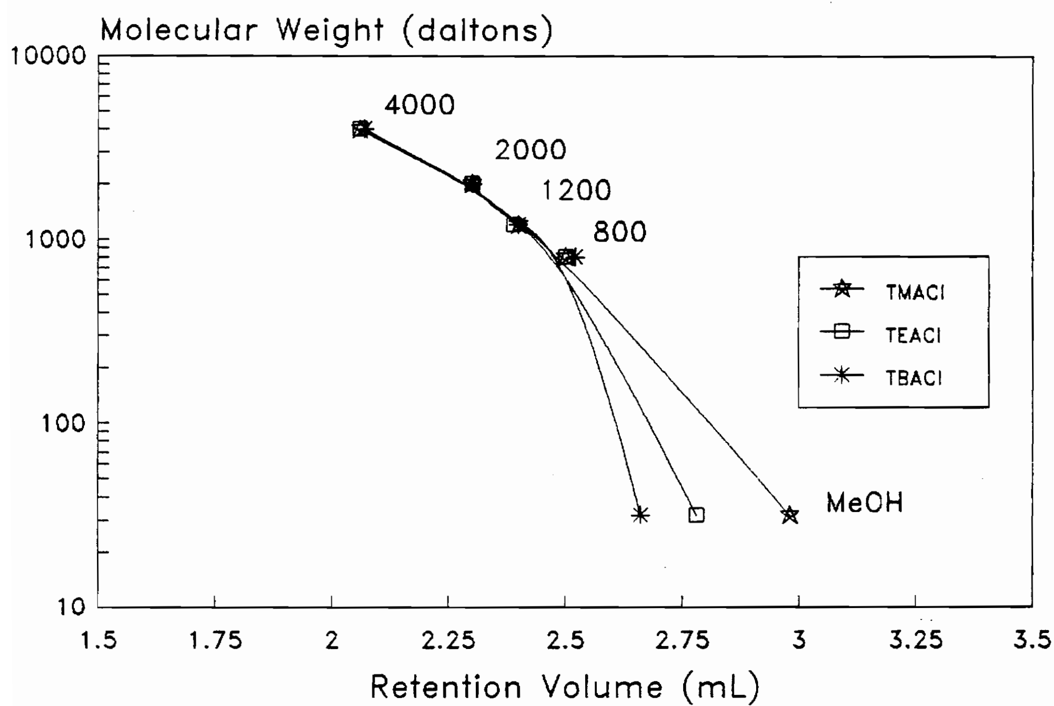


Figure 20. Effect of Different Salts on SEC Calibration Curves of Polypropylene Glycol Standards (4000, 2000, 1200, 800) and Methanol.
TMACl: tetramethylammonium Chloride
TEACl: tetraethylammonium Chloride
TBACl: tetrabutylammonium Chloride

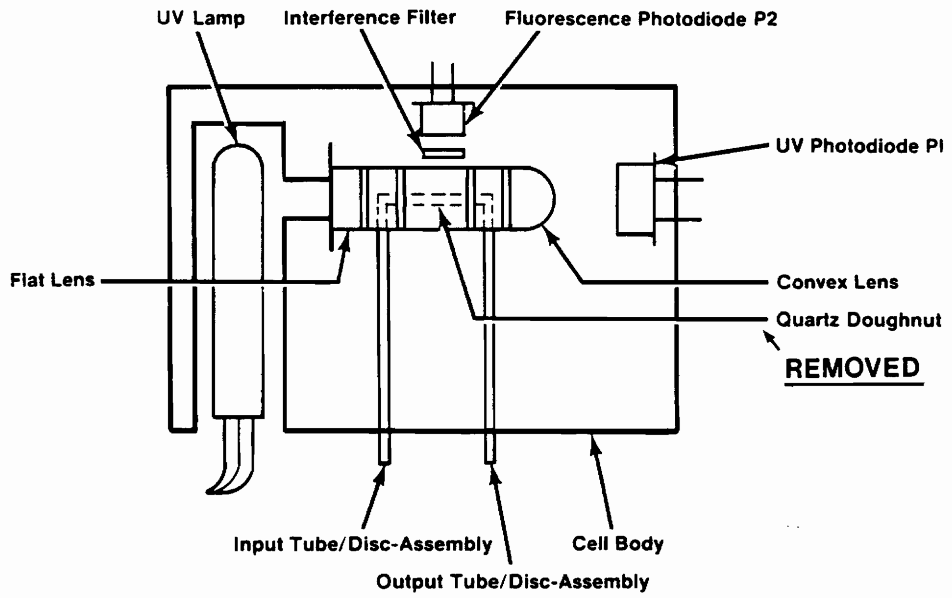


Figure 21. Detector Cell Schematic.

two electrodes (conductors) was of approximately 2 mm and the internal cell volume was reduced from 2.3 uL to 1.0 uL. This modification offers two advantages. First, a reduction in the cell volume and consequently a reduction in peak broadening which is always desirable in high performance chromatography. Second, an increase in the background conductivity which eventually produces a greater sensitivity. This last advantage is explained with the basic conductance equation:

$$G = k \cdot \frac{A}{L} \qquad (4)$$

where, G = conductance of the medium

k = specific conductance of the medium

A = cross-sectional area of the conductors and

L = separation of the conductors

From the equation, a reduction in the separation of the conductors would have the result of increasing the conductance of the mobile phase.

In order to validate this relationship a baseline or background profile was obtained with both cells and the same mobile phase solution. With the modified cell the zero of the detector needed to be readjusted to a new level.

Figure 22 shows the background or noise level of the detector with a mobile phase flow rate of 1.0 mL/min. and a 10 mM tetramethyl-ammonium chloride solution. The top profile A was obtained with the original cell design and the bottom profile B with the modified one, both at the same detector attenuation (4X). A less noisy baseline was

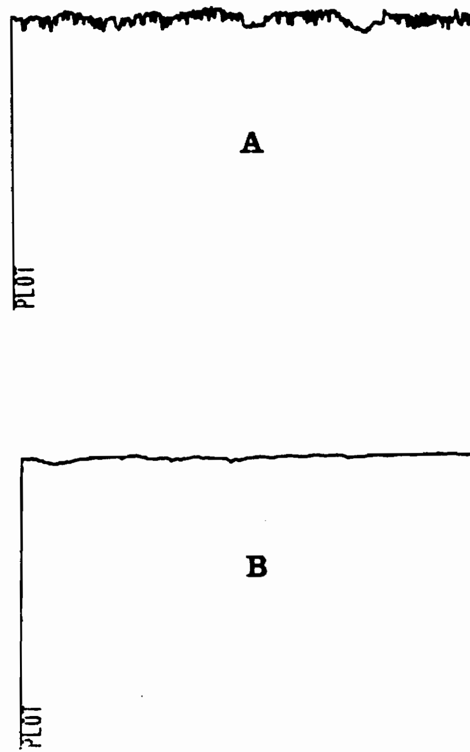


Figure 22. Noise Level Detector with the Original Cell (A) and the Modified Cell (B).

observed with the modified cell. The same mobile phase solution and equilibration time was used to obtained both baselines. In a second experiment, a polypropylene glycol (PPG) standard of 2000 molecular weight was injected and its response analyzed with both cells. Figure 23 illustrates the separation of the 2000 PPG standard (approximately 100 ug.) and methanol under identical conditions (attenuation 8X) using both cells. The top chromatogram A (original cell) shows a more noisy baseline than the bottom B (modified cell) as shown before. But surprisingly, the area of the PPG standard and methanol is 30% larger with the original cell than with the modified one. According to these results, the modified cell offers an improvement in noise level but a slight decrease in sensitivity. Figure 24 shows the chromatograms of the same 2000 molecular weight PPG standard and methanol. In this case, the detector attenuation when using the modified cell was reduced in order to produce a similar noise level as compared to the original cell. The net result was an improvement in the signal to noise ratio (S/N) with the cell modification. There was not effect in peak broadening with the cell modification.

These results can be explained in terms of the response time of the detector. By comparing the two detector cells, the original has the electrodes more separated than the modified one and consequently the 'residence time' of the solutes in the original cell is longer. Since, the response time is set by the detector electronics, with the modified cell the detector is actually observing a more 'averaged signal' because of the smaller solute residence time in the cell. This would explain the

Figure 23. Separation of 2000 PPG and Methanol (MeOH) at Detector Attenuation 8X with Original Cell (A) and Modified Cell (B).

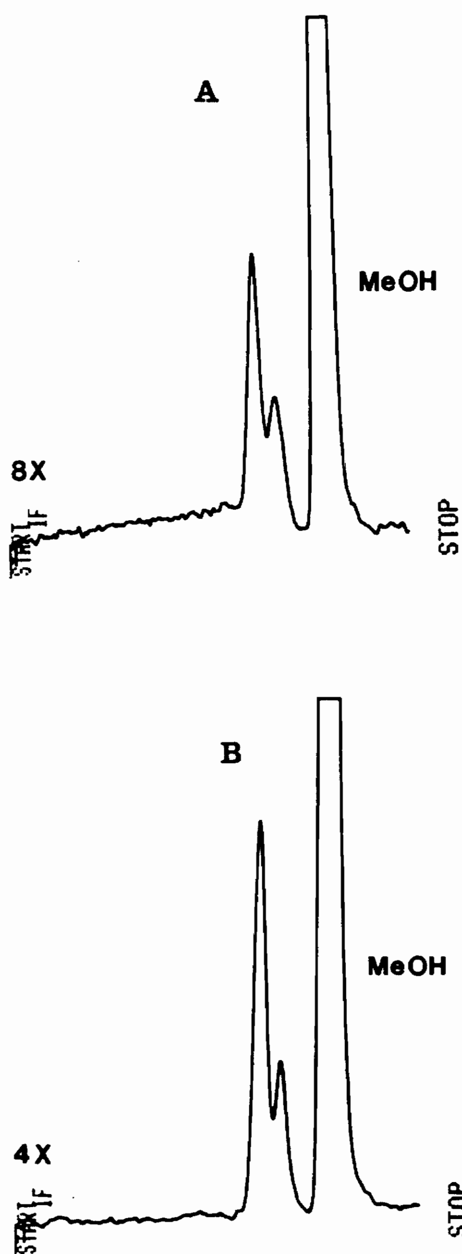


Figure 24. Separation of 2000 PPG and Methanol (MeOH) at Similar Noise Level.
A: Original Cell (Attenuation 8X)
B: Modified Cell (Attenuation 4X)

lower noise of the modified cell. For the original cell, the detector has enough time to observe any variation in mobile phase composition and more noise in background is generated. Thus, the observed increase in S/N with the modified cell is the result of a closer placement of the conductors.

CHAPTER IV

CONCLUSIONS

Indirect conductivity detection provides a new alternate detection method for size exclusion chromatography. The application of this detection mode in the separation of small molecules has been demonstrated.

The concentration of the probe added to the mobile phase affects the response, column efficiency and elution time; a concentration of 10 mM with tetramethylammonium chloride was found optimum in terms of response and column efficiency.

Flow rate did not influence resolution as usually expected in the separation of small molecules by SEC.

Selected conditions -stationary phase, mobile phase and conductivity probe- did not alter the size separation as shown in the calibration curves of different functional groups. However, the SEC curves did not lie in a single line, which may be explained by adsorption of the more polar groups, differences in hydrodynamic volumes and by solvation effects as well.

The response factors of the different functional groups increased with the molecular weight. This increase is more pronounced for the molar response factors.

The limits of detection for small molecules were found to be in the 20 nanoliter range, which is comparable to conventional refractive index detection.

The effect of the probe sizes on the elution time of methanol suggested a possible blockage of the silica gel micropores by the larger

probes. This pore size alteration by the mobile phase additive brings an important idea concerning a new way to possibly tailor the stationary pore size for more selective separations.

The detector cell construction permitted a reduction in the electrodes separation which enhanced the signal to noise level but did not affect the peak broadening; this modification is supported by the fundamental conductance equation.

Suggestions for further work are to use other solvents in combination with appropriate probes for the conductivity background to offer a wider applicability of the present method to other samples. Methanol has the disadvantage that a limited number of polymers are soluble in this solvent. Improvements in sensitivity could be achieved by increasing the temperature of the conductometric cell. The use of columns with different pore sizes (coupled in series) would extend the range of molecular weights which could be analyzed in complex mixtures. A comparison of the molecular weight distribution profiles and molecular weight averages for several standard samples could be developed between this detection method and refractive index; molecular weight comparisons would dictate which detector offers the most accurate analysis.

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