THE DETERMINATION OF PHENOLIC-TYPE COMPOUNDS IN WATER BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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THE DETERMINATION OF PHENOLIC-TYPE COMPOUNDS

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LIQUID CHROMATOGRAPHY

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PREFACE

The objective of this investigation was to develop a concentration and analysis procedure for phenolic materials in natural water. Evaluation of the resulting method was made with laboratory and real aquatic samples.

The investigation described should provide guidelines for future studies involving the analysis of organic materials in natural systems and will hopefully contribute to the advancement of environmental analysis.

This study was made possible by Dr. Louis P. Varga, who served as major adviser. I want to thank Dr. Varga for his guidance and friendship during my graduate work. Drs. E. J. Eisenbraun, T. C. Dorris, T. E. Moore, and Harold Zallen served on the advisory committee. Special appreciation goes to Dr. Eisenbraun for his assistance and advice during this investigation. Gratitude is also extended to Dr. Billy G. Hudson for equipment and analysis support. I wish to thank Wayne Adkins and Ewald Friedle for help with construction and maintenance of equipment. I also want to acknowledge the help extended by Allen Faust, Heinz Hall, and my fellow graduate students.

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INTRODUCTION

In recent years, increasing attention has been given to the problem of water pollution control in natural waters and waste water. Coinciding with this trend in more stringent pollution abatement is the need for analytical procedures which permit identification and quantification of phenolic-type contaminants in the milligrams-per-liter to micrograms-per-liter, or lower, concentration range.

Phenols, defined as hydroxy derivatives of benzene and its condensed nuclei,[1] are introduced into surface waters from a variety of sources. Industrial effluents, municipal disposal, and chemical spills constitute important sources of man-made origin for phenolic chemical pollutants. Stringent discharge limitations have been adopted by public agencies because phenols increase oxygen demand and cause unpleasant taste and odor in potable water supplies. Also, chlorination often produces substituted reaction products of greater taste and odor than the original substances. This process takes place when phenol-contaminated water is chlorinated in purification processes. The toxicity of phenols to fish and other aquatic organisms is also well known.[2]

Analytical studies of trace phenols have been deterred by their low concentration and the complexity of the organic constituents in aqueous media. Most methods require a minimum of one milligram of a

single compound for positive identification. Instrumental procedures which are most promising at present involve spectrophotometric and chromatographic techniques. These systems lack the sensitivity to analyze the organic directly at the level found in natural and waste waters. Preliminary concentration of phenols which does not alter the individual constituents or their relative distribution is essential.

This investigation includes an evaluation of existing procedures using prepared standards containing alkyl, chloro, amino, and naturally occurring phenols. Information obtained will be utilized in the development of an improved fluorescence technique employing high-pressure liquid chromatography.

CHAPTER II

PHENOL ANALYSIS

Pretreatment

A number of screening procedures have been developed to improve the applicability of original analytical methods to determine phenols. Domestic and industrial waste waters may contain such interferences as phenol-decomposing bacteria, oxidizing and reducing substances, and alkaline pH.[1] Some of the major interferences can be eliminated as follows:

Since oils and tars contain phenols, an alkaline extraction (pH 12-12.5) with carbon tetrachloride removes this form of interference.

Acidification, along with aeration, eliminates H_2S and SO_2.

Copper sulfate effectively preserves the phenols prior to analysis and alleviates any bacterial oxidation.

A distillation step prevents interference from color, turbidity, and metallic ions.

Concentration

The identification and quantification of phenolic compounds pose an analytical problem. Instrumental techniques are not capable of operation at levels normally found in natural waters and in industrial effluents. Concentration is necessary to increase phenolic content to minimum detectable levels, but this process must not alter the phenolic

molecular structure and must be quantitative. Procedures which have been used include activated carbon filtration and solvent desorption, liquid-liquid extraction, freeze concentration, distillation, ion exchange, or some combination of these.

Concentration of phenols from aqueous solution by carbon adsorption was introduced at the National Environmental Research Center, Cincinnati, Ohio.[3,4] The procedure is based on organic adsorption on granular activated carbon and desorption with suitable solvents. Phenol is quantitatively adsorbed, but desorption has been reported to be less than 70 percent complete.[5] Identification and measurement of individual phenols in a carbon chloroform extract (CCE) are extremely difficult because of large amounts of other organic materials which are desorbed from the carbon. Further, the high volatility of many phenols results in large losses when the CCE is evaporated to apparent dryness. A *Florisil* column cleanup preceded by a sodium hydroxide extraction of the CCE has been proposed as an efficient method of isolation for many phenols.[6] Thirty-seven phenols were investigated with satisfactory results for thirty-one of these (80 percent or higher recovery efficiency). The procedure has the potential for chemical and biological degradation of adsorbed phenols prior to extraction.

Liquid-liquid extraction is an efficient method for extracting certain classes of organic microcontaminants from water.[7] Although large scale liquid-liquid extraction procedures offer some improvement over carbon adsorption techniques,[5] separation of solvent from recovered organics poses a significant problem.

Microorganics in water may be concentrated by freezing.[8-10] The process involves the slow freezing of water with the first ice crystals

formed relatively pure and clear. Dissolved solids and organic solutes remain in the residual liquid portion. If the freezing process is interrupted prior to completion, the residual volume containing the concentrates may be collected for analytical purposes. The procedure does not induce obvious biological, chemical, or physical transformations inherent in the previously mentioned techniques. The efficiency of the freeze-out concentration technique is dependent on several factors including rate of freezing, ionic concentration of the sample, and particular organic constituents of the sample. Volumetric concentrations up to tenfold were quite efficient in preliminary tests. Experiments with phenol, m-cresol, and 2,4-dichlorophenol showed recovery in excess of 90 percent.[9] This technique has one serious disadvantage: it is usually performed on small volumes of water (400 mls) and is limited for concentrating trace organic compounds from large volumes of water. Application of the procedure to waste water analyses shows concentration by freezing to be a useful supplement to other analytical methods.[6]

The oldest and best known concentration technique is distillation. Vacuum, steam, and fractional distillation techniques are useful for concentrating relatively volatile compounds from an aqueous solution. This method has no practical value for concentration purposes but is excellent for purification since the rate of volatilization of the phenols is gradual and the volume of distillate must equal that of the sample being distilled.[1]

The use of ion exchange for trace analysis is essentially a mild and speedy method of concentration. It has been used successfully for measuring trace elements in plant material and in urine, for the anal-

ysis of natural waters, and for determining copper in milk.[11] If a substance is retained quantitatively by an ion-exchange resin, a considerable volume of solution may be passed through the resin until sufficient material has been collected. The required substance can then be stripped from the resin in a small volume of eluent. Phenols are bound more strongly than their weak ionization would suggest because of the "solvent" action of the resins.[12] They are absorbed from dilute aqueous solution to an amount considerably exceeding the ion-exchange capacity and are absorbed as neutral molecules as well as ions. Rieman,[13] utilizing a technique known as "elution solubilization chromatography," separated phenols by the addition of acetic acid to the aqueous eluent. This increases the solubilities and decreases the distribution ratios, thus making the separation possible. This separation is useful in recovering phenols from waste waters and in other industrial applications.

Analytical Methods

Presently, there are several methods for the determination of phenolic material in water. Of these, spectrophotometric and chromatographic techniques are the most promising.

Analysis in the visual range of the spectrum is the oldest and most widely used of the spectrophotometric procedures. One method embraces all hydroxybenzene materials which react with 4-aminoantipyrene to form a colorimetrically detectable complex.[1] This method, developed by Emerson,[14] is seriously limited by its insensitivity to para-substituted phenols, especially para-cresol in which the necessary coupling site is occupied.[15,16] Three forms of the procedure exist,

and one is sensitive enough for use in waters containing less than 1 mg/1.[1] Another modification of the test is used to detect para-substituted phenols.[2] Water samples containing mixtures of phenols are reacted with 4-aminoantipyrene and then passed through an ion-exchange column containing the hydroxide-form of Dowex 1-X8. The dye complexes pass through the column while the remainder is sorbed. Para-substituted phenols are eluted, diazonized, and determined spectrophotometrically. This method has many reaction interferences, and many phenols do not react.

Another widely used method described by Stoughton[17] and modified by Lykken[18] is based on the chemical treatment of extracted phenols to form nitrosophenols which are tautomerized by the addition of ammonium hydroxide to form the colored quinoid salts. The colored solutions which exhibit large intensity variations among positioned isomers are measured colorimetrically. The limitations of the method are obvious because of the complex nature of phenolic solutions.

An ultraviolet absorption method[19] has been developed for the determination of total phenolic materials. The procedure is characterized by a shift in the absorbance band system from the 270 nm to 280 nm region (bathochromic shift) to 292.5 nm after the addition of sodium hydroxide. Since the presence of small amounts of extraneous materials interfere with direct analysis, an aliquot of untreated sample is used as a reference material. Experimental evidence has shown that the method is non-selective, precise, and rapid. Increased sensitivity may be accomplished by extraction with tributylphosphate (ultraviolet transparency). Again, however, the method lacks specificity, and the extraction procedure is 80 percent effective. Martin, et al,[15]

developed a continuous monitoring system based on ultraviolet absorption (Figure 1).

Table I summarizes the spectrophotometric methods presently utilized for phenolic determinations. Infrared is also reported although its usefulness is now primarily that of a supplement to other techniques such as GLC.

Gas-liquid chromatography has become a valuable tool for the qualitative and quantitative determination of trace organics. Baker,[20] in the gas chromatographic analysis of phenols, employed a direct aqueous injection method utilizing a flame ionization detector. This type of detector is relatively insensitive to water. The technique is applicable to waste waters or to concentrates which contain more than 1 mg/l of phenolic compounds. However, the detection and measurement of phenols from the weak-acid fraction of a CCE is difficult because of the interfering substances in these fractions.[21] The technique is only useful when the phenol is a part of a relatively pure, simple mixture.

Kawahara[21-23] developed a rapid and sensitive method by converting the unidentified phenols to pentafluorobenzyl ethers. The compounds were separated and determined quantitatively by electron capture gas chromatography.

Coupled mass spectrometer-gas chromatograph instruments are receiving increased attention.[24] The mass spectrophotometer is used as the gas chromatographic detector and is comparable to the most sensitive GLC detectors.[25]

Increased interest has been shown for the determination of phenolic compounds by coulometric titration. This method is based on

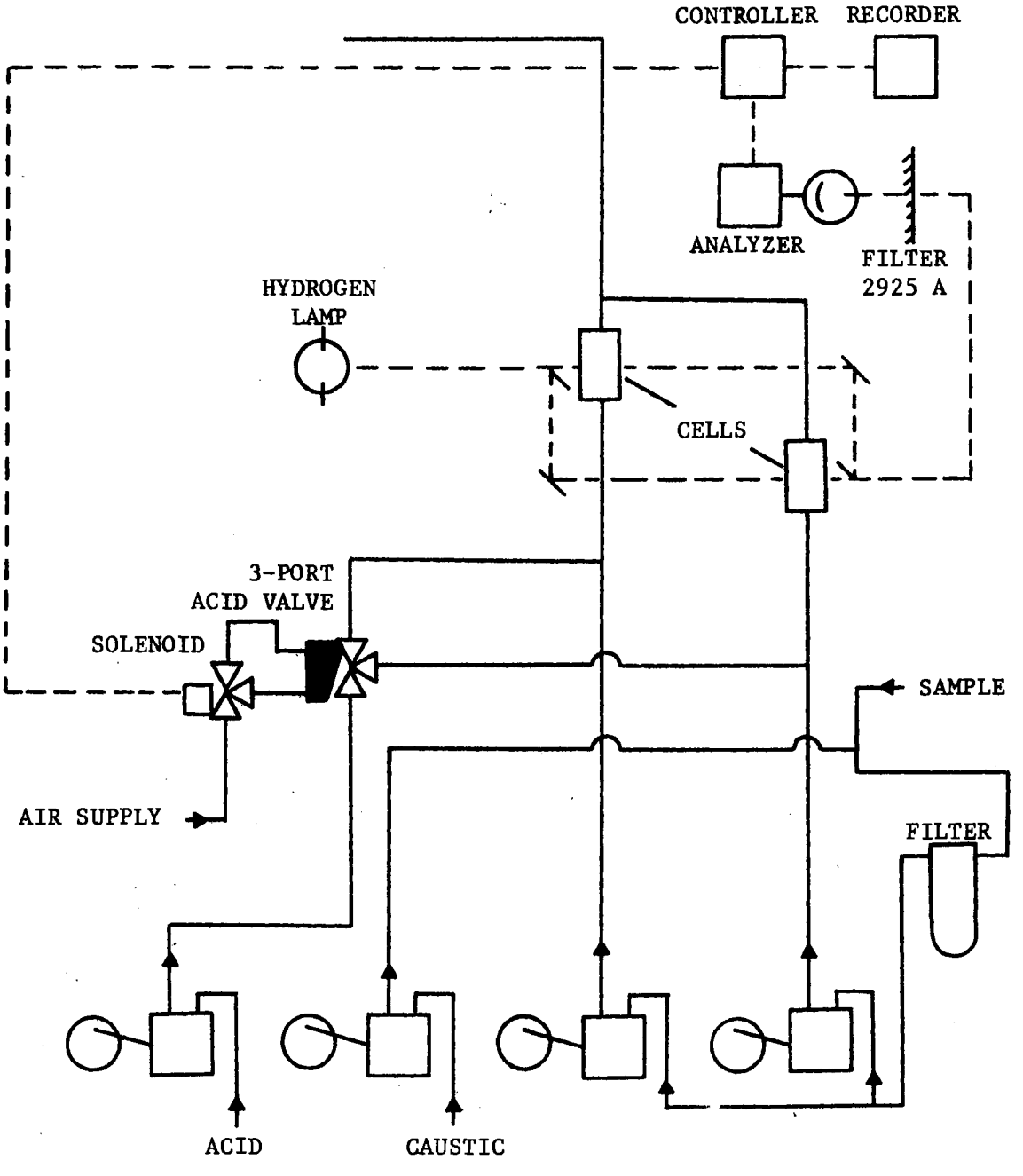


Figure 1. Phenols Analyzer Flow Scheme[16]

TABLE I

SUMMARY OF METHODS STUDIES FOR DETERMINATION OF PHENOLS IN WASTE WATERS[16]

	Gibbs	Nitrosophenol	4-Aminoantipyrine	Infrared	Ultraviolet
Analytical field	Colorimetry	Colorimetry	Colorimetry	Absorption spectrophotometry	Absorption spectrophotometry
Special equipment	None	None	None	Spectrographic equipment (72° lithium fluoride prism)	Standard ultraviolet spectrophotometer
Special reagents	Borate buffer, 2,6-dibromoquinone chlorimide	Acetic acid-potassium hydroxide reaction solvent	4-Aminoantipyrine potassium ferricyanide	None	Tri-n-butyl phosphate quaternary amine hydroxide
Concentration range	0 to 10 ppm, 0 to 100 ppb	0 to 10 ppm	0.1 to 2.0 ppm, 0 to 100 ppb	5 to 100 ppm; used for as low as 10 ppb, possible extension to 5 ppb	0.1 to 0.9 ppm, possible extension to ppb range
Interferences	Sulfides, reducing agents, thiocresol	Inorganic salts, water content, aniline, xylidine	Color, turbidity, sulfur compounds, metallic ions	Ortho-substituted and sterid-hindered phenols, organic acids	Dihydric phenols, phenols with butyl or larger group in 2 or 6 position
Precautions	Temperature, time	Temperature, time	pH, temperature, time, avoid use of stopcock grease	Add enough potassium bromide, avoid use of stopcock grease	Avoid use of stopcock grease
Time requirement	6 to 24 hours	4 to 24 hours	15 min. to 1 hour	Approx. 1 hour	1.5 to 2.5 hours
Precision repeatability	0.65 to 7.8 ppm: std. dev., 1.03; 95% confidence limits, 3.17	1.6 to 9.6 ppm: std. dev., 1.45; 95% confidence limits, 4.35	0.49 to 1.93 ppm: std. dev., 0.022; 95% confidence limits, 0.065 18 to 75 ppb: std. dev., 3.3; 95% confidence limits, 10.7	No data on waste water	0.119 to 0.485 ppm. std. dev., 0.008; 95% confidence limits, 0.028

TABLE I (continued)

	Gibbs	Nitrosophenol	4-Aminoantipyrine	Infrared	Ultraviolet
Reproducibility	1.0 to 5.6 ppm: std. dev., 1.85; 95% confidence limits, 6.73 7 to 78 ppb: std. dev., 6.47; 95% confidence limits, 23.54	No data	0.95 to 1.86 ppm: std. dev., 0.25; 95% confidence limits, 0.91	No data on waste water	No data
Accuracy	No data	No data	± 2% of true value	± 10% of true value (synthetic blends)	± 8% of true value
Advantages	None	p-Cresol is determined	Ease of manipulation Speed of analysis Reproducible standard curves Used by regulatory bodies Applicable over wide concn. range Stable reagents	Para-substituted phenols are determined Oil and phenol are determined with one procedure Brominated derivatives have absorption maxima at same wave length	Ortho-, meta-, and para-substituted phenols are determined Elimination of interferences by bathochromic shift Repeatability good
Disadvantages	Standard curves not repeatable Time p-Cresol does not react Instability and variability of reagent	Coolant needed Time Incomplete recovery of phenols by ether extraction Low results	Para-substituted phenols not determined Sensitivity varies with pH	Ortho-substituted hindered phenols are not determined Questionable recovery of brominated derivatives when extracted Need of special equipment Time	Only 80% phenol recovered Time

the reaction of phenol with bromine. Thielemann[26] studied the shortcomings of this method and reported on the materials which gave false tests (cresols, xylenols, and aromatic amines). Krause and Kratochvil[27] investigated this method in propylene carbonate in the presence of pyridine and found that phenols and amines could be determined with accuracies of 1%.

CHAPTER III

HIGH-PRESSURE LIQUID CHROMATOGRAPHY

Introduction

Liquid chromatography was the original form of chromatography developed by Tswett.[28] This method was extended by Martin and Synge[29] through the development of liquid-liquid partition chromatography in 1941. Paper,[30] thin-layer,[31] and gas[32,33] chromatographic methods were developed as a result of this work. The application of gas chromatography was an immediate success with rapid development of techniques and theoretical aspects of the methods. The theory and methods of liquid chromatography then remained static until 1963.[34]

Giddings[34,35] applied gas chromatographic theory with minor modifications to liquid chromatography. He showed that the performance of liquid chromatography could be greatly improved by attention to certain key features such as the interrelation between pressure drop, particle size, eluent velocity, and column efficiency. The theory indicated that liquid chromatography could rival gas chromatography in both speed and resolving power by using high pressures and very small particles.

Golay[36] and Purnell[37] first suggested that support materials composed of thin porous layers of adsorbent surrounding solid impenetrable cores (porous layer beads, PLB) could be useful as packing materials for rapid analysis. Halasz and Horvath[38,39] were the first to prove

the success of these materials in GC. Kirkland[40] showed how high speed separations could be achieved in liquid chromatography by using layered particles of 30 μm in diameter and pressures up to 3,000 psi.

Many of the new support materials used in high-pressure liquid chromatography are basically porous layer beads. These materials are *Zipax* (developed by Kirkland[40]), pellicular ion-exchange resin beads,[41,42] surfaced etched beads,[43] and *Corasil* I and II.[44] These beads are spherical in shape to give good packing density and are not deformed under pressure. Figure 2 is a diagrammatic representation of these materials.

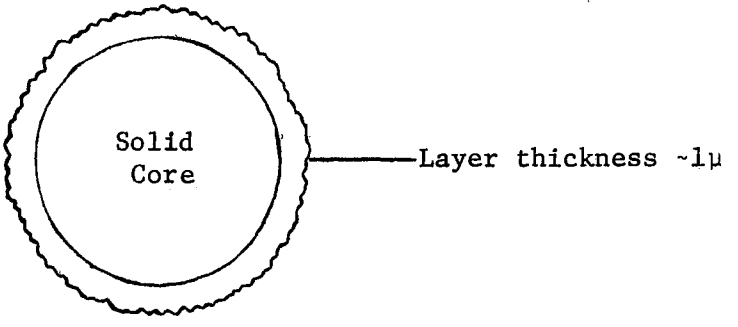


Figure 2. Diagrammatic Representation of a Porous Layer Bead. Particle Diameter ~40μ, Porous Shell ~1μ

Porous layer beads diminish the path length for diffusion in the stagnant mobile phase by eliminating the deep pores. Diffusion occurs only in the thin layer on the surface allowing rapid mass transfer. Rapid velocities can be achieved while equilibrium conditions are maintained.

Two procedures of high-pressure liquid chromatography are currently receiving significant attention. The methods involved are liquid-solid (adsorption) and liquid-liquid (partition) chromatography.

Liquid-Solid Chromatography

Liquid-solid chromatography (LSC) is potentially useful for high-pressure separations but until recently has found limited practical application. This limited use was due to the success of thin-layer chromatography (TLC) which can be used for the same types of samples separated by LSC columns. LSC will eventually become as widely used as TLC due to the advantages of LSC such as greater speed and separation efficiency.

Separations by LSC are usually performed on polar adsorbents. The primary factor in determining the relative adsorption of a sample molecule (k') is its functional groups. Relative adsorption increases as polarity and number of functional groups increase. Uniqueness of retention and selectivity in LSC arises from two characteristic features of adsorption from solution:

1. Competition between sample and solvent molecules for a place on the adsorbent surface.
2. Multiple interactions between functional groups on the sample molecule and corresponding rigidly fixed site on the adsorbent surface.

LSC normally shows little selectivity among homologs (no molecular weight selectivity). There is a pronounced tendency toward compound type selectivity which leads to the possibility of compound type separation. Analysis of lipids[45] and petroleum[46] exemplifies this type of separation (Table II).

TABLE II

COMPOUND TYPE SEPARATIONS BY LSC OF LIPIDS OF PETROLEUM

A. Lipids	
Hydrocarbons	
Cholesterol esters	($R-CO_2-$)
Triglycerides	(three $R-CO_2-$ groups)
Free sterols	(-OH)
Diglycerides	(two $R-CO_2-$ groups plus -OH)
Monoglycerides	($R-CO_2-$ plus two -OH groups)
Free fatty acids	(-COOH)
B. Petroleum	
Saturated hydrocarbons	
Monoaromatics (alkyl benzenes, thiophenes)	
Diaromatics (alkyl naphthalenes, benzothiophenes)	
Triaromatics (alkyl phenanthrenes, anthracenes, etc.)	
Tetraaromatics	
Polar heterocompounds (phenols, carbazoles, etc.)	

Liquid-Liquid Chromatography

Liquid-liquid chromatography (LLC, partition) has been a powerful technique for high-resolution separations. Theoretical insight, specialized column packings, sensitive detectors, and reproducible pumping systems have all been combined to make high-pressure LLC a practical means of separation.

The LLC column consists of a bed of finely divided solid (support), usually inert, on which a stationary partitioning phase is fixed. The mobile phase flowing through the column is in contact over a large interface with the stationary phase. Equilibrium distribution of the

solute between the phases rapidly takes place. The primary mode of operation is elution chromatography where the sample components migrate down the column with different velocities.

LLC is one of the most versatile forms of chromatography. Versatility is based on the large selection of partitioning phases which may be used to accomplish separation. Unique chemical combinations may be used to perform certain separations which are difficult by other techniques. LLC can be applied to a wide variety of sample types ranging from polar to nonpolar.

Stationary phases are polar materials and mobile phases considerably less polar in the most widely used form of LLC. This arrangement is used for separating polar compounds which are preferentially retained in polar stationary phases. Many polar compounds have been separated in this manner.[47-56]

The separation of nonpolar compounds requires that the polarity of phases be reversed. Nonpolar solutes may be effectively separated by using nonpolar stationary phases (either liquid or bonded) with polar carriers (often aqueous methanol). This form of reversed-phase LLC can be extended to more polar solutes by increasing the concentration of water in the carrier. Applications of this procedure have been demonstrated by Kirkland.[50]

Conventional LLC has a significant procedural limitation. Solubility of the stationary phase in the mobile phase requires presaturation with the stationary liquid to avoid gradual stripping. Also, the high flow rates generate forces which tend to remove the stationary phase from the support. Column packings with bonded stationary phases have been developed to overcome these disadvantages.

The esterification of siliceous supports with a monomolecular organic layer of alcohols produces chromatographic packings with a chemically bonded stationary phase. Esterification of porous glass, *Porasil* C, with alcohols has resulted in phases called "brushes." These materials are available as *Durapak* from Waters Associates. Separations with these materials probably do not involve liquid-liquid interactions but adsorption chromatography at the surface of an organic modifier. These materials give excellent resolution but have poor hydrolytic and thermal stability.

Octadecyl (ODS) *Permaphase* is a permanently bonded silicone based on the work of Kirkland.[50] This stationary phase has the advantage of uniform coating on a solid support and thermal stability in a wide variety of mobile phases. The main use of this material is in reverse-phase situations because of the hydrocarbon nature of the material. *Permaphase* ETH is a more polar form of this material (bonded ether). Most of the previously mentioned separations utilized these two materials.

CHAPTER IV

RELATIONSHIP OF THEORY TO PRACTICE IN HIGH-PRESSURE LIQUID CHROMATOGRAPHY

Introduction

Chromatography involves separation due to differences in the equilibrium distribution of sample components between two immiscible phases. One phase is a moving or mobile phase and the other is a stationary phase. The sample components migrate through the chromatographic system only when they are in the mobile phase. Separation results from different velocities as a consequence of differences in equilibrium distribution. Although the chromatographic process is very complex, it is possible to devise simple models and equations which closely resemble chromatographic separation. The following theoretical development was taken from Giddings.[34,35]

Retention

Symmetrical Gaussian peaks are obtained as the components elute from the column if sample sizes are sufficiently small. It is possible to relate the time of elution of the peak maximum to the equilibrium distribution coefficient. This time is called the retention time (t_R) as illustrated in Figure 3. The assumption is made that the average solute molecule maintains distribution equilibrium in its travel down the chromatographic column. The retention time is independent of the

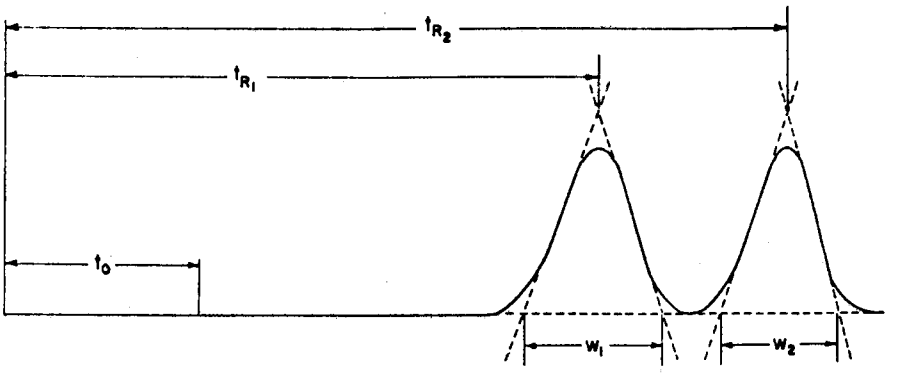


Figure 3. Chromatogram illustrating the definitions of retention time, t_R; nonsorbed time, t_o; and band width, w

amount of sample injected when a symmetrical peak is obtained and the distribution isotherm is linear. This method is called linear elution chromatography.

The time for elution of a nonretained component (t_o) is illustrated in Figure 3. This parameter is the total volume of the mobile phase in the column (dead volume) if extra-column volumes are negligible. Conversion to a fundamental parameter, retention volume (V_R), is easily accomplished by multiplying the retention time by the mobile-phase flow rate.

The fundamental relationship between retention and equilibrium, column length, and mobile phase velocity is given by Equation 1,

$$t_R = \frac{L}{V}(1 + k'), \tag{1}$$

where $\frac{L}{V} = t_o$

and $k' = \frac{t_R - t_o}{t_o} = \frac{\text{amount of solute in stationary phase}}{\text{amount of solute in mobile phase}} = \text{capacity factor.}$

Resolution

The achievement of high performance in chromatography requires an understanding of how resolution can be controlled and improved. Resolution (R_s) is the quantification of the degree of separation. Two characteristics determine the degree of band overlap--the distance between peak maxima and widths of the bands. A reasonable measure of resolution is the following:

$$R_s = 2\left(\frac{t_{R_1} - t_{R_2}}{W_1 + W_2}\right) \qquad (2)$$

The larger the value of the resolution the better the bands are separated. The only assumption made in Equation 2 is that the peaks are symmetrical. Peak width for Gaussian bands is equal to 4σ where σ is the standard deviation of the Gaussian. Assuming equal peak widths, Equation 2 becomes:

$$R_s = \frac{t_{R_2} - t_{R_1}}{4\sigma} \qquad (3)$$

When R_s equals 1, the peak to peak separation is equal to 4σ. This is called a 4σ separation. For a two component system this would correspond to a 2% overlap. When R_s is less than 1, the bands overlap more. The separation is unsatisfactory when R_s is less than 0.8.

Probably the most important equation in chromatography relates resolution and three fundamental chromatographic parameters. Assuming closely spaced peaks, this equation is:

$$R_s = \tfrac{1}{4}\left(\frac{\alpha-1}{\alpha}\right)\left(\frac{k_2'}{1+k_2'}\right)(N_2)^{\frac{1}{2}}, \qquad (4)$$

where α = relative retention $= \dfrac{k_2'}{k_1'} = \dfrac{t_{R_2} - t_o}{t_{R_1} - t_o}$,

and N = number of theoretical plates (subscript 2 denotes second component).

In effect, α relates to the peak to peak separation of the two components and is a measure of the thermodynamic differences in their distributions:

$$\Delta(\Delta G) = -RT \ln \alpha \qquad (5)$$

where $\Delta(\Delta G)$ is the difference in free energies of distribution for the two components.

The second parameter in the resolution equation is N, the number of theoretical plates:

$$N = 16 \left(\frac{t_R}{W}\right)^2 = \left(\frac{t_R}{\sigma_t}\right)^2 \qquad (6)$$

where σ equals the standard deviation of the Gaussian function in time units. The number of theoretical plates is related to the relative band broadening in the chromatographic column and to the column efficiency. The resolution equation is simplified by introducing the number of effective theoretical plates:

$$N_{eff} = \left(\frac{k'}{1+k'}\right)^2 (N) = 16\left(\frac{t_R - t_o}{W}\right)^2 = \left(\frac{t_R - t_o}{\sigma_t}\right)^2 \qquad (7)$$

The number of effective plates gives the true separating efficiency of the column and substitution into the resolution equation gives

$$R_s = \tfrac{1}{4}\left(\frac{\alpha-1}{\alpha}\right) (N_{eff})^{\frac{1}{2}} \qquad (8)$$

This equation relates resolution to two approximately independent factors. The functionality of α versus N_{eff} can be plotted assuming the resolution equals 1 (Figure 4).

When α is small, a large number of theoretical plates are needed. When α increases the number of theoretical plates required decrease. The slope of the curve reflects the fact that a small change in α when α is close to 1 results in a large decrease in the N_{eff} required.

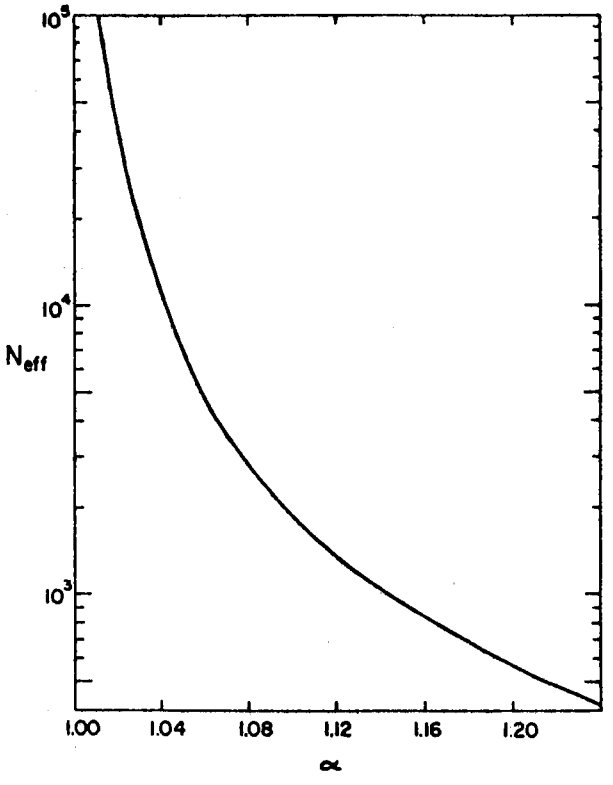


Figure 4. Plot of Log N_{eff} Required For Achievement of a Resolution = 1 as a Function of Relative Retention, α

Since N_{eff} is approximately proportional to time, larger α values mean a shorter separation time.

Height Equivalent to a Theoretical Plate

Optimum separation is achieved when adequate resolution occurs in the minimum amount of time. Incomplete resolution may be improved either by making individual peaks narrower without changing their relative retention times, or by increasing peak separation without changing peak widths. An increase in separation without changing peak widths requires a change in the distribution coefficient ratios of the

substances. Alterations in relative retention can be obtained only by changing the stationary phase and/or the mobile phase. It is therefore vital to examine how resolution can be improved by making peaks narrower.

There are two sources of band spreading in any form of chromatography. One is slow axial diffusion and the other is the disturbance of equilibrium caused when one fluid flows over another fluid or over a solid. The second source of spreading is important in currently practiced liquid chromatography. Three distinct regimes within any column are important in flow-generated dispersion:

1. Stationary phase which is the support or permanently held by the support.
2. Stagnant zone of the mobile phase which fills the interstices of the support.
3. Free flowing mobile phase in the interparticle space.

A spectrum of fluid velocities exists both across the column and along the column within the flowing part. Band spreading is produced by velocity variations within and between these interstitial regions. Variations can be countered by molecular diffusion across the direction of flow and by the tortuous nature of the flow itself. No velocity, however extreme, is maintained for more than a few particle diameters downstream from any point in the column. Dispersion is reported in terms of a quantity called the "height equivalent to a theoretical plate" or plate height (H). This term is simply expressed as the mean downstream equilibration distance within the column. Height is defined as:

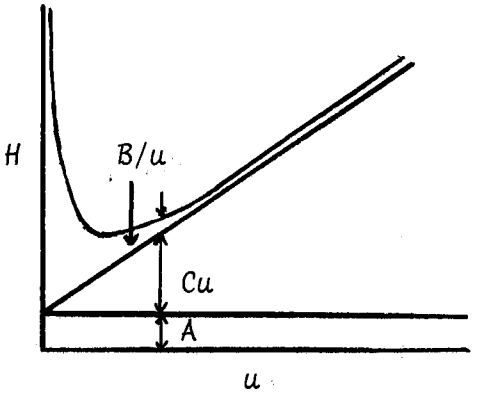
$$H = \frac{L}{N} = \sigma_L{}^2/L \qquad (9)$$

where L = length of the column and σ_L is the standard deviation of the Gaussian function in length units.

When flow variations are countered only by lateral diffusion the equilibration time is constant and the plate height is proportional to the fluid velocity, u. If dispersion is countered only by tortuous nature of the flow, fluid velocity will have no effect on plate height because equilibration distance is governed by geometry of the column. In practice both counter-dispersion mechanisms are important and the dependence of H on u is found to follow a fractional power of u over two magnitudes of u,

$$H = Cu^n \text{ where } 0<n<1 \qquad (10)$$

Generally, a low exponent is desirable. At lower velocities than commonly used in high-pressure liquid chromatography, longitudinal diffusion becomes important. This dispersion produced increases with the time of retention in the column. Figure 5 is a schematic plot of van Deemter's equation with arbitrary values of important parameters.



$H = A + B/u + Cu$

B/u = longitudinal molecular diffusion term

Cu = nonequilibrium term

A = eddy-diffusion term

Figure 5. Schematic Representation of the van Deemter Equation

This non-equilibrium theory of dispersion explains peak broadening in terms of a balance between the dispersive effects of flow variations across the column and the countering effects of the tortuous downstream flow and transverse diffusion. Since these effects depend on particle diameter, dp, better resolution should be obtained with smaller particles. Improvement does not always materialize because of packing difficulties. Also, the driving pressure required for comparable elution speed increases as $1/dp^2$. Therefore, particle diameter does not have to be reduced much before the maximum pressure capability of most equipment is exceeded.

CHAPTER V

APPARATUS AND MATERIALS FOR HIGH-PRESSURE LIQUID CHROMATOGRAPHY

Introduction

The primary concern when constructing a liquid chromatographic system is the type of liquid chromatography to be done. It is difficult to design a system which will simultaneously yield optimum conditions for steric exclusion, large-scale preparative, and high-pressure analytical chromatography. The system utilized in this study was designed and constructed to perform high-pressure analytical chromatography. A block diagram is shown in Figure 6.

Mobile Phase

Separations by liquid chromatography depend on the column and the solvent. The solvent plays a critical role in maximizing the performance of liquid chromatography and the selection of the right solvent system for a given application is therefore important. Practical requirements of the solvent include:

1. column stability,
2. detector compatibility,
3. adequate solvency for the sample, and
4. noninterference with sample recovery.

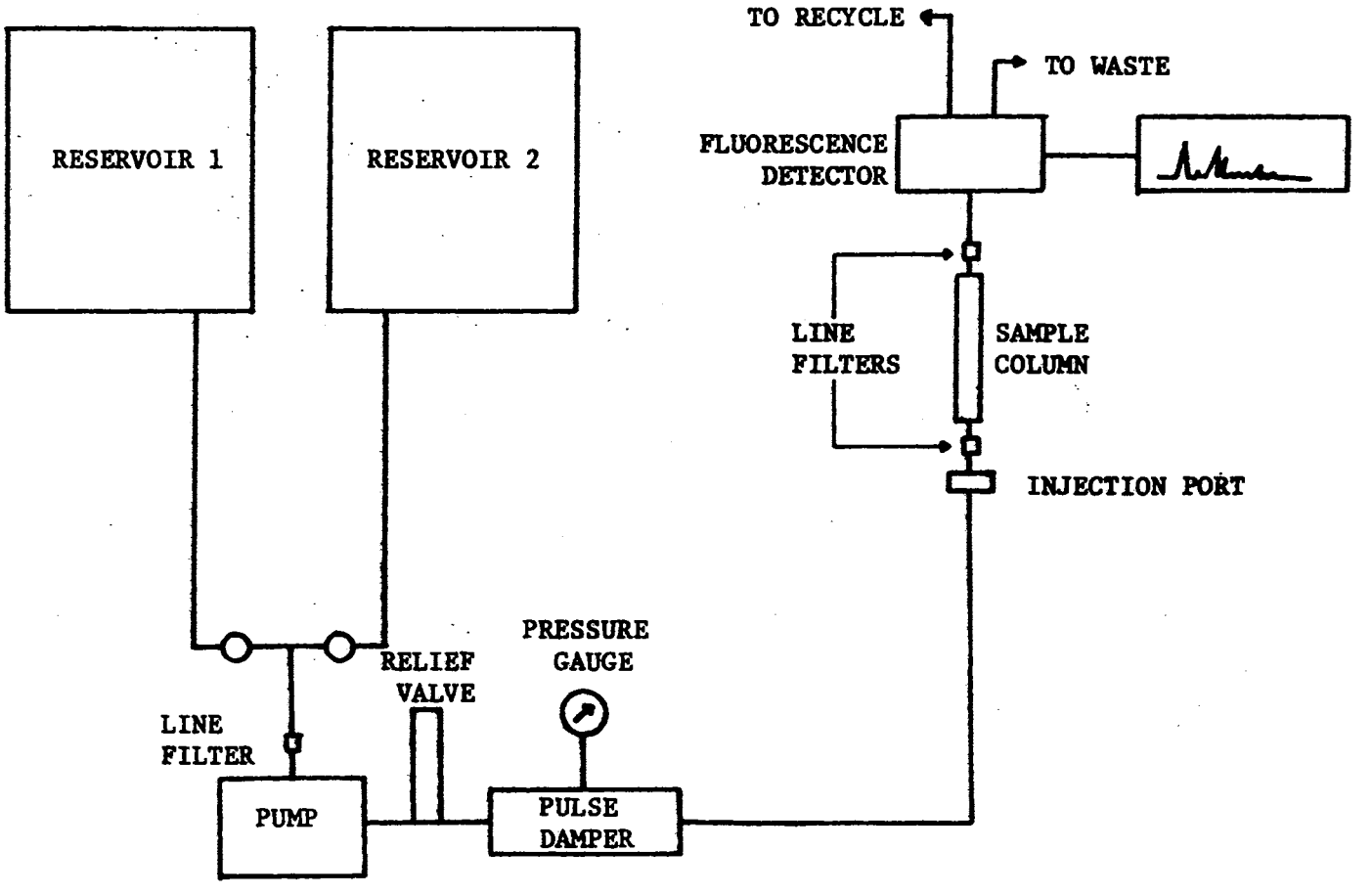


Figure 6. Block Diagram of Liquid Chromatographic System

The solvents used were n-hexane, cyclohexane, ethanol, methanol, acetonitrile, and water. The first two solvents were used as the mobile phase for the partition separation whereas the last four were used for the reverse-phase separation.

Solvent Reservoirs

Solvent reservoirs were constructed of glass with volume capacities of 300 and 1000 milliliters. Flexible tygon tubing was used to connect the reservoirs to the pump, and stopcocks were utilized for solvent selection. Reservoirs were positioned above the pump to flood the suction of the pump.

Pumping System

One common type of mechanical pump is the reciprocating piston (Milton-Roy Company, St. Petersburg, Florida) which delivers a pulsating supply of liquid. Specific advantages of this pump are that internal volume is small and delivery is continuous so that there is virtually no restriction on reservoir size. Another advantage is that this pump delivers a constant volume regardless of small variations in pressure drop across the rest of the system. A relief valve or rupture disc must be included with this type of pump to protect components from damage if a blockage occurs in the system. Several commercial instruments incorporate this pump design (Waters Associates, Framingham, Massachusetts; Varian Aerograph, Walnut Creek, California).

Line Filters

Line filters should be used to prevent particulate matter from

clogging the chromatographic system. Filter porosity should be small enough to collect harmful particles, yet large enough so that the pressure drop across the filter element is negligible. A pore size of 10μ is a good compromise.

Small-pore filters were obtained from Waters Associates (Framingham, Massachusetts) as part of their reducing union assembly. Three filters were incorporated into the system between the reservoir and pump, injection port and column, and column and capillary detector line.

Damping Device

A damping device was used to eliminate pulsations caused by the reciprocating pump and to reduce noise and variations due to the pulsating mobile phase. Approximately 90% of the pulse was damped by incorporation of a restrictor (3' x .01" i.d. line), capacitor (1/8" *Swagelok* tee), and restrictor (20' x .01" i.d. line). The latter restrictor was wound into a 4 cm diameter coil and hung free so that pulses were absorbed by the flexing of the coil. The remainder of the pulse was eliminated by incorporation of a pressure gauge at the capacitor tee.

Injection Port

The injection port was designed to allow on-column as well as swept injection. The sample in on-column injection is directly deposited by the syringe which extends through the septum and into the column packing. A diagram of the injection port is shown in Figure 7. A troublesome feature of on-column injection is that syringes tend to plug frequently because the diameter of the column-packing material is

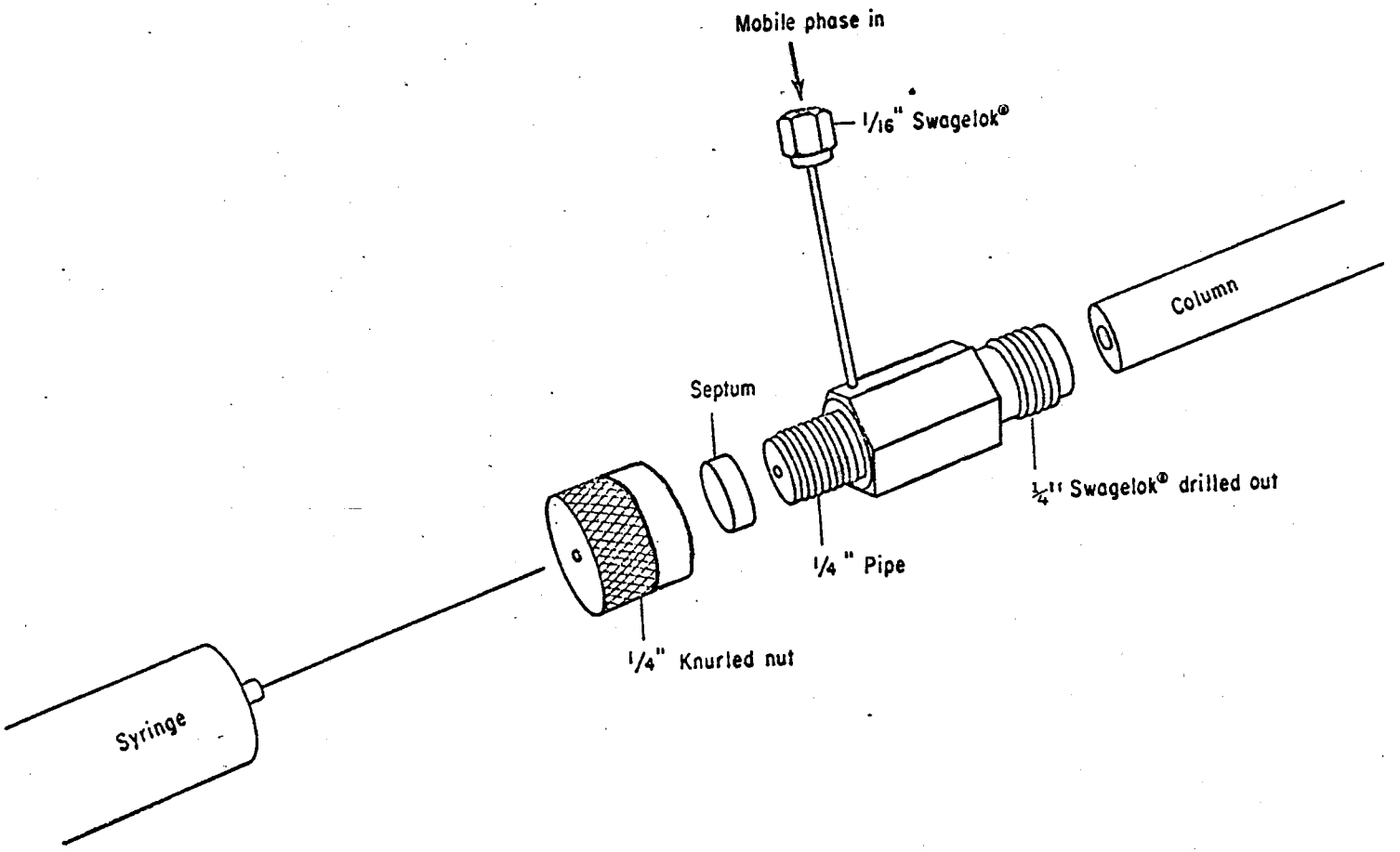


Figure 7. Injection Port for High-Pressure Liquid Chromatograph (Modified after Kirkland[48])

small and packing may be forced into the syringe needle. The sample in "swept injection"[50] is deposited just before the column inlet and carried into the packing by the mobile phase. Figure 8 illustrates the swept injection design utilized to adapt the injection port for one-eighth inch diameter columns. Band spreading was minimized by packing the adapter with silanized glass wool. Septum material utilized in the injection port was BUNA-N (Waters Associates, Framingham, Massachusetts).

Stop-flow and on-stream injection was carried out with both types of injection port. Efficiency was found to be identical in both cases and was probably due to the slow rate of diffusion in a liquid mobile phase.

Columns

The type of tubing used to construct high-pressure liquid chromatographic columns greatly affects their efficiency. Columns in this study were constructed of precision-bore stainless steel and precision-bore capillary glass tubing. Columns made of precision-bore stainless steel result in a more homogeneous dry-packed column. Glass columns were found to be useful only when using large diameter (>75μ) materials due to the difficulty in column plugging.

Packing Materials

The primary material used in this study was a support with chemically bonded silicone polymers. Column packings with chemically bonded, nonextractable, thermally and hydrolytically stable polymeric

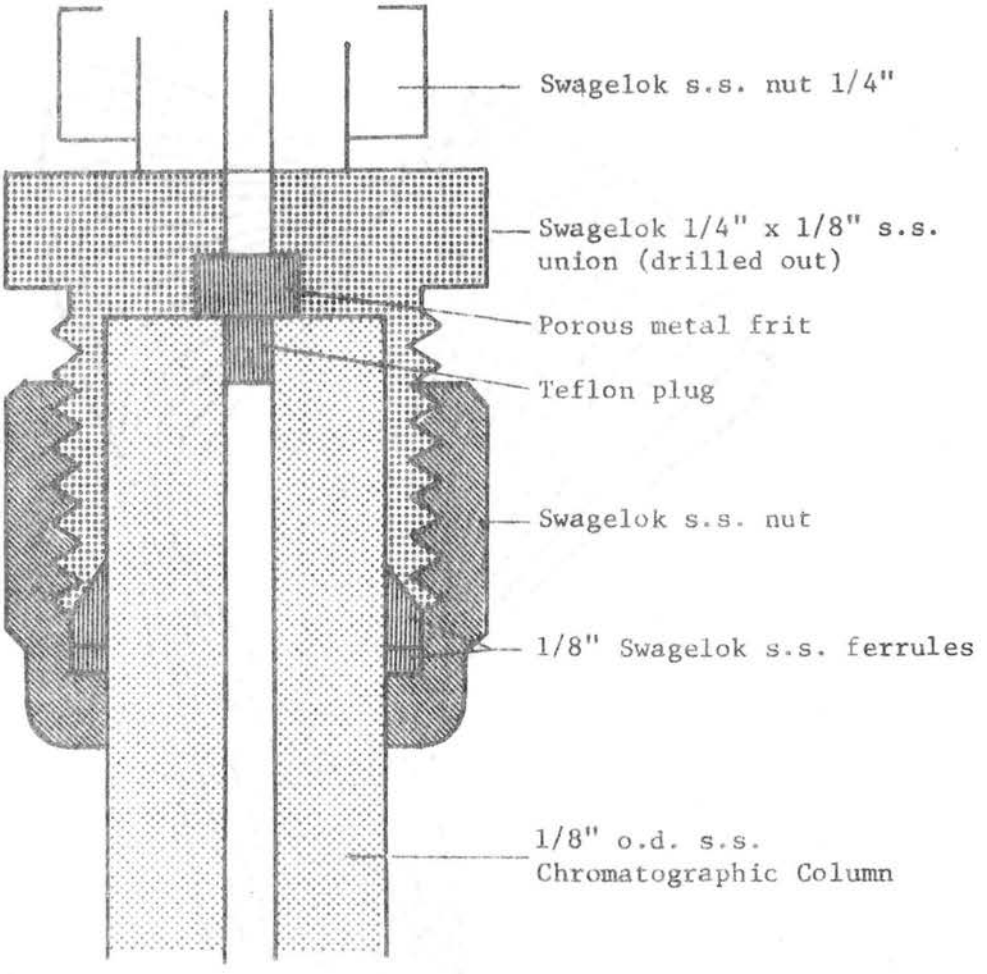


Figure 8. Injection Port Adapter

silicone stationary phases have shown important liquid chromatographic applications.

Permaphase ETH (E. I. du Pont de Nemours and Company) is one form of this bonded-phase packing. This material was prepared by reacting silane reagents with the surface of the porous shell of *Zipax* support and then polymerizing the reagents to give the desired coating. The unique surface of this material which contains a highly polar ether group provided unusual selectivities. Other materials used are listed in Table III.

Column Loading

Since the column is the heart of the chromatographic system, maximum efficiency must be built into the column in its preparation. Column materials were loaded into the column depending on the rigidity of the packing. The rigid materials (*Porasil*, *SAX*, *Permaphase*) were dry-packed into straight columns. A porous dise of sintered *Teflon* was forced into the internal diameter of the outlet to retain the packing. Small amounts (100-200 mg) of packing were then introduced into the top of the vertical column. The tubing was tapped vertically on the bench top and the side of the tube tapped (2-3 times a second) at the level of the packing. Addition and tapping was repeated until the column was full. The inlet of the column was then sealed by forcing in another *Teflon* plug.

The cation exchange resins and *Porapak* were packed by the slurry technique. A suspension was forced to flow into the column at a rapid rate by applying suction to the outlet of the column. Glass wool plugs held the packing material in place.

TABLE III

SUPPORT MATERIALS FOR LIQUID-LIQUID AND LIQUID-SOLID CHROMATOGRAPHY

Name	Type	Supplier
Porasil A, C, D, E, F (difference is pore size)	Porous silica beads	Waters Associates
Strong Anion Exchange (*SAX*)	Polymer-coated *Zipax*	E. I. du Pont de Nemours and Company
Dowex 50W-X12	Strongly acidic cation exchanger	Dow Chemical Company
Amberlite CG-120	Strongly acidic cation exchanger	Rohm and Haas Company
Porapak Q	Porous polymer beads	Waters Associates
Zipax	Controlled surface porosity on solid core beads	E. I. du Pont de Nemours and Company

Detector

A detector is used in liquid chromatography to continuously monitor concentration of the solute in the mobile phase as it leaves the column. Two types of detectors are commonly used in liquid chromatography. Bulk property detectors (Refractive Index) measure the overall physical property of the mobile phase. The solute property detector is sensitive to physical properties of the solute.

The fluorescence detector is sensitive to physical properties of the solute and measures the fluorescent energy from a solute which has been excited by UV radiation. Spectral distribution of fluorescence radiation is a physical and absolute characteristic of a given substance which is useful for qualitative considerations. Emission intensity of fluorescence at a given wavelength is useful for quantitative analysis with a given instrument after careful standardization. Theoretical aspects of fluorescence are further discussed in Appendix A. The instrument used in the present study was a Farrand spectrofluorometer and is further discussed in Appendix B.

The flow-through cell used in the detector was custom designed for the Farrand spectrofluorometer (Figure 9). The cell has an approximate volume of 30 microliters with a 5 mm path length. The cell was made of Spectrosil quartz (Q) by Markson Science, Del Mar, California.

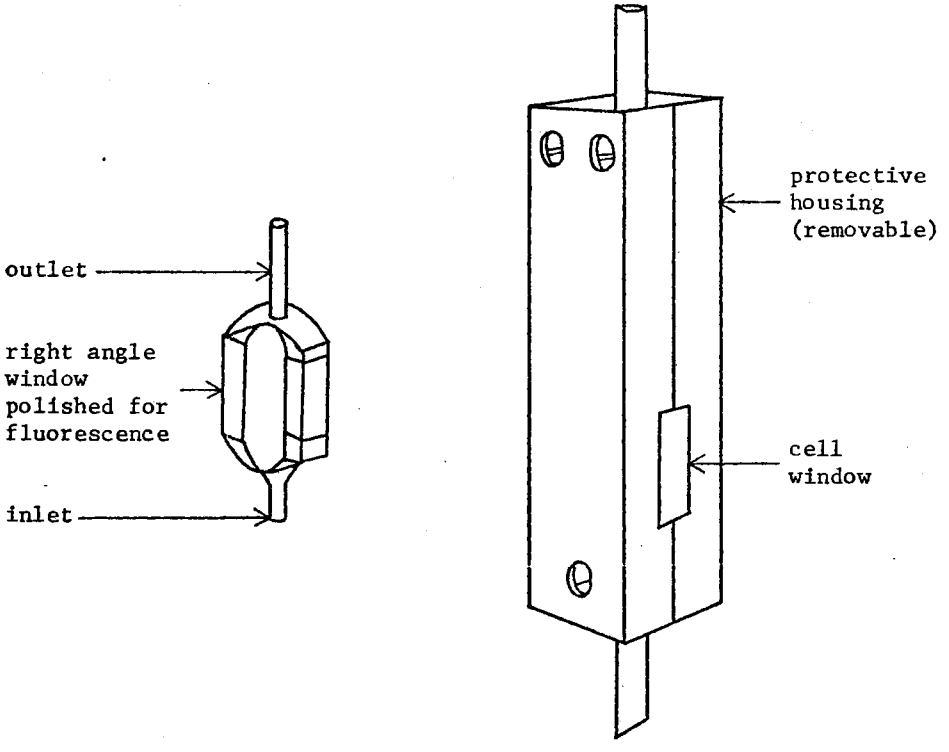


Figure 9. Flow-Through Cell

CHAPTER VI

RESULTS AND DISCUSSION

Pretreatment

Two screening procedures were used to improve the applicability of the analytical methods used to determine phenols. Distillation efficiency was investigated to prevent interference from color, turbidity and metallic ions. Figure 10 illustrates the recovery efficiency of this method. Filtration was used with samples which did not require distillation. Preservative steps such as acidification and cold storage were also used to eliminate interferences.

Concentration

Phenolic content was raised to a minimum detectable level by concentration. The procedures evaluated included carbon filtration and solvent desorption, freeze concentration, distillation, ion exchange, and liquid-liquid extraction.

Force-flow techniques were used to evaluate the activated carbon procedure. The column was packed with coarse activated carbon and the sample was forced through the column by the action of a peristaltic pump. Adsorption of the solute was enhanced by making the solution acidic with boric acid (1%) and high in salt concentration (1%). Desorption required changing the solvent to 0.01 M sodium hydroxide. Samples were collected in 50 ml increments and analyzed. It required

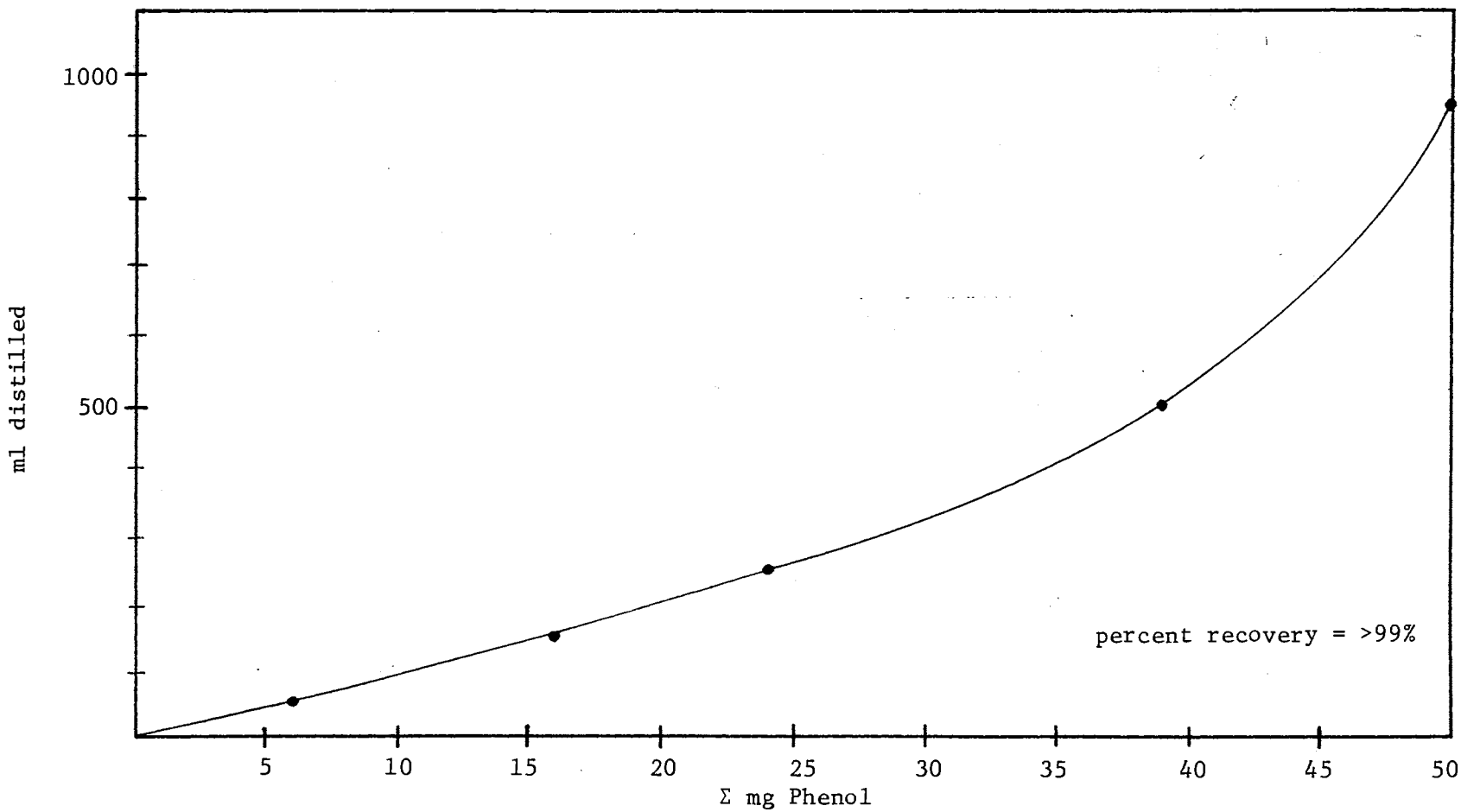


Figure 10. Recovery Efficiency of Phenol Distillation

100 mls of solvent to successfully elute 99% of the phenolic constituents. The column employed in this investigation was glass tubing with dimensions of 16" x 1/4" x 5/32".

Two methods were eliminated from further consideration. Freeze concentration was found to be time consuming and difficult to reproduce. Distillation (at pH = 12) was found to alter the sample composition.

Force-flow techniques were employed to evaluate an ion-exchange procedure. The column was packed with 50-100 mesh *Dowex* 50W-X12 cation exchange resin. The sample solution was forced through the system by the action of a peristaltic pump. Adsorption was enhanced by making the solution acidic with boric acid (1%). Desorption required changing of the solvent to water. Increments (50 ml) were collected and analyzed. It required 100 mls of solvent to elute 99% of the phenolic constituents. Aqueous ethanol (95%) eluted these materials after 25 milliliters. The column employed in this experiment was glass tubing with dimensions of 40" x 1/4" x 5/32".

Liquid-liquid extraction was evaluated using tributylphosphate (TBP) which extracts phenols from aqueous solutions. The technique incorporated the work of Schmauch and Grub.[19] A 25 ml aliquot of TBP extracted 91% of the phenol present in 1000 mls of aqueous solution. Table IV represents the distribution of phenols between TBP and water at pH 5.

TABLE IV

PARTITION COEFFICIENTS FOR VARIOUS PHENOLS BETWEEN TRIBUTYLPHOSPHATE AND WATER

Substance	Partition Coefficient (K)*
phenol	420**
o-cresol	1500**
p-cresol	1400**
m-cresol	1410
o-chlorophenol	1300
p-chlorophenol	1800
catechol	400

*Determined by: percent extracted = $100K/K + V_w/V_o$
**The values are comparable with reference 19.
The other compounds were not reported.

Tributylphosphate proved to be an inadequate solvent for chromatographic purposes. A second extraction of the TBP with 1.0M NaOH eliminated this problem. The extraction from the acidic aqueous phase to the organic phase was the limiting extraction after cyclohexane was added to the organic phase. Figure 11 illustrates the extraction efficiency of this method.

Spectrophotometric Methods of Analysis

An ultraviolet absorption method[15,19] was used to determine total phenolic materials. The procedure utilized the bathochromic shift in the absorbance band system after the addition of sodium hydroxide.

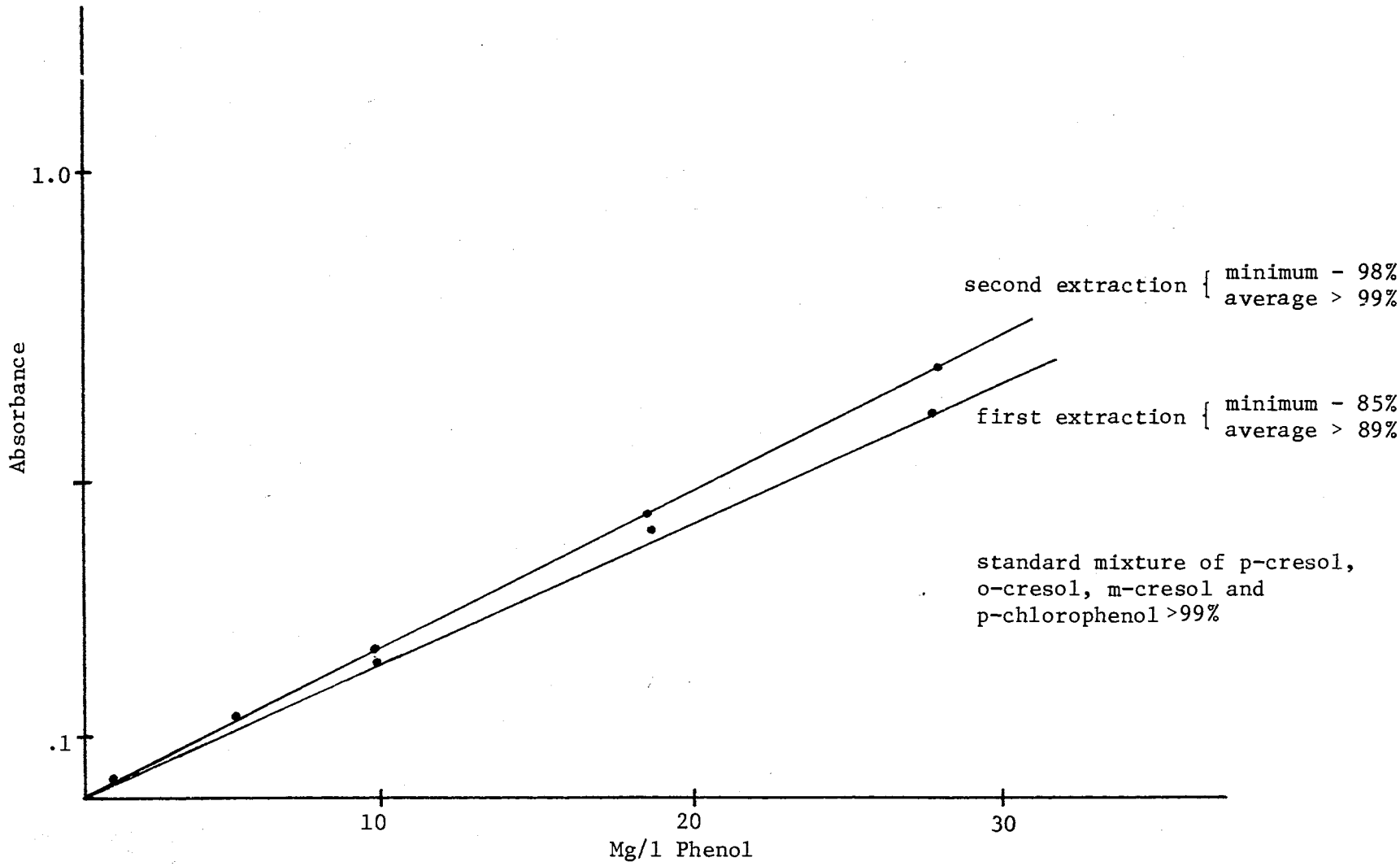


Figure 11. 100-fold Concentration Efficiency of Liquid-Liquid Extraction Procedure

This characteristic shift is illustrated for phenolic materials in Figure 12. The calibration curve for phenols based on standardized phenol is depicted in Figure 13. This method was used to characterize the recovery efficiencies of the previously mentioned methods.

Moderate-Pressure Liquid Chromatographic Method

Initial investigations into the liquid chromatographic behavior of phenolic compounds were conducted on a medium-pressure system. Nitrogen tank pressure (150 psi max.) was utilized to drive the solvent in this apparatus. *Dowex* cation exchange resin, *Porapak* Q, and *Porasil* A were investigated using this medium-pressure system.

Dowex cation exchange resin (50W-X12) and *Porapak* Q were found to have similar chromatographic selectivities for phenols. Both materials exhibited similar separation characteristics with the separation on *Porapak* Q illustrated in Figure 14. These materials consist of cross-linked polystyrene with a matrix which imbibes water. The major difference in separation efficiency is caused by the ionic functional groups of the exchange resin which exert a salting-out effect on organic materials. Salting-out counteracts the adsorptive effect of the resin matrix.

Porasil A was used as the support material in the determination of total phenols by medium-pressure liquid chromatography. The procedure was developed to utilize the concentration procedure previously mentioned. The response and linearity of this method is illustrated in Figure 15. No interaction of the solutes with this support material was noted when water was used as the mobile phase.

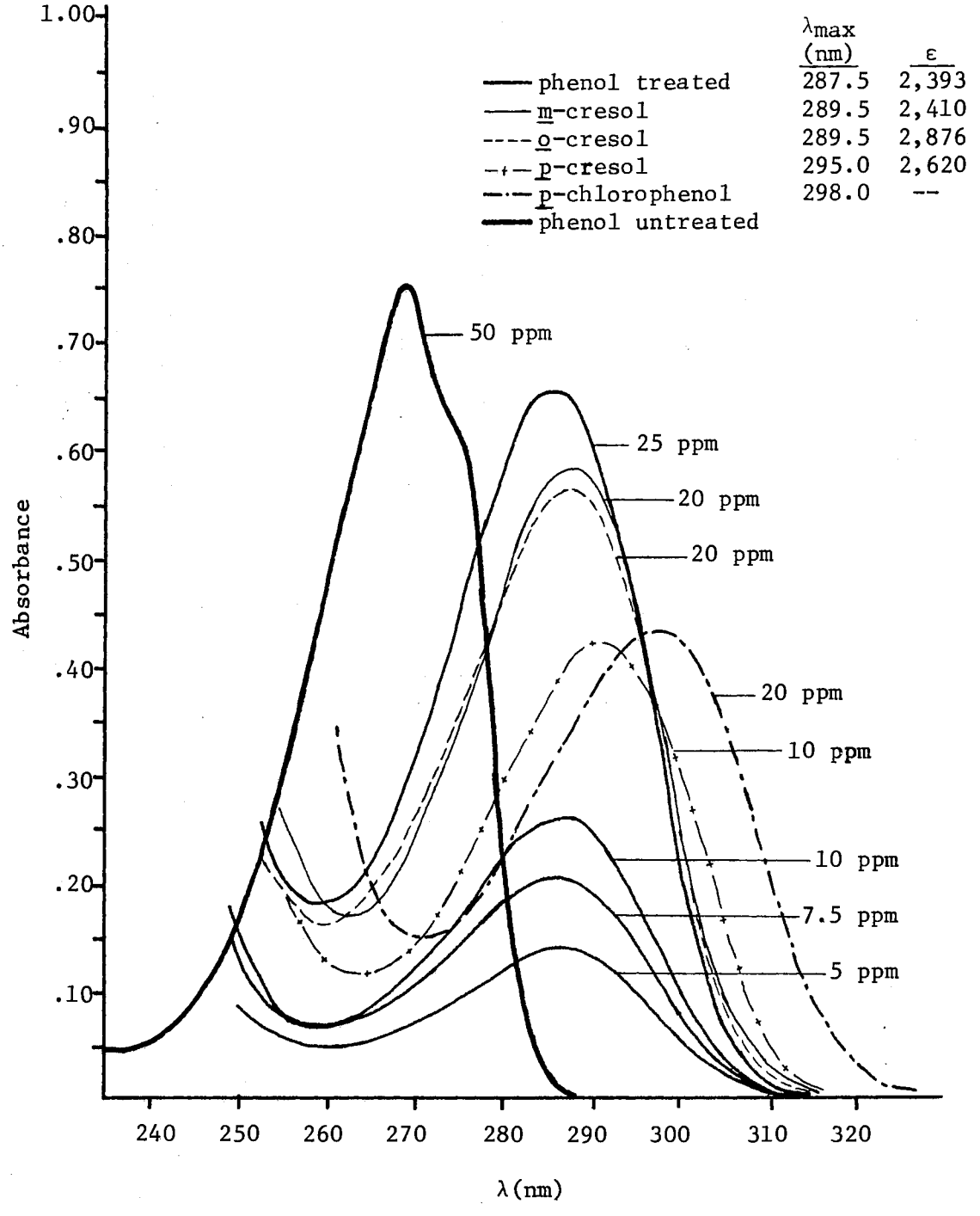


Figure 12. Bathochromic Shift Characteristics of Selected Phenolic Materials

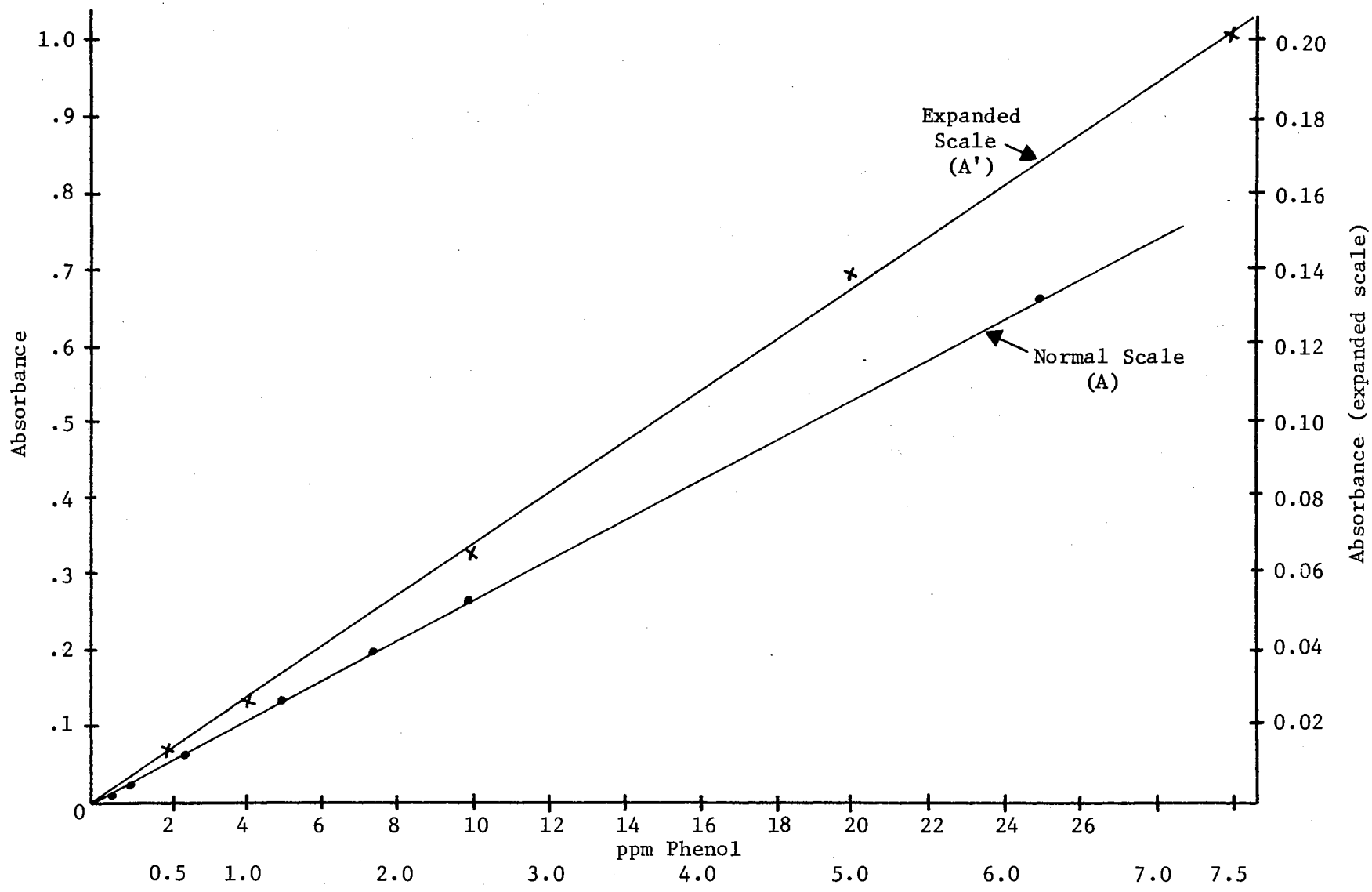


Figure 13. Standard Curve for Phenol

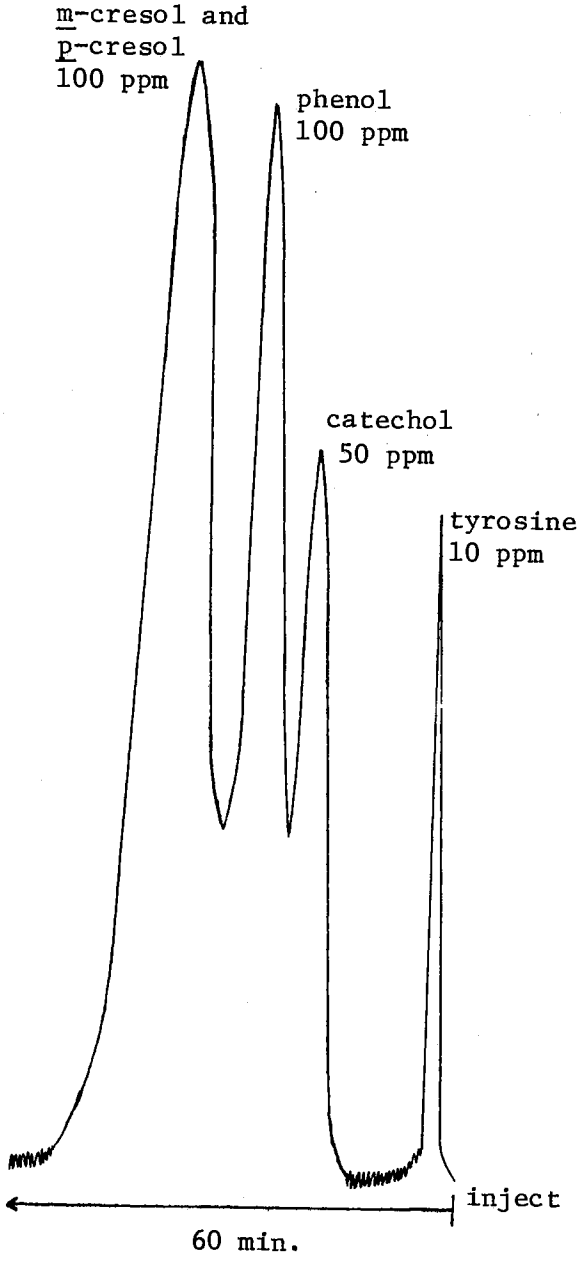


Figure 14. Separation of Phenolic Materials on *Porapak* Q

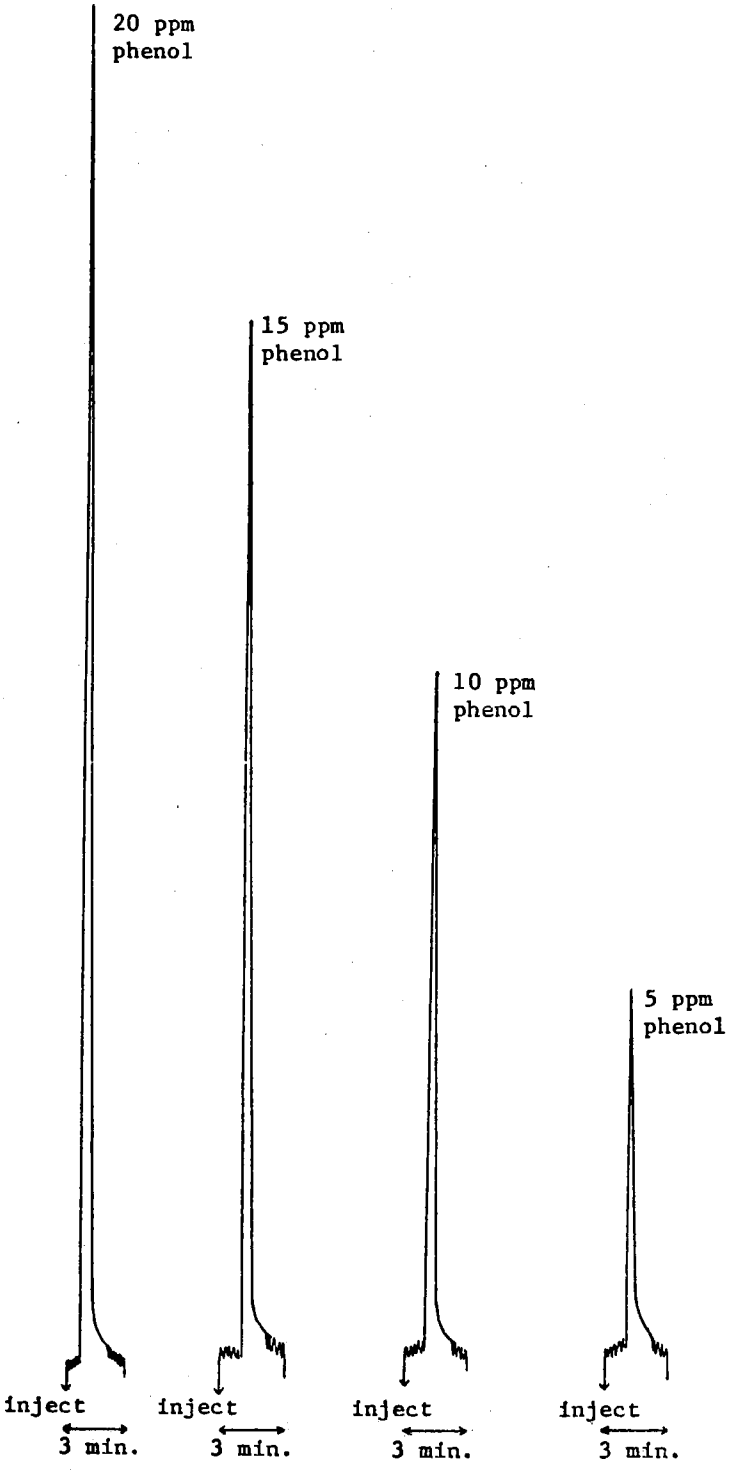


Figure 15. Phenol Analysis on *Porasil* A

High-Pressure Liquid Chromatographic Method

Liquid-partition chromatography was used to separate phenolic materials using the apparatus described in Chapter V. This technique was applied for the analytical purpose of determining the phenolic constituents in natural water. Both forms of liquid partition chromatography were investigated with the column material (*Permaphase* ETH) identical in both cases.

The stationary phase (*Permaphase* ETH) was polar and the mobile phase (n-hexane) nonpolar in the initial investigation. This separation was based on the work of Kirkland.[50] Solute solvents were investigated in order to achieve a practical separation to coincide with a concentration procedure. Solute solvents investigated were n-hexane, chloroform, methanol, TBP, and water. The separation characteristics using chloroform as a solvent is illustrated in Figure 16. Basic chromatographic variables associated with the separations found to be compatible with the mobile phase (n-hexane) are listed in Table V. Water was also eliminated as a compatible solvent due to tailing of solutes. The separation of m-cresol from p-cresol in all solvents tested was nonexistent when both were injected into the system as a solute mixture. Spectrophotometric analysis of a two-component mixture was used for identification and quantification of these simultaneous eluting substances.

A variable wavelength fluorometer was used in this investigation. This capability allowed analysis at two emission wavelengths (305 nm and 315 nm) with fixed excitation wavelength (270 nm). The method of analysis involved obtaining a chromatogram at one emission wavelength with subsequent analysis at the other wavelength. The two compounds of

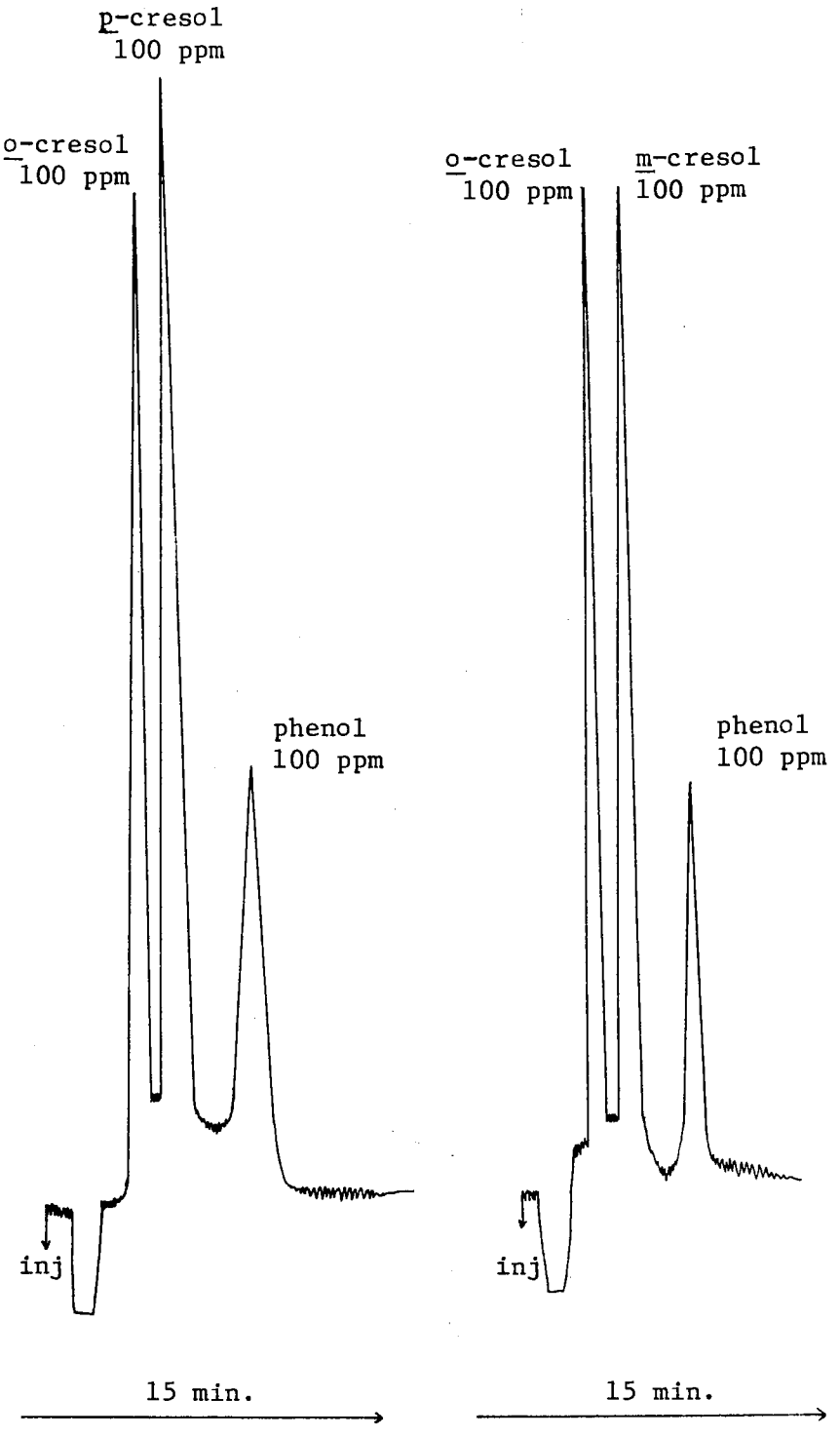


Figure 16. Separation of Phenolic Materials With Chloroform as Solute Solvent v = 1 ml/min.

TABLE V

CHROMATOGRAPHIC VARIABLES OF COMPATIBLE SOLVENTS†

Solvent	Solute	t_R	w_R	$\alpha=\left(\frac{t_{R_2}-t_o}{t_{R_1}-t_o}\right)$	$k'=\left(\frac{t_R-t_o}{t_o}\right)$	$R_s=2\left(\frac{t_{R_2}-t_{R_1}}{W_1+W_2}\right)$	$N=16\left(\frac{t_R}{W}\right)^2$	$H=L/N$
n-hexane	o-cresol	6.2 min. (0.31")	0.069"	-	5.9	-	323	0.07
(v=1 ml/min.)	p-cresol	7.5 min. (0.38")	0.075"	1.26	7.3	0.97	411	0.06
(t_o=0.9 min.)	phenol	12.5 min. (0.63")	0.145"	1.76	12.9	2.27	302	0.08
chloroform	o-cresol	8.3 min. (0.415")	0.095"	-	8.2	-	305	0.08
(v=1 ml/min.)	m-cresol	11.6 min. (0.580")	0.150"	1.45*	11.9	1.35*	241	0.10
(t_o=0.9 min.)	p-cresol	11.8 min. (0.590")	0.160"	1.47*	12.1	1.37*	218	0.11
	phenol	18.0 min. (0.900")	0.185"	1.60,1.57**	19.0	1.91,1.80**	381	0.06
water	o-cresol	7.5 min. (0.375")	0.075"	-	7.3	-	400	0.06
(v=1 ml/min.)	p-cresol	10.5 min. (0.525")	0.220"	1.45	10.7	1.02	91	0.26
(t_o=0.9 min.)	phenol	17.2 min. (0.860")	0.250"	1.70	18.1	1.43	189	0.13
methanol - noncompatible solvent								
tributyl phosphate - noncompatible solvent								

†for descriptions and typical values see Appendix D

*relative to o-cresol

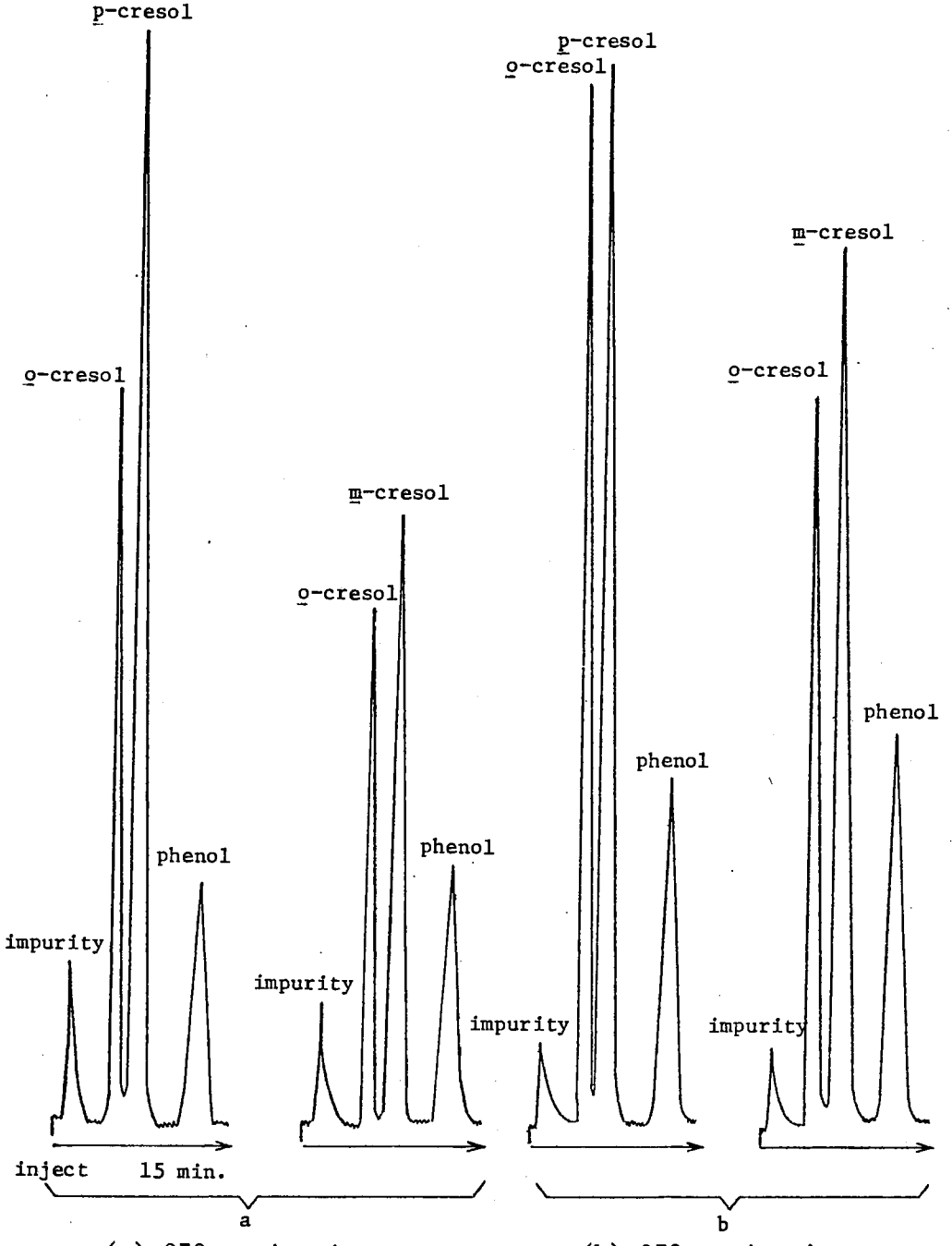
**relative to m-cresol, p-cresol

interest (p- and m-cresol) have different excitation and emission characteristics. Table VI lists the parameters necessary for this determination and Figure 17 illustrates the chromatograms obtained. Excitation at 270 was predetermined to yield maximum change with the change in emission wavelength. Stop-flow and scanning procedures were attempted and found insufficient in that peak areas could not be obtained. This method also provided qualitative information for the other phenols present. Figure 18 illustrates the excitation characteristics at fixed emission wavelength and emission characteristics at fixed excitation wavelength for p- and m-cresol in n-hexane. The sensitivity and linearity of the separation and detector response is illustrated in Figure 19. The signal to noise ratio at 1 ppm phenol was determined to be 10 to 3. The detector response in this study was found to be linear from 0.5 to 500 ppm phenol. Both requirements (additivity and Beer's law) were met for this determination by variable wavelength spectroscopy.

TABLE VI

VARIABLE WAVELENGTH CHARACTERISTICS OF PHENOLS

Compound	λmax excitation	λmax emission	% change = $\frac{\text{pk area at 315}}{\text{pk area at 305}} \times 100$
m-cresol	280 nm	305 nm	72%
p-cresol	285 nm	315 nm	103%
o-cresol	275 nm	305 nm	71%
phenol	270 nm	310 nm	68%



(a) 270 excitation
315 emission

(b) 270 excitation
305 emission

Figure 17. Chromatograms Representing Chromatographic Spectrophotometric Identification Procedure

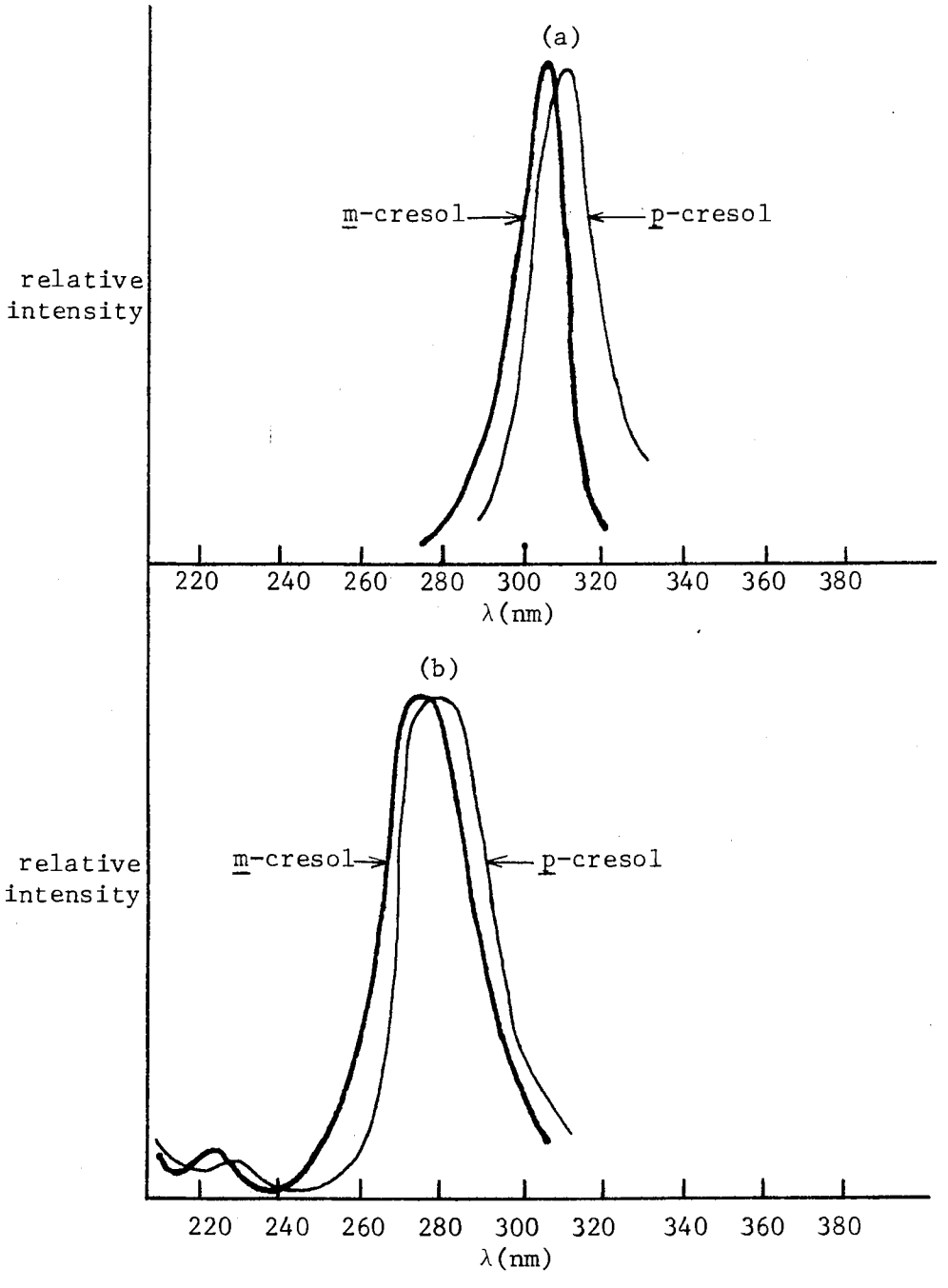


Figure 18. Excitation and Emission Wavelength Characteristics of Para and Meta Cresol
(a) Emission at Fixed Excitation
(b) Excitation at Fixed Emission

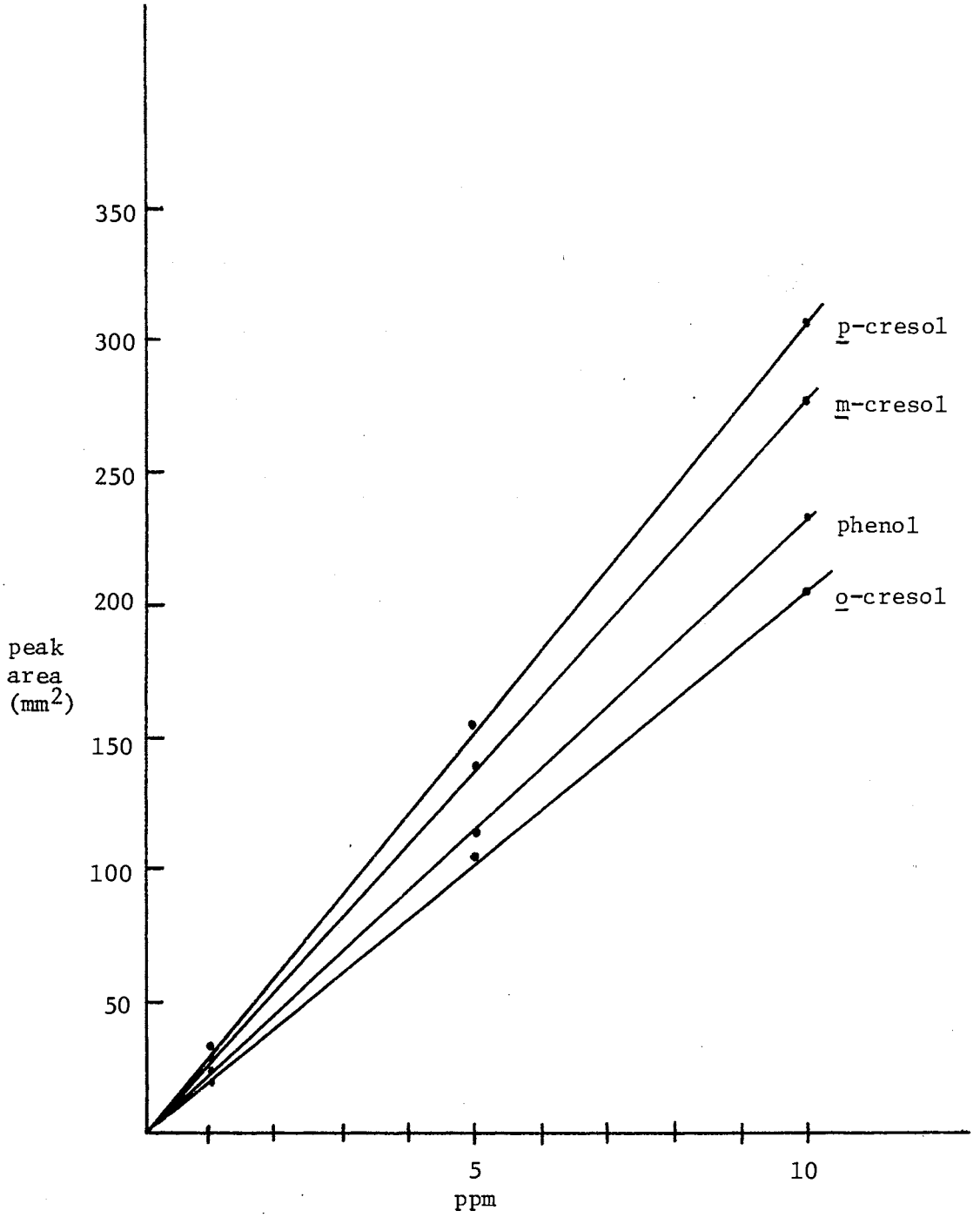


Figure 19. Standard Curve for Phenols by Peak Area Analysis

The reverse-phase separation was based on previous investigations using ion-exchange resins and porous polystyrene. In this mode of partition chromatography the stationary phase is nonpolar and the mobile phase polar. This approach to partition chromatography was generally used to separate nonpolar solutes. In this investigation reverse-phase liquid-liquid chromatography was extended to separate polar solutes based on solubility in water (Table VII).

TABLE VII

SOLUBILITY OF SELECTED PHENOLS IN H_2O AT 25°C

Compound	g/100g H_2O
phenol	9.3
o-cresol	2.5
m-cresol	2.6
p-cresol	2.3
catechol	45
p-chlorophenol	2.7

The column material (*Permaphase* ETH) was the same as for the partition separation. Organic partition phases are basically nonpolar when used with water as the mobile phase. Reverse-phase systems are generally less efficient because of slower diffusion in more polar (and viscous) mobile phases. The separation of phenols at pH 5 buffered is

illustrated in Figure 20. The basic chromatograph parameters are listed in Table VIII for this separation.

Methanol was used as a modifier to increase the efficiency of the reverse-phase system. The modifier produced a less viscous mobile phase. Methanol decreased the capacity factor (k') for the solutes uniformly up to 20% by volume. This separation at buffered pH 5 is illustrated in Figure 21. Chromatographic parameters are listed in Table IX.

The adjustment of pH was investigated to determine the effect on separation. Since phenols are acids with slightly different pK_a values, a change in pH will affect solubility in the aqueous phase which changes k' values. Ionized materials do not interact with the stationary phase which causes competing equilibria within the column at high pH values. Figure 22 illustrates the dependence of separation of phenolic materials on pH. The response to catechol and p-chlorophenol was significantly diminished at these pH values.

Salting-out was investigated as a separation tool for the chromatographic separation of phenols. This approach had a negative effect on the separation efficiency of the system by causing peak spreading.

The previous procedures were used to develop a separation procedure which yielded maximum information about the compounds investigated. Initial chromatographic separation was performed at pH 5 and 20% methanol. More complex separations require analysis at pH 9.8 and 20% methanol using spectrophotometric analysis of simultaneous eluting solutes. The separation at pH 9.8 and 20% methanol is illustrated in Figure 23. Figures 23 and 24 illustrate the response characteristics of this system at two emission wavelengths with constant excitation

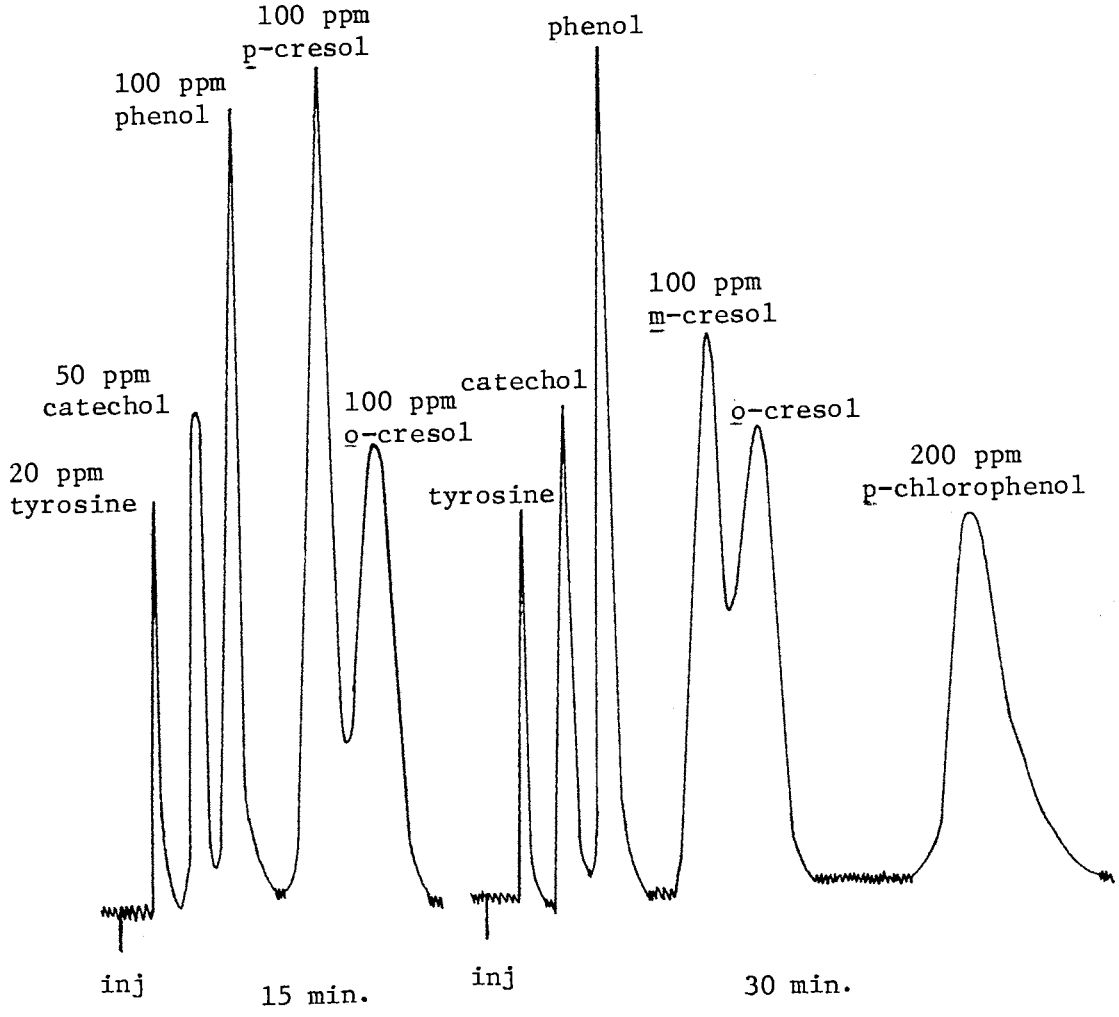


Figure 20. Separation of Various Phenols at pH 5 Buffered

TABLE VIII

BASIC CHROMATOGRAPHIC PARAMETERS FOR THE SEPARATION OF PHENOLIC MATERIALS BY REVERSE-PHASE LLC AT pH 5 BUFFERED†

Solute	t_R	W_R	α relative to tyrosine	k' relative to tyrosine	R_s	N	H
tyrosine	1.80 min. (0.180")	0.115"	-	-	-	25	0.96
catechol	3.90 min. (0.390")	0.115"	-	1.17	1.83	54	0.44
phenol	5.75 min. (0.575")	0.157"	1.88	2.19	1.36	59	0.41
p-cresol	9.80 min. (0.980")	0.230"	2.03*	4.44	2.09*	68	0.35
m-cresol	10.05 min. (1.005")	0.250"	2.09*	4.58	2.11*	64	0.38
o-cresol	12.90 min. (1.295")	0.310"	1.39,1.33**	6.19	1.17,1.04**	67	0.36
p-chlorophenol	24.70 min. (2.470")	0.475"	2.05	12.70	1.50	83	0.29

† for descriptions and optimum values see Appendix D
* relative to phenol
** relative to p-cresol, m-cresol

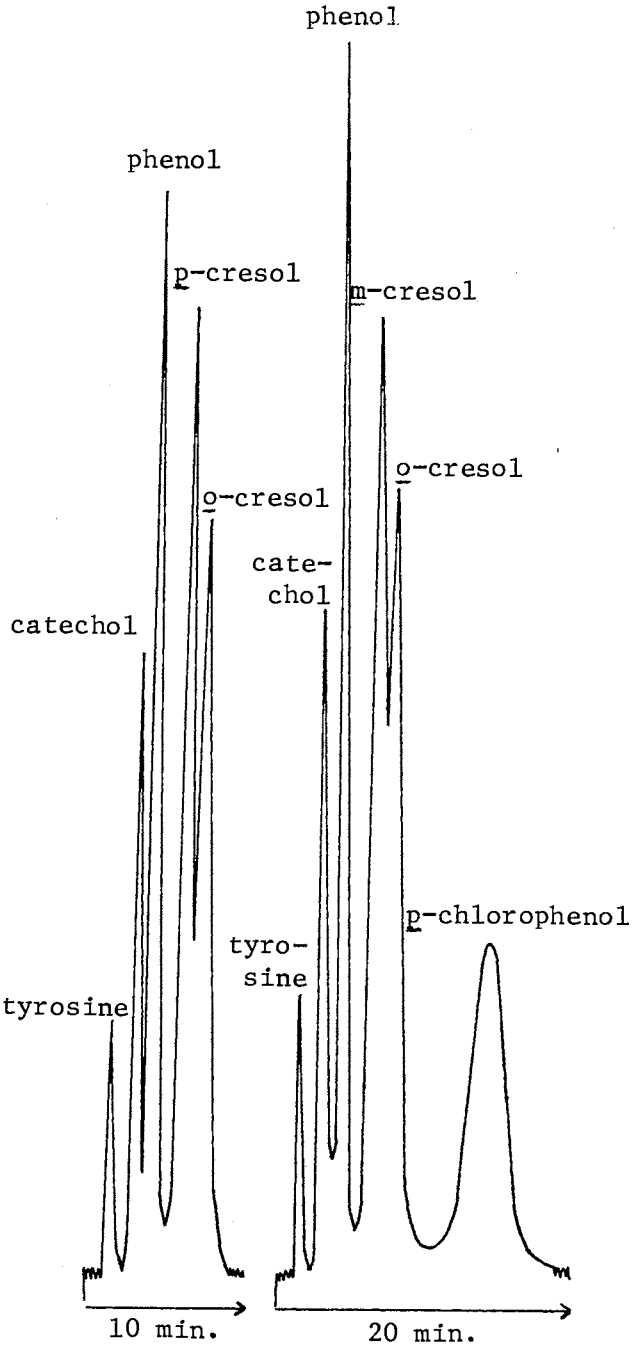


Figure 21. Separation of Various Phenols at pH 5 Buffered - 20% MeOH

TABLE IX

BASIC CHROMATOGRAPHIC PARAMETERS FOR THE SEPARATION OF PHENOLIC MATERIALS BY REVERSE-PHASE LLC AT pH 5 BUFFERED AND 20% MeOH†

Solute	t_R	W_R	α relative to tyrosine	k' relative to tyrosine	R_s	N	H
tyrosine	1.6 min. (0.156")	0.050"	-	-	-	50	0.48
catechol††	3.3 min. (0.330")	0.080"	-	1.06	2.68	66	0.36
phenol	4.1 min. (0.410")	0.110"	1.47	1.56	0.90	60	0.40
p-cresol	6.2 min. (0.620")	0.140"	1.84*	2.90	1.68*	71	0.34
m-cresol	6.3 min. (0.630")	0.150"	1.88*	2.94	1.76*	67	0.36
o-cresol	7.7 min. (0.773")	0.160"	1.33,1.30**	3.81	0.99,0.92**	77	0.31
p-chlorophenol	14.2 min. (1.420")	0.410"	2.03	7.75	2.27	55	0.44

† for descriptions and optimum values see Appendix D
†† resorcinol elutes simultaneously with catechol
* relative to phenol
** relative to p-cresol, m-cresol

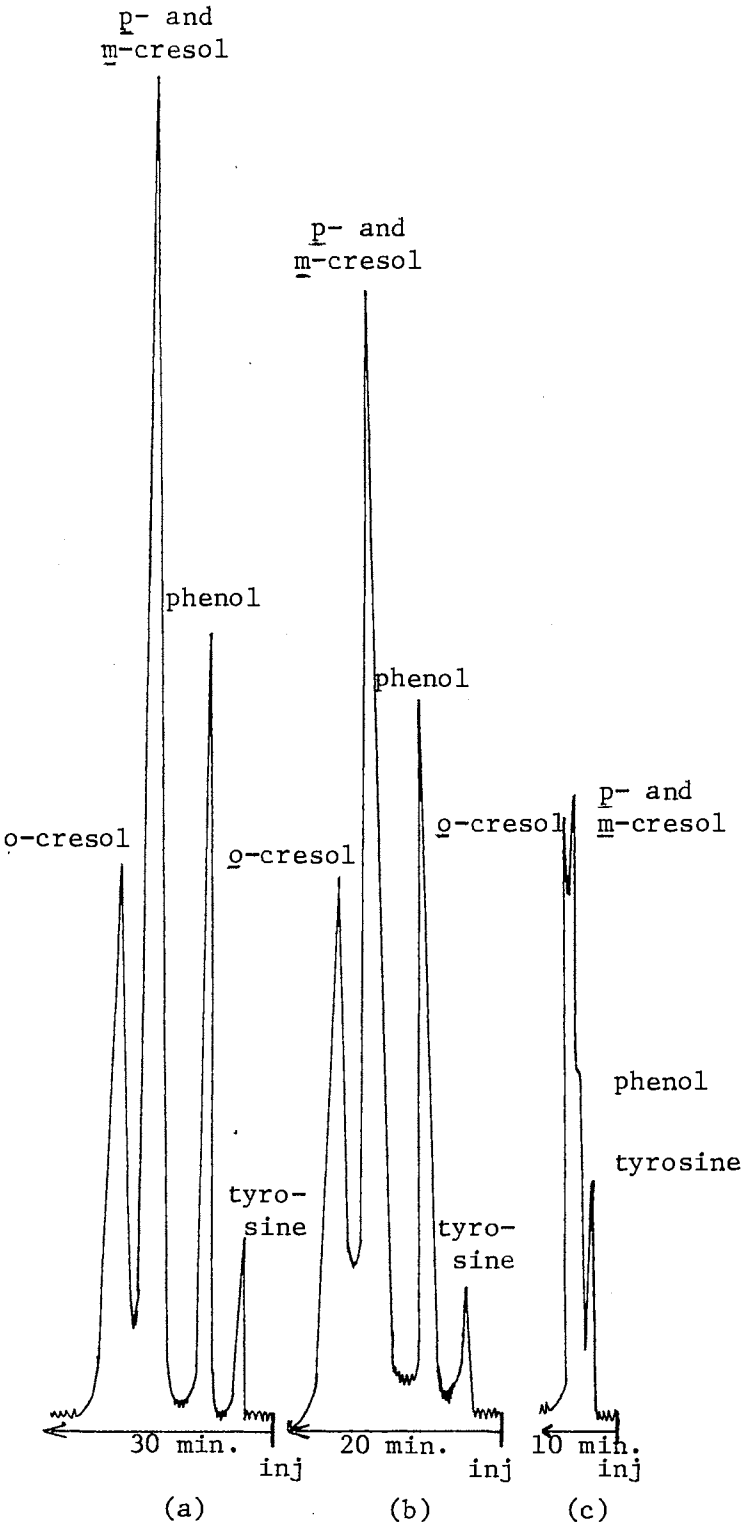


Figure 22. Separation of Phenols by pH
(a) pH = 9.8, (b) pH = 10,
(c) pH = 10.5

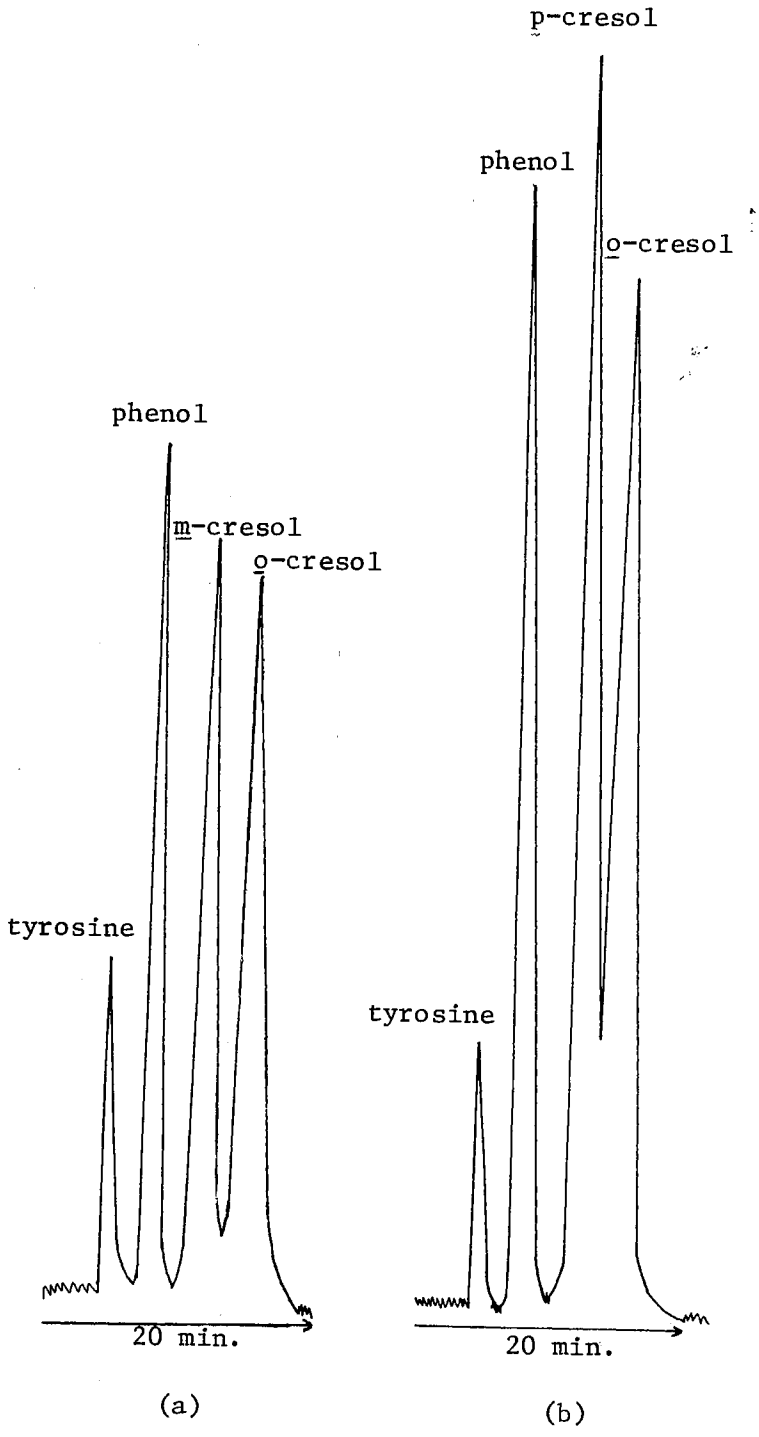


Figure 23. Separation of Phenols at pH 9.8 and 20% MeOH
v = .5 ml/min.
(a) 270 - 315; (b) 270 - 305

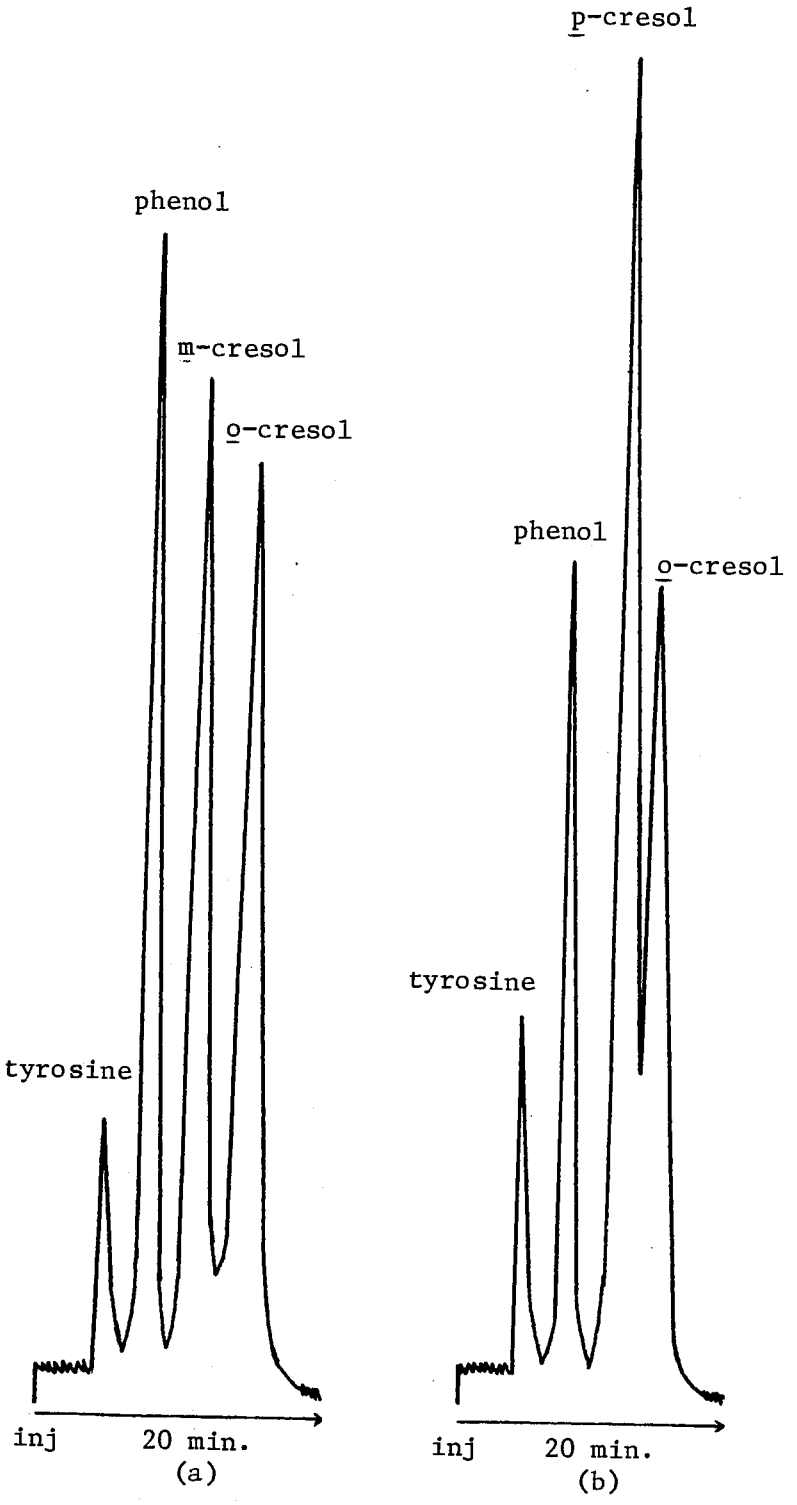


Figure 24. Separation of Phenols at pH 9.8 and 20% MeOH
v = .5 ml/min.
(a) 270 - 305; (b) 270 - 315

wavelength. The sensitivity and linearity parameters of the system response were found to compare favorably with those for the regular partition separation. These parameters are illustrated in Figure 25 and Table X.

Strong anion exchange (*SAX*) was investigated as a stationary phase for the reverse-phase separation of phenols. The chromatographic separation is illustrated in Figure 26. The investigation was terminated because of the failure to separate the three cresols.

Practical Application

Little has been reported on the practical application of high-pressure liquid chromatography. The methods investigated have been applied to the analysis of phenolic materials in natural systems. These methods incorporated solvent extraction for concentration and analysis by high-pressure liquid chromatography.

The initial system investigated was Lake Carl Blackwell, located 11 kilometers west of Stillwater, Oklahoma. The main input to the lake is Stillwater Creek with most water derived from natural runoff. The main outflow is the intake to the water supply for the City of Stillwater and Oklahoma State University. The drainage basin consists mainly of pastured grassland and wheat farmland.

The chromatographic analysis of a sample concentrated 500-fold is illustrated in Figure 27. By peak analysis and comparison with a standard of 1 ppm, this system was found to contain less than 1 ppb phenol (~.8) and less than 1 ppb p-cresol (~.6). A gas chromatographic-mass spectrometer analysis (LKB-9000) was attempted but provided inconclusive evidence for the presence of these compounds. This was due in

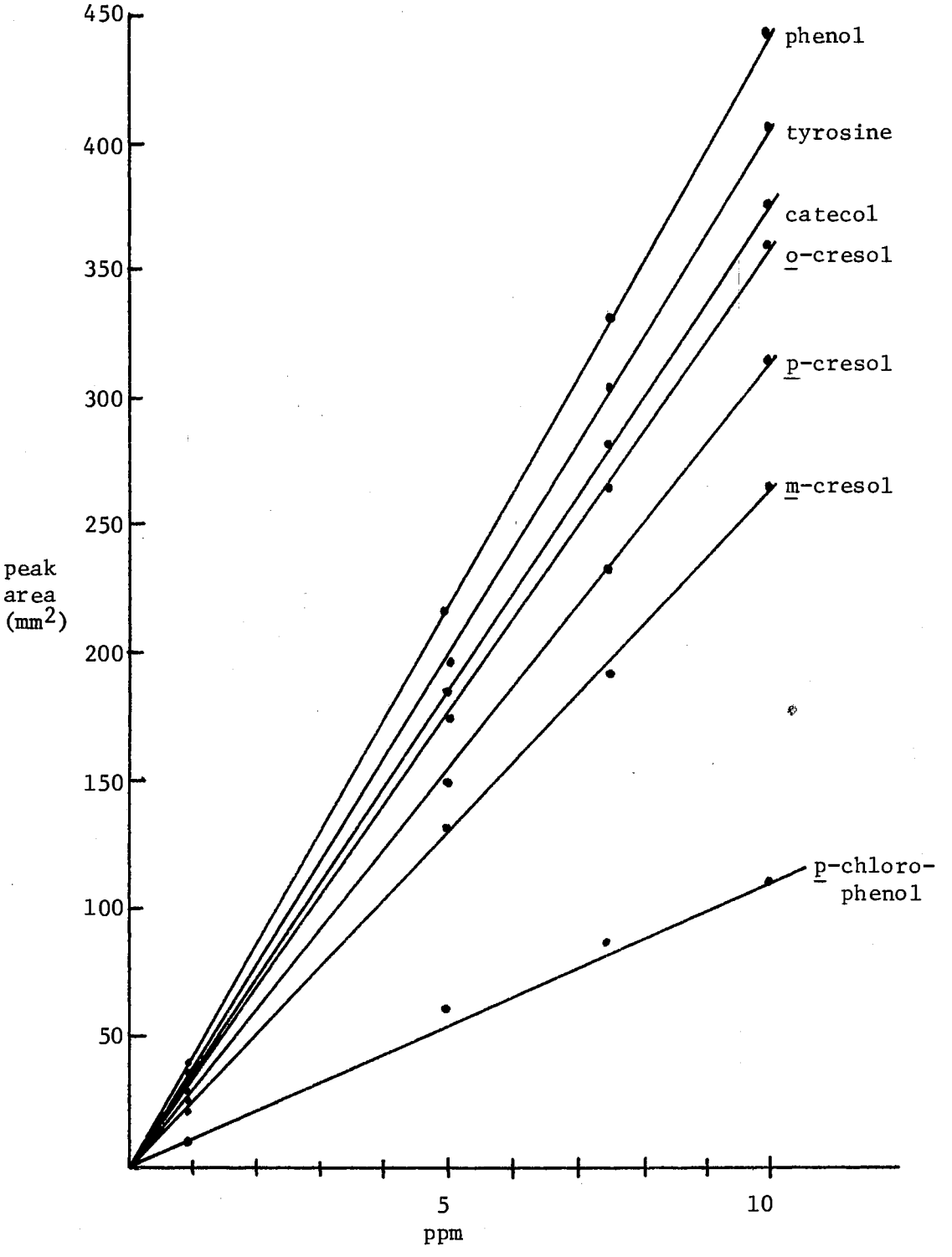


Figure 25. Standard Curve for Phenols by Peak Area Analysis
(a) tyrosine at pH 5, (b) catechol at pH 5,
(c) phenol at pH 5, (d) o-cresol at pH 9.8,
(e) p-cresol at pH 9.8, (f) m-cresol at pH 9.8,
(g) p-chlorophenol at pH 5

TABLE X

SEPARATION PARAMETERS FOR PHENOLS AT pH 9.8 AND 20% MeOH†
v = .5 ml/min

Solute	t_R	W_R	α relative to tyrosine	k' relative to tyrosine	R_s	N	H
tyrosine	3.55 min. (0.355")	0.075"	-	-	-	76	0.32
phenol	6.05 min. (0.605")	0.080"	-	0.71	3.22	121	0.20
m-cresol	8.85 min. (0.885")	0.148"	2.12*	1.50	1.16*	96	0.25
p-cresol	8.90 min. (0.890")	0.150"	2.14*	1.51	1.19*	95	0.25
o-cresol	11.23 min. (1.123")	0.175"	1.50,1.44**	2.16	1.47,1.43**	103	0.23

† for descriptions and optimum values see Appendix D
* relative to phenol
** relative to m-cresol, p-cresol

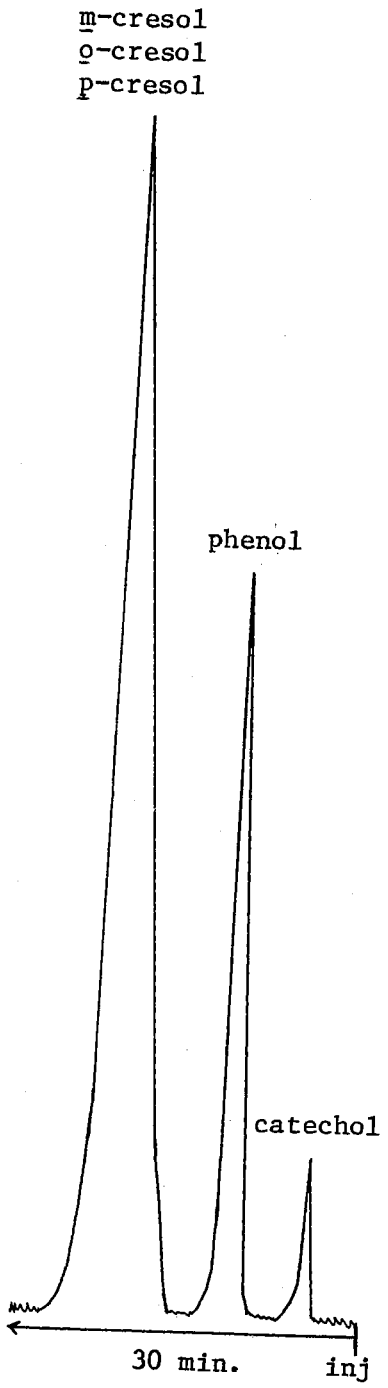


Figure 26. Separation of Phenolic Materials on *SAX* at pH 5

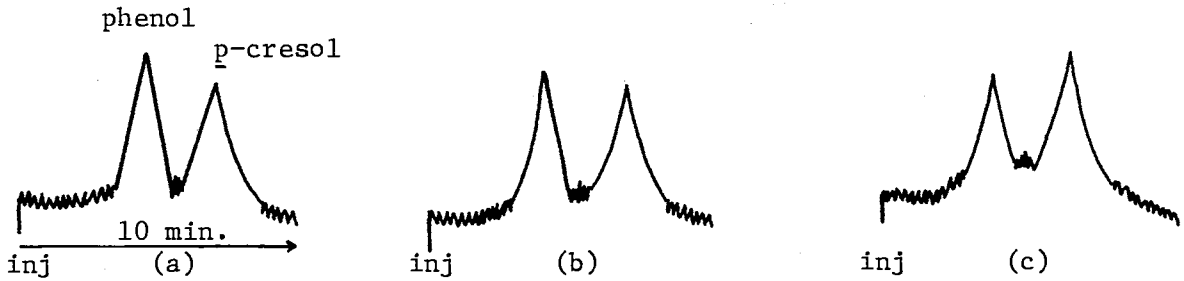


Figure 27. Analysis of Lake Carl Blackwell Sample
v = .5 ml/min. - 25λ injection
(a) pH = 5 270 nm - 305 nm
(b) pH = 9.8 270 nm - 305 nm
(c) pH = 9.8 270 nm - 315 nm

part to the small amount of material present which required further concentration (100-fold) and the poor gas chromatographic characteristics of these compounds (severe tailing and ghost peaks). The presence of p-chlorophenol was exhibited by GC-MS which did not appear in the liquid chromatographic analysis.

An analysis was performed on an effluent from a refinery in Oklahoma. The result is illustrated in Figure 28. Phenol was the only material identified. The analysis determined that approximately 9 ppb (±4.5) phenol was present since the sample was concentrated 100-fold.

The final analysis was performed on a sample from an aerobic decomposition of algae. This system was constructed to illustrate a natural source for phenolic materials. The algae decomposed was anabaena. The chromatographic results are illustrated in Figure 29. The materials identified include tyrosine, phenol, and p-cresol. Since the analysis was performed on a 100-fold concentrated sample, these materials were present at approximately 3 ppb (±1.5).

Summary

The primary objective of this research was to develop an analytical procedure including concentration and analysis to selectively measure individual phenolic materials in natural systems. The concentration procedure and high-pressure liquid chromatographic analysis method have undergone extensive laboratory evaluation and found to be sensitive and accurate. This research showed significant application to the analysis of field samples.

The use of fluorimetry as a detection mode has been shown to be highly specific in the measurement of phenolic content. The major

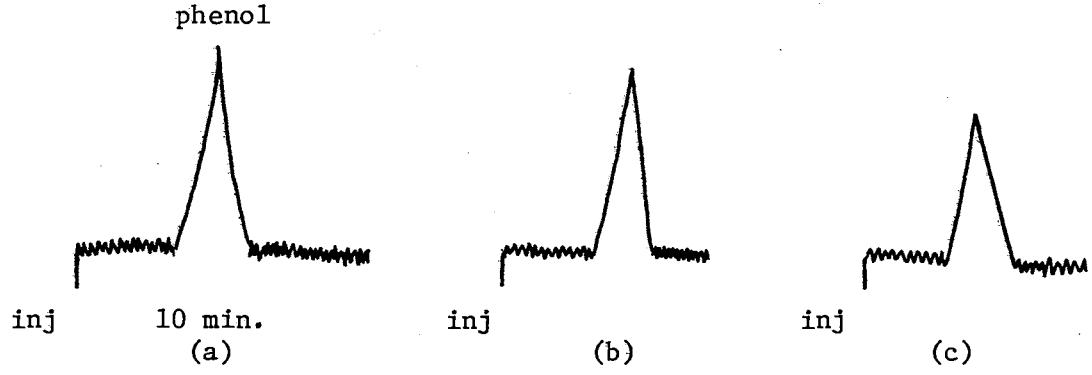


Figure 28. Analysis of Refinery Effluent Sample
v = .5 ml/min. at pH = 5
(a) pH 5 270 - 305
(b) pH 9.8 270 - 305
(c) pH 9.8 270 - 315

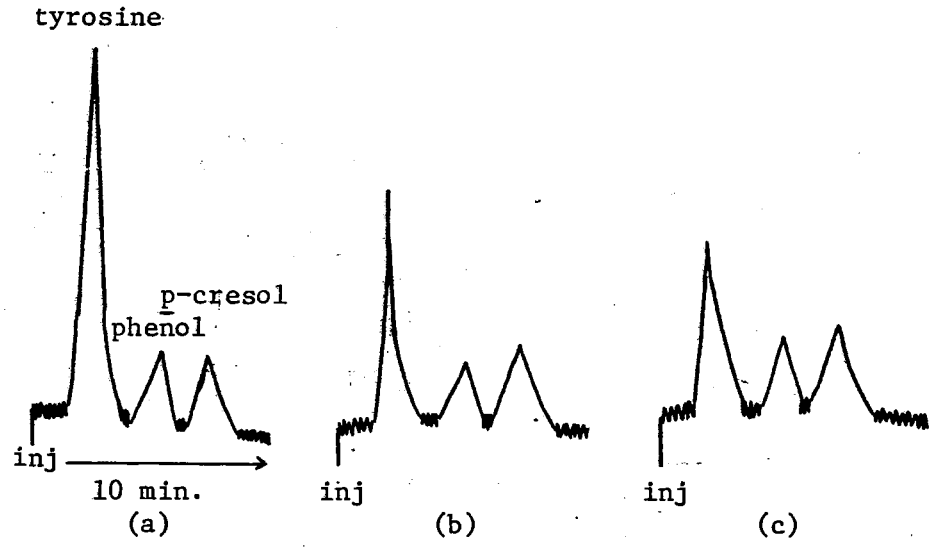


Figure 29. Analysis of Aerobic Decomposition Sample
v = .5 ml/min. - 25λ injection
(a) pH 5 270 - 305
(b) pH 9.8 270 - 315
(c) pH 9.8 270 - 305

advantages of fluorescence monitoring are the very low detectable sample size and specificity. Incorporation of variable emission wavelength into the analysis procedure permitted further identification in the evaluation of the sample. This was significant in the analysis of simultaneously eluting solutes because of the specificity inherent in two spectral requirements instead of one as in absorption spectroscopy.

The development of a reverse-phase separation procedure for the analysis of phenols was significant because it illustrated the versatility of this mode of partition chromatography. The use of *Permaphase* ETH as a stationary phase for this separation was unique. The effect of methanol content and pH on separation of phenolic materials also demonstrated the versatility of this approach.

Improvement in detection requires decrease in the noise level because a finite signal is always measured. The limit of sensitivity is only approached when the fluorescence signal strength falls to the noise level of the detector circuit. Improvement in the detector circuit requires:

1. replacement of the present power supply for the photomultiplier tube with a battery pack,
2. replacement of the present photomultiplier tube (RCA model 1P28) with a more sensitive photodetector, and
3. cooling of the photomultiplier tube to reduce noise.

The separation efficiency of the present chromatographic system can only be improved by using smaller diameter packing material. This can be accomplished by sieving the present material to decrease the average particle diameter from 28 μ to 20 μ. This will decrease the particle size range and decrease void volume. It will also increase the separation efficiency of the system by having a favorable effect on

mass transfer. This approach will also require changing the present pumping system with one that will withstand greater pumping pressures in order to maintain mobile phase velocity.

The development of elegant methods for the separation of complex mixtures (chromatographic methods) makes it possible to utilize fluorescence in instances formerly thought impossible. Analytical methods have been described for many organic compounds. A selective list of compounds which absorb ultraviolet radiation and re-radiate at longer wavelengths is given in Appendix C. The principal analytical application of fluorescence analysis has been drug analysis. The determination of metal ions after complexation is the most frequent application of fluorescence spectroscopy in inorganic chemistry. Nonfluorescent compounds can be converted into fluorescing substances by suitable reactions producing a fluorophor. Investigations should be attempted in these areas for possible use of the present reverse-phase chromatographic system.

Analysis by reverse-phase and direct partition chromatographic systems requires information on solubility in water, polarity, and the pK_a or pK_b in the case of acids and bases. Organic compounds with high solubility in water are generally polar materials. Polar compounds elute from the reverse-phase system with less retention volume than nonpolar materials. Methanol modifier is used to decrease the retention volume for less polar materials by increasing solubility in the mobile phase. Comparisons can be made for the solubility parameter by using Table VII. For organic acids and bases the respective pK_a and pK_b must be known since ionic materials do not interact significantly with the column. This parameter can also be used to separate

weak organic acids and bases as in the cases of the cresols. Adjustment of methanol modifier and pH can be used to increase resolution for the eluting solutes.

Nonpolar solutes may be effectively separated by using an aqueous methanolic mobile phase. Highly polar materials require analysis by direct partition chromatography using a polar stationary phase and nonpolar mobile phase. Separation parameters are listed in Table XI. At present two bonded phase materials exhibit general applicability to most partition separations. ODS *Permaphase* is used as a nonpolar support for reverse-phase and *Permaphase* ETH is used for reverse-phase and direct partition applications.

To ascertain whether an unknown material can be determined by the fluorescence detection system, a UV scan must be obtained prior to analysis. The maximum absorbance wavelength for the compound is then used to determine an emission scan for the unknown. The fluorometer is then set at these excitation and emission wavelengths for chromatographic analysis. Once the chromatogram has been obtained, basic chromatographic procedures can be used to optimize the system. These procedures include the resolution parameters. Identification requires standardization with known materials. Fraction collection of the eluting unknown solutes can be used to determine the unknown by other methods (infrared, gas chromatograph-mass spectrometer, etc.) if sufficient material is present to allow these analyses.

TABLE XI

GENERAL SEPARATION PARAMETERS FOR PARTITION CHROMATOGRAPHY

Separation Type	Compound Type	Stationary Phase	Mobile Phase	Modifier	Examples
Direct Partition	Polar	*Permaphase* ETH	Di-n-butyl ether	Acetonitrile Methanol Tetrahydrofuran	Fenuron Monuron Hydroquinones
	Moderately polar	*Permaphase* ETH	N-hexane	Acetonitrile Dioxane Ethanol	Phenol Ortho, para, and meta Nitro aniline Xylenols
Reverse-Phase	Moderately polar	*Permaphase* ETH	Water	Methanol (0-20% v/v) Acetonitrile	Phenolic compounds
	Weak acid	*Permaphase* ETH	Acidic medium buffered (dependent on pK_a)	Methanol (0-20% v/v) Acetonitrile	Phenol Anthranilic acid P-amino benzoic acid
	Weak base	*Permaphase* ETH	Basic medium buffered (dependent on pK_b)	Methanol (0-20% v/v) Acetonitrile	Aniline Methylaniline Amphetamines
	Nonpolar	ODS *Permaphase*	50-50 methanol-water (v/v)	Methanol	Substituted anthraquinones Chrysene Benzpyrene

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APPENDIX A

FLUORESCENCE METHODS

Fluorescence is characterized by two spectra. The excitation spectrum is the absorption spectra of the molecule and may be different from the usual absorption spectra as a result of instrumental artifacts. The fluorescence spectra is the radiation emitted at a different wavelength after the molecule has absorbed radiation anywhere in the excitation spectrum. Fluorescence is specific for a given molecule and can be used to qualitatively characterize a sample. The emission intensity of a given molecule can be used in quantitative determinations.

In order for fluorescence to occur, a molecule must be exposed to radiation in the portion of the spectra in which the molecule is capable of absorbing. The absorption process occurs in about 10^{-15} seconds. If the energy is increased sufficiently, an electronic transition will occur according to the Frank-Condon principle. The Frank-Condon principle requires that the transition be vertical to the excited state. The transition usually occurs from a ground singlet state to an excitated singlet state. This singlet-singlet transition gives rise to the ultraviolet absorption spectra or excitation spectra.

The excited singlet state may last for 10^{-8} to 10^{-4} seconds. During this time energy may be lost in collisions with other molecules. This leaves the molecule in a lower vibrational state than when the transition originally occurred. When the transition back to the ground

state occurs, the Frank-Condon principle is again obeyed. This results in a transition of lower energy and longer wavelength. This gives rise to the fluorescence spectrum.

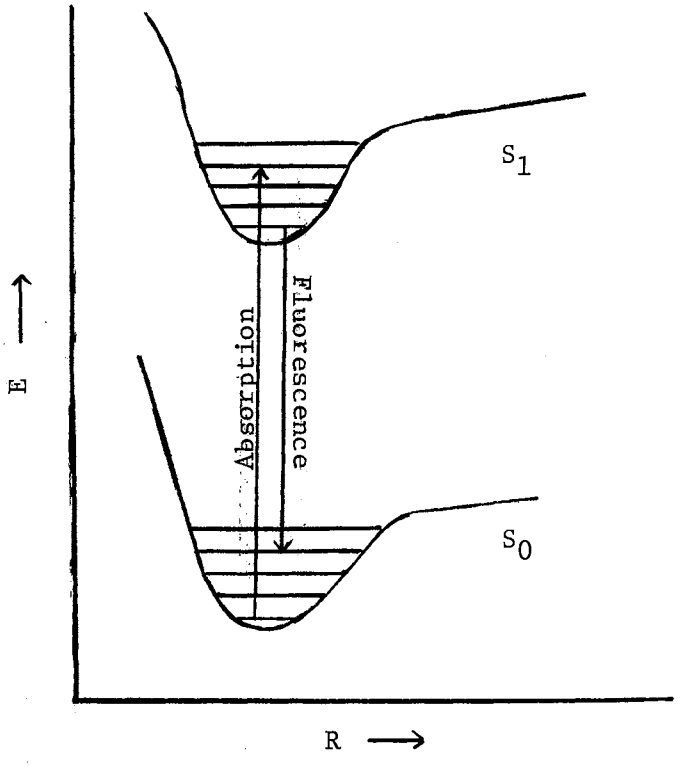

Figure 30. Electronic Excitation and Fluorescence in a Diatomic Molecule

The excitation spectrum usually consists of wavelengths in the ultraviolet. The fluorescence spectrum usually consists of wavelengths in the visible region.

The first requirement for fluorescence is absorption of light. In general the stronger the absorption the stronger the fluorescence. Fluorescence may be expected in molecules that contain multiply conju-

gated double bonds with a high degree of resonance stability (aromatic and heterocyclic compounds). Many factors affect fluorescence such as substituent groups, pH of solution, and solvent system.

Fluorescence is not as common as light absorption because of quenching effects which tend to decrease the quantum efficiency of the molecule and thus the quantum yield of fluorescence. Quenching occurs when an electron returns to the ground state by some other mechanism than fluorescence.

Fluorescence depends upon the concentration of fluorophors present in solution. From Beer's law the fraction of light transmitted is:

$$\frac{P}{P_o} = e^{-\varepsilon bc} \tag{1}$$

The corresponding fraction of light absorbed is:

$$1 - \frac{P}{P_o} = (1 - e^{-\varepsilon bc}) \tag{2}$$

Rearranging we have

$$P_o - P = P_o\ (1 - e^{-\varepsilon bc}) \tag{3}$$

The fluorescence intensity is equal to the light absorbed times the quantum efficiency of fluorescence (Φ)

$$F = (P_o - P)\Phi \tag{4}$$

or,

$$F = P_o\ (1 - e^{-\varepsilon bc}) \tag{5}$$

For dilute solutions only a small amount of light is absorbed. If the term εbc is less than about 0.05, we have

$$F = k\ P_o \varepsilon bc \tag{6}$$

where k is an instrumental constant. Therefore, fluorescence is linear at low concentrations (parts per million).

APPENDIX B

THE FARRAND SPECTROFLUOROMETER

The fluorometer in this experimentation is a Farrand spectrofluorometer. The instrument shown in Figures 31 and 32 is a single beam dual monochromator spectrofluorometer arranged for right angle illumination.

The light source consists of Hanovia 150 Watt D.C. Xenon Arc Lamp. The lamp is focused on the entrance slit by an off axis ellipsoidal mirror. The power supply for the lamp is a solar unit which provides a momentary potential of 25K to 40K volts to start the lamp. It normally operates at about 20 volts. Two grating monochromators are used, one for excitation of the sample and the other for analysis of fluorescent radiation from the sample. The monochromators operate from 220 to 700 mμ. The wavelength is read directly from a linearly calibrated dial. The monochromators have a focal length of 160 mm and a focal ratio of F/3.5. Replica gratings were taken from a Farrand master having 14,400 lines per inch. Dispersion is 11 mμ/mm in the first order. Filters may be used to block undesired radiation.

Four interchangeable slits are used, two in each monochromator. Slits available are 0.1, 0.5, 1.0, and 210 mm. In general, doubling the slit size doubles the signal observed. The use of narrow slits gives best resolution. By changing all slits from 0.5 mm to 2.0 mm increases the signal 100 times.

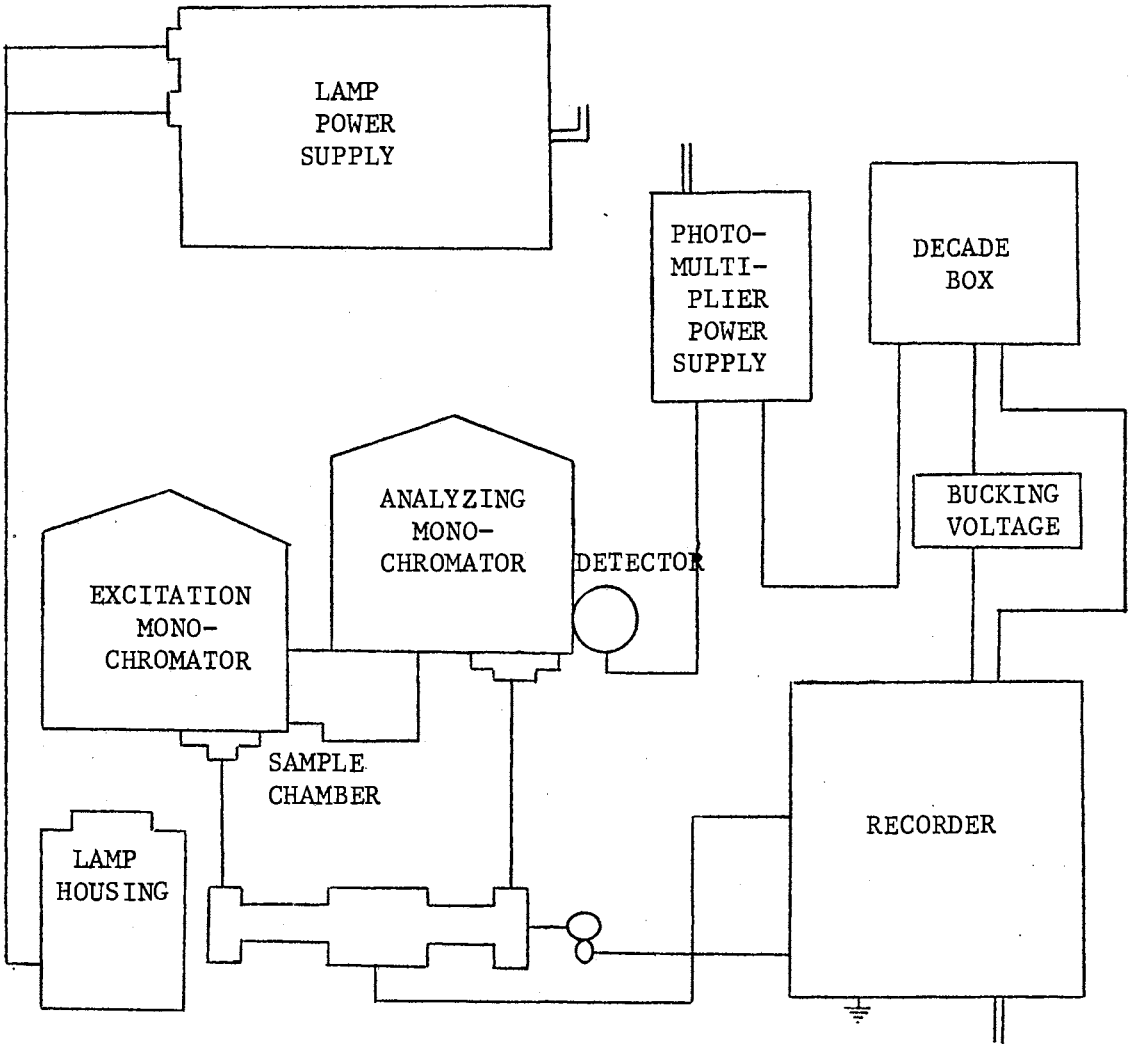

Figure 31. Block Diagram of Farrand Spectrofluorometer With Modifications

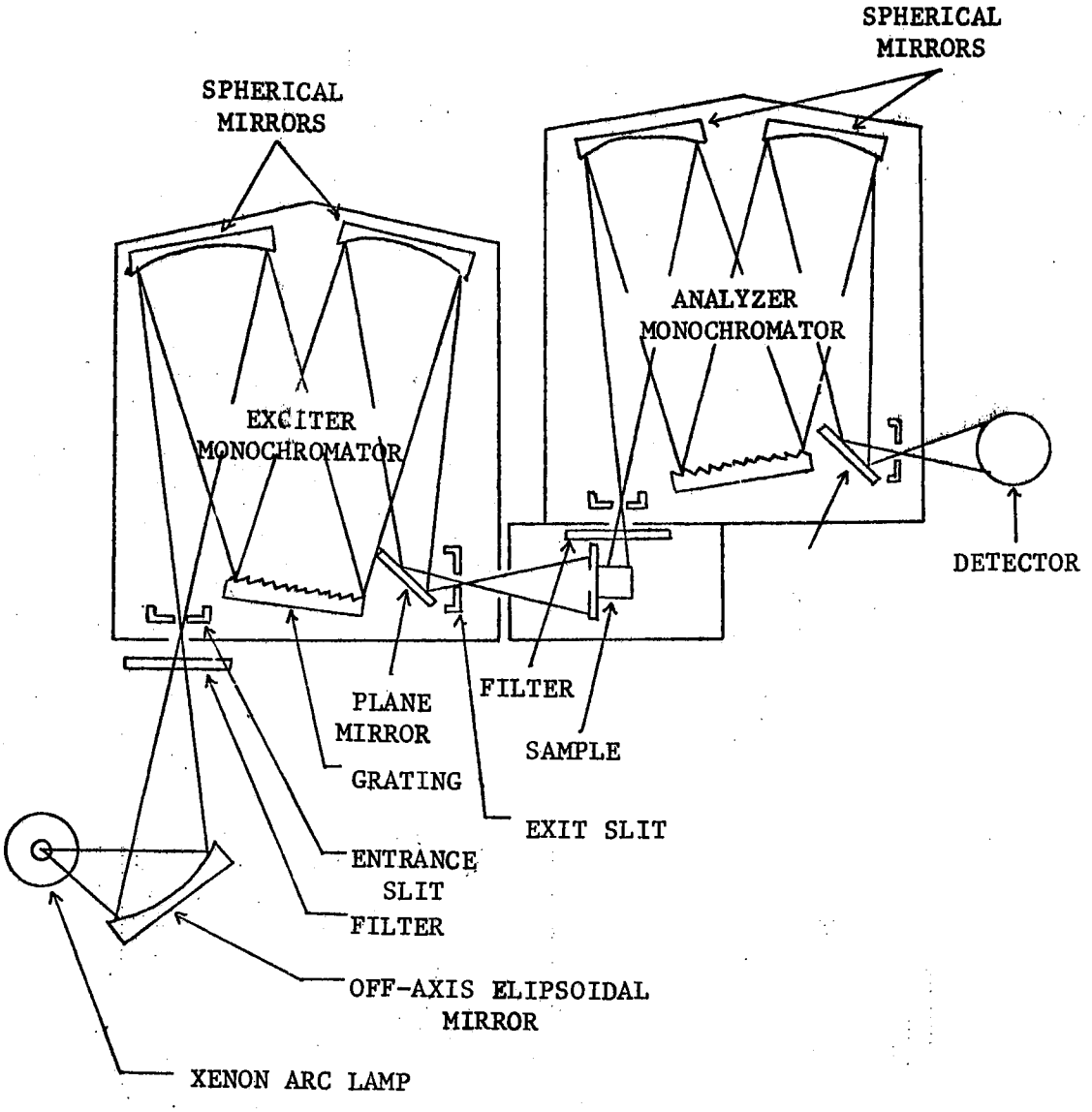

Figure 32. Optical Diagram of Spectrofluorometer

The photomultipliers available are interchangeable 1P21 and 1P28 photomultiplier tubes. In this experimentation the ultraviolet sensitive 1P28 photomultiplier was used. The photomultiplier power supply is an A.C. line operated type. It provides 400 to 900 volts D.C. The power supply was operated at maximum voltage.

For automatic operation a Texas Instruments Model PWS ServoRiter recorder with a 10.0 mv scale and 0.5 second response time was used. A Heathkit Model EVW-30 decade resistance box connected in parallel between the recorder and microammeter allowed additional selectivity in signal attenuation. Bucking voltage was applied to the signal as a zeroing device.

The wavelength calibration of the monochromators was checked with a General Electric G4T4-1 mercury lamp. The lamp is placed in the sample compartment with the Xenon lamp off. The peaks from the mercury lamp are quite sharp and can be compared with literature values. It was found that the wavelength accuracy was 1 mm or better in all cases.

Quinine sulfate in dilute (0.1 N) sulfuric acid was used to check the instrumental sensitivity. Quinine sulfate (Sargent-Welch) was used without further purification. Sulfuric acid was prepared by dilution of concentrated reagent sulfuric acid (Fisher). Concentrations of 10^{-5} g/ml were barely detectable with concentrations of 10^{-3} g/ml readily detectable.

TABLE XII

INSTRUMENTAL SENSITIVITY TO QUININE SULFATE

Concentration µg/ml	Fluorescence (on relative scale of 100)
10^{-1}	Off scale
10^{-2}	44
10^{-3}	25
10^{-4}	9
10^{-5}	2

APPENDIX C

FLUORESCENCE CHARACTERISTICS OF SELECTED COMPOUNDS[57]

Compound	Activation Peak nm	Fluorescence Peak nm
acetylcolchinol	265	380
achromycin	390	515
adenine	280	375
adenosine	285	395
adenylic acid	285	395
adrenalin	295	335
allylmorphine	285	355
p-aminobenzoic acid	295	345
p-aminosalicylic acid	300	405
aminopterin	280,370	460
amytal	265	410
aniline	280 (240)	340
anisole	270 (220)	300
anthranilic acid	300	405
1-arterenol bitartrate	280	320
ATP	285	395
aureomycin	355	445
azaguanine	285	405
benzanthracene	280,340	390,410
benzanthrene	245,325,340	385,400
benzo[j]fluoranthrene	295,330,385,405	480,515
benzoin	370	480
benzperylene	305,375,395	430
brominated LSD	315	460
pyrocatechol	270 (220)	325
2,3-dihyroxybenzoic acid	305 (240)	440
chloroquine	335	400
chlorpromazine	350	480
chlorpromazine sulfoxide	335	400
chrysene	250,300,310	360,380
cinchonidine	315	445
cinchonine	320	420
m-cresol	280 (220)	305
o-cresol	275 (220)	305
p-cresol	285 (230)	315
dibenzo[def,mno]chrysene	310,335,390,410	480,510
dibenzo[rst]pentaphene	255,290,300,395,415	455,485
dibenzo[def,p]chrysene	230,285,305,325,340	440,470

Compound	Activation Peak nm	Fluorescence Peak nm
Be complex of 1,4-dihydroxyanthraquinone	530,570	630
2,5-dihydroxybenzoic acid	290	340
3,4-dihydroxybenzoic acid	280 (230)	330
2,3-dihydroxyindole	315	400
3,4-dihydroxyphenethylamine	285	325
3,4-dihydroxyphenylacetic acid	280	330
3,4-dihydroxyphenylalanine	285	325
3,4-dihydroxyphenylserine	280	320
dimethylaniline	255 (285)	370
DPNH	340	435
dromoran	275	320
Be complex of 1-amino-4-hydroxyanthraquinone	530-560	620
Th complex of 1-amino-4-hydroxyanthraquinone	550-580	660
epinephrine	295	335
l-epinephrine bitartrate	280	320
equilenin	250,290,340	370
equilin	290	345,420
eserine	300	360
estradiol	285	330
estrone	285	325
N-ethylharmine	300,365	450
Zr complex of flavonol	400	465
flexin	280	320
folic acid	365	450
folinic acid	370	460
2,5-dihydroxybenzoic acid	325 (235)	455
guanine	285	365
guthion	250,312	380
harmine	300,365	400
homogentisic acid	290	340
homovanillic acid	270	315
3-hydroxyanthranilic acid	320	415
5-hydroxyanthranilic acid	340	430
p-hydroxycinnamic acid	350	440
p-hydroxycinnamic acid	350	440
o-hydroxycinnamic (cis) (coumarinic)	365 (280)	510
o-hydroxycinnamic (trans) (coumaric)	365 (380)	500
o-phenylphenol	320 (250)	420
p-phenylphenol	310	410
indol-5-ol	290	355
5-hydroxyindoleacetic acid	300	355
3-hydroxykynurenine	365	460
5-hydroxykynurenine	375	460
p-hydroxymandelic acid	300	380

Compound	Activation Peak nm	Fluorescence Peak nm
3-hydroxy-4-methoxyhomovanillic acid	270 (225)	315
p-hydroxyphenethylamine	285	325
p-hydroxyphenylacetic acid	280	310
p-hydroxyphenylpyruvic acid	290	340-350
p-hydroxyphenylserine	290	320
indole	280	350
indoleacetic acid (in methanol)	285	345
indoxyl	310	395
indoxyl acetate	285	375
kynurenic acid	325	405
kynurenic acid	325	440
kynurenine	370	490
LSD	325	465
"McNiel 485"	280	320
menadione	335	480
methotrexate	280,375	460
methylaniline	290 (240)	360
1-methylfluorene	280,290,365	460-480
2-methyl indole	280	355
1-naphthaleneacetamide (in methanol)	230,286	327
1-naphthaleneacetic acid	230,282	325
1-naphthol	330 (250)	480
2-naphthol	350 (280)	460
1-naphthacene	290,310	480,515
neocinchophen	275,345	455
neosynephrine	270	305
norepinephrine	295	335
oxindole	300	345
oxychloroquin	335	380
Li complex of oxine	370	580
paredrin	275	300
pentobarbital	265	440
pentothal	315	530
phenobarbital	265	440
phenol	270 (215)	310
piperonyl butoxide (in methanol)	248,292	320
piperoxan	290	325
plasmoquin	300,370	530
podophyllotoxin	280	325
potasan (in methanol)	320	385
procaine	275	345
procaine amide	295	385
3,4-dihydroxybenzoic acid	300	370
pteroic acid	365	450
4-pyridoxic acid lactone	360	440,445
pyridoxal	330	385
pyridoxamine	335	400

Compound	Activation Peak nm	Fluorescence Peak nm
pyridoxine	340	400
quinacrine	285,420	500
quinine	250,350	450
hydroquinone	285 (220)	340
rescinnamine	310	400
reserpine	300	375
resorcinol	265 (215)	315
2,6-dihydroxybenzoic acid	340-370	455
2,4-dihydroxybenzoic acid	295 (250)	400
riboflavin	270,370,445	520
salicylic acid	310	435
3-hydroxybenzoic acid	315 (250)	425
4-hydroxybenzoic acid	295	350
serotonin	295	340
skatole	290	370
surital	310	530
synephrin	270	310
terramycin	390	520
thiophenobarbital	280	470
thiochrome	370	445
thymol	265	300
tocopherol	295	330
tolserol	280	315
3,4,5-trimethoxybenzoic acid	270 (295)	375
tryptamine	290	360
tryptophan	285	365
tyramine	275	310
tyrosine	275	310
uric acid	325	370
vitamin A	325	470
vitamin B 12	275	305
warfarin (in methanol)	320	385
xanthine	315	435
xanthurenic acid	350	460
yohimbine	270	360
2,4-xylenol	285	
2,6-xylenol	275 (230)	305
3,4-xylenol	280 (230)	310

APPENDIX D

BASIC VARIABLES IN LIQUID CHROMATOGRAPHY AND SOLVENT EXTRACTION

Symbol	Name	Description	Desired Range
K	Distribution coefficient	Related to extraction efficiency	>100
V_w	Volume of raffinate	-	-
V_o	Volume of extractant	-	-
R_s	Resolution	Resolving power of system for adjacent peak	1.0 → 2.0
k'	Capacity factor	Retention characteristics of chromatographic system	0.2 → 10
α	Relative retention	Resolving power of system	>1.10
H	Height equivalent to theoretical plate	Efficiency of system	Small as possible (.08-3.0 mm)
N	Number of plates	Separation capabilities of system	Large (>50)
N_{eff}	Number of effective plates	Effective separation characteristics of system	Large (>50)
ΔP	Pressure drop	Required system pump pressure	100-8000 psi (1000 maximum for present system)

t_R	Retention time	Elution time	Fast as possible (20 sec.-1 hr.)
V_R	Retention volume	Elution volume	-
W_R	Peak width	Column broadening	Narrow (10 sec.-1 hr.)
d_p	Particle diameter	-	Small
v	Mobile phase velocity	-	Fast as possible (2 ml/min maximum for present system)

VITA

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Doctor of Philosophy

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