METHODS FOR IMPROVED DETECTION AND SEPARATION IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

METHODS FOR IMPROVED DETECTION AND SEPARATION IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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This dissertation consists of two parts that are related by the need for methods to facilitate the analysis of complex samples. Two different approaches have been taken. The first area of research involves increasing the sensitivity and selectivity of the detector through post-column derivatization. The second approach has been to improve chromatographic separation by recycle chromatography.

An on-line post-column reaction detector for phenols has been developed. This system links together two highly versatile techniques - high performance liquid chromatography and air segmented continuous flow analysis. The resulting system contains the combined advantages of each system. The reaction employed involves the coupling of phenol and diazotized sulfanilic acid to form an azo dye. The optimum reaction conditions were initially determined by a univariate method and subsequently by simplex optimization. Other studies performed included compatibility checks between the post-column reaction detector and HPLC systems. The application of this detector to the determination of phenols in various sample matrices has been described to illustrate its versatility.

A new approach to recycle chromatography, recycle heartcut chromatography, has been developed for applications involving complex sample matrices. In this approach, a heartcut is taken from an eluting peak which is then recycled onto the column. The sequence of heartcut and recycle is repeated until an 'adequate' separation is obtained. This technique has been applied to a number of applications to demonstrate its potential. The results show small improvements in resolution and simpler chromatograms due to the isolation of the components of interest for further separation. The amount of band broadening was studied as a function of the heartcut loop tubing dimensions. A microcomputer controlled HPLC system with time and detector-based heartcutting capabilities was employed.

To my family,

for all their love and support.

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CHAPTER I

INTRODUCTION

The determination of compounds in complex sample matrices often poses a formidable challenge to the chromatographer. Complications can arise in both the separation and detection stages. They include chemical and spectral interferences, even after an extensive sample workup; inadequate resolution, which requires the use of an elaborate separation scheme; and a lengthy overall analysis time. Two solutions to these difficulties are investigated in this work. In one approach, methods for increasing detector selectivity and sensitivity are studied, while in the other, a new technique for improving chromatographic separation is investigated. The methods that are employed here to achieve these objectives are post-column derivatization and a new form of recycle chromatography. The bases of these methods are discussed in the following sections.

A. REACTION DERIVATIZATION IN LIQUID CHROMATOGRAPHY

A limitation encountered in modern liquid chromatography is the lack of sensitive and specific detectors in applications involving complex sample matrices. Conventional HPLC detectors, such as the UV-visible and refractive index detectors, often lack the selectivity and sensitivity required for such samples. Fluorescence and electrochemical detectors can be more selective and sensitive, but they are not as widely

applicable and are more susceptible to interferences. These problems may be circumvented by reacting the analyte to form a derivative with different and/or enhanced characteristics. Reaction derivatization may be divided into two categories depending on the objectives of the derivatization. If the goal is to alter the separation characteristics, pre-column derivatization is utilized. If the purpose is to enhance the detection, the situation calls for post-column derivatization, which is the method employed in this work.

1. Pre-Column Derivatization

Pre-column derivatization involves a chemical reaction of the sample components prior to separation. This method has been extensively employed in gas chromatography (1,2) before its application to liquid chromatography (1,3,4). The major advantage of pre-column derivatization over post-column derivatization is greater freedom in the selection of reaction conditions. The primary disadvantage is the possible formation of multiple products, which would further complicate a separation, especially one involving a complex sample matrix. Also, a different separation scheme may have to be developed for these derivatives.

2. Post-Column Derivatization

Post-column derivatization involves a chemical reaction of the sample components after chromatographic separation and prior to detection. Selective reagents can enhance detectability of the desired

compounds by forming derivatives which are more readily detected and/or possess more distinct spectral characteristics. The presence of different substituent groups on the reagent as well as the reactant can impart different spectral characteristics to the derivatives, thus making reaction detectors very flexible and readily tailored to meet the needs of a particular situation. The reaction conditions such as pH and time, may also be used to obtain an additional degree of selectivity. Other advantages of post-column reaction (PCR) detectors are: (1) in comparison to pre-column derivatization, the reaction does not need to go to completion, which results in reduced analysis time and decreased band broadening; (2) different types of detectors can be used in series with the post-column reaction detector and; (3) since the compounds are separated in their original form, separation procedures from the literature can be employed.

Disadvantages of the PCR detector arise mostly from the incompatibilities between the separation and detection systems. The solvents required for the optimum chromatographic separations are often not suitable as reaction media. This can result in precipitation or decomposition of the reagent, mixing problems arising from viscosity differences and lower detector response due to altered reaction kinetics and mechanisms. Other incompatibilities that may be observed are the flow rates of the two systems and the chemical inertness of the PCR detector materials to the separation solvents. Thus, these potential incompatibilities have to be investigated in the development of a PCR detector. Despite these complications, the benefits of post-column derivatization are numerous and it has become a widely used method.

3. Post-Column Reaction Detection of Phenols

Phenols are an important class of widely used chemicals consisting of a variety of substituted compounds, some of which have undesirable characteristics. They are present in many industrial effluents and occur as a consequence of environmental transformation of both natural and synthetic chemicals. Chlorinated phenols are considered to be water pollutants as they impart a disagreeable taste to drinking water. The presence of phenols in petroleum products such as paint and varnish thinners causes a retardation in the drying rate of these products. On the desirable side, phenols are used as antiseptics for cleaning purposes as well as in medications. They have also been used extensively as antioxidants in fuels to prevent gum formation and in food products as preservatives.

Due to the presence of phenols, wanted or unwanted, numerous analytical methods have been reported for the qualitative and quantitative analysis of phenolic compounds (5-10). Some of these have involved post-column derivatization of phenols with fluorescence and electrochemical detection (8-10). Some of the problems distinctive to these detection methods are quenching, as witnessed by Wolkoff and Larose (8), and electrode fouling. UV-visible absorption is an extremely versatile and extensively used detection mode because a large number of compounds absorb in this region of the spectrum. Also, the detectors needed for routine HPLC work are generally of a simple and inexpensive design. For these reasons, it is surprising that there have been no reports of on-line post-column reaction systems with colorimetric detection for phenols.

The primary objective of this research has been to develop a colorimetric based reaction detector for use in conjunction with HPLC in the determination of phenols. The characteristics of the ideal PCR detector include on-line detection, high sensitivity and selectivity, high precision and accuracy, total compatibility with the separation system, no band broadening and high sample throughput. With these goals in mind, a post-column reaction detector was developed for the colorimetric determination of phenols. A detailed account of this work is given in Chapter III.

B. RECYCLING METHODS IN LIQUID CHROMATOGRAPHY

In the following discussion, a review of the various aspects of chromatographic resolution is provided. The parameters that govern resolution and the changes in experimental conditions that need to be made to achieve an improvement in resolution are described. The situations or types of separation problems that are best handled by the use of recycle chromatography are then presented along with a systematic approach for obtaining improved resolution. Finally, this discussion provides a basis for the introduction of recycle heartcut chromatography.

1. Resolution Enhancement in Liquid Chromatography

Resolution is a quantitative measure of the degree of separation between two adjacent bands. The fundamental relationship between resolution and parameters which in turn can be related to experimental conditions is given by the equation:

$$R_s = (1/4)\ (\alpha - 1)\ N^{1/2}\ \frac{k'}{k' + 1} \qquad \text{Equation 1.1}$$

The resolution, R_s, is expressed as a function of the separation selectivity, α, the separation efficiency as expressed by the number of theoretical plates, N, and the capacity factor, k'. The experimental conditions that can affect each of these parameters are listed in Table 1.1.

A flow diagram outlining the routes for improving resolution is shown in Figure 1.1. This diagram, representing one school of thought, utilizes the capacity factor as the point of origin. This is due to the ease with which k' can be altered by changing the solvent strength. As k' is increased, R_s would also increase as shown in Equation 1.1. However, this is accompanied by an increase in retention time, R_t, and peak width, t_w. Eventually a point is reached where the benefits of improved resolution are offset by the longer separation times and the increased peak width. Also, from Equation 1.1, it is observed that the fraction, k'/(k' + 1), approaches unity asymptotically as k' increases. Thus, the gain in R_s becomes increasingly smaller. The optimum range of k' values, as suggested by Snyder (12), is between 1 and 10. The first question of the flow diagram verifies this condition. If k' is not in

Table 1.1. Factors that affect α, N and k'.

Parameter	Symbol	Method of Variation
Separation selectivity	α	1. Mobile phase 2. Stationary phase 3. Mobile phase pH 4. Temperature 5. Special chemical effects
Separation efficiency	N	1. Solvent velocity 2. Column length 3. Column packing
Capacity factor	k'	1. Solvent strength

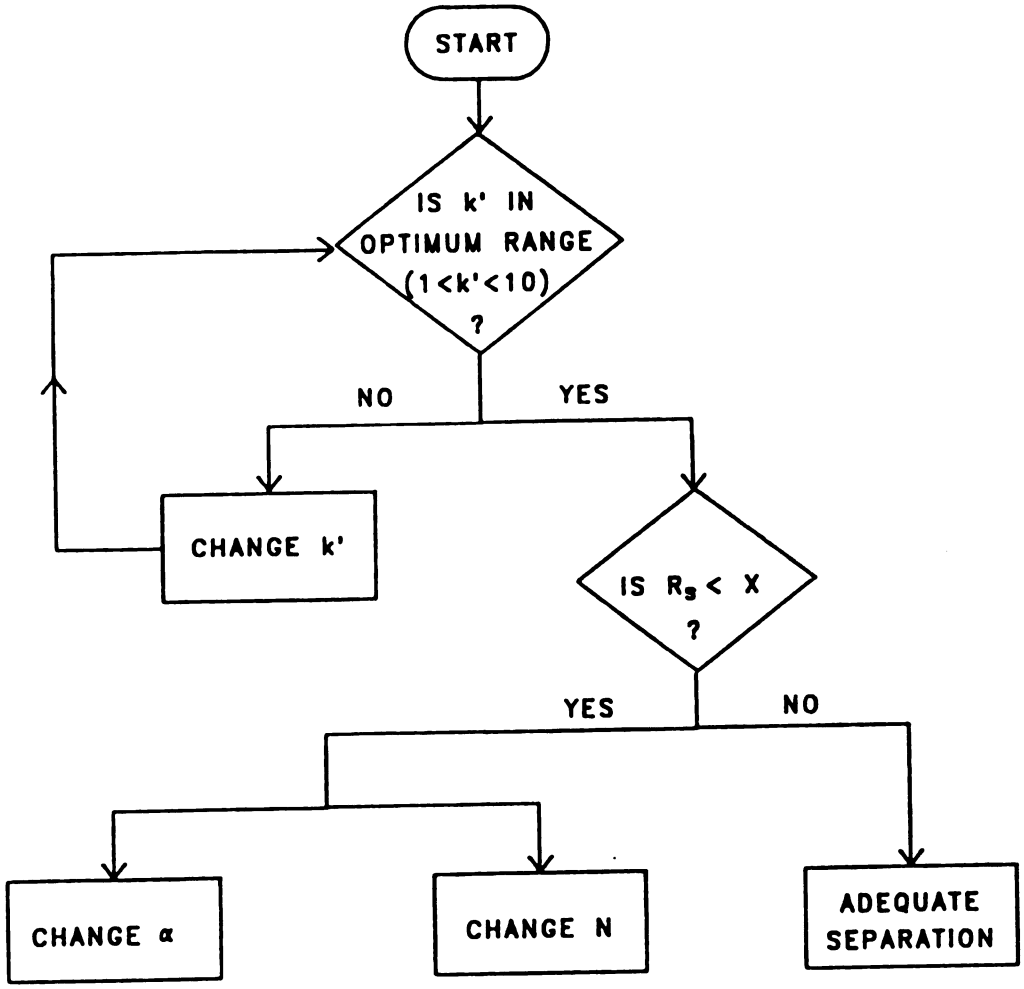


Figure 1.1. Routes for improving chromatographic resolution.

this range, improvements in resolution should be achieved by first changing the solvent strength. If k' is already in the optimum range and an additional gain in resolution is still needed, the next parameters to be considered are α and N. The only clear cut situation which calls for a change in α rather than N is when $(\alpha - 1)$ is equal to zero. Under this circumstance, $R_s = 0$, and any changes in N would prove ineffective. The separation selectivity is a difficult parameter to vary, especially when dealing with complex samples, because the prediction of the resulting effects on the separation is an intricate procedure. This often results in a trial and error type of approach to obtain satisfactory separation. The number of theoretical plates, on the other hand, produces a much more straightforward effect on the resolution and is often a simpler route to take.

The number of theoretical plates can be increased by employing columns with smaller size packings, decreasing the flow rate and increasing the column length. The first method may not be a feasible choice especially if the column being used already contains the smallest size particles that current column technology offers. The second alternative, decreasing the flow rate, provides a limited gain in the number of theoretical plates. This can be explained by recalling the equation, analogous to the Van Deemter equation for GC, that was derived by Snyder (13) for LC

$$H = Au^{0.33} + \frac{B}{u} + Cu + Du$$

where H is the theoretical plate height, u is the flow rate and A, B, C and D are constants for a given column. A plot of H vs u for a liquid chromatographic column containing small porous packings (8 μm) shows the characteristic plot with an optimum flow rate. Flow rates higher or

lower than this optimum result in an increase in theoretical plate height. The third alternative, increasing the column length, may be accomplished by using a longer column, by coupling of columns in series or by recycling the sample through the same column. The coupling of multiple columns in series results in an expensive system as well as one with high column inlet pressures. The latter is not a desirable consequence, particularly, when working with columns containing packing materials that cannot tolerate high pressures. An example of this is the gel-based packing used in size exclusion chromatography columns. Under these circumstances, recycle chromatography is the method of choice.

To summarize, recycle chromatography is a technique that should be used when: (1) the path chosen to achieve a gain in resolution is through increasing the number of theoretical plates; (2) if changing the column packing and mobile phase flow rate are not possible or show no improvement in resolution; and (3) if multiple column lengths have to be employed and an increase in column back pressure is undesirable.

2. Recycle Heartcut Chromatography

The analysis of complex samples can be facilitated through modifications to the separation system that would result in an improved chromatographic separation. As discussed in the previous section, one route to improving resolution is through increasing the number of theoretical plates. This, in turn, can be accomplished with recycle chromatography. However, a problem with recycle chromatography is the eventual overlap of early and late eluting peaks as the sample plug broadens to occupy an entire column volume. Thus, samples containing

components representing a broad k' range (e.g., complex samples) may not be successfully separated because of the resulting limitation on the number of recycles.

The objective of this research was to develop a separation system that incorporated the method of increasing resolution used by recycle chromatography and was applicable to the determination of specific compounds in complex matrices. Some of the desirable characteristics are versatility, simplicity, ease of implementation and inexpensiveness. Such a system, recycle heartcut chromatography, has been developed as described in detail in Chapter IV.

CHAPTER II

HISTORICAL BACKGROUND

This chapter presents the historical background for the research described in this dissertation. The first section provides a brief discussion of the development and basic principles of air segmented continuous flow analysis, the technique employed in this post-column detection work. An extensive literature review of post-column derivatization from its early days to the present is then presented. This is followed by a short survey of the colorimetric reactions which have been used in the determination of phenols. The last section contains a description of the research that has been performed in the area of recycle chromatography.

A. CONTINUOUS FLOW ANALYSIS

The purpose of the following discussion is to provide a brief review of the historical development and basic principles of CFA. Due to the breadth of the subject matter and the existence of a number of reviews (14-18), this discussion will focus primarily on the early years of CFA and the basic principles and developments that have made CFA suitable for use in post-column reaction detection.

The need for methods that would alleviate the ever increasing load of samples to be analyzed in the clinical laboratory prompted the introduction of continuous flow analysis in the 1950's by Skeggs (19,20).

In this thesis, the term continuous flow analysis (CFA) is used to describe the technique that uses a network of tubing to perform the sequential analysis of samples in a flowing liquid stream segmented at regular intervals by air. Continuous flow analysis involves the uninterrupted treatment and monitoring of a train of samples contained in a network of tubing. These samples can be diluted, mixed with various reagents, heated, dialyzed, extracted and delivered to the detector without operator intervention. This sequential analysis of samples in a flowing stream provided the desired increase in sample throughput without the use of complex instrumentation. In contrast to the batch analyzers used at the time, the automated continuous flow analyzers had very few moving parts, the liquid being the moving component of the system. As a result, the latter was mechanically simpler, easier to construct and less expensive. This form of automated chemical analysis proved to be a flexible way to perform operations that would be encountered in a routine chemical analysis. The concept and prototype instruments that were built by Skeggs were purchased by Technicon Corporation, in 1956. The following year, the first CFA analyzer was marketed under the trade name, Autoanalyzer. This marked the beginning of a line of instruments that were to become widely used in clinical and industrial laboratories.

In order to present a complete picture, it is necessary to mention the related technique of flow injection analysis (FIA). Until the early 1970's, air segments were considered necessary for successful analyses in flow streams, and the term continuous flow analysis referred exclusively to air-segmented CFA systems. In the mid-1970's, two independent groups of researchers, Stewart and coworkers (21), and

Ruzicka and Hansen (22) demonstrated the potential of nonsegmented continuous flow analysis. It was shown that if the dispersion observed in CFA could be controlled and reproduced, nonsegmented streams could be used for analytical purposes. This technique, presently known as flow injection analysis (FIA), has grown tremendously in popularity in the last decade. A book on this subject has been written by Ruzicka and Hansen (23) and numerous reviews have been published (24-28). The relative performance of CFA and FIA have been discussed in a number of papers (16,29-33).

A major problem that was encountered by Skeggs in his early experiments with nonsegmented flow streams was axial dispersion. This spreading of the sample bands along the flow axis placed a limit on the throughput rate because the samples had to be separated by a minimum distance. The effect of air bubbles on axial dispersion was a serendipitous discovery that resulted in an important breakthrough by Skeggs. The output of the two types of flow streams are compared to that of the ideal case in Figure 2.1. The sample and wash solutions are alternately introduced into the flow stream, which produces the rectangular input. Under ideal circumstances, the peak profile at the output would be identical to that at the input (Figure 2.1a) i.e., the introduction of a rectangular sample plug should yield an identical rectangular output. When dispersion takes place, the rise and fall of the sample peaks are no longer instantaneous and a Gaussian peak shape is obtained (Figure 2.1b) as in the case of a nonsegmented flow stream. (The sample zone spreads as it moves downstream and changes from the original square plug to an asymmetric shape and eventually to the Gaussian form.) Figure 2.1c shows the characteristic 'flat top'

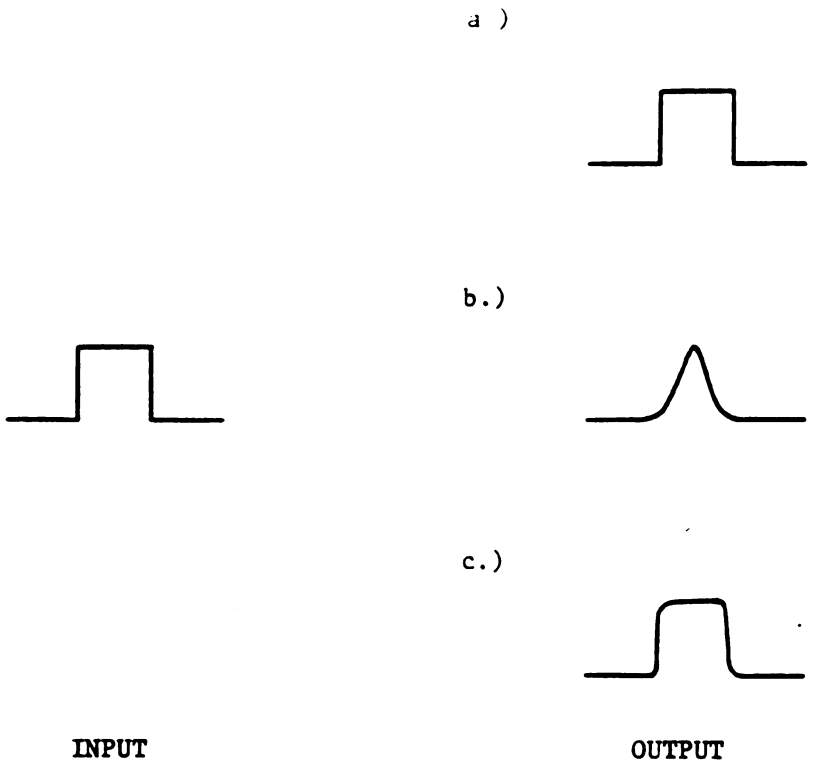


Figure 2.1. Continuous flow analysis output for three different cases a.) ideal b.) nonsegmented and c.) air segmented.

output for CFA. The dispersion observed is less than that for the nonsegmented case, but is significant when compared to the ideal case.

The differing peak shapes obtained for the nonsegmented and air segmented cases can be explained by examining the sample plug profile as it flows through a narrow open tube (Figure 2.2). When the stream is nonsegmented, (Figure 2.2b), significant axial dispersion of the sample is observed. This is due to the parabolic velocity profile that is characteristic of laminar flow (34). The liquid in the center of the tube is moving at approximately twice the mean speed of the fluid while the liquid layers near the wall are moving at progressively slower velocities. The resulting dispersion is counteracted by radial diffusion. (A radial concentration gradient is created by this process, which in turn, causes radial diffusion. The molecules along the walls diffuse into the center of the tube and vice versa. Thus, the sample plug is not dispersed throughout the tube.) When the flow stream is segmented with air, as in Figure 2.2a, there is a significant decrease in dispersion because the air bubbles act as physical barriers that break up the laminar flow profile. In addition, the presence of the air segments introduces a toroidal flow within each liquid segment as illustrated in Figure 2.2a. These circular motions result in homogeneously mixed liquid segments (35,36).

The use of air segmentation successfully reduces the axial dispersion that is caused by laminar flow. However, in CFA, there is a second source of band broadening which arises from the transference of small amounts of sample from one segment to the next via the liquid layer that wets the tube wall. This dispersion causes, in part, the nonideality of the CFA output shown in Figure 2.1c. Equations have

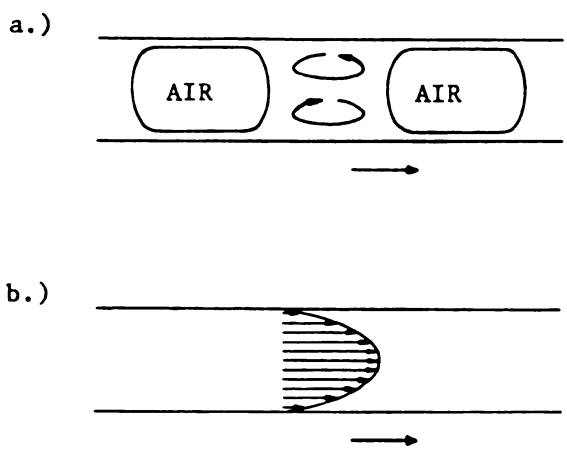


Figure 2.2. Flow profiles for a.) air segmented and b.) nonsegmented flow streams.

been developed to describe the dispersion observed in the flow stream and to relate them to experimental parameters (35,36). Axial dispersion can be minimized by the use of shorter lengths of tubing, high flow rates and high segmentation frequencies. Slow mass transfer between the stagnant liquid film and the liquid segments, on the other hand, is fundamental to air segmented CFA, as it is currently practiced. The liquid film cannot be eliminated without having detrimental effects on the hydraulic stability of the segmented stream. Nord and Karlberg (37) have studied the effect of different materials that are used for constructing manifold components on dispersion.

Since the conception of CFA, there have been a large number of developments in the instrumentation used in this field. Patton (17) has traced the development of continuous flow analyzers, in particular the Autoanalyzer, from the initial one channel unit to the present day computer controlled, multichannel, multifunction system. Furman (15) gives a detailed account of the technological advances made for each component of a continuous flow analyzer e.g., pumps, detectors, flow cells, tubing materials, and dialyzers. One important advance that should be specifically mentioned is the bubble gate. The feature of air segmented CFA that is often referred to as a disadvantage or complication is the removal of the air segments for the purposes of spectroscopic detection. The early methods that employed physical removal of the air bubbles from the flow stream prior to detection (38) resulted in significant amounts of dispersion because of mixing in the debubbler chamber and in the tube between the point of debubbling and detection. In addition, a fraction of each liquid segment is lost with each bubble removal. An alternative method to physical debubbling is

to remove the bubbles electronically by gating the signal from the detector. Habig et al. (39) designed a bubble gating flow cell that employed a conductance-activated relay to detect the presence of air bubbles in the flow cell. When the conductivity was below a preset value, the relay opened and disconnected the power supply from the recorder servo motor, thus inactivating the pen. In 1974, Neeley and coworkers (40) introduced an electronic bubble gate, which they modified as reported in a subsequent paper (41). This bubble gate made use of the difference in the colorimetric detector output when an air bubble enters the flow cell e.g., erratic spikes are observed instead of a constant signal. Two measurements were made within a time interval and their values compared by a window comparator circuit. A difference between the two signals that is within the set limits of the comparator, indicates that the flow cell is filled with liquid. The real time signal was then stored and output to a chart recorder.

In 1982, Patton and coworkers (17,43) described the design and development of a simpler bubble gate unit that monitored the transmittance of the flow stream for the presence of an air segment. The periodic fluctuations of the detector signal as air and liquid segments passed through the flow cell were used to synchronize data acquisition and storage. When an air segment traverses the flow cell, it reflects most of the incident light and the transmittance approaches zero. A comparator with a set threshold is used to determine whether air is present in the flow cell. Measurements were made only when the transmittance was above the threshold value. This gating of the signal from the detector eliminates the problems previously observed when the air bubbles were physically removed.

In post-column reaction work, it is desirable to have a system that introduces an insignificant amount of band broadening to the chromatographic peak. A second desirable trait is good mixing between the reagents and the sample. These features, characteristic of CFA, are very important, especially for post-column reaction detectors that employ reactions with multistep addition of reagents and long delay times. The advantages in using CFA will become evident as the work done for this dissertation involving post-column reaction detection is discussed in Chapter III.

B. POST-COLUMN REACTION DETECTORS

Post-column derivatization has become an extensively used technique for improving the detection characteristics of a selected analyte. There have been numerous reviews assessing the current status of reaction detectors in liquid chromatography (44-54) and publications reporting new applications. Several comprehensive books have been written on this topic including those by Krull (55), Lawrence and Frei (56,57) and Blau and King (1). Jupille (58), in his article on UV-visible absorption derivatization in liquid chromatography, provides a listing of manufacturers who provide reagents and technical support for derivatization techniques. More recently, reaction units constructed specifically for post-column derivatization have become commercially available (59). The chemistry developed for Technicon Autoanalyzers (60) has often been combined with known or newly developed LC separation techniques to produce new post-column reaction detectors.

The applications of post-column derivatization have been so extensive that it is not practical to report, in this thesis, all the work that has been done. Instead, some representative examples are given in Table 2.1 to demonstrate the broad range of possibilities of this technique. Reviews have been published discussing the role of chemical derivatization in the areas of pharmaceuticals (72), pesticides analysis (73), food additives (74) and nuclear materials (75). Post-column derivatizations have also been used in conjunction with a number of detection modes. Kissinger et al. (76) and Krull and coworkers (77) have reviewed the role of PCR detectors in electrochemistry. Lawrence (78) and Reh and Schwedt (79,80) have presented overviews of chemical derivatization techniques coupled with fluorescence detection. The possibility of post-column derivatization with detection by mass spectrometry, nephelometry and flame and plasma spectrometry are discussed by Frei (81).

Numerous developments in the area of post-column reaction detectors have resulted from the need for selective and sensitive detectors and the efforts to overcome the disadvantages of this type of detector. As previously mentioned, these shortcomings include band broadening due to inappropriate choice of reactor and poor mixing between sample and reagents, and dilution of the sample. One method of reducing band broadening is through the use of an appropriate reactor design based on the kinetics and complexity of the reaction. The three commonly used reactors are the open tubular reactor, packed bed reactor and segmented stream reactor. The theoretical aspects of band broadening and the performance of each type of reactor have been discussed in detail by a number of authors (52-54,82-84)).

Table 2.1. Applications of post-column reaction detectors.

Analyte	Reagent	Reactor Type	Detection Mode	Ref
Organic acids	Ce^{4+}	PBR	F	61,62
Penicillin	Imidazole	SSR	UV-Vis	63
Carbamates	hydrolysis, o-phthaldehyde	OTR	F	64,65
Phenolic ethers	Br_2	OTR	EC	66
Sugars	Copper phenanthroline	OTR	EC	67
Phenolic glycosides	ß-glucoronidase	SPR	EC	68
Cholesterol	Cholesterol oxidase	SPR	UV	69
Cocaine	hν	OTR	EC	77
Enzymes	various	PBR	F	71

OTR - open tubular reactor
PBR - packed bed reactor
SSR - segmented stream reactor
SPR - solid phase reactor

F - fluorescence
UV - ultraviolet
Vis - visible
EC - electrochemical

The open tubular reactor (OTR) is the simplest reactor and is usually made from stainless steel or Teflon tubing. These reactors can be straight, coiled or knitted. (A knitted OTR consists of a piece of PTFE tubing that has been knitted into a series of figure eights. The two loops of the 8 occupy planes that are at a 120° angle from each other, thus yielding a three dimensional structure of half loops. Due to the increased radial mass transfer, band broadening is decreased.) It has been reported that the latter two configurations result in reduced band broadening due to a secondary flow effect (85-87). Open tubular reactor designs are usually recommended for fast (< 30 s) reactions. However, Engelhardt (86) has shown that it is possible to use a knitted OTR for a reaction lasting several minutes.

Packed bed reactors (PBR) have been recommended for reactions with intermediate kinetics (0.5 - 4 min) (88-92). They are usually made from glass or stainless steel columns (1 mm i.d.) that are packed with small nonporous glass beads (40 - 150 μm). These reactors resemble liquid chromatography columns with no retentive properties. The sources of band broadening in these reactors are similar to those encountered in LC. However, because of the absence of a retention mechanism, the equations describing dispersion in PBR are much simpler (88,93).

Segmented stream reactors (SSR) have generally been recommended for use with slow reactions (up to 20 min) or in place of packed bed reactors. However, Scholten et al. (83) have shown, in a comparison of the three post-column reactor designs, that not only can segmented stream reactors be used for fast reactions, but they also yielded the least amount of band broadening. Segmented stream

reactors are based on segmentation of the flow stream with either air or an immiscible solvent. As previously discussed, the segmentation reduces dispersion by inhibiting laminar flow and at the same time enhances mixing within each sample containing segment. Dispersion in an air-segmented reactor can be approximated by the equation derived by Frei and Scholten (54). A more detailed equation, derived by Snyder (35,36), takes into account various experimental parameters specific to the reaction system, such as viscosity, liquid surface tension and sample diffusion coefficient. The use of immiscible solvents for segmentation has opened up a new area in PCR detection. Aside from reducing sample dispersion, the immiscible solvent segments can be used as reagent carriers, reaction media and as an extraction media (94-99). Apffel et al. (100) have used a solvent segmentation system for post-column cleanup in the analysis of urine for antineoplastic agents. The column effluent was segmented with dichloroethane which served as an extraction medium for the analyte. The two phases were separated on-line, and the dichloroethane layer containing analyte was directed to the detector. This system, which is not a reaction detector in the strictest sense, allows the urine sample to be used without any other sample pretreatment. Other areas to which these two phase reactors have been applied are LC/MS (101) and LC/GC (102). The objective in both cases was to make the analyte more compatible with the detection systems.

In using immiscible solvents for segmentation, the problem of compressibility of gases is not encountered. Compressibility leads to a noisy signal in air segmented systems because the pump pulsations are transmitted down the length of the tube. Another problem of air segmentation, band broadening due to wetting of the tube walls, can be

reduced when using solvent segmentation. The key is to employ tubing materials that are not wetted by the phase that contains the compounds of interest. An alternative to this is to use extraction solvents that do not wet the tubing material that is being used. The effectiveness of solvent segmentation in reducing band broadening was demonstrated by Werkhoven (97) who utilized this reactor type in a 24 min derivatization reaction. Lawrence et al. (96) have published some interesting findings concerning the influence of glass versus Teflon on band broadening as a function of flow rate for air segmented and solvent segmented reactors.

A disadvantage of post-column reaction detectors, especially when using OTR and PBR is inefficient mixing. Mixing, which has to take place in a short period of time and without significant band broadening, is not readily achieved when the column effluent and reagents have different flow rates and physical properties (e.g., viscosity and density). Mixing units of different geometries have been designed by several workers (47,91,103,104). Frei et al. (105) have compared the performance of some mixing units in a derivatization system using OTR and a 10 s residence time. Use of the traditional 90° tee mixer resulted in layering effects and thus band broadening. When a mixing unit that combined the column effluent and reagents at 60° angles was used, band broadening was significantly reduced. This was due to the turbulence produced in the mixing area which resulted in enhanced mixing. Jonker and coworkers (91,92) have proposed the use of narrow diameter tubing filled with large glass beads to enhance mixing. This is a practice commonly employed in flow injection analysis (23).

A disadvantage of post-column reaction detectors is that the detection limits are increased due to dilution by reagent addition. Recently, reaction units have been developed that minimize or eliminate the need for reagent addition. They include solid phase reactors (106-110), electrochemical reactors (10,66,111), photochemical reactors (70,112,113) and thermosensitized reactors (114). Solid phase reactors are essentially the same as packed bed reactors. The difference is that instead of using inert glass beads, a reagent or catalyst is immobilized on the glass surface. One such system is the immobilized urease reactor used to convert urea to ammonia which then reacts with o-phthaldehyde to produce a fluorescent derivative (109). Electrochemical reactors have been used to generate reagents needed for derivatization. King and Kissinger (10) have described the use of an amperometric generator electrode to generate bromine from the potassium bromide that had been added to the mobile phase. The bromine undergoes reaction with phenols in the space between the upstream generator electrode and downstream detector electrode. The analyte concentration is proportional to the difference in reagent concentration between these two points. A thermosensitized reaction detection was reported by Le Page and Rocha (114). Their work involved the ninhydrin reaction commonly used for the determination of amino acids and other primary amines. Since this reaction takes place extremely slowly at ambient temperatures, the ninhydrin reagent may be added to the mobile phase. When the column effluent, containing a homogeneous mixture of sample and reagent, is subjected to an increase in temperature, the reaction then proceeds at a rapid rate.

From this review, it is evident that post-column reaction detectors have become widely used. This is reflected by both the number of applications and the extent of coupling of post-column derivatization to different detection modes. With the development of new reaction units (e.g., solid phase, electrochemical and two phase reactors), comes endless possibilities for expansion into different application areas.

C. COLORIMETRIC REACTIONS FOR PHENOLS

Many colorimetric reactions for the qualitative and quantitative determination of phenols have been developed. Veibel (115), Siggia and Hanna (5), and Pesez (116) have summarized some of these methods. Cheronis (7) and Knapp (117) have listed some important derivative classes for phenols along with recommendations concerning the derivatives that would be most suitable for different phenol types. Reagents used in conjunction with various chromatographic methods for the detection of phenols and organic acids were summarized by Hanai (118). The reactions that have been used for the quantitative determination of phenols by colorimetric means can be divided into two categories based on whether a coupling reaction takes place or not. Coupling reactions produce highly conjugated derivatives with correspondingly high molar absorptivities. The derivatives most commonly formed are azo, indophenol and antipyrine analogues. Koppe et al. (119) have published a comparison of the reactivity of four different reagents with 126 phenols.

The classical method for the determination of phenols is the coupling of phenol with a diazonium salt to form a colored azo dye. An

extensive amount of work has been done with this type of reaction because of the numerous possible diazonium-phenol combinations. Some of the diazonium reagents that have been used are p-aminobenzoic acid analogues (20), p-nitroaniline (119,122-125), p-arsanilic acid (121) and sulfanilic acid (119,122-125). Also, the importance of azo compounds in the dye industry has resulted in thorough investigations of the formation mechanism of these compounds (6,127-130). Diazo coupling reactions have found wide analytical use because of: (1) good reproducibility, (2) high selectivity (due to the relatively weak electrophilic nature of the diazonium ion), (3) insensitivity to small variations in reaction conditions, (4) predominantly monosubstituted reaction products and (5) the aqueous reaction medium. Since aromatic diazonium reagents are not highly reactive electrophiles, coupling takes place only with activated aromatic compounds (primarily phenols and anilines). The reactivity of the diazonium group can be altered by ring substitutions (131). Electron withdrawing substituents enhance the electrophilic nature of the diazonium group, whereas electron donating substituents decrease the reactivity of the this group.

Indophenols can be formed by the oxidative coupling of phenols with various reagents such as diamines (132) and hydrazones (119,133) or by reaction with quinoneimines (134-139). Some reagents that have been used for the oxidative coupling of phenols are N,N-dimethyl-p-phenylenediamine (131) and 3-methyl-2-benzothiazolinone hydrazone (119,132). The most well known quinoneimines are 2,6-dichloro- and 2,6-dibromoquinone chlorimide, also known as Gibb's reagent (133,134). For many years, the latter was considered the standard reaction for the quantitative determination of phenols.

Although it is very sensitive, Gibb's reagent does not yield reproducible results. Svobodova et al. (138,139) have studied the phenolic reaction with this reagent extensively and have reported that a reagent to sample ratio of 30-50 to 1 is essential for reproducibility. Other reagents similar to Gibb's reagent, such as benzenesulfonyl quinoneimine, have also been used (140).

The third class of derivatives, antipyrines, are formed by the coupling reaction of phenolic compounds with 4-aminoantipyrine (also known as Emerson's reagent) in the presence an oxidizing agent (141-143). This reaction is currently the most commonly used and accepted colorimetric reaction for quantitative phenol determinations (60,145,146). Potassium ferricyanide is the oxidizing agent most commonly used in this reaction. However, as reported by Blo et al. (147) in their work on pre-column derivatization of phenols, this oxidizing agent causes the production of a large number of reaction products that interfere in the analysis. These authors have investigated the use of silver chloride as the oxidizing agent and compared its performance to potassium ferricyanide. Their findings show that, in comparison to the system in which potassium ferricyanide was employed, the reaction time was greatly increased (40 min) and there was a reduction in sensitivity. The advantages of the 4-aminoantipyrine reaction (with potassium ferricyanide as the oxidant) are sensitivity, speed, and approximately the same λ_{max} for all derivatives. The latter may be a disadvantage if information is desired for specific phenols, since the different phenol derivatives cannot be spectrally resolved.

Several reactions are used for phenol determinations that do not require coupling with the reagent to form derivatives with extended conjugation. These include nitrosation (148) and halogenation (149,150) of the phenols. In the former case, phenols are treated with a sodium nitrite solution in sulfuric acid to form a nitrosophenol which rearranges in the presence of excess alcoholic ammonium hydroxide to form a highly colored quinoid radical. This reaction has been used in the analysis of gasoline and allied products for phenols (151). Quantitative and qualitative analyses for phenols have also been carried out by halogenation with iodine or bromine (149,150). Since substitution may take place at more than one position on the ring, experimental conditions have to be carefully controlled when doing quantitative work.

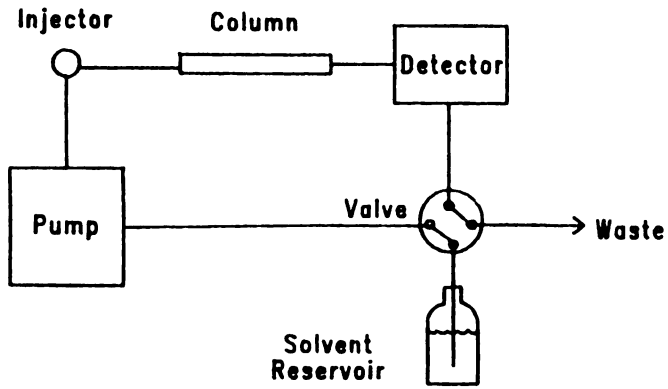
D. RECYCLE CHROMATOGRAPHY

The idea of recycling was first suggested for use in gas chromatography (152). In 1962, this concept was applied to liquid chromatography for the first time by Porath and Bennich (153), who showed that it was possible to achieve very large column plate numbers without the use of additional column packing. Recycling has been a successful alternative in complex separations when a change of columns and/or mobile phases have failed to provide the necessary resolution and in cases where the coupling of columns in series is not a feasible option. Since its introduction, recycle chromatography has been widely used in size-exclusion chromatography (SEC) (154-165). The use of long columns and the coupling of multiple columns to obtain an increase in the number of theoretical plates is not a feasible option in SEC due to

the inability of the gel based column packing to withstand the resulting increase in back pressure.

Recycle chromatography has been carried out using two different approaches - the closed loop (CL) principle (153) and the alternate pumping (AP) principle (161), both of which are shown in Figure 2.3. Most of the research done in recycling has involved the use of one of these approaches, sometimes with slight modifications. The unique characteristic of the CL configuration, illustrated in Figure 2.3a, is the connection of the pump, injector, column and detector in a continuous loop. The injected sample is separated on the column and detected as it elutes from the column. The valve is used to divert the effluent to a fraction collector or back to the pump for recycling. A major advantage of the closed loop configuration when it is used to recycle the entire sample is minimum dilution since the mobile phase that the sample is eluting in is being fed back into the column. Thus, there is no additional dilution with each recycle. Its major drawback is the band broadening that occurs when the partially separated sample flows through the pump chambers. This places a limitation on the type of pump that can be employed with CL recycling. In order for negligible band broadening to occur in the pump, the internal volume of the pump that should be used has been calculated to be about 5 μl (166). In most cases, this is not a realistic requirement as the routine LC pumps have a much higher internal volume. Therefore, the CL configuration has been used mainly for slow analyses performed with wide columns and has not been used in HPLC. A theoretical and experimental study of the closed loop principle was published by Martin et al. (167) in 1976. In this work, the theoretical predictions and the experimental observations

a.)



b.)

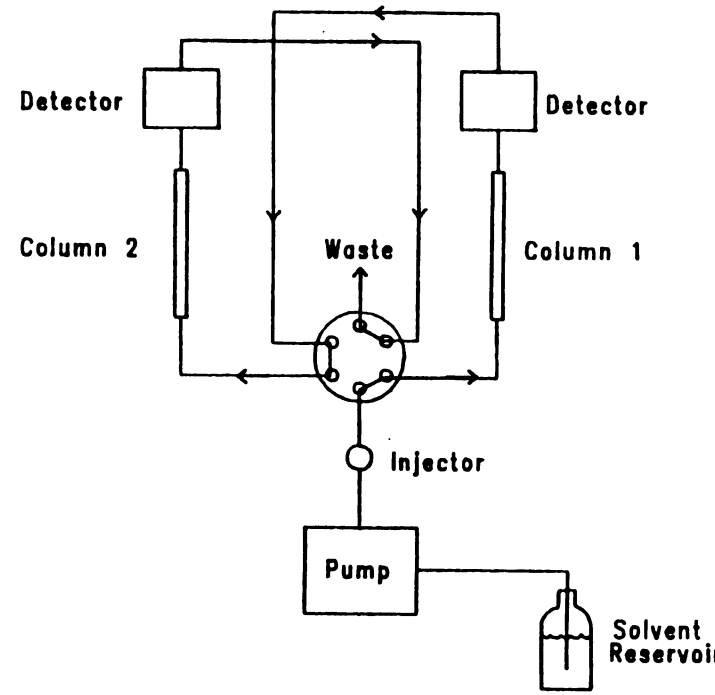


Figure 2.3. Two configurations of recycle chromatography a.) alternate pumping and b.) closed loop.

of a number of factors were compared (e.g., the relationship between resolution and the number of recycles and the effect of sample concentration on resolution). The advantages of recycling over one cycle systems that yielded equal resolution were noted.

The alternate pumping principle was initially introduced for use in SEC by Biesenberger et al. (161) and later adapted to high performance liquid chromatography (HPLC) by Henry et al. (168). The apparatus used for alternate pumping recycle chromatography is shown in Figure 2.3b. Sample is introduced via the injection port and initially separated on column 1. The six port valve is used to divert the effluent either to waste or on to column 2. In this way, undesirable components can be removed from the system. As the last peaks elute from column 1, the valve switches so that the pump is now directly connected with column 2. The effluent from column 2 is then diverted onto the head of column 1 after passage through cell 2. This sequence of steps is continued until the sample peaks have broadened to occupy the entire length of the column or an adequate resolution has been achieved. The advantages of AP over the closed loop approach are: (1) decreased band broadening, (2) all types of pumps can be used with AP recycling since the sample does not recycle through the pump and (3) a reference cell through which the mobile phase flows is maintained throughout the separation. The disadvantages, on the other hand, are the necessity for two identical columns and two high pressure flow cells. The number of times that a sample can be recycled is limited by the eventual broadening of the sample peaks to occupy an entire column volume. When this happens, the early eluting components start to overlap with the late eluting components. Columns of the same type, but with

significantly different separation characteristics can result in a further limitation on the number of recycles. In addition, the valve switching sequence can become very complicated, especially if time is the parameter used to initiate the valve switchings.

A third configuration for recycle chromatography was introduced by Minarik et al. (166) in 1981. In this approach, the eluate from the column outlet is repeatedly transported into the column inlet with a circulation valve that is turned by a stepper motor. This approach attempts to combine the advantages of the AP and CL recycling systems. The dead volume in the valve is of the same order as that encountered in the capillary connections used in AP. The recycling path, however, is similar to that used in the CL pumping principle. This system has been successfully tested in the separation of a mixture of isophthalic acid and its esters by gel permeation chromatography.

A technique that should also be mentioned in this section is boxcar recycle chromatography (BRC). This technique which was introduced by Snyder in 1981 (169) combines boxcar chromatography with the alternate pumping method of recycling. Boxcar chromatography is a form of column switching that enables increased sample throughput for routine applications in GC and LC. This is accomplished by continuously injecting samples which are serially separated on the first column. The partially separated components of interest for each sample injection are diverted onto a second column via a switching valve. A second pump is used to effect separation on the second column. In this way, the second column is filled with a train of samples which is further treated. The instrumentation requirement for BRC are two pumps, two switching valves and three columns. The first and second columns are

used for performing boxcar chromatography. When the second column is loaded with a train of sample fractions, the third column is switched in and these last two columns are used for recycling.

Recycling has been used in gel permeation chromatography for the separation of oligomers and polymer additives (154), diastereomers (156) and phenols (155). Henry et al. (168) applied the AP method to the separation of endo/exo isomers of methyl-1,1-(spirocyclopropyl)-indene by high speed liquid chromatography. Recycle chromatography has also been used in microbore liquid chromatography as reported by Kucera and Manius (170).

CHAPTER III

DESIGN AND CHARACTERIZATION OF A POST-COLUMN REACTION DETECTOR FOR PHENOLS BY AIR-SEGMENTED CONTINUOUS FLOW ANALYSIS

A. OVERVIEW

The development of an on-line post-column reaction detector for phenols is described from the initial reaction characterization experiments to the final evaluation and application stage. The technique of air-segmented continuous flow analysis (CFA) was chosen due to its unique characteristics and suitability as a post-column reaction detector system. The presence of air-segments has two major consequences - reduced longitudinal dispersion due to the break down of the parabolic flow profile and enhanced mixing within each liquid segment due to the toroidal flow in each of these segments.

The reaction employed in this work involves coupling between a phenol ($Ar'OH$) and a diazotized aromatic amine ($ArN_2^+Cl^-$) to form an azo dye. The general diazotization and coupling reactions are summarized in Equations 3.1 and 3.2, respectively.

$$ArNH_2 + NaNO_2 + 2HCl \rightleftharpoons ArN_2^+Cl^- + 2H_2O + NaCl \quad (3.1)$$

$$ArN_2^+Cl^- + Ar'OH \rightleftharpoons ArN{=}NAr'OH + HCl \quad (3.2)$$

The aromatic amine ($ArNH_2$) used in this case is sulfanilic acid. The performance of diazotized sulfanilic acid (DSA) and several other coupling reagents have been evaluated for 126 different phenolic compounds by Koppe et al. (119). Studies by Whitlock et al. (125) and Baiocchi et al. (126) to characterize the reaction and to determine the optimum reaction conditions for DSA have not produced consistent results. It was therefore necessary to undertake an extensive study of the effects of experimental conditions on the reaction detector response. Simplex optimization was employed to confirm the optimum reaction conditions. The potential of the post-column reaction detector for use with reverse phase liquid chromatography was investigated by studying the effects of organic solvents on the diazo coupling reaction. Other aspects of interest were the influence of flow rates on resolution and the degree of band broadening introduced by the detector system. The analytical figures of merit were determined and compared to that of other systems. To evaluate the usefulness of the system, the PCR detector was employed for the determination of phenols in fuel and spiked river water samples.

B. EXPERIMENTAL

1. Apparatus

A schematic of the instrumentation employed in this work is shown in Figure 3.1. The main components of the HPLC unit were a Spectra-Physics SP8700 solvent delivery system (Santa Clara, CA), a Rheodyne 7120 injection valve (Cotati, CA) with a 20 µl sample loop and a

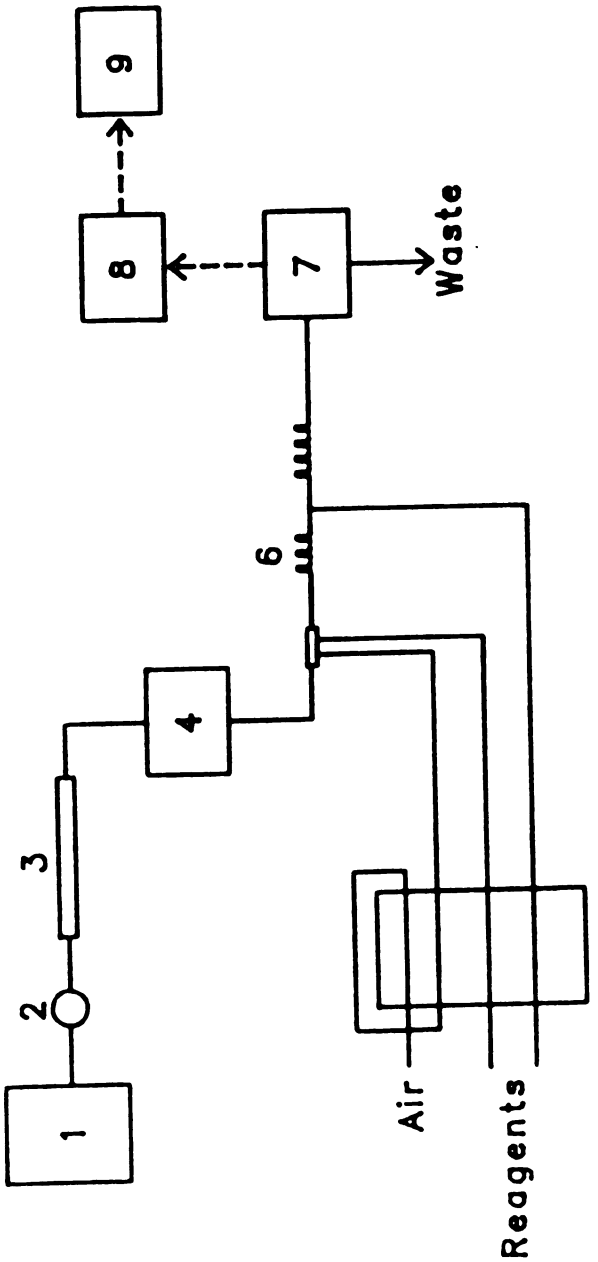


Figure 3.1. Apparatus employed for the post-column detection of phenols using an air-segmented mCF analyzer. 1: solvent reservoir; 2: injection valve; 3: analytical column; 4: UV detector; 5: peristaltic pump; 6: reaction coil; 7: colorimeter; 8: bubble gate; 9: readout device. Flow stream path is denoted by —— and the electrical signal path by ---.

Chromatronix 220 UV detector (Berkeley, CA). Separations were carried out on a 5 μm Spherisorb ODS column (25 cm x 4.5 mm) purchased from Alltech Associates (Deerfield, IL). The PCR system is a miniaturized continuous flow (mCF) analyzer designed in our laboratory (17,33,43). The peristaltic pump (Model IP-12, Brinkmann Instruments, Westbury, NY), used to proportionate reagents to the column effluent, was modified from the original 8 roller assembly to one with 16 rollers for a smoother flow. A dual channel colorimeter (17) with narrow bandpass filters for wavelength discrimination was used to monitor the absorbance of the flow stream at 450 nm. Light was transmitted from the source to the colorimeter through a bifurcated fiber optic. The signal from the colorimeter was directed into a "bubble gate" circuit where the effect of the air segments was electronically removed (43) and the resulting signal observed on a digital recorder.

As shown in Figure 3.1, the sample is injected into the HPLC system where it undergoes separation on an analytical column followed by UV absorption detection. The column effluent is then directed to the PCR detector. The interface between the HPLC and PCR systems is a short piece of 0.01" i.d. stainless steel HPLC tubing, which is connected at one end to the output of the UV detector and at the other to a stainless steel reagent addition block on the PCR manifold. The flow stream is immediately air-segmented using the dual pump tube method described by Habig, et al. (39). Reagents are added to the column effluent via a 90° glass tee. Mixing and time delays are accomplished by means of glass coils of various lengths. In the final stage, the analytical stream containing derivatized products passes into a low

volume bubble-through flow cell (2 µl) mounted on a colorimeter and then out to waste.

Temperature studies were performed with a 30 kΩ thermistor (Unicurve™, Fenwal Electronics, Inc., Framingham, Mass) and a thermostatted water bath. The simplex optimization program (OPTIMA) obtained from Dr. Adrian P. Wade (British Petroleum Research Centre, Middlesex, Great Britain) was run with an IBM compatible microcomputer (PC Designs, Tulsa, Oklahoma).

2. Preparation of Solutions for the Determination of Phenols

a. Reagents

Commercially available chemicals were used without further purification. Diazotized sulfanilic acid was prepared using the procedure described by Whitlock et al. (125) with some modifications. A 5 mmol amount of the sodium salt of sulfanilic acid was dissolved in 50 ml of distilled water. A 10 ml aliquot of a 50 mM sodium nitrite solution was transferred to this solution followed by cooling to 0°C. After the addition of 2 ml of 2 M HCl, the final volume was adjusted to 100 ml with chilled, distilled water. The diazotized sulfanilic reagent was tested for the presence of excess nitrous acid using starch-potassium iodide paper. If the test was positive, either sulfamic acid or urea was added to decompose the nitrous acid. The reagent was transferred to an amber bottle and stored at 5°C.

The pH of the 0.05 M sodium borate buffer was adjusted by the addition of HCl or NaOH. A surfactant, Brij® 35 (Fisher Scientific

Company, Fair Lawn, NJ), was added to all solutions in quantities of 0.5 ml per liter of solution.

b. Phenol Standards

Standard solutions of phenol were made from a 1 mg/ml phenol stock solution (Sigma Chemical Company, St. Louis, MO). Solutions of higher concentrations were made from phenol crystals. All phenolic compounds used were purchased from Aldrich Chemical Company (Milwaukee, WI), Eastman Organic Chemicals (Rochester, NY), and K & K Laboratories (Cleveland, OH). Brij® (0.5 ml/l) was added to all the standards.

c. HPLC Solvents

Spectral grade HPLC solvents (Burdick and Jackson, Muskegon, MI) were filtered through a 0.45 μm filter and degassed with helium prior to use.

3. Sample Preparation

a. River Water Samples

Water samples collected from the Grand River (Lansing, MI), were filtered through a 0.45 μm filter prior to injection into the HPLC system. The samples were stored at 4°C with no added preservatives. The recommended technique (171) for the preservation of phenols in waste waters is the addition of copper sulfate and phosphoric acid to samples, followed by storage at 4°C. These conditions inhibit the breakdown of phenols due to microbiological activity. As the effect of this

preservation method on organic moieties has not been studied and the water samples were used within 48 hours of collection, no preservatives were added. Ettinger et al. (172) have reported a 15% loss of phenolic content in an unpreserved river water sample that had been stored at 4°C for four days.

b. Residual Fuel Oil Fractions

The residual fuel oil (RFO) samples were obtained from the National Aeronautics and Space Administration (NASA). Sample preparation consisted of an initial fractionation step by means of sequential elution solvent chromatography (173,174). This method involves the use of nine solvent mixtures of varying polarities to elute compounds containing different functional groups. The solvent mixtures and the functionalities to be found within each fraction are summarized in Table 3.1. This step was carried out using a flash chromatography apparatus (J. T. Baker Co., Phillipsburg, NJ) which consisted of a 2 cm column packed with 17 cm of 40 μm 'Flash' silica gel. A 20 g quantity of silica gel on which 0.5 g of sample had been deposited was placed over the packed bed. A 300 ml amount of each solvent mixture was sequentially added to and eluted from the column. A back pressure of nitrogen (2–5 psi) was used as the driving force to speed up the eluting process. Each fraction was collected and the solvent evaporated to yield a concentrated sample. An RFO fraction containing phenols, fraction #7, was employed in this work.

Table 3.1. Sequential Elution Solvent Chromatography.

Fraction no.	Solvent Mixture	Compound Types
1	Hexane	Saturates, Alkenes
2	Hexane/15% Benzene	Aromatics
3	Chloroform	Polar aromatics
4	Chloroform/10% Ether	Monophenols
5	Ether/3% Ethanol	Basic nitrogen heterocycles, (diphenols)
6	Methanol	Highly functional molecules
7	Chloroform/3%Ethanol	Polyphenols
8	Tetrahydrofuran/ 3% Ethanol	Phenolics, O - compounds
9	Pyridine/3% Ethanol	Heterocycles

C. RESULTS AND DISCUSSION

1. Introduction

The results of the work done in the various developmental stages of a post-column reaction detector for phenols are presented and discussed in this section. The effect of changes in variables such as pH, reaction time, reagent-to-substrate ratio and temperature on the diazo coupling reaction was investigated. Studies were carried out via both univariate and simplex optimization. These findings have been compared with those of other researchers. The extent of compatibility between the separation (HPLC) and PCR detection (air-segmented CFA) systems was studied. The aspects of interest were the compatibility between the media and the flow rates of the separation and detection systems as well as the degree of band broadening introduced by the PCR detector. The statistical figures of merit are reported along with a comparison of this method to other methods for determining phenols. This section concludes with some applications to actual samples; these help to demonstrate the advantages of post-column reaction detection of phenols separated by HPLC.

2. Reaction Characterization

The reaction characterization and optimization studies were carried out on the mCF reaction system without coupling to the HPLC unit. The manifold employed throughout these studies, unless otherwise stated, is

shown in Figure 3.2. Phenol was used in all of the characterization experiments.

a. Optimization via the Univariate Approach

The following optimization with the univariate method was performed by changing one reaction condition at a time (keeping all others constant) and recording the resulting absorbance value.

i. pH Effects. Borate buffers of different pH values were used to attain the necessary solution pH. Pump speeds and coil lengths were varied as needed to produce the desired reaction times. The effect of pH on the detector response at varying reaction times is shown in Figures 3.3 - 3.5. For all the curves, an optimum can be seen at approximately pH 10. The general peak shapes can be explained by considering the following equilibria:

$$Ar'OH \rightleftharpoons Ar'O^- + H^+ \qquad pK_1 = 9.89 \qquad (3.3)$$

$$ArN_2^+ \rightleftharpoons ArN_2OH \rightleftharpoons ArN_2O^- + H^+ \qquad pK_1 + pK_2 = 20.96 \qquad (3.4)$$

The reaction is an electrophilic aromatic substitution with the reactive species being the diazonium, ArN_2^+, and the phenolate, $Ar'O^-$, ions. These two species predominate at conflicting pH conditions. Thus, the response increases to an optimum and then decreases as the ratios of the reactive species change with pH. The plateau regions which extend over a range of pH values in Figure 3.4 can be varied by changing the amount of DSA added. Higher precision should be obtained in this region as small fluctuations in pH would cause no changes in absorbance

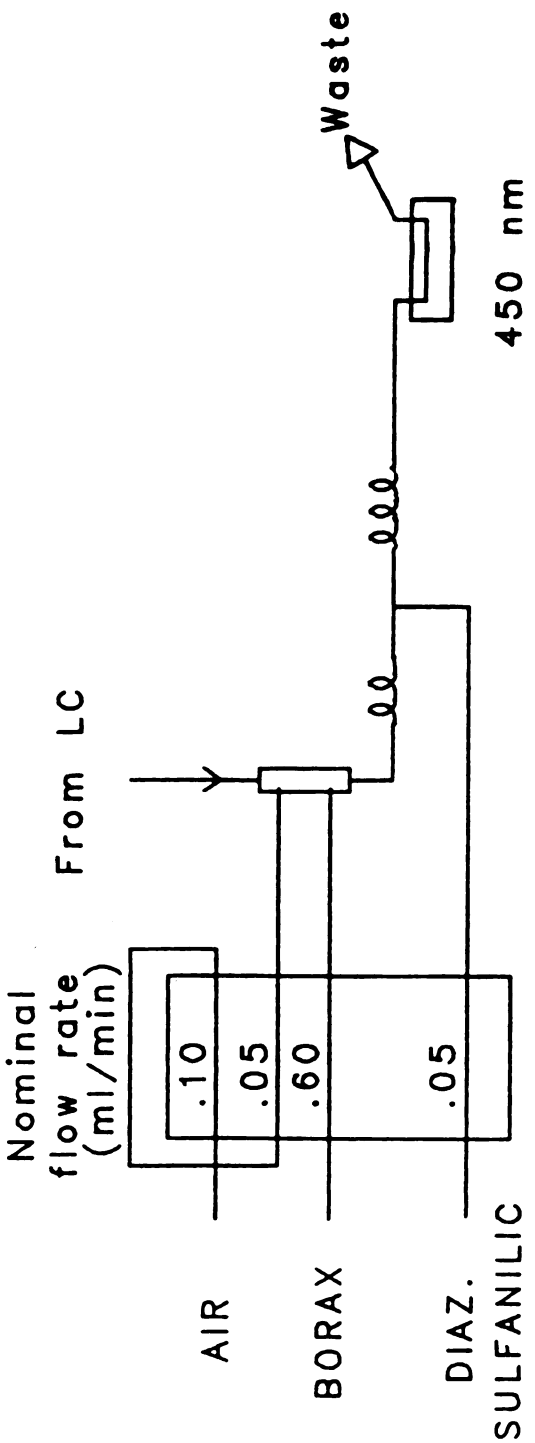


Figure 3.2. CFA manifold employed in this post-column reaction work.

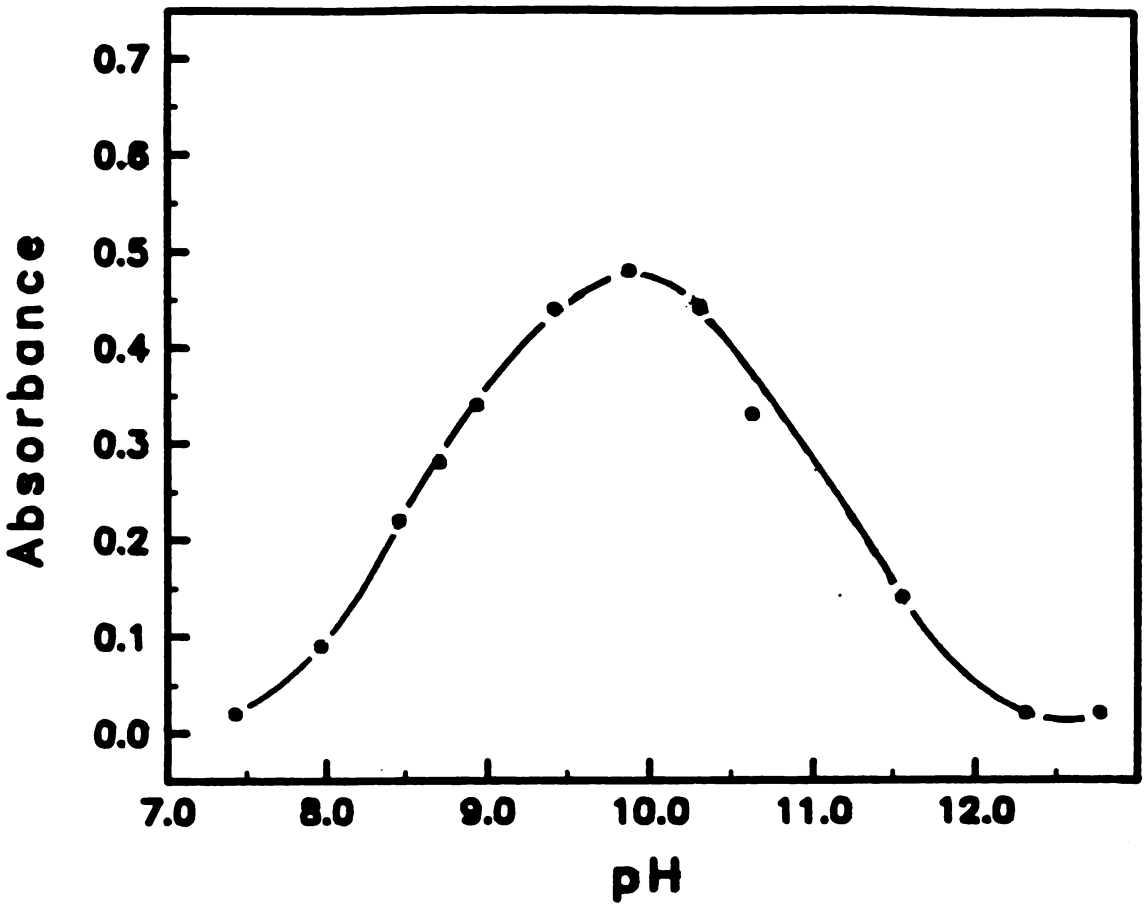


Figure 3.3. Effect of pH on PCR detector response for a reaction time of 15 s. Conditions: 0.05 M sodium borate buffer adjusted to appropriate pH with NaOH or HCl; flowrates: 30 μM phenol in borax buffer - 0.6 ml/min; DSA - 0.03 ml/min.

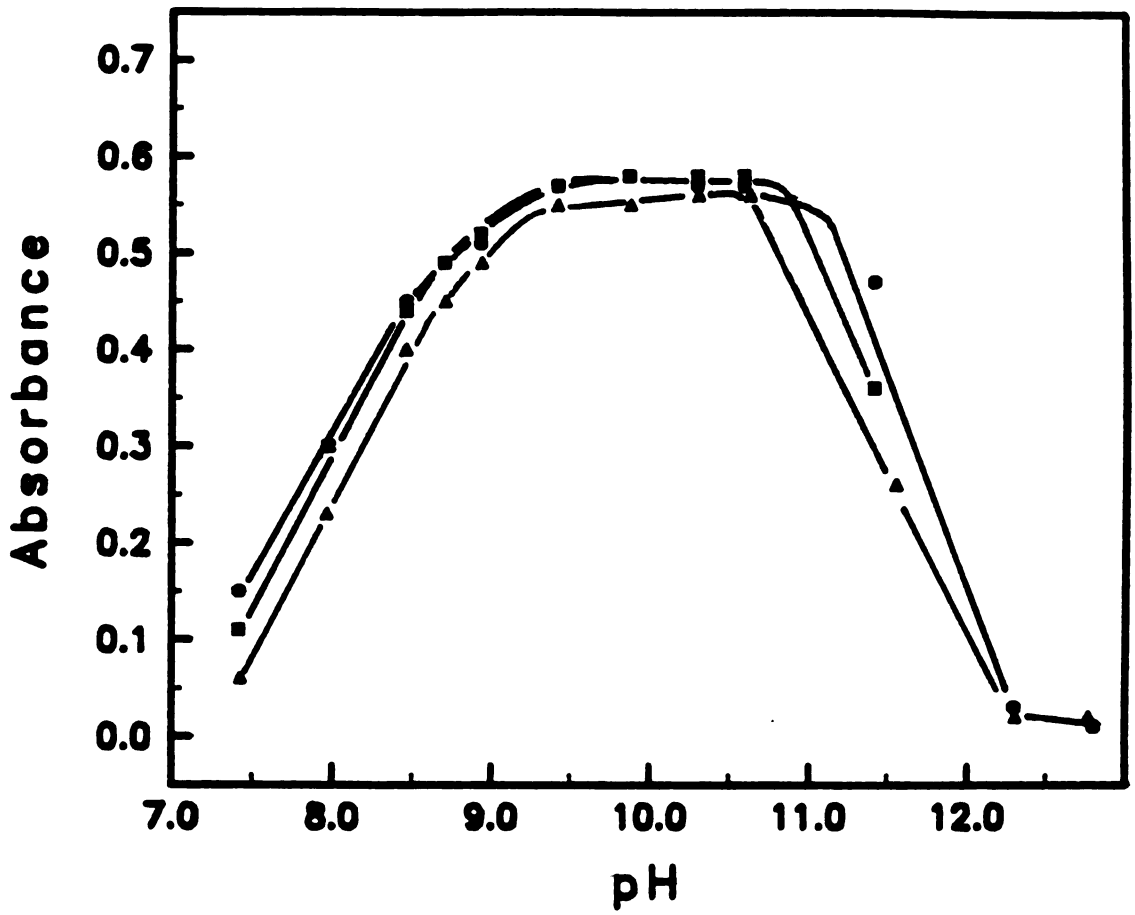


Figure 3.4. Effect of pH on PCR detector response for reaction times of 35 s (▲), 55 s (■) and 130 s (●).

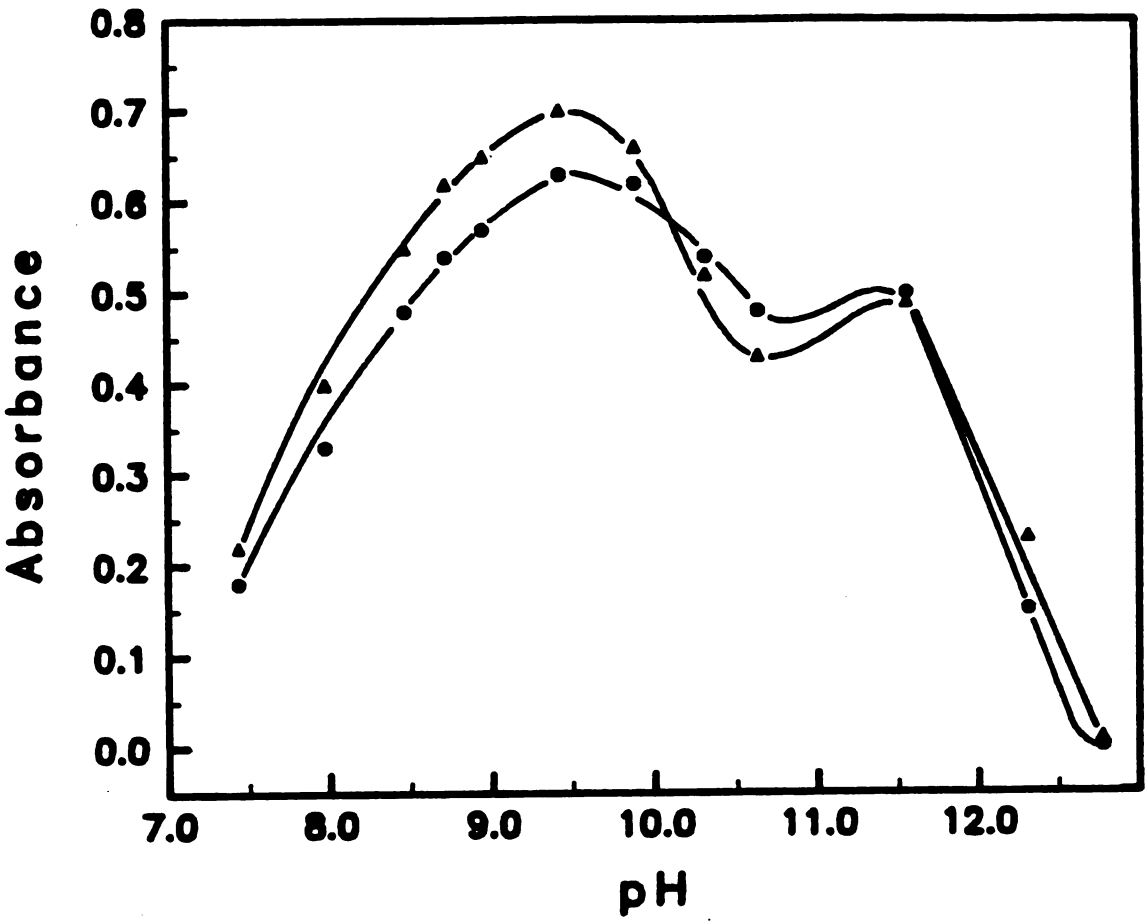


Figure 3.5. Effect of pH on PCR detector response for reaction times of 10 min (●) and 30 min (▲).

readings. The various shapes of the curves arise from the different reaction times used. This aspect is discussed in a later section.

A distribution diagram for the diazonium and phenolate ions as a function of pH is shown in Figure 3.6. The standard Henderson-Hasselbach equation,

$$pH = pK + \log \frac{[A^-]}{[HA]}$$

was used for computation of the mole fraction of phenolate. A derived expression was used for calculations involving the diazonium ion. The necessity of a derived equation arises from the differences between the hydrolysis characteristics of the diazonium ion and those of the 'classic' dibasic acids, in that, for the former case, $K_2 \gg K_1$. Neutralization curves of aqueous solutions of diazonium salts do not yield two inflection points with an intermediate pH region as is normally observed for dibasic acids (175). Instead, one step that extended over two equivalents of base per diazonium ion was observed. This anomalous behaviour was first reported by Schwarzenbach (176) who proposed possible explanations for this phenomenon. Since $K_2 \gg K_1$, individual constants were not available and the overall equilibrium constant was used in the calculation of the mole fraction of diazonium ions at different pH values. Shown below is the derivation of an equation

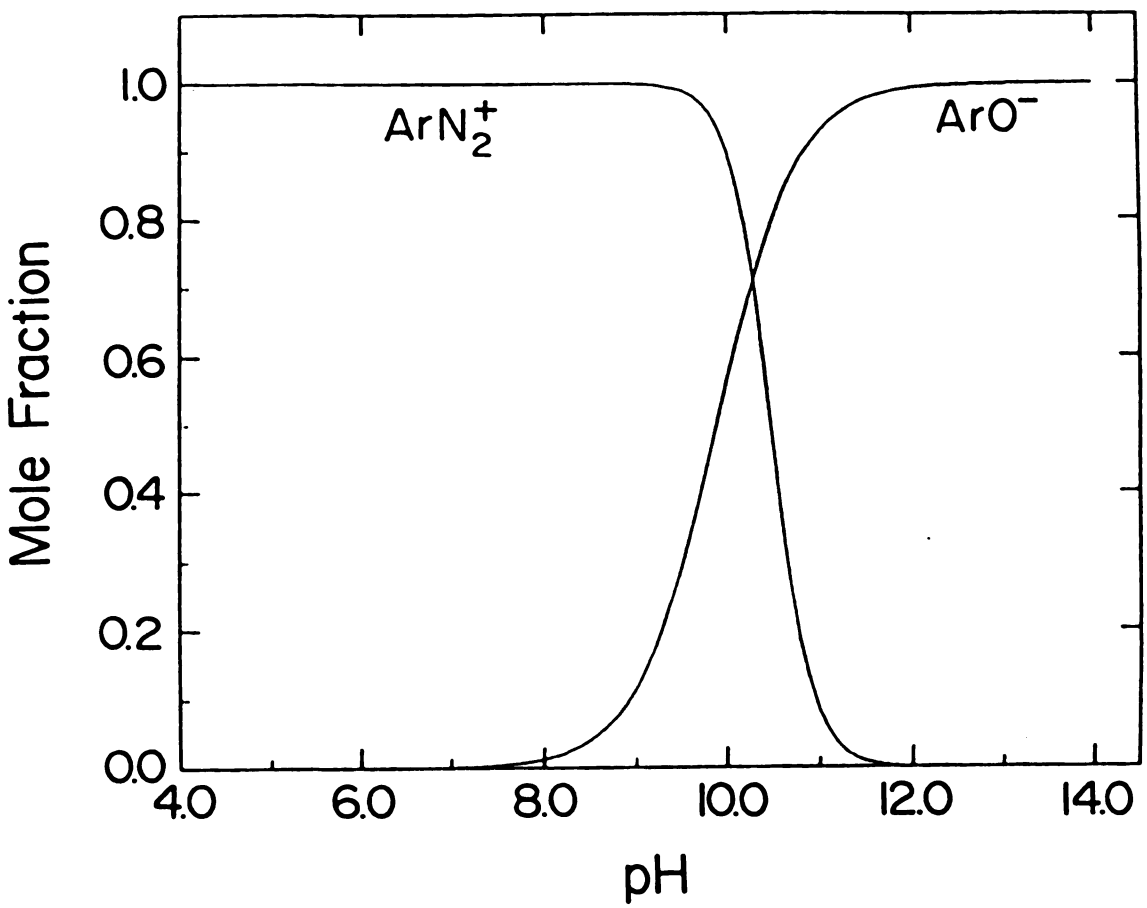


Figure 3.6. Distribution diagram for diazonium, ArN_2^+, and phenolate, $Ar'O^-$, ions at various pH values.

which makes possible the use of the overall equilibrium constant, $pK_1 + pK_2$, in this calculation. Referring to Equation 3.4,

$$K_1 = \frac{[ArN_2OH][H^+]}{[ArN_2^+]}$$

$$K_2 = \frac{[ArN_2O^-][H^+]}{[ArN_2OH]}$$

$$K_1 K_2 = \frac{[H^+]^2[ArN_2O^-]}{[ArN_2^+]}$$

$$pK_1 + pK_2 = 2pH - \log \frac{[ArN_2O^-]}{[ArN_2^+]}$$

$$pH = \frac{pK_1 + pK_2}{2} + \frac{1}{2} \log \frac{[ArN_2O^-]}{[ArN_2^+]}$$

Calculations employed the equilibrium constants given alongside Equations 3.3 and 3.4 (177,178). The resulting distribution diagram, Figure 3.6, shows that the amounts of diazonium and phenolate ions are maximized relative to each other at pH 10.2. (The intersection of the lines mark the point of optimum pH.) It should be noted that the presence of an optimum pH does not dictate that the reaction will proceed as other aspects have to be taken into account. These include the types and positions of substituents on the aromatic ring. In general, electron withdrawing substituents do not promote the diazo coupling reaction. Substitutions occur at the para and ortho positions, with preference for the former. Thus, phenols with substituents at both the ortho and para positions do not undergo reaction with diazotized sulfanilic acid. Large and bulky substituents may also inhibit the reaction due to steric hindrance.

ii. Influence of Reagent-to-Substrate Ratio. The influence of the reagent-to-substrate concentration ratio was investigated by the addition of diazotized sulfanilic acid solutions of different concentrations to the buffered flow stream containing phenol. The absorbance values obtained at different reagent-to-substrate ratios and reaction times are shown in Figure 3.7. Pump speeds and coil lengths were varied as needed to produce the desired reaction times. Theoretical calculations of the mole fraction of diazonium and phenolate ions, Figure 3.6, show that the two species are present in equal amounts (0.7 mole fraction) at a pH of 10.2. Thus, working at this pH, a DSA:phenol ratio greater than one should produce no increase in the maximum absorbance. However, a definite kinetic effect is observed as demonstrated in Figure 3.7. There was a decrease in the time required for the reaction to go to completion as the amount of reagent added was increased. Results indicate that the mole ratio of DSA to phenol should be greater than or equal to three for the reaction detector to attain a maximum response in less than 60 s.

iii. Effect of Reaction Time. The dependence of detector response on reaction time when different DSA:phenol ratios are used is demonstrated by Figures 3.3 - 3.5 and 3.7. From the data presented in Figures 3.3 - 3.5, the degree to which the reaction has proceeded to completion, given by % Abs_{max}, is calculated for the different reaction times. These values are summarized in Table 3.2. The reaction was 97% complete when a delay time of 35 s was employed and 100% complete for a 55 s and 130 s delay. When a reaction time of 10 min and greater was used, secondary reactions (179) may be taking place as evidenced by the increase in maximum absorbance and the appearance of a second

Table 3.2. The extent of reaction completion, % Abs_{max}, for various reaction times.

Time (s)	Abs	% Abs_{max} [a]
15	0.48	83
35	0.56	97
55	0.58	100
130	0.58	100
600	0.63	109
1800	0.70	121

[a] Assuming that the reaction is complete at 130 s.

peak. These observations are supported by the results shown in Figure 3.7. The time needed for the reaction to go to completion is less than 45 s when DSA:phenol ratios greater than 2.8 are used. The results shown in Figures 3.3 - 3.5 and Table 3.1 were obtained with a reagent-to-substrate ratio of 3.3. Based on these results and considerations of throughput and sensitivity, the optimum reaction time selected was in the range of 35 s to 55 s.

iv. Effect of Temperature. Temperature studies were carried out with glass reaction coils built into plexiglass water jackets. A thermistor was used to measure the temperature of the thermostatted flow stream as it exited the flow cell. The thermistor was calibrated via the Skinhart-Hart equation:

$$\frac{1}{T} = A + B(\ln R) + C(\ln R)^3$$

where T is temperature and R is the resistance. The coefficients A, B and C were experimentally determined for different temperature ranges using the following procedure (180). The thermistor was submerged in a thermostatted bath of known temperature. Resistance readings were recorded at three different temperatures in 10°C intervals. This yielded a solvable set of three equations with three unknowns. The values for A, B and C at the different temperature intervals are given in Table 3.3. To prevent erroneous readings as air and liquid segments flowed pass the thermistor, the flow stream was debubbled prior to measuring temperature. The previously established optimum reaction conditions were used in this study. A plot of the results shown in Figure 3.8 indicates little change in detector response between 21.0°C and 28.0°C. Therefore, normal fluctuations in room temperature should have little

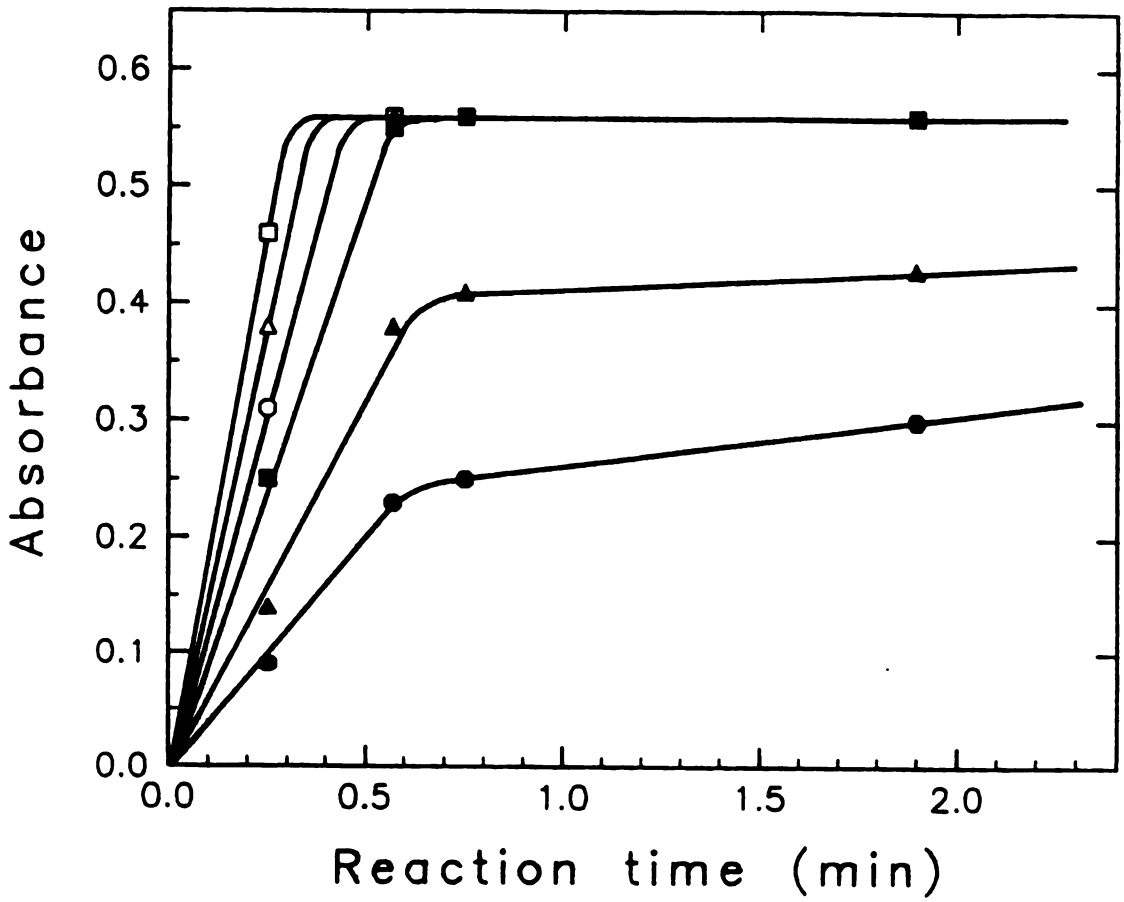


Figure 3.7. Influence of DSA:phenol ratios and reaction times on the PCR detector signal. DSA:phenol ratios: 0.7 (●); 1.2 (▲); 2.8 (■); 6.7 (○); 12.4 (△); 27.8 (□).

Table 3.3. Values of the coefficients in the Skinhart-Hart equation for various temperature ranges.

Temperature Interval (°C)	A	B	C
21 - 30	0.1326	-0.05954	0.002789
31 - 40	0.05351	-0.02261	0.001594
41 - 50	0.03578	-0.01446	0.001373
51 - 60	0.01001	0.001121	0.0005273

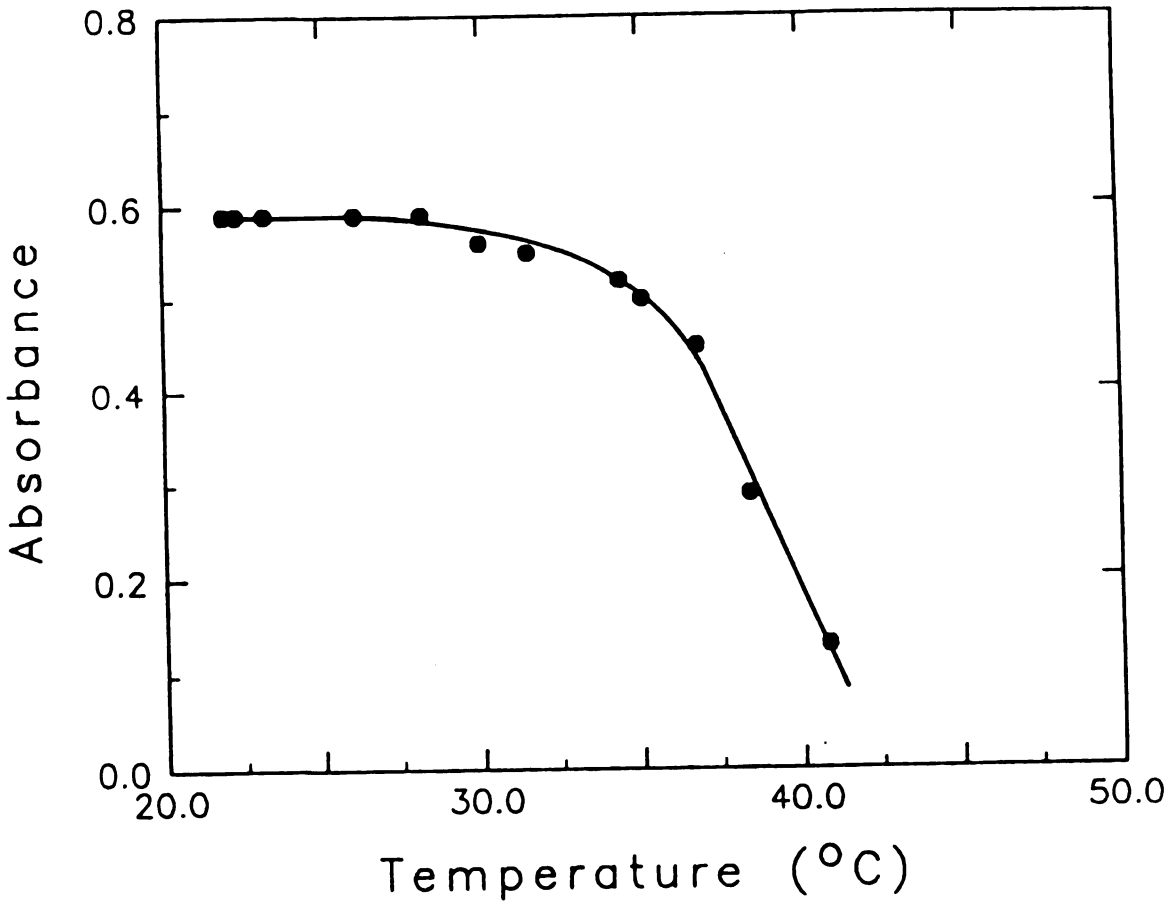


Figure 3.8. Temperature studies. PCR flow rates: 80 μM phenol - 0.30 ml/min; 5.9 mM DSA - 0.05 ml/min; pH 10.1 borax buffer - 0.60 ml/min. Reaction time of 37 s.

effect on the absorbance signal, and thermostatting is not essential. A decreased signal observed at temperatures above 28.0°C can be attributed to the increased thermodynamic and kinetic feasibility of secondary reactions.

b. Simplex Optimization

A univariate optimization in which one variable is changed while all others are held constant is the scheme most commonly employed for establishing the best reaction conditions. The assumption made in this approach is that all the variables are independent of each other. A false optimum may be found if this assumption is incorrect. Therefore, simplex optimization was used to test the optimum conditions established by the univariate approach.

For a better understanding of the work done in this section, it is necessary to present a brief discussion on the historical progression and the basic rules of simplex optimization.

Simplex optimization was first presented by Spendley et al. (181). This method suffered a number of limitations, some of which were the inability to accelerate a simplex over the response surface when searching for an optimum and the lack of verification procedures to confirm that the optimum found is a true optimum. In 1965, Nelder and Mead (182), proposed the 'modified simplex optimization' which introduced new operations that would overcome some of the limitations of the original procedure. These operations included expansion and contraction of the simplex. Since these early works, a large number of papers have been published concerning applications of simplex optimization and further modifications to the basic method (183-190). In 1985, Betteridge

and coworkers (191) introduced the 'composite modified simplex' (CMS) method which incorporated many of the modifications previously proposed by other workers. A second paper by this group (192) evaluated and compared the performance of the CMS method to that of other modified simplex methods. The program developed by Betteridge et al., OPTIMA, was the one employed in this work.

A simplex is a geometric figure whose number of vertices is equal to one more than the number of variables (or dimensions) being investigated. A simplex in two dimensions is a triangle. In three dimensions, it is a tetrahedron. Higher dimensions are often used, but the figures are not as easily visualized. For the purpose of introducing the principles of simplex optimization, a two dimensional simplex will be used. Figure 3.9 shows the progress of the two dimensional simplex on a contour map as it moves towards the optimum. The contour lines (circles) on the response surface represent different combinations of the two parameters being studied that would give equal responses. The objective is to rapidly move the simplex towards the region of optimum response. This progression is guided by a set of rules, of which only the basic ones are discussed here. First, after the evaluation of the initial simplex, a move is made after each response. Second, the point that gives the worst response is discarded and its mirror image across the face of the remaining points becomes the new vertex of the simplex. In Figure 3.9, the initial simplex is a triangle defined by vertices 1, 2 and 3. Point 1 gives the worst response of the three and is reflected across the face 2-3 to generate point 4. The new simplex has as its vertices, 2, 3 and 4. The response at point 4 is obtained and the next move is made based on this value. This process continues until the

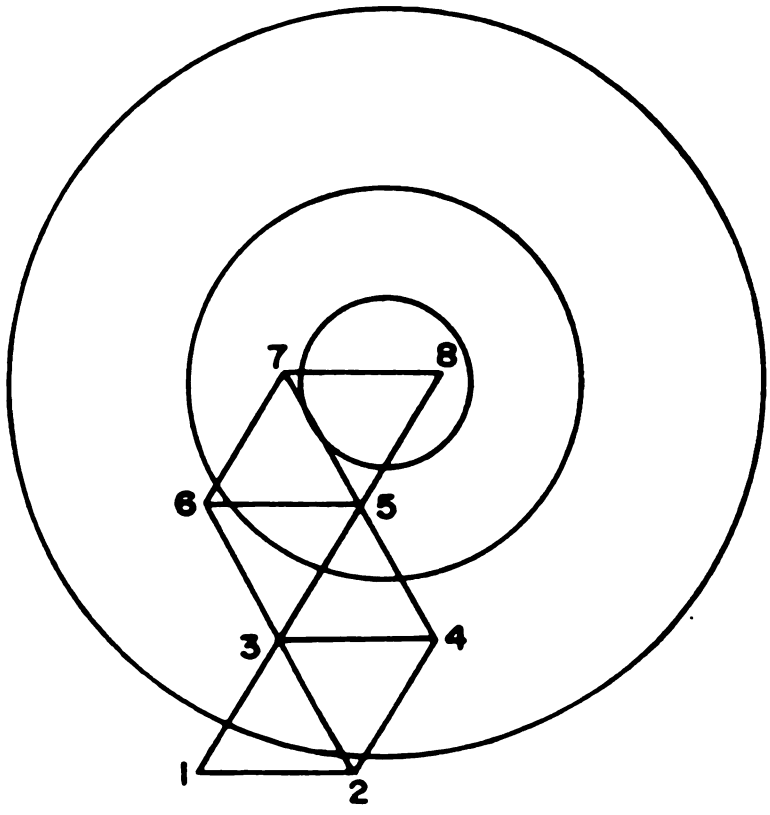


Figure 3.9. Progression of a two dimensional simplex over a response surface. Circles represent iso-response conditions.

simplex starts to circle which signifies that an optimum has been found. This optimum may be a local optimum rather than a global optimum. This could be verified by restarting with a larger simplex. A third rule is used to prevent the simplex from oscillating when the reflected point yields the lowest value in the new simplex. When this happens, the second to lowest response in the new simplex is reflected to obtain the new simplex.

A large number of rules not mentioned above are used in the OPTIMA program to handle the pitfalls of the original simplex method. These include the expansion, contraction, polynomial fitting and redirection of the simplex. However, rather than going into extensive explanations, this part is best left to the interested reader to pursue. The three rules mentioned above should be sufficient information for the reader to follow the progression of the three dimensional simplex used in this work.

Before starting the simplex optimization, the boundary conditions listed in Table 3.4 were established for the variables to be tested. This was based on a general knowledge of the system. For instance, the diazo coupling reaction with phenol does not proceed in acidic solutions. This places a lower limit on the pH region that was studied. Sodium borate has a buffering range between pH 8.0 and 11.8. Thus, the pH interval to be investigated was set at 7.0 to 12.0. The peristaltic pump has an arbitrary speed setting which ranges from 1 to 99. The lower boundary was set based on the flow rates at pump settings < 20 being too slow. The upper limit of the DSA concentration was established by the decomposition observed at high concentrations.

Table 3.4. Boundary conditions for simplex optimization with the composite modified simplex method.

Variable	Lower Limit	Upper Limit
pH	7.0	12.0
Pump speed	20	99
[DSA] (mM)	1.5	16.6

The reaction manifold was thermostatted at 26.5°C throughout these experiments. The phenol solution employed was 30 μM and the sampling and wash times were 40s and 20s, respectively. The blank was borax buffer and DSA.

The parameters varied were reaction time, pH of the reaction medium and the reagent concentration. The response optimized was absorbance. The readings obtained for each set of conditions were entered into the microcomputer and the next set of conditions was generated. The results of the simplex optimization are summarized in Table 3.5.

The final simplex has as its corners, experiments that have yielded the highest absorbance readings, namely, numbers 3, 7, 13 and 14. The largest absorbance value of 0.31 was measured for simplex no. 3. The response obtained when the reaction was carried out using conditions determined univariately, 0.30 AU, was not significantly different from that obtained at the optimum conditions established by simplex optimization. The precision of the absorbance readings is ±0.01 AU. The four points of the final simplex were all obtained in the pH interval of 10.2 ±0.5 indicating an optimum pH region. This observation coincides well with that made in the univariate experiments as well as the value predicted using dissociation constants. The magnitude of this pH interval will, once again, depend on the amounts of DSA added.

The major factor influencing the response of the PCR detector is pH as demonstrated by the scatter diagrams shown in Figure 3.10. A definite optimum is observed for this variable. However, there appears to be no clear trends in the effects of reaction times and DSA concentrations on the PCR response in the intervals studied.

Table 3.5. Optimization of the phenol/sulfanilic reaction system by the composite modified simplex method.

Expt no.	Simplex Vertices	Time[a] (s)	pH[b]	[DSA] (mM)	Abs[c] (AU)
1		116	8.06	1.50	0.09
2		49	8.06	1.50	0.06
3		67	10.08	1.50	0.31
4		67	8.69	7.70	0.25
5	3,4,1,2	127	10.67	4.27	0.28
6	3,5,4,1	56	11.40	5.58	0.20
7	3,5,4,6	67	10.58	4.56	0.30
8	3,7,5,4	83	11.61	1.50	0.05
9	3,7,5,4	72	9.44	5.30	0.29
10	3,7,9,5	48	9.40	2.07	0.29
11	3,7,9,10	127	10.48	4.27	0.26
12	3,7,9,10	56	9.66	2.62	0.30
13	3,7,12,9	60	9.78	2.82	0.30
14	3,7,13,9	60	10.64	1.50	0.30
15	3,7,13,14	72	9.62	3.65	0.29
Univariate Method		45	10.10	5.90	0.30

[a] Time was measured for each pump setting used.
[b] pH of solution measured after reaction has proceeded.
[c] Average of triplicate measurements.

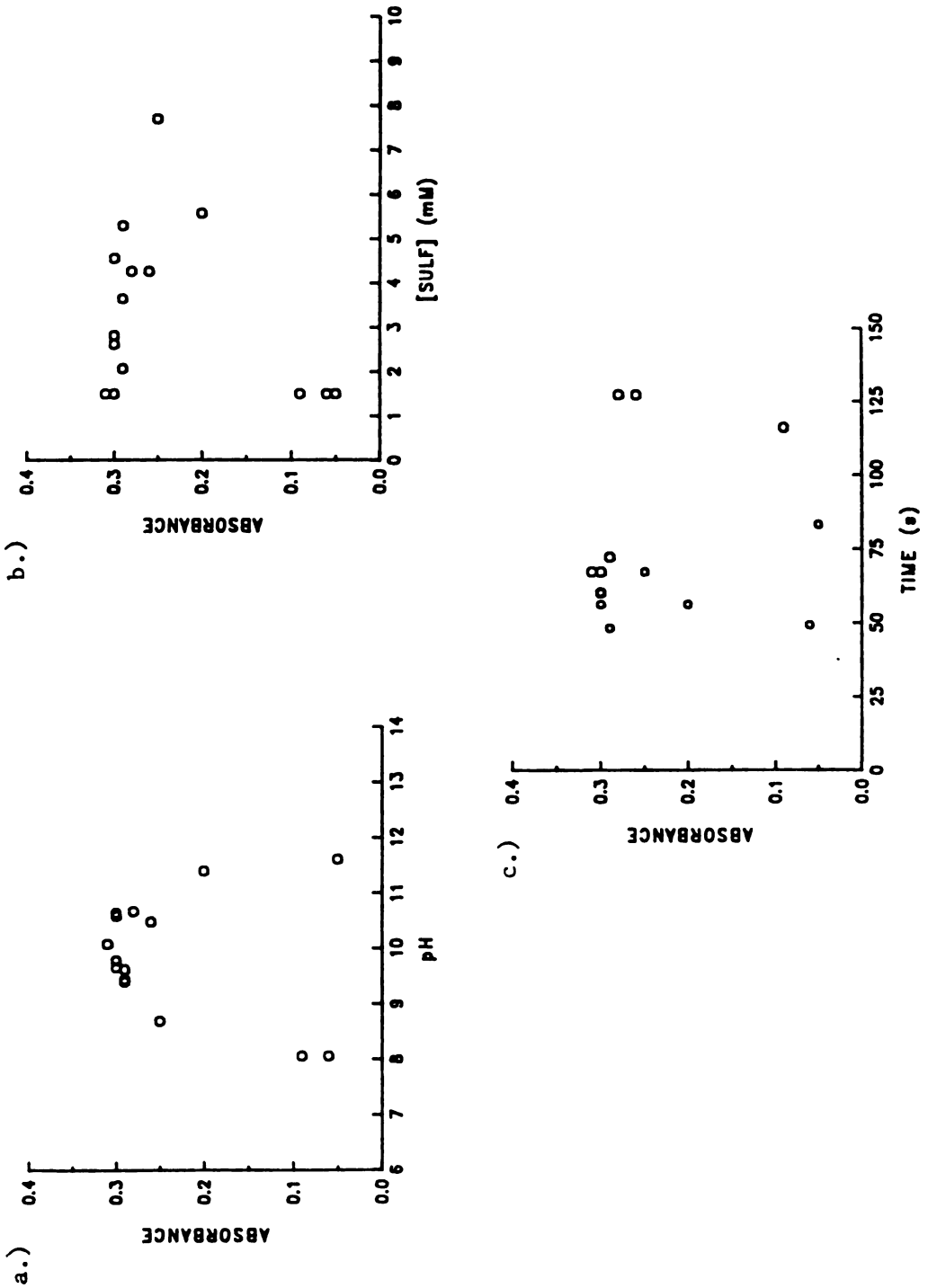


Figure 3.10. Scatter diagrams of absorbance vs. the parameters studied.
a.) pH b.) DSA concentration c.) time.

c. Comparison With Other Workers

It should be noted that the optimum reaction conditions reported by other workers for this reaction are not entirely in agreement with those established here. Whitlock et al. (125) reported that, for phenol, the optimum pH was 8.5. A sodium bicarbonate buffer was used, and the reaction required 2 min for completion. Preliminary experiments performed in our laboratory to characterize the reaction also employed sodium bicarbonate as the buffer. The optimum pH determined with this buffer agreed with that reported by these workers. However, the buffer was changed to sodium borate due to the poor buffering capacity of sodium bicarbonate at pH 8.5. The two different optimum pH values obtained indicate that one or both buffers are not inert. Other observations made by Whitlock et al., such as the effect of excess reagent and the stability period for the DSA reagent are in agreement with our findings.

Baiocchi et al. (126), reported an optimum pH of 11.0 in their work on pre-column derivatization of phenols by sulfanilic acid. In this case, NaOH was added to obtain the desired pH. Their reported reaction time was 15 min, and the optimum DSA:phenol ratio was 40:1; both of these are much larger than values determined in this work. In addition, the reagent had to be used within 10 minutes of its preparation. These observations may be due to improper preparation of the diazotized sulfanilic acid. If the starting compound is sulfanilic acid, rather than its sodium salt, an indirect diazotization needs to be carried out (130). That is, prior to diazotization, sodium carbonate is added to the solution

containing sulfanilic acid to convert it to the water soluble form. This step was not reported by Baiocchi and coworkers.

3. Evaluation of the HPLC/PCR System

The optimized PCR detector for phenols was used in conjunction with an HPLC apparatus with UV detection for system characterization studies. The performance of the PCR detector was evaluated with respect to its compatibility with the chromatographic conditions, band broadening contributions, detection limit, linear dynamic range and precision.

a. Mobile Phase Effects

The effect of varying amounts of common reverse phase solvents on the detector response was investigated. A 75 μM phenol solution prepared in pH 10.1 borax buffer was continuously added to and mixed with the column effluent. The manifold employed was identical to that shown in Figure 3.2. The UV and PCR detector responses were monitored while running a gradient of water/acetonitrile (Figure 3.11) or water/methanol (Figure 3.12). A drift of 1% in the detector signal is observed as the acetonitrile composition of the mobile phase spanned 0% to 80%. The upper limit was set by the solubility of sodium borate in acetonitrile. A decrease in the signal was observed when the percentage of methanol was increased beyond 60%. This effect may be attributed to a combination of different factors among which are solvent polarity and proton donating ability. The blank signal shows no change for either solvent. From these results, it was concluded that

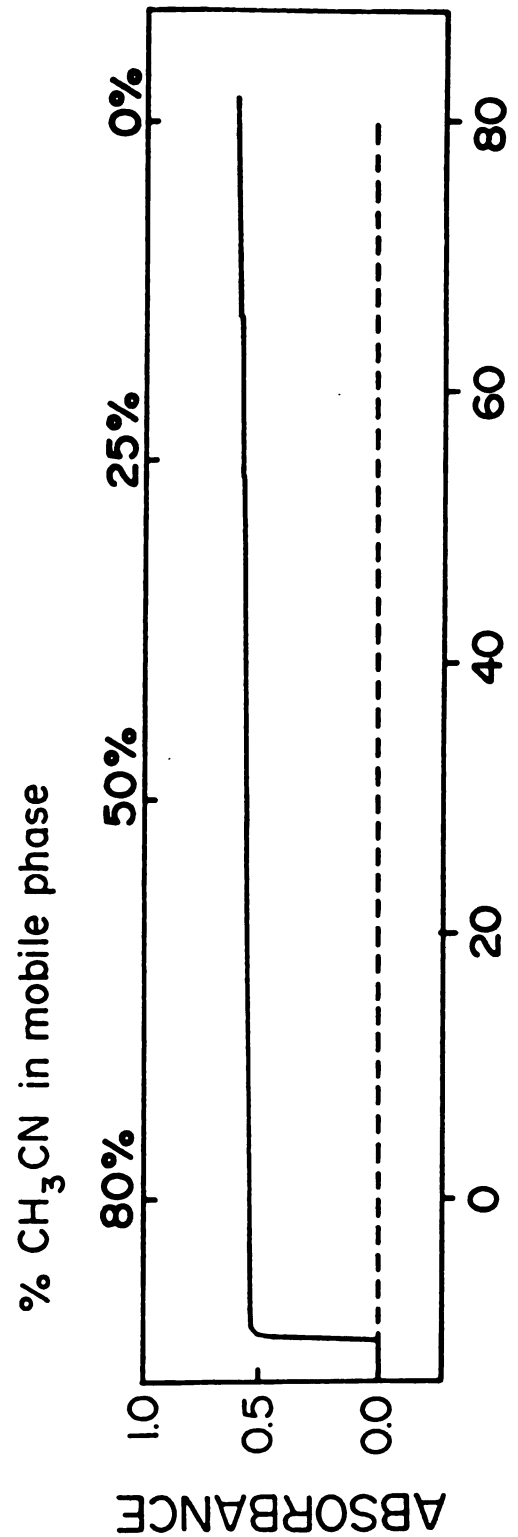


Figure 3.11. Influence of varying CH_3CN mobile phase composition on the diazo coupling reaction. HPLC flow rate is 1.0 ml/min with a 1%/min gradient.

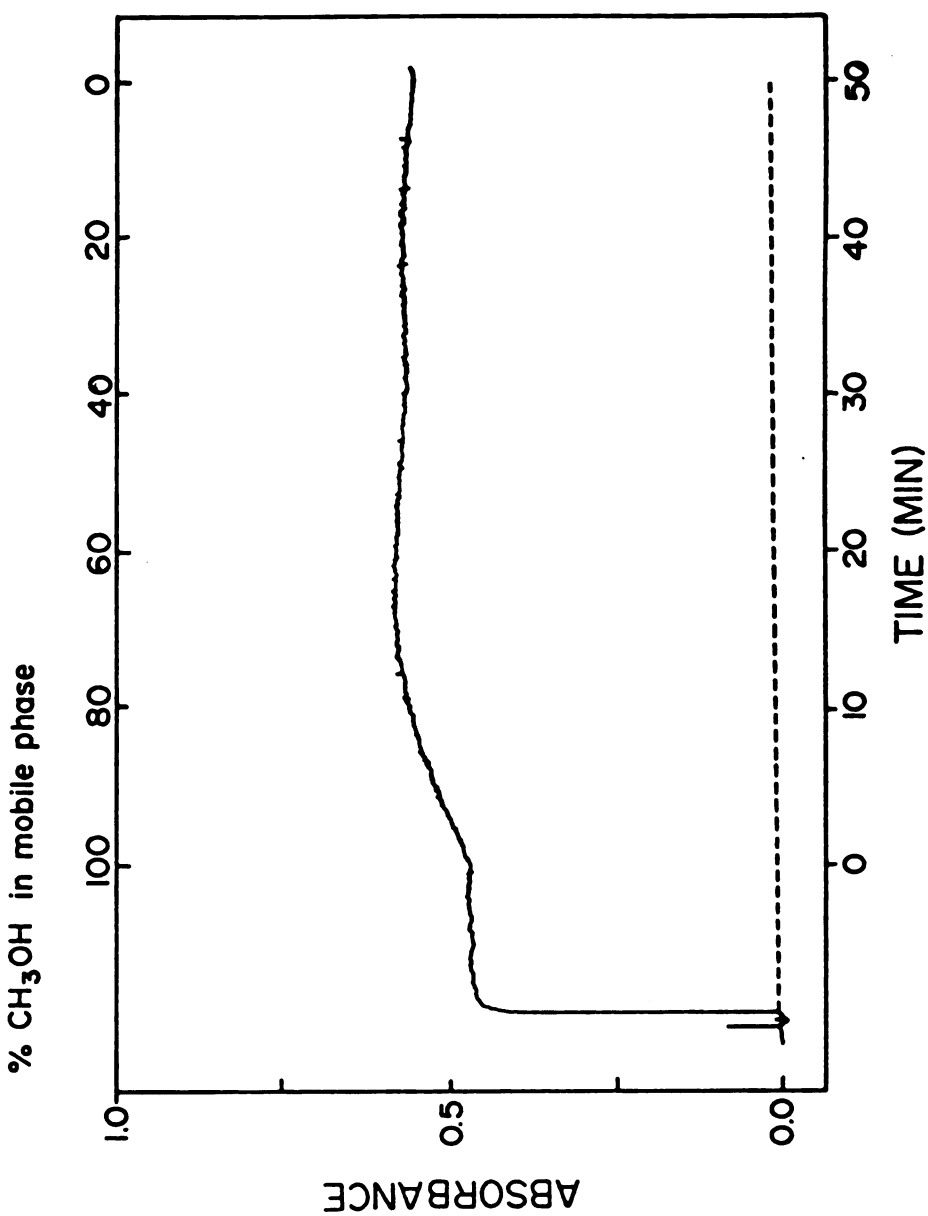


Figure 3.12. Influence of varying CH_3OH mobile phase composition on the diazo coupling reaction. HPLC flow rate is 1.0 ml/min with a 2%/min gradient.

acetonitrile is the more versatile solvent, especially for separations requiring gradient elution. Also, a slightly higher response was obtained in acetonitrile (0.56 AU) than in methanol (0.51 AU). This may be due to a shift of λ_{max} from 430 nm for acetonitrile to 420 nm for methanol.

b. Band Broadening Contributions

Laminar flowing streams in open tubes have a characteristic parabolic flow profile. The particles that are adjacent to the walls of the tubing travel at a slower velocity than those towards the center of the tube (due to viscous drag along the walls). The result of this phenomenon is longitudinal dispersion in the tube. When air segments are added to the flowing stream, this parabolic flow profile is broken down and thus dispersion is reduced. This reduction of dispersion is particularly dramatic when long sections of tubing have to be used.

The degree of band broadening introduced by the PCR detector was examined. For the most accurate results, pyrogallol was employed. This trihydroxy phenol is weakly retained by the ODS column. A typical peak width comparison for the two detectors is shown in Figure 3.13. From the ratio of the peak widths at half height of the UV and PCR responses, the amount of band broadening was found to be 5%-20%. This value demonstrates the effectiveness of air-segmentation and the miniaturized CFA instrument in limiting dispersion.

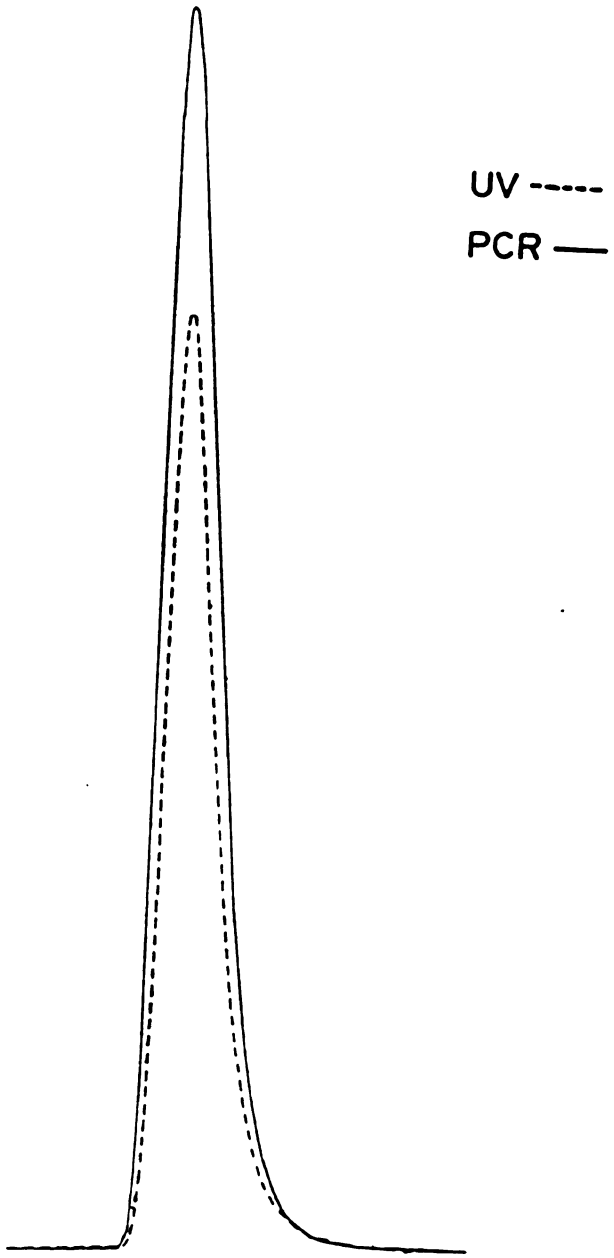


Figure 3.13. Comparison of peak widths at half height, $w_{1/2}$, for the UV and PCR trace.

c. Flow Rate Compatibility

Studies were done to determine whether there was a limitation placed on the flow rate of the HPLC system by the PCR system or vice versa. The flow rates were varied in the range normally used for HPLC, that is, 0.5 ml/min to 2.0 ml/min. A sample mixture of phenol, 3,5-dimethylphenol, and 2,6-dichlorophenol was injected in 20 µl quantities. The responses for flow rates of 0.5 ml/min, 1.0 ml/min and 2.0 ml/min are illustrated in Figure 3.14. The mobile phase was 40%:60% water:acetonitrile.

The resolution of the separated peaks is maintained by the PCR system within this flow rate interval. This demonstrates the efffectiveness of air-segmentation in limiting dispersion. The reason for the varying relative peak heights at different flow rates originates from the difference in reaction kinetics for each phenolic compound. Since the manifold was not changed, the reaction time used decreased with increasing flow rates. Thus, the coupling reaction of each compound was reaching a different degree of completion.

The conclusion to be drawn from these results is that there is a compatibility in flow rates between the two systems for this particular reaction. This conclusion should be extended to other systems with some caution. This is because the flow rate and the reaction time needed are the factors that determine the coil length that has to be used. For instance, for a flow rate of 2 ml/min and a reaction time of 5 min, the coil length needed would be 330 cm (coil i.d. of 0.1 cm). This is not a 'practical' length.

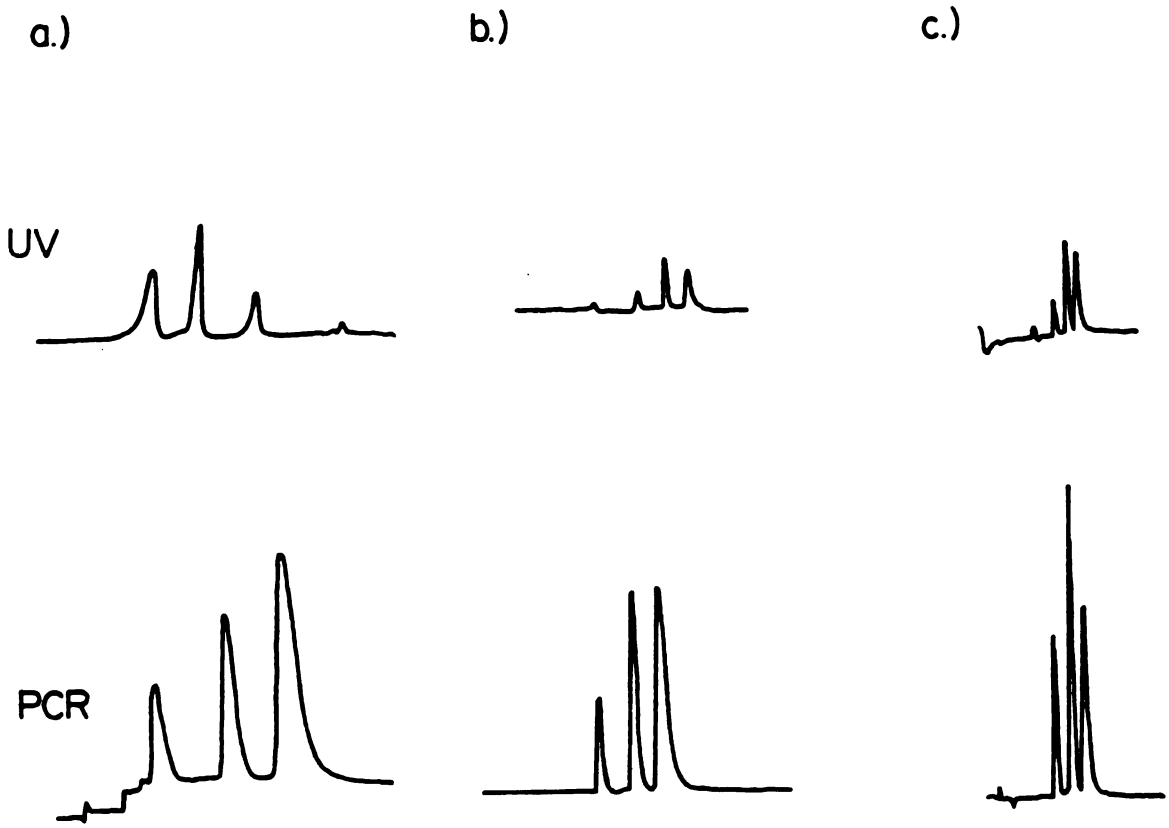


Figure 3.14. Flow rate compatibility between the HPLC and PCR systems. Sample consists of phenol, 3,5-dimethylphenol and 2,6-dichlorophenol, with elution in this order. Mobile phase is 60/40 CH_3CN/H_2O. Flow rates are a.) 0.5 ml/min b.) 1.0 ml/min and c.) 2.0 ml/min.

d. Statistical Figures of Merit

The detection limit (S/N = 2) for phenol with this PCR detector is 0.75 μM. The molar absorptivity of the phenol derivative (22500 l mol^{-1} cm^{-1}) (calculated) is 16 times that of phenol itself (1400 l $mole^{-1}$ cm^{-1}) (193). The linear dynamic range of the reaction, shown in Figure 3.15, spanned over 2 orders of magnitude, from 0.75 μM to 150 μM. The upper limit was established by deviations from Beer's law which resulted from instrumental effects rather than chemical. Reproducibility, based on 15 injections of 100 μM phenol standards into the HPLC, was ±0.64%.

e. Performance Comparison with Other Systems

As mentioned earlier, no colorimetric-based post-column reaction detectors for phenols have been developed. For this reason, it is not possible to compare the performance of the developed detector to other post-column colorimetric systems. Instead, the following discussion will focus on a comparison of the post-column derivatization of phenols with fluorescence detection and precolumn derivatizations with colorimetric detection to the system developed in this work.

The systems that were compared included two post-column derivatization schemes with fluorescence detection that have been reported by Cassidy et al. (194) and Wolkoff and Larose (8). The PCR detector reported by the first set of investigators measured the fluorescence of derivatives formed from the reaction between hydroxyphenyls and dansyl chloride (1-dimethyl-aminonaphthalene-5-sulfonyl chloride). The second PCR system, described by Wolkoff and Larose, involved the indirect detection of phenols through the reduction of cerium (IV) by phenols to the fluorescing species, cerium (III). An

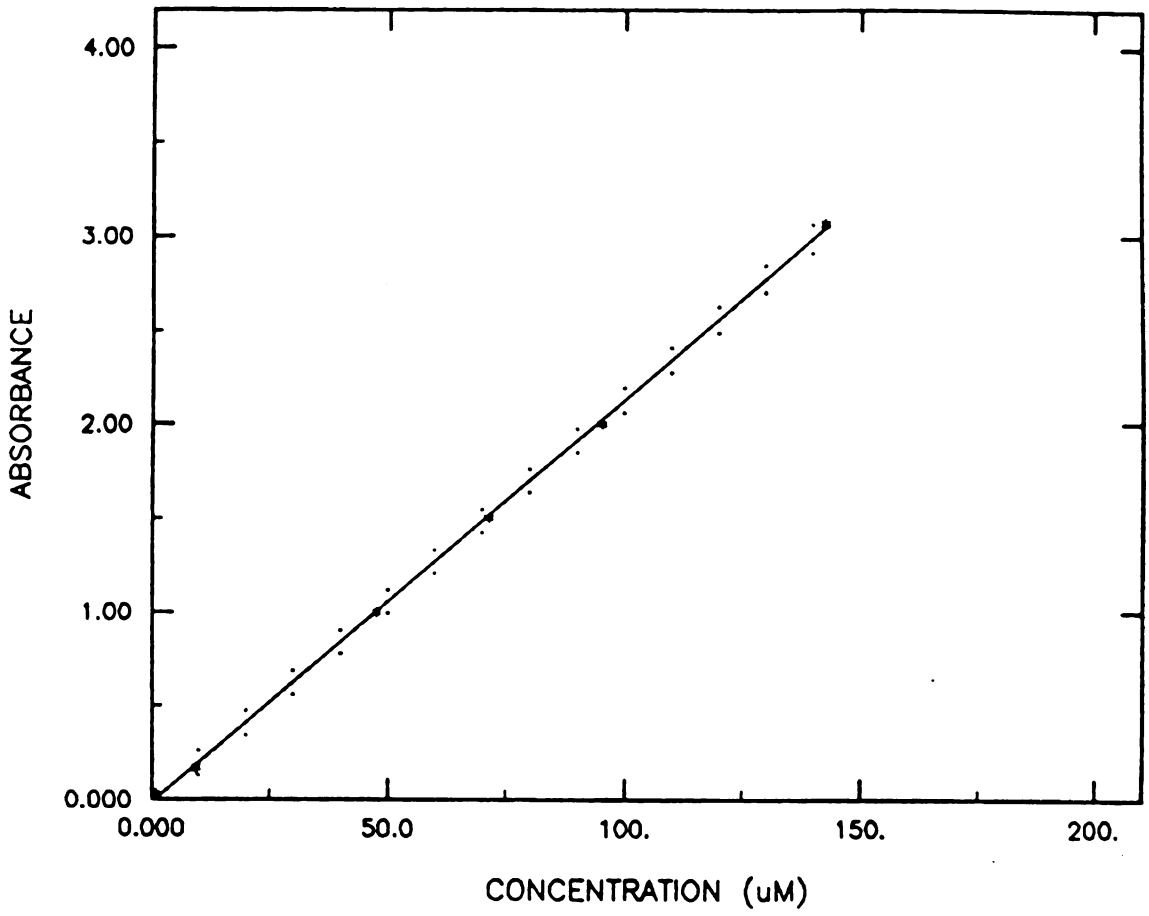


Figure 3.15. Linear dynamic range of the phenol-sulfanilic reaction.

air-segmented continuous flow analyzer was used as the PCR system. Two other systems included in this comparison involve precolumn derivatization of phenols with 4-aminoantipyrine (147) and sulfanilic acid (126) to form derivatives that absorb in the visible region. The reported analytical figures of merit for the fluorescent and colorimetric systems are summarized in Table 3.6. The figures quoted in this table for the fluorescent systems do not represent an accurate comparison with the colorimetric based detectors because different compounds were used. For example, phenol was used for the colorimetric systems, hydroxyphenyl for the dansyl chloride reaction and o-chlorophenol for the cerium system.

The analytical figures of merit that are listed in Table 3.6 indicate that post-column derivatization with diazotized sulfanilic acid, when compared to the other systems, shows the best overall results. The linear dynamic range spans over two orders of magnitude, and the precision (determined by multiple injections of phenol and measurement of the peak height) is a factor of ten better than that achieved by pre-column derivatization with 4-aminoantipyrine (4-AAP). The higher detection limit observed for post-column derivatization with sulfanilic acid, in comparison to the other colormimetric procedures, is due to dilution of the separated sample as reagents are added.

It should be noted, that although fluorescence in general, is a more sensitive detection mode than UV-visible absorption, it suffers to a larger extent from interferences. As a result, fluorometric detection may not be suitable for analyses of complex samples without extensive sample pretreatment. Fluorescence also suffers from a number of inherent problems, one of which is self absorption. This is the factor that limits

Table 3.6. Comparison of the statistical figures of merit for the determination of phenols through pre- and post-column derivatization.

Reagent	Type of Derivatization	Detection Principle	Detection limit (μM)	Linearity Range (μM)	%RSD	Ref
Dansyl Chloride	PCR	F	*	1.0 - 250	1.0	194
Cerium	PCR	F	0.04	0.1 - 2.3	*	125
Sulfanilic	PCR	C	0.75	0.75 - 150	0.64	195
Sulfanilic	Pre	C	0.08	*	*	126
4-AAP	Pre	C	0.32	*	7	147

* not available
Abbreviations are: F - Fluorescence, C - Colorimetric, Pre - pre-column

the linear dynamic range of the cerium PCR detector. Self absorption has been observed at high concentrations of cerium (IV) sulfate (196).

4. Applications

The performance of the reaction detector as a sensitive and selective HPLC detector for phenols was compared with that of the UV detector in the analysis of real samples.

a. Determination of Phenols in River Water

Grand River (Lansing, MI) water, collected downstream from a coal driven power plant, was analyzed for the presence of phenols. No phenols were detected. The sample was then spiked with 1.9 ppm phenol, 2.2 ppm o-cresol, 2.4 ppm 3,5-dimethylphenol and 2.2 ppm resorcinol. The detector responses, both prior to (UV) and following derivatization (PCR) are shown in Figure 3.16. The chromatographed peaks were barely discernible by UV detection but clearly distinguishable by PCR detection despite the sensitivity of the former being set at a lower scale than the latter. The figure clearly demonstrates the increased sensitivity obtained when using post-column derivatization.

The diazo coupling reaction is an electrophilic aromatic substitution reaction. Thus, the aromatic compounds that are most likely to undergo this reaction would be those with a strongly activating substituent such as phenols and aromatic amines. The class selectivity of the PCR detector is demonstrated by the chromatogram shown in Figure 3.17 where the UV and PCR responses are compared for a water

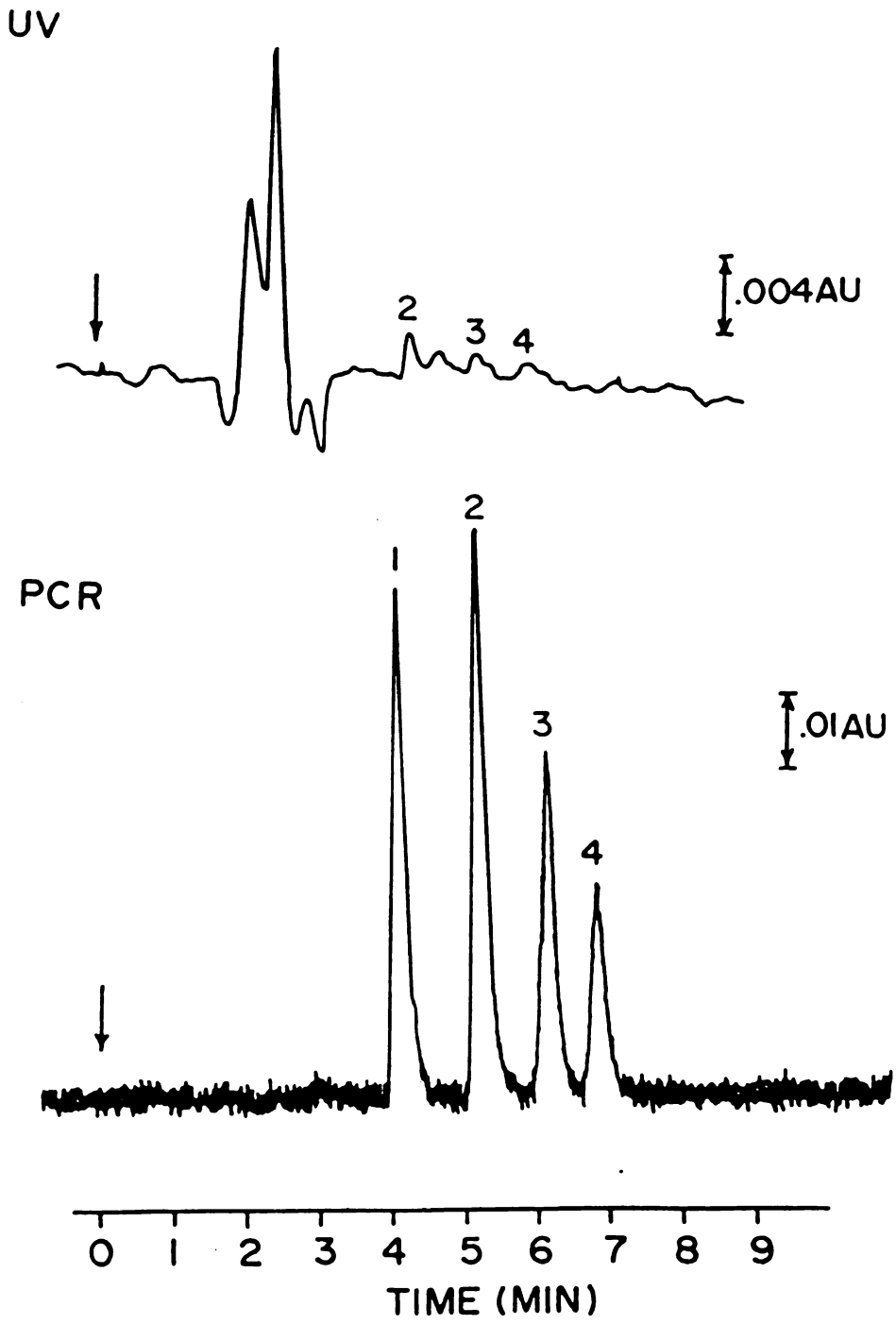


Figure 3.16. Comparison of UV and PCR detection for the separation of phenols in a spiked river water sample (Grand River, East Lansing, Michigan). 1.) resorcinol, 2.2 ppm 2.) phenol, 1.9 ppm 3. o-cresol, 2.2 ppm 4.) 3,5-dimethylphenol, 2.4 ppm. HPLC: Spherisorb ODS column; acetonitrile/water (50:50) mobile phase; flow rate of 1.0 ml/min; UV detection at 254 nm; 0.04 AUFS. PCR: manifold as shown in Figure 3.2; detection at 450 nm; 0.10 AUFS.

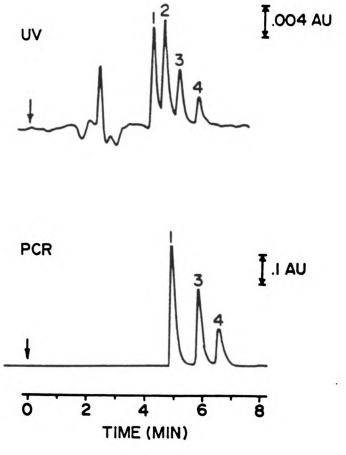


Figure 3.17. Comparison of selectivity of UV and PCR detection for phenols in a spiked river water sample. 1.) phenol, 18.8 ppm 2.) aniline, 19.6 ppm 3.) o-cresol, 21.6 ppm 4.) 3,5-dimethylphenol, 24.4 ppm. HPLC: Spherisorb ODS column; acetonitrile/water (50:50) mobile phase; flow rate of 1.0 ml/min; UV detection at 254 nm; 0.04 AUFS. PCR: manifold as shown in Figure 3.2; detection at 450 nm; 1.0 AUFS.

sample that had been spiked with phenol, aniline, o-cresol and 3,5-dimethylphenol. UV detection of the chromatographed sample showed the presence of all four compounds, with aniline as a major peak. The PCR detector showed only three peaks which corresponded to the phenols. This is a result of the different optimum reaction conditions needed for DSA coupling to phenol and to aromatic amines.

It may be observed in Figures 3.16 and 3.17, that the column void volume peak was not present in the PCR detector trace. This is due to the toroidal flow within each liquid segment that is characteristic of air-segmented flow streams. The result is enhanced mixing which leads to homogeneity within each liquid segment.

b. Phenols Determination in Residual Fuel Oil

A residual fuel oil sample was separated into fractions of different functional group types by sequential elution solvent chromatography. The fraction containing phenols was chromatographed under isocratic conditions with a 50:50 acetonitrile:water mobile phase. A series of peaks were observed in the UV trace as shown in Figure 3.18. Only two peaks were evident in the PCR detector recording. These peaks have been tentatively identified by retention times and spiking experiments to be phenol and a cresol. Figure 3.19 shows the responses obtained when the sample was spiked with 90 ppm and 400 ppm phenol. At the lower concentration, there is no measureable change in relative peak heights (ratio of phenol containing peak, peak b, to peak a on the chromatogram) for the UV detector response, whereas an 11 fold increase is observed in the PCR trace. When the sample was spiked with 400 ppm phenol, a two fold increase in the relative peak height is

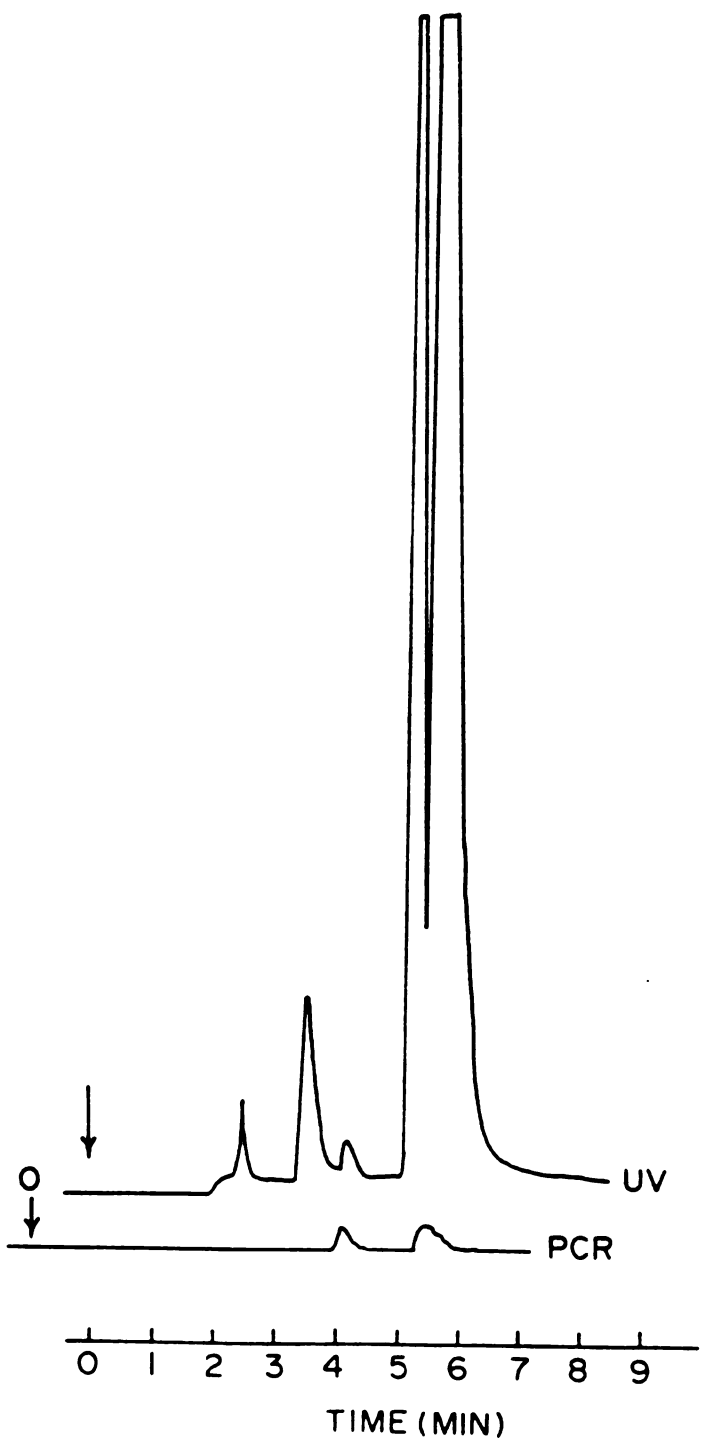


Figure 3.18. Reverse phase separation of RFO, fraction #7. HPLC: Spherisorb ODS column; acetonitrile/water (50:50) mobile phase; flow rate of 1.0 ml/min; UV detection at 254 nm; 0.64 AUFS. PCR: manifold as shown in Figure 3.2; detection at 450 nm; 1.0 AUFS.

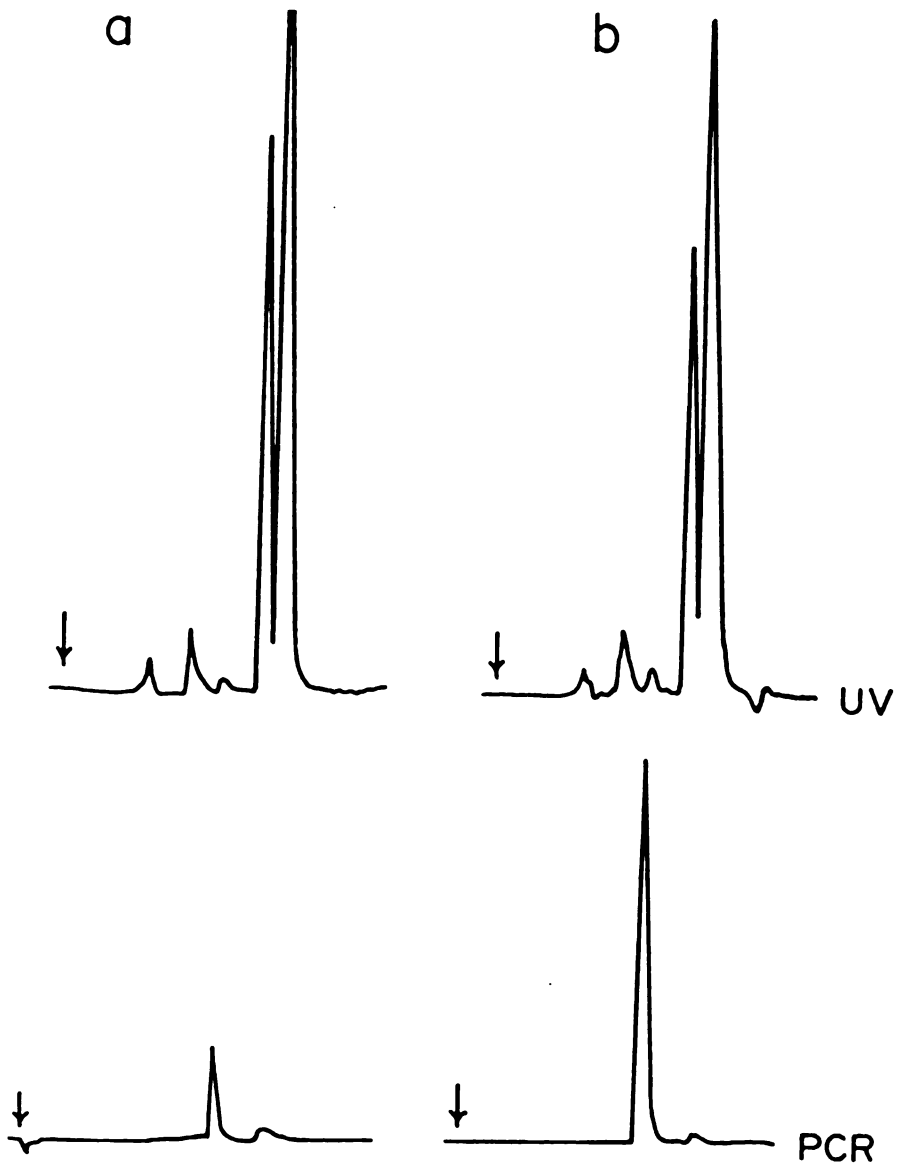


Figure 3.19. Chromatogram of RFO, fraction #7, spiked with a.) 90 ppm phenol b.) 400 ppm phenol.

observed in the UV trace and a 46 fold increase is obtained for the PCR response. This further demonstrates the greater sensitivity of the PCR detector over the UV detector. In the identification of cresol by spiking, the sample was diluted 20 times and the resultant solution spiked with 32 ppm o-cresol. The detector responses are shown in Figure 3.20. From the change in relative peak heights (UV) and the corresponding changes in the PCR response, it was apparent that other compounds were coeluting with phenol and o-cresol and were not detected by the derivatization detector.

D. Conclusions

The advantages of using post-column derivatization in HPLC are apparent. The gain in selectivity and sensitivity have been demonstrated with applications to phenols determination in water and fuel samples. The latter application also shows that a potentially complicated separation may be greatly simplified by using a detector of this type. In addition, the advantages of using air-segmented continuous flow analysis such as minimized band broadening due to longitudinal dispersion in the PCR detector and enhanced mixing within each liquid segment have been illustrated. Other positive points, particular to this system, include on-line detection and compatibility between the separation and reaction systems with respect to flow rates and varying acetonitrile/water mobile phase composition.

Post-column reaction detection of phenol derivatives formed from the diazo coupling reaction with sulfanilic acid does have its limitations, however. The first of these disadvantages is dilution, which is inherent

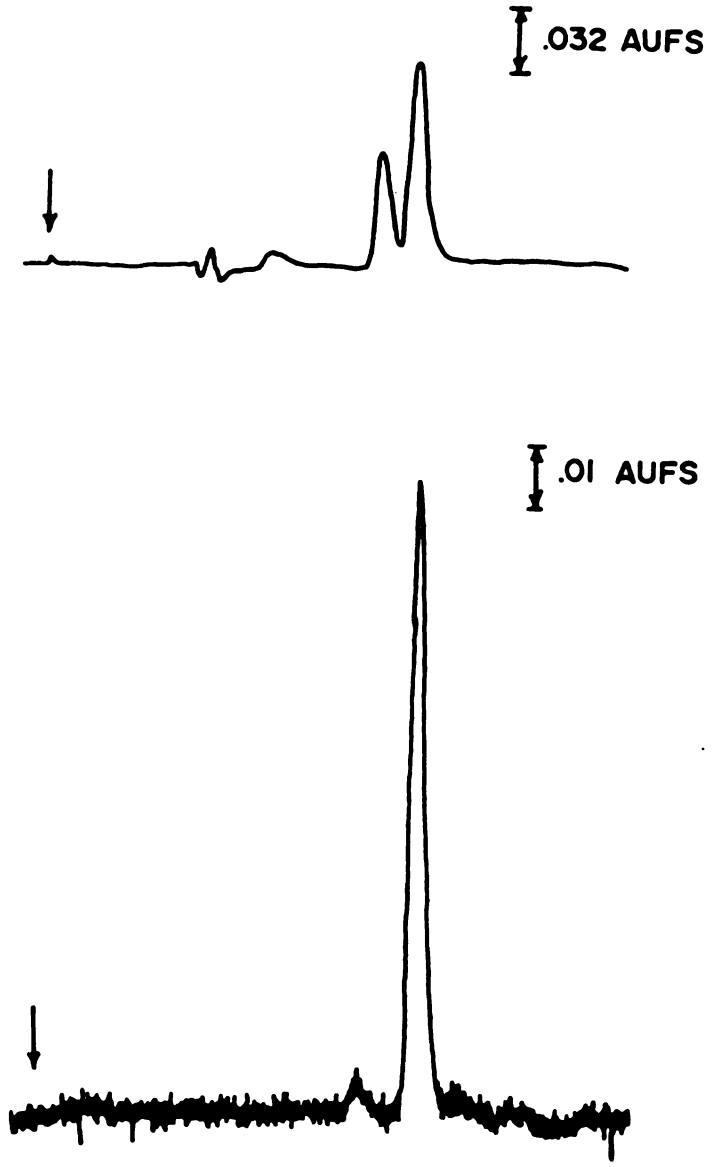


Figure 3.20. Chromatogram of RFO fraction #7 diluted 20x and spiked with 32 ppm o-cresol.

to PCR detector systems that involve the addition of reagents to the column effluent. Therefore, the volume of reagents added should be kept to a minimum. A second limitation is that the diazo coupling does not occur for all phenols and therefore, cannot be used for the determination of total phenols. As with other colorimetric reactions for phenols that involve electrophilic aromatic substitution, in general no reactions occur for phenols containing electron withdrawing groups. Phenols with substituents occupying both the ortho and para positions to the hydroxyl group also yield no response to this reaction as the positions at which electrophilic aromatic substitutions would take place are blocked. This lack of response from all phenols may be regarded as a disadvantage or an advantage depending on the desired information.

A survey of the reactivity of 126 phenols to diazotized sulfanilic acid, as measured by the presence of a measureable absorbance, was reported by Koppe et al. (119). In general, this work provides a good guide to the types of phenols that undergo this reaction. However, detectable responses have been obtained in our laboratory for some phenols that have been listed by Koppe et al. as having no responses ie. 2,4-dichlorophenol and p-tert-butylphenol. This may be a result of the reaction conditions that were used by Koppe et al. being different from that employed by us e.g., NaOH was added to obtain a basic solution. As previously noted, the buffering systems are not inert.

CHAPTER IV

THE DEVELOPMENT OF RECYCLE HEARTCUT CHROMATOGRAPHY (RHC) FOR ANALYSES IN COMPLEX SAMPLE MATRICES

A. INTRODUCTION

The development of a new valve switching technique, which we call recycle heartcut chromatography (RHC), is described. Preliminary results are presented that characterize the RHC system. Several potential applications are explored.

The method of recycle heartcut chromatography involves the repetitive isolation of specific components and the recycling of these components through the column for an improved separation. The HPLC components used to implement RHC are shown in Figure 4.1. In addition to the two six port valves, the system consists of a single detector, a pump and one column. The recycled sample does not flow through the pump so that the problem of peak dispersion in the pump cavity is not observed. Such dispersion is encountered in the direct pumping approach to recycle chromatography. The use of the same column for recycling eliminates any complications that may arise from employing more than one column as with the alternate pumping principle. Such complications originate from variations in column characteristics.

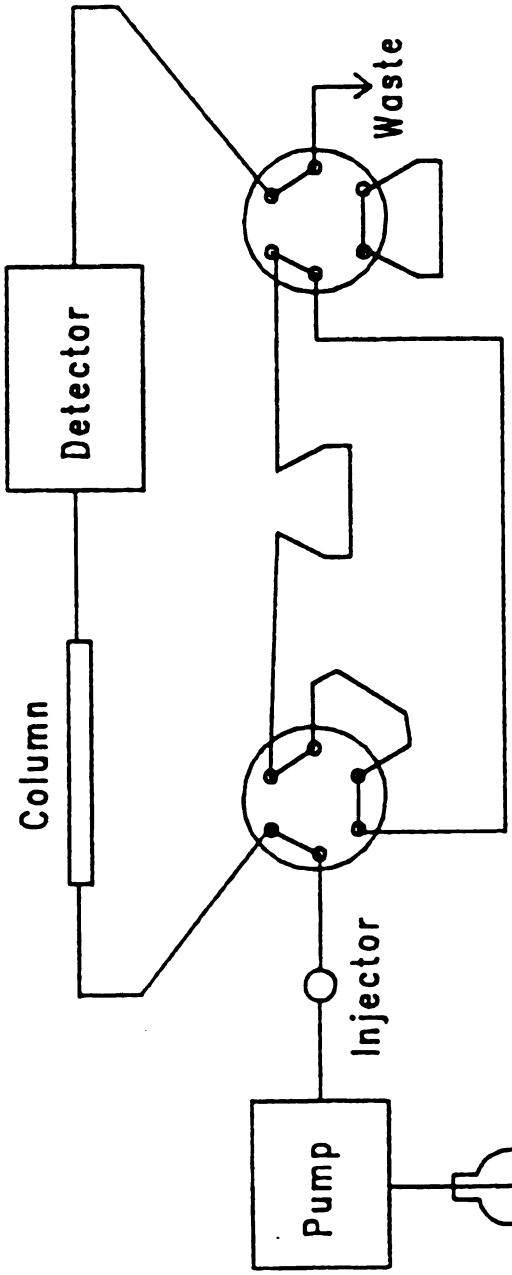


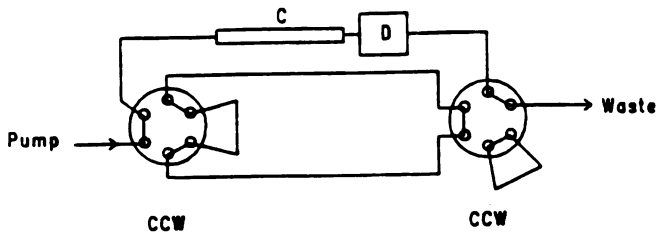
Figure 4.1. HPLC system and valving configuration used to implement recycle heartcut chromatography.

The valve switching sequence used in RHC is demonstrated in Figure 4.2. When the sample is first injected, valves 1 and 2 are both in the counterclockwise (CCW) position as shown in Figure 4.2a. The column effluent is directed to waste until the desired fraction begins to elute. At this point, valve 2 switches clockwise (CW) (Figure 4.2b) to divert this fraction to a heartcut loop that is made from stainless steel tubing. After an appropriate delay, valve 2 switches back counterclockwise (Figure 4.2c) to allow the column to flush. Figure 4.2d shows the final stage when valve 1 is turned clockwise to divert the mobile phase flow through the heartcut loop and sweep its contents onto the head of the column. This valve switching sequence is repeated in order to obtain a 'cleaner' and more resolved fraction with each recycle.

There are a number of advantages that arise from the positioning of the detector in this configuration. First, only one detector is needed to monitor the column effluent continuously. The detector output can be used to trigger a valve switch, as discussed in a later section. Third, a high pressure flow cell is not needed because one end of the detector is always connected to ambient air.

In the following sections, the instrumentation used in RHC is described. The methods used to trigger valve switchings are illustrated. The various contributions to band broadening are discussed for the specific heartcut loops that were used in this work. Finally, some applications of RHC are presented.

a.)



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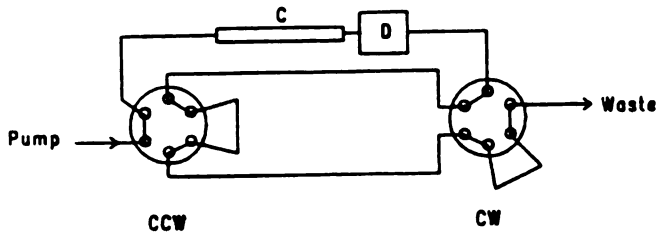

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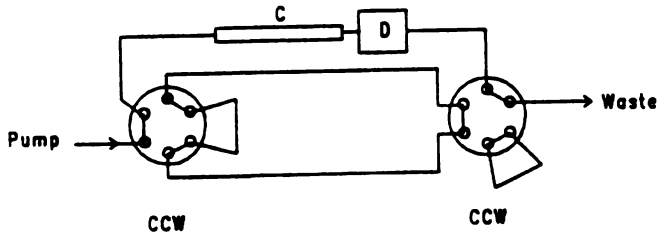

d.)

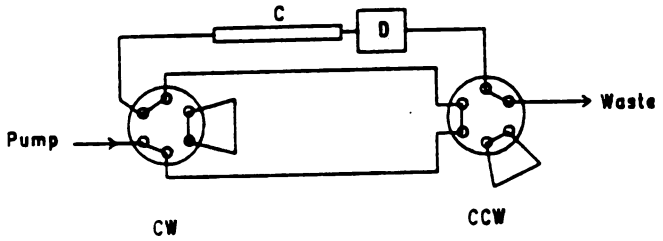

Figure 4.2. Valve switching sequence employed in RHC, a.) start-up b.) heartcut c.) column flush and d.) recycle heartcut.

B. EXPERIMENTAL

1. Instrumentation

The description of the instrumentation used to implement RHC is divided into two sections. First, details are given of the HPLC apparatus that was employed. This is followed by a discussion of the role of the microcomputer in experiment control.

a. HPLC Apparatus

A block diagram of the apparatus employed for recycle heartcut chromatography is shown in Figure 4.1. The HPLC system is assembled from a Spectraphysics SP8700 ternary solvent delivery system (Santa Clara, CA) with a Z80 microprocessor, a Rheodyne 7120 injection valve (Cotati, CA) with a 20 μl sample loop, a 4.6 mm x 25 cm Zorbax ODS column (Dupont, Wilmington, DE), a Chromatronix 220 UV absorbance detector (Berkeley, CA) and two Rheodyne valves (7010 and 7040) with pneumatic actuators for diverting the solvent flow stream along various paths. The pneumatic actuators are operated by 2-port and 2-position solenoid valves (Scovill Corporation, Wake Forest, NC) which direct the flow of air or nitrogen to either side of the actuator piston. The gas pressure (50 psi) provides the driving force that turns the valves. For details on the circuitry employed, the reader is referred to the original work (197).

b. Microcomputer Controlled HPLC System

The HPLC system is controlled by an Intel 8085 based microcomputer that was built in our laboratory (197). As shown in Figure 4.3, the microcomputer can be used to control the pump, the chart recorder drive and the valve switchings as well as to facilitate a number of other functions.

Communication between the pump and the microcomputer is achieved through the use of a keypad emulator (198) which allows the computer to simulate a keystroke on the keypad of the SP8700 solvent delivery system. This set-up enables the option of a synchronized separation and data acquisition scheme consisting of changing separation conditions as well as valve switchings and data acquisition based on the same time clock. The AM9513 timer (Advanced Micro Devices), used for this purpose, is installed as part of the microcomputer system. Data taken by the microcomputer are stored and processed with a DEC LSI 11/23 minicomputer. The chromatogram is displayed on a chart recorder which may also be placed under microcomputer control. The valves are switched as directed by the TTL signal sent by the microcomputer to an optically isolated relay that is connected to the solenoid valve. Valve positions are sensed by the microcomputer via the absence or presence of a current through a reed switch that is mounted on the valve housing. A magnet mounted on the valve shaft causes the switch contacts to close as it moves near the switch when the valve is turned clockwise. The state of this reed switch, sensed by the microcomputer, enables it to determine the position of the valve and whether the valve turning had been successfully carried out.

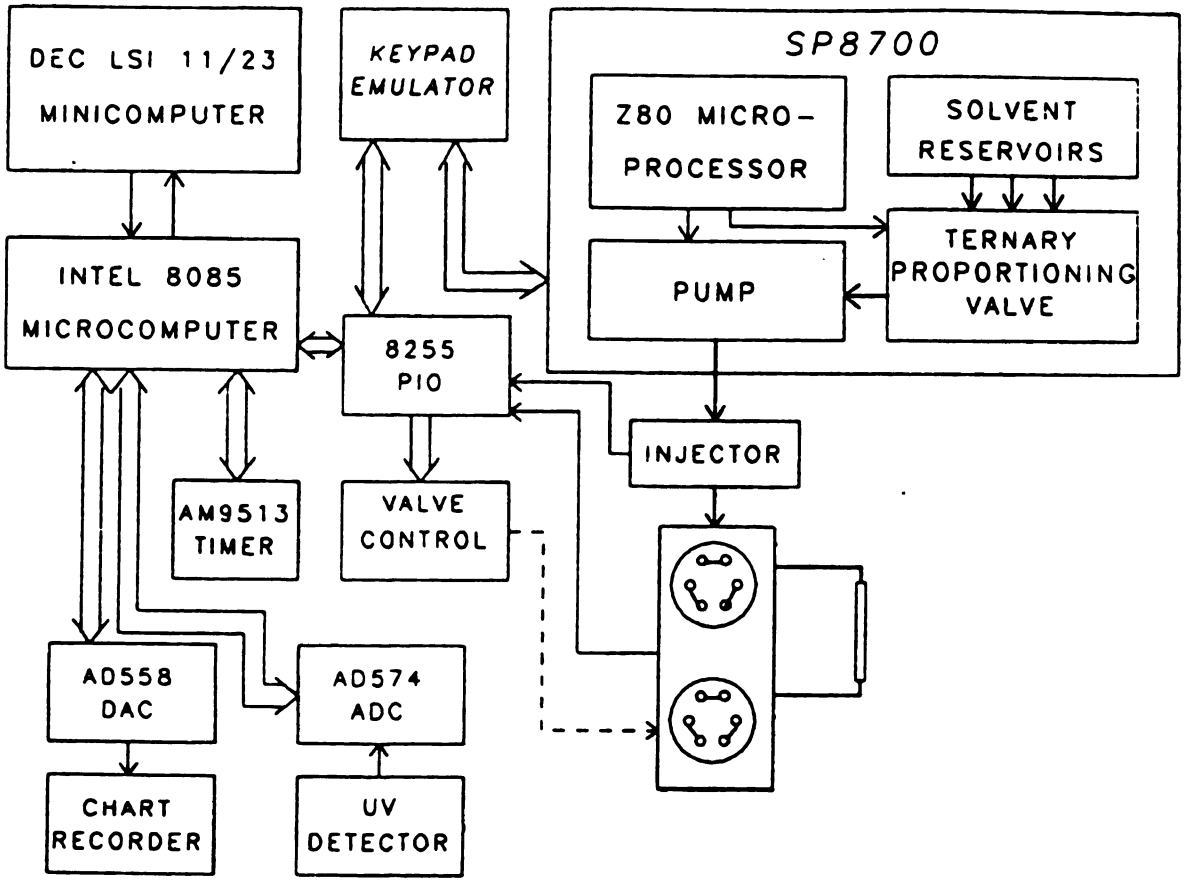

Figure 4.3. Block diagram of microcomputer controlled HPLC system used in RHC.

This system, aside from the initial sample injection, is completely automated. Its construction also allows for the manual control of each of the functions mentioned above.

2. Software

The software developed by P. M. Wiegand (197) employed polyFORTH as the operating language at the microcomputer level. FORTH is a threaded language that allows a new word to be defined either by stringing together existing words or by building the word from the basic kernal commands. A single word may be used to carry out a complex sequence of events which makes this language particularly suitable for instrument control. In addition, the FORTH routines are easy to read, write and alter. An example of a FORTH routine that is used to take a cut when a specific peak is sensed and to recycle it is shown in Figure 4.4. When the command " 1CUT " is entered, the following events take place:

1.) Valves 1 and 2 are turned counterclockwise (CCW).
2.) When the mobile phase flow has stabilized, a message is displayed on the terminal prompting the operator to inject a sample.
3.) When the sample is injected, the microcomputer clock starts timing and the recorder chart drive is turned on.
4.) At 2 min 30 s, the data acquisition and peak counting routines are initiated.
5.) When the peak count = 1, valve 2 is switched CW for a heartcut.
6.) After a 12 s delay, valve 2 is turned CCW to allow the column to flush.
7.) At 4 min 30 s, valve 1 is turned CW and the heartcut is recycled.
8.) At 8 min 30 s, the message " END OF RUN " is displayed on the terminal.
9.) The pump, chart drive and peak finding and data taking routines are ended.

```
Block Number: 85
0       (One cut)
1       : 1CUT 1 CCW 2 CCW
2       FLOW? ." FLOW IS READY, INJECT SAMPLE."
3       INJECT DIGREC ON
4       2 30 EVENT TAKEDATA Z#PEAKS ?PEAK
5       BEGIN 0 RFLAG C! PAUSE #PEAKS @ 1 = END 2 CW
6       1200 WAIT 2 CCW
7       4 30 EVENT 1 CW
8       8 30 EVENT ." END OF RUN."
9       STP PRESS OFF 1STOP 4STOP ENDDATA ;
```

Figure 4.4. An example of a FORTH block used to take a heartcut based on peak count which is then recycled through the column.

3. Solution and Sample Preparation

Polyaromatic hydrocarbons were purchased from Chem Service (West Chester, PA). Steroids were obtained from Steraloids, Inc. (Wilton, NH). All solutions were filtered through a 0.45 μm filter prior to injection into the HPLC system.

Urine samples were pretreated using a C_{18} Sep-Pak cartridge (Waters Associates, Milford, MA) which had been prewetted with 2 ml of methanol followed by 5 ml of water. The cartridge was then charged with 20 ml of urine and washed with 5 ml of water to remove salts. A 3 ml amount of methanol was used to elute the organic components off the cartridge. This fraction was collected and spiked with 10 μM of 1,4 androstadiene-3,17-dione. As a final step, the urine samples were filtered through a 0.45 μm filter.

Spectral grade HPLC solvents (Burdick and Jackson, Muskegon, MI) were filtered through a 0.45 μm filter and degassed with helium prior to use.

C. Evaluation of the RHC System

1. Reproducibility of Valve Switchings

The microcomputer software has been developed so as to allow valve actuation to be initiated based on the fulfillment of a variety of conditions. These conditions are peak number, detector threshold and time. Heartcuts that are initiated by the first two methods are referred to as detector-based cuts since the detector output is monitored to

determine whether the conditions for a heartcut are met. Excerpts of FORTH routines used to implement valve switchings in the determination of valve switching precision are listed in Figure 4.5. The first case, Figure 4.5a, is set up to count the number of peaks, with instructions to turn valve 2 CW for a heartcut when the first peak is sensed. Valve actuation can also be initiated by setting a threshold based on detector output as shown in the Figure 4.5b. In this example, a cut is taken when the detector response has reached 10% of the full scale value. The third type of heartcut is one that is based on the time after injection. As demonstrated by the third case, Figure 4.5c, this is accomplished by specifying a time (e.g. 4 min 30 s).

The reproducibility of the valve switchings was determined by injections of a 300 μM naphthalene solution followed by a 12 s heartcut that corresponds to a 200 μl volume. This heartcut was sent back through the column, and the peak height of the recycled peak was recorded for use in precision calculations. The mobile phase was 100% methanol at a flow rate of 1.0 ml/min. The detector was set at 0.16 absorbance units full scale (AUFS). Table 4.1 lists the peak height measurements made for the first and second cycles through the column. The percent relative standard deviation (%RSD) was calculated from the ratio of peak heights of cycle 2 to that of cycle 1 to eliminate the contribution from injection inconsistencies. Ideally, the sample should be injected as a sharp plug. However, mixing of the sample and mobile phase can occur due to imperfect injector design, badly made connectors and poor injection technique. Any of these factors could lead to a variation in peak height.

a. 2 30 EVENT TAKEDATA Z#PEAKS ?PEAK

BEGIN 0 RFLAG C! PAUSE #PEAKS @ 1 = END 2 CW

b. 2 30 EVENT TAKEDATA

10 %FSCHG 2 CW

c. 2 30 EVENT TAKEDATA

4 30 EVENT 2 CW

Figure 4.5. Examples of FORTH commands used to initiate valve actuations by a.) peak count b.) threshold and c.) time after injection.

Table 4.1. Determining the precision of valve switchings triggered by peak count, detector threshold (10% full scale) and time.

Peak Height (A/D units)		Relative peak height	% RSD
Cycle 1	Cycle 2		
Peak Count			
792	304	.384	
849	311	.366	
829	325	.392	4.54
825	318	.385	
819	340	.415	
Detector Threshold			
824	483	.586	
830	475	.572	
809	501	.619	3.23
821	496	.604	
818	501	.612	
Time			
840	423	.504	
840	448	.533	
830	373	.449	7.25
868	428	.493	
875	397	.454	

The retention time, R_t, of a compound can differ from day to day as a result of small fluctuations in separation conditions such as temperature and flow rate. Over an extended length of time, these variations may arise from the deterioration of the analytical column. Regardless of the source, this change in R_t causes a different portion of the chromatogram to be isolated when time-based cuts are employed. This results in a variability of the peak height and area of the recycled peak. The advantage of detector-based heartcuts over time-based cuts is evident. The variations in retention times do not affect the detector-based cuts as they do a time-based system. This is reflected by the %RSD values that are listed in Table 4.1 which shows better precision for detector-based cuts. Even though detector-based cuts are preferred, certain applications will require the use of time-based cuts.

2. Band Broadening in the RHC System

Band broadening is a crucial factor in RHC. Improvements in resolution may be lost if significant amounts of dispersion are introduced in recycling the heartcut. Band broadening can originate from components of the HPLC system such as the injection valve, tubing, column, detector and any of the numerous connectors.
In this section, the dispersion that occurs in the tubing used as the heartcut loop is described. The peak widths at half height of the recycled peak are compared when tubing of different dimensions are used. The feasibility of using a heartcut loop of larger volume than needed and of partially filling it is discussed.

A 450 μM naphthalene solution was used in these studies. The mobile phase was 100% methanol and the flow rate employed was 1.0 ml/min. Time-based heartcuts were utilized throughout.

a. Heartcut Loop Tubing Dimensions

The heartcut loops were made from stainless steel tubes of various lengths and internal diameters (i.d.). A cut of the eluting naphthalene peak was diverted into the heartcut loop. This cut was then recycled through the column, and the peak width at half height, $w_{1/2}$, measured. The values for $w_{1/2}$ for both the first and second cycle through the column are summarized in Table 4.2. Note that tripling the length of the 0.01" i.d. tube (cases 1 and 3) produces a 15% increase in $w_{1/2}$ whereas tripling the length of the 0.02" i.d. tube (cases 4 and 5) produces a 65% increase. Equal volume cuts with the two different i.d. tubes (cases 2 and 4) yield approximately equal $w_{1/2}$ values. This leads to the conclusion that long lengths of 0.02" i.d. tubing should not be used as the heartcut loop. This is confirmed when the $w_{1/2}$ values of the recycled peaks are compared for the cases in which long lengths of the two tubes are used (cases 3 and 5). The 0.02" i.d. tube yields a recycled peak width that is 60% higher than that obtained for the 0.01" i.d. tubing. Therefore, if large volume cuts need to be made, the 0.01" tubing should be employed.

The trends observed between peak width and tubing dimensions are as expected. The degree of band broadening in tubing is directly

Table 4.2. Effect of tubing dimensions on the peak width at half height, $w_{1/2}$, of a recycled peak.

Heartcut Tubing Dimensions			$w_{1/2}$ (± 0.03 cm)	
Length (cm)	i.d. (in)	Volume (ml)	Cycle 1 (cm)	Cycle 2 (cm)
60	0.01	34.0	2.45	2.20
72	0.01	36.8	2.45	2.19
183	0.01	92.6	2.45	2.49
18	0.02	36.8	2.45	2.32
60	0.02	121.6	2.45	3.93

proportional to the tube length and proportional to the fourth power of the tube radius as expressed in the equation derived by Scott and Kucera (199).

$$\sigma_{tb}^2 = \frac{\pi r^4 l}{24 D_m Q} \qquad \text{Equation 4.1}$$

The symbols in the above equation denote the following:

σ_{tb} is the standard deviation of the Gaussian peak that represents band broadening due to the tubing (s),

r is the internal radius of the tubing (cm),

l is the length of the tubing (cm),

D_m is the diffusitivity of the solute in the mobile phase (cm^2/s) and

Q is the flow rate of the mobile phase (cm^3/s).

A second expression, Equation 4.2, can be derived to calculate the relative amount of band broadening introduced by heartcut loops made from tubes of different lengths and diameters. This equation assumes identical separation conditions for the cases being compared.

$$\frac{(\sigma_{tb2})^2}{(\sigma_{tb1})^2} = \frac{(r_2)^4 l_2}{(r_1)^4 l_1} \qquad \text{Equation 4.2}$$

The calculated and experimental values for band broadening are compared in Table 4.3. The calculated values were higher than those obtained experimentally. A possible explanation for this observation lies in the necessary coiling of the long lengths of tubing used as the heartcut loop. In continuous flow analysis systems, coiling a piece of tubing will result in a smaller amount of dispersion than that observed for the same length of straight tubing (87).

Table 4.3. Comparison of the calculated and experimental band broadening in tubing of various dimensions.

Heartcut Tubing Dimensions		% Band Broadening*	
Length (cm)	i.d. (in)	Calculated	Experimental
60	0.01	0	0
72	0.01	110	0
183	0.01	170	113
18	0.02	220	105
60	0.02	400	179

* calculated relative to values obtained for the case of 60 cm x 0.01" i.d.

b. Partial Filling of the Heartcut Loop

With the configuration described above, the heartcut volume is fixed and determined by the dimensions of the tubing used as the heartcut loop. One method that would allow different heartcut volumes and increase the flexibility of the system is to use a large volume heartcut loop which is only partially filled. Studies were performed to determine whether this approach would be analytically useful. Two pieces of tubing, 0.01" i.d. x 60 cm and 0.02" i.d. x 60 cm, were used as heartcut loops. A 450 μM naphthalene solution was injected and cuts of different sizes (Figures 4.6 and 4.7) were taken so as to fill the loop to various extents. The peaks resulting from recycling the partial loop fills of two heartcut loops are shown in Figures 4.8 and 4.9. The same behaviour was observed for both the 0.01" and 0.02" i.d. loops. First, the peak maximum occurs at a progressively longer time as the percentage of the loop volume that is used, V_{loop}, decreases. In partially filling the heartcut loop, the sample displaces the mobile phase present in the loop beginning with the section that is closest to the column outlet. The remaining portion of the loop that is not occupied by sample (i.e., the side attached to the column inlet) remains filled with mobile phase. The distance between the center of the heartcut sample plug and the column inlet increases when the percentage of the loop volume that is used, V_{loop}, decreases. This causes the small offset in retention times that is observed (Figures 4.8 and 4.9) when the heartcuts of different V_{loop} values are recycled. Second, a disproportionality is observed between peak height and V_{loop}, i.e., the peak height for the 50% loop fill is not half that of the 100% loop fill. The reason for this becomes clear when Figures 4.6 and 4.7 are

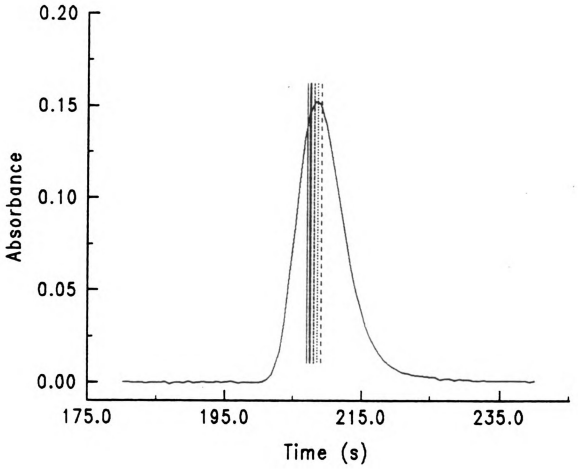

Figure 4.6. Heartcuts taken to fill a 60 cm x 0.01" i.d. loop to various extents - 100% loop fill (——), 75% loop fill (····), 50% loop fill (-·-·) and 25% loop fill (——). 450 μM naphthalene with a 100% CH_3OH mobile phase and a 1.0 ml/min flow rate. Detection is at 254 nm and 0.16 AU full scale.

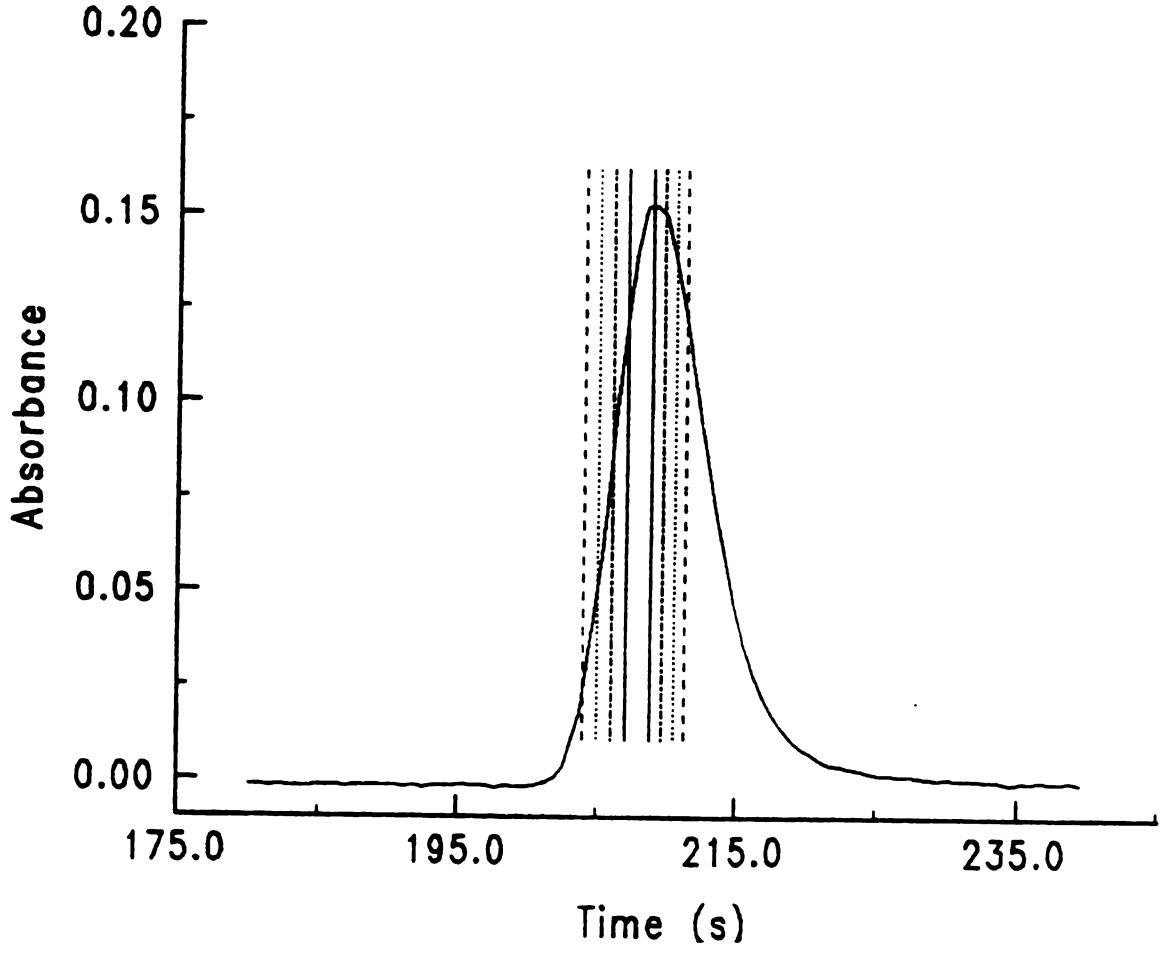

Figure 4.7. Heartcuts taken to fill a 60 cm x 0.02" i.d. loop to various extents - 100% loop fill (——), 75% loop fill (····), 50% loop fill (-·-) and 25% loop fill (——). 450 mM naphthalene with a 100% CH_3OH mobile phase and a 1.0 ml/min flow rate. Detection is at 254 nm and 0.16 AU full scale.

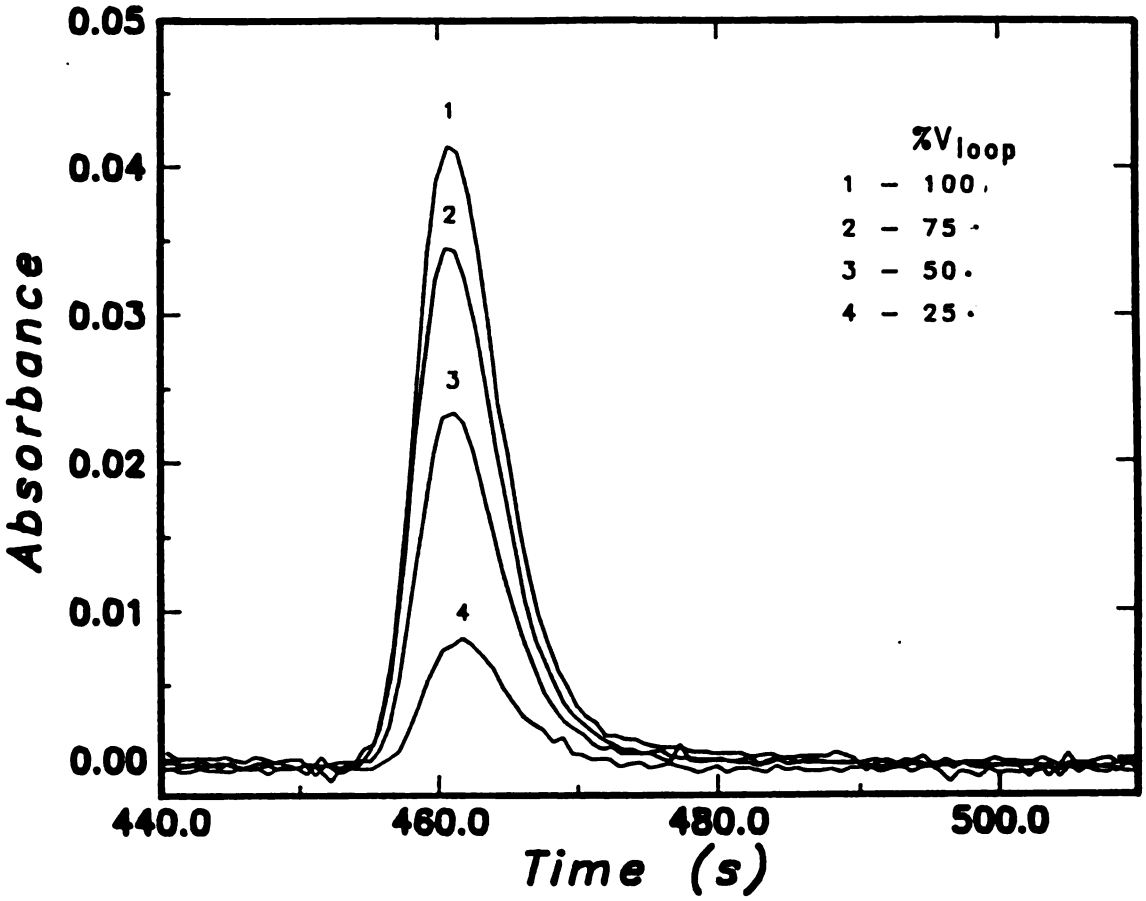

Figure 4.8. Chromatograms of the recycled peaks that resulted from the partial filling of the heartcut loop (60cm x 0.01" i.d.).

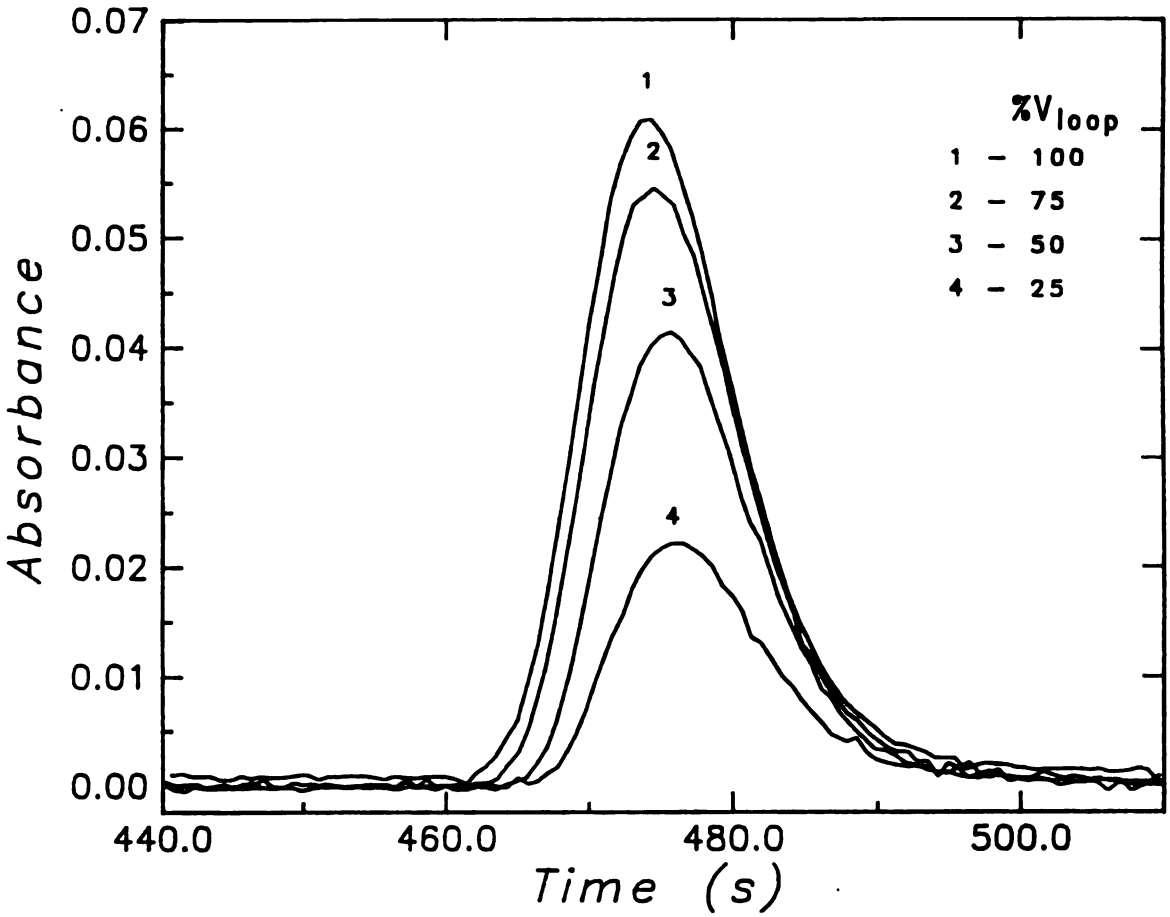

Figure 4.9. Chromatograms of the recycled peaks that resulted from the partial filling of the heartcut loop (60cm x 0.02" i.d.).

reexamined. The cuts were taken on the slope of the peak. Thus, the amount of sample within each heartcut is not proportional to the volume of the cut. Third, the $w_{1/2}$ values of peaks that result from recycling heartcuts of different volumes that partially filled a tube are approximately equal for each tube. The $w_{1/2}$ values of the recycled peaks in the cases where tubes of different i.d.s are filled to various extents are summarized in Table 4.4. In the case of no sample dispersion, the expected result is a decreasing $w_{1/2}$ value from 100% V_{loop} to 25% V_{loop} for each piece of tubing. The fact that the values for $w_{1/2}$ listed within each set of peaks are not significantly different indicates that dispersion is taking place. Work needs to be done to determine whether this dispersion is occurring predominantly in the heartcut loop or in other parts of the separation system. Despite the dispersion, the plots show that partial loop fills are a feasible alternative.

D. Applications

Preliminary experimental work on the application of RHC to various problems has been carried out. The following discussion illustrates the experimental approach used in RHC and shows the potential of the technique.

Table 4.4. Values of peak width at half height, $w_{1/2}$, of recycled heartcuts that partially filled tubes of different i.d.s to varying extents.

V_{loop} (%)	$(w_{1/2})_{0.01}$ (cm)	$(w_{1/2})_{0.02}$ (cm)
100	1.29	2.28
75	1.29	2.25
50	1.27	2.20
25	1.27	2.20

1. Component(s) Isolation

There are numerous instances in which the purpose of the analysis is to verify the presence of specific compounds and to provide quantitative information about them. Examples of such cases are the testing of body fluids for specific drug metabolites and the analysis of body fluids and tissue for certain chemicals that are produced by the body as a sign of disease. In both cases, the sample matrices are very complex. One way to address this type of analysis is to extensively pretreat the sample with hopes of removing all possible interferences before subjecting the sample to a final analytical separation. The complication often encountered is the presence of interferences which have not been removed in the sample pretreatment step because of their similar chemical and physical characteristics to the compounds of interest. An intricate and time consuming separation scheme may have to be used to circumvent this problem. An alternative method of analysis is to continuously isolate and separate the compounds of interest from the matrix. This can be accomplished with recycle heartcut chromatography. Only minimal amounts of pretreatment are needed prior to injection into the HPLC system. The heartcut step provides any further fraction isolation that may be necessary. The recycling step should provide an increase in the number of theoretical plates and thus, improved resolution.

The analysis of a urine sample for the steroid, 1,4-androstadiene-3,7-dione, is illustrated in Figure 4.10. The term, cycle 1, is used when referring to the first pass through the column and cycle 2, when referring to the second pass. The portion of the chromatogram

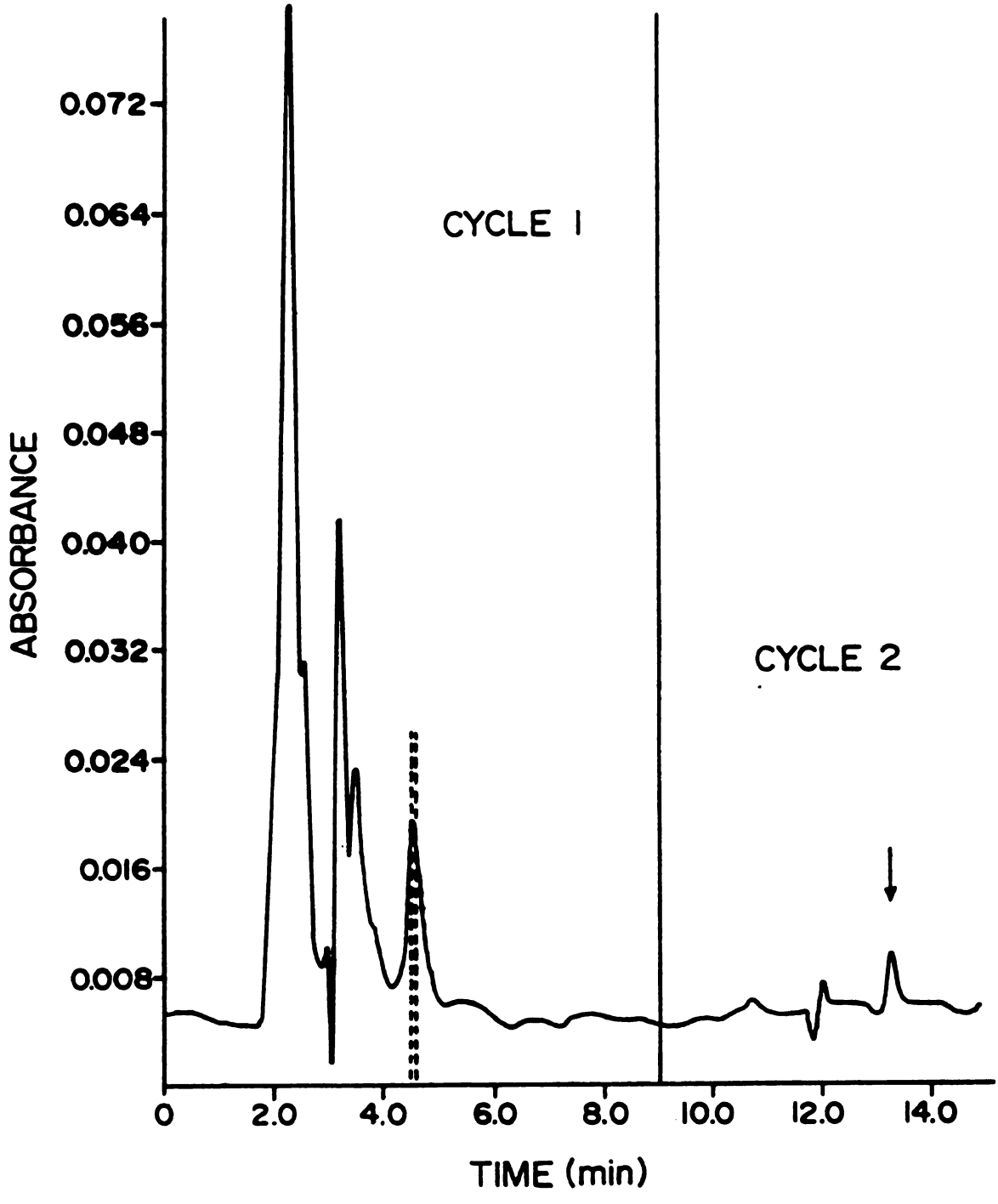

Figure 4.10. Isolation of 1,4-androstadiene-3,17-dione from a spiked urine sample. Separation conditions: mobile phase is 85/15 CH_3OH/H_2O; flow rate is 1.0 ml/min. Heartcut is time-based and for a volume of 100 µl.

corresponding to cycle 1, shows the elution of a number of compounds, some of which are unresolved. The heartcut that was taken is denoted by the dotted lines shown in Figure 4.10. The position of this heartcut was based on the retention time of a previously injected steroid standard. The chromatogram for cycle 2 shows a baseline disturbance due to the solvent front at 12 min and the isolated steroid component eluting at 13 min 10 s (indicated by arrow). The single peak obtained does not show any indication of other components being present. In this example, a minimum amount of sample pretreatment (e.g. the urine was passed through a Sep-Pak cartridge as described in the experimental section) and an isocratic separation (85/15 CH_3OH/H_2O) were employed in the isolation of a specific compound present in a complex matrix. These are some of the advantages that are offered by RHC.

The technique of recycle heartcut chromatography also has many potential uses in the area of preparative liquid chromatography. The analytical column can be replaced by a preparative LC column with only minor modifications to the separation system. Since such a column was not available, an analytical column was used instead in the demonstration of potential applications in this area. RHC can be used either to remove a contaminant present in a final product or to isolate a desired component. An example of the latter case is demonstrated in Figure 4.11. A mixture of polynuclear aromatic hydrocarbons is partially separated as shown in the cycle 1 portion of the chromatogram. The desired compound is naphthalene which corresponds to the peak eluted at 210 s. A heartcut was taken of this peak as denoted by the lines drawn in cycle 1 and the elution of the recycled sample is shown in

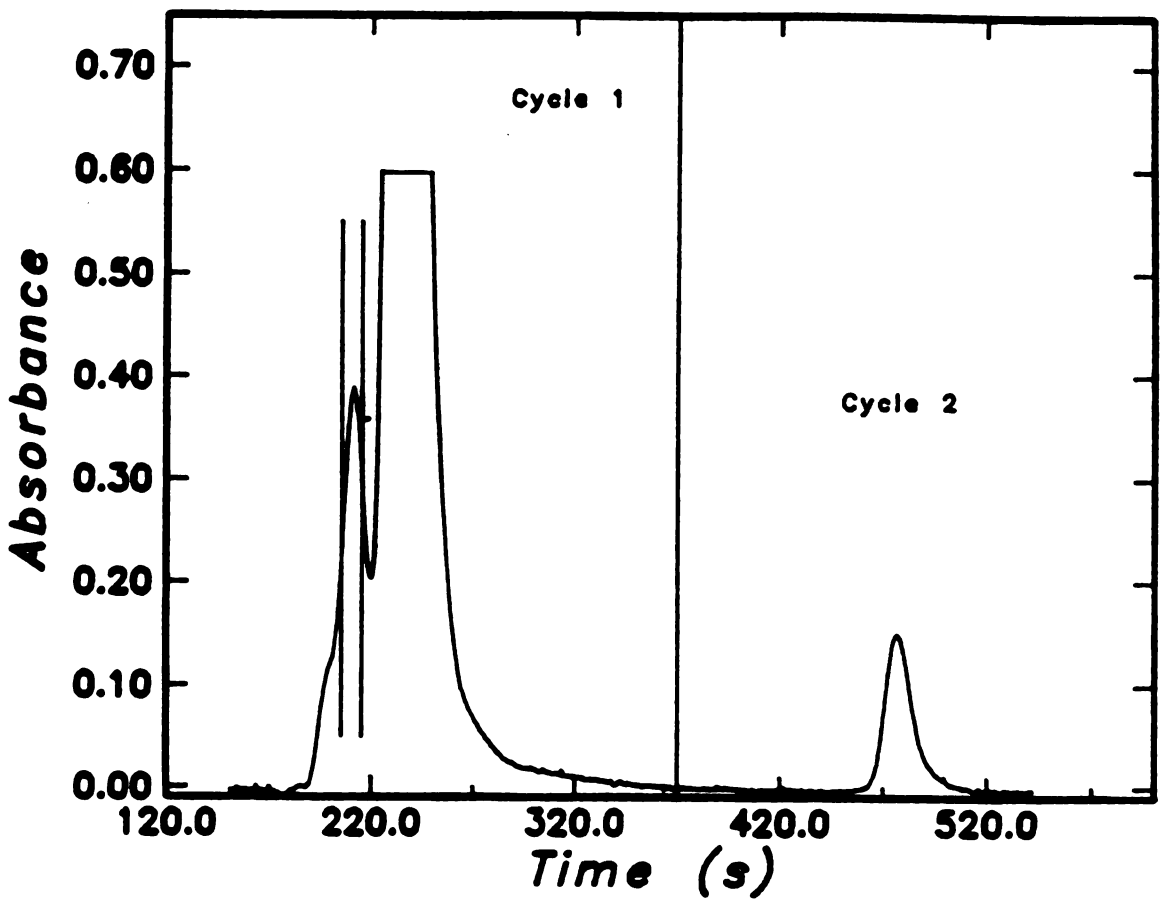

Figure 4.11. Isolation of naphthalene from a mixture of polynuclear aromatic hydrocarbons consisting of naphthalene, biphenyl and phenanthrene. Separation conditions: mobile phase 90/10 CH_3OH/H_2O; flow rate is 1.0 ml/min.

cycle 2. The heartcut step has been used, in this example, as a fractionation step to expedite the isolation of a single component that is surrounded by closely eluting impurities. Note that the column has been somewhat overloaded to demonstrate a practice commonly employed in preparative work to maximize the amount of purified sample per unit time (especially when the desired compound is a minor component).

2. Verification of Peak Purity

The potential of employing RHC as a verification technique for chemicals listed as 'chromatographically pure' is demonstrated in Figure 4.12. The chemical system that was employed is a model mixture of biphenyl and naphthalene. The chromatogram that results from the first pass through the column, Figure 4.12a, shows a peak with a shoulder. A 430 µl cut is made, as shown by the dotted lines, and recycled through the column to obtain the chromatogram shown in Figure 4.12b. This step is repeated and the chromatogram that resulted from this second recycle is illustrated in Figure 4.12c. With the second recycle, it is clear that the suspect peak consisted of more than one component. The resolution of the two sample bands can be estimated via a visual comparison with the set of standard resolution curves for different band-size ratios that have been introduced by Snyder (200). A small increase in resolution (0.5 to 0.6) is observed after two recycles. The final resolution is lower than the expected value of 0.85. (Resolution, as defined by Equation 1.1, is proportional to the square root of the number of theoretical plates, N. If the sample is cycled through the column three times, there would be a three fold increase in N, assuming

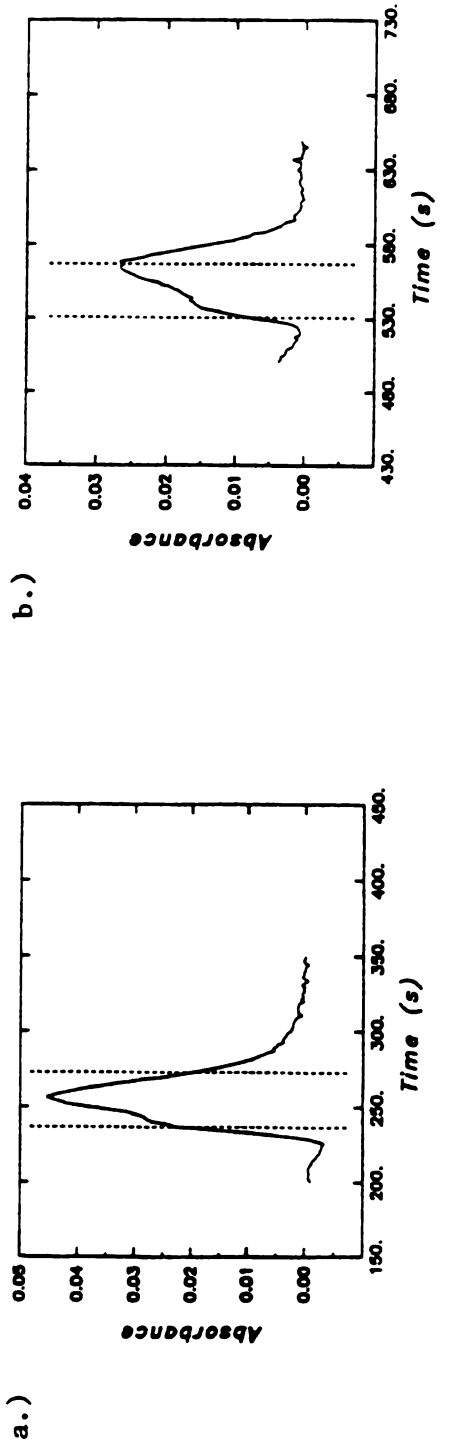

Figure 4.12. Verification of peak purity a.) cycle 1 b.) cycle 2 and c.) cycle 3. Sample is 1.8×10^{-4} M in naphthalene and 4.7×10^{-4} M in biphenyl.

that N is additive. Resolution should then increase by a factor of $(3)^{1/2}$ or 1.7.). This smaller gain in resolution can be attributed to void volumes in the RHC system or the use of poorly designed HPLC components that cause mixing of the partially separated sample. In the worst case, any separation that has been achieved by the first pass through the column is lost. The sample that is recycled is then equivalent to the homogeneous sample plug that was initially injected; thus no improvement in resolution will be observed with each recycle. Further studies need to be done to locate the major sources of dispersion in the RHC system.

3. Quantitative RHC

The feasibilty of employing RHC as a quantitative technique was investigated by plotting calibration curves for naphthalene standards that had undergone a series of two heartcuts. The two successive heartcuts were taken at 10% detector full scale and were 234 µl in volume. Figure 4.13 demonstrates the calibration curves obtained when the absorbance at maximum peak height for each of the three cycles and each standard was plotted. Linearity was observed for all three cases. The statistics related to each calibration curve are summarized in Table 4.5. A problem that is inherent to the technique of RHC becomes evident with this data. With each recycle, the sample becomes more diluted by the mobile phase and with each heartcut, a smaller portion of the peak is taken. This results in a decrease in the slope of the calibration curves and thus, a decrease in sensitivity from cycle 1 to

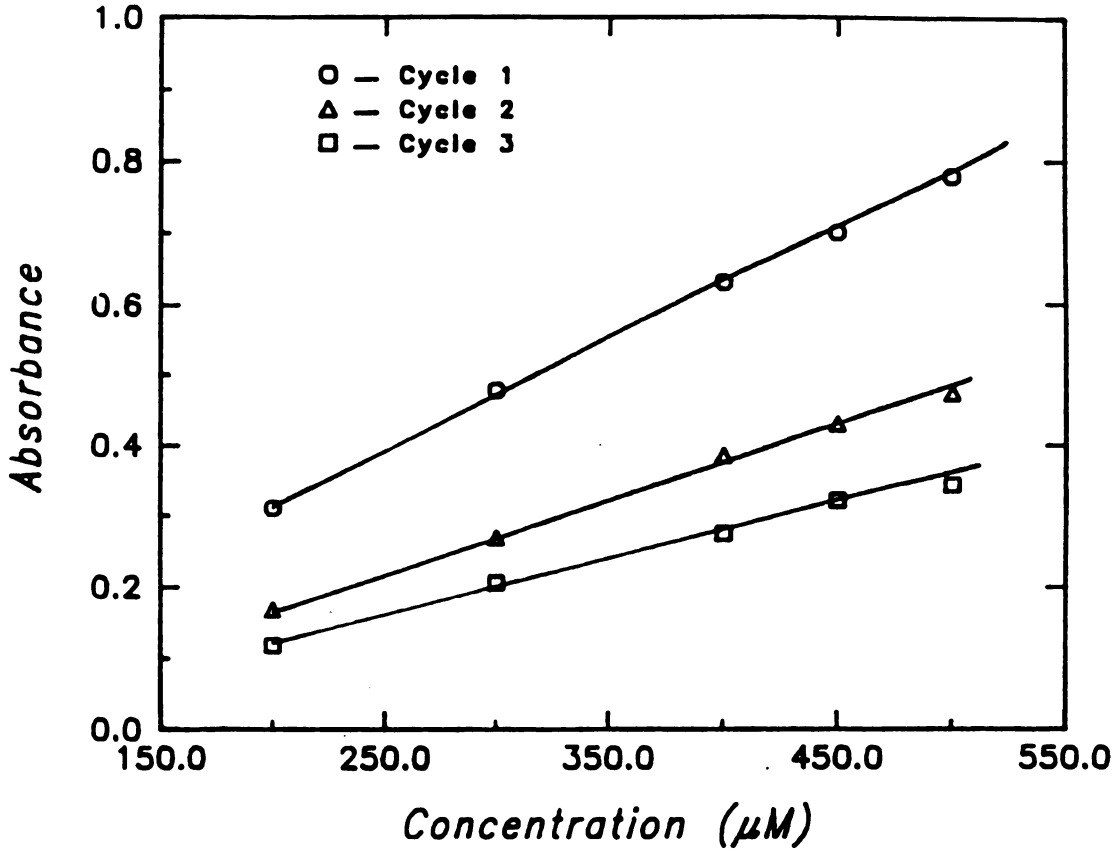

Figure 4.13. Calibration curves for naphthalene standards of which two heartcuts have been taken and recycled.

Table 4.5. Linear regression analysis of calibration curves for quantitative REC.

	Cycle 1	Cycle 2	Cycle 3
Slope	1.7×10^{-3}	1.0×10^{-3}	6.9×10^{-4}
Std. error of estimate	0.0001	0.0003	0.0004
Correlation coefficient	.9992	.9914	.9822
y-intercept	-0.002	-0.016	-0.005

cycle 3. The observed increase in the standard error of the estimate is a manifestation of the decrease in S/N ratio with each heartcut.

E. Conclusions

An automated HPLC system which integrates a heartcut step for fraction isolation with a recycling step for increasing the separation efficiency has been described. The performance of this recycle heartcut chromatography system has been evaluated with respect to the precision of the various methods that can be used to initiate a heartcut. The method of partially filling a large heartcut loop to provide a more flexible system was examined and determined to be a viable alternative. The versatility and advantages of RHC were demonstrated with a number of general applications. First, in the analysis of complex mixtures for specific compounds, it was shown that an extensive pretreatment of the sample prior to introduction to the HPLC was not necessary. Nor was it essential to employ an intricate separation scheme. Second, the fraction isolation capabilities of RHC make its extension to preparative LC a logical move. This system would be capable of isolating fractions of very high purity. The gain in purity with each heartcut will be offset by the loss of sample. A balance between the two factors will have to be established for each specific case. Third, RHC can be used as a quantitative technique.

In addition to the advantages mentioned above, the RHC system is simple and easy to implement as most of the components needed are standard accessories used in liquid chromatography. The automated features are desirable, but not essential for all applications. The cost

of an RHC system is minimal as only one pump, one column, one detector with a low pressure flow cell and two valves are needed. (The first three components are readily available in most laboratories that routinely utilize liquid chromatography.) It should also be mentioned that even though RHC has been discussed in this work as pertaining to HPLC, this valve configuration may be extended with ease to other liquid chromatographic techniques.

The RHC system has its limitations, a major one being the loss of sensitivity with each recycle, due to a smaller fraction of the initial sample plug being used and dilution by the mobile phase. This problem could be somewhat alleviated with the use of gradient elution. A gradient can be employed either in the last recycle to sharpen the final peaks or in each recycle to compress the broadened peaks back to the same volume as the peak that resulted from the first pass through the column. Using this approach, there should be a minimal loss of the desired component with each recycled heartcut. A second problem that has been encountered is band broadening in the system. As a result, the expected gain in resolution has not been achieved. As mentioned earlier, work needs to be done to determine the major source of dispersion and if possible, to remedy it. In this chapter, some preliminary results obtained using recycle heartcut chromatography have been presented. Further work also needs to be done to characterize and investigate the advantages and limitations of the method. In particular, the trade-off between resolution enhancement and decreased S/N ratios should be studied.

CHAPTER V

FUTURE PROSPECTIVES

A. OVERVIEW

The recent developments in post-column reaction detectors have enabled the use of this type of detector in conjunction with a variety of different techniques (e.g., miniaturization of PCR systems which has led to the first work with microbore columns (201)). There is still room for growth, both in the applications area and the development of improved manifold components and instrumentation. The preceding statement also applies to the area of recycle heartcut chromatography.

B. POST-COLUMN REACTION DETECTORS

1. Tandem Analyses

Even though most of the reactions that have been used with PCR detectors are very selective, often more than one class of compounds is able to react. The reaction conditions can be used to favor the reaction of one class of compounds over another, thus providing an additional degree of selectivity. It is conceivable to develop a detector system that would employ a change of reaction conditions after the first set of compounds has been detected to favor reaction with a second set of

compounds. In this way, a tandem analysis of different compounds could be carried out. A specific example is the formation of azo dyes which, for phenols, take place in basic media, but for anilines, under acidic conditions. If the reaction medium was initially alkaline, phenols would undergo the coupling reaction with the diazonium reagent. After the absorbance of the phenol derivative formed is measured, acid is added to the flow stream and aniline would be able to react with the diazonium reagent. The absorbance of the aniline derivatives would then be measured on a second detector. It should be noted that in a tandem analysis, different detection principles could be employed.

2. Solvent Segmentation

The use of solvent segmentation as the post-column reaction system provides a powerful and flexible detector. As discussed earlier, advantages of segmentation with solvents over that with air include reduced sample interaction and background noise. The use of the solvent segment as an extraction medium imparts an additional degree of selectivity to the reaction detector. The possibility of choosing an optimum solvent with respect to viscosity, solubility and spectral characteristics is another factor that makes this system very versatile.

In order for this system to reach its full potential, the behaviour of sample and solvent molecules and the flow dynamics in a solvent segmentation have to be well understood. There is a need for such investigations. The extent to which the band broadening equations that have been developed for air segmentation can be used in predicting the dispersion in a solvent segmented system remains to be determined.

Also needed, is the derivation of equations that can be used to calculate the extraction efficiency between solvent segments in a flow stream. This should then lead to a study of the effects of coiling on dispersion and extraction efficiencies.

3. Biological Detectors

Solid phase reactors have recently been introduced in PCR systems. This was a natural progression because of the existing methodology concerning immobilization of reagents onto surfaces. In particular, the techniques for enzyme immobilization have been well researched (202,203). Enzymes are among the most selective reagents known. This degree of selectivity may be achieved through the use of reactors containing materials that are coated with biological reagents such as antibodies and proteins. These specific reaction detectors have many potential applications, especially with the present interest in biotechnology and the increasing number of syntheses of biological substances that need to be separated and detected.

C. RECYCLE HEARTCUT CHROMATOGRAPHY

Future research in recycle heartcut chromatography can take place in many areas. One possibility is the use of the commercially available multiport, multiposition valves for analyses that require multiple heartcuts to be taken. This system could also be employed in a similar fashion to boxcar chromatography. Samples could be continuously injected and the fraction of interest isolated and deposited

on the different heartcut loops. When all the heartcut loops are filled, sample injection is put on hold, and the fractions on the heartcut loops are recycled. The emptying out of each loop is spaced by a short time interval. The result would be a train of sample peaks, similar to that observed in boxcar chromatography. A second area would be in reducing dispersion in the heartcut loop. Long lengths of tubing are not recommended in HPLC because of the band broadening due to laminar flow. In flow injection analysis systems, single bead string reactors have been employed to break up the parabolic flow profile. The use of an equivalent system (to a single bead string reactor) in recycle heartcut chromatography could reduce the band broadening that occurs in the heartcut loops. Finally, the nature of the dispersion and diffusion in the heartcut loop require further investigation so that the full potential of recycle heartcut chromatography can be evaluated. Presently, computer simulations are being done (204) to clarify this aspect.

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