On-line Multidimensional HPLC: Development, Theory and Applications

by

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INTRODUCTION

1.1 SCOPE AND GOALS

This century might well be characterized by the phenomenal growth of science and the resulting growth of man's understanding of the world around him. This accumulation of knowledge has in turn resulted in an ever increasing ability to control or manipulate that world. In order to understand or manipulate his environment, or even to understand the effects of these manipulations, it has been neccesary for man to study the structure and composition of his material world. In many areas of science, this study is directed toward chemical systems, and as the depth of man's understanding in these fields has advanced, so has the sophistication demanded of techniques in analytical chemistry. An excellent example of this trend is the growth of chromatography, a technique for the separation and identification of components in complex mixtures. Progress in chromatography has paralleled the needs of a wide range of disciplines. From its earliest uses when Mikhail Tswett first separated the major components in a plant pigment, chromatography has progressed

to its current status in which as many as four hundred components may be separated in a single sample by capillary gas chromatography, and trace levels of components as low as femtograms (10^{-15}g) may be detected through the use of specialized detector systems. However, just as science has continued to progress, so has the complexity of the analytical demands made upon chromatography. The analyses required by such diverse fields as the biological, clinical, environmental and industrial sciences require the resolution of increasing numbers of components at decreasing concentration levels.

Resolution in chromatography, given by equation 1 (2),

$$R=\tfrac{1}{4}\sqrt{N}(\alpha-1)(k'/k'+1) \qquad (1)$$

is a function of three factors: the column efficiency, N; the selectivity, α ; and the capacity factor, k'. The last of these parameters is the most accessible experimentally and is often used in the optimization of a separation. On a more fundamental level, however, the first two factors, selectivity and column efficiency, are the most critical. During the last decades, the main thrust in chromatography has been the optimization of the column efficiency of chromatographic systems. Although this has resulted in such breakthroughs as capillary gas chromatography, the column

efficiency available today is approaching a theoretical limit. It is therefore neccesary to turn to the remaining factor, selectivity, in order to increase the resolution available. As Dr. J.F.K. Huber (1) pointed out in his acceptance address of the 1981 Dal Nogare Award presentation at the 1981 Pittsburgh Conference for Analytical Chemistry and Applied Spectroscopy, the benefits of being able to optimize the selectivity are almost limitless and one technique able to capitalize on this fact is multidimensional chromatography.

Multidimensional chromatography is a technique in which two or more chromatographic modes are coupled together to affect a single separation. The chromatographic systems which are coupled can be similar or different, and thus, in the two dimensional case, through the coupling of gas chromatography (GC) and High Performance Liquid Chromatography (HPLC), a family of possible techniques results including GC/GC, HPLC/HPLC, HPLC/GC and GC/HPLC.

The objectives of this work are essentially threefold; the development, the application and the theoretical evaluation of quantitation of on-line multidimensional high performance liquid chromatography. The development of the on-line multidimensional technique has been limited to two systems, one of which couples liquid and gas chromatography

(LC/GC), and one which couples two liquid chromatographic modes (LC/LC). Each of these systems has been applied to several analytical problems to demonstrate the scope and usefulness of each. The applications are from a range of fields and include the analysis of biological markers of cancer, the analysis of drugs in biological fluids, the analysis of hydrocarbon group types in petroleum related products, and the analyses of substances of environmental interest such as polycyclic aromatic hydrocarbons (PAH's) and pesticides.

Although work has appeared in the literature in several of the multidimensional techniques, particularly LC/LC and GC/GC, there has been little development of fundamental theoretical considerations. In order to take full advantage of such techniques, it is neccesary to understand the factors which affect performance characteristics such as quantitation. The third objective of this work, therefore, is the evaluation of the quantitation in on-line multidimensional chromatography from both theoretical and experimental standpoints. The two systems discussed here (LC/GC and LC/LC) have been applied in such a way that these theoretical considerations are applicable to generalized on-line multidimensional chromatographic systems.

1.2 CONCEPTS, DEFINITIONS AND TERMS

The following concepts, definitions and terms represent a basic background, knowledge of which will be assumed in subsequent sections. A more complete discussion of these concepts, and of chromatography can be found in Snyder and Kirkland (2).

1.2.1 Chromatographic Concepts

A typical chromatographic elution profile is shown in Figure 1.

The retention time, t_r, is the time from the point of injection to the time of the peak maximum.

The interstitial time, t_o, or "dead time", is the time from the injection point to the time at which the solvent front elutes.

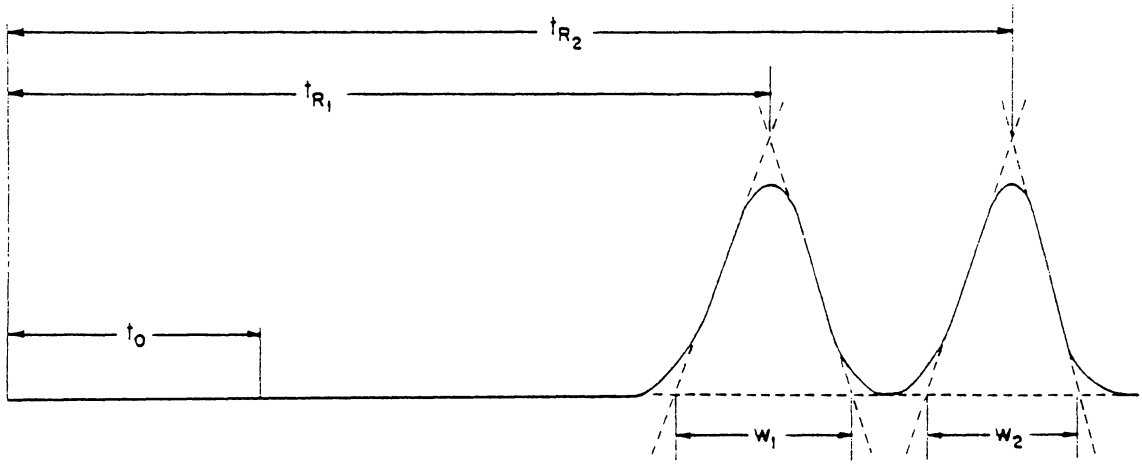
The width at base, w_b, is the time between the two points at which the two tangents intercept the baseline.

The efficiency or effect of band broadening is described by N, the number of theoretical plates, given by

$$N=16(t_r/w_b)^2 \qquad (2)$$

The number of theoretical plates is a measure of how narrow the peak is with respect to its retention time.

The capacity factor, k',is given by the equation



$$R_s = \frac{2(t_{R_2} - t_{R_1})}{w_1 + w_2}$$

Figure 1. Typical Chromatogram (2)

$$k' = (t_r - t_o)/t_o \quad (3)$$

The capacity factor describes how strongly the peak is retained.

The selectivity, α, is given by the equation

$$\alpha = (t_{r1} - t_o)/(t_{r2} - t_o) \quad (4)$$

The selectivity is a measure of how well the peaks are discriminated by the chromatographic phase system.

Resolution, R, is calculated by the equation

$$R = 2(t_{r2} - t_{r1})/(w_{b1} + w_{b2}) \quad (5)$$

and is related to chromatographic factors by equation 1;

$$R = \tfrac{1}{4}\sqrt{N}(\alpha - 1)(k'/k' + 1) \quad (1)$$

The effects of these factors on the chromatographic separation are shown in Figure 2. Figure 2 (a) represents a hypothetical chromatogram. Figure 2 (b) shows the effect of increasing the capacity factor. The peaks have increased in retention with a corresponding increase in band width. The selectivity, however, has not changed. That is to say that the relative retention of the two peaks is the same. Figure 2 (c) shows the effect of increasing the number of theoretical plates, or efficiency of the column while holding all other factors constant. The peaks maintain

their position, but they become narrower. In Figure 2 (d), the selectivity has increased.

Two additional definitions will be helpful; "normal phase chromatography" (NPC) refers to a system in which the mobile phase is non-polar with respect to the polar stationary phase; "reverse phase chromatography" (RPC) refers to a system where the converse is true; the mobile phase is polar with respect to the non-polar stationary phase.

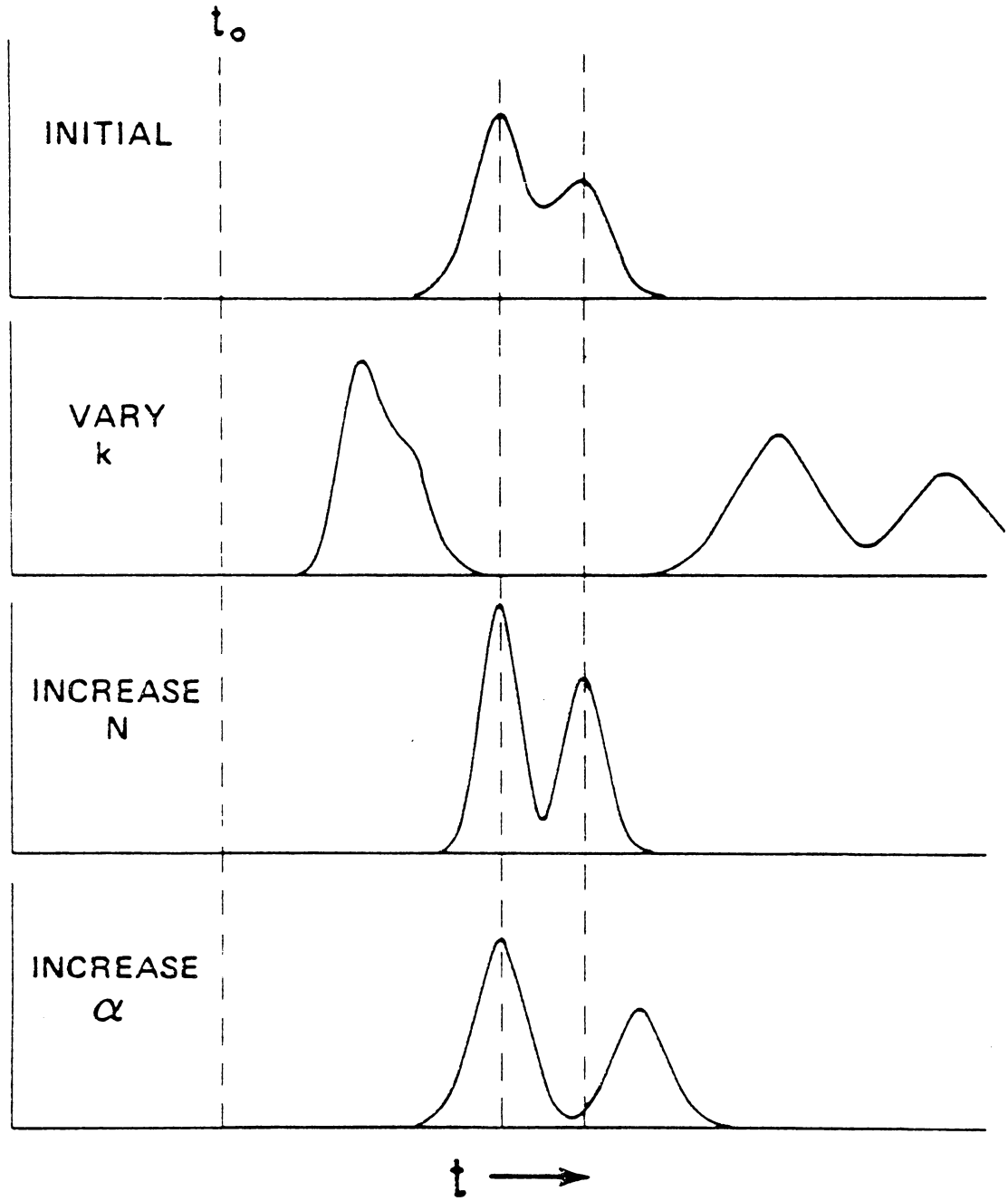


Figure 2. Variables Effecting Chromatographic Resolution (2)

1.2.2 On-line Multidimensional Chromatography

In the following sections, the phrase On-line Multidimensional Chromatography will refer to the sequential coupling of two or more chromatographic columns by automated means. For the most part, reference here is made to the two dimensional case in which two chromatographic modes are coupled together.

In the following sections, operation of on-line multidimensional chromatographic systems is divided into two distinct classes; "Trapping", in which a small portion (<10%) of the peak volume is transferred from one chromatographic mode to another; and "Switching", in which a major portion (>10%), usually most, of the peak volume is transferred. There is no established terminology for this distinction. Trapping has, in the literature, been refered to as "heartcutting", "front cutting" and "end cutting", depending on which portion of the chomatographic peak is transferred. Switching has been referred to as "quantitative cutting", "column switching", "peak transfer" and "on-column concentration". The use of the terms trapping and switching in this work is primarily motivated by the instrumental methods employed.

The coupling of two liquid chromatographic modes will be referred to as LC/LC, column switching in HPLC or on-line

multidimensional HPLC. The coupling between modes in LC/LC is usually accomplished through the use of high pressure switching valves. These valves can be used either to divert the flow from the primary column into the secondary column, a switching technique, or the valve can be used as a loop injector for the secondary column, which is filled by the effluent of the primary column, a trapping technique.

The coupling of two gas chromatographic modes will be referred to as GC/GC, column switching in GC, or on-line multidimensional GC. Coupling between modes in GC can be accomplished in a number of ways: mechanical valves can divert the effluent from a primary column onto a secondary column; flow can be diverted by relative pressure differences in the system; or the use of fluidic logic can control flow. Although GC/GC is usually performed as a switching technique, through the use of high speed switching, small portions of a peak can be transferred. Such a system can be run in a trapping mode.

The coupling of liquid and gas chromatography will be referred to as LC/GC. Coupling between these modes as described in this work is accomplished via an automated syringe injector. Effluent from the liquid chromatograph continuiously flows through the injection syringe of the gas chromatograph which automatically traps and injects a

portion of this effluent into the GC on a time programmed command. With typical peak volumes in conventional HPLC (>1mL), and typical injection volumes in GC (<50μL), this coupling is operated as a trapping system.

Chapter II

HISTORICAL DEVELOPMENT

2.1 INTRODUCTION

On-line multidimensional chromatography involving liquid chromatography is in its infancy. Although reports appeared as early as 1973, its real growth has taken place in the last several years. The application of on-line multidimensional techniques is somewhat older, however, and has become an established tool in several areas. The technique first gained widespread popularity after the use of valveless switching introduced by Deans (3) in 1968. Using "Deans' Switching", sample and carrier flow are directed by means of the adjustment of pressure differences in the system. Applications have appeared in a number of fields using systems which couple two packed columns (4), packed and capillary columns (5) and two capillary columns (6). Theoretical work on these systems examines such aspects as band broadening (7) and information content (8) involved in these techniques. An excellent review of this field has recently been published by Bertsch (9,10,11). Although the instrumentation used in these systems bears little resemblance to that used in multidimensional HPLC,

many of the techniques and concepts used are applicable to multidimensional chromatography in general.

2.2 LC/LC

The use of on-line multidimensional techniques in liquid chromatography has gained a great deal of interest in the last several years. An indication of this is given by the appearence of a number of review articles on the subject. Majors (12) reviewed the applications of both LC/LC and LC/GC. Freeman (13) has reported some hypothetical and theoretical aspects of multidimensional liquid chromatography in general. In this work, Freeman states that separations of 10^{12} components could, in principle, be obtained through the exploitation of the diverse selectivities available in modern HPLC. Although from a practical point of view, this may not be feasible (or neccessary), it does indicate the power of the technique. Hirschfeld (14) has discussed the use of "Hyphenated Methods", including LC/GC, but disregarding LC/LC. Snyder and Kirkland (2) have also discussed the subject of LC/LC.

Applications of on-line multidimensional liquid chromatography have been aimed primarily at the solution of specific problems. With the appearance of work of a more general nature, the technique can be viewed from a more

technique oriented outlook as a sub-discipline of modern liquid chromatography.

2.2.1 Off-line Applications of LC/LC

Before discussing the work which has been done in on-line LC/LC, it is useful to examine the applications which have been performed off-line. This work can be classified from a number of points of view. One useful classification separates multidimensional chromatographic applications into two main groups: those which are used primarily to clean up and isolate an analyte in a complex or "dirty" matrix; and those which exploit the selectivities of both chromatographic steps to accomplish an analytical separation. Another approach groups these applications according to the chromatographic modes which have been coupled together, and it is this approach which is used in this thesis.

Review of this material offers several benefits. Many of the multidimensional separations which have been done off-line could easily be transferred on-line with a significant improvement in sample throughput and reproducibility and are useful sources of applications. On the other hand, the application of off-line techniques does not preclude the use of immiscible or incompatible mobile

phases which is the case in most conventional on-line procedures. Off-line procedures are often applied to analytical applications that on-linr techniques cannot approach.

Much of the work which has been reported in off-line applications of multidimensional LC/LC couples similar chromatographic modes usıng similar or identical mobile phases. For example, the coupling of two normal phase separations is particularly common, owing to the use of adsorbents such as Florisil in sample preparations. The use of mobile phases of high volatility allows a concentration step by solvent evaporation to be performed in between the two chromatographic separations.

Some recent applications of off-line LC/LC a which couple two normal phase separations include the separations of urea herbicides on silica gel following a Florisil clean-up (15); the separation of plant hormones on silica gel following a preparative separation also on silica gel (16); the separation of aflatoxins on silica following a clean-up step on a mixture of silica, alumina and sodium sulfate (17); and the analysis of lipid soluble vitamins in milk following clean-up on alumina (18).

Similarly, the coupling of two reverse phase columns has also been used. This is less common than coupling

normal phase systems because the mobile phases in reverse phase chromatography are typically mixtures of water and methanol or acetonitrile, and due to the reduced volatility, concentration of samples following separation is more difficult than in the case of the two normal phase columns. An example of this system is the analysis of oligosaccharides on a microparticulate octadecylsilane bonded phase (ODS), following clean up on a preparative ODS column (19).

Although the coupling of normal phase and reverse phase systems requires the phase transfer from an organic mobile phase to a aqueous mobile phase, there are many examples of such systems. The dissimilarity of these two chromatographic modes offers unique advantages to their coupling. In an off-line system, if the primary mode is normal phase using a highly volatile mobile phase, this solvent can easily be evaporated from a collected fraction and the sample redissolved in a aqueous mobile phase and injected into the second mode. As this phase transfer is difficult in an on-line procedure, the off-line applications are particularly useful. Recent applications include the analysis of aflatoxins by reverse phase chromatography following a clean-up separation on alumina (20); the determination of vitamin D precursors in human serum by an

analytical reverse phase separation following clean-up separations on silica and diatomaceous earth (21); the analysis of avermectins by reverse phase chromatography following two normal phase separations on Florisil and silica, respectively (22); and the analysis of catechols in rat tissue by reverse phase following an initial fractionation on silica (23).

Size exclusion chromatography (SEC) has been coupled off-line to most other liquid chromatographic modes. This is a particularly attractive combination due to the versatility and ease of operation of size exclusion techniques. SEC can be applied to a wide range of samples including very complex and "dirty" matrices with a minimum of sample preparation. In addition, SEC exhibits little dependence upon mobile phase characteristics allowing optimization of non-chromatographic characteristics of the mobile phase such as volatility. Although selectivity of SEC between compounds of similar size is low, this can often be an advantage, allowing analysis by a second mode of a specific size fraction. These factors make SEC an ideal technique for the clean-up of samples for subsequent analytical separation.

Aqueous SEC has frequently been coupled off-line to reverse phase separations. One of the earliest examples by

Little (24) is significant because of the emphasis placed on the use and exploitation of "Sequential Chromatography", using differing chromatographic selectivities. Applications in this area include the analysis of endogeneous vitamin D in human plasma (25); the analysis of neuropeptides in brain extracts (26); and the analysis of catechol-o-methyl tranferase and related products in liver homogenates (27).

The use of organic mobile phases with high volatility for the SEC separation allows reconcentration of the collected fraction. For this reason there are many applications of off-line coupling of organic SEC with both normal phase and reverse phase chromatography. Since the concentrated samples can often be redissolved in an aqueous mobile phase, reverse phase can be effectively coupled with with SEC. Examples include the analysis of sterigomatocystin by reverse phase chromatography after clean-up of an extract of oats and grains by both normal phase and size exclusion chromatography (28); the reverse phase separation of pesticides in butter following isolation by SEC isolation (29); and the analysis of retinyl palmitate by non-aqueous reverse phase (NARP) after sample preparation by SEC (30).

Normal phase chromatography has been coupled to organic SEC as well. Some examples are the analysis of lipoproteins

and lipids in serum (31); the analysis of photo-oxidation products of bilirubin in infant serum (32); and the analysis of zearelenone in animal feeds (33).

Ion exchange chromatography (IEC) has been coupled with a number of other chromatographic modes. The primary advantage of this technique is the ability to fractionate samples according to their ionic characteristics prior to analytical separation. This is particularly useful in the analysis of amino acids, proteins and peptides in biological matrices. Both cation and anion exchange systems have been used in this connection. IEC has been used to fractionate samples prior to reverse phase separation for a number of applications including the analysis of alkylbenzene sulfates in river water (34); the analysis of neuropeptides in brain extracts (26); and the analysis of pyrimidine ribotide and deoxyribotide pools in cultured cells and liver extracts (35).

Similarly, IEC has been used to fractionate samples prior to normal phase separations. The application of this combination to the analysis of proteins is less common than the coupling of IEC with reverse phase due to the potential denaturation of proteins in organic solvents. Furthermore, the need for aqueous ionic solutions in IEC requires a phase transfer to organic solvent prior to most normal phase

separations. Due to the relatively low volatility of aqueous solutions, solvent transfer from aqueous to organic mobile phases generally must be done via some solvent extraction technique. Thus use of this technique must be reserved for favorable cases where the analytes can be efficiently extracted from aqueous to organic solvent. An example is the analysis of adenyl cyclase in enzyme mixtures (36).

An interesting combination of chromatographic modes is the coupling of anion and cation exchange. This technique allows the analysis of both anionic and cationic substances in a single analysis. Thus, rather than a simple clean-up or fractionation step followed by an analytical step, this approach couples two analytical processes, relying on the efficiency and selectivity of each chromatographic mode for a particular separation. Examples include the analysis of inorganic ions (37); the analysis of ionic metabolites in biochemical mixtures (38); the analysis of caffeine ad trigoneline in beverages (39); and the analysis of biogenic amines in biological fluids (40).

Examples of off-line LC/LC discussed above are summarized in Table I.

TABLE 1

Off-line Applications of LC/LC

Application	LC Modes used	Ref.
Urea herbicides	NP/NP	15
Plant Hormones	NP/NP	16
Aflatoxins	NP/NP	17
Fat soluble vitamins in Milk	NP/NP	18
Oligosaccharides	RP/RP	19
Aflatoxins	NP/RP	20
Vitamin D in Serum	NP/RP	21
Avermectins	NP/NP/RP	22
Catechols in Rat Tissue	NP/RP	23
Vitamin D in Plasma	Aq.SEC/RP	25
Neuropeptides in Tissue Extracts	Aq.SEC/RP	26
Catechol O-methyl Tranferase in Liver	Aq.SEC/RP	27
Sterigmatocystin in Grains	Org.SEC/RP	28
Pesticides in Butter	Org.SEC/RP	29
Retinyl Palmitate	Org.SEC/NARP	30
Lipoproteins	Org.SEC/NP	31
Bilirubin Metabolites in Serum	Org.SEC/RP	32
Zearelenone in Feeds	Org.SEC/NP	33
Alkylbenzene Sulfonates in River Water	IEC/RP	34
Neuropeptides in Tissue Extracts	IEC/RP	26
Nucleotides in Liver Extracts	IEC/RP	35
Adenyl Cyclase	IEC/NP	36
Inorganic Ions	IEC/IEC	37
Ionic Metabolites	IEC/IEC	38
Caffeine and Trigonellene in Beverages	IEC/IEC	39
Biogenic Amines	IEC/IEC	40

2.2.2 *On-line Applications of LC/LC*

Historically, the on-line two-dimensional chromatographic techniques to receive the most attention have been the "homogeneous" systems, that is two step processes involving only a single technique such as LC/LC or GC/GC. Confronted with separation problems beyond the scope of a single chromatographic mode, the next easiest procedure is to repeat the initial step. If conditions of the second step are identical to those of the first and all of the sample is transferred between the modes, a technique called "Recycle Chromatography" results (41). The goal of recycle chromatography is to increase the number of theoretical plates in a system without increasing the actual column length and consequently the pressure drop. If, on the other hand, conditions of the second separation differ from the first, or only a selected part of the sample is transferred, a multidimensional chromatographic technique results which allows the adjustment of resolution through both selectivity and capacity factor. Implementation of such "homogeneous" systems is more straight forward than the "heterogeneous" multidimensional chromatographic systems (LC/GC or GC/LC), because, in general, following the initial separation, the sample and mobile phase are in a form that is compatible with the second separation. In contrast, heterogeneous

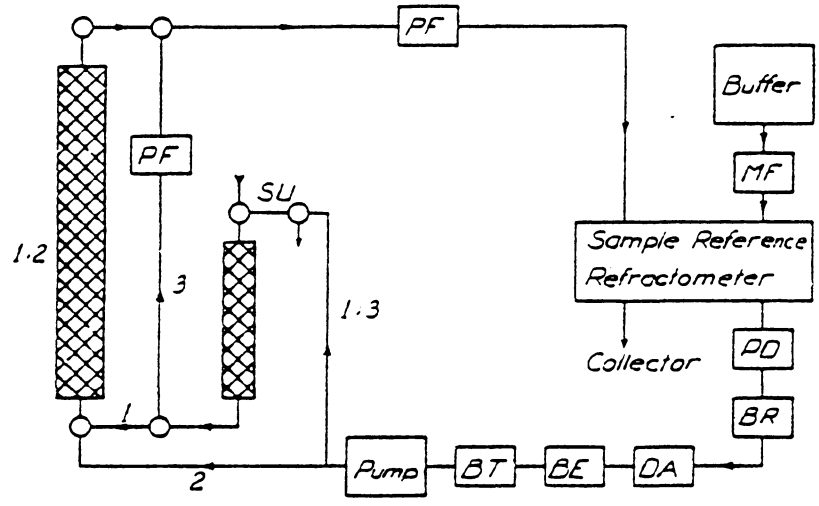
systems usually require a phase transfer step. LC/GC will require, for instance the transfer from a liquid to a gaseous mobile phase.

The on-line coupling of two liquid chromatographic systems has received a good deal of attention since 1970. The literature has dealt with several key aspects in the development of this technique. Although emphasis has been on the development of hardware and applications, there have been a number of works dealing with theoretical aspects, principally evaluating band broadening effects and increased resolution obtainable in such systems.

The earliest report of an on-line multidimensional LC/LC system was made by Snyder in 1970 (42). This work compared the characteristics of a number of techniques including normal elution, gradient elution, flow programming, temperature programming and coupled column chromatography in the solution of the "General Elution Problem" in liquid chromatography. The goal of such a solution was the resolution of compounds of widely differing partition coefficients by a single technique in the minimum time. In his system, column switching between two identical phase systems was used to adjust the capacity factors of the eluting compounds. Although the purpose of the experimental work was to validate theoretical considerations, and

utilized relatively low pressure columns, this work did demonstrate the feasibility of such a technique. In 1971, Liljamaa and Hellen (43) reported a practical application of such a system in the separation of borate complexes of sugars by ion exchange chromatography. In their system, schematically shown in Figure 3, a two column system was used to adjust the capacity factors of the samples by varying the effective column length experienced by the compounds. In this technique, the chromatographic phases are identical, but by directing the sample elution through either one or both columns, the length of the column through which any sample compound passes could be adjusted.

The first example of an LC/LC system to be used to adjust selectivity of the chromatographic phase system was reported by Scott *et al.* (44) in 1972. In the analysis of biochemical samples, anion exchange and cation exchange systems were coupled together. Instrumentally, this system is shown in Figure 4. The procedure involved two steps: (1) initially, samples were injected and sample ions were adsorbed onto one column or the other depending on their charge; (2) by adjusting the valving and the pressure restrictions, the two columns were then eluted simultaneously. The effluents were monitored separately by two UV photometers. In this system, the eluent could be



Flow scheme of the two-column chromatographic system. The buffer passes through a 0.3 μ Gelman membrane filter (MF), the reference cell of the refractometer, the pulsation damper (PD) a buffer refiner (BR) consisting of a 1 × 4 cm column with AG 1 × 4 renewed every second month a deaerator (DA) in which the buffer is heated to 100°, a buffer equilibrator (BE) consisting of a 500 ml vessel provided with a magnetic stirrer, a bubble trap (BT), the pump, the columns (alternative routes 1, 2 and 3), backpressure filters (PF) and the sample cell of the refractometer to the collector.

Figure 3. LC/LC Apparatus of Liljamaa and Hellen (43)

delivered in a gradient, but pumped from a single pump. The flow was split to the two columns, flow rate being adjusted by pressure restrictors. Thus the elution conditions for the two columns were identical. It should be noted that although it is significant that the selectivity is being adjusted, it is a very broad adjustment using cation and anion exchange columns. This should be kept in mind in comparison with later, more subtle uses of selectivity.

The earliest report of work in this area by the research group of J.F.K Huber appeared in 1973 (45). This work contained several unique features. All previous reported work had utilized low pressure, low efficiency columns. Huber's work introduced column switching to HPLC. Furthermore, an excellent evaluation of band broadening effects of the system from both theoretical and experimental standpoints was presented. The general design, shown in Figure 5, remains essentially unchanged in all of Huber's subsequent work. It allows the columns to be eluted in series, or either column to be eluted separately while the other is isolated, using a single pump. One important feature of this system is the use of a pneumatic switching valve then manufactured by the Siemens Corporation which, unfortunately, is no longer commercially available. Huber's work was directed at the adjustment of capacity factors by

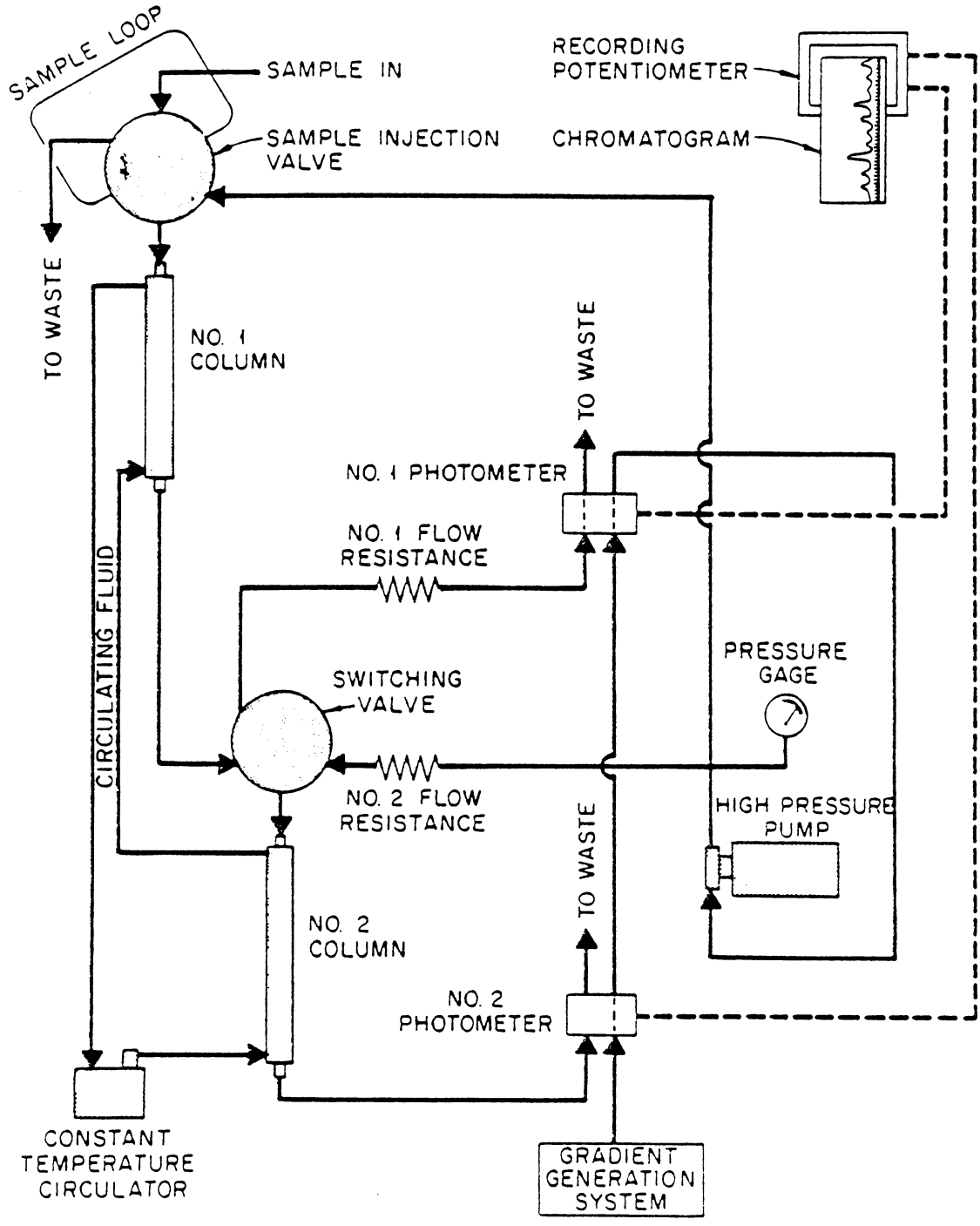


Figure 4. LC/LC Apparatus of Scott (43)

the adjustment of effective column length and phase ratios, in a manner similar to that reported by Liljamaa and Hellen (43).

Following Huber's work there was a period of little development in the field extending to 1976. In 1976, however, two papers appeared by Dolphin and Willmott (46,47). Schematically, Dolphin's hardware is shown in Figure 6. In terms of the intrumentation, there are several innovations that should be noted. As in Huber's earlier work, Siemens valves were used. However, in this system, two high pressure pumping systems were used. In addition to the coupling between the two liquid chromatographs, an on-line coupling was also made to an electron capture detector. This work is application oriented, dealing with the analysis of pesticides in milk. The multidimensional HPLC system is used to adjust the capacity factor between a short prefractionation column and an analytical column, both packed with 10 m silica. There is some theoretical work (47) evaluating the band broadening and the minimum length required in the precolumn for adequate fractionation.

In 1978, Huber *et al.* (48) published work dealing with a theoretical evaluation of the system developed earlier. The effect on the capacity factor of coupling HPLC systems which differ only in their stationary phase ratio was

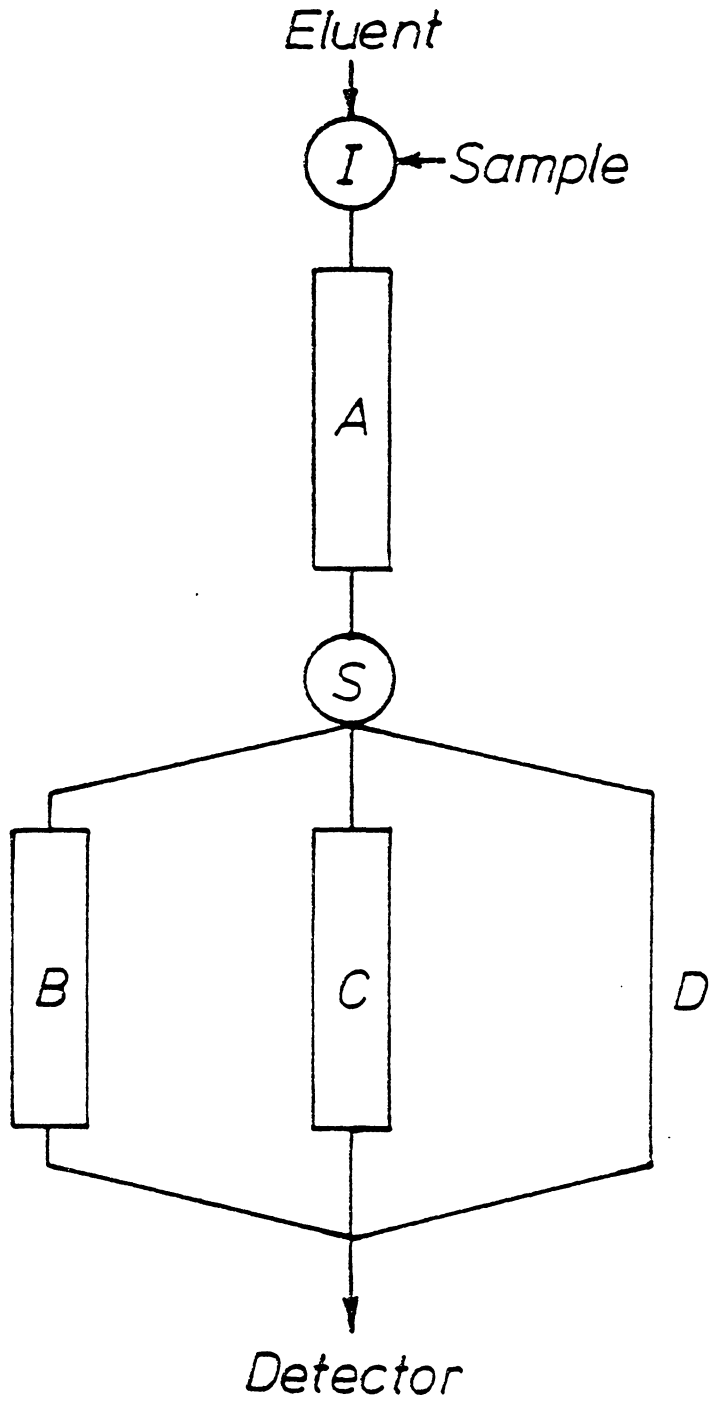


Figure 5. LC/LC Apparatus of Huber (45)

examined theoretically and verified using the separation of PAH's and the separation of dyes as examples. In these experiments, the phase ratios of the stationary phases were adjusted by varying the specific surface area of the adsorbent used.

One of the first genuine two dimensional HPLC systems was developed by Erni and Frei (49). In this work, they recognized the significance of using different types of stationary phases to adjust the selectivity of the separation. Except for the earlier low pressure system of Scott (44), this is the first report of the coupling of two dissimilar liquid chromatographic phase systems, and in this sense it is actually a two dimensional system. Instrumentally, this system coupled size exclusion chromatography and reverse phase chromatography in the apparatus shown schematically in Figure 7. Significant features of the hardware include the use of two high pressure pumps and the use of pneumatically actuated multiport valves manufactured by Valco Instrument Company (Houston, Tx.). These loop valves were used to inject a trapped volume of the effluent of the first column into the second colomn. The technique used a phenomenon known as "on-column concentration". By using a mobile phase in the first chromatographic column which is weak with respect to

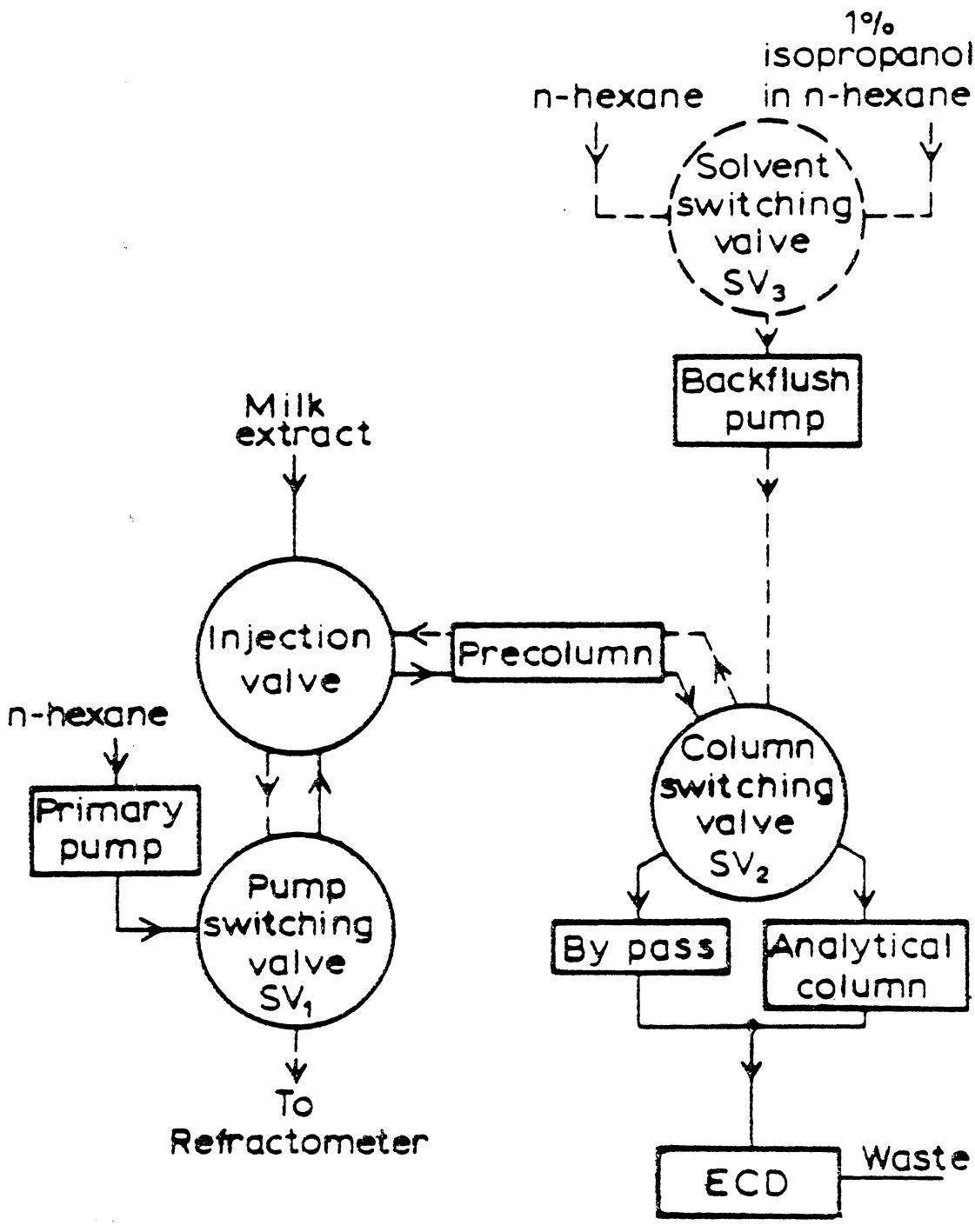
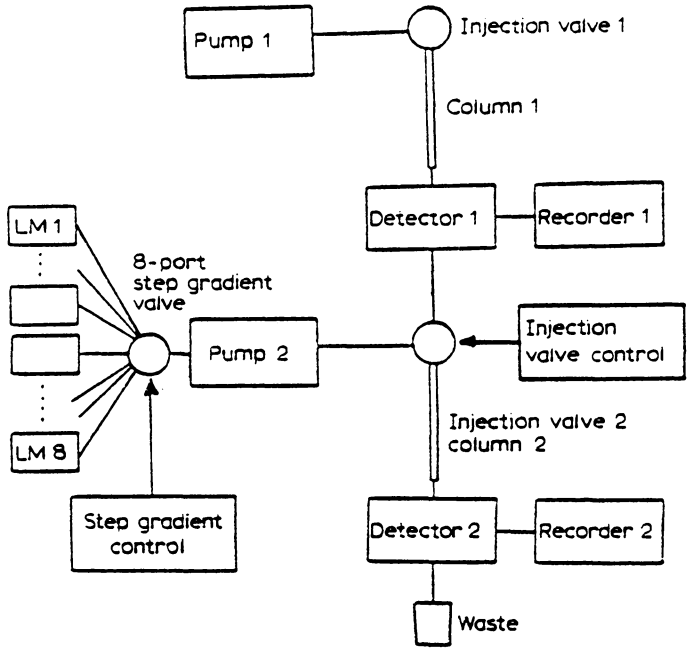


Figure 6. LC/LC Apparatus of Dolphin (46)

the second chromatographic column, compounds entering the second column concentrate in a narrow band at the head of the column. The effect of this is to nullify any band broadening due to the column switching hardware. The use of this technique had not been applicable in the earlier systems since the similar phase systems used demanded similar mobile phase strengths for both columns. Another significant feature of Erni and Frei's work is their use of information theory to evaluate the information content of multidimensional HPLC systems.

Johnson *et al.* (50) reported applications using a very similar system. In this system, two HPLC's are coupled via Valco loop valves. Applications included the analysis of malathion in tomatoes, additives in compounded rubber and limonin in grapefruit.

In 1978, Willmott (51) reported improvements in previously reported instrumentation (46,47). This consisted of a single high pressure pump and several switching valves interfaced with a microprocessor to control the HPLC and the valves. This work is significant in that it places the control of the switching valve under a microprocessor. All previous work and most subsequent work controls the valves on a time based system. The use of a peak recognition based control has several advantages, foremost of which is the



Schematic diagram of on-line two-dimensional HPLC with step-gradient elution and automatic injection control of loop 2. Pump 1: Lewa Model FL 1 (Lewa, Herbert Ott AG, Leonberg Stuttgart, G.F.R.). Injection valve 1: Valco loop, 7000 p.s.i., 50 μl (Valco Instruments, Houston, Texas, U.S.A.). Column 1: stainless steel, 200 × 0.4 cm, filled with CPG (controlled pore glass), 200–400 mesh, 88A/113/A 170A/240A (Electro-Nucleonics, Fairfield, N.J., U.S.A.). Detector 1: LC 55 UV detector (Perkin-Elmer, Norwalk, Conn., U.S.A.). Recorder 1: W – W Model 600 recorder (Kontron, Zürich, Switzerland). Injection valve 2: Valco loop, 7000 p.s.i., 1.777 ml. Control unit for the injection valve: home-made, two type RDF time relays (Summerer, Zürich, Switzerland). Pump 2: Altex Model 100 (Altex, Berkeley, Calif., U.S.A.). Step gradient valve: Labotron eight-port valve, No. 2581 (Kontron). Step gradient control unit: home-made, RS 21 × PG time relays (Comateletric, Worb, Switzerland). Column 2: stainless steel, 25 × 0.4 cm, filled with Nucleosil C_{18} reversed-phase material, 5 μm (Machery, Nagel & Co., Düren, G.F.R.). Detector 2: L 55 UV detector (Perkin-Elmer). Recorder 2: W – W Model 600 recorder (Kontron).

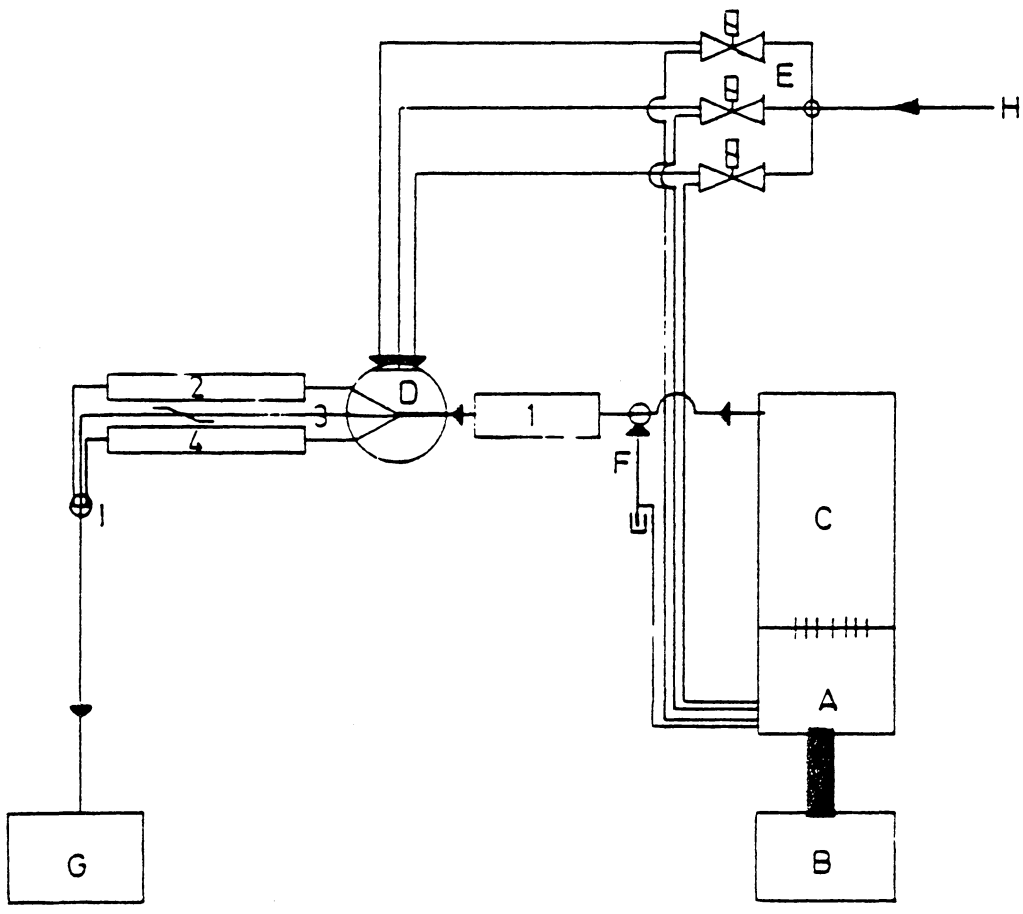
Figure 7. LC/LC Apparatus of Erni and Frei (49)

increase of reproducibility. Unfortunately, in a more generalized on-line multidimensional system, there are inherent problems in the use of a peak recognition system. The goal of multidimensionl HPLC as applied to complex samples is to analyze a sample by first fractionating the complex matrix and susbsequently analysing the appropriate fraction. In such analyses, there are a number of cases which would preclude the use of peak recognition valve control. In many cases, the initial fractionation produces a very complex chromatographic signal which may vary significantly sample-to-sample and may easily overwhelm the simple peak recognition algorithms used by the microprocessor. Another problem case involves the analysis of trace components in complex matrices. In such an analysis, the signal produced by the matrix may completely obliterate the signal produced by the trace analyte. Triggering valving from such "buried" peaks would be difficult.

It is also significant that this system utilizes a single high pressure pump. Although several other earlier reports used single pumps, they were also using identical or similar mobile phase for both chromatographic steps. Willmott's system utilized two separate mobile phase systems.

In 1979, Hulpke and Werthmann (52) published work involving the analysis of trace organics using multidimensional HPLC. This work incorporated many of the features of previous developments. Schematically, the hardware is shown in Figure 8 . This system utilized a single high pressure pump and Siemens switching valves. Distinctions are made between the concepts of "heart cutting", "quantitative cutting" and "backflushing". Heart cutting is a trapping technique in which only a portion of a peak of interest is transferred between chromatographic modes. Quantitative cutting is a switching technique in which an entire peak is transferred. Although these two procedures fit into the categories of trapping and switching as defined in the introduction, both techniques were accomplished via valve switching rather actually trapping a sample in a valve loop. Backflushing is a technique by which strongly retained compounds can be eluted from a column by reversing the direction of the mobile phase flow. Later, Hulpke and Werthmann (53) reported experimental evaluation of reproducibility.

Two reports were made by Davis and Kissinger in 1979 (54,55), describing a column switching system utilizing electrochemical detection. Two columns of similar phase systems were used to adjust capacity factors. Their system



1 Precolumn, 2/4 Analytical column, 3 Adjustable restriction and capillary.
A Microprocessor, B Terminal, C Chromatograph, D Column switching valve, E Magnet valve, F Injection system, G Detection system, H compressed air, I Union.

Figure 3. LC/LC Apparatus of Hulpke and Werthmann (52)

is shown in Figure 9. The main goal was the clean-up of complex biological samples in order to protect the electrochemical detector from poisoning by unwanted matrix components. Two applications were reported;(1) the analysis of serum dopamine hydroxylase; and (2) the analysis of serotonin in plasma (55).

Balke and Patel (56) described a system coupling two size exclusion chromatographic steps in the evaluation of polymer composition. The valving was arranged such that the first column fed an injection valve for the second column. Two high pressure pumps were used. This work constitutes essentially an application of the systems described earlier by Erni and Frei (49) and Johnson *et.al.* (50). An interesting suggestion is made concerning nomenclature in multidimensional HPLC, which the authors felt should be called "orthogonal chromatography" as opposed to "multidimensional chromatography". The distinction is a reference to the use of the term multidimensional in thin layer chromatography (TLC). In two dimensional TLC, a thin layer plate is developed along one axis and the turned and redeveloped along a second perpendicular axis. All points along the first axis are developed along the second axis. In multidimensional HPLC, on the other hand, only selected points of an initial separation are analyzed by the second

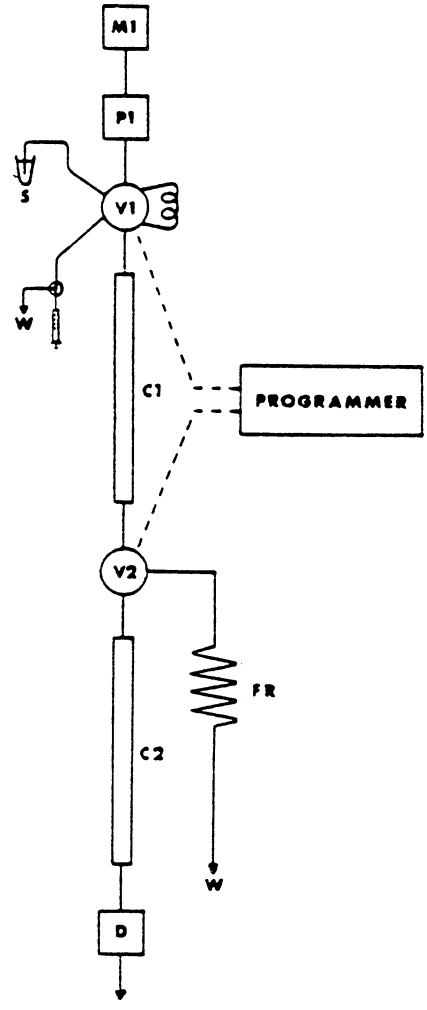


Diagram of the split column liquid chromatograph. M1 is the mobile phase reservoir; P1 is the pump; V1 is a six-port injection valve; V2 is a three-way valve; C1 and C2 are the analytical columns; S is the sample; W is a waste receptacle; FR is a flow restrictor; and D is the detector. The two valves are controlled by the digital programmer

Figure 9. LC/LC Apparatus of Kissinger

"orthogonal" chromatographic mode. Although this nomenclature may be valid, it has not been widely accepted.

Huber *et al.* (57, 58) published a two part report on applications of multidimensional HPLC to the analysis of carbamate pesticides in fruits and vegetables. Although it is significant that this is the first report by Huber on the adjustment of selectivity, this report is primarily applications oriented. The hardware used is unchanged from his earlier work (45). In a later paper in 1980 (59), Huber *et al.* discuss some conceptual considerations of the adjustment of selectivity by multidimensional HPLC. He generalizes the operation into three major categories: 1) adjustment of the separation by gradual adjustment of the resolution parameters, including column length, phase ratio and distribution coefficient; 2) increase of separation power for specific components by adjustment of their selectivity; and 3) improvement of the degree of separation by reducing the relative concentration of interfering components. Essentially these three techniques are the adjustment of capacity factor, selectivity and sample clean-up respectively.

Wheals *et al.* (60) and Rose and Schwartz (61) both presented the use of separate HPLC columns run in parallel on a single sample using multiple high pressure valves.

Although examples of column switching, these are not truly multidimensional chromatographic techniques as defined in the introduction.

In 1980, Apffel *et al.* (62) described the use of aqueous size exclusion columns as a preliminary on-line clean-up step for the analysis of water soluble samples. Although primarily applications oriented, this work did examine the relative advantages and disadvantages of trapping systems as opposed to switching systems. Instrumentally, Apffel's work involved the use of a single high pressure pump and pneumatically actuated valving. Through the coupling of SEC with several other chromatographic modes, including reverse phase, normal phase and ion exchange, not only were "dirty" samples cleaned-up on-line, but use of selectivity optimization was demonstrated as well.

Gfeller and Stockmeyer (63) reported in 1980 a similar on-line clean-up system applied to the analysis of medicated feeds. This apparatus, however, utilized two separate pumping systems. In this technique, two reverse phase columns were coupled via a trapping system. In other words, the effluent from the primary column loaded a loop valve which injected a trapped sample into the second column. The general approach was identical to that of Apffel *et al.* (62)

and Erni and Frei (49). The coupling of the two reverse phase columns resulted in an adjustment of capacity factor.

Miller *et al.* (64) reported on the application of column switching to the analysis of PAH's in complex samples. In this system, a reverse phase column was used as a switched guard column to protect a secondary SEC separation from column degradation. Backflushing was also used.

In 1981, Erni *et al.* (65) elaborated on earlier work, describing a generalized system for HPLC. Schematically, the hardware is shown in Figure 10. Although a single pump delivers flow to both columns, an auxiliary pumping system is used to backflush the primary column. In this system, the effluent from the first column is switched either to the second column or to a by-pass to the detector. This switching system is different from the trapping system reported in their earlier work (49). In terms of methodology, the concept of column switching was divided in this work into "front cutting", "heart cutting" and "end cutting", depending on which portion of the peak is transferred between the chromatographic systems. Although Erni's work is performed instrumentally by a switching process, only a portion of the peak is transferred.

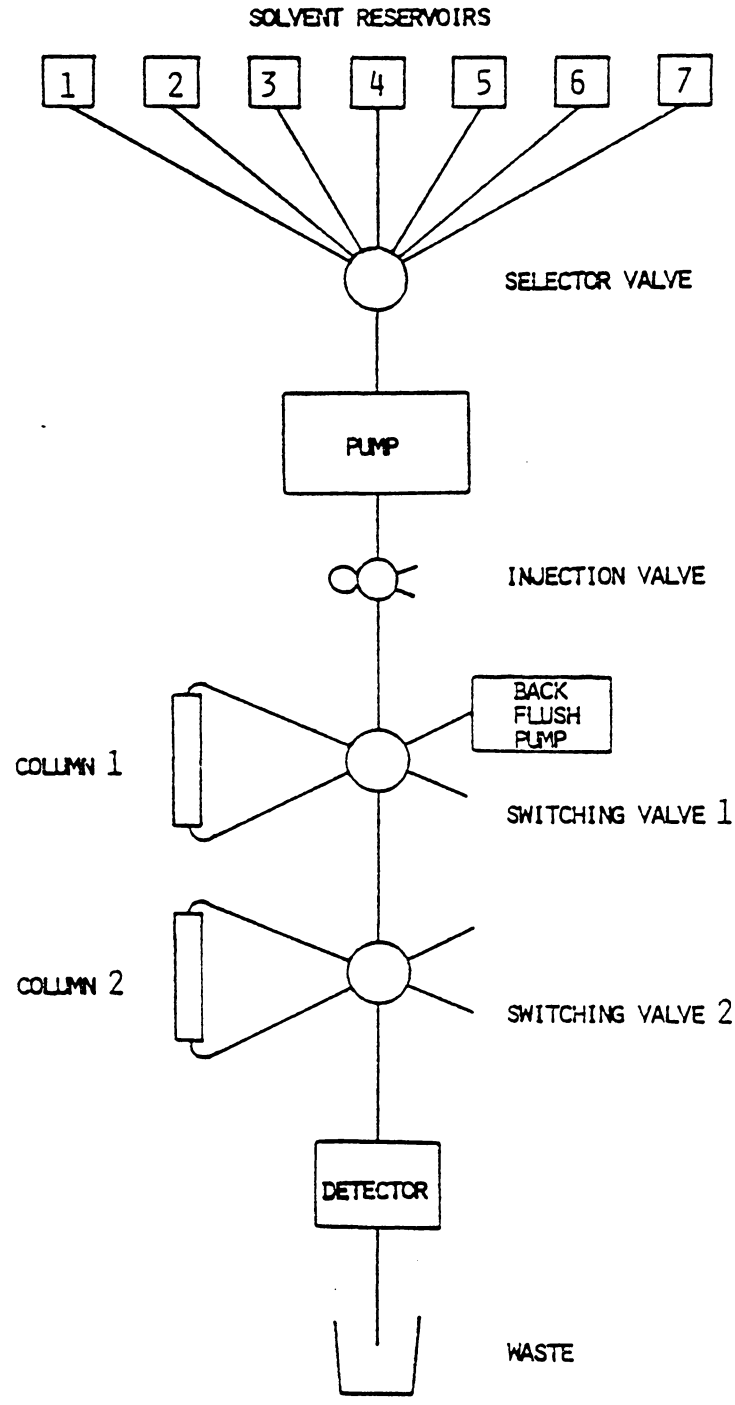


Figure 10. LC/LC Apparatus of Erni and Frei II (65)

In January 1981, a special symposium on Multidimensional Chromatography was held at the Middle Atlantic Regional Meeting of the American Chemical Society. Freeman (66) presented an overview of progress in the field. Apffel and Zimmerman (67) presented elaboration on previous work coupling SEC and other HPLC modes in the analysis of water soluble samples. Katz and Ogan (68) presented a system for the clean-up and fractionation of complex samples by SEC prior to reverse phase analysis of PAH's. This was actually a three dimensional system; a reverse phase column was used as a switched guard column prior to an SEC fractionation; fractions from the SEC were collected and transferred off-line to a second reverse phase column for the analytical separation. May (69) and Sonnefield *et al.* (70) reported an on-line multidimensional system coupling normal phase and reverse phase systems in the analysis of PAH's in shale oil. This work is significant because it couples two systems which use immiscible solvents. All previous work used miscible and compatible mobile phases. In their system, fractions eluting in hexane from an aminopropyl bonded silica column were selectivily concentrated on a short diamino bonded silica concentrator column. The concentrator column was then heated while a flow of nitrogen removed the hexane solvent. The samples

were then eluted in water from the concentrator column and re-concentrated at the head of the reverse phase column, minimizing band broadening due to the transfer. The PAH's were then separated via gradient elution on the reverse phase column.

Several reports of Multidimensional HPLC systems appeared at the 1981 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy. As referred to in the introduction, J.F.K. Huber (1) in his acceptance presentation for the Dal Nogare Award, noted the significance of the use of on-line multidimensional chromatographic techniques. He also reviewed some of the work which had been previously reported elsewhere. Apffel and McNair (71) reported the application of multidimensional HPLC to the analysis of hydrocarbon groups in fuel samples. In this work, column switching between a silica column and a silver nitrate impregnated silica column was used to separate saturates, unsaturates and aromatics in gasolines. Instrumentally, this system is similar to Apffel's earlier work (67) except for the addition of a backflushing feature through the valving scheme. Schmidt and Slavin (72) reported the use of coupled columns in biochemical analysis. This system concentrated ionic samples on a cation exchange pre-column. These ionic sample components were subsequently

eluted from the precolumn and separated on a similar analytical column.

At the 1981 American Chemical Society Southeast Regional Meeting held in Atlanta, Apffel, Chen and McNair (73) reported the analysis of PAH's in solvent refined coal using column switching between an aminopropyl bonded silica column and a column packed with picramidopropyl bonded silica, which formed charge transfer complexes with the PAH's.

The uses of on-line multidimensional HPLC as described above have followed a number of trends summarized in Table 2. Early systems utilized multiple pumping systems, but there has been a trend toward single pumps. Although earlier systems tended to adjust capacity factors, more recent systems optimize both selectivity and capacity factors. In many recent examples there is a tendency to utilize on-column concentration techniques to overcome band broadening effects. Two main types of transfer systems have been used: trapping, in which a sample valve loop of a second column is loaded by the effluent of the first column; and switching, in which the effluent flow from the first column is diverted by valving either to the second column or to bypass.

TABLE 2

Trends in On-Line LC/LC

Author (Ref)	High(H) or Low(L) Pres.	Trap.(T) or Switch.(S)	Capacity Factor(F) or Select.(S)	Single(S) or Mult.(M) Pump
Snyder(42)	L	S	C	M
Liljamaa(43)	L	S	C	M
Scott(44)	L	S	S	S
Huber(45)	H	S	C	S
Dolphin(46)	H	S	C	M
Huber(48)	H	S	C	S
Erni(49)	H	T	S	M
Johnson(50)	H	T	S	M
Willmott(51)	H	S	S	S
Hulpke(52)	H	S	S	S
Kissinger(54)	H	S	C	M
Balke(56)	H	T	S	M
Huber(57)	H	S	C+S	S
Apffel(62)	H	T+S	S	S
Gfeller(63)	H	T	S	S
Erni(65)	H	S	C	S
Apffel(67)	H	T+S	S	S
Katz(68)	H	S	S	S
May(69)	H	S	S	S
Apffel(71)	H	S	S	S
Schmidt(72)	H	S	C	S
Apffel(73)	H	S	S	S

2.2.3 Trace Concentration Methods

Trace organic concentration methods are a group of techniques closely related to multidimensional HPLC. As mentioned above, some on-column concentration techniques are used to counteract the band broadening which takes place in column switching. These techniques have been used alone, nowever, to effect the analysis of extremely low levels (ppb) of organics. Some of the earliest work in this area of HPLC utilized an on-column concentration approach (74). This procedure, which is still widely used today, involves pumping large volumes of a sample through the analytical column. Analytes are adsorbed at the head of the column and are subsequently eluted, usually via a solvent gradient. Huber and Becker (75) examined this technique from both theoretical and practical standpoints. Some recent examples of analyses employing on-column concentration include the analysis of plasticizers and polychlorinated biphenyls (76), pthalate esters (77) and PAH's in water (78). The use of on-column concentration suffers from a major potential problem in that in extremely "dirty" samples, the performance of the analytical column may be severely degraded. In an attempt to alleviate this, short concentrator columns have been used to collect the sample which is subsequently eluted onto the analytical column and

separated (79,80,81). The relationship of this type of technique to on-line multidimensional HPLC is really one of emphasis. While the hardware used is very similar to that discussed above, the concentrator column does not generally contribute effectively to the actual separation. Usually the concentrator is packed with the same material as the analytical column. There are exceptions to this, and one of the most significant was described by May *et al.* (82) and utilizes an effect called "elution focusing". If the rate of migration of the solutes is greater in the concentrator column than in the analytical column, then the solutes will tend to reconcentrate at the head of the analytical column, reducing any band broadening introduced into the system by the concentrator column. This is essentially the effect described earlier in its use in Multidimensional HPLC.

Organic concentration analyses have been performed off-line as well. Recently, a number of small disposable concentrator cartridges have become commercially available. The "Sep-pak" cartridge, marketed by Waters Associates, was the first of these systems and is the most widely used. Some examples of applications using disposable concentrator cartridges include the analysis of chlorphenoxy esters in drinking water (83) and pesticides in drinking water (84,85).

2.3 LC/GC

GC analyses have also employed preliminary liquid chromatographic separations as sample preparation steps. The pre-analytical LC steps fall into three main categories; clean-up, concentration and fractionation. In sample clean-up procedures, the analytes are isolated from the sample matrix. This is often accomplished by injecting the sample with conditions under which the analytes are strongly retained. After the unretained components have eluted, the conditions can be altered so that the analytes are eluted and collected with relatively little dilution. Similar procedures are used in sample concentration techniques, except that the samples are usually injected as much larger volumes. In sample fractionation techniques, the analytes are eluted in several groups for subsequent characterization.

2.3.1 Off-line Applications of LC/GC

Almost exclusively, the combination of LC and GC has been performed off-line and the use of such procedures is quite common. An exhaustive review of the uses of this technique is not neccessary in building a foundation for the use of on-line LC/GC and is, furthermore, beyond the scope of the present work. The following, therefore comprises only a review of some representative examples.

The use of LC as a clean-up step prior to GC analysis has been widely used, involving most LC phase systems. Uses of normal phase chromatography are, however, the most common, particularly in the isolation of polar biological metabolites. Some examples include the analysis of urinary testosterone (86); tocopherols in red blood cells (87); and amino acids in the urine of mental retardates (88). Polar pesticides are commonly isolated using normal phase chromatography. Examples such as the analysis of DDT in flowers (89); residues of carbophenothion in mice (90); pyrethroid insecticides in crops (91); bentazon in soil and plants (92); and toxaphene in soil (93) are in the recent literature. Other examples of the use of normal phase chromatography include the clean-up of enzymatic esterification products in sunflowers (94); the analysis of particulate combustion products of polymers (95); and the analysis of chlorinated aromatics in biological matrices (96).

Fractionation of samples by normal phase chromatography has been applied to similar types of analyses. In many cases, the fractionation is an expanded clean-up step, but there are also numerous examples of group fractionations. Normal phase chromatography has found great use in separating samples into chemical classes or groups. This is

particularly useful in the petroleum industry where characterization of hydrocarbon group distributions (saturates, unsaturates, aromatics and polars) is required. The separation of hydrocarbon groups has been performed routinely on silica adsorbents (97) and this technique has been coupled with GC in characterizing individual groups. This has been applied to shale oil (98); solvent refined coal (99); crude oil (100); and HYGAS oil samples (101). A similar type of procedure has been applied to the analysis of oxygen containing monoterpenes in essential oils (102-105). Similar analyses using bonded normal phase chromatography have been reported in the fractionation of complex environmental samples. HPLC on aminopropyl bonded silica columns has been used to fractionate samples containing PAH's according to the number of aromatic carbons in the PAH ring system (106). This is one of the procedures used in the certification of the National Bureau of Standards' Standard Reference Material for shale oil (SRM 1580)(107).

Ion exchange chromatography is often used in the isolation of ionic species prior to GC analysis. Some examples are the analysis of bile acids in urine (108); organic acids and amino acids in amniotic fluid (109); amino acids in blood (110); natural steroids in urine with GC/MS

identification (111); trehalose in cellular slime mold (112); serum bile acids (113); and captoril in human blood (114). Of course, these clean-up procedures can become quite complex. One reported procedure uses not only ion exchange but also a reverse phase and a thin layer separation in the clean-up of metabolites of prostaglandins for GC analysis (115). Ion exchange has also been used to fractionate samples according to their ionic character prior to GC separation. Examples include the analysis of acids in serum (116); screening for amino acid metabolic disorders in infant urine (117); and the analysis of pear acids and sugars. In this last report, the advantages of HPLC clean-up are compared to the classical lead acetate precipitation method(118).

Reverse phase liquid chromatography has been used as a preliminary step in the analysis of hydrophobic substances by GC. As a clean-up procedure reverse phase techniques have been used in the analysis of both biological and environmental samples. Some examples include the analysis of benzoylecgonine in urine (119); tetrahydrocannabinol in biological fluids (120); and hexachlorocyclohexane pesticides in wool fat (121). Reverse phase systems have also been used to fractionate complex samples, particularly in environmental applications. Some examples include the

analysis of lanvadin essential oils (122); the analysis of PAH's in water (123); and in smoke particulates (124); and identification of organics in smoke particulates by GC/MS (125).

Size exclusion chromatography has been used extensively to both clean-up and fractionate samples prior to GC analysis. As previously mentioned, SEC is particularly well suited as a clean-up procedure due to its ease of operation and versatility. Recent examples include the analysis of carotenoids in mandarin oil (126); the analysis of PAH's in gas and air particulates (127); purification of pesticides in fats, feeds and vegetables (128); the isolation of organochloro plant treatment materials in tobacco (129); analysis of hypoglycin A in amino acid mixtures (130); the analysis of fenitrothion in bivalves (131); the analysis of sterols in edible fats and oils (132); and the analysis of oxygenated neutral constituents of the tumor inhibiting fraction of cigarette smoke (133). SEC has been of great use in fractionating samples according to molecular weight ranges. Some examples include the analysis of PAH's in water (134); biliary bile acids in rats (135); and urinary steroids in pathological pregnancy (136).

In addition to the techniques discussed above, some special cases of off-line LC/GC should be mentioned. The

liquid chromatographic clean-up of samples for subsequent analysis, has recently been facilitated by the availability of disposable chromatographic catridges such as Waters Associates' "Sep-pak". These systems have been discussed previously in the preparation of samples for LC analysis and are used in an identical capacity for GC.

Another special technique which can be considered an off-line application of LC/GC is the preparation of samples by trace concentration techniques in HPLC. Considerable use has been made of ion exchange resins such as XAD-2 (Rohm and Hass, Philadelphia, Pa.) and Tenax GC (Akzo, Deventer, The Netherlands). Although these adsorbents are more typically used to concentrate samples from vapor, they have been used directly with liquid samples. These systems usually desorb the concentrated samples thermally rather than via solvent displacement (137,138).

These applications of off-line LC/GC are summarized in Table 3 .

TABLE 3

Off-line Applications of LC/GC

Application	LC Mode	Ref.
Urinary Testosterone	NP	86
Urinary Amino Acids	NP	88
Tocopherols in Blood	NP	87
DDT in Chamomilla	NP	89
Carbophenothion in Mice	NP	90
Pyrethroid Insecticides	NP	91
Bentazon in Plants	NP	92
Toxaphene in Soil	NP	93
Enzymatic Esterification Products	NP	94
Particulate Combustion Products	NP	95
Chlorinated Aromatics in Biological Matrices	NP	96
Hydrocarbon Groups in:		
Shale Oil	NP	98
Solvent Refined Coal	NP	99
Crude Oil	NP	100
"Hygas" Oil	NP	101
Monoterpenes	NP	102-105
PAH's in Shale Oil	NP	106
Urinary Bile Acids	IEC	108
Urinary Steroids	IEC	111
Organic Acids in Amniotic Fluid	IEC	109
Amino Acids in Blood	IEC	110
Serum Bile Acids	IEC	113
Captoril in Blood	IEC	114
Prostaglandins	IEC/RP/TLC	115
Serum Uric Acid	IEC	116
Urinary Amino Acids	IEC	117
Trehalose in Slime Mold	IEC	112
Pear Sugars and Acids	IEC	118
Urinary Benzoylegonine	RP	119
THC in Biological Fluids	RP	120
Pesticides in Fat	RP	121
Lanvadin Essential Oils	RP	122
Organics in Smoke	RP	125
PAH's in:		
Water	RP	123
Smoke Particulates	RP	124
Gas and Air	SEC	127
PAH's in Water	SEC	134
Carotenoids in Mandarin Oil	SEC	127

TABLE III CONT'D.

Pesticides in Fats	SEC	128
Organochlorics in Tobacco	SEC	129
Hypoglycin A	SEC	130
Fenitrothion in Bivalves	SEC	131
Steroids in Fats	SEC	132
Bile Acids	SEC	135
Urinary Steroids in Pathological Pregnancy	SEC	136

2.3.2 On-line LC/GC

Compared to the application of on-line techniques to LC/LC, on-line LC/GC is an untouched area. The primary reason for this is the relative complexity of the hardware required. In the case of LC/LC, the mobile phases of both chromatographic modes are compatible, i.e. they are both liquids, and portions of the effluent from the primary mode can be transferred directly to the inlet of the secondary mode. In the case of LC/GC, however, the two mobile phases are in different physical states, liquid and vapor, and therefore, some phase transfer step is required in the coupling of the two systems. Although conventional sample introduction systems for GC can accomodate either gases or liquids, GC systems usually introduce relatively small volumes of sample (<10 ul) and thus the direct continuious flow of the LC effluent into the GC inlet system is not feasible. This is to be compared with the simple valving requirements in coupling two HPLC systems.

The first example of an on-line LC/GC system has been recently developed and introduced by Varian Associates as the Model 8070 LC/GC Interface. This system consists of a modified GC Autosampler injection module equipped with a flow-through side arm syringe. The LC effluent flows continuiously through this syringe, and at a given signal,

traps a small portion of the effluent and injects it into the GC. This unit was introduced at the 1979 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy and two papers were given in relation to it. In the first, Cram *et al.* (139) described the development and operation of the system. This report described the use of the interface in coupling HPLC with both packed column and capillary GC. In the second paper, Majors *et al.* (140) described applications of the system including the analysis of trace pesticides in butter and pesticides in vegetable extracts.

At the time of this writing, the only other on-line multidimensional LC/GC work reported has been by the author in relation to the present work and utilizing the system developed by Varian Associates.

In 1980, Apffel *et al.* (141) reported the use of the LC/GC interface in the analysis of pesticides in butter. This analysis was largely based on the previous work by Majors *et al.* (140), and utilized the coupling of organic SEC and packed column GC with electron capture detection. Further refinements were made in this work with respect to the earlier work in the area of quantitation. In 1981, Apffel and McNair (71) reported the use of on-line LC/GC in the analysis of hydrocarbon group types. This separation utilized the preliminary fractionation of a hydrocarbon

sample into saturates, unsaturates and aromatics by normal phase HPLC with subsequent characterization of the individual groups by capillary GC. At the 1981 Southeast Regional ACS meeting, Apffel *et al.* (73) reported the application of LC/GC to the analysis of PAH's in solvent refined coal. This analysis was performed by the initial normal phase fractionation of the sample according to the number of aromatic carbons in the PAH ring system followed by capillary GC separation of the individual fractions.

There are several reported techniques which, although not strictly multidimensional chromatography as the term has been used, do have bearing on LC/GC. The first is the use of Tenax GC in concentrating liquid samples for GC analysis. This has been discussed in a previous section as an off-line technique, but has been used on-line as well. In this technique, after a sample has been adsorbed on the Tenax GC column by passing a liquid sample through the column, the solvent is evaporated leaving only the adsorbed sample which is the thermally desorbed. Thermal desorption elutes the sample in the vapor phase directly onto the GC column at a relatively low temperature such that the sample is re-concentrated at the head of the GC column. Temperature programming is then used to analyze the sample by GC. As discussed earlier, May (69) used a similar procedure to

effect a phase transfer from an organic to an aqueous mobile phase in coupling two HPLC systems. Although in May's work, the sample was desorbed via mobile phase gradient onto a second LC column, there is no reason why this technique could not be used to couple LC and GC.

Another technique which is tangential to LC/GC is the use of GC detectors with LC systems. There are several systems used in this type of coupling and, with some redesign, they may in the future be employed for the coupling of LC and GC. One approach utilizes a nebulizer to spray the LC effluent into a GC flame ionization detector. The major difficulty with this system is the background signal due to the LC mobile phase. A second approach has been that of transport detectors. In these systems the LC effluent is delivered on some type of transport mechanism from which the mobile phase is evaporated. The transport system then introduces the sample into the detector by either thermally desorbing the dried sample or redissolving the sample in an appropriate solvent. This is one of the principle methods used in coupling HPLC with mass spectrometry. Finnigan Instruments has introduced this type of system in which the transport mechanism is a moving wire (142). The major limitation is that a solvent of high volatility must be used for optimum results. This limits

the use of aqueous mobile phases common to reverse phase HPLC. Buffers present problems in such a system as well.

Chapter III

THEORY

3.1 INTRODUCTION

An important feature of on-line multidimensional chromatography which has not recieved adequate attention is a theory of quantitation. If on-line multidimensional sytems are to be used effectively in quantitative analysis, it is critical that the effect of the various operating parameters on the characteristics of quantitation be understood. It is therefore the aim of the following to develop a model which can be used to predict quantitation factors such as reproducibility, accuracy and recovery for a given set of experimental conditions as a function of the chromatographic variables.

In general, most on-line multidimensional chromatographic techniques can be modelled as follows. A sample is introduced into a primary column from which the components elute at some later point depending on their retention characteristics. For a given analyte or group of analytes, some set volume of the effluent of this primary column is transferred to the secondary column and undergoes separation. The critical operation which differentiates the

quantitation of multidimensional systems from conventional chromatographic processes is the transfer of the analyte from the primary mode to the secondary. Although there are variations in experimental designs, most multidimensional chromatographic systems transfer a volume of effluent directly from the outlet of the primary mode to the inlet of the secondary. Furthermore, most systems program this transfer on a time basis. There are systems which do not fit this model such as Sonnefeld's transfer system which utilized an intermediate concentrator column (70) and Willmott's peak detection based triggering system (51), but the model above adequately describes the operation of the majority of reported systems and will be used here to describe the quantitation in such systems.

A major variation in operation of multidimensional systems involves the volume of sample transferred relative to the total peak volume. As described earlier, two categories will be used here: "Trapping", in which a small portion (<10%) of the total peak volume is transferred; and "Switching", in which a large fraction (>10%), usually most, of the analyte peak is transferred between modes. These distinctions are motivated by two considerations. From an instrumental point of view, as the names imply, trapping systems usually act by trapping a portion of effluent in a

syringe or sample loop prior to it's introduction into the second column, and switching systems usually operate by switching the primary column effluent flow directly into the secondary column inlet for a fixed time. Although small fractions can be transferred by a switching mechanism, for simplicity, the above operations will be assumed. The second motivation for using these two categories is that the mathematics involved in modelling such systems are simplified by making these assumptions.

The equations which are developed below describe a generalized on-line multidimensional chromatographic system. The term "generalized" here is meant to indicate a system which operates according to the model outlined above. Application of these equations is independent of the chromatographic modes which are coupled together. The theory, therefore, will be equally applicable tothe LC/LC and LC/GC systems which have been evaluated experimentally.

3.2 QUANTITATION AND STATISTICS

Before proceeding further, it is neccesary to describe what is meant by "quantitation" and to define some statistical systems used in evaluating quantitation. In the evaluation of the performance of analytical systems it is possible to define a large number of terms to describe many

facets of the quantitation (143) including run-to-run reproducibility and accuracy, day-to-day reproducibility and accuracy, inter- and intra-laboratory comparisons of reproducibility, recoveries, detection limits, linearity, accuracy and interlaboratory transferability. Several of these have no theoretical basis but are rather empirical results. In the operation of a generalized on-line multidimensional chromatographic systems it is adequate to describe the run-to-run reproduciblity, accuracy and transfer efficiency. The evaluation of performance characteristics such as detection limits and linearity are highly method dependent and are, at any rate, related to recoveries. Interlaboratory evaluations are primarily personnel related rather than technique evaluations.

In dealing with statistical populations, the reproducibility of an event is described by variance, s, given by

$$s=\Sigma(x_i-\mu)^2 p(x_i) \qquad (6)$$

where

x_i=i^{th} value of x

μ=mean x value

$p(x_i)$=probability function for x

The concept of variance, however, assumes an infinite number of trials. In order to apply this to experimental data, an approximation is made to describe the standard deviation given by

$$\sigma=\sqrt{\Sigma (x_i-\bar{x})^2/(n-1)} \tag{7}$$

where

$\bar{x}$=average x value

n=number of trials.

The goal of this work is to predict the standard deviation of a multidimensional system based on *a priori* considerations. From equation 6,

$$s\leq(\bar{x}-x_m)^2 \tag{8}$$

where $\bar{x}$ is a expected value and x_m is the maximum value obtainable. It follows that

$$\sigma\leq|\bar{x}-x_m| \tag{9}$$

There can be, therefore, defined a parameter, R, as the range

$$R=|\bar{x}-x_m| \tag{10}$$

R is a predictable measure of the reproducibility of the experimental system. The relative range, R', can also be defined

$$R' = (|\bar{x} - x_m| / \bar{x}) 100 \qquad (11)$$

The range and relative range can therefore be predicted and compared with the maximum expected value of the standard deviation and the relative standard deviation for experimental data.

In predicting the recovery of a sample in the multidimensional transfer process, the transfer efficiency can be defined,

$$T = (A_s / A_t) 100 \qquad (12)$$

where ;

A_s=amount of sample transferred

A_t=total amount of sample.

In describing the accuracy in a multidimensional chromatographic system, the error, E, is described as

$$E = x_t - \bar{x} \qquad (13)$$

where ;

x_t=true value

$\bar{x}$=average value.

and the relative error, E', as

$$E' = (x_t - \bar{x})/\bar{x} \cdot 100 \qquad (14)$$

Finally, a mathematical model must be used to describe the chromatographic elution profile. Although several functions have been used, the most common is a Gaussian distribution. This function is generally assumed to describe the elution profile of most chromatographic processes adequately according to the equation (2)

$$P(v) = (\sqrt{N}/V_r)\,(w/\sqrt{2\pi})\exp\{-N/2(1-V/V_r)^2\} \qquad (15)$$

where ;

N=number of theoretical plates

V_r=retention volume

V=elution volume

w=weight of sample.

P(v), is given in units of concentration.

3.3 REPRODUCIBILITY

3.3.1 The Trapping System

According to the equations given above, the reproducibility in multidimensional chromatographic systems can be described by the range, R, given in equation 10. This requires a theoretical evaluation of the expected amount and maximum amount to be transferred. This has not been treated previously in the literature. It was therefore neccessary to derive the following equations. Referring to Figure 11, if an amount A corresponding to a volume s of effluent at elution volume V is to be trapped and transferred, then any variation Δv in the retention volume of the peak, V_r will lead to a variation in the amount trapped. Mathematically, the range will be given by

$$R=|A_t-A_1|=|A_t-A_2| \tag{16}$$

where A_1 and A_2 are the maximum and minimum amounts which will be trapped due to the retention variation Δv. Therefore,

$$R=\int_{v-s/2}^{v+s/2} P dv - \int_{v+\Delta v-s/2}^{v+\Delta v+s/2} P dv \tag{17}$$

A mathematical difficulty arises at this point in that the Gaussian function has no closed form solution to its

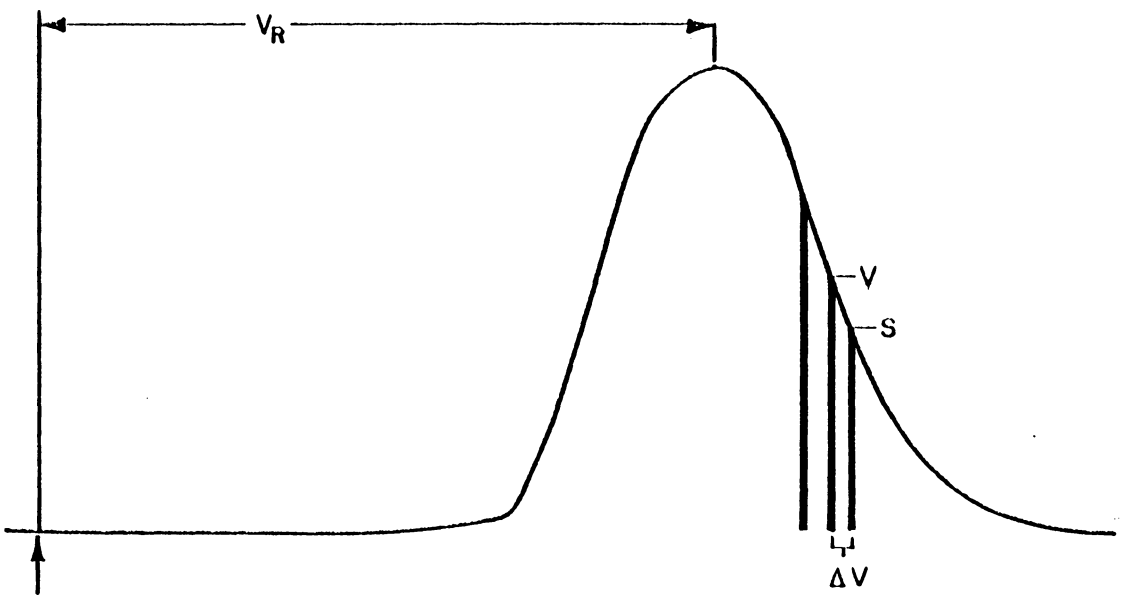


Figure 11. The Trapping System

integral. The integrals in equation 17 must therefore be approximated. In the trapping system this can be done by assuming that the transfer volume, s, is small relative to the peak volume. If it is also assumed that the retention variation Δv is small relative to the peak width, then the following approximations can be made

$$\int_{v-s/2}^{v+s/2} P dv \simeq sP(v) \tag{18a}$$

and

$$\int_{v+\Delta v-s/2}^{v+\Delta v+s/2} P dv \simeq sP(v+\Delta v) \tag{18b}$$

Equation 17 then becomes

$$R = s\{P(v) - P(v+\Delta v)\} \tag{19}$$

by substituting equation 15 into equation 18,

$$R = s\sqrt{N}/V_r (w/\sqrt{2\pi}) (\exp\{-N/2(1-V/V_r)^2\} - \exp\{-N/2(1-(V+\Delta V/V_r)^2\}) \tag{20}$$

Some further substitutions can be made according to the following assumptions:

1. If the triggering system is time based, there is some fixed time resolution which will lead to a fixed volume error, V_E. Therefore,

$$V=V_s+V_E \tag{21}$$

or

$$V=V_s+T_EF \tag{22}$$

where; V_s=intended sampling point
T_E=timing error
F=flow rate

2. The variation of the sampling point with respect to the retention volume will consist of two components, one based on retention precision, ΔV_r and one based on trapping device precision ΔV_t. Thus,

$$\Delta V=\Delta V_r+\Delta V_t \tag{23}$$

or

$$\Delta V=pV+\Delta t_sF \tag{24}$$

where;

Δt_s=device timing precision
p= percent retention precision.

Substituting equations 22 and 24 into equation 20 yields

$$R=s\sqrt{N}/V_r(w/\sqrt{2\pi})(\exp\{-N/2(1-(V_s+T_EF)/V_r)^2\} -\exp\{-N/2(1-(V_s+T_EF+pV+\Delta t_sF)/V_r)^2\}) \quad (25)$$

The relative range, R', can be obtained by dividing the range in equation 25 by the average amount transferred as given in equation 18a with the appropriate substitutions of equations 15, This simplifies to

$$R'=1-\frac{\exp\{-N/2(1-(V_s+T_E+pV+\Delta t_sF)/V_r)^2\}}{\exp\{-N/2(1-(V_s+T_EF)/V_r)^2\}} \quad (26)$$

3.3.2 The Switching System

As opposed to the trapping system in which a single event is required in the transfer process, the switching system requires two discrete events (on and off) to transfer a sample from one chromatographic mode to another. As shown in Figure 12, if V_a and V_b are the intended switching points

and the area under the peak between V_a and V_b is the intended transfer amount, then variation in V_a and V_b can lead to a maximum transfer volume of $V_a - \Delta V_a$ to $V_b + \Delta V_b$, and a minimum transfer volume of $V_a + \Delta V_a$ to $V_b - \Delta V_b$. In analogy to the previous development for the trapping system, the range for a switching system is given by

$$R = \left| \int_{V_a}^{V_b} P dv - \int_{V_a}^{V_b + \Delta V_b} P dv \right| \tag{27}$$

Unlike the trapping system, however, these integrals cannot be approximated by rectangulation. Referring to Figure 12 it can be shown that R can be given by the sum of the areas V_a to $V_a + \Delta V_a$ and V_b to $V_b - \Delta V_b$ instead of the difference given in equation 28. If it is assumed that ΔV_a and ΔV_b are small compared with the peak width, R can be approximated using rectangulation;

$$R = \left| \int_{V_a - \Delta V_a}^{V_a} P dv + \int_{V_b}^{V_b - \Delta V_b} P dv \right| \tag{28}$$

$$R = V_a P(V_a + \Delta V_a)/2 + V_b P(V_b + \Delta V_b)/2 \tag{29}$$

where $(V_a - \Delta V_a)/2$ and $(V_b - \Delta V_b)/2$ are taken to be average areas over the interval V_a to $-\Delta V_a$ and V_b to ΔV_b. Substituting equation 15 for P(v) yields

$$\begin{aligned} R = \sqrt{N}/V_r \, (w/\sqrt{2\pi}) \, (\Delta V_a \exp\{-N/2 (1 - (V_a + \Delta V_a)/2V_r)^2\} \\ + \Delta V_b \exp\{-N/2 (1 - V_b + \Delta V_b)/2V_r)^2\}) \end{aligned} \tag{30}$$

Analogously to the substitutions made in equations 21-26 for the trapping case, the following substitutions can be made:

$$V_a = V_{s_a} + T_E F \tag{31a}$$

$$V_b = V_{s_b} + T_E F \tag{31b}$$

$$\Delta V_a = pV_a + \Delta t_s F \tag{32a}$$

$$\Delta V_b = pV_b + \Delta t_s F \tag{32b}$$

Substituting these into equation 31 yields

$$\begin{aligned} R = \sqrt{N}/V_r (w/\sqrt{2}) \cdot \\ \{(pV_a + \Delta t_s F)\exp\{-N/2(1-(V_{s_a} + T_E F + pV_a + \Delta t_s F)/2v_r)^2\} \\ + (pV_b + \Delta t_s F)\exp\{-N/2(1-(V_{s_b} + T_E F + pV_b + \Delta t_s F)/2V_r)^2\}\} \end{aligned} \tag{33}$$

It should be noted that in substituting equations 31 and 32 into equation 33, it is assumed that the timing error, T_E, and the device precision Δt_s, are the same at V_a and V_b and that ΔV and ΔV are small enough that not substituting equations 32 into equations 33 will result in negligible error.

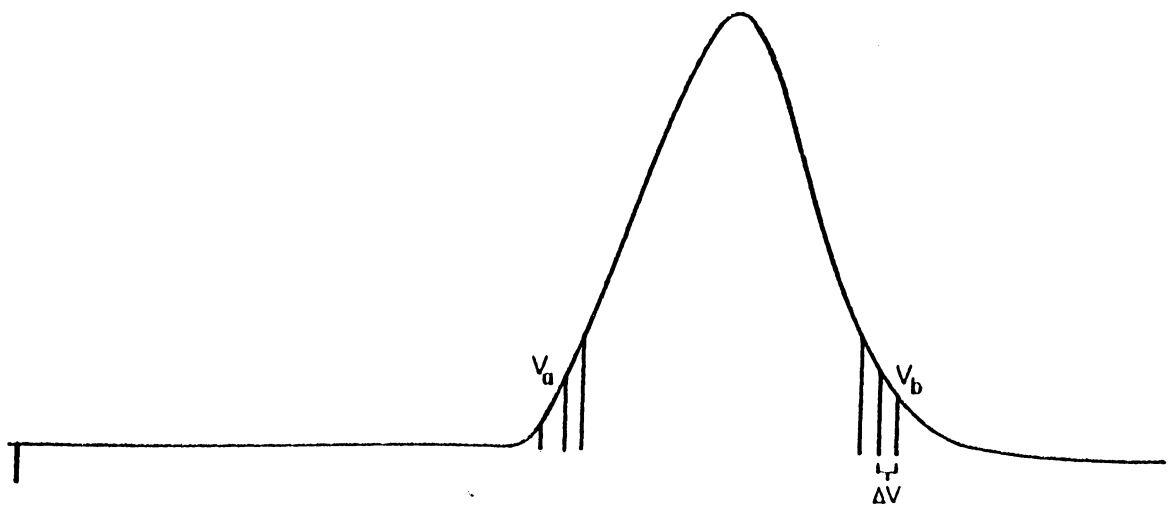


Figure 12. The Switching System

The relative range for the switching system requires that the actual area under the curve, P(v), must be determined, and this requires the integration of a Gaussian function. This integral can be determined either from mathematical compilation tables or by a numerical approximation. For the present work, an approximation is made using the trapezoidal rule. If the area thus determined is Q, then the relative range, R' will be

$$R'=(R/Q)100 \tag{34}$$

3.4 TRANSFER EFFICIENCY

3.4.1 The Trapping System

The transfer efficiency as given in equation 12 can be evaluated for the trapping system by substituting equation 18a into equation 12 for A_s. A_t is given by the total weight of sample present, w. Therefore

$$T=sP(v)/w \tag{35}$$

$$T=s\sqrt{N}/V_r\sqrt{2\pi}\exp\{-N/2(1-(V_s+T_EF)/2V_r)^2\}100 \tag{36}$$

3.4.2 The Switching System

As is the case for the range in switching systems, the derivation of an expression for the transfer efficiency requires the determination of the area under a Gaussian function. The total amount of the analyte can be taken as w, as above. The transfer efficiency for switching systems therefore becomes

$$T=(w/Q)\cdot 100 \qquad (37)$$

3.5 ACCURACY

The accuracy of a determination is evaluated as an error or a relative error as given in equations 13 and 14. From a fundamental basis, the true value, x_t, cannot be determined. However, standards of high confidence can be determined and compared to accepted values. The role of a quantitative technique, therefore is to calibrate values obtained experimentally to yield accurate values. Mathematically,

$$A_t=A\cdot C \qquad (38)$$

where

A_t=the true value
A=the experimental value
C=the calibration factor.

C, the calibration factor, can be determined by a number of methods and used in quantitation techniques such as internal standard and external standard. From a theoretical standpoint, there is no reason for the multidimensional chromatographic transfer process to affect the accuracy of a determination. The introduction of error would require that the transfer process caused some perturbation in the analysis of calibration standards which did not occur in the analysis of samples. Therefore, for both trapping and switching systems,

$$A_t=\overline{A} \tag{39}$$

$$E=0 \tag{40}$$

$$E'=0 \tag{41}$$

3.6 EXPERIMENTAL CONSIDERATIONS

It should be noted that the development of the equations describing the quantitation in multidimensional chromatographic systems describes only the component of quantitation due to the multidimensional transfer process. In any analytical system there is some lack of

reproducibility and accuracy and this will have an effect in addition to that described by the equations above. This is to say that the equations developed here describe only the effect of the transfer process on quantitation.

The only factor which needs to be considered in this respect is the reproducibility since, by definition, the transfer efficiency is determined only by the transfer process and therefore will have no component in the rest of the system and the accuracy as discussed above has no component in the transfer process.

Variances are additive. Thus for a complete on-line multidimensional chromatographic system,

$$\sigma_T^2=\sigma_{c1}^2+\sigma_{I/F}^2+\sigma_{c2}^2 \qquad (42)$$

where

σ_T^2=total variance
σ_{c1}^2=variance due to column 1
$\sigma_{I/F}^2$=variance due to interface
σ_{c2}^2=variance due to column 2

The relative standard deviation is given by

$$RSD=(\sigma/\bar{x})\cdot 100 \qquad (43)$$

Therefore

$$\sigma=RSD\cdot\bar{x}/100 \qquad (44)$$

Substituting equation 45 into equation 43 yields

$$(RSD\cdot\bar{x})^2_T=(RSD\cdot\bar{x})^2_{c1}+(RSD\cdot\bar{x})^2_{I/F}+(RSD\cdot\bar{x})^2_{c2} \quad (45)$$

In examining the quantitation of the transfer efficiency, the portion of the sample examined is the same in all components of the system. The fraction of the sample which undergoes the transfer step is the same portion which is analyzed by the second step and is a portion of the first step which can be conceptually isolated from the rest of the sample. Therefore,

$$\bar{x}_T=\bar{x}_{c1}=\bar{x}_{I/F}=\bar{x}_{c2} \quad (46)$$

and thus

$$RSD^2_T=RSD^2_{c1}+RSD^2_{I/F}+RSD^2_{c2} \quad (47)$$

This means that from experimental results, the relative standard deviation due to the transfer process can be calculated from

$$RSD^2_{I/F}=RSD^2_T-RSD^2_{c1}-RSD^2_{c2} \quad (48)$$

since all factors on the right side of equation 49 can be determined experimentally. The relative standard deviation due to the transfer process can thus be determined experimentally and compared to the theoretical values of the relative range.

Chapter IV

EXPERIMENTAL

4.1 EQUIPMENT AND APPARATUS

4.1.1 Liquid Chromatography

The liquid chromatography reported in this work was performed either on a Varian Model 5020 Liquid Chromatograph or a Varian Model 5060 Liquid Chromatography (Varian Associates, Walnut Creek, Ca.) Both systems were equipped with a 254nm ultraviolet absorption detector, column heater, three solvent input and a Valco CV-6-UHPa-N60 sample valve (Valco Instrument Company, Houston, TX). The primary difference between the two instruments is that the Model 5060 can generate ternary solvent gradients while the Model 5020 can generate only binary gradients. Both systems are microprocessor controlled.

In addition to the 254nm UV detector, the following additional detector systems were used: a Varian Varichrom variable wavelength UV detector; a Varian Fluorichrom filter fluorometric detector equipped with a deuterium lamp; a Varian Refractive Index Detector; and a Bioanalytical Systems Model LC-16 electrochemical detector (Bioanalytical Systems, West Lafeyette, IN.) equipped with a glassy carbon

working electrode and a Ag/AgCl reference electrode. The controller for the electrochemical detector was constructed by the VPI&SU Department of Chemistry Electronics Shop according to a design presented by Kissinger *et al.* (144).

Unattended automatic operation of the liquid chromatograph was accomplished using a Varian Model 8050 LC Autosampler equipped with pneumatically actuated sample valve (Valco Model AH-CV-6-UHPa-N60).

Data processing, including peak integration, determination of retention times and automatic quantitation routines was performed using a Varian CDS 111L data system. The analog detector response was recorded using either a Varian Model 9176 Dual Channel Recorder or a Linear Instruments Model 885 Dual Channel Recorder (Linear Instruments, Irvine, CA.).

4.1.2 Gas Chromatography

The gas chromatography reported in this work was performed on a Varian Model 3700 gas chromatograph. This instrument has two interchangeable injection systems allowing both packed and capillary column operation. In addition, the capillary inlet system has several inserts to accomodate split, splitless and direct injection techniques.

The Model 3700 gas chromatograph was equipped with both a flame ionization detector (FID) and an electron capture detector (ECD), with a Ni^{63} foil ionization source.

Data processing was accomplished using a Varian CDS 111C data system. This data system automatically determines retention times and peak areas and can generate simple quantitation methods such as internal and external standard.

Analog detector signals were recorded using a Varian Model 9176 dual pen recorder with a 1 mv span module.

4.1.3 Multidimensional Chromatography: LC/LC

All LC/LC was performed using the liquid chromatographic equipment described above. In addition, two Valco air actuated six port high pressure valves, Model AH-CV-6-UHPa-N60, were used in column switching. The valves were actuated by two Humphrey solenoids (Varian Associates, Walnut Creek, CA.) which delivered 60 psi gas pressure. The solenoids in turn were operated by two external events from the 5000LC microprocessor. Events 3 and 4 are powered events which operate at 110 volts. Through the use of these events, the valve activation can be controlled independently by a program executed by the microprocessor. Although the powered events require the installation of two optical relays on the main power board of the chromatograph, this is

a standard procedure. Both solenoids and valves were mounted on the interior wall of the side cabinet of the 5000LC, allowing the connection of columns with a minimum of connecting tubing. A schematic of the system is shown in Figure 13 .

The six port valves operate in two positions which will be termed "on" and "off" In the "on" position, one set of adjacent ports are connected, and in the "off" position, the other set of adjacent ports are connected. This is illustrated in Figure 14 . Thus, through the combination of two valves, there are four possible flow paths corresponding to the four combinations; on/on, on/off, off/on, and off/off. With the capabilities of the two valves, there is a wide variety of possible valving schemes. No universal valving scheme was used which satisfied all requirements of all applications. However, three main configurations that were used.

Valving Scheme A (shown in Figure 15) is the simplest and most general used in this work. In position a, both columns are eluted in series. Switching either valve from the position shown in position a isolates the column connected to that valve. Thus in position b, column two is isolated, and in position c, column one is isolated. If both valves are switched from position a, then both columns are bypassed.

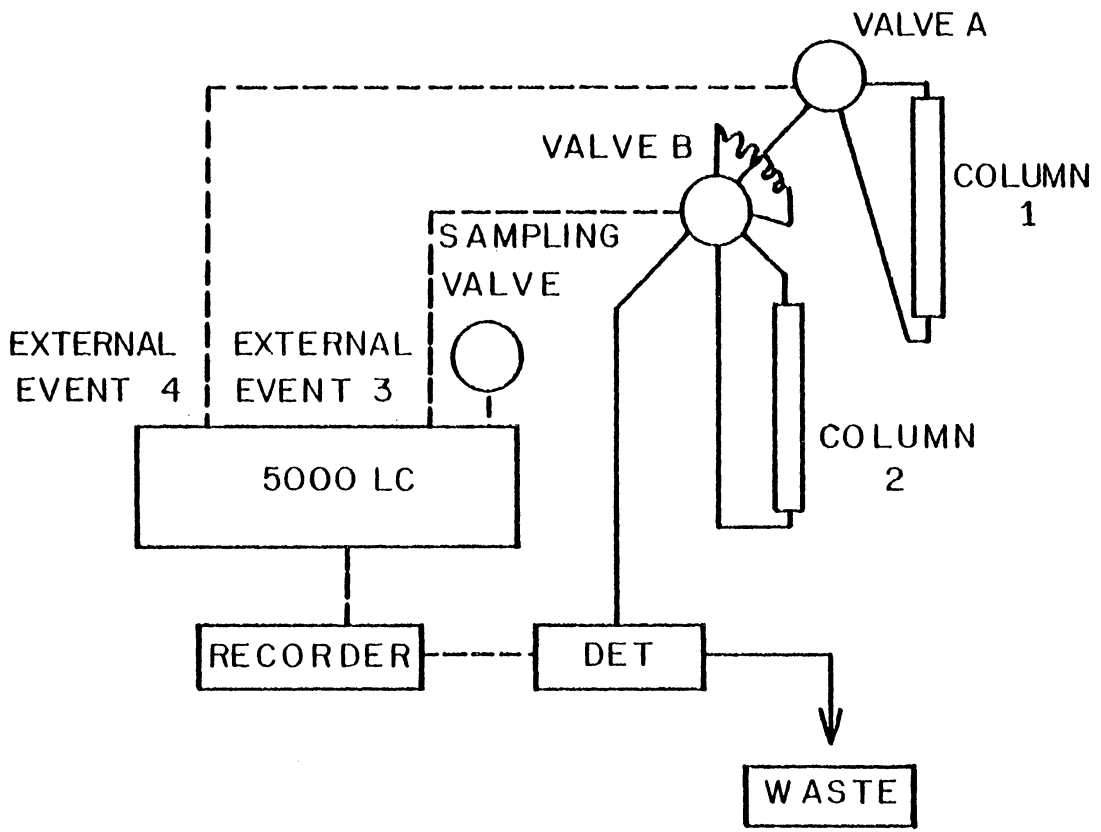


Figure 13. Schematic of LC/LC System

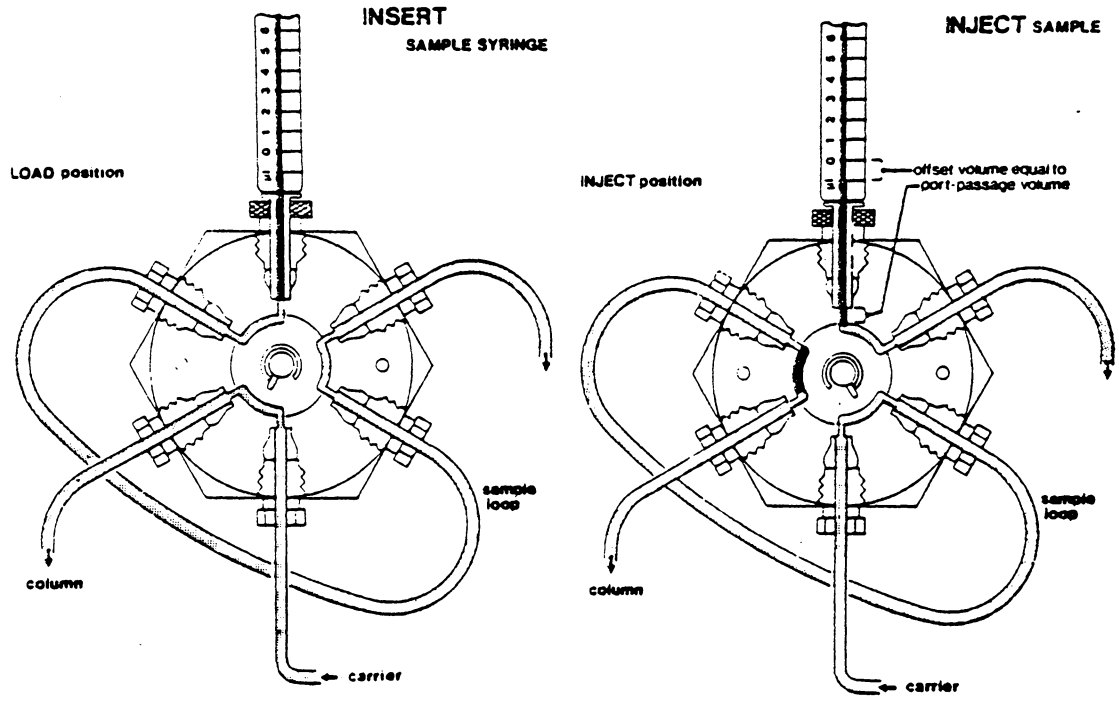


Figure 14. The Six Port Valve

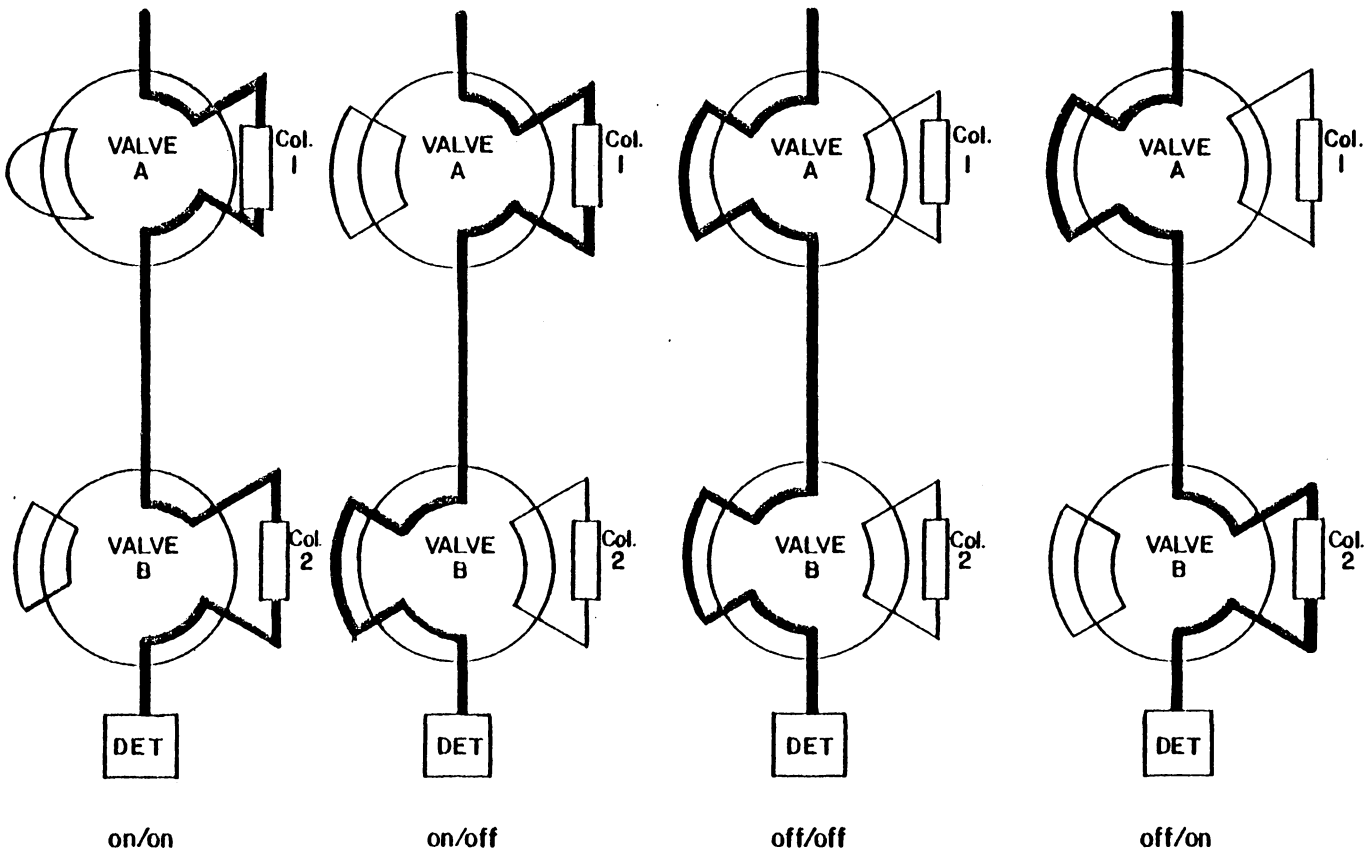


Figure 15. LC/LC Valving Scheme A

Valving Scheme B, shown in Figure 16, is similar to Scheme A with the addition of the ability to be used in a trapping mode. In this mode of operation, the trapping loop in valve b is loaded from the effluent of column one. The volume of effluent in this loop is introduced into column two when the valve is switched.

Valving Scheme C, shown in Figure 17, is used in cases where it is neccesary to backflush a column to elute the strongly retained components. This scheme can be run in the following ways; column one and column two run in series (figure 17 a) column two isolated (figure 17 b) and column two isolated and column one backflushed (figure 17 c).

Scheme B

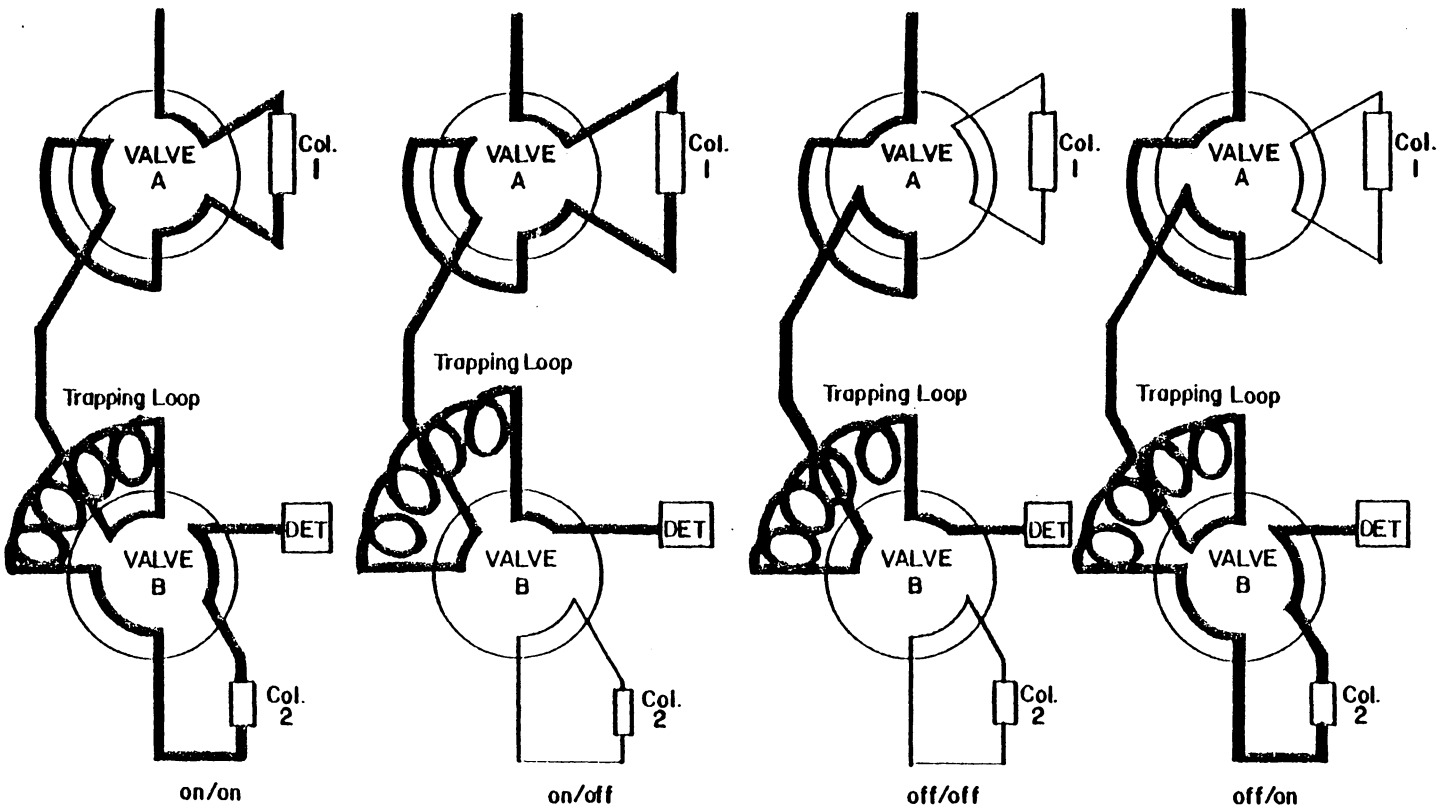


Figure 16, LC/LC Valving Scheme B

SCHEME C

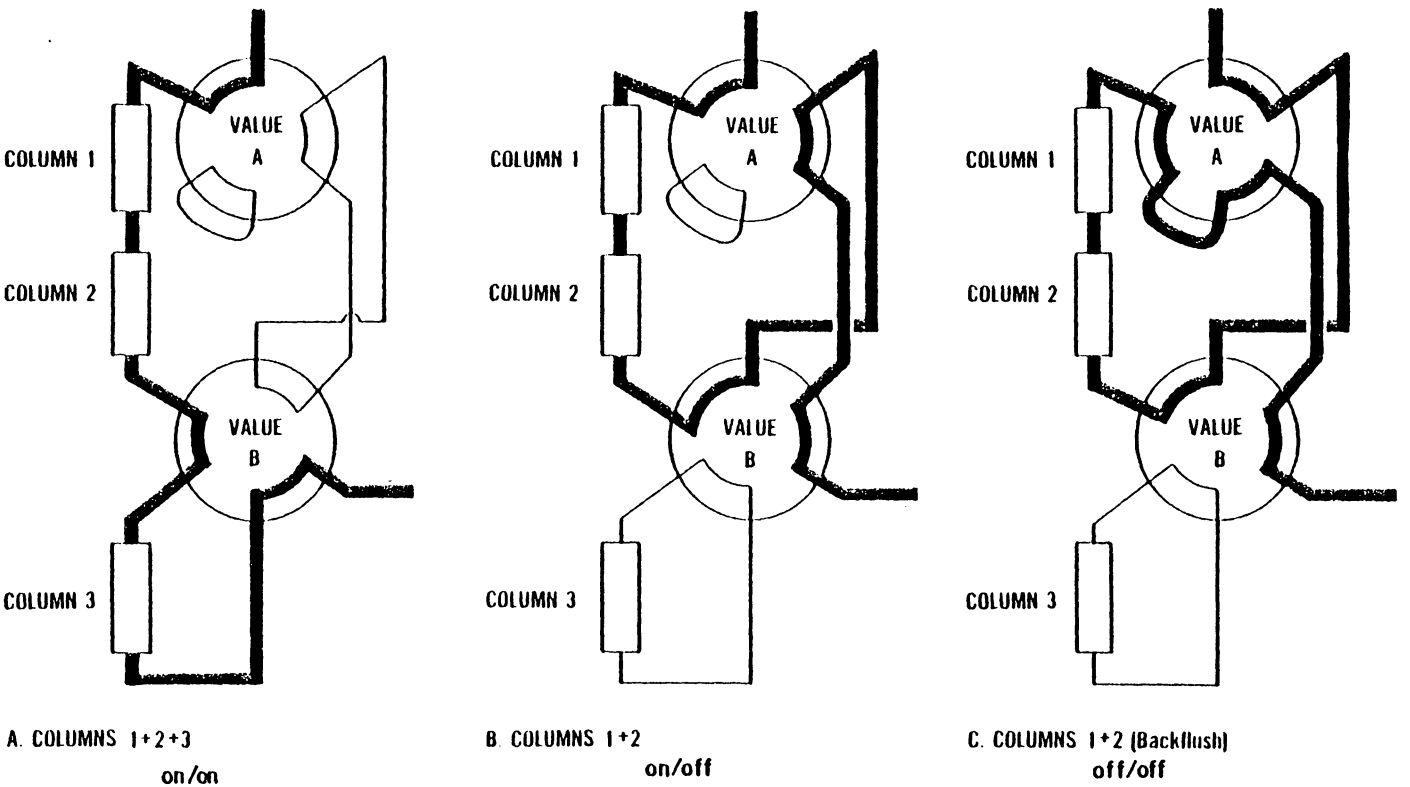


Figure 17. LC/LC Valving Scheme C

4.1.4 Multidimensional Chromatography: LC/GC

The LC/GC performed in this work utilized both the liquid chromatographic and the gas chromatographic apparatus described above. In addition, a Varian Model 8070 LC/GC Interface was used. The LC/GC Interface is the modified injection module of the Varian GC autosampler. The system utilizes a flow-through side arm syringe through which the effluent from the LC continiously passes. Figure 18 is a photograph of the interface.

Figure 19 shows the principle of operation for the interface. When the interface is triggered , a rapid sequence of actions takes place. From the standby position shown in Figure 19 a, the syringe assembly lifts, drawing the needle out of the waste receptacle. The syringe plunger is lowered, sizing the trapped sample to be injected. After the syringe has lifted, the waste receptacle is swung out of the way. The syringe is rapidly lowered, causing the needle to pierce the GC injection port septum. The plunger is lowered, making the injection. This inject position is shown in Figure 19 b. Following the injection, the syringe is returned to its standby position. The entire process is driven pneumatically and takes less than five seconds to complete the cycle. The waste receptacle is constantly purged with gas flow.

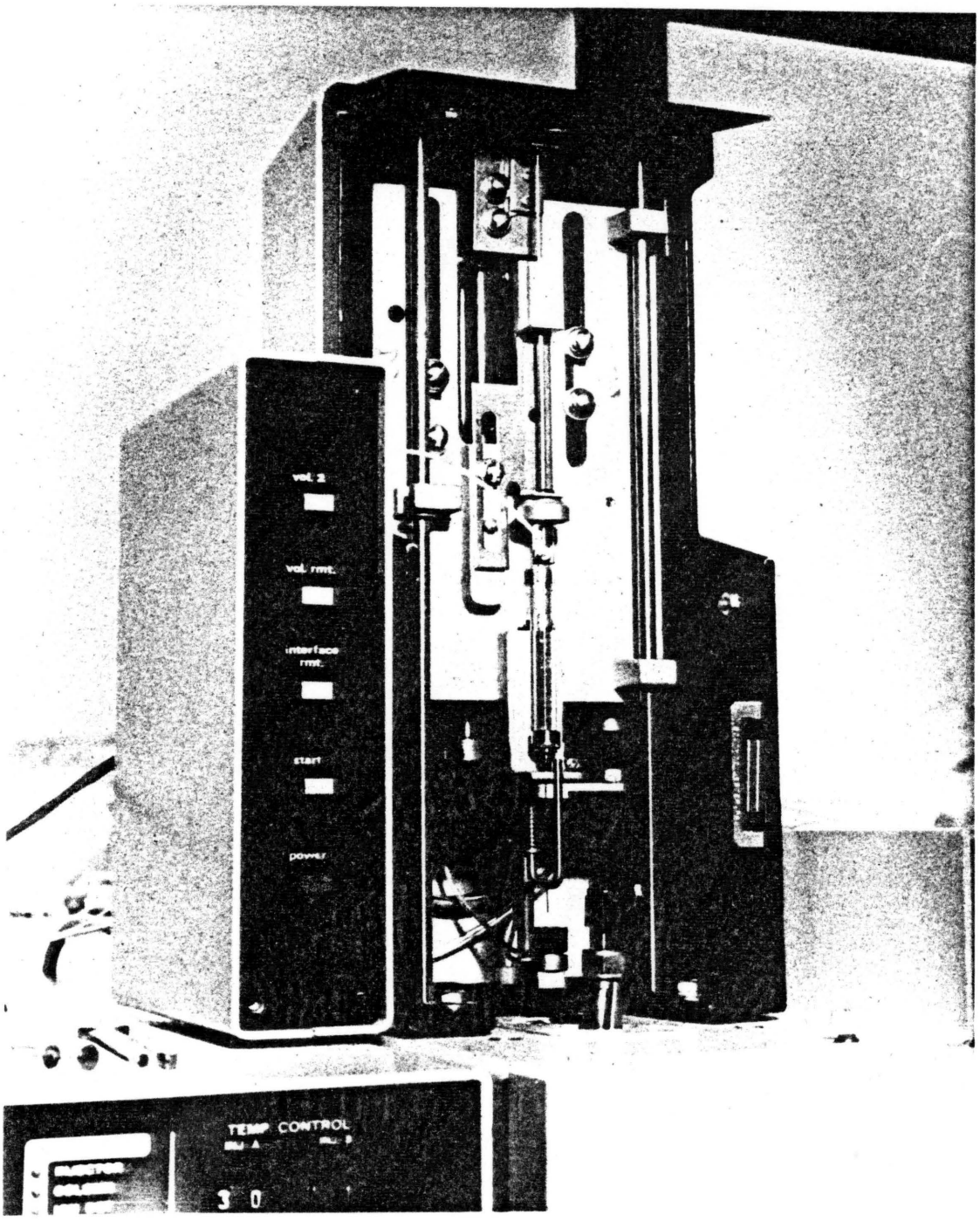


Figure 18. Photograph of the LC/GC Interface

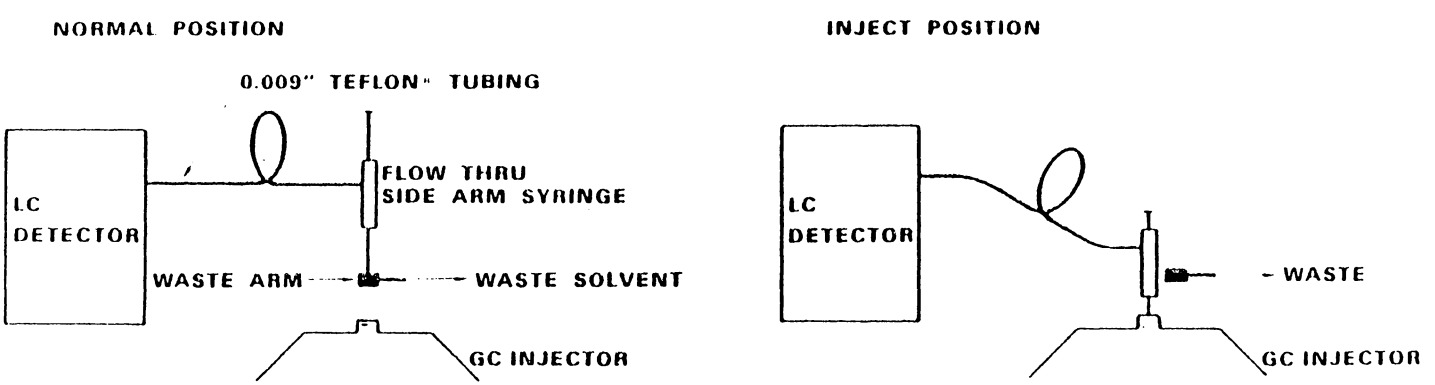


Figure 19, Principle of Operation of the LC/GC Interface

The LC/GC interface can be triggered in several ways. The activation can be accomplished manually by depressing the "inject" button on the front panel of the interface. The activation can also be controlled remotely by a contact closure. In the system used, the contact closure was generated either by the external events "one" and "two" of the Varian 5000LC microprocessor, or by the external events of the Varian CDS 111L data system.

A schematic of the LC/GC system is shown in Figure 20 In this arrangement, the activation of the interface is accomplished by the Varian 5000LC. The effluent flows from the LC detector to the interface via a length of 0.009' I.D. teflon tubing. The LC/GC interface operates in this system with a number of control circuit "ready" signals. For an injection to take place, the interface must recieve "ready" flags from the GC, the GC data system, the LC and the LC autosampler.

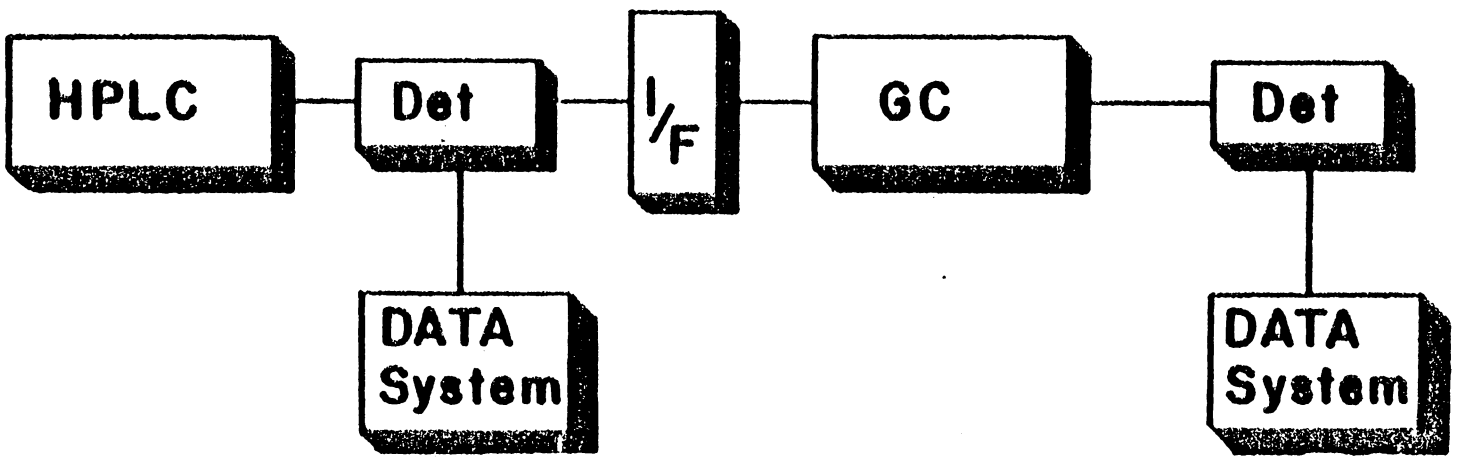


Figure 20. Schematic of the LC/GC System

4.2 *REAGENTS AND MATERIALS*

All chromatography was performed using HPLC grade solvents. Acetonitrile, methanol, tetrahydrofuran, hexane and chloroform were obtained either from MCB Manufacturing Chemists Inc. (Cincinatti,OH.) as "Omnisolve" glass distilled quality or from Burdick and Jackson Laboratories, Inc. (Muskegon, MI.) as "distilled-in-glass" quality. Diethyl ether was obtained from Fisher Scientific Company (Fairlawn, NJ.). HPLC grade water was prepared from the conventional carbon adsorption/ion exchange treated distilled water from a Barnstead Nanopure system (Barnstead Company, Boston, Ma.). This water was further purified by passing it through a preparative reverse phase column (E. Merck Lobar size B, 31cm x 0.25 mm I.D. semi-preparative column, E.M. Laboratories Inc. Gibbstown, N.J.). This procedure has been described elsewhere by Marsh *et al.* (145).

HPLC columns used include a variety of Varian Micropak Columns, 30cm x 4.6mm i.d. in dimensions. These include reverse phase columns consisting of 10 µm monomeric octadecylsilane (C-18) bonded silica (Micropak MCH-10) and 5 µm C-18 (Micropak MCH-5) columns. Normal phase columns included 5 µm silica (Micropak Si-5), 10 µm cyanopropyl bonded silica (Micropak CN-10), and 10 µm aminopropyl bonded silica (Micropak NH_2-10). Organic size exclusion columns

consisted of a polystyrene divinylbenzene copolymer based column (Micropak TSK 1000H). The aqueous size exclusion column used was a Micropak TSK 2000SW, a silica based material with a bonded diol functionality.

Two additional columns were used; a 15cm x 4.6 mm i.d. Supelcosil RP-8 obtained from Supelco Inc. (Bellafonte, PA.); and a 25cm x 4.6 mm i.d. RP-8 obtained from Hewlett Packard (Avondale, PA.). Both of these columns were 5 µm octylsilane bonded silica columns.

All packed gas chromatographic columns were prepared in the laboratory using 2 m x 2.2mm i.d. glass column obtained from the Glass Shop of the Department of Chemistry, VPI&SU. The columns were packed using a combination of vibration and suction from a faucet aspirator. Columns were packed with 1% SE-30 on Chromosorb W-HP, 80/100 mesh obtained from Supelco Inc..

All capillary gas chromatographic columns were obtained from Supelco Inc., and included; a 30m x 0.25 mm i.d. WCOT SP-2100; a 30m x 0.5mm i.d. WCOT SE-52 and a 30m x 0.5mm i.d. WCOT SE-54. All capillaries were borosilicate glass.

4.2.1 Reagents

Silver nitrate was obtained from Alfa Division, Ventron Corporation (Danvers, Ma.). Heptane sulfonic acid, sodium salt (HSA), camphor sulfonic acid, sodium salt (CSA) tetramethyl ammonium chloride (TMAC) and dimethoxypropane were obtained from Aldrich Chemical Company (Millwaukee, Wi.). Monobasic potassium phosphate monobasic was obtained from J.T. Baker Company (Phillipsburg, N.J.). Disodium ethylenediaminetetraacetic acid was obtained from Eastman Kodak Company (Rochester, N.Y.).

4.2.2 Analyte Standards

Pesticide standards listed in Table 4 and hydrocarbon standards listed in Table 5 were obtained from Polyscience Corporation (Niles, Il.). Polynuclear Aromatic Hydrocarbon (PAH) standards listed in Table 6 were obtained from RFR Corporation (Hope, R.I.). Catecholamine standards listed in Table 7 were obtained from Aldrich Chemical Company.

4.2.3 Samples

Butter, gasoline and diesel fuel were obtained locally. Samples of Cyclogen "L" oil were supplied courtesy of John Partanen of Arizona Refining Company (Phoenix, Arizona). Solvent Refined Coal (SRC) samples were obtained as an Amax

TABLE 4

Pesticide Standards

a BHC
lindane
b BHC
heptachlor
aldrin
heptachlor epoxide
pp'DDE
dieldrin
op'DDE
endrin
pp'DDT
pp'DDD
op'DDT

TABLE 5

Hydrocarbon Standards

Paraffins	Olefins
n-heptane	hexene-1
n-octane	hexene-2 (cis, trans)
n-nonane	2-methyl pentene-1
n-decane	4-methyl pentene-1
n-undecane	4-methyl pentene-2 (cis, trans)
n-dodecane	2-methyl butene-1
n-tridecane	heptene-1
n-tetradecane	heptene-2 (cis, trans)
n-pentadecane	heptene-3
n-hexadecane	octene-1
n-heptadecane	octene-2
n-octadecane	2-ethyl hexene-1
n-nonadecane	nonene-1
	decene-1

Naphthenes	Aromatics
cyclopentane	benzene
methylcyclopentane	toluene
cyclohexane	o-xylene
methylcyclohexane	m-xylene
1,2-dimethylcyclohexane	p-xylene
cis 1,3-dimethylcyclohexane	ethyl benzene
1,1-dimethylcyclohexane	cumene
cyclooctane	mesitylene
cyclododecane	p-cymene
	n-propyl benzene
	n-butyl benzene
	n-hexane benzene
	n-octyl benzene
	n-decyl benzene

TABLE 6

PAH Standards

naphthalene
acenaphthalene
acenaphthylene
fluorene
phenathrene
anthracene
2-methyl anthracene
fluoranthene
pyrene
benz(a)anthracene
chrysene
benzo(a)pyrene

TABLE 7

Catecholamine Standards

3,4 dihydroxymandelic acid (DOMA)
vanilmandelic acid (VMA)
3,4 dihydroxyphenylalanine (DOPA)
norepinephrine (NE)
epiniphrine (E)
3,4 dihydroxyphenylacetic acid (DOPAC)
dopamine (DA)
5-hydroxyindoleacetic acid (5-HIAA)
5-hydroxyindolecarboxyllic acid (5-HICA)
homovanillic acid (HVA)

production run from Southern Services Pilot Plant (Wilsonville, Al.). Certified standards of solvent refined coal (SRC-II), shale oil (SRM 1580) and crude oil (Wilmington Crude) were supplied courtesy of Dr. Willie May from the National Bureau of Standards in Gaithersburg, Maryland. Urine samples of patients with disseminated malignant melanoma were supplied courtesy of Dr. Phil Banda of the Department of Dermatology, University of California, San Francisco. Normal urine, blood and serum samples were obtained locally.

4.3 METHODS

4.3.1 LC/LC Applications

4.3.1.1 Caffeine and Theophylline in Biological Fluids

The analysis of caffeine and theophylline in biological fluids was carried out using both trapping and switching systems. In both instances, the first column was a Micropak TSK 2000SW exclusion column (50cm x 7.5mm I.D.) and the second column was a Micropak MCH-10 reverse phase column (30cm x 4,6mm I.D.). The effluent from both columns was monitored at 272nm. Exclusion chomatography was performed using water as a mobile phase at 1 ml/min. Caffeine and theophylline were co-eluted from the aqueous exclusion column at 18.8 ml under these conditions. In both the

trapping and switching approaches valving Scheme B was used (see Figure 16).

In the trapping approach used for the analysis of caffeine, 50 µl of raw urine was injected onto the exclusion column. At the elution volume of the caffeine peak from the exclusion column, Valve A was switched to trap a portion of the analyte peak in a 50 µl loop. Solvent flow was switched to the reverse phase column by appropriate valve actuation and, after an equilibration period for the second column of 4 minutes at 4 ml/min, the trapped solute was injected onto the reverse phase column. Reverse phase analysis of caffeine was performed isocratically using a mobile phase consisting of 20 mM tetramethylammonium chloride, 10 mM minobasic potassium phosphate in 20% acetonitrile in water at a flow rate of 1 ml/min. The elution volume of caffeine is slightly under 4 ml using these conditions.

In the switching method for blood, plasma and urine analysis, a 2.8 ml volume corresponding to the elution volume of caffeine and theophylline from the exclusion column, was switched onto the head of the reverse phase column. The caffeine and theophylline were then separated via gradient elution.

4.3.1.2 Hydrocarbon Group Analysis

The analysis of hydrocarbon groups was performed as follows. Three columns were used in series in the following order; a 10 μm cyanopropyl bonded silica; a 5 μm silica column; and a silver nitrate impregnated 5 μm silica column. Preparation of the silver nitrate impregnated column is described below. Valving Scheme C (see Figure 17) was used. After injection of a hydrocarbon sample, the saturates were allowed to elute from the first two columns onto the silver nitrate impregnated silica column, which was then isolated from the mobile phase flow path. The aromatics were then eluted from the first two columns which were then backflushed to remove the polar compounds. Detection was by UV at 200 nm and refractive index detectors.

Hexane was used as the mobile phase throughout. The hexane mobile phase was dried in the following way; activated 10-16 mesh Molecular Sieve 5A was added to the hexane and after at least one hour, the hexane was filtered though a Millipore 1.5 μm filter. The silica column was dried in one of the following ways to ensure a high activation. Either the column was heated at 200°C overnight in a gas chromatograph oven with a slow flow (2 ml/min) of nitrogen gas continiously passing through it, or the silica was activated in-situ as described by Bredeweg *et al.* (146).

In this procedure, 100 ml of a 96:2:2 v/v solution of methylene chloride: acetic acid: and dimethoxypropane is passed through the column followed by 100 ml methylene chloride and 100 ml hexane.

Silica was impregnated in-situ with silver nitrate as follows. The silica columns were activated overnight at 200°C with a slow flow of nitrogen gas. A 500 ml solution of silver nitrate was then passed through the column at 0.5 ml/min at room temperature. The following silver nitrate solutions were evaluated separately: a)0.5% silver nitrate in methanol; b) 10% silver nitrate in acetonitrile and c) 1.5% silver nitrate in water. Following the impregnation solution, 50 ml of the solvent was pumped through the column followed by 100 ml isopropanol and finally 500 ml hexane. In the case of the aqueous impregnation solution, it was neccesary to follow the isopropanol with 100 ml diethyl ether, 100 ml tetrahydrofuran and finally the 500 ml hexane. These additional solvents were neccesary in order to remove any residual water adsorbed on the impregnated surface.

4.3.1.3 Catecholamines in Urine

The analysis of catecholamines in urine was performed using three approaches: both trapping and switching techniques coupling aqueous size exclusion chromatography

(SEC) and ion pair chromatography (IPC), and a switching technique coupling two IPC columns. In all three cases, electrochemical detection was used with the glassy carbon working electrode potential at 720 mv vs Ag/AgCl.

The trapping system coupled a Micropak TSK 2000SW SEC column and a Micropak MCH-10 reverse phase column. The mobile phase for both columns consisted of 20 mM camphor sulfonic acid and 0.1 mM disodium EDTA in a aqueous solution buffered to pH 3.5 with 20 mM monobasic potassium phosphate pumped at 1 ml/min. Valving Scheme A was used (see Figure 15) with an initial sample volume of 10 µl of undiluted urine and a trapping loop volume of 500 µl. Samples were initially injected into the system with the valves in the on/off position; with the IPC column isolated. When the analyte DOPA had eluted from the SEC column and was in the trapping loop, the valving was switched to the off/on position, injecting the trapped volume into the IPC column. Following the elution of DOPA, the valving was returned to the on/off position and the remainder of the sample eluted.

In coupling SEC and IPC, a Micropak TSK 2000SW column was used as above. The second column was a HP RP-8 reverse phase column. The mobile phase consisted of 5 mM heptane sulfonic acid and 0.5 mM disodium EDTA in a solution of 9 % methanol in water, bufferred to pH 3.5 with monobasic potassium phosphate.

The coupling of two IPC separations used a 15 cm Supelcosil RP-8 column for the initial fractionation. The second column was the HP RP-8. The mobile phase was the same as in the coupling of SEC and IPC.

Both the switching systems utilized Valving Scheme A (see Figure 15) in an identical manner. When the components of interest began to elute from the primary column, the valving was switched from an inital on/off position to an on/on position allowing the analytes to flow directly from the primary column to the secondary column. Once all the analyte peaks had been thus transferred, the valving was switched to an off/on position and the secondary column was eluted. Finally, the valving was returned to the on/off position and the remainder of the sample allowed to elute.

4.3.2 LC/GC Applications

4.3.2.1 Pesticides in Butter

The analysis of pesticides in butter was performed as follows. A 40 g sample of butter was dissolved in 100 ml tetrahydrofuran and then filtered through a 5 μm Millipore filter. This sample was injected into a Micropak TSK 1000H organic size exclusion column using THF as a mobile phase at flow rates between 0.5 and 2.0 ml/min. The column was thermostatted at 30°C. The retention volume of a pesticide

standard, pp'DDT, was determined. LC detection was by UV at 214 nm. Using the retention time plus a correction for the delay between the LC detector and the LC/GC interface as a triggering time, the pesticides were injected into the GC as a 10 µl aliquot and analyzed by packed column GC using electron capture detection. The GC analysis was performed on a 2m x 2mm I.D. glass column packed with 1% SE-30 on 80/100 mesh Chromosorb W-HP. The analysis was operated isothermally at 200°C. The GC carrier gas was nitrogen, flowing at 30 ml/min with an additional 10 ml/min nitrogen make-up gas to the detector. The injector was operated at 250°C and the detector at 280°C.

4.3.2.2 Hydrocarbon Group Analysis

In the analysis of hydrocarbon groups by LC/GC, a 10 µl undiluted hydrocarbon sample was initially fractionated using a 5 µm silica column with a 30 µm cyanopropyl bonded silica guard column (5 cm x 4,6 mm I.D.). The mobile phase was dry hexane at 0.5 to 1.0 ml/min flow rate. The column was thermostatted at 30°C. LC detection was by refractive index detection. Points corresponding to the maxima of the individual hydrocarbon groups (saturates, unsaturates, and aromatics) were sampled by the LC/GC interface and analyzed by capillary GC with flame ionization detection. The

capillary chromatography was performed on a 30m x 0.25mm I.D. WCOT SP-2100 glass capillary column. The carrier gas was helium at 2 ml/min. 2 µl samples were injected into the capillary column using the splitless injection technique described by Grob and Grob (147). In this technique, the sampled is injected at a low column temperature (40°C) and after 30 seconds, the splitter flow is turned on. The hydrocarbon groups were analyzed using the following temperature program: isothermally at 40°C for 15 minutes followed by a temperature program of 2°C/min to 220°C. Helium make-up gas to the detector was added such that the total flow to the detector was 45 ml/min. Detector hydrogen flow was 30 ml/min, and detector air flow was 300 ml/min. Injector temperature was 270°C and the detector temperature was 300°C.

4.3.2.3 PAH's in Fuels

The initial fractionation of a 500 µl sample of a 10% solution of sample in hexane was performed on a 10 µm aminopropyl bonded silica column with a 30 µm cyanopropyl bonded silica guard column. The analytical column was thermostatted at 30°C. The mobile phase consisted of 5% chloroform in hexane pumped at 1 ml/min. Detection was by UV at 280 nm. Fractions containing specific PAH's were

sampled by the LC/GC interface and analyzed by capillary gas chromatography. Capillary GC was performed on either a 30m x 0.5mm I.D. WCOT SE-52 glass column or a WCOT SE-54 column of the same dimensions. Direct injection of 10 µl samples were made with no splitter flow and a Varian direct injection insert. Both isothermal and temperature programmed analyses were used. The temperature program used consisted of an isothermal hold at 40°C for 2 minutes followed by a linear temperature gradient at 8°C/min to 275°C. Isothermal analyses were performed with temperatures optimized for the specific analyte as follows; naphthalene,acenaphthalene, acenaphthylene and fluorene at 120°C; phenanthrene and anthracene at 150°C; 2 methyl anthracene, fluoranthene and pyrene at 180°C; and benzo(a)anthracene, chrysene and benzo(a)pyrene at 260°C. Injections for isothermal analysis were made at the analysis temperature. Nitrogen was used as a carrier gas at 2 ml/min. Detection was by FID. Nitrogen make-up gas was added to the detector input so that the total flow into the detector was 45 ml/min. Detector hydrogen flow was 30 ml/min. Detector air flow was 300 ml/min. The injector temperature was 250°C and the detector temperature was 300°C. For isothermal analyses, the injector temperature was 290°C.

4.3.3 Preparation of Standards

All standards were prepared using a Mettler H5 analytical balance (Mettler Instrument Corporation, Hightstown, N.J.). Stock solutions of appropriate concentrations were prepared in 100 ml volumetric flasks and then diluted using a Finnpipette Model FP12TE 50-200 µl automatic micropipette (Lab Systems OY, Helsinki, Finland) into 10 or 50 ml volumetric flasks.

Caffeine and theophylline were prepared in stock solutions of 1 mg/ml each in the LC mobile phase; 20 mM tetramethylammonium chloride, 10 mM monobasic potassium phosphate in 24% acetonitrile/ water.

Pesticide standards were prepared as stock solutions of 10 mg/ml in tetrahydrofuran and diluted to prepare calibration studies.

Hydrocarbon standards were prepared in 1 mg/ml stock solutions of each component in hexane. Group standards were prepared using the hydrocarbons listed in Table 4 .

Catecholamine standards were prepared as solutions of 10 µg/ml in 50 mM monobasic potassium phosphate, pH 3.2.

PAH standards listed in Table 5 were prepared as a stock mixture at concentrations of 1 mg/ml each.

4.3.4 Preparation of Samples

Urine samples were filtered through a 1.5 m Millipore filter and stored in a refrigerator at 10°C in amber flasks. Blood and serum samples were kept refigerated at 10°C after the addition of oxalate as an anticoagulant.

Butter samples were prepared by dissolving 40g of butter in 100 ml of tetrahydrofuran and filtering with a 1.5 m Millipore filter. Butter solutions were stored in a refrigerator at 10°C.

Shale oil, solvent refined coals and crude oil samples were prepared by stirring overnight at room temperature in 10% w/w hexane. The samples were then filtered through a 1.5 μm Millipore filter and stored in amber flasks at 10°C.

Gasoline and diesel fuel samples were used as obtained.

In cases in which analytes were spiked into sample matrices, a Finnpipette was used to add appropriate amounts into 100 ml volumetric flasks to samples prepared as above.

4.3.5 Computer Calculations

All computer calculations were performed on the IBM 370 computer system operated by the Computer Center at VPI&SU. All programs were written in VSBASIC, an IBM basic language.

Chapter V

RESULTS AND DISCUSSION

5.1 INTRODUCTION

The following presentation of results and the discussion of these results is divided into two sections: applications and quantitation. Each of these sections si further divided into a section covering LC/LC and a section covering LC/GC. Three applications were performed using each of the multidimensional systems. The LC/LC applications included: the analysis of theophylline and caffeine in biological fluids; the analysis of hydrocarbon groups; and the analysis of catecholamines in urine. The LC/GC system was applied to the analysis of pesticides in butter, the analysis of hydrocarbon groups, and the analysis of polycyclic aromatic hydrocarbons in petroleum related samples. The effects of operating variables on the quantitation in both trapping systems and switching systems has been evaluated using the equations developed in Chapter III. The effects of several of the key operating variables have been experimentally investigated for both systems and these results have been compared to those predicted by theory. The trapping system was experimentally evaluated

using the analysis of polycyclic aromatic hydrocarbons as a model. The switching system has been experimentally evaluated using the analysis of catecholamines as a model.

5.2 APPLICATIONS

5.2.1 LC/LC

5.2.1.1 Theophylline and Caffeine in Biological Fluids

Theophylline (1,3 dimethylxanthine) is used in the treatment of bronchial asthma and has been applied as a CNS stimulant and a diuretic. HPLC has been applied to the analysis of theophylline and a common interferent, caffeine (1,3,7 trimethylxanthine) in biological samples such as blood, serum and urine. Analyses of this nature have received a great deal of interest recently. Most of the HPLC analyses have required extensive sample preparation. The techniques have been of a varied nature, and have included extractions (148), off-line column chromatography fractionations (149) and preconcentrations (150). In the case of clinical analysis, sample throughput and low labor intensity are critical. Manual sample preparation clearly limits sample throughput and requires trained technicians. The following application is designed to demonstrate the use of on-line multidimensional LC/LC techniques to the automation of sample clean-up procedures.

In this analysis, both trapping and switching approaches were used. In both cases, the initial fractionation used aqueous size exclusion chromatography (SEC) with water as the mobile phase. The analytical

separation was performed by reverse phase chromatography (RPC) using a buffer and tetramethylammonium chloride (TMAC) in mixtures of water and acetonitrile. In both approaches, Valving Scheme B was used (see Figure 16).

On the SEC column, theophylline and caffeine co-elute. Thus, by sampling this peak, either by trapping a small fixed volume or by switching the entire peak, only the fraction of the total sample which includes the analytes can be separated on the RPC column.

Using the trapping system, the analytical sequence was as follows. Referring to the schematic of Valving Scheme B (figure 16), the valves were initially in an on/off configuration. An untreated sample was injected onto the SEC column, column one. After 18.8 ml have eluted from the SEC column, the valves were switched to an off/on position. This time was determined from the elution volume of caffiene and theophylline standards. Thus after 18.8 ml, the maximum of the elution peak for these two analytes was in the trapping loop. By switching the valves to the off/on position, the effluent trapped in the loop was flushed onto the RPC column, column two. The system was immediately switched to the off/off position, isolating both columns so that the entire pumping and connection system could be equilibrated with a different mobile phase for the second

separation. After an equilibration period, the valves were returned to the off/on position, and the analytes were eluted from the RPC column, isocratically. A rapid gradient to 100% methanol was then run to clean off any undesirable components which might have been transferred to the second column with the analytes.

Figure 21 shows the analysis of a caffeine standard, and Figure 22 shows the analysis of caffeine in urine. Using this method, the minimum detectable concentration (MDC) for theophylline and caffeine of 5 x 10^{-3} mg/ml was obtained with linearity up to 10 mg/ml. In this and the following applications, the MDC and linearity have been determined from calibration curves. A calibration plot for the analysis of theophylline and caffeine using the above method is shown in Figure 23. This figure represents typical data obtained using the multidimensional chromatographic system. The MDC in these applications is taken as the concentration of analyte which produces a detector signal twice that of the detector noise. The linearity is primarily dependent on the detector characteristics, while the detection limit depends on a combination of the detector sensitivity and the transfer efficiency of the multidimensional transfer process. The trapping loop volume in this application was 50 µl. While

this volume did not contribute significantly to the overall band broadening of the second separation, it was found that increasing the loop volume to 100 µl or greater contributed noticeably to the overall band broadening. The MDC in this case is not sufficiently low for the analysis of theraputic levels of theophylline. It is for this reason that the switching approach was applied.

The analytical sequence employed for the switching method was as follows. Again, referring to the schematic of Valving Scheme B (Figure 15), the valves were initially in the on/off position. As the leading edge of the peak corresponding to theophylline and caffeine began to elute from the SEC column, the valves were switched to the on/on position and the analyte peak was allowed to flow onto the RPC column. Once the entire volume containing the analytes had entered the RPC column, the valves were switched to the off/on postion. Theophylline and caffeine were then separated on the RPC column using gradient elution with methanol and water. Following the elution of the analytes, the valves were returned to the on/off position and the remainder of the sample was flushed off the SEC column.

Figure 24 shows the analysis of a blood serum sample and Figure 25 show the analysis of a urine sample. The samples shown in these figures were spiked with 50 ng of

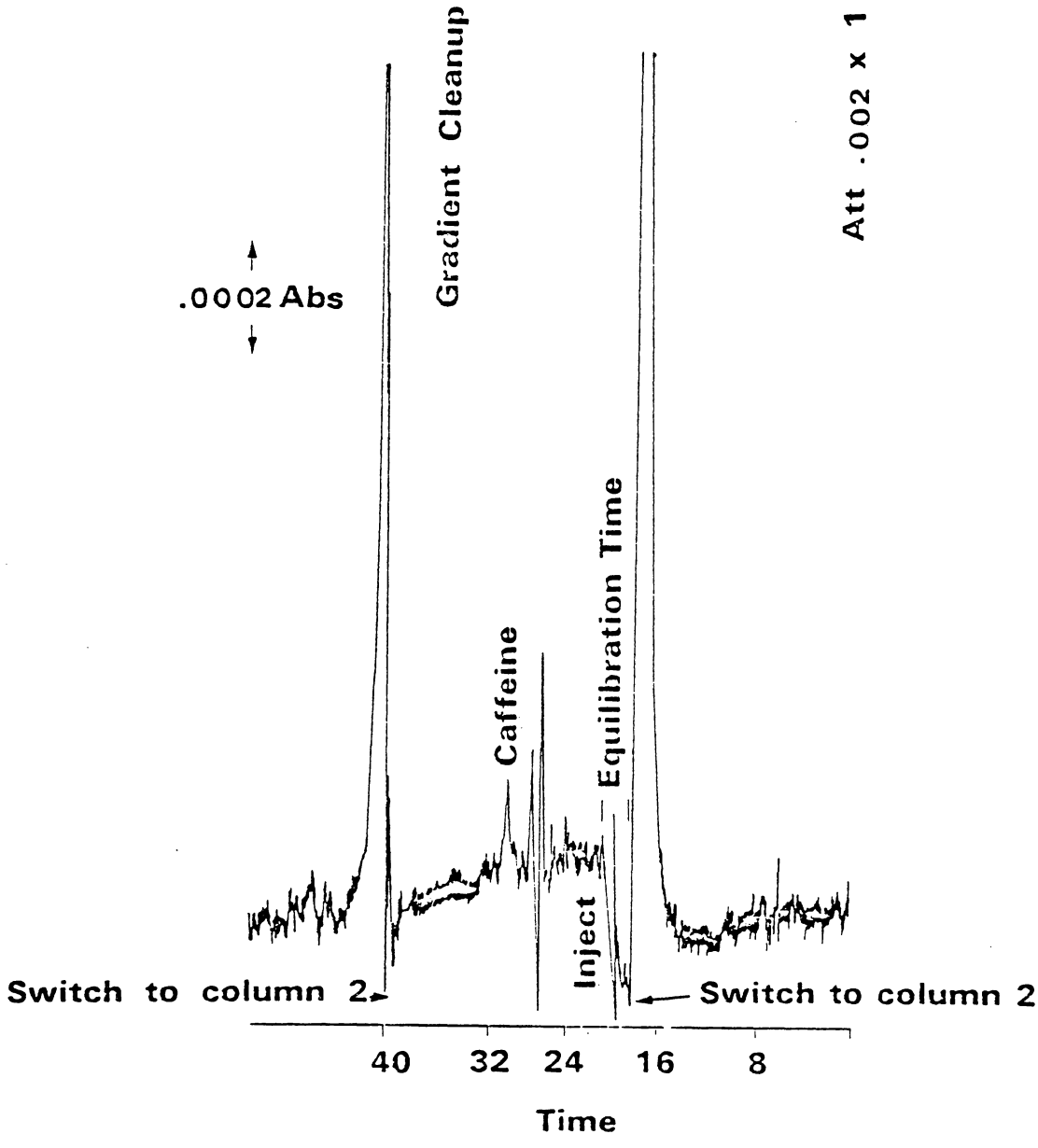


Figure 21. LC/LC Analysis of Caffeine Standard: Trapping System

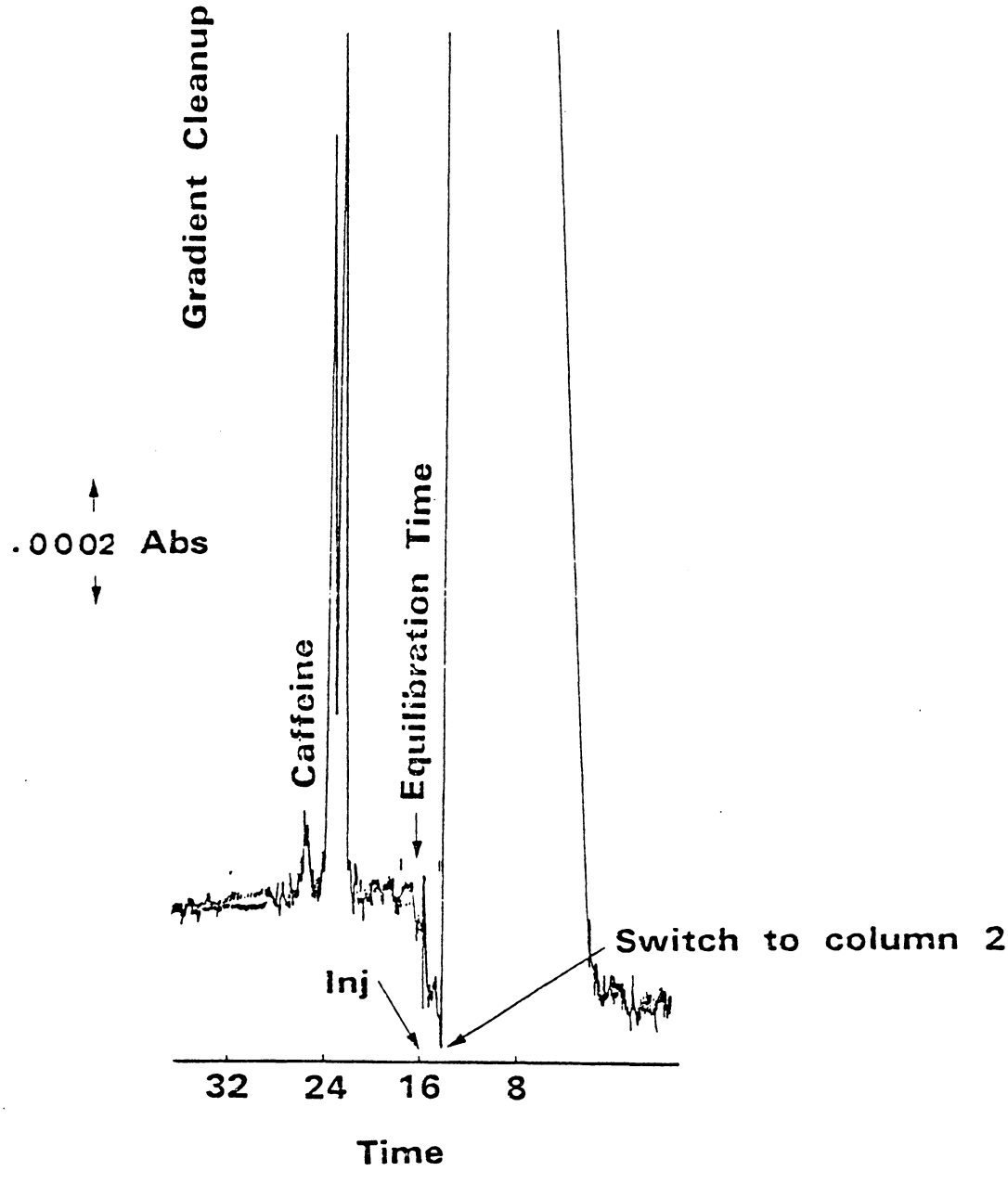


Figure 22. LC/LC Analysis of Caffeine in Urine: Trapping System

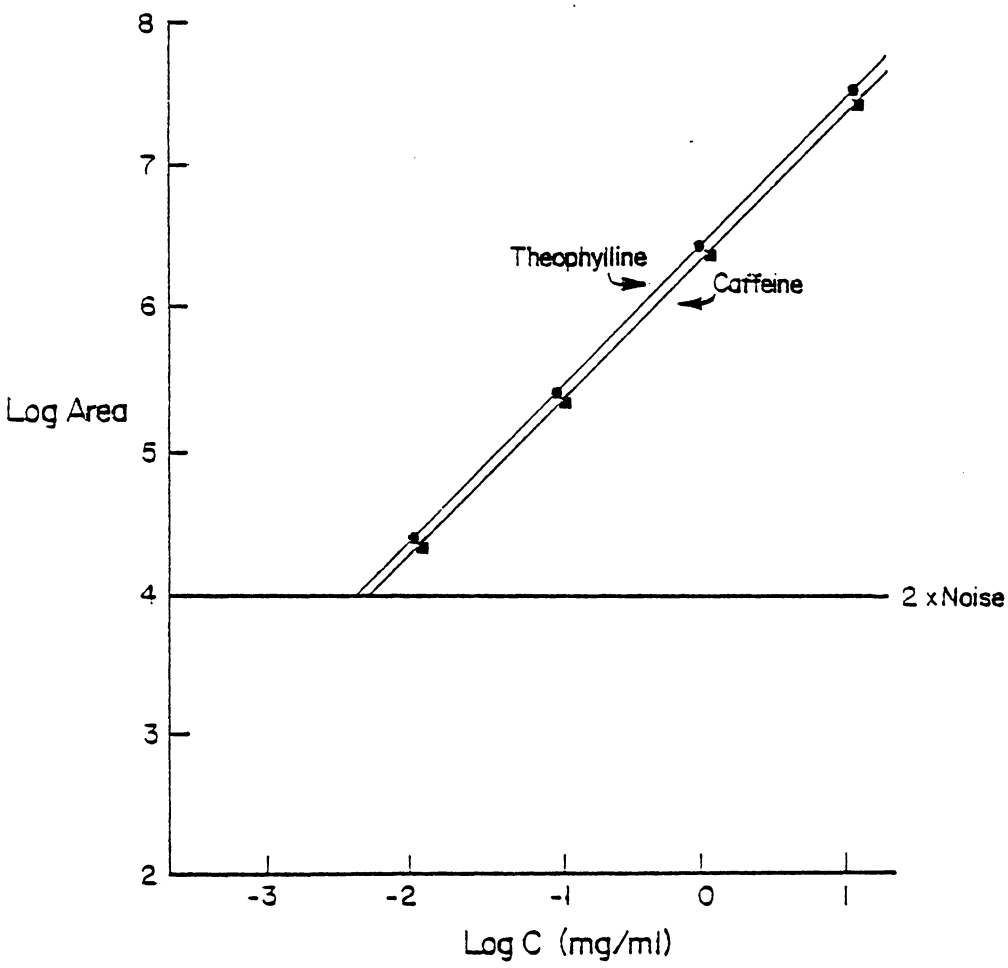


Figure 23. Calibration Curve for the Analysis of Theophylline and Caffeine

theophylline and caffeine after having been found to contain less than detectable amounts of these compound by this method. The technique was found to yield a MDC for both theophylline and caffeine of 2 x 10^{-4} mg/ml with linearity up to 1 mg/ml.

Comparison of these two approaches to on-line multidimensional chromatography points out several important characteristics of both. Comparing Figures 21 and 24, it can be seen that while the switching system exhibits improved detectability, the trapping system is significantly more selective. In the trapping system, only two peaks other than the analyte appear. In fact, although a gradient clean-up was used in early experiments, it was later found to be unneccesary. In the switching system, on the other hand, small peaks can be noted which are are not totally resolved from the analyte peaks.

The detection limits for the switching system are somewhat higher than those reported in the literature, which are typically on the order of 2 x 10^{-5} mg/ml (149). The switching system is capable of analyses of drugs in the theraputically useful range which is above 10^{-4} mg/ml. It should be noted here that, although a great deal of emphasis is placed on detection limits, the final critereon for a technique is the capability to measure samples in a specific

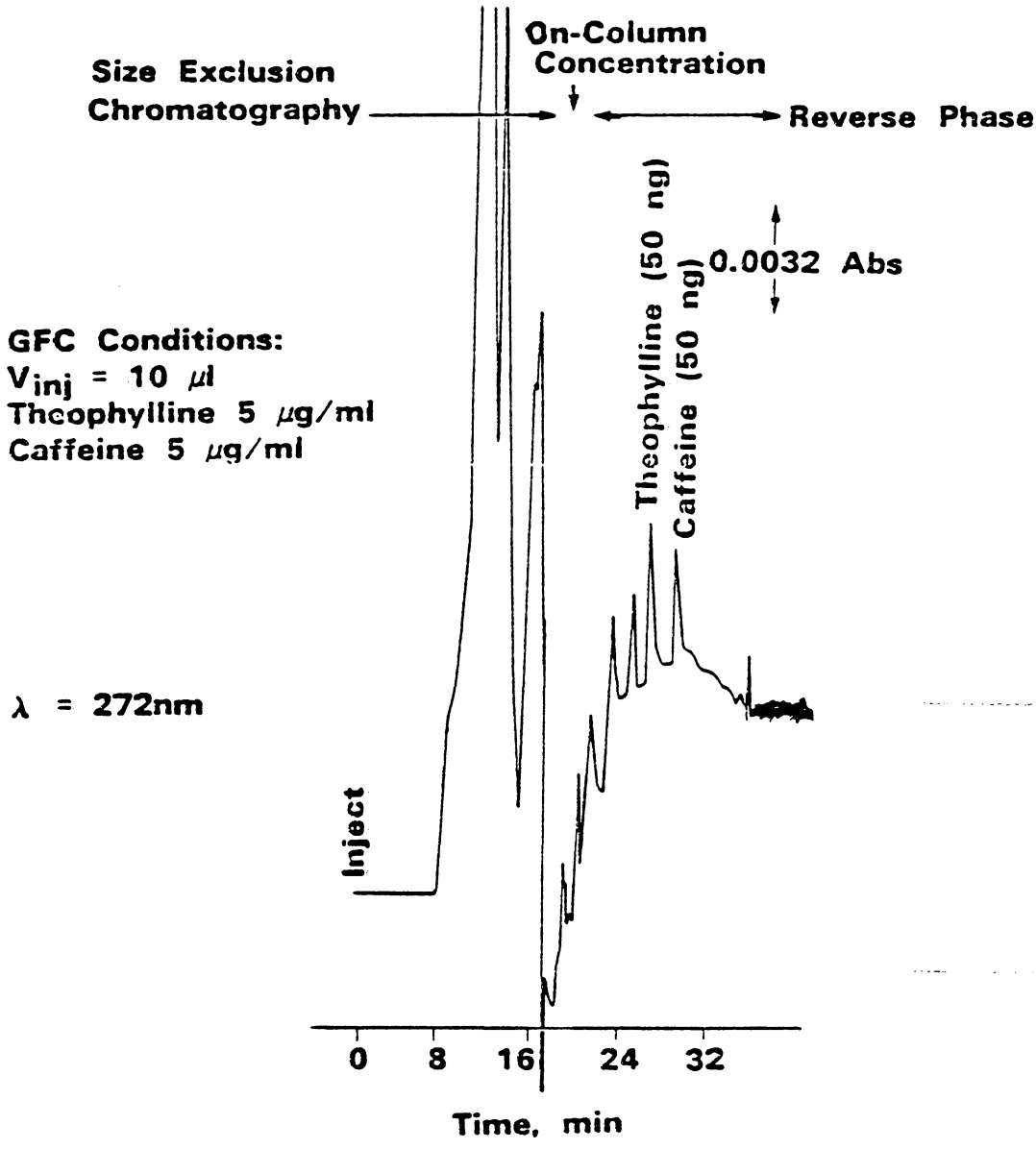


Figure 24. LC/LC Analysis of Theophylline and Caffeine in Serum: Switching System

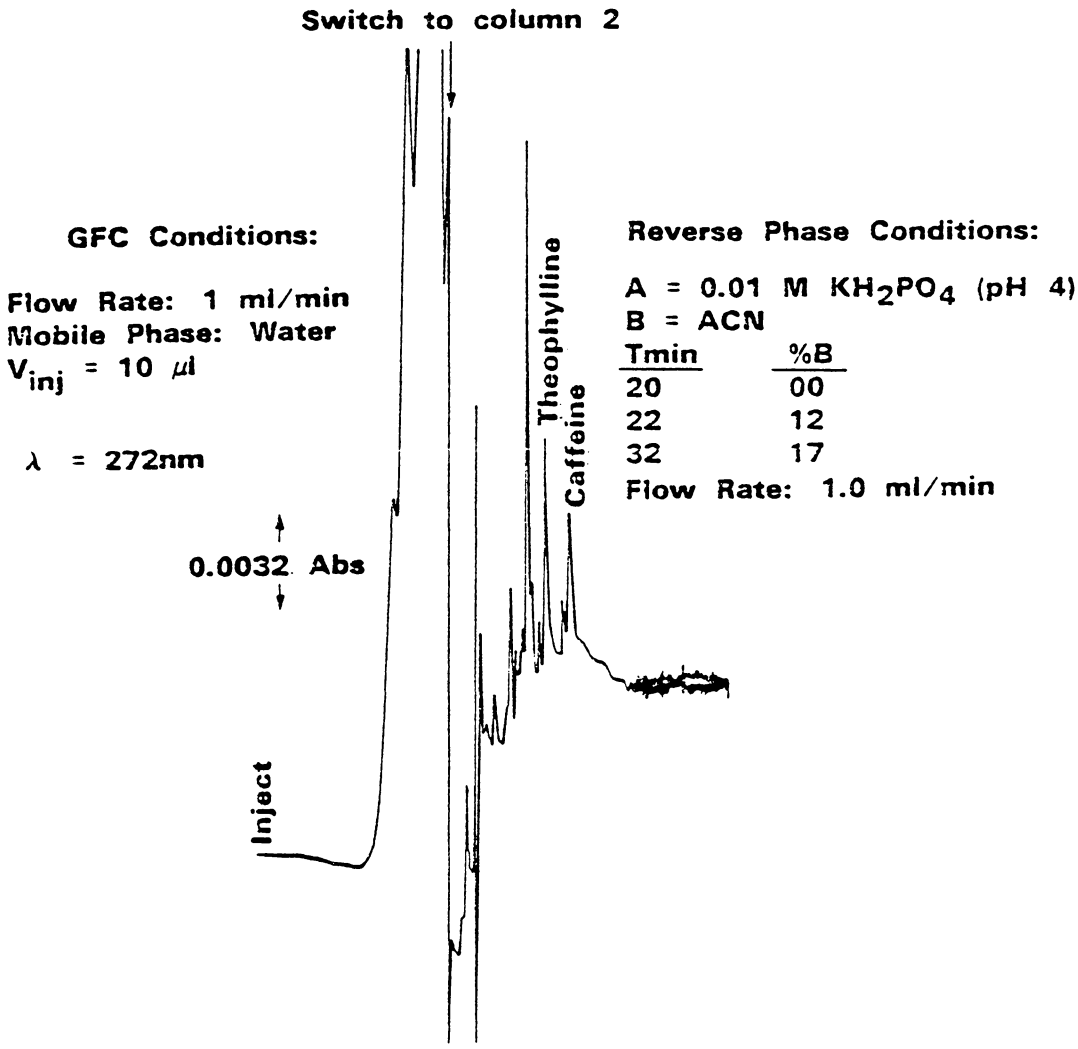


Figure 25. LC/LC Analysis of Theophylline and Caffeine in Urine: Switching System

range of interest. This system is completely automated and can analyze samples with varying matrix characteristics at the rate of one sample every 40 minutes.

5.2.1.2 Hydrocarbon Group Analysis

In industrial applications utilizing petroleum sources, it is often critical that the chemical make-up of the product and/or raw material be characterized. One approach to this characterization is the determination of the relative distributions of chemical classes or groups. There are a variety of such group separations including: the analysis of paraffins, olefins, naphthenes and aromatics (PONA); the analysis of saturates, aromatics, resins and asphaltenes (SARA); and the analysis of saturates, unsaturates, aromatics and polars, which is the subject of this application.

HPLC, and in particular, normal phase HPLC has found great utility in the analysis of chemical groups. Separations have been reported using silica as a stationary phase and non-polar mobile phases such as hexane (97) and freon (151). In such a system, groups elute in order of increasing polarity; saturates, unsaturates, aromatics, and polars. The separation of aromatics fom both non-aromatics and from polars is easily accomplished. The separation of

saturates from unsaturates, on the other hand requires a highly activated silica surface. The activation of a silica surface depends, in part, on the removal of water adsorbed to the polar silanol groups. For this reason, water must be rigorously excluded from the chromatographic system for the separation of saturates and unsaturates by adsorption on silica. The process of maintaining such an active surface reproducibly is both difficult and inconvenient (152).

The use of "argentation" chromatography, which takes advantage of the charge transfer complexes formed between silver ions and olefins, has been used in thin layer chromatography (153) and has recently been applied to HPLC (154-157). Argentation HPLC has been applied to the separation of olefinic isomers (154) and the separation of saturates and unsaturates (155). In this last report, olefins and aromatics were found to co-elute, so an off-line two dimensional system was used in which aromatics were initially separated from both polar compounds and non-aromatics, followed by a separation of the non-aromatic fraction into saturates and unsaturates using a silver nitrate impregnated silica column. The purpose of the present application is to use a similar technique in an on-line multidimensional chromatographic analysis.

The analytical system employed consisted of three columns run in series using Valving Scheme C (see Figure 17). The three columns used were a cyanopropyl bonded silica column, a silica and a silver nitrate impregnated silica column. Since they are run in series, the effect of the three columns is cumulative. However, for the purposes of visualization, they can be thought of as each performing a separate function. The cyanopropyl bonded silica was used as a guard column to retain the highly polar compounds which might otherwise become irreversibly adsorbed to the following silica column. The polar compounds were subsequently backflushed from this column. The silica column was used to separate the aromatics from the non-aromatics, and the silver nitrate impregnated silica was used to separate the saturates from the unsaturates. Hexane was used as the mobile phase. Referring to Figure 17, the analytical sequence was as follows. The hydrocarbon sample was injected into the system with the valves in an on/on configuration. After the saturates and unsaturates had entered the silver nitrate impregnated silica column (column three), but before the aromatics had eluted from the silica column, the valving was switched to an on/off position, isolating the silver nitrate impregnated silica column. The aromatics were then eluted from the silica column and the

valving are switched to an off/off position, backflushing the polar compounds off the cyanopropyl bonded silica column. The valving is then returned to the on/on position and the saturates and unsaturates were eluted from the silver nitrate impregnated silica column.

Figure 26 shows the analysis of a synthetic hydrocarbon mix using only the first two columns in series, without the silver nitrate impregnated silica column and without the use of the valving described above. The components in this mixture are listed in Table 5. The upper trace is for a refractive index detector and the lower trace, offset one minute to the right is the UV detector operated at 210 nm. This separation is typical of the resolution one might expect from a conventional single dimensional technique. In this separation, the saturates and unsaturates are not resolved. Figure 27 shows the analysis of the same sample using the multidimensional chromatographic technique described above. With the switching system, all three groups, aromatics, saturates and unsaturates were well resolved. Note that the synthetic mixture does not contain any polar compounds, so the backflushing step was not used.

Figure 28 shows the LC/LC analysis of Texaco unleaded gasoline. Note the backflushing at 14 minutes and the elution of the polar compounds at 22 minutes. The olefins

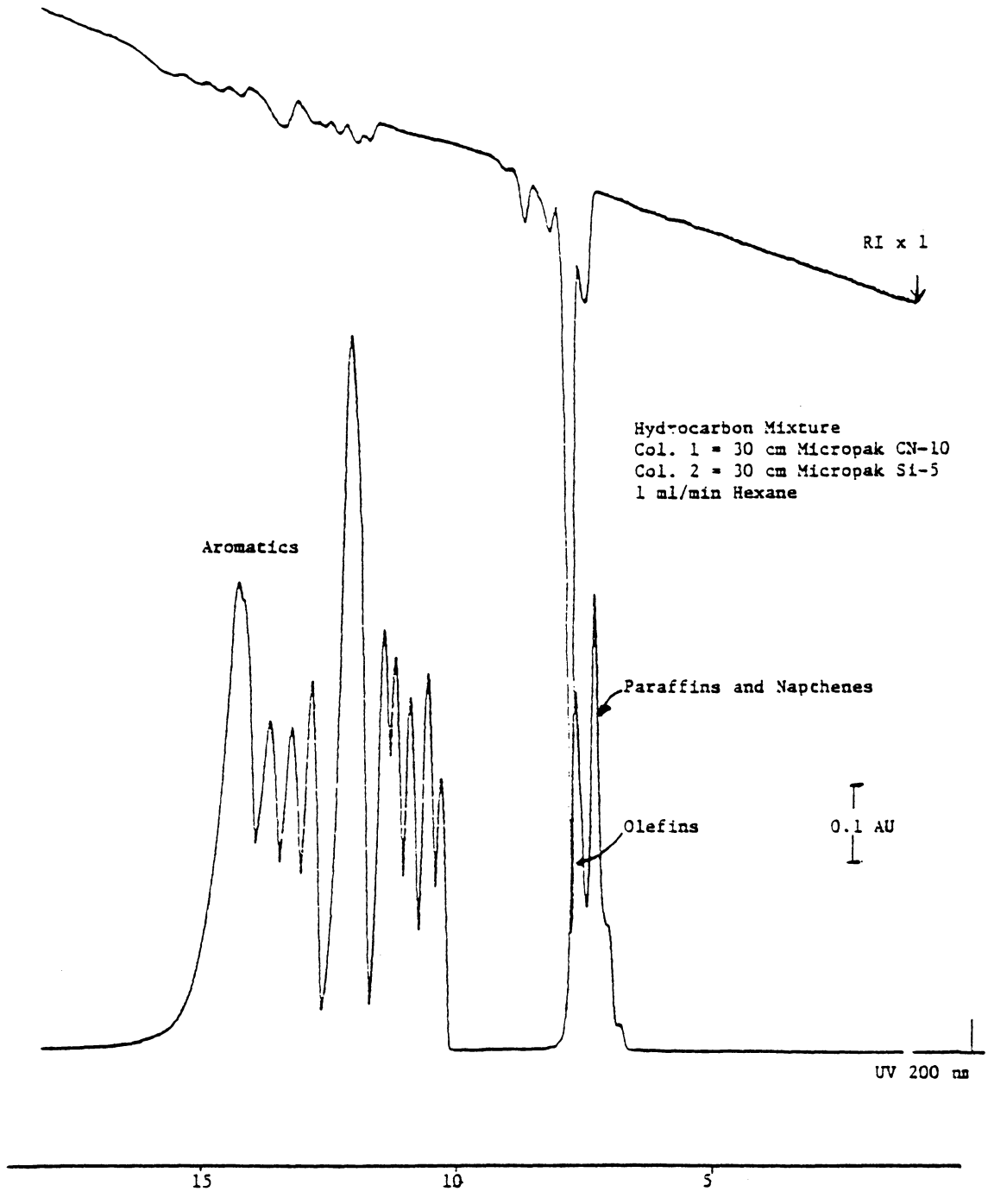


Figure 26: LC Analysis of Hydrocarbon Standard Mix

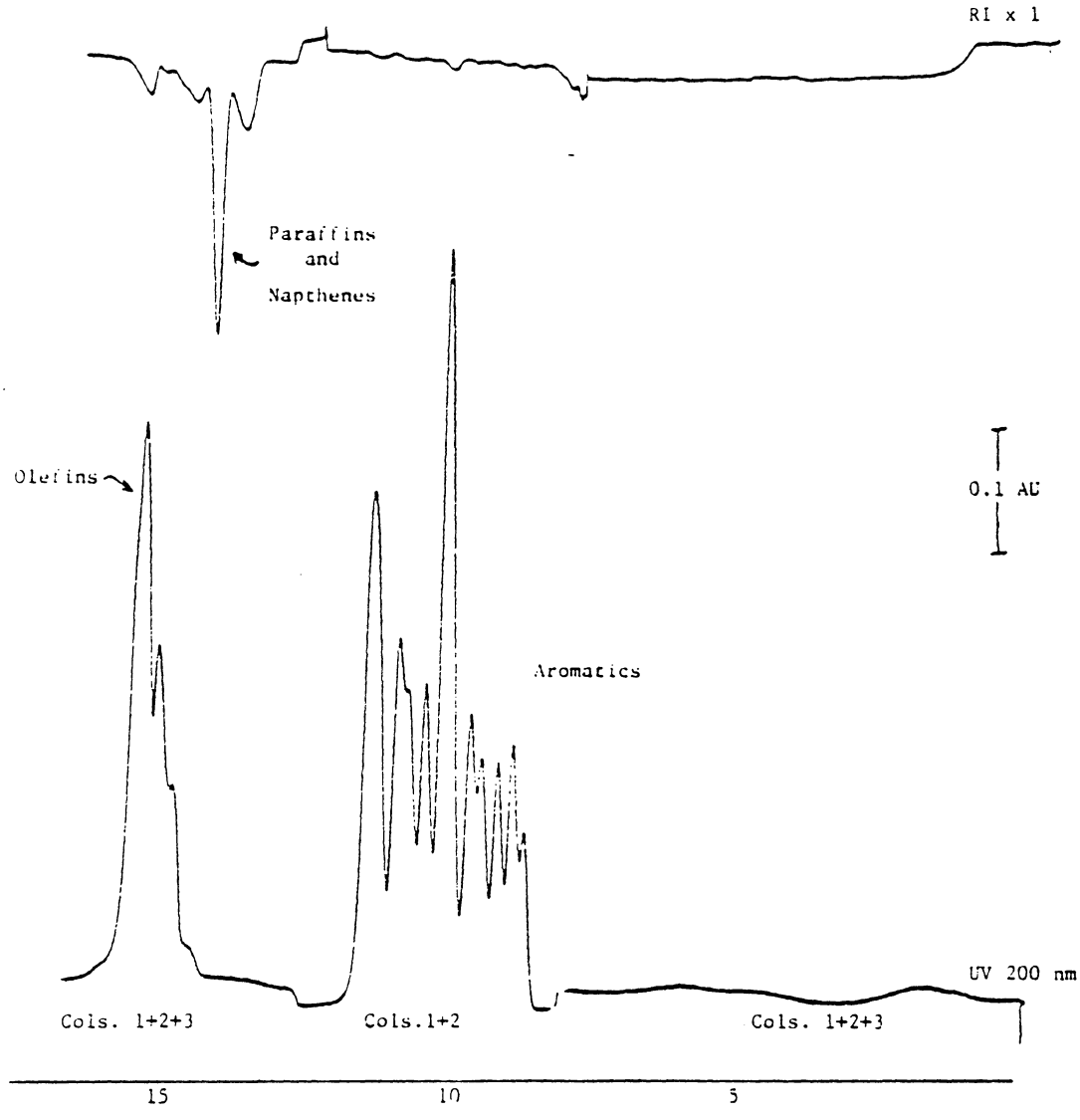


Figure 27: LC/LC Analysis of Hydrocarbon Standard Mix

in this separation were not only separated from the saturates, but were further separated into two groups. Analysis of standards shows that the retention time of the trans olefins corresponded to the first peak and the retention time of the cis olefins corresponded to the second peak.

Figure 29 shows the application of this analysis to a 10% solution of Cyclogen "L" in hexane. Cyclogen "L" is an oil added to asphalts to improve their thermal characteristics. As in the last figure, saturates, unsaturates, aromatics and polars were well resolved.

One of the major problems in hydrocarbon group analysis is quantitation. The main reason is that each peak contains many individual components, and as such, it is not possible to determine an absolute detector response factor. It is therefore not possible to determine the absolute amount of the individual groups present. It is possible to determine relative amounts present between samples, and this approach can be reasonably accurate if the samples being analyzed are similar in nature and therefore differ only in quantitative aspects. This problem of quantitation is compounded since there is no universal and sensitive detector available to HPLC. While aromatics can be detected by UV between 200 and 300 nm, olefins can only be detected below 220 nm and

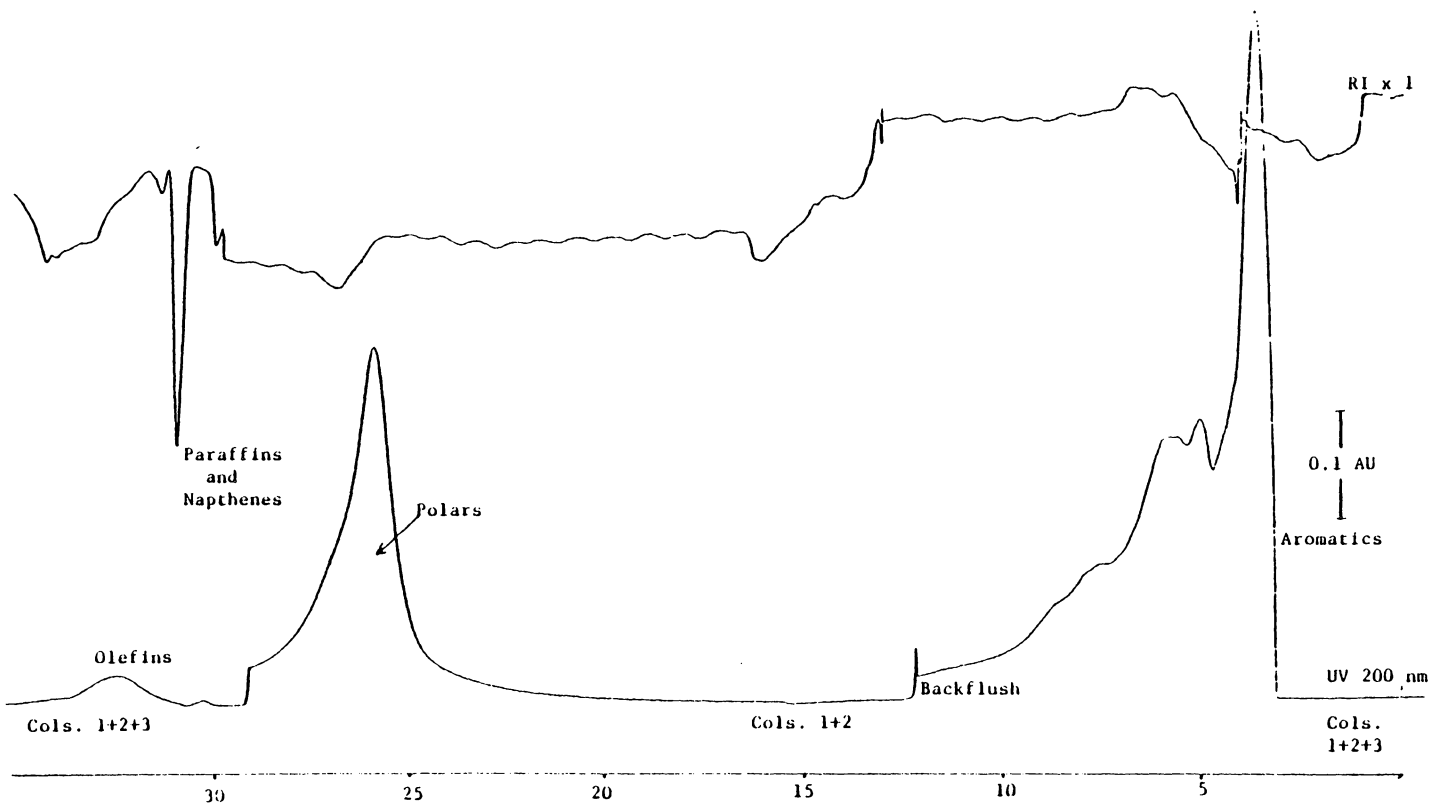


Figure 28: LC/LC Analysis of Texaco Unleaded Gasoline

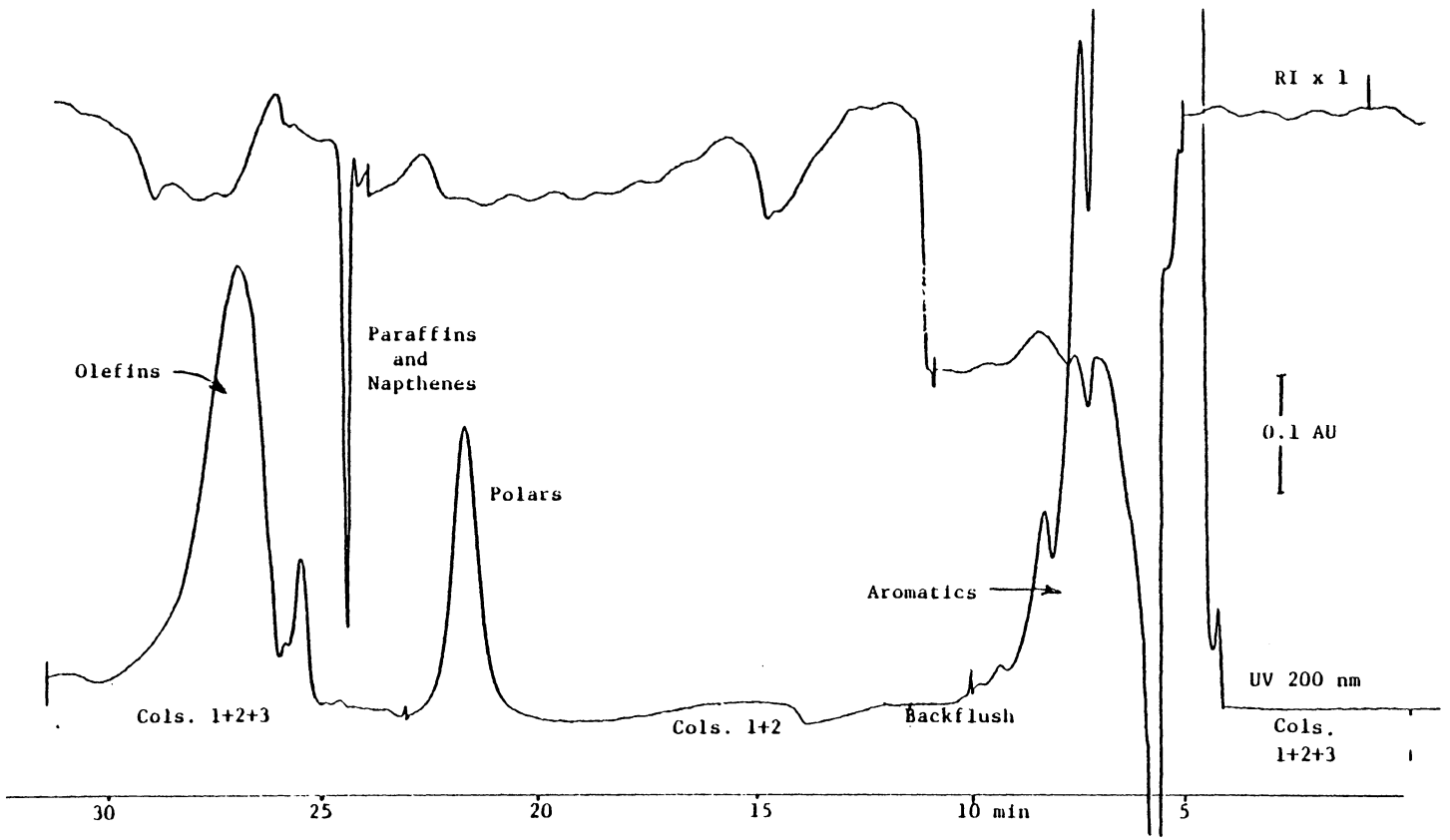


Figure 29: LC/LC Analysis of Cyclogen "L"

saurates do not absorb in a useful region. Therefore, in this application both UV and RI detection systems are neccessary. No quantitation studies were performed in this application, but comparison of Figures 28 and 29 show, for instance, that the relative distribution of saturates to unsaturates is considerably lower in Texaco unleaded gasoline than in Cyclogen "L".

This application exhibits several unique features which were not present in the previous application. One of these is the backflushing feature available to Valving Scheme C. Backflushing in this multidimensional chromatographic system depends on two characteristics of HPLC. The first requirement is that the column be capable of eluting samples in either direction. Although this may seem self-evident, it is a requirement which is not fulfilled by all columns. If the packing bed is not uniform, tight, and without voids, reversing the flow through the column can cause the packing to shift and thus lead to the formation of voids, which will severely degrade column performance. In this respect, the Varian Micropak columns were found to be well packed and applicable to this analytical system. A second characteristic of HPLC which is taken advantage of in this analysis is the ability to stop the flow in a column without degrading the resolution of the components. This

characteristic is possible because of the low diffusion of molecules in liquids.

The majority of reports on the preparation of silver nitrate impregnated silica columns have prepared the packing in bulk and then packed chromatographic columns by a slurry packing technique (154,155). The typical procedure is as follows. The silver nitrate is dissolved in acetonitrile at a concentration of 1 to 10 % (w/w). To 50 ml of this solution is added 10 g of silica gel. The acetonitrile is then removed using a rotary evaporator, leaving the silver nitrate precipitated, and presumably impregnated on the silica surface. This procedure is carried out in opaque vessels. The material is then slurry packed into a column. There are two disadvantages to this technique. First, it is possible that not all the silver nitrate is adsorbed on the silica surface. If the silver nitrate is present in too high a concentration, it may precipitate as crystals among the silica particles, rather than being adsorbed on the silica surface. Secondly, this procedure requires both slurry packing equipment and expertise to produce high efficiency columns.

Several approaches (156,157) have been published concerning the in-situ impregnation of silica with silver nitrate. Heath and Sonnet (156) reported a procedure in

which a small volume (15 ml) of a 3% silver nitrate solution in acetonitrile was pumped through a packed column followed by 30 ml each of hexane and benzene. Heftmann *et al.* (157) reported a procedure in which 250 ml of a 0.5% w/w solution of silver nitrate in methanol was pumped through a packed column followed by overnight recylcing of 750 ml of the same solution. The column was rinsed with 200 ml of methanol and then equilibrated with hexane.

Neither of the above procedures yielded acceptable results when applied to the preparation of columns for this application. A procedure was therefore applied which was a modification of Heftmann's. Solutions of silver nitrate were recycled through columns containing 5 and 10 μm silica. Three different impregnation solutions were evaluated: 10% w/w silver nitrate in acetonitrile; 1.5% w/w silver nitrate in water; and 0.5% w/w silver nitrate in methanol. Following the impregnation solution, a series of solvents were pumped through the column: 50 ml of the impregnation solvent (without silver nitrate); 100 ml of isopropanol; and finally 500 of ml hexane. For the aqueous impregnation solution, it was neccesary to follow the isopropanol rinse with 100 ml of diethyl ether and 100 ml of THF before equilibrating the column with 500 ml of hexane.

Sample chromatograms are shown for these columns in Figure 30. This figure shows the separation of a synthetic mixture containing the paraffins and olefins listed in Table 5 . Figures 30 a, b and c show the performance of the columns impregnated using the aqueous, the methanol and the acetonitrile impregnating solvents, respectively. As can be seen, the aqueous impregnating solvent yielded the best results. Running individual samples shows that the first peak in the olefin group consists of trans olefins and the second peak is made up of cis olefins. This is as predicted, since the cis double bond can interact more effectively with the silver ion. The column impregnated in a methanol solution gave very little separation and, although the column impregnated in an acetonitrile solution shows complete resolution of the paraffins and olefins, it does not show the separation between the olefins that the aqueous column does. When the procedure outlined above was performed without the diethyl ether and THF rinses, no separation between the paraffins and olefins was obtained using the column impregnated with an aqueous solution. However, the use of these additional rinses had no effect on the other two columns. This effect is attributed to the solvation of the adsorbed silver ions by water molecules interfering with the interaction of the silver ions with

olefins. This water is not completely removed without the additional rinses.

5.2.1.3 Catecholamines in Urine

Catecholamines are a group of amino acid related compounds which are of interest in a number of biochemical pathways and in relation to a number of metabolic disorders. Several catecholamines, primarily epinephrine (adreniline), norepinephrine (noradreniline) and dopamine, act as neurotransmitters in the sympathetic nervous system. These compounds are synthesized in biological systems from the amino acid tyrosine, leading through several intermediates including 3,4-dihydroxyphenylalanine (DOPA). Of similar and related interest are the hydroxyindoles such as 5-hydroxyindole acetic acid (5-HIAA) and 5-hydroxyindole carboxylic acid (5-HICA). The metabolism of catecholamines is depressed in such diseases as pheochromocytoma. It has been recently shown by Banda, Blois *et al.* (158-161), that the levels of certain catecholamines appear elevated in the urine of patients with malignant melanoma, a type of skin cancer. These compounds, termed "Melanogens", can therefore be used as biochemical markers in monitoring the progress of the disease.

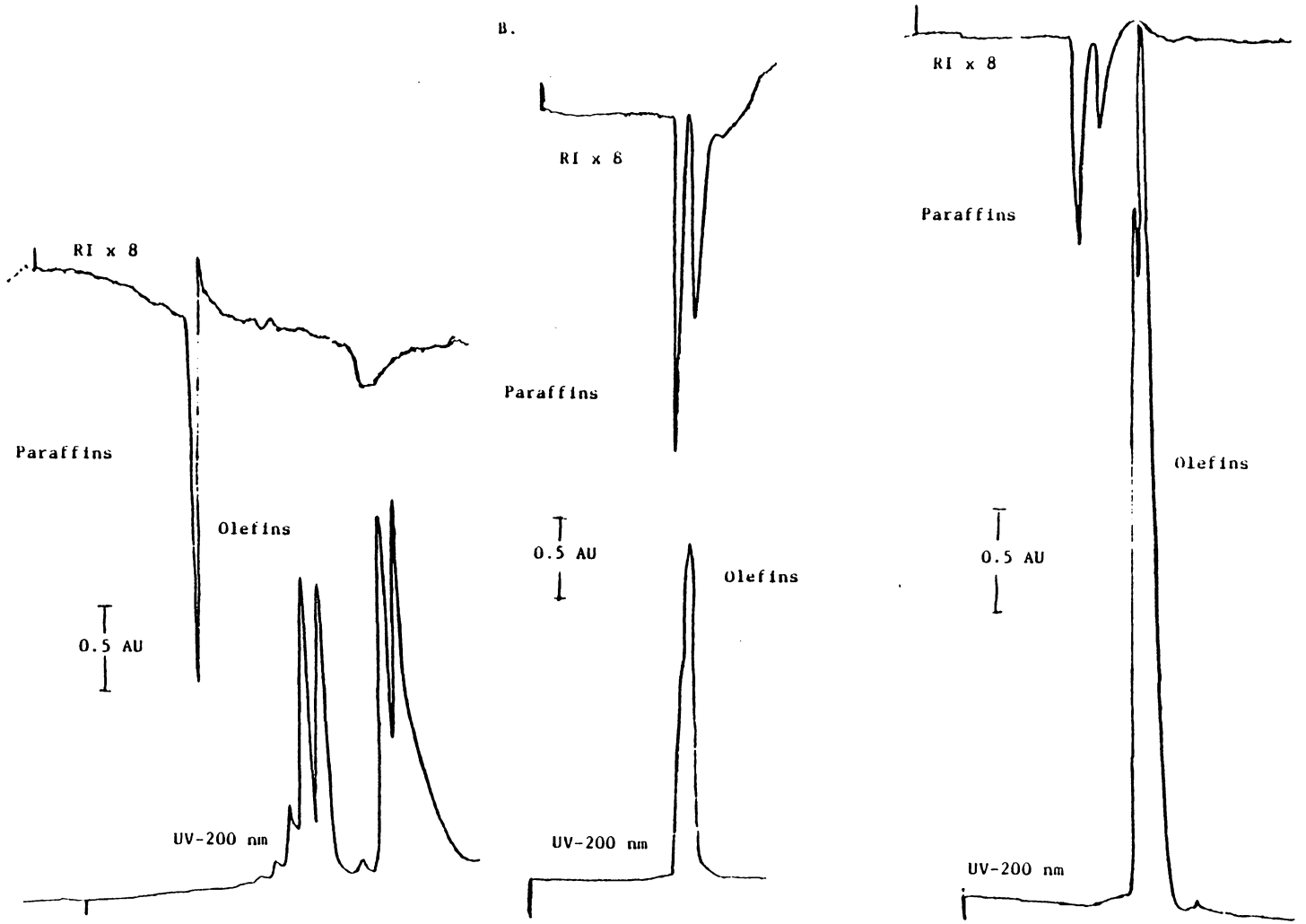


Figure 30: Evaluation of In-Situ Impregnated Silver Nitrate Silica Columns

The analysis of catecholamines has received considerable attention in the literature. A number of separation techniques have been applied including cation exchange chromatography (162-164), anion exchange chromatography (165), ion pair chromatography (166) and reverse phase chromatography with ion supression (167). In addition, a number of detection systems have been used including flouresence detection (165), electrochemical detection (163) and colorimetric detection of diphenylpicrylhydrazyl (DPPH) derivatives (159-161).

The advent of commercially available electrochemical detector systems had caused much interest in their use for the highly sensitive and selective detection of catecholamines. With currently available systems, the electrochemical detector suffers from a major problem in that the working electrode is easily poisoned leading to poor detector stability. Kissinger *et al.* (54,55) have applied column switching systems to the analysis of biochemical samples in an effort to isolate the electrochemical detector from sample components responsible for electode poisoning. To date, however, no such system has been applied to the anaysis of catecholamines. The goals of this application are two fold: first, to isolate catecholamines from a complex biological matrix in order to

utilize an electrochemical detection system with improved stability; and second, to optimize the resolution of these catecholamines by isolating a fraction of the total sample containing them, and separating them using a second chromatographic mode. The structures of the ten catecholamines analyzed in this application are shown in Figure 31 .

Three multidimensional chromatographic techniques were evaluated in the approach to this analysis. The coupling of aqueous size exclusion chromatography (SEC) and ion pair chromatography (IPC), was used in both trapping and switching modes. In addition, the coupling of two IPC systems was used in a switching mode. The quantitation in switching systems, which will be discussed later, was evaluated experimentally using the application of this last approach in the analysis of DOPA.

The application of IPC in all three analytical approaches was similar. Either heptane sulfonic acid (HSA) or camphor sulfonic acid (CSA) was used to ion pair the protonated amine groups of the catecholamines. The mobile phase was buffered to pH 3.5 to ensure that the amino groups were protonated. For those analytes which possessed no amino functionality, the separation was performed simultaneously through the suppression of ionization of the

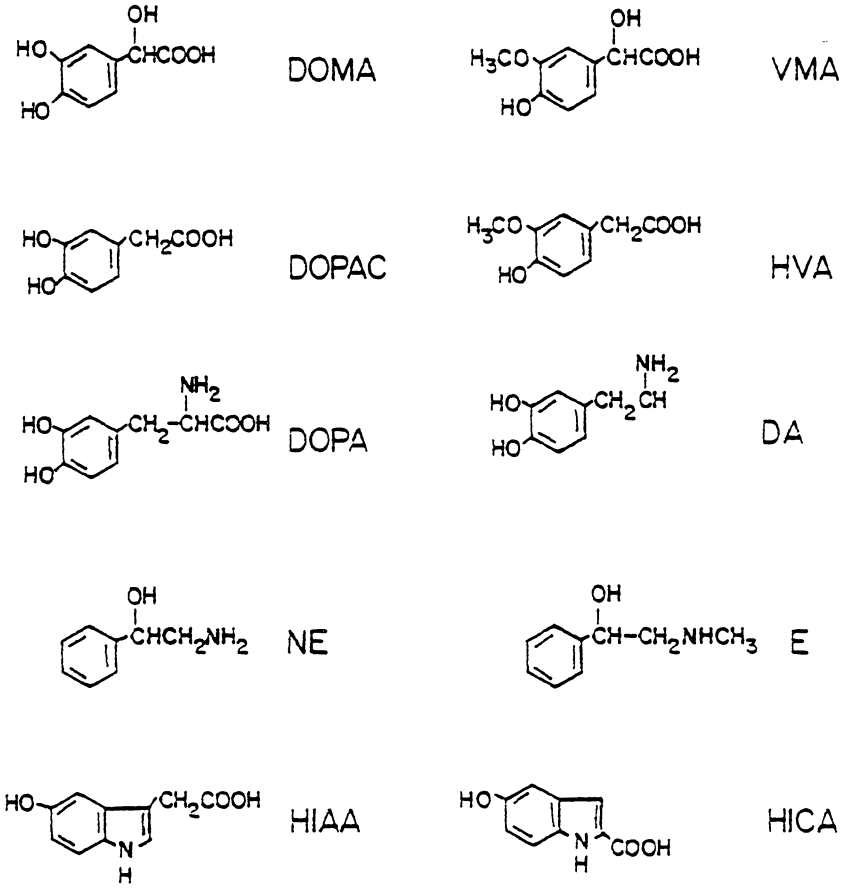


Figure 31. Stuctures of Catecholamines

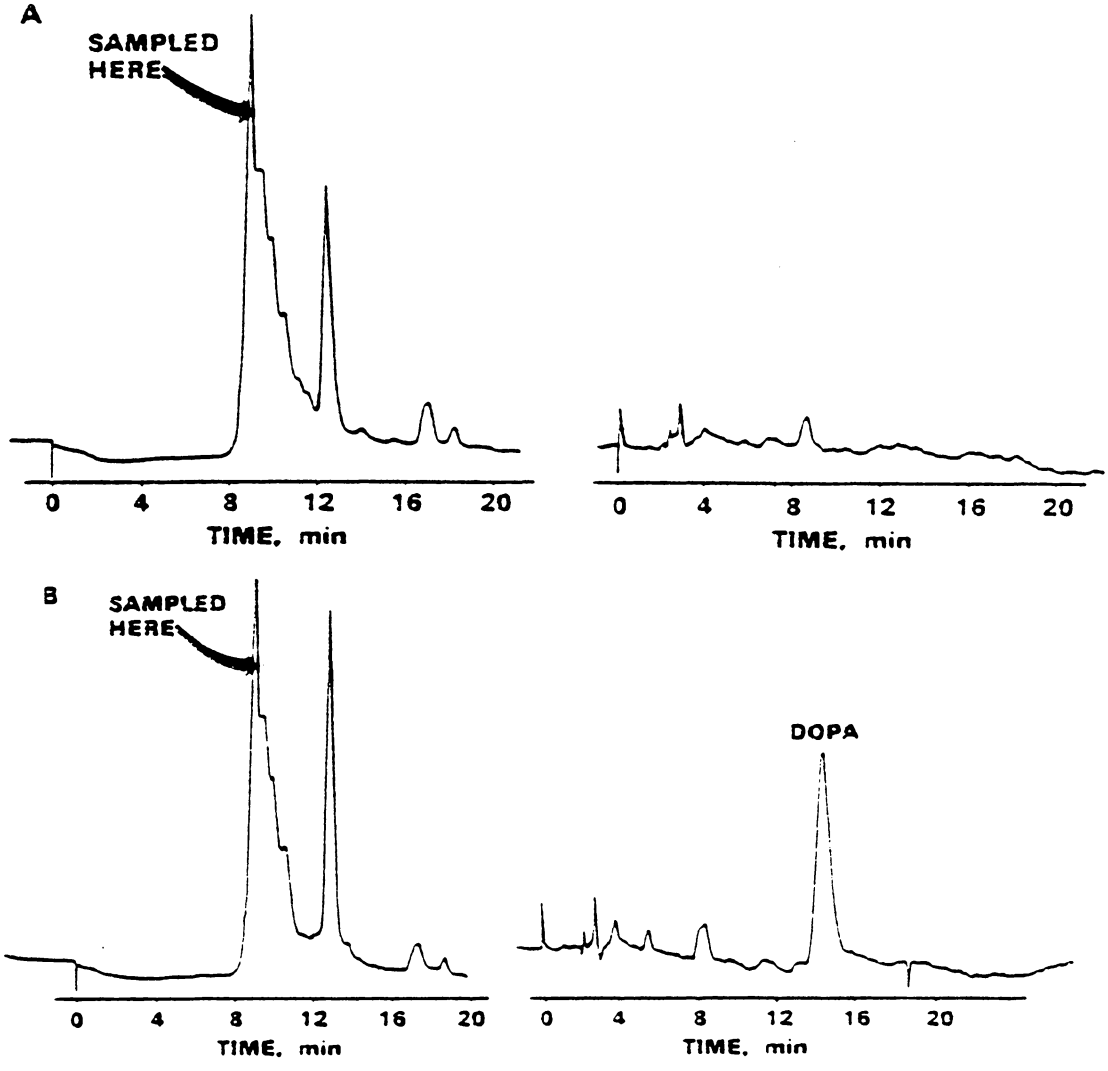
carboxylic acid groups present in all ten analytes. Thus the separation of both anionic and cationic species could be carried out on a single column. The use of electrochemical detection precludes the use of mobile phase gradients, so all IPC separations were isocratic. The reason for this restriction is that changes in solution conductivity due to mobile phase changes cause severe baseline shifts. Small amounts of methanol were added to the mobile phase to adjust the capacity factors of the analytes. Disodium EDTA was also added to the mobile phase to improve detector stability.

For the first two analytical approaches, the size exclusion chromatography was performed using a Micropak TSK 2000SW column. The objective of the primary SEC separation was to transfer a fraction containing the analytes to the secondary IPC column.

The analysis coupling SEC and IPC in a trapping system employed Valving Scheme B (see Figure 16). This method was applied to the analysis of a single component, DOPA. A minor modification of the valving configuration was made, such that the effluent from the IPC column went directly to the electrochemical detector rather than being reconnected to valve B. In this way, only the effluent of the IPC column passed through the electrochemical detector and the

remainder of the sample passed through a separate UV detector operated at 230 nm. With this modification, the analysis proceeded as follows. The sample was injected into the SEC column with the valving in an on/off position. In this configuration both the IPC column and the electrochemical detector were isolated from the flow path. At the retention volume of DOPA (8.9 ml, determined from standards), the valving was switched to an off/on position, simultaneously isolating the SEC and injecting the volume of effluent in the trapping loop onto the IPC column. After the analyte was eluted from the IPC column, the valves were returned to the on/off position and the remainder of the sample was eluted from the SEC column.

Figure 32 shows the analysis of DOPA in urine using this method. Figure 32 a shows the analysis of normal urine and Figure 32 b shows the analysis of the urine of a patient with deseminated malignant melanoma. The chromatograms on the left correspond to the effluent from the SEC column as detected by the UV detector, and the chromatograms on the right show the electrochemical detector response to the effluent from the IPC column. Note the elevated level of DOPA in the melanoma urine relative to that in the normal urine. The DOPA peak in the melanoma urine corresponds to 200 ng/ml. The MDC for DOPA by this method was found to be 5 ng/ml with linearity to 100 μg/ml.



. (A) Normal urine; (B) abnormal urine. EC analysis (left): column, MicroPak TSK 2000SW (30 cm × 7.5 mm I.D.); water at 1.0 ml/min; injection volume, 10 μl raw urine; detection at 230 nm; 2.0 a.u.f.s. RPC analysis (right): column, MicroPak MCH-10 (30 cm × 4 mm I.D.); water with 20 mM camphorsulfonic acid, 100 mM NaH_2PO_4 and 0.1 mM NaEDTA at 1 ml/min; detection, electrochemical detector with glassy carbon electrode operated at 0.720 mV *vs.* Ag–AgCl; attenuation, 2 nA/V; sample, 100 μl.

Figure 32. LC/LC Analysis of Dopa in Urine: Trapping System

The application of the coupling of SEC and IPC in a switching system used Valving Scheme A (see Figure 15). A similar modification as that described above was used in this valving configuration to isolate the electrochemical detector from the SEC effluent. The analytical sequence proceeded as follows. A 10 µl sample of undiluted urine was injected into the SEC column with the valving in an on/off position. In this position, the IPC column is isolated. At a point at which the analytes began to elute from the SEC column, the valving was switched to an on/on position. In this position, the effluent of the SEC column flows directly onto the IPC column. When the fraction containing the catecholamine analytes had eluted from the SEC column, the valving was switched to an off/on position, eluting the IPC column and isolating the SEC column. Finally, following the elution of the analytes, the valves were returned to the original on/off configuration and the remainder of the sample allowed to elute from the SEC column. With the mobile phase conditions used, the analytes did not all co-elute from the SEC column, so a series of SEC effluent volumes were transferred between the chromatographic columns. The volumes which did not contain analytes were not transferred. This simply required a series of valve changes from an on/on position for transfer to an on/off position for the volumes which were not to be transferred.

Figure 33 shows the SEC separation of the Catecholamine standards. Note that as stated above the analytes do not co-elute. Figure 34 shows the separation of the same catecholamine standard mixture using the SEC column and the IPC column in series, without switching. This is shown instead of the IPC separation alone, because the actual analytical separation benefits from the selectivity of both columns. Figure 35 shows the SEC separation of the urine samples. Figure 35 a shows the analysis of normal urine and Figure 35 b show the analysis of a 1:10 dilution of melanoma urine.

Figure 36 shows the application of the multidimensional chromatographic switching system described above to the analysis of catecholamine standards. Similarly, Figures 37 and 38 show the LC/LC separations of the normal urine and the diluted melanoma urine, respectively. This method yielded an MDC for DOPA of 1 ng/ml with linearity to 100 µg/ml.

The final approach to the multidimensional chromatographic analysis of catecholamines in urine coupled two IPC separations. Both separations utilized 5 µm octylsilane bonded silica columns, but the primary column was a 15cm Supelcosil RP-8 column and the second column was a 25 cm HP RP-8 column. The analytical system was otherwise

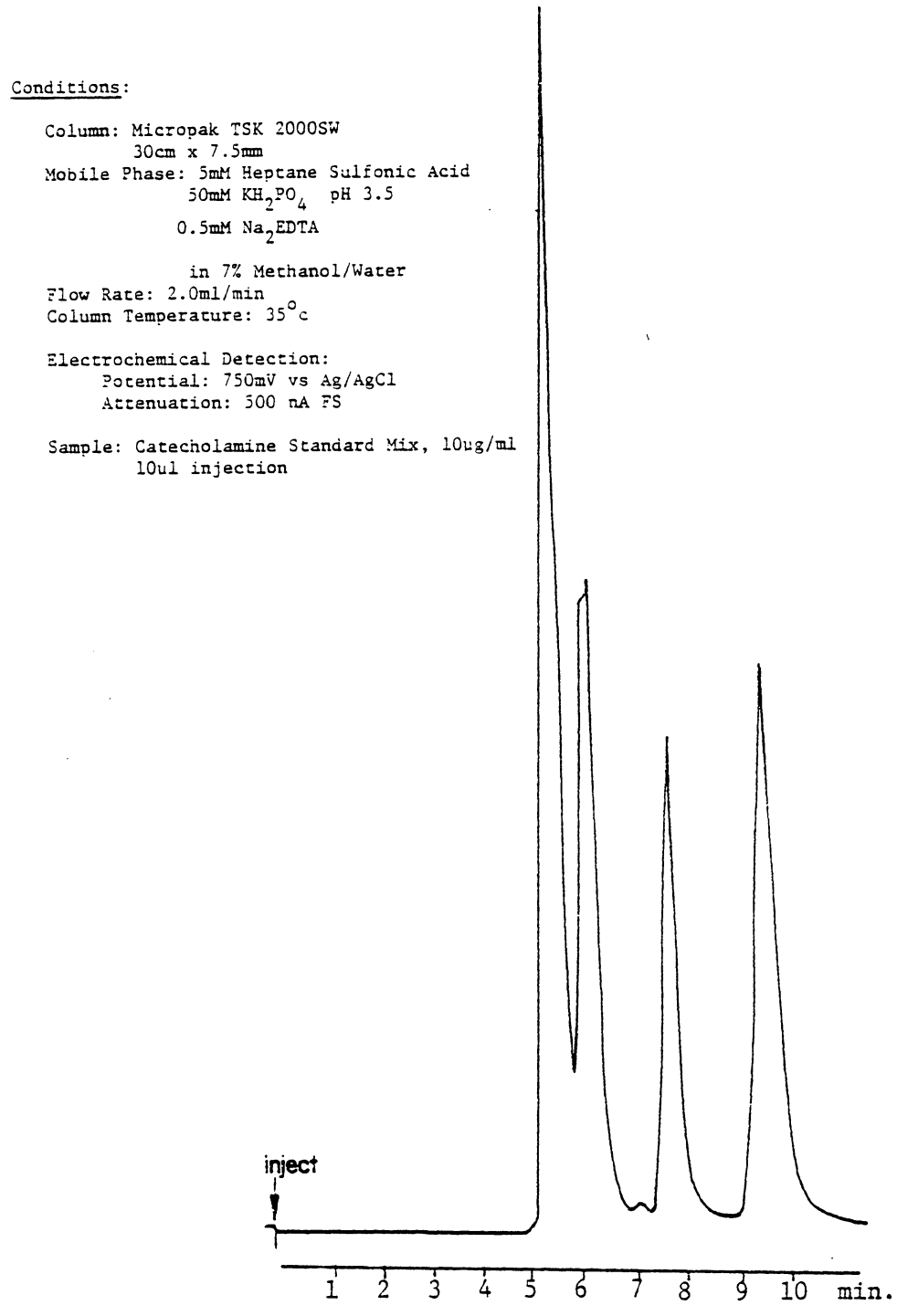


Figure 33. SEC Fractionation of Catecholamine Standards

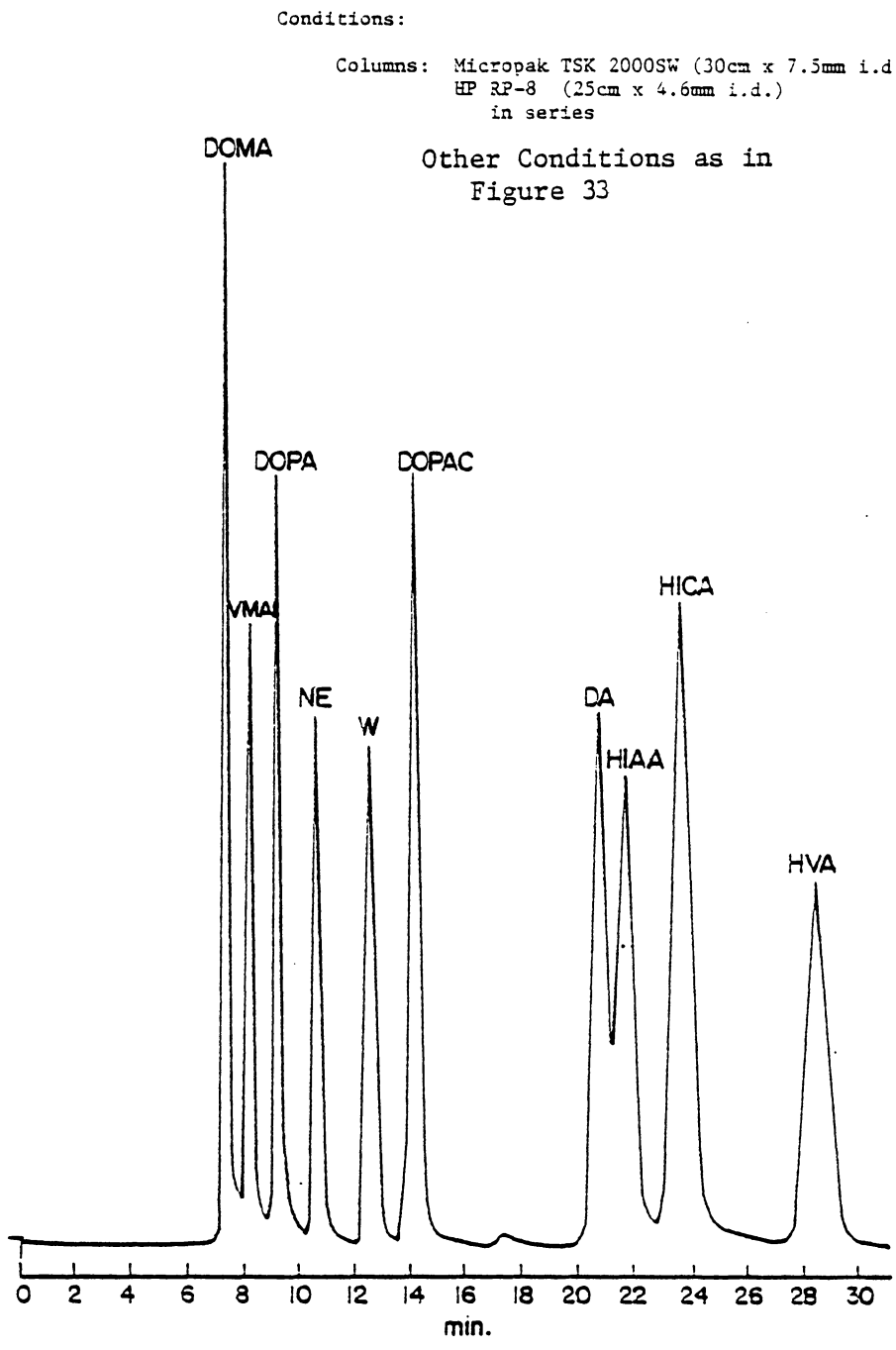


Figure 34. Separation of Catecholamine Standards (Without Switching)

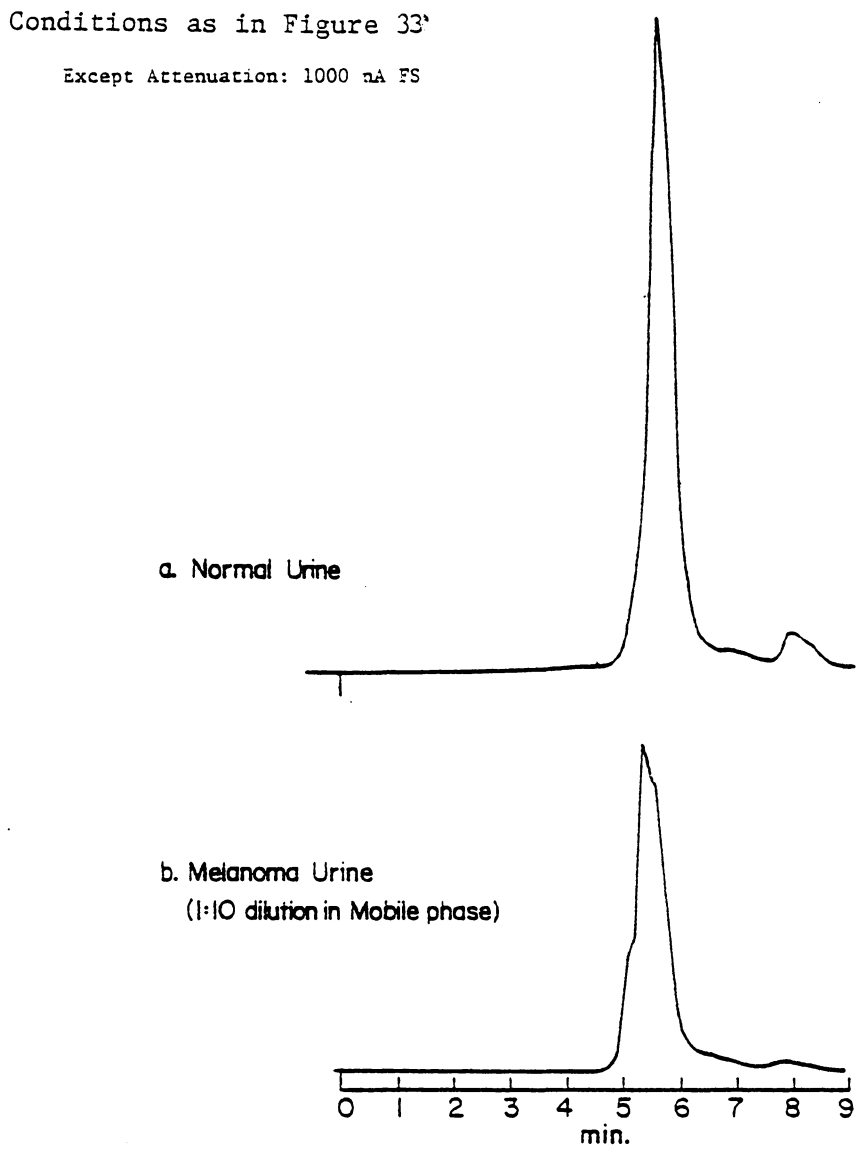


Figure 35. SEC Fractionation of Urine Samples

Conditions as in Figure 33

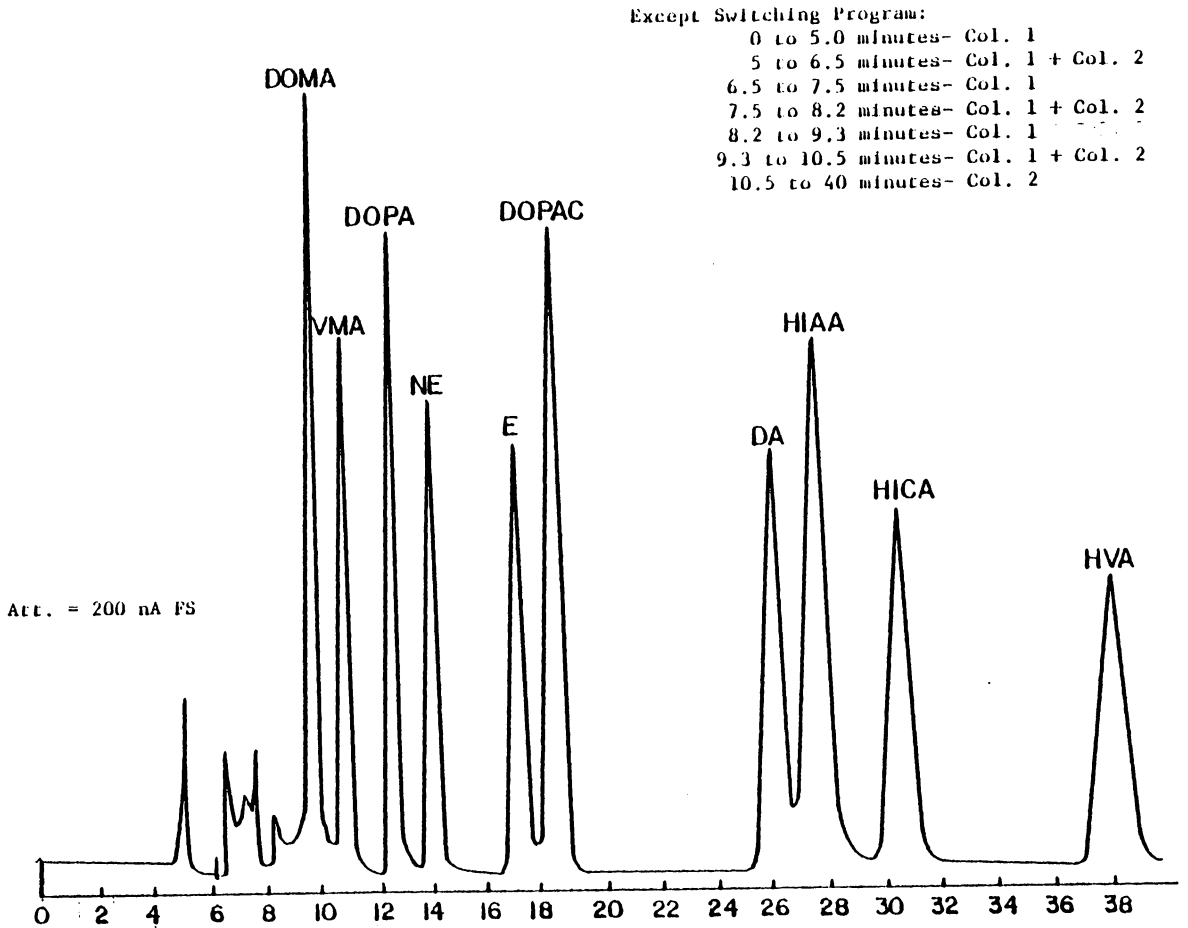


Figure 36. LC/LC Analysis of Catecholamine Standards

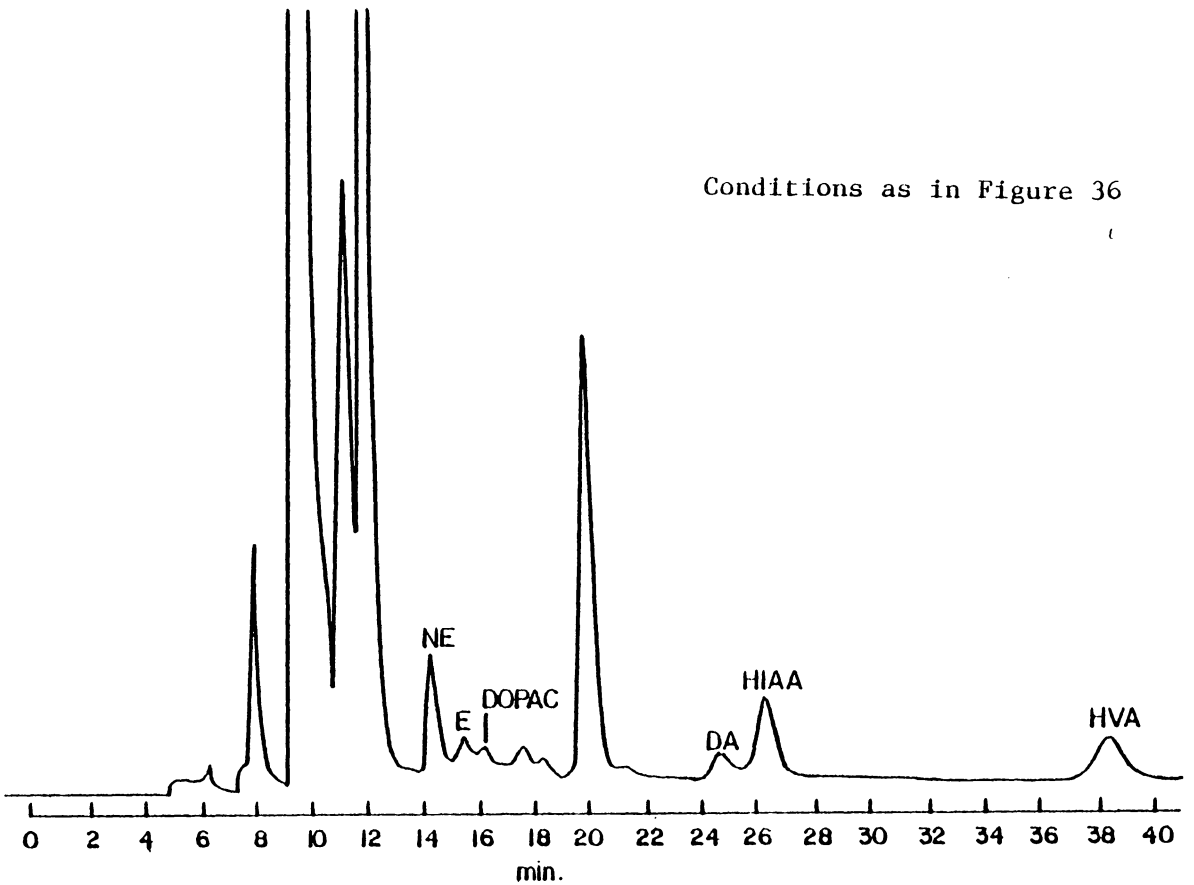


Figure 37. LC/LC Analysis of Normal Urine

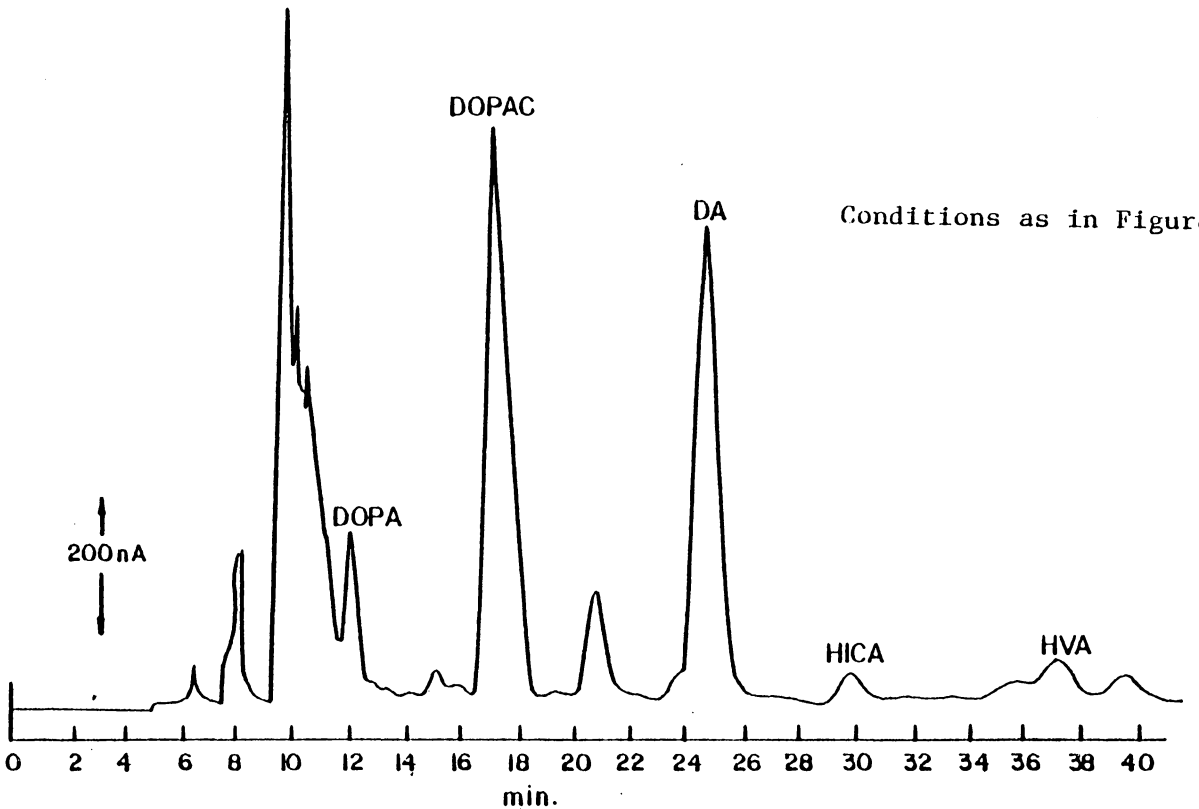


Figure 38. LC/LC Analysis of Melanoma Urine

identical to that described for the switching analysis coupling SEC and IPC. The analytical sequence followed the same pattern as the earlier technique, with only the switching times adjusted to correspond to the analyte elution times from the shorter IPC column. One major change was made, however. It was determined that using the IPC column, many polar sample components eluted with the first two analyte peaks from the IPC column. This is because the first two eluting analytes, DOMA and VMA are themselves polar compounds and elute very close to the solvent front. These polar matrix components are partially responsible for the electrode poisoning, and therefore, this fraction was not transferred to the secondary IPC column.

Figure 39 shows the separation of catecholamine standards on the 15 cm IPC column. Figures 40, 41 and 42 show the multidimensional chromatographic separation of catecholamine standards, normal urine and melanoma urine respectively using the coupling of two IPC columns in a switching mode. It should be noted that in these three figures, both column effluents are detected by the electrochemical detector. For these examples, the unmodified Valving Scheme A was used. This is shown only for the sake of illustration and in the routine analyses, the modification described above was used.

Conditions as in Figure 33

Except:
Column: Supelcosil RP-8
(15cm x 4.6mm i.d.)

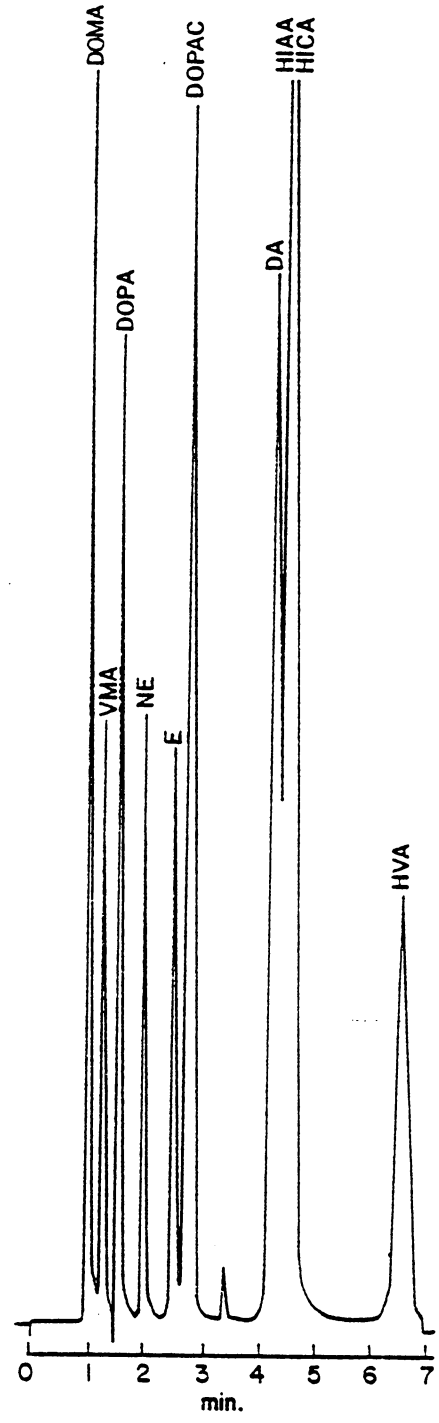


Figure 39. IPC Fractionation of Catecholamine Standards

Conditions as in Figure 33

Except:
Columns: Supelcosil RP-8 (15cm x 4.6mm i.d.)
HP RP-8 (25cm x 4.6mm i.d.)
in series
Switching Program:
0 to 1.1 minutes- Col. 1
1.1 to 5.5 minutes- Col. 1 + Col. 2
5.5 to 30 minutes- Col. 2

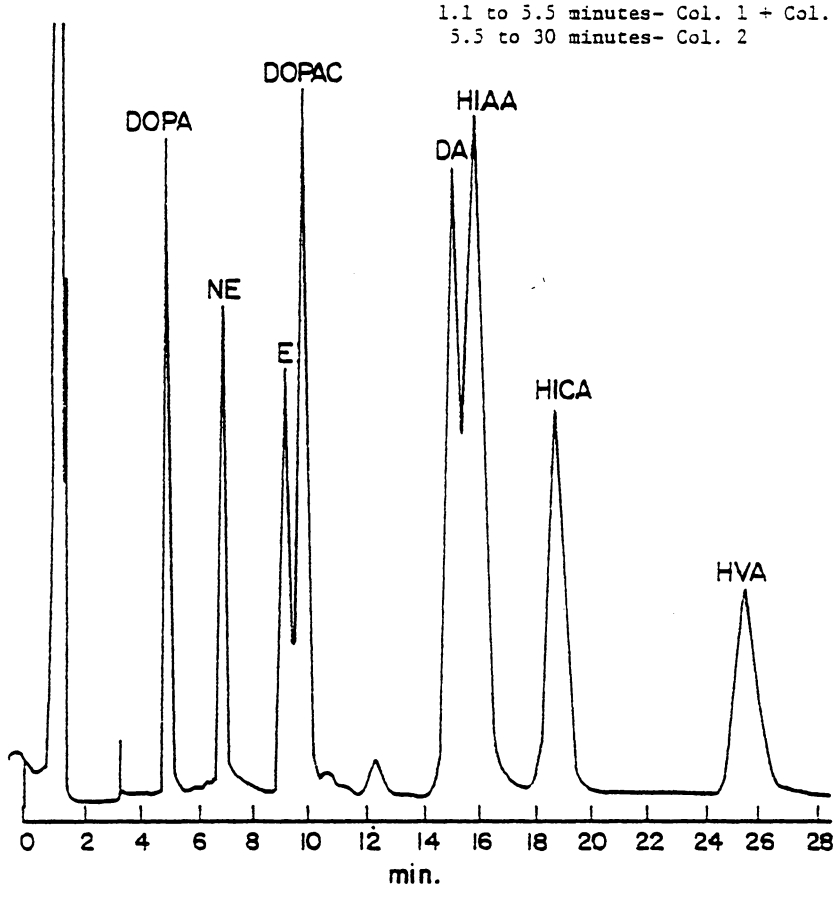


Figure 40. IPC/IPC Analysis of Catecholamine Standards

Conditions as in Figure 40

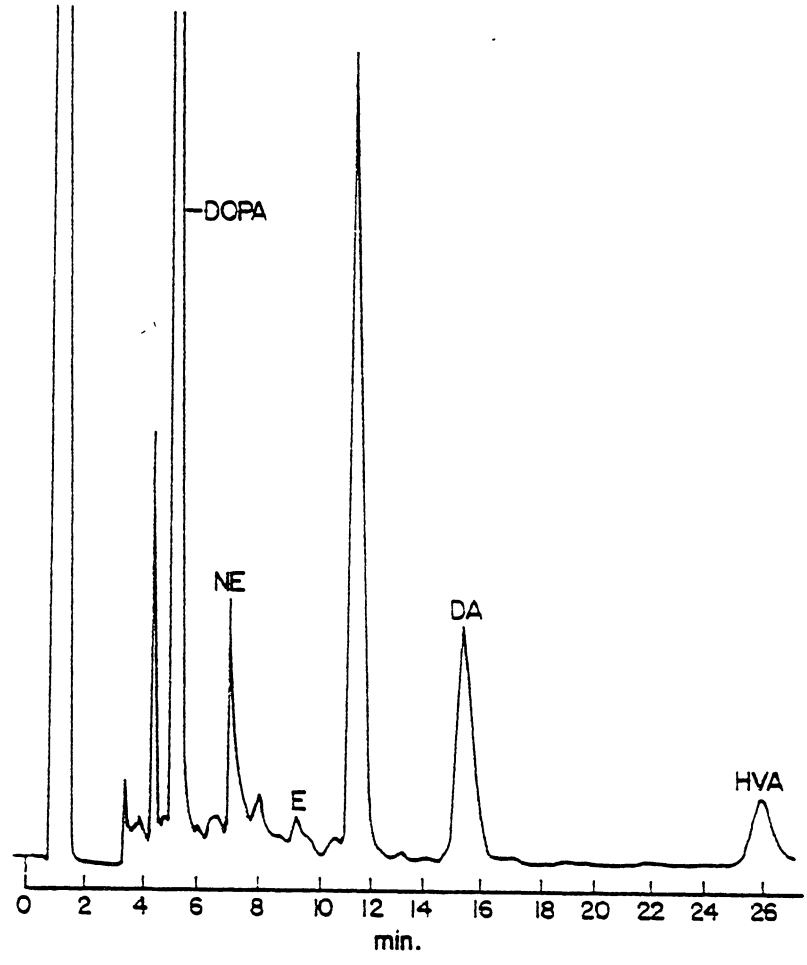


Figure 41. IPC/IPC Analysis of Normal Urine

Conditions as in Figure 40

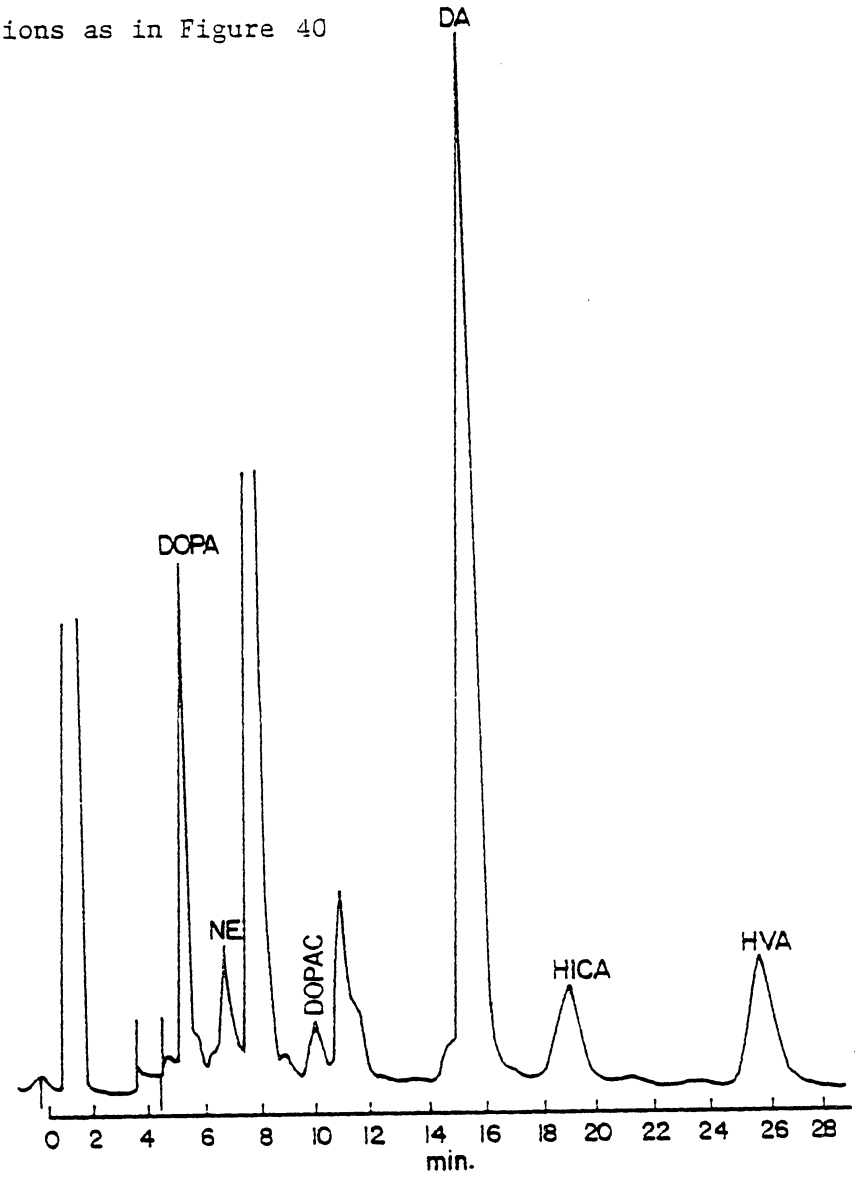


Figure 42. IPC/IPC Analysis of Melanoma Urine

The evaluation of the above three techniques allows advantages and disadvantages of the different approaches to be examined. Comparing the application of switching techniques to trapping techniques in the coupling of SEC and IPC reiterates conclusions which have been made from the analysis of theophylline and caffeine. Namely, switching techniques yield better detectability, while trapping techniques are more selective. In these analyses, this is very pronounced. The use of the trapping mode allows specificity for DOPA. In Figure 35 , while there are other components present in the IPC separation, they are at concentrations far below that of the analyte. In the case of the switching technique, however, the selectivity is poor. It is not possible to quantitate all ten analytes due to the lack of resolution. On the other hand, the trapping system yields MDC's an order of magnitude less than the switching system. Both the enhanced selectivity and poor detectability in the trapping technique are due to the transfer of only a small portion of the analyte from the effluent of the SEC column. It should be recognized that the enhanced selectivity present in this system is not always desirable. In many applications, it will be the objective to analyze a group of similar compounds. In the case of this trapping system, however, only a single

component was determined. Examining the structures of the catecholamines would suggest that these compounds should all co-elute on the SEC column. This, however, is not the case, due to the non-exclusion mechanisms present in the Micropak TSK Type SW SEC column described previously.

Comparison can also be made between the use of SEC and IPC as primary separations. The comparison of these two techniques is most easily made by examining the motivation behind the use of the two systems. In the trapping system, the use of SEC resulted in high selectivity, as described in the previous paragraph. The injection of a trapped fraction introduced an initial peak volume in the secondary IPC separation of 100 µl. This volume was not great enough to interfere with the overall efficiency of the separation. The use of larger volumes, however, did add significantly to the band broadening. The switching system can be thought of as an extension of the trapping system to very large transfer volumes. In the IPC separation, there are no conditions under which the analytes were strongly retained. Thus, on-column concentration techniques could not be exploited as in the case of the analysis of theophylline and caffeine. In the absence of these ancillary techniques, the large peak width of the SEC separation has severe effects on the overall resolution of the IPC separation. For this

reason, an alternate fractionation step was used. Comparing Figures 33 and 39 , it can be seen that overall peak volume of the individual peaks is considerably less in the case of the IPC separation. The comparison of the results of using the IPC separation instead of the SEC separation can be seen in Figures 36 and 40. The resolution obtained using the two IPC columns in series is considerably better than that obtained using the SEC and the IPC column in series. Obviously, this increase in resolution is carried over in the analysis of real samples. Thus it can be seen that without techniques to compensate for the band broadening introduced by the primary separation, this effect can be overwhelming. Wherever possible, on column concentration methods should be used, and, in the absence of this possibility, it is essential that the band broadening be minimized.

As stated in the introduction to this application, one of the primary reasons for using multidimensional chromatography in the analysis of catecholamines in urine was to protect the electrochemical detection system from electrode poisoning. According to Riggins and Kissinger (164), the poisoning of the electrodes in the analysis of biological fluids has two major components. The first is the pseudo-irreversible adsorption of polar compounds on the

surface of the glassy carbon working electrode. The term "pseudo-irreversible" refers to the fact that while the adsorbed materials can be removed by polishing techniques and soaking in methanol, they do not freely desorb in aqueous solutions. The use of the multidimensional chromatographic techniques discussed here can minimize this effect by isolating the electrode from the eluting fractions containing these polar compounds. The second poisoning effect is due to the analytes themselves, and this cannot be avoided through the use of LC/LC. The effect in this case is due to the polymerization of the catechols and phenols on the surface of the electrode. The analysis of dilute samples will minimize this effect.

The use of multidimensional chromatography effectively decreases the electrode poisoning effect due to the adsorption of polar compounds on the surface of the glassy carbon working electrode. Figure 43 shows the effect of this technique on the electode noise as a function of the number of analyses. The lower curve in this figure shows the increase in the detector noise in a multidimensional LC/LC system. The upper curve shows the increase in the detector noise without the use of switching techniques. The analysis used to evaluate this effect was the coupling of two IPC systems in a switching mode described above. The

samples run consisted of 10 μl samples of undiluted normal urine. The detector noise was measured as peak to peak noise in nanoamps (nA) at the base line between samples. It can be seen that the reduction in electrode poisoning is significant. It should be noted that even with the switching technique, there is an increase in the detector noise, which is attributed to the polymerization of catecholamines and phenols in the sample.

5.2.1.4 Summary: LC/LC

One of the characteristics which differentiates the methods described in the preceding section is the method of sample transfer, ie. trapping or switching. In all cases it can be seen that the trapping system shows high selectivity. but poor detectibility. Conversely, the switching system shows improved detectibility, but poor selectivity. Another factor is the use of on-column concentration techniques in the sample transfer process. Although this is a critical step in the switching system, it may improve the overall resolution in a trapping system as well. If the peak width in the initial fractionation system is excessive, it will lead to reduced resolution. This was the case in the coupling of SEC and IPC in the analysis of catecholamines in urine. This effect can be circumvented in three ways. A

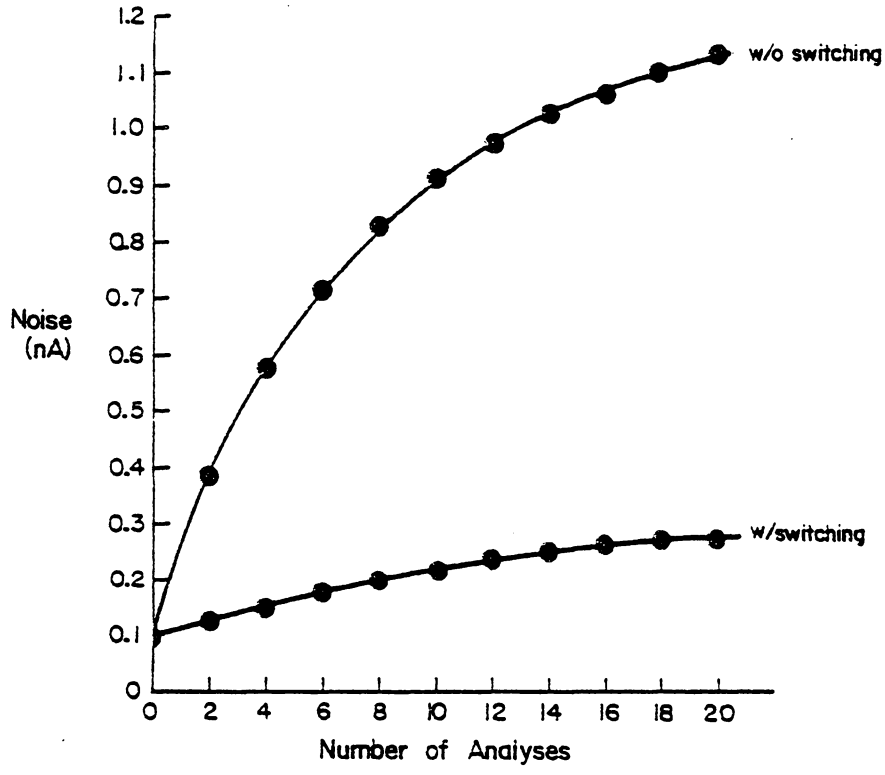


Figure 43. Effect of LC/LC Operation on Detector Noise

trapping system can be used. This was one approach used in the catecholamine analysis. On-column concentration methods can be used. This was employed with favorable results in the case of the theophylline and caffeine separation. Finally, if the initial fractionation separation has the same efficiency as the analytical separation, the band broadening contribution may not be severe. This can be seen in the hydrocarbon group analysis and the coupling of two IPC systems in the catecholamine analysis.

In terms of methodology, the objective of the three major applications differed. The analysis of theophylline and caffeine in urine was used exclusively as a sample clean-up technique. Since the analytes co-eluted from the initial column, it did not contribute effectively to the resolution. In this clean-up procedure, only a small portion of the total sample, containing theophylline and caffeine, was of analytical interest. In the analysis of hydrocarbon groups, on the other hand, the entire sample was analyzed and all three columns contributed significantly to the separation. Finally, in the analysis of catecholamines in urine, the system was used for the protection of the electrochemical detector. In terms of the separation, the objectives of this analysis were a combination of clean-up and selectivity optimization.

Each of the three analyses utilized differing mechanisms of separation to achieve their objectives. The analysis of theophylline and caffeine used the adjustment of selectivity to effect the sample clean-up involved. The hydrocarbon group analysis relied almost exclusively on the optimization of selectivity to achieve an otherwise difficult separation. The analysis of catecholamines in urine utilized two mechanisms. In the coupling of SEC and IPC, selectivity optimization was used and in the coupling of two IPC systems, the capacity factor was adjusted.

The mobile phases used point out some interesting characteristics of these three analyses. The analysis of theophylline and caffeine used the mobile phase to contribute to the adjustment of selectivity. The analysis of hydrocarbon groups on the other hand depended on the stationary phases almost entirely. Due to the detector restrictions in the analysis of catecholamines, the adjustment of both selectivity and capacity factor depended on stationary phase characteristics.

Some of these applications are more successful than others. The analysis of hydrocarbon groups was succesful and acheived all the goals expected of it. The analysis of theophylline and caffeine was also highly successful in its switching form. The trapping system was not successful, but

it does contribute to the understanding of the optimum use of LC/LC. While the analysis of catecholamines in urine was successful in its goal of protecting the electrochemical detector, it was not succesful in its switching forms for the analysis of a broad spectrum of catecholamines. The trapping system on the other hand, not only protected the detector, but was also useful in the analysis of a single analyte.

5.2.2 LC/GC Applications

It will be noted that the discussions of the following applications are somewhat shorter than those of the LC/LC applications. This is because the operation of LC/GC systems is conceptually more direct than the complex valving schemes and flow patterns used in LC/LC. In this connection, it is interesting to observe that although LC/GC appears conceptually simpler than LC/LC, reports in the literature are much less common for LC/GC than LC/LC.

5.2.2.1 Pesticides in Butter

Chronologically, the analysis of pesticides in butter by LC/GC is the first application performed in this research. It served as a model for both the development of techniques and the preliminary evaluations of the effects of operating parameters on quantitation.

Many GC analyses of complex environmental and biological samples require extensive sample clean-up procedures involving extractions, distillations and column chromatographic clean-up. The objective of this application is to demonstrate the use of LC/GC as an automated sample clean-up technique for the analysis of pesticides in butter.

The initial fractionation of a butter sample dissolved in THF was performed on a Micropak TSK 1000H organic SEC column using THF as a mobile phase. At a fixed time following the injection of the sample into the SEC column, 10 µl of the LC effluent was injected into a packed GC column. The injection time was determined from the retention time of pp'DDT standards. The GC separation was isothermal and GC detection was by electron capture detection (ECD). The system was completely automated and the entire analysis of a sample required twenty minutes.

Figure 44 shows the analysis of a pp'DDT standard by this method. The upper trace in this figure represents the LC UV detector operated at 214 nm and the lower trace represents the GC ECD response. Figures 44 a and b show the analysis of a blank and a sample containing the pesticide, respectively. The MDC for pp'DDT for this method was found to be 4.5×10^{-4} mg/ml with linearity to 0.1 mg/ml.

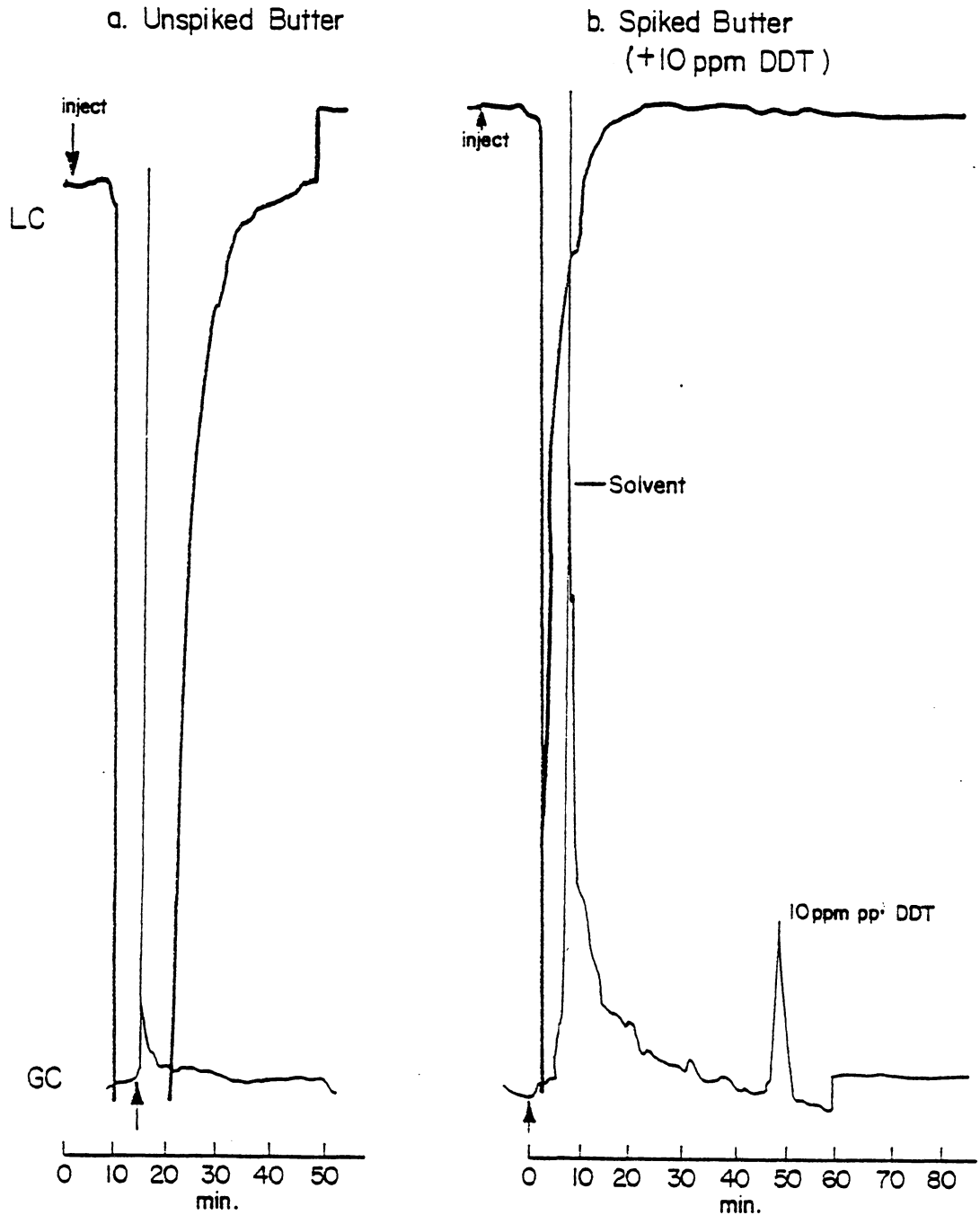


Figure 44. LC/GC Analysis of pp'DDT Standard

Unlike the Micropak TSK Type SW aqueous SEC column, the Type H organic SEC columns are a polystyrene divinylbenzene polymer and the separation mechanism is primarily one of exclusion. This means that compounds of a similar molecular size will co-elute. Therefore, in the presence of other pesticides similar to DDT, multiple pesticide analyses can be performed from a single LC/GC sampling point. Figures 45 and 46 show the analysis of a butter sample containing the thirteen pesticides in an EPA pesticide mixture listed in Table 4. Figure 45 shows the SEC analysis of a butter sample and Figure 46 shows the GC analysis of a sample injected at point marked "A" on the SEC analysis. Note that although there are thirteen pesticides present in the sample, only five of them co-elute near enough to pp'DDT in the SEC fractionation to be included in the LC/GC sample.

The use of strong solvents such as THF can lead to rapid column degradation. This is shown in Figure 47 In this graph, the number of theoretical plates in the GC column is plotted as a function of the number of days of use. Up to fifty 10 µl injections were made per day. The column efficiency decreases steadily. This demonstrates a significant difference between on-line and off-line LC/GC procedures. In the application of this technique, off-line, the pesticide containing fractions could have been collected

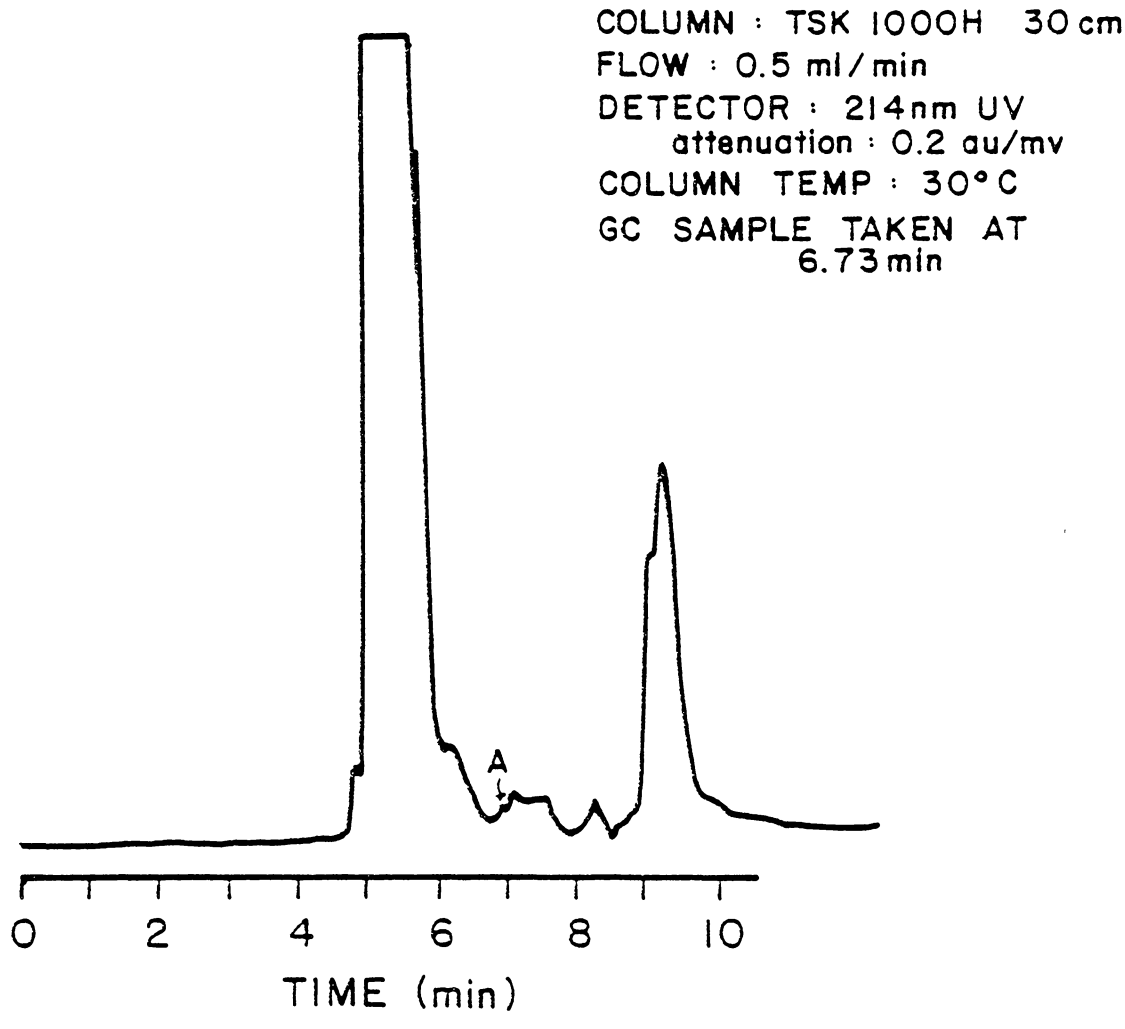


Figure 45. LC Fractionation of Butter Sample

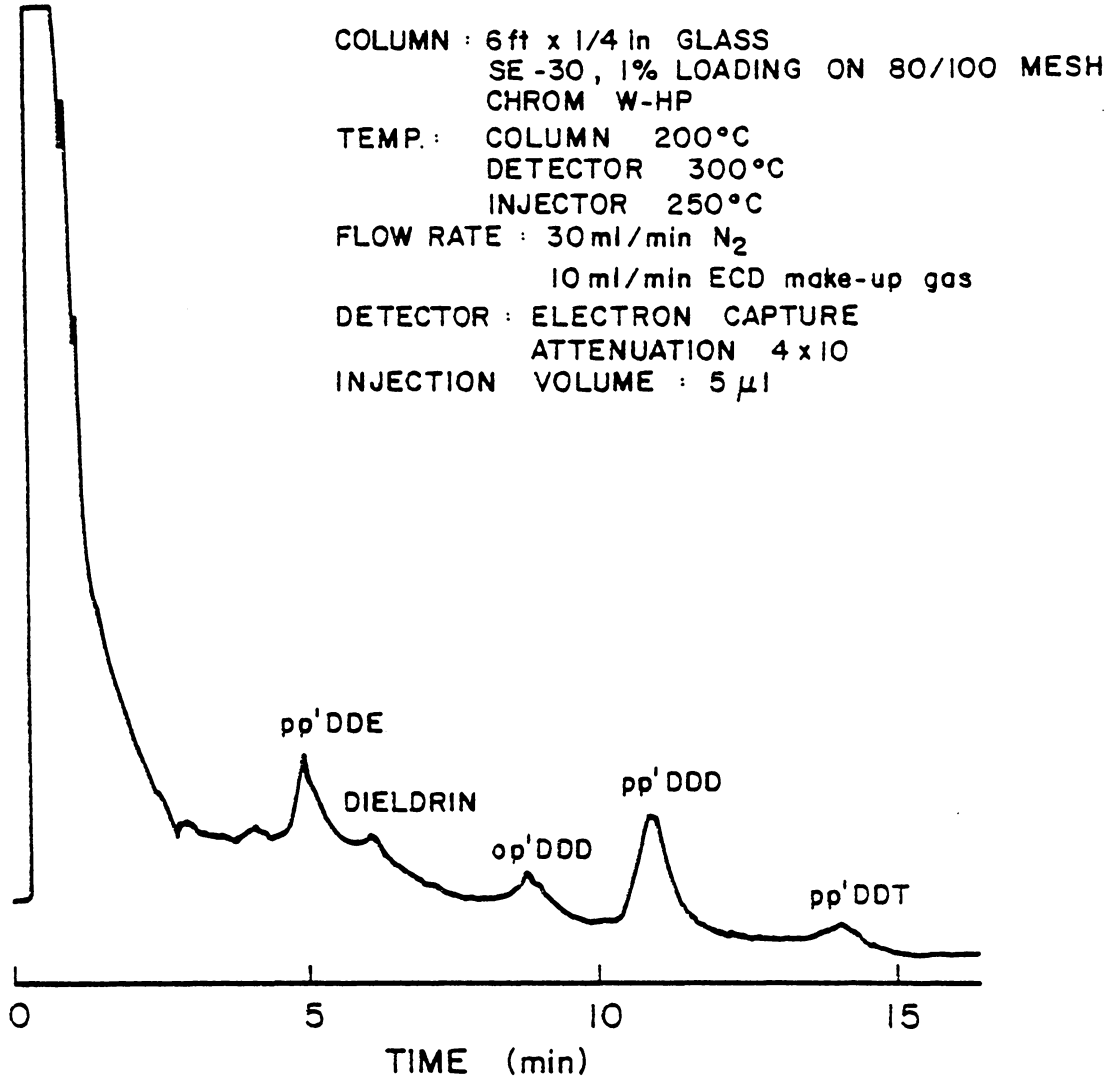


Figure 46. LC/GC Analysis of Pesticides in Butter

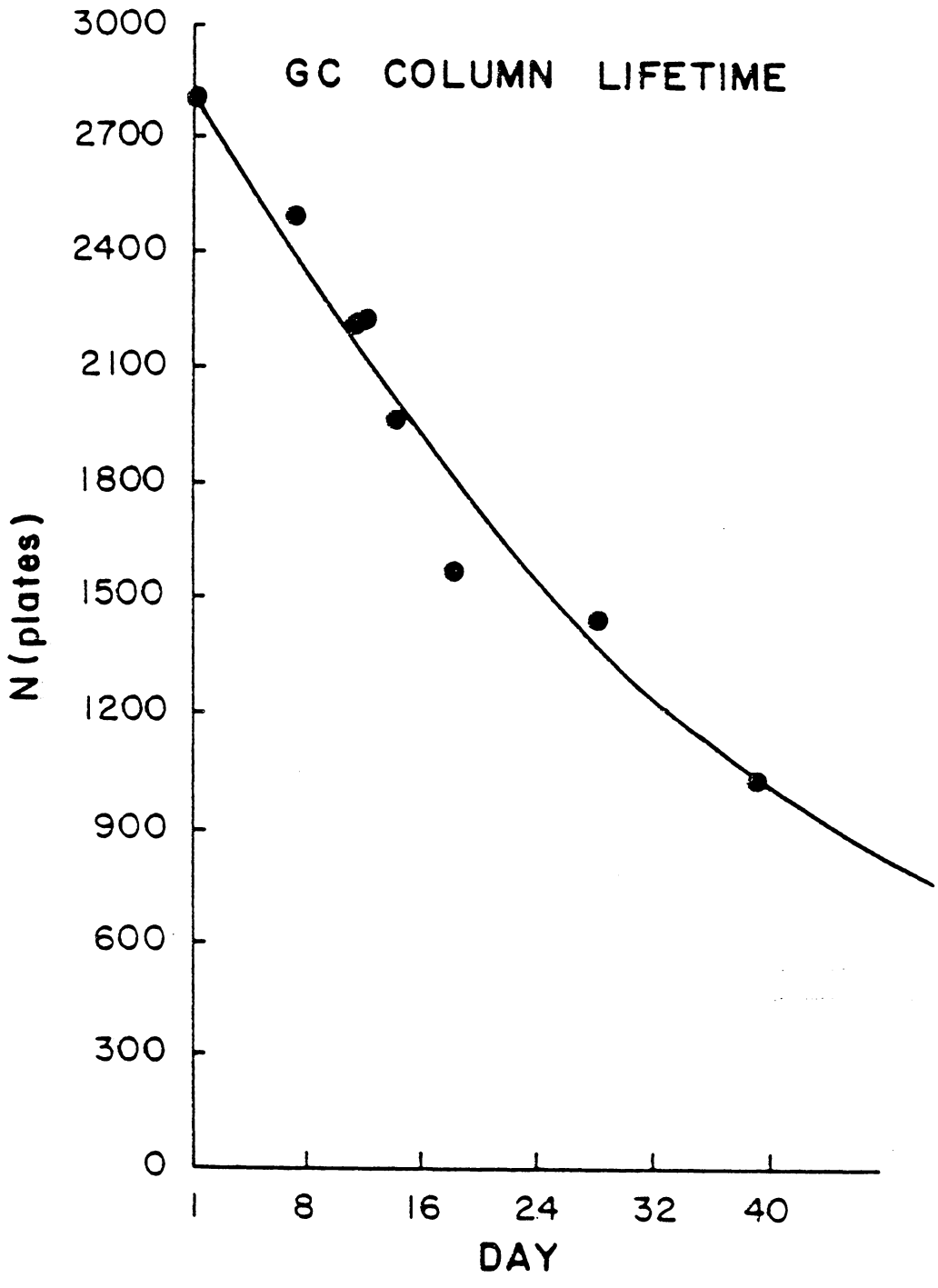


Figure 47. Effect of LC/GC on Column Lifetime

and the THF evaporated. The sample could then be redissolved in a solvent more compatible with the GC column and injection procedure. On the other hand, off-line procedures would not be capable of the high selectivity obtained when the on-line procedure accurately and reproducibly samples a 10 µl portion of the LC effluent.

This application provided a fast simple automated analysis for selected pesticides in butter. Because of the stability and versatility of the SEC column used, this analysis could be easily transferred to the analysis of pesticides in similar samples with minor modifications. Due to the high selectivity of the LC/GC transfer process, the overall analytical application is relatively independent of the sample matrix analyzed.

5.2.2.2 Hydrocarbon Group Analysis

The application of LC/LC to the analysis of hydrocarbon groups has already been discussed. On-line multidimensional LC/GC can be applied to this type of analysis as well. In th case of LC/LC, the objective was to determine the relative amounts of the individual hydrocarbon groups. In the following LC/GC application, the analysis is taken a step further to characterize the distribution of components within the individual groups according to carbon number.

The initial fractionation in this application was performed on a 30 cm cyanopropyl bonded silica column and a 30 cm silica column in series. This column series corresponds to the first two columns used in the LC/LC hydrocarbon group analysis. In this application however, the silver nitrate impregnated silica column is omitted. At times corresponding to the maxima of the individual hydrocarbon groups, 3 µl volumes of the effluent from the LC fractionation were trapped by the LC/GC interface and injected into the GC. The GC separation was performed on a 30m x 0.25mm i.d. WCOT SP-2100 glass capillary using temperature programming.

Figure 48 shows the LC analysis of 10 µl of gasoline. Figure 49 shows the capillary GC analysis of a fraction sampled by the LC/GC interface at the maximum of the LC saturates peak for gasoline. The evenly spaced large single peaks correspond to the normal paraffins. Between each pair of normal paraffin peaks there are two smaller groups of peaks which correspond to the 1- and 2- branched paraffins. Figure 50 shows the GC analysis of the fraction sampled at the maximum of the LC unsaturates peak. This chromatogram is more complex than that obtained for the saturates distribution, and while the individual peaks are not identified, it is possible to identify the chain length of

each peak group. Figures 51 and 52 show the GC analysis of the fractions sampled at the two maxima of the LC aromatics peak. The aromatics do not conform to a simple carbon number distribution as in the case of the saturates and unsaturates, and in this distribution only a few of the peaks have been identified by comparison of retention times with standards.

Figures 53 through 56 show the corresponding analyses for diesel fuel. In Figure 53, the LC fractionation of diesel fuel is shown. Note the change in attenuation. The saturates peak for diesel fuel is an order of magnitude larger than the corresponding peak for gasoline. The unsaturates in diesel fuel, on the other hand, are present in low concentration. Figure 54 shows the GC analysis of the saturates fraction, sampled by the LC/GC interface. Comparing this to Figure 50, it can be seen that the saturates distribution in diesel fuel is heavier than in gasoline, the maximum being around C-15 compared to C-11 for gasoline. Figures 55 and 56 show the GC analyses of four aromatic fractions, sampled at one minute intervals from the LC effluent by the LC/GC interface. As in the case of gasoline several of these peaks have been identified.

In this application, the fractions transferred from the LC to the GC were injected using splitless injection

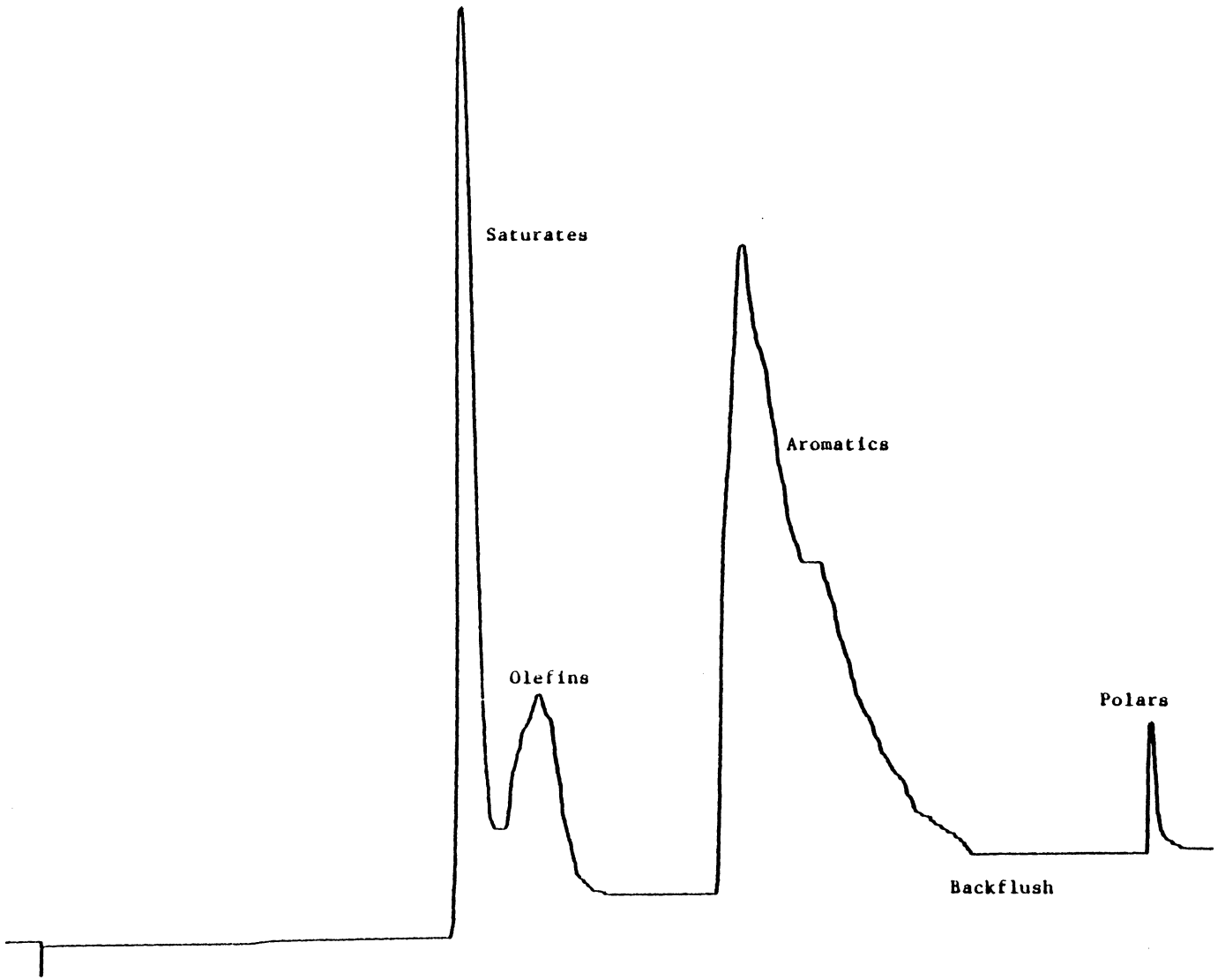


Figure 48: LC Fractionation of Gasoline

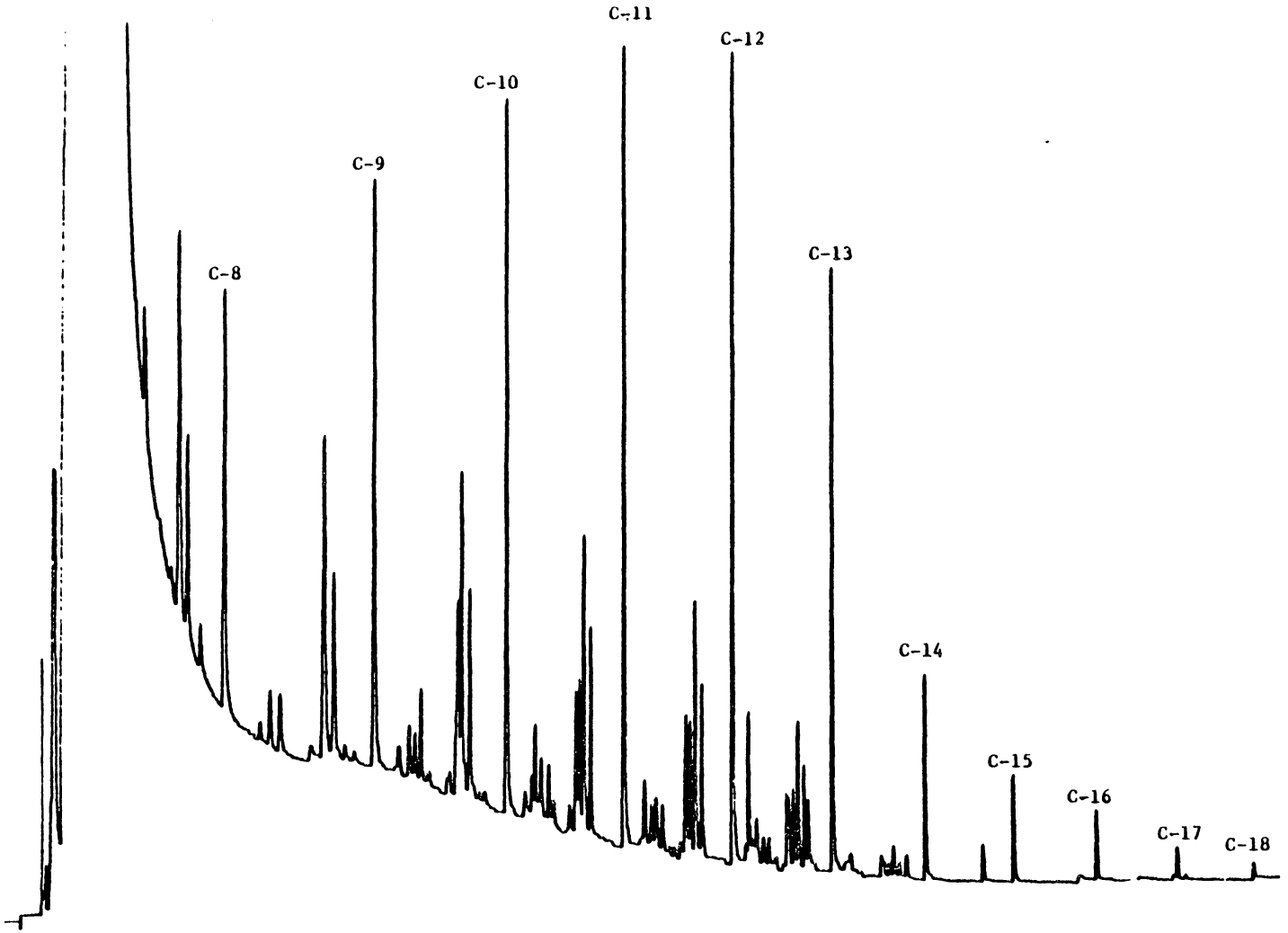


Figure 49: LC/GC Analysis of Gasoline Saturates

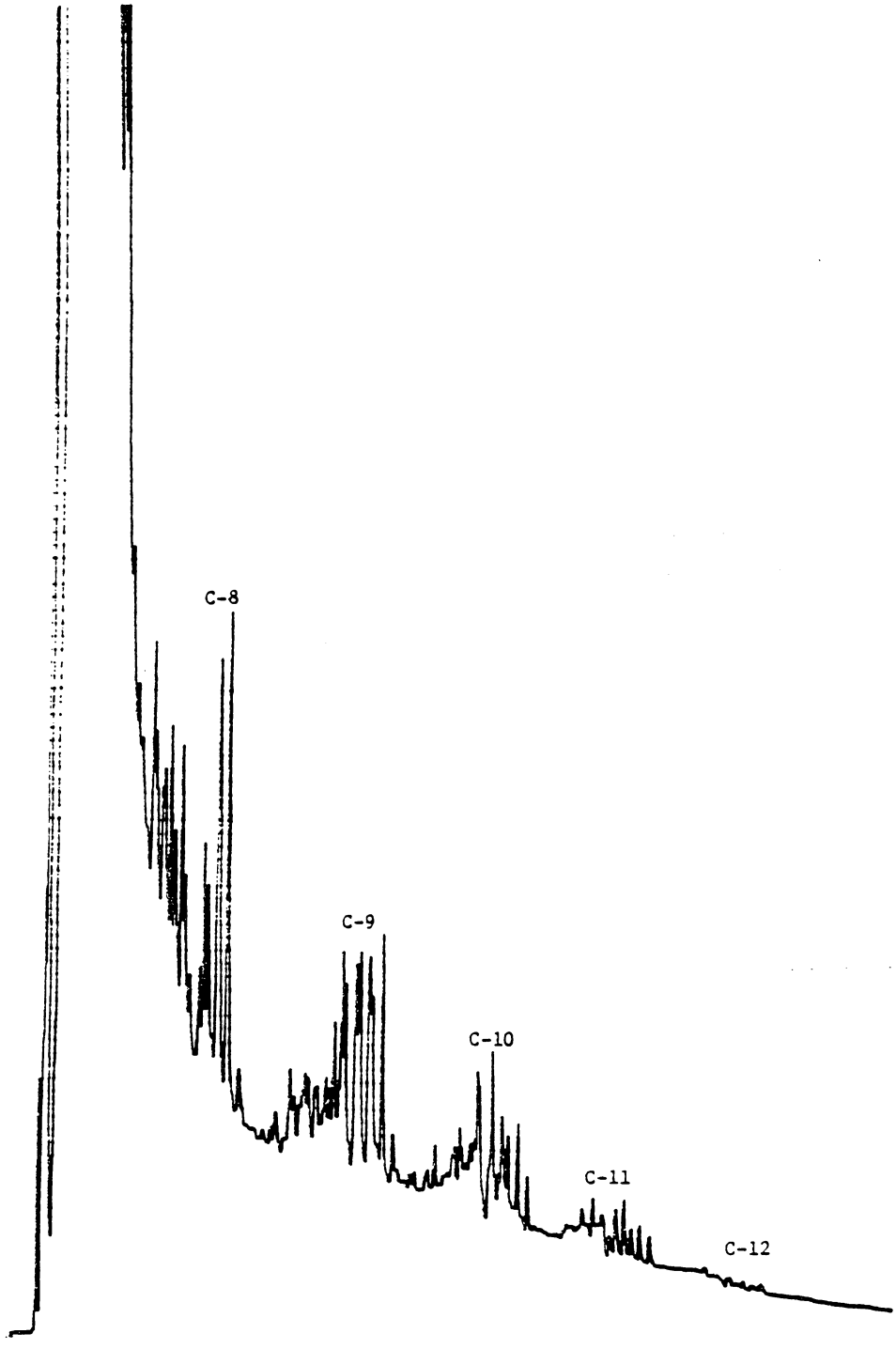


Figure 50: LC/GC Analysis of Gasoline Unsaturates

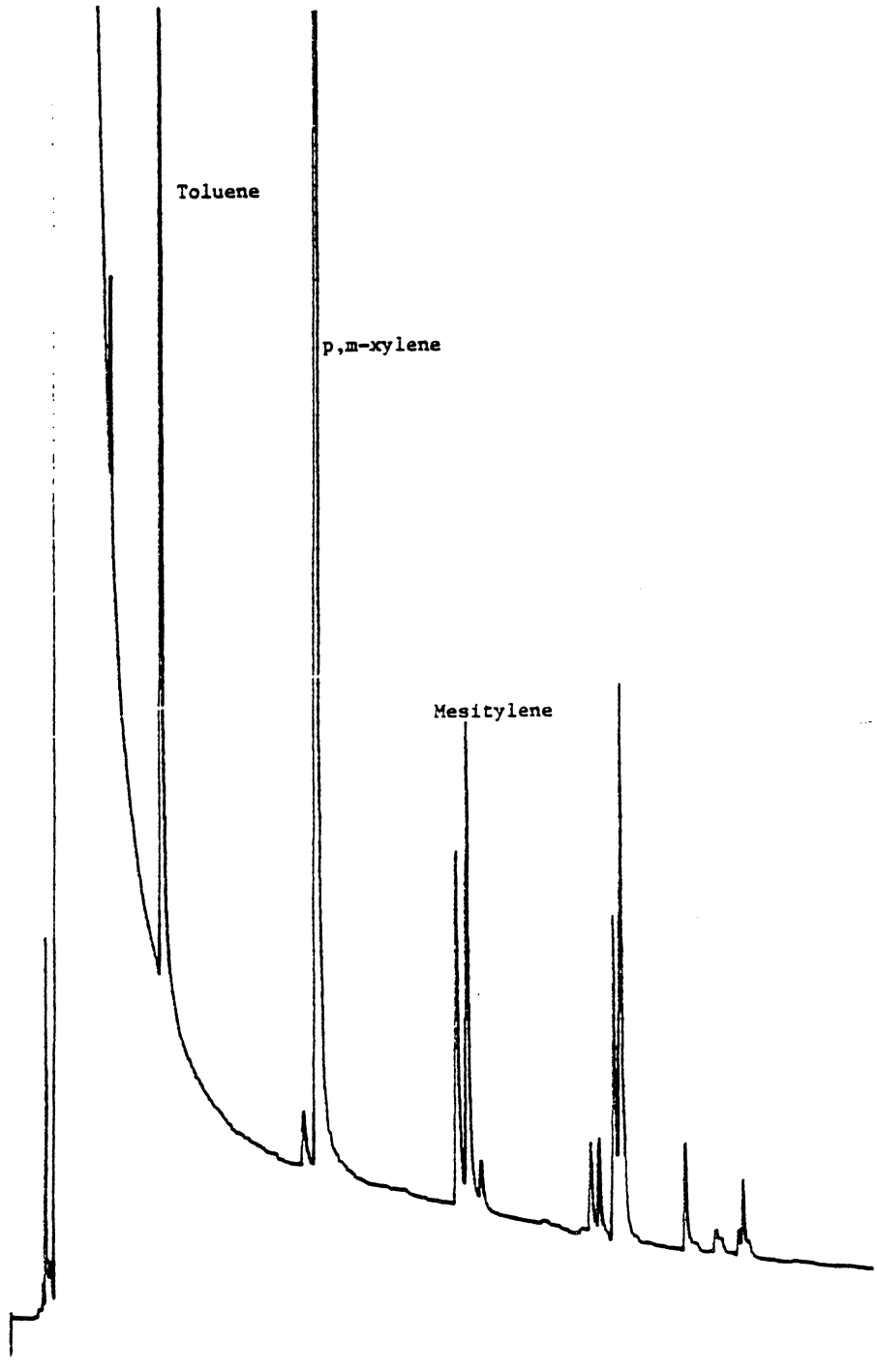


Figure 51: LC/GC Analysis of Gasoline Aromatics: I

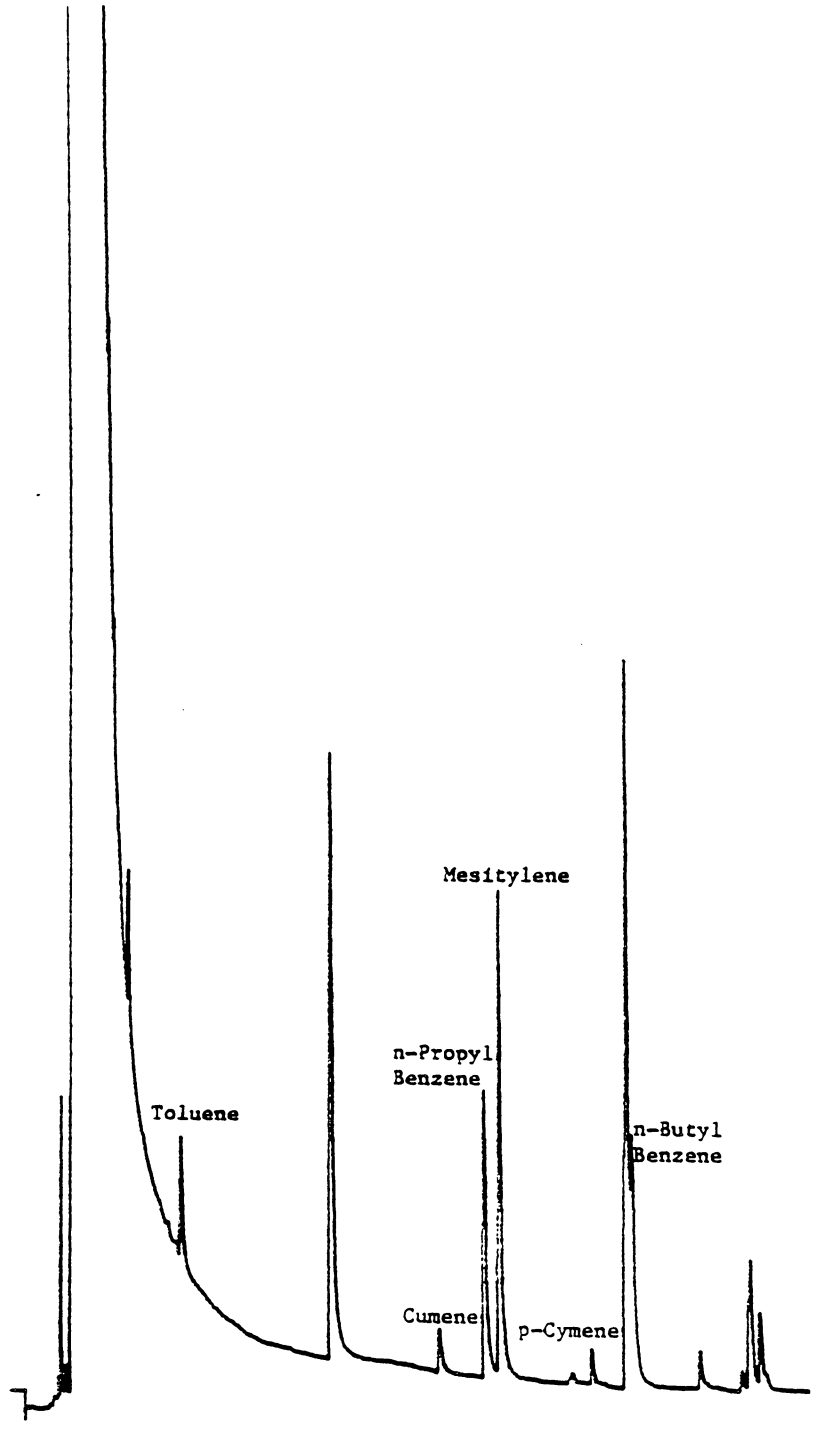


Figure 52: LC/GC Analysis of Gasoline Aromatics: II

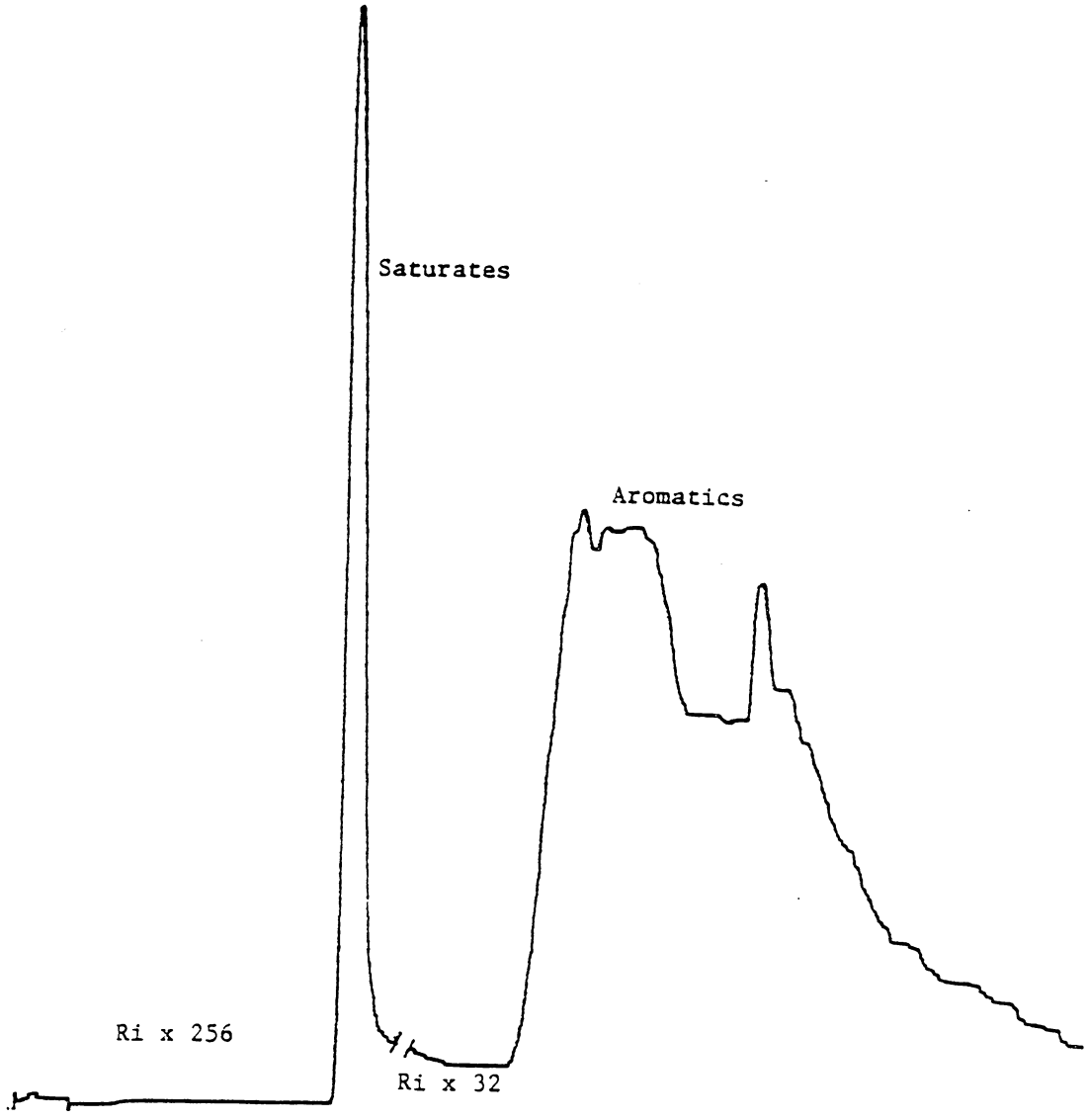


Figure 53: LC Fractionation of Diesel Fuel

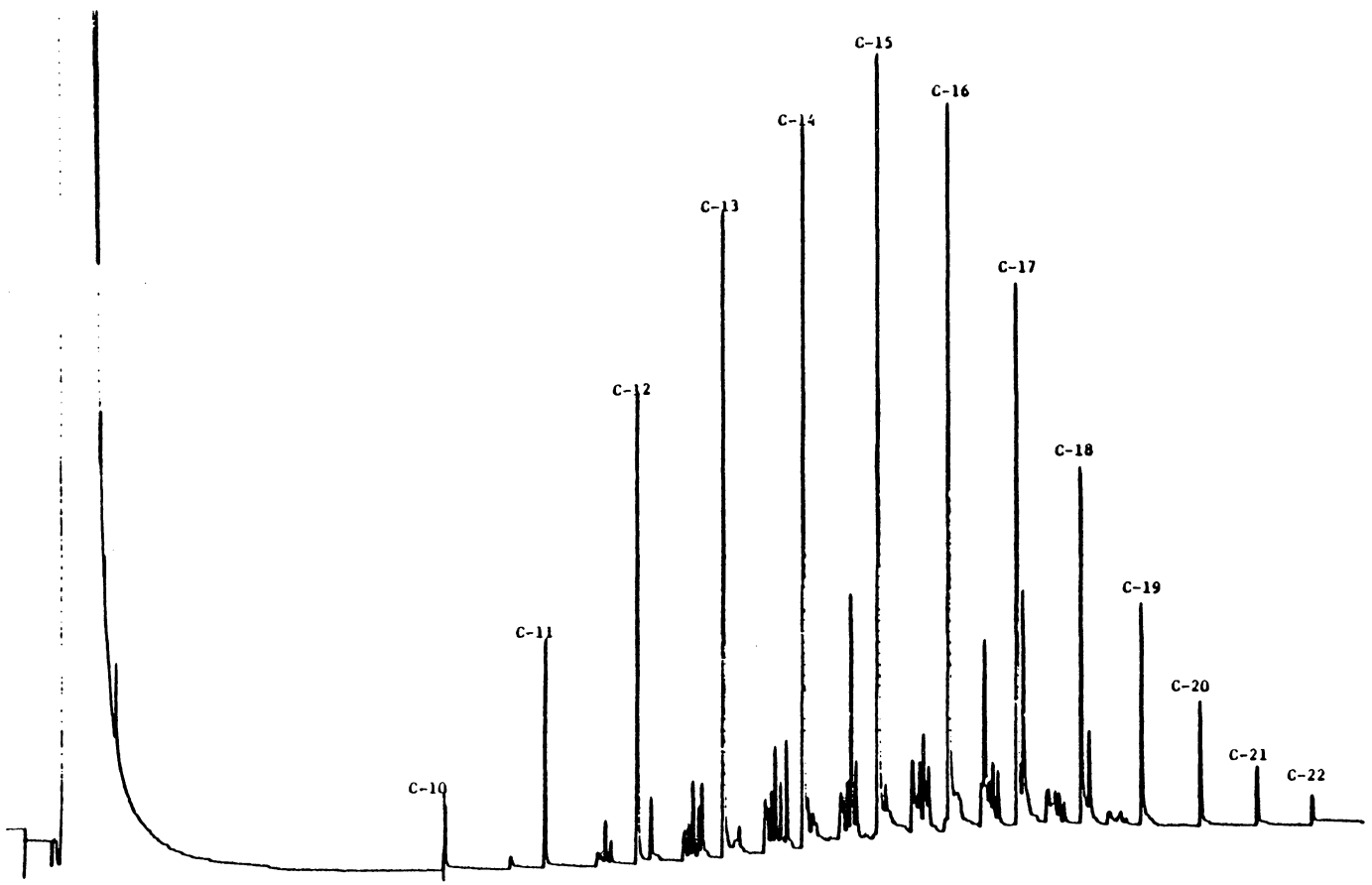


Figure 54: LC/GC Analysis of Diesel Fuel Saturates

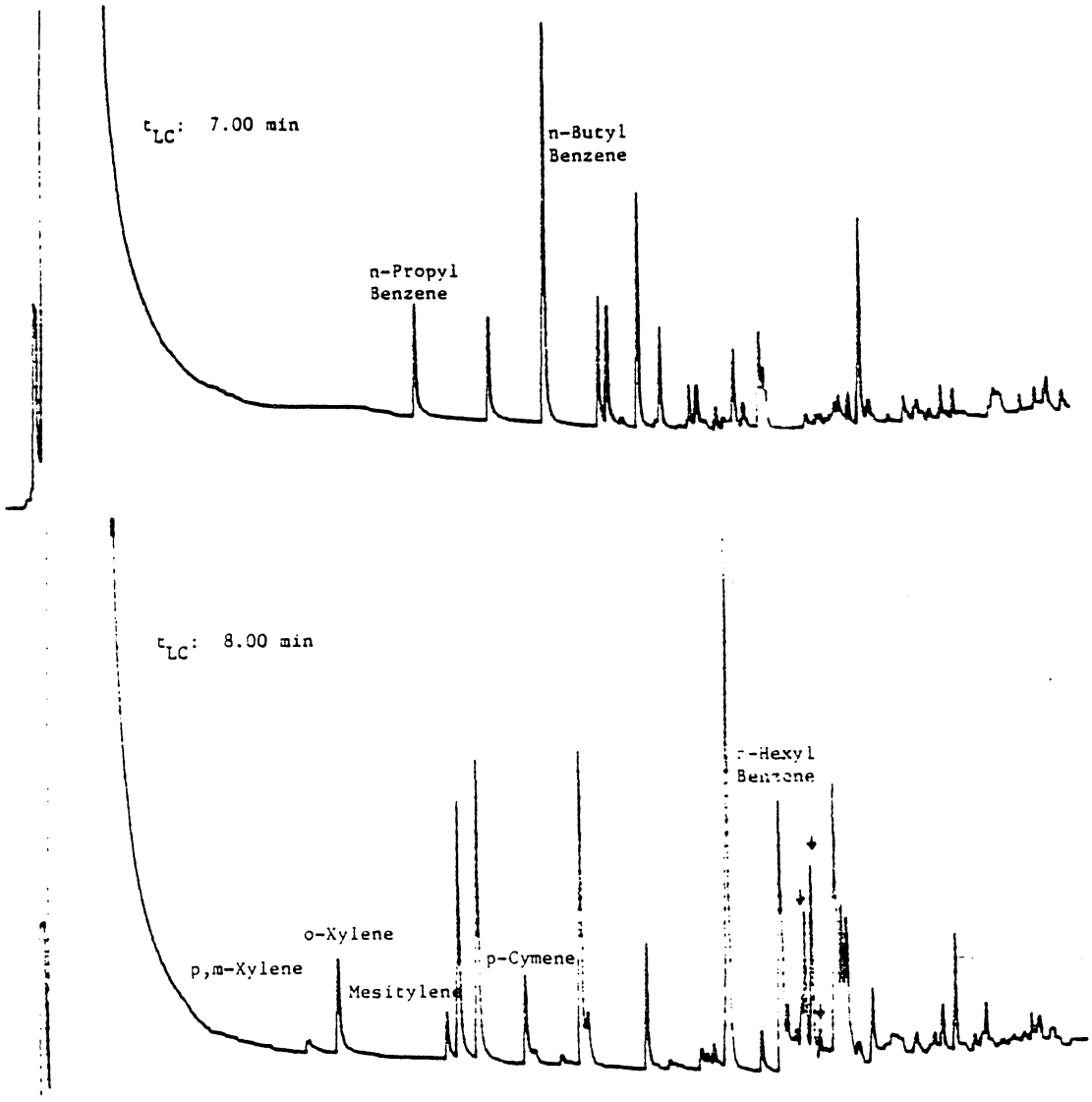


Figure 55: LC/GC Analysis of Diesel Fuel Aromatics: I

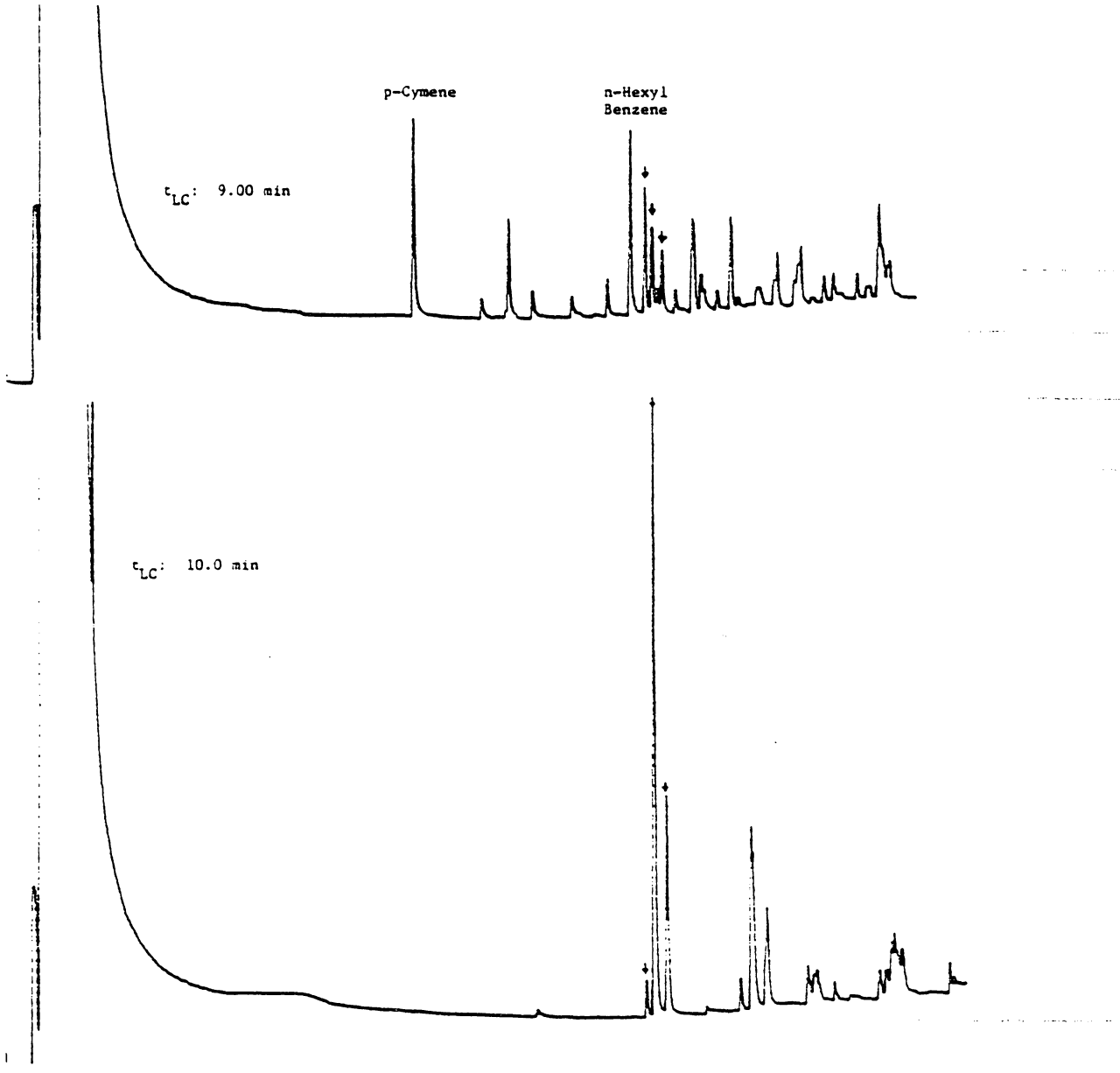


Figure 56: LC/GC Analysis of Diesel Fuel Aromatics: II

techniques. As previously discussed, this is an ideal system for the injection of samples from the LC. Using hexane as a mobile phase allows the use of the solvent effect to improve the band broadening characteristics of the injection process. Samples were initially injected in 10 µl undiluted samples into the LC. After dilution takes place in the LC separation, the individual components are present in concentrations which are sufficiently low that the capacity of the capillary column is not exceeded.

Referring to Figure 48, it can be seen that the saturates and unsaturates are not completely resolved by the LC separation. It is not neccesary, however, for complete separation to take place. The resolution of the two groups is sufficient that GC analysis of fractions sampled at the two maxima show little overlap between groups. By sampling small fractions, the LC/GC system greatly enhances the selectivity of the overall separation. This can be seen in the GC analysis of the aromatic fractions, as well. The efficiency of the LC column is sufficient that GC analysis of fractions sampled 1 ml apart for the most part contain different aromatics. Referring to Figures 55 and 56, an exception to this can be seen in that the peaks marked with arrows appear in all four chromatogram. The major peak in this group must therefore be present in high concnetration

leading to a broad LC peak. Although the aromatic distribution shows some resolution within the LC peak, the saturates and unsaturates elute from the LC as homogeneous peaks. Sampling at different points on these peaks changes the overall concentration of the sample injected into the GC but not the distribution of the components within the sample.

In order to emphasize the high selectivity of the LC/GC analysis, Figure 57 shows the analysis of a gasoline sample by capillary GC without prior LC fractionation. The chromatogram is complex and it is not possible to determine any trends present in the distribution of peaks.

A final point which should be mentioned about this application concerns the quantitation. As mentioned in the LC/LC approach to hydrocarbon group analysis, quantitation in such systems is difficult. This is primarily due to the inability to determine an absolute calibration factor for a peak containing numerous components. In the LC/GC case, though, this is not a problem. The absolute concentration of a particular group can be determined by summing the absolute amounts of individual components determined by the capillary GC separation. Although this may seem a laborious and time consuming proceedure, through the use of modern chromatographic data systems, this entire analysis, including quantitation, can be performed automatically.

5.2.2.3 PAH's in Fuel Related Sources

In the last decade, the interest in the health effects of a wide range of organic pollutants has grown considerably. The recognition of the carcinogenic effects of polynuclear aromatic hydrocarbons (PAH's) has prompted the development of a number of analytical techniques capable of detecting these compounds at trace levels in a variety of samples. Two of the most widely used techniques are HPLC and Capillary GC.

The analysis of PAH's by HPLC is particularly attractive with the use of highly sensitive and selective fluoresence detection systems. Another attractive feature of reverse phase HPLC systems is the applicability of trace on-column concentration methods, which have been discussed previously.

The use of normal phase HPLC has also been used in the analysis of PAH's. Normal phase HPLC does not yield the higher resolution obtained by reverse phase systems in the separation of PAH's, but is extremely useful in fractionating PAH samples according to the number of aromatic carbons in the PAH ring system.

Capillary GC analyses have been used extensively in the analysis of PAH's. In terms of resolution, capillary GC is significantly more powerful compared to HPLC. There has

been a great deal of work performed on the separation of PAH's with capillary columns coated with phenyl silicone stationary phases such a SE-52 and SE-54. Lee *et al.* (168) have shown the resolution of 209 PAH's and have developed a retention index system for temperature programming analysis of PAH's. Separations have utilized both flame ionization detection (169) and electron capture detection (170). ECD shows high sensitivity for larger PAH ring systems but for two and three ring systems is of equal or less sensitivity to the FID.

The main disadvantage to the analysis of PAH's by capillary GC is the need for extensive sample preparation. GC analyses of complex petroleum samples require either extractions or column chromatographic separations prior to GC separation.

The aim of this application is to couple normal phase HPLC and capillary GC in order to take advantage of the excellent fractionationg power of normal phase HPLC and the high selectivity and resolution of capillary GC.

The initial fractionation of samples in this application was performed on an aminopropyl bonded silica column using mixtures of hexane and chloroform as a mobile phase. A guard column packed with pellicular cyanopropyl bonded silica was used to prevent damage to the

fractionating column by the complex fuel samples. Fractions of the effluent of this column were automatically sampled by the LC/GC interface and the PAE's present in these fractions were separated by capillary GC on a 30m x 0.5 mm i.d. WCOT SE-52 or SE-54 glass capillary column. Both temperature programming and isothermal analyses were used.

Figure 58 shows the separation of a 500 μl sample of the PAH standards listed in Table 6 . Although band broadening due to column overload is present, the resolution is still nearly complete. The larger sample volume is used to optimize detectibility. The peaks present in this figure correspond to ten, twelve, fourteen, sixteen, eighteen and twenty aromatic carbon fractions. Figure 59 shows the capillary GC separation of these PAH's.

Figures 60 through 63 show the application of the LC/GC procedure to the analysis of Amax Solvent Refined Coal (SRC). Figure 60 shows the LC fractionation of 500 l of a 1:40 dilution of SRC in hexane. Figures 61, 62 and 63 show the capillary GC separation of fractions from the LC peaks 1, 3 and 4, sampled by the LC/GC interface.

Figure 64 shows the direct capillary GC analysis of the Amax SRC sample without prior LC fractionation. Although the three major components, anthracene, fluoranthene and pyrene can be identified, the lower concentration PAH's are

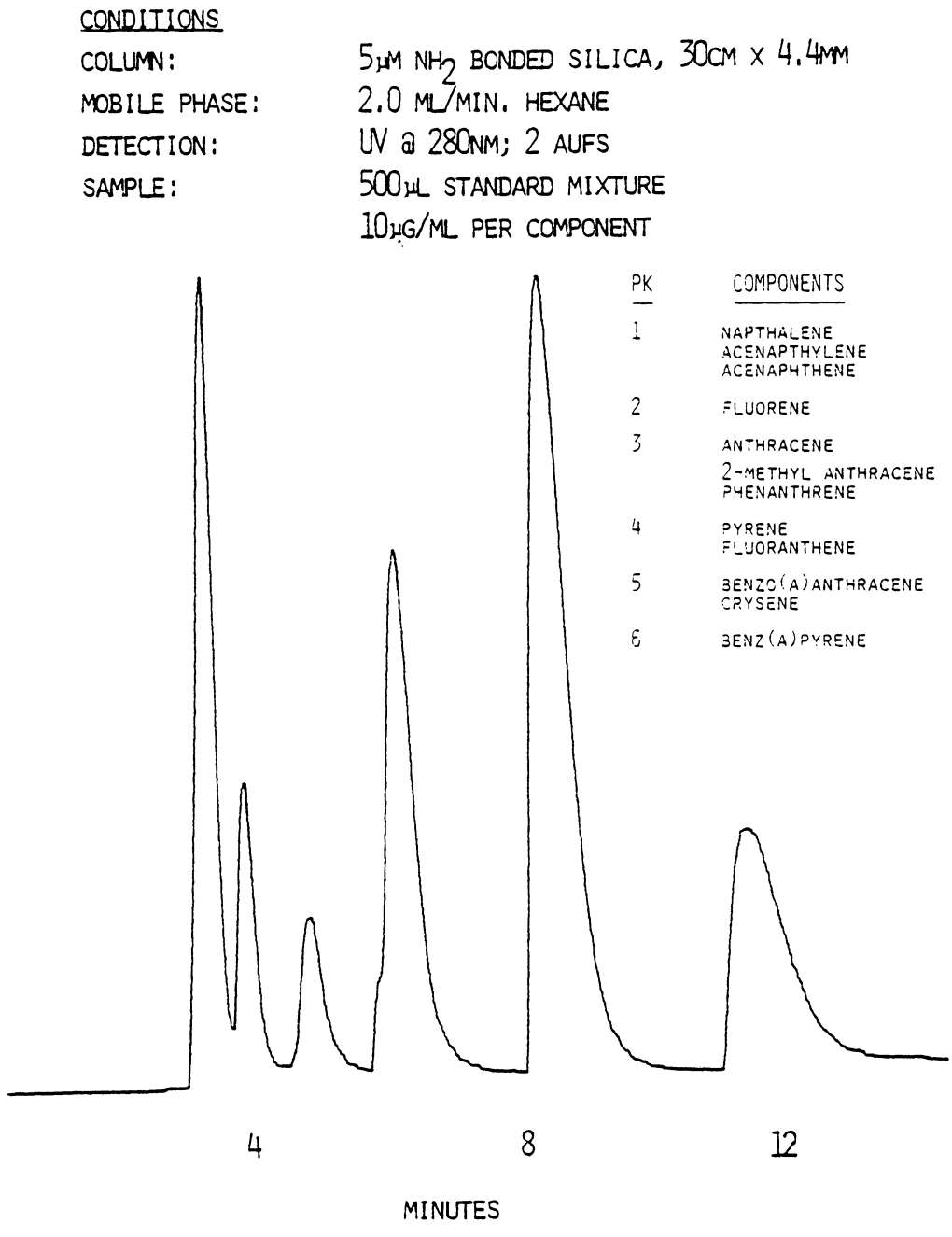


Figure 58. LC Fractionation of PAH Standards

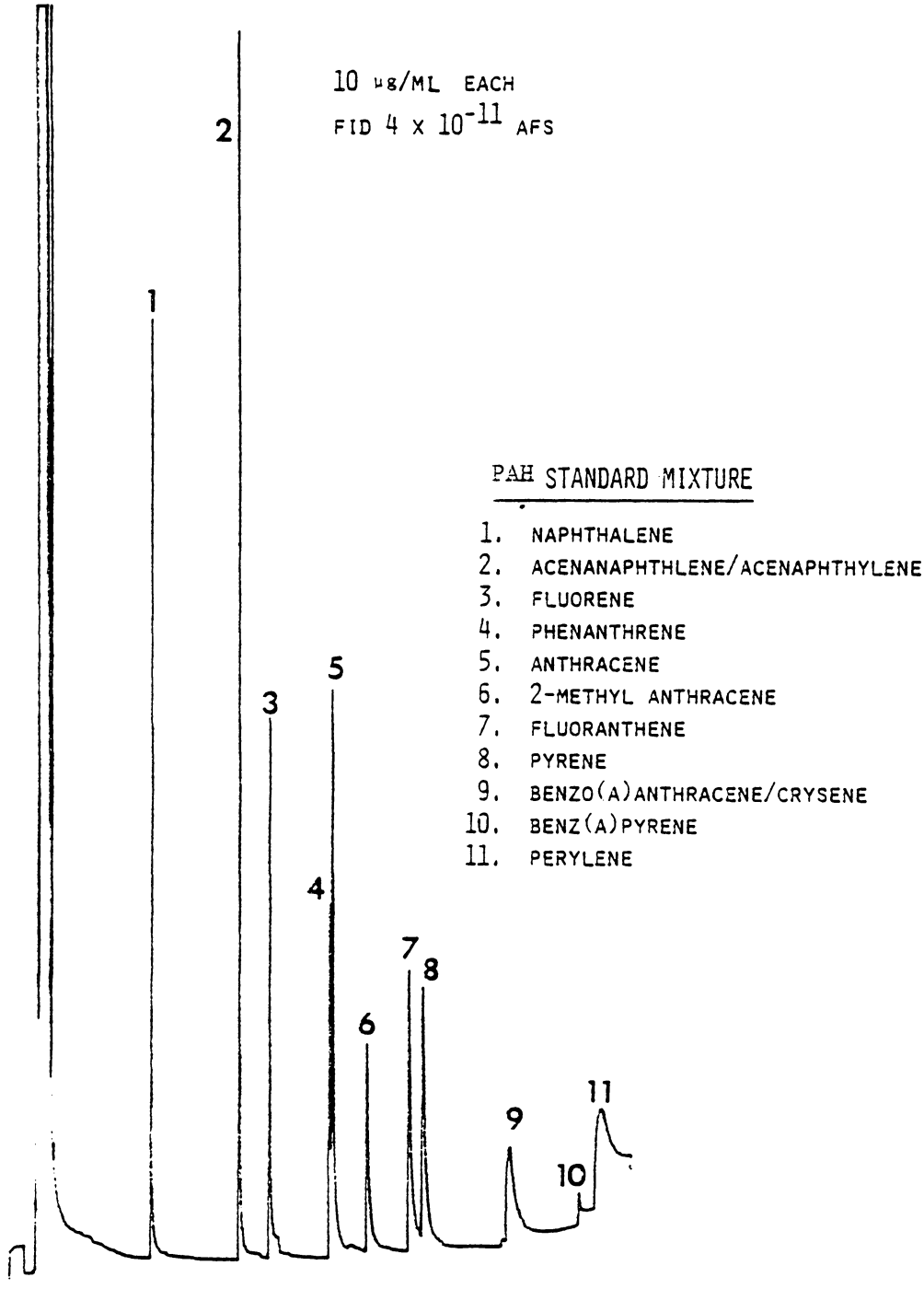


Figure 59. Capillary GC Analysis of PAH Standards

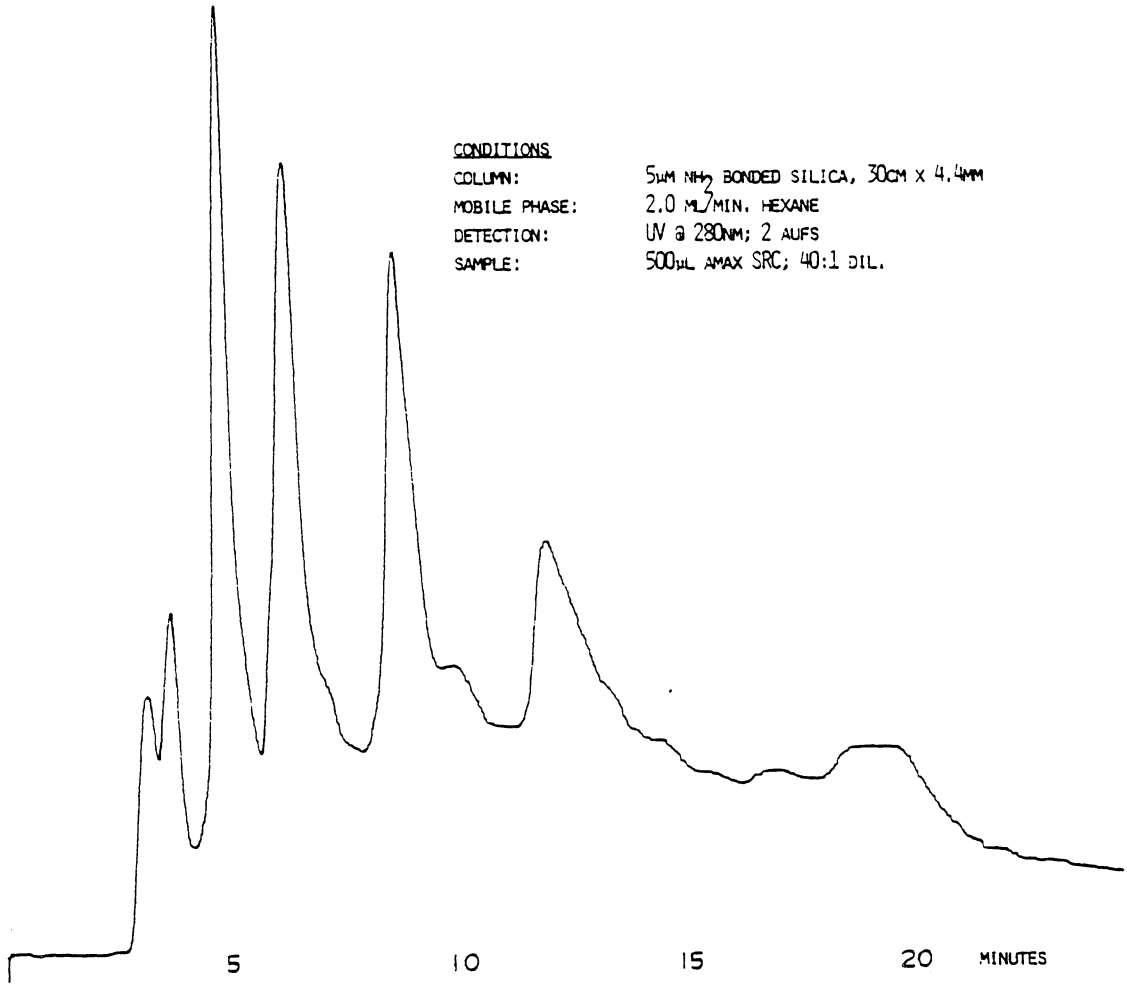


Figure 60. LC Fractionation of AMAX SRC

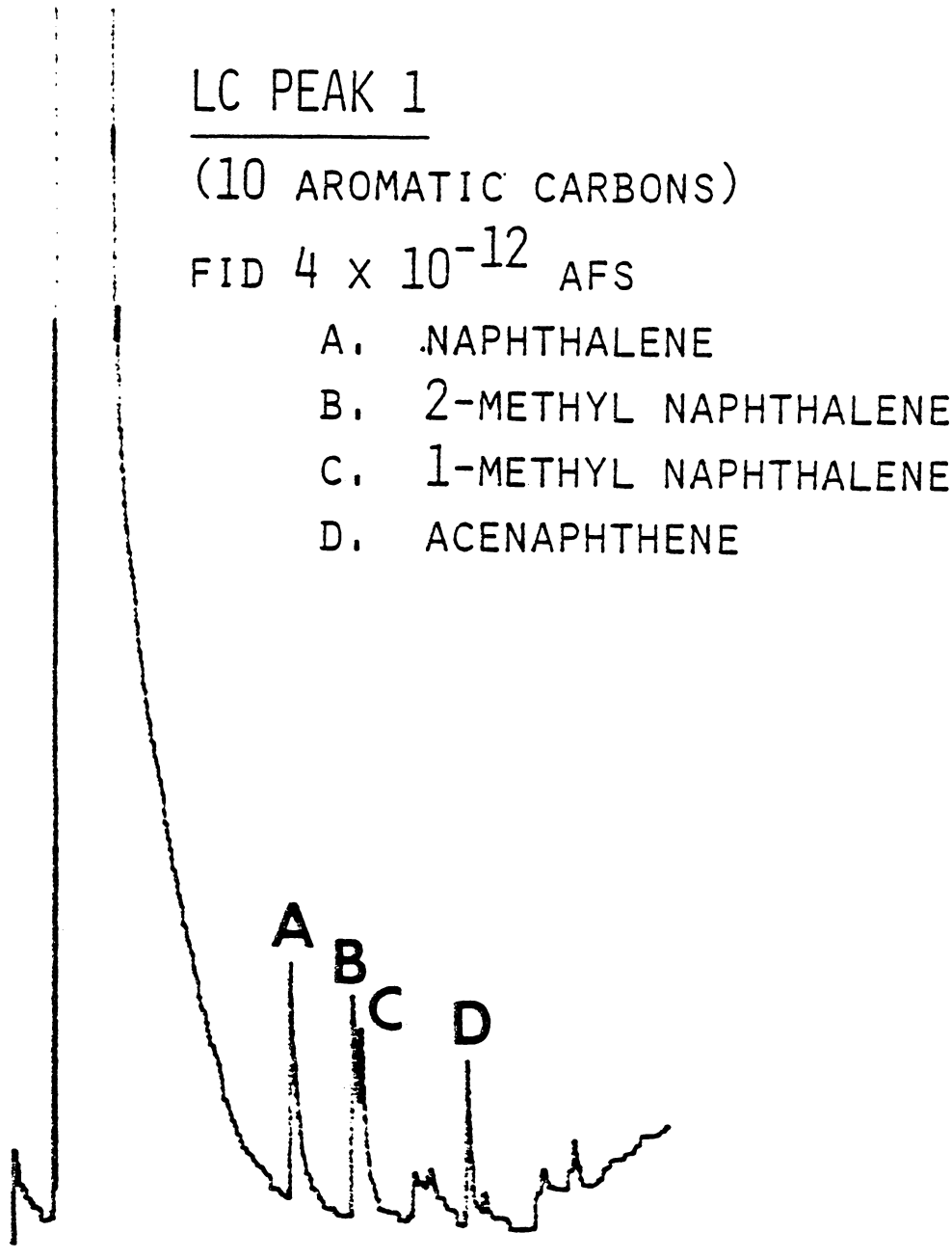


Figure 61. LC/GC Analysis of AMAX SRC: I

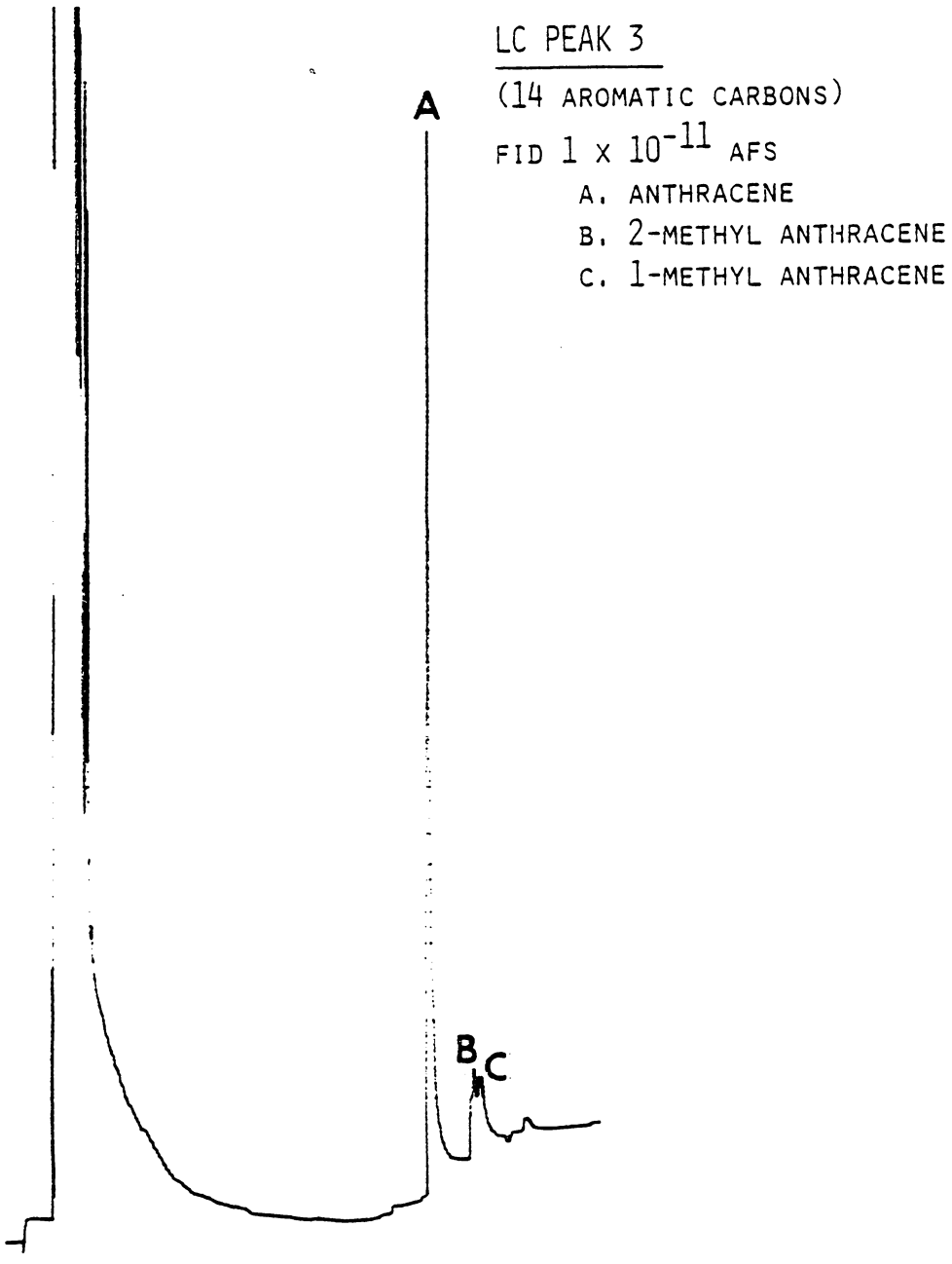


Figure 62. LC/GC Analysis of AMAX SRC: II

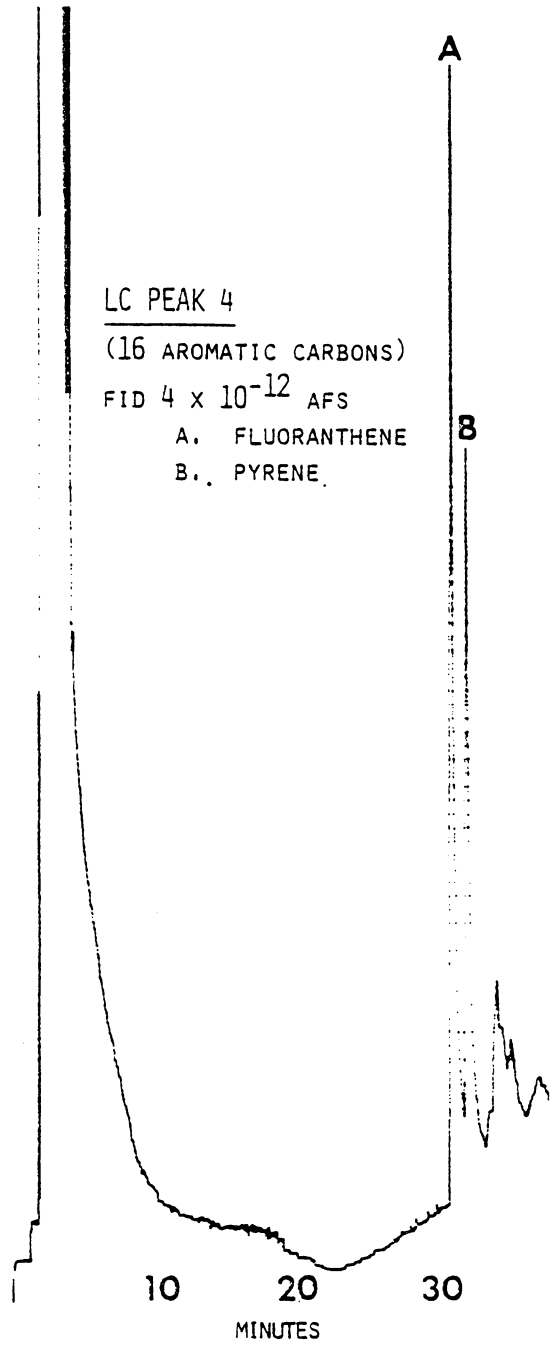


Figure 63. LC/GC Analysis of AMAX SRC: III

difficult to identify in the sample background. Comparing this with the LC/GC analysis, the improvement in selectivity can easily be seen. The major disadvantage is the poor detectability. The detection of naphthalene in this sample, as shown in Figure 62, is very close to the detection limit. This is due to the fact that only a fraction of the total peak volume is transferred.

The analysis of the first LC peak, shown in Figure 61, points out the high selectivity of this method compared to the HPLC technique. In the normal phase HPLC analysis, naphthalene, 2-methyl naphthalene and acenaphthalene cannot be separated, but the capillary GC resolves these three components well.

This application was used as a model for the evaluation of quantitation in trapping systems. Although this will be discussed in detail in the next section, analytical results of this technique are of interest here. For the study of quantitation, the method was modified slightly from the above to optimize reproducibility and detection limits. Two major changes were made; 1) all capillary GC analyses were performed isothermally; and 2) samples were diluted 1:10 in hexane rather than 1:40. The samples analyzed in this study were provided courtesy of Dr. Willie May of the National Bureau of Standards. These samples had been analyzed at the

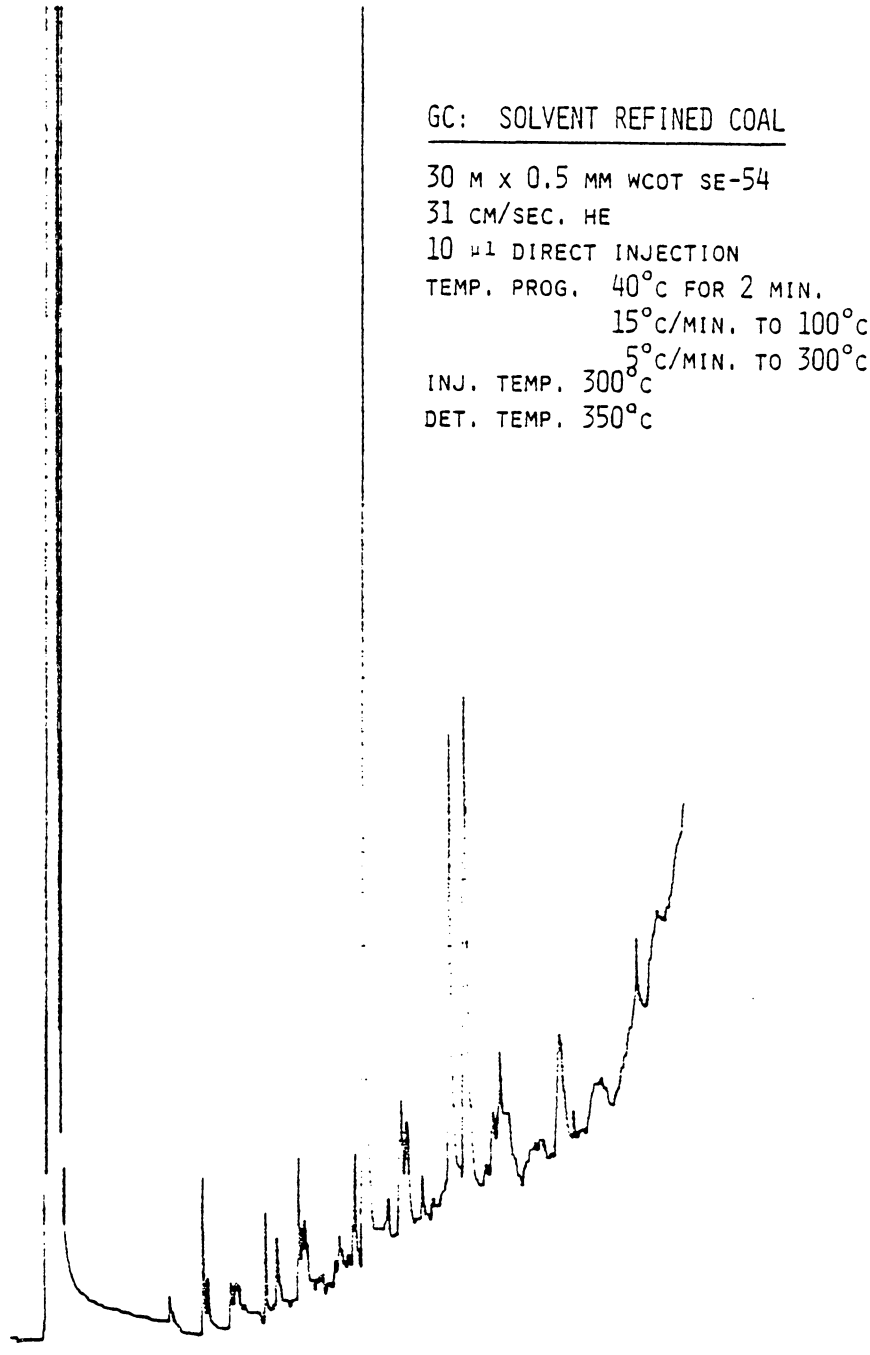


Figure 64. Capillary GC Analysis of AMAX SRC

NBS and could therefore be used by comparison to evaluate accuracy of the LC/GC analysis. These samples included shale oil (SRM 1580), solvent refined coal (SRC-II) and crude oil (Wilmington Crude). For the evaluation of quantitation, the analyses of three model compounds were performed; fluoranthene, pyrene and benzo(a)pyrene (B(a)P). The reasons for selecting these three compounds were that all had been analyzed by the NBS in the samples used, and thus values were available for comparison, and the three compound exhibited differing LC elution characteristics, covering a range of LC retention times. Furthermore, in the case of fluoranthene and pyrene, the LC resolution is incomplete, therefore, the effect of sampling partially separated peaks by LC/GC could be investigated.

Figure 65 shows the GC analyses obtained when the LC/GC interface sampled the LC effluent at 13.25 minutes, a point corresponding to the maximum of the fluoranthene peak. Figures 66 and 67 show similar analyses of pyrene and B(a)P. In these figures, the LC/GC interface was triggered at 13.4 and 18.9 minutes, respectively. The GC analyses of these samples were performed isothermally to eliminate any irreproducibility due to with the execution of a temperature program. The GC column temperature for both fluoranthene and pyrene was 180°C; for B(a)P, 260°C was used.

Conditions:

LC conditions as in Figure 58

GC Conditions:
Column: 30m x 0.25mm i.d. WCOT SE-52
Carrier: N_2 @ 45 cm/sec
Col. Temp.: 180°c
Inj. Temp.: 270°c
Det. Temp.: 300°c
FID Detection
Att.: 8×10^{-12} AFS
H_2 Flow: 30 ml/min
Air Flow: 300 ml/min
Det. Make-up: N_2 @ 30 ml/min

LC/GC Interface Triggering Time: 13.25 min.

Sample: 500 ul PAH Standard Mix
(100 ug/ml each)

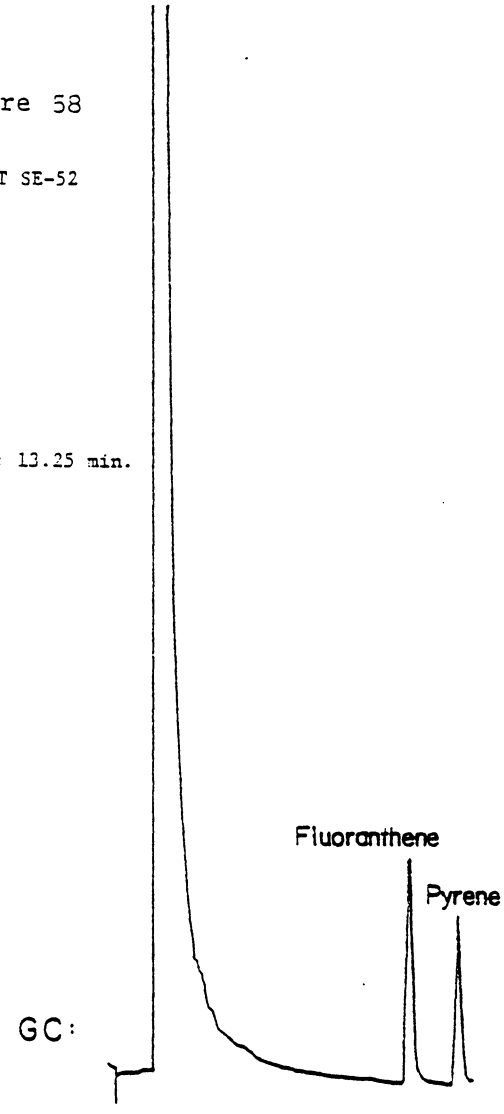


Figure 65. LC/GC Analysis of Fluoranthene Standard

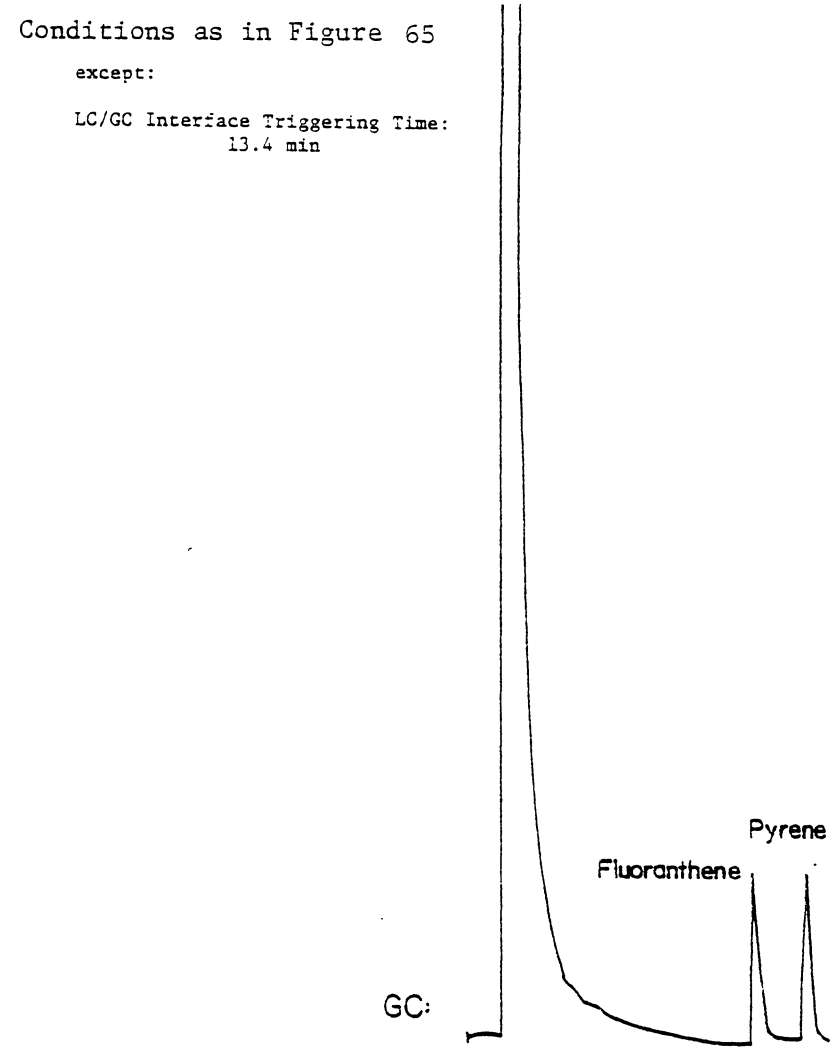


Figure 66. LC/GC Analysis of Pyrene Standard

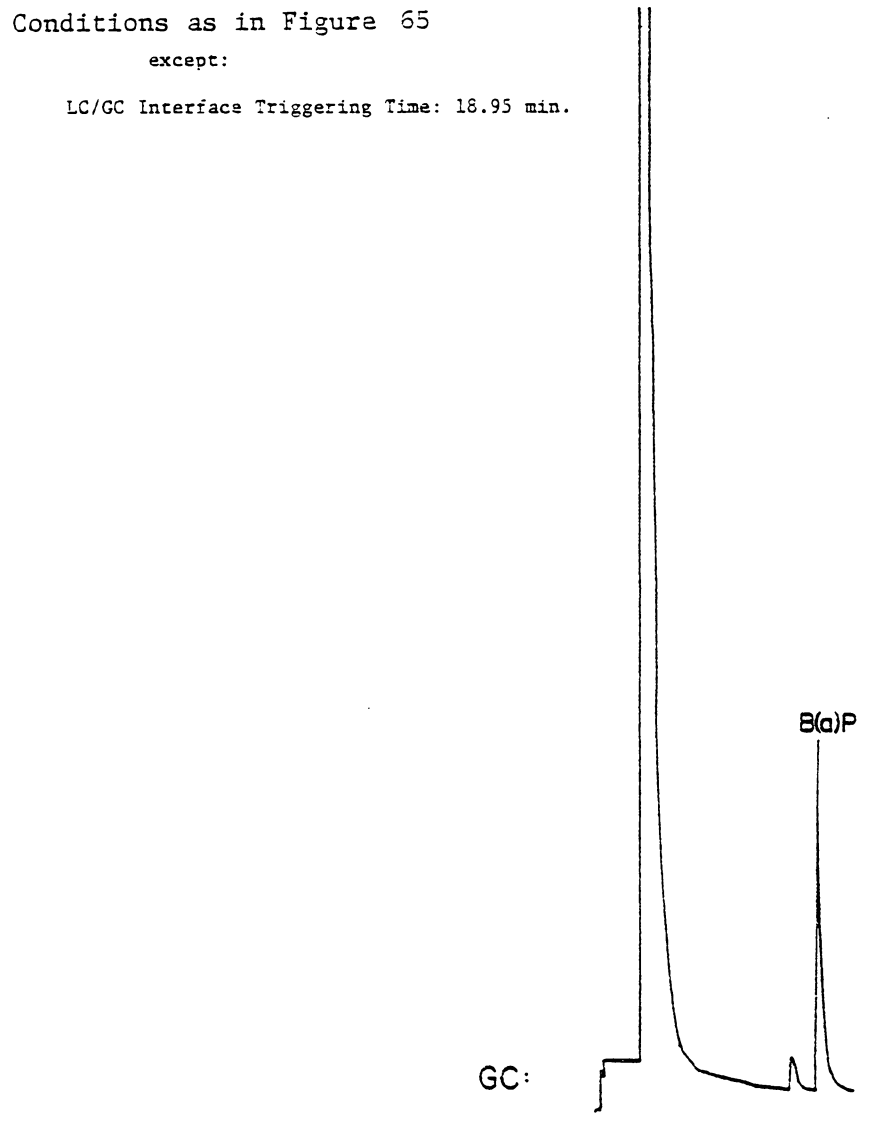


Figure 67. LC/GC Analysis of Benzo(a)Pyrene Standard

Figures 68 through 71 show the application of this analysis to SRM 1580 shale oil sample. Figure 68 shows the LC fractionation of a 1:10 dilution of SRM 1580 in hexane. This chromatogram was made using a 10 µl injection so that the peak would remain on scale for the purposes of illustration. The actual LC fractionation for LC/GC analysis, however, used a 500 µl injection volume. Figures 69, 70 and 71 show the GC analysis for the LC/GC interface sampling of fluoranthene, pyrene and B(a)P, respectively. These analyses used the same conditions as those above for standards. In the analyses of fluoranthene and pyrene, both peaks are present in both chromatograms.

Similarly, in Figures 72 through 75, this technique is applied to the analysis of SRC-II solvent refined coal, and Figures 76 through 79 show the analysis of Wilmington Crude.

Although the effect of system variables on the quantitation factors of reproducibility and transfer efficiency will be discussed in detail in the next section, it is appropriate to discuss the accuracy of this technique in this section. Tables 8, 9 and 10 show a comparison of the results obtained by this method to the results reported by the NBS methods for SRM 1580, SRC-II and Wilmington Crude, repectively. The results compare very well, with a few exceptions. In most cases, the LC/GC results are within

Conditions:

Columns: Cyanopropyl bonded Silica Guard
(5cm x 4.6mm i.d.)
Micropak NH_2-10 (30cm x 4.6mm i.d.)
in series
Mobile Phase: 10% Chloroform/Hexane
Flow Rate: 1.0 ml/min
Column Temperature: 35°c
UV detection @ 280nm
Attenuation: Max.

Sample: 10 ul Shale Oil (SRN-1580)
1:10 dilution in Hexane

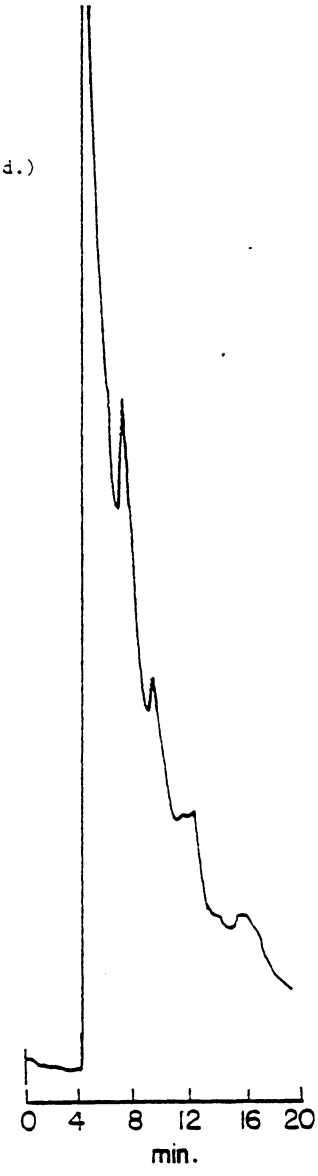


Figure 68. LC Fractionation of Shale Oil

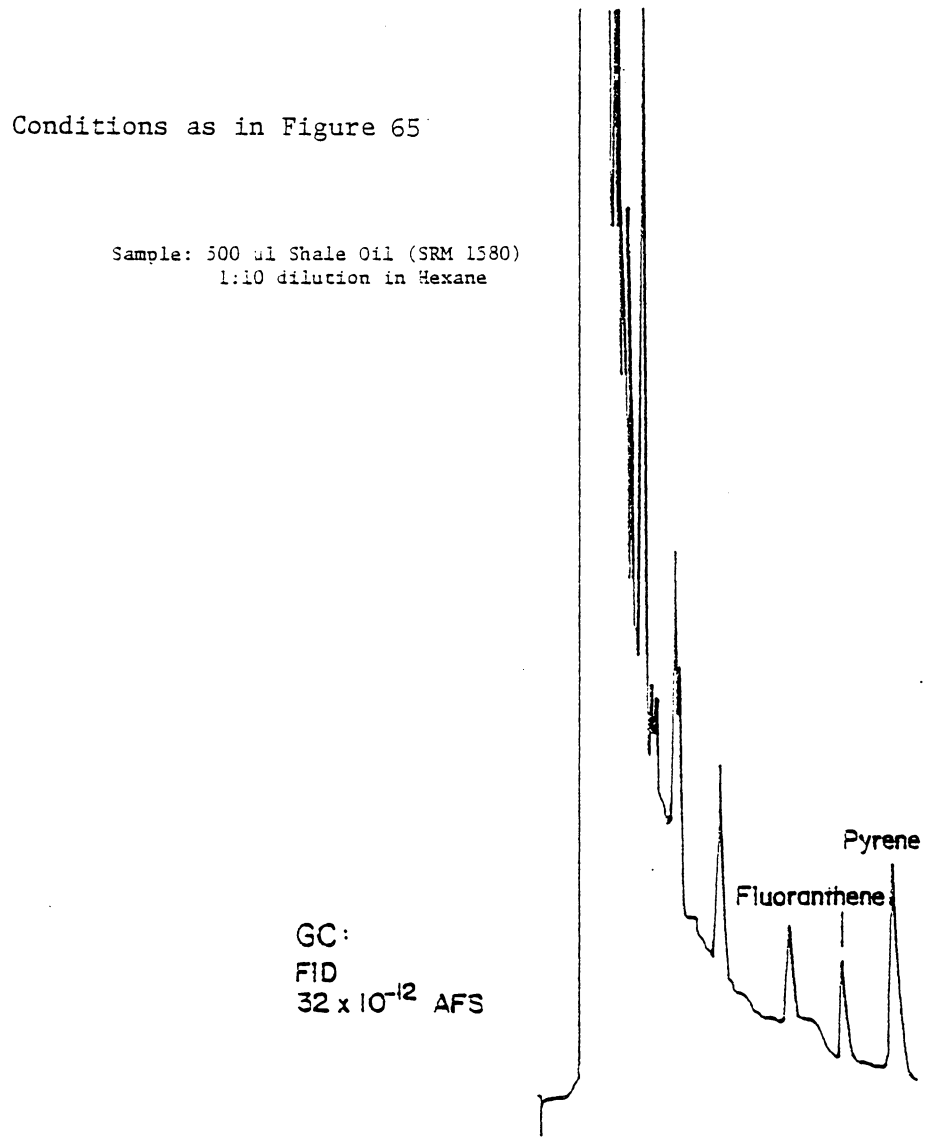


Figure 69. LC/GC Analysis of Fluoranthene in Shale Oil

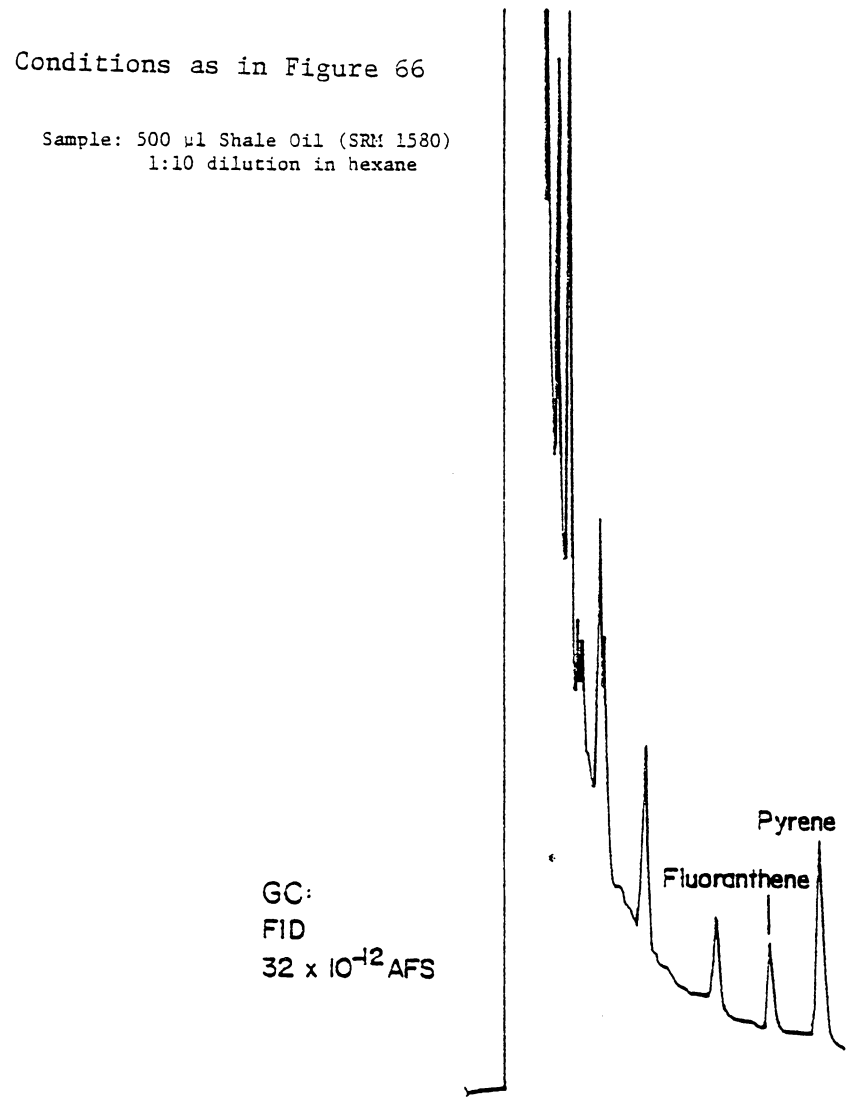


Figure 70. LC/GC Analysis of Pyrene in Shale Oil

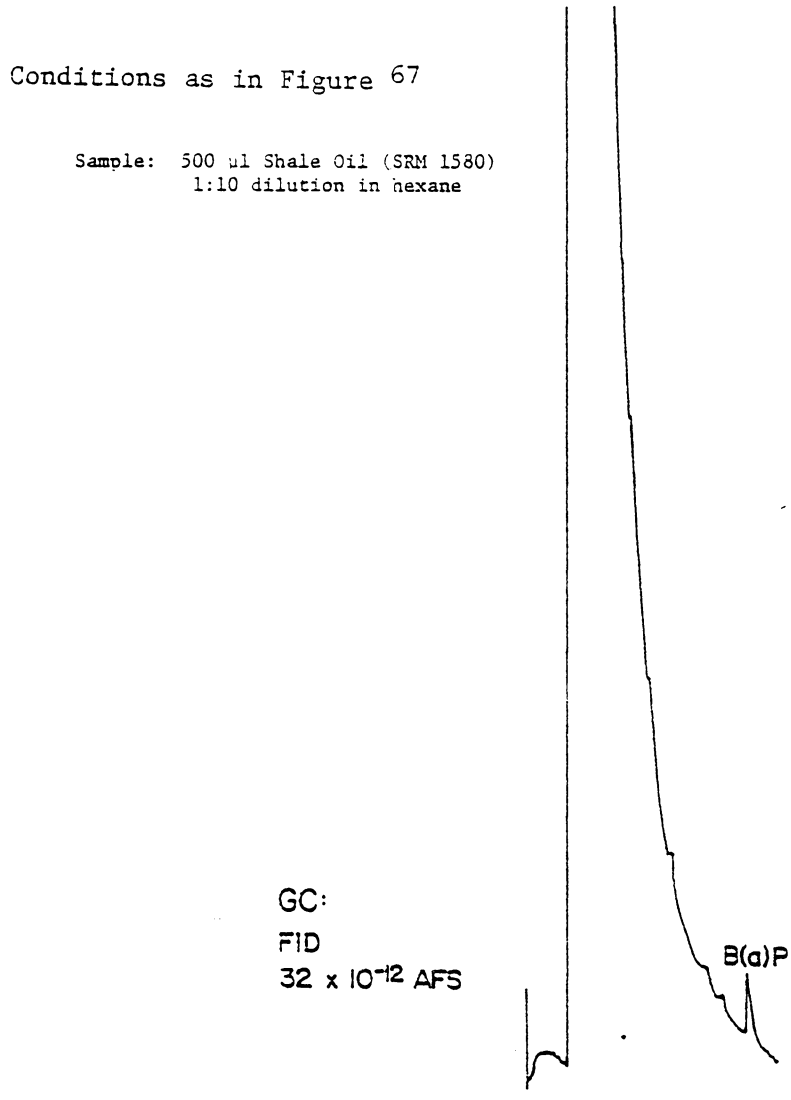


Figure 71. LC/GC Analysis of Benzo(a)Pyrene in Shale Oil

Conditions as in Figure 68

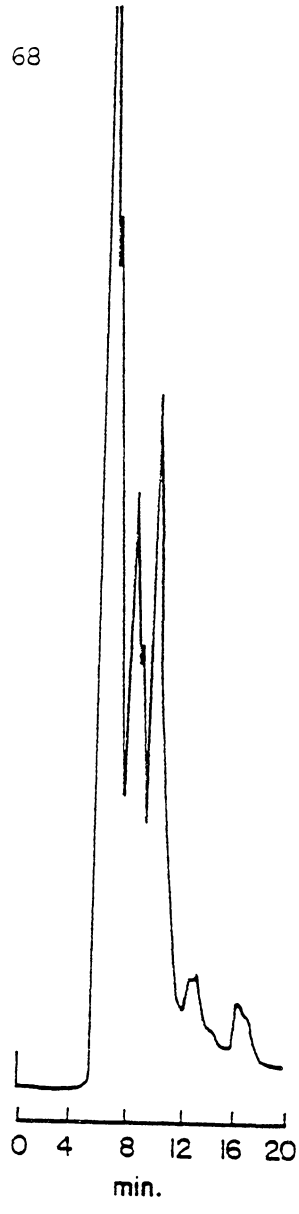


Figure 72. LC Fractionation of Solvent Refined Coal

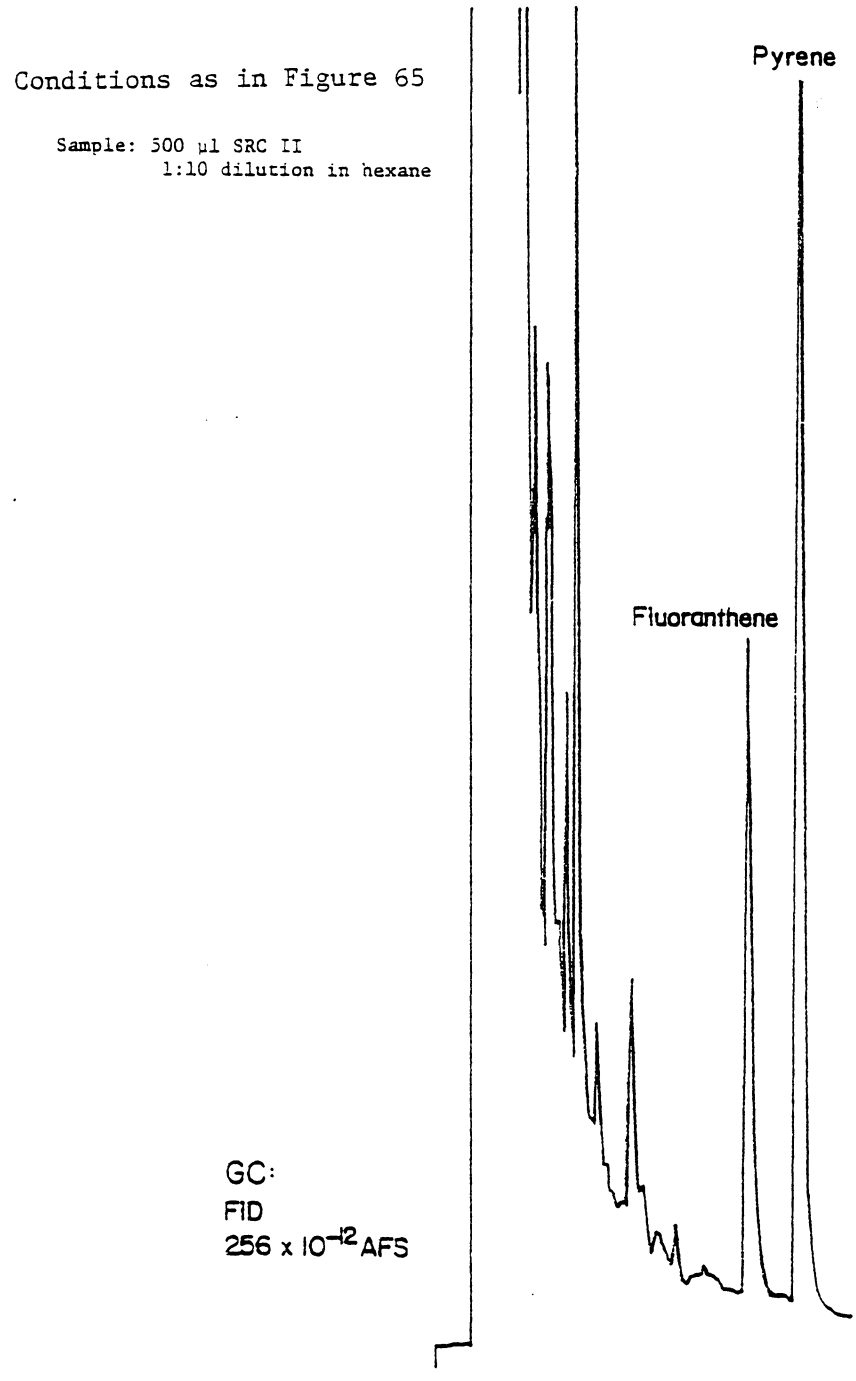


Figure 73. LC/GC Analysis of Fluoranthene in Solvent Refined Coal

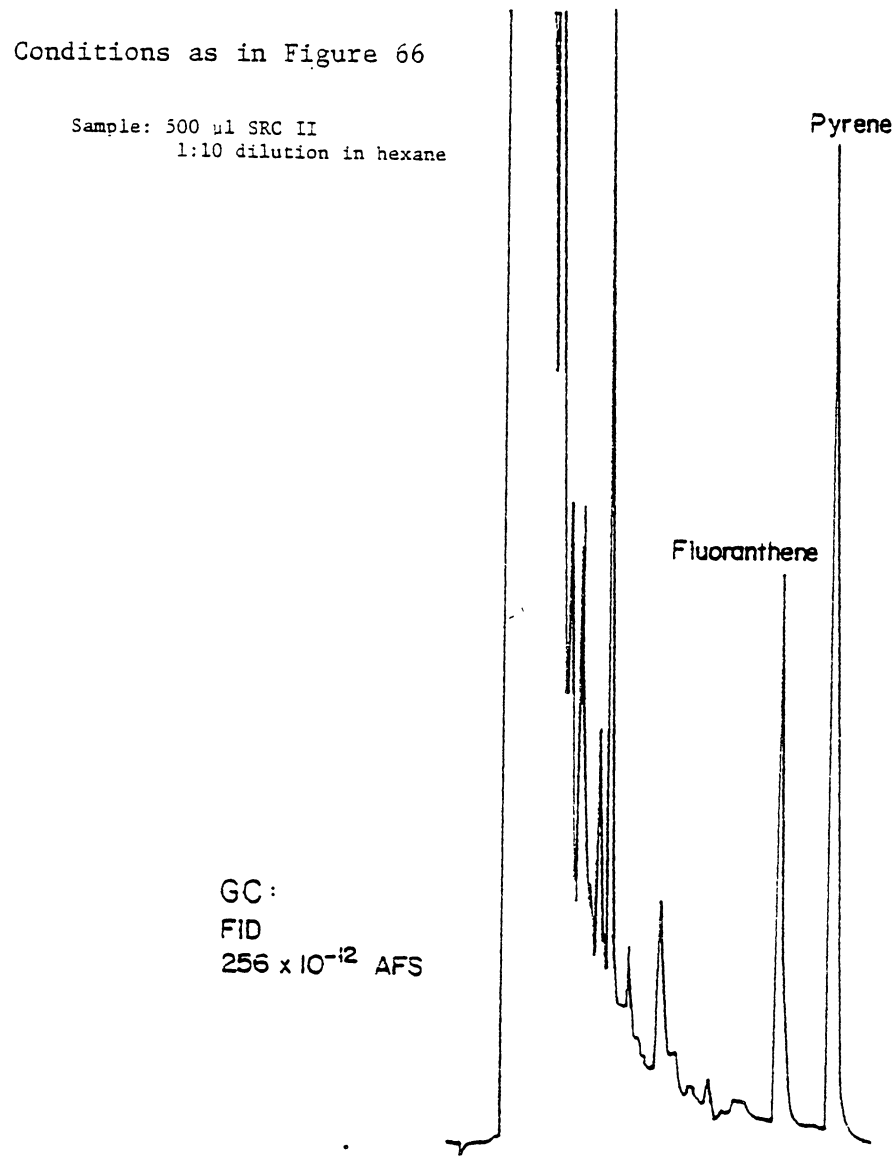


Figure 74. LC/GC Analysis of Pyrene in Solvent Refined Coal

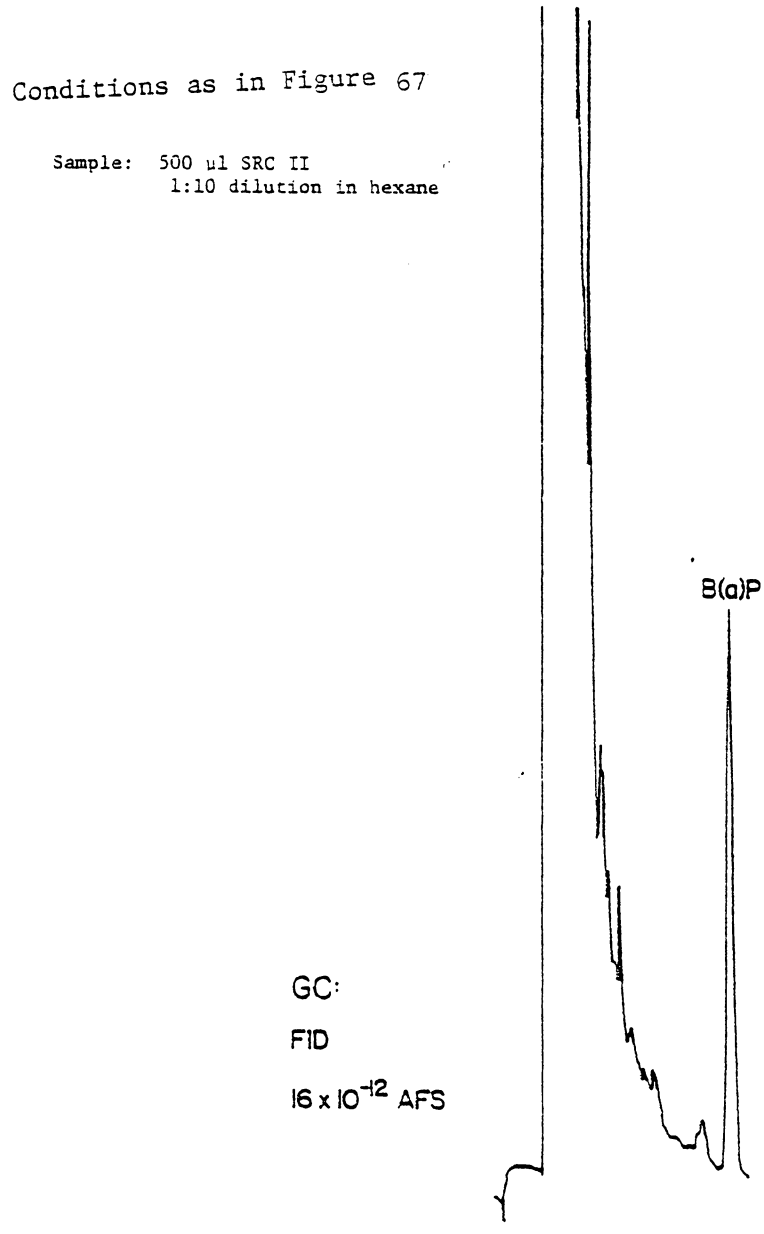


Figure 75. LC/GC Analysis of Benzo(a)Pyrene in Solvent Refined Coal

Conditions as in Figure 68

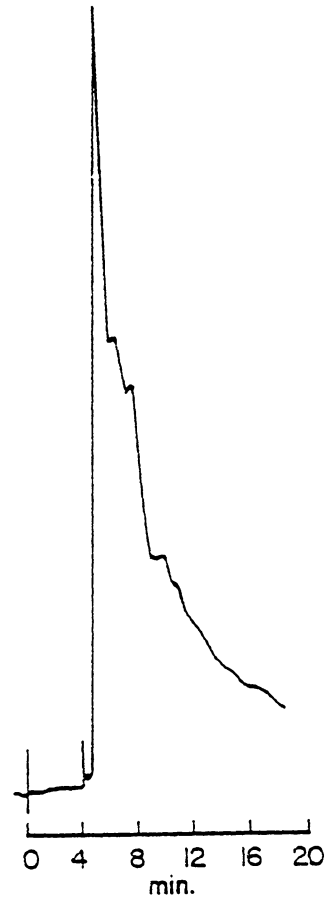


Figure 76. LC Fractionation of Crude Oil

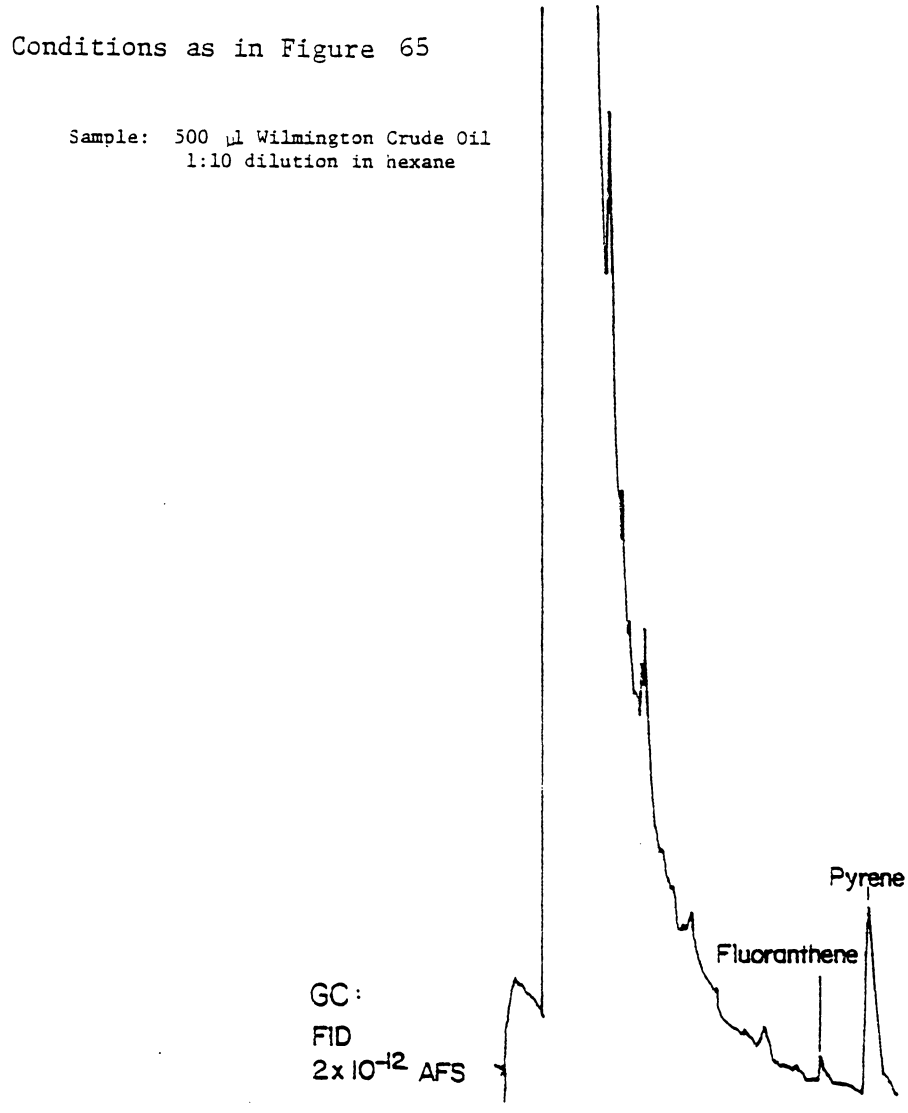


Figure 77. LC/GC Analysis of Fluoranthene in Crude Oil

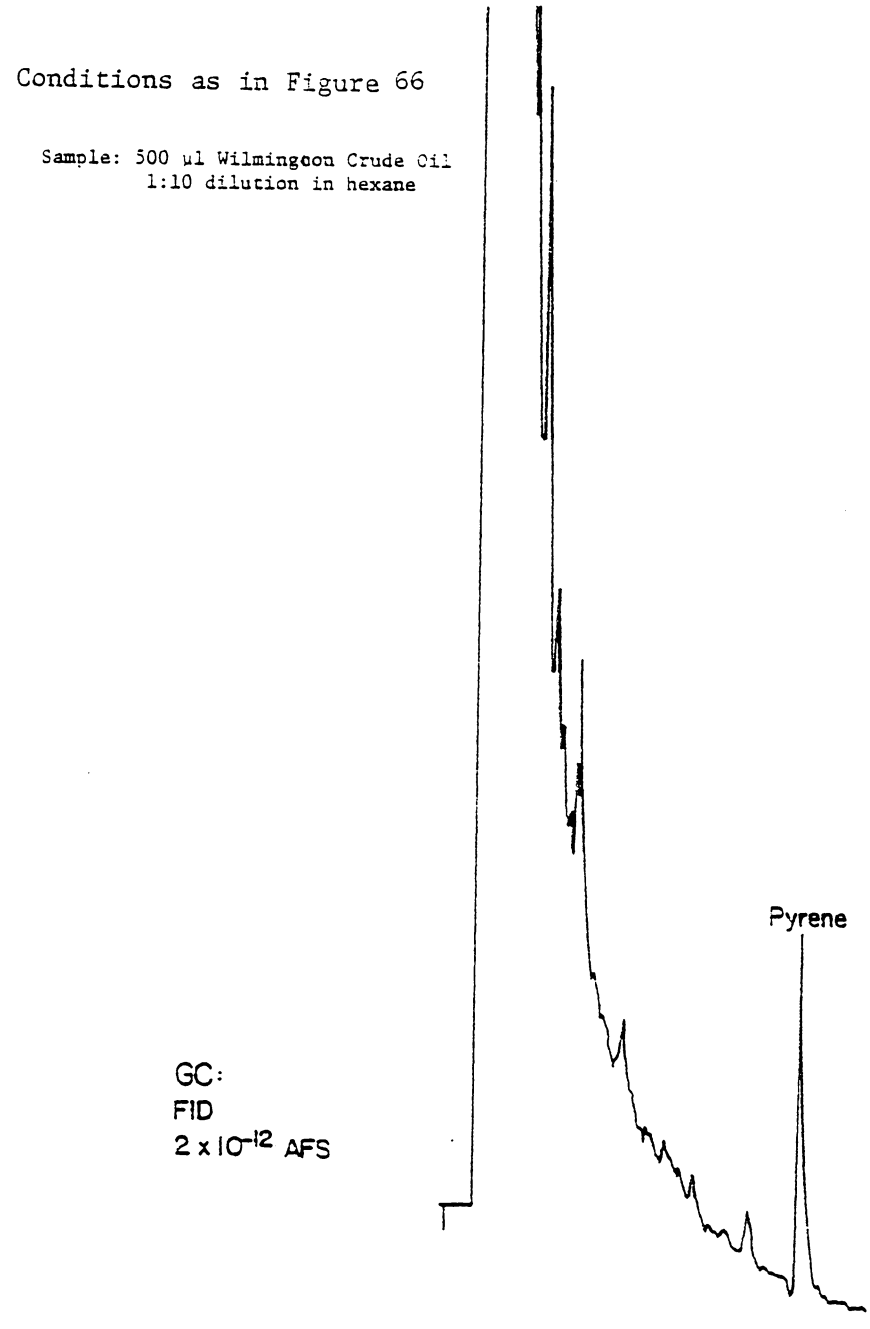


Figure 78 . LC/GC Analysis of Pyrene in Crude Oil

Conditions as in Figure 67

Sample: 500 µl Wilmington Crude Oil
1:10 dilution in hexane

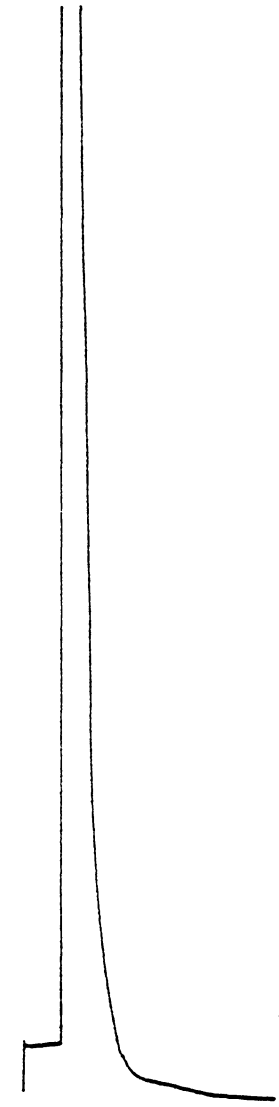


Figure 79. LC/GC Analysis of Benzo(a)Pyrene in Crude Oil

the confidence levels of the NBS results. The four main exceptions are; pyrene in SRM 1580, fluoranthene in SRC-II and fluoranthene and B(a)P in Wilmington Crude. Comparison of these deviations to those obtained in an NBS interlaboratory study (171), shown in Table 11, indicate that the values obtained by LC/GC are as good or better than those obtained by other methods.

The deviations in the values obtained for crude oil are due to the relatively poor detection limits of the LC/GC method. As has been emphasized previously, in the trapping mode, multidimensional chromatographic techniques have poor detection limits due to the fact that only a small fraction of the total amount of analyte present is transferred, and therefore detected. For this method, the MDC for all three PAH's determined was found to be in the range of 10 μg/ml.

In summary, this method yields excellent results for the analysis of PAH's in a variety of a samples containing the analytes in concentration ranges >10 μg/ml. It is accurate, reproducible, requires little sample preparation and can be fully automated. Although only three PAH's were examined in this study in detail, the analysis of other PAH's using this system should be just as successful.

TABLE 8

Comparison of LC/GC and NBS Values for Shale Oil

Compound	LC/GC (5 samples) (μg/g)	NBS (Ref. 171) (μg/g)
Fluoranthene	51.88 + 3.2	54 + 6
Pyrene	138.8 + 8.3	104 + 9
B(a)P	20.7 + 2.1	21 + 2

TABLE 9

Comparison of LC/GC and NBS Values for SRC-II

Compound	LC/GC (5 samples) (µg/g)	NBS (REF.171) (µg/g)
Fluoranthene	2628 + 134	3300 + 160
Pyrene	6478 + 327	6000 + 200
B(a)P	136 + 8.3	134 + 7

TABLE 10

Comparison of LC/GC and NBS Values for Wilmington Crude Oil

Compound	LC/GC (5 samples) (µg/g)	NBS (REF. 171) (µg/g)
Fluoranthene	---	3 + 1
Pyrene	14.4 + 2.2	14 + 2
B(a)P	---	2 + 5

TABLE 11

Interlaboratory Comparison of Quantitation[e]

Compound	NBS[a]	2	3 b	3 c	3 d	4	5	6	7	8	9
Flouranthene	61±8	102	247	75		80	108	126	112	116	380
Pyrene	102±5	155	161	138	147	150	168	177	141	185	620
Benzo(a)pyrene	22±2	-		19	13				3.3		-
Benzo(e)pyrene	21±1								1.3		

Table from Ref. 171, (concentrations in ppm).

a Results reported by NBS represent the mean of values obtained by GC, GC/MS and HPLC. Uncertainties represent the standard deviations of the mean.

b Quantitation by GC/MS.

c Quantitation by HPLC.

d Quantitation by Laser Excited Spectroscopy.

e These data are representative only. Results in tables 8, 9 and 10 are for different samples.

5.2.2.4 Summary: LC/GC Applications

There are a number of characteristics of these three applications of LC/GC that should be emphasized. As in the case of the applications of LC/LC, the basic objectives of each analysis were different. In the analysis of pesticides in butter, LC/GC was applied as an on-line clean-up procedure. Although the hydrocarbon group analysis and the PAH analysis utilized very similar instrumental systems, their emphases were different. In the hydrocarbon group separation, all components of the sample were of interest. In the analysis of PAH's, on the other hand, only a specific group of compounds were determined. In both the analysis of hydrocarbon groups and the analysis of PAH's, both chromatographic steps were essential in their contribution to the overall separation. In the case of the hydrocarbon group separation, the selectivities of the two chromatographic modes were exploited in obtaining the resolution of the analytes. Without the use of the primary LC separation, the distributions of compounds within the individual groups could not be determined. In the analysis of PAH's, without the initial LC fractionation, the presence of extraneous sample components interfered with the determination of the minor PAH's.

In terms of the LC modes applied, only two types were used, adsorption and size exclusion. Both of these used organic mobile phases. No applications were performed using aqueous LC mobile phases. Systems utilizing aqueous LC mobile phase introduce problems in coupling the LC mobile phase with conventional GC columns. Although approximately 90% of reported LC analyses are currently performed using reverse phase sysems and aqueous mobile phases, these systems were omitted in this study. The reason for this is that since LC/GC is a new field, it was felt the greatest utility would be obtained by exploiting more ideal systems. The use of SEC and normal phase applications as initial separations for GC analysis, for instance, have been commonly performed as off-line procedures, and both, as has been demonstrated, are very useful in on-line application. The emphasis of these applications has been to illustrate the use of on-line multidimensional chromatographic techniques as powerful analytical tools, rather than to demonstrate all possible combinations of chromatographic techniques. This is not meant to imply that the use of reverse phase HPLC techniques are not applicable to LC/GC. However, there are potential complications in the use of RPC, and it will be neccesary to apply special procedures to circumvent these problems.

5.3 QUANTITATION

In the Theoretical section, equations were developed to describe the reproducibility and transfer efficiency of both switching and trapping modes of on-line multidimensional chromatographic techniques in terms of chromatographic variables. In this section, these equations and their results will be evaluated. Hypothetical chromatographic systems will first be evaluated in order to describe the effects on quantitation of the individual chromatographic variables. These equations will then be applied to two of the applications described in the previous section in order to verify the theoretical considerations. Experimentally, quantitation in a switching system was evaluated using the analysis of catecholamines by LC/LC as a model. The quantitation in trapping systems was evaluated using the analysis of PAH's by LC/GC as a model.

Before proceeding with this discussion, it should be noted that the purpose of deriving the equations based on theoretical models was to describe the effects of those variables which are critical in the operation of a multidimensional chromatographic system. Although these equations could be used to predict the reproducibility and transfer efficiency for a specific application, this would not be of great utiltiy. The reason for applying these

theoretical equations to the applications stated above is to verify the conclusions drawn from the model.

5.3.1 The Trapping System

5.3.1.1 Effects of Operating Variables

In on-line multidimensional chromatographic systems, the quantitation factors of reproducibility and transfer efficiency are determined by two characteristics of the system; the sampled peak and the sampling process. The fate of the transferred fraction after the transfer does not affect the quantitation due to the transfer process. The effects of the chromatographic variables can be evaluated separately by varying one parameter at a time using a hypothetical peak and sampling process. In the analysis of the quantitation for the trapping system, the hypothetical system used is outlined in Table 12. The relative range and transfer efficiency were calculated by using these conditions and equations 27 and 38 in Chapter III.

In a trapping system, a small portion of a peak eluting from the primary chromatographic system is trapped and transferred to the secondary chromatographic system. The characteristics of the peak which affect this transfer process are the flow rate of the initial separation, the reproducibility of the flow rate and peak retention and the

TABLE 12

Hypothetical Conditions for Trapping system

Retention Volume	10 ml
Sampling Point	10.03 ml
Flow Rate	1 ml/min
Theoretical Plates	5000
Transfer Volume	5 µl
Device Precision	0.6 seconds
Retention Precision	1%
Sample Volume	10 µl
Sample Concentration	10 µg/ml

peak width, which will be determined by the column efficiency and the retention volume. The characteristics of the transfer process which effect the quantitation are the position of the sampling point relative to the peak maximum, and the sampling volume relative to the total peak volume.

Of the variables stated above, the most critical is the position of the sampling point. The effect of the sampling point on the reproduciblity and transfer efficiency is shown in Figure 80 . In this graph, the x axis represents the elution volume at which the peak is sampled. For the hypothetical peak being considered, the retention volume of 10 ml correspond to the peak maximum. Therefore, the origin of the graph represents the peak maximum and as the x values increase so does distance from the peak maximum that the peak is sampled. The peak is defined as a symmetrical Gaussian distribution, and therefore the peak center corresponds to the peak maximum. Since the peak is symmetrical, only sampling points greater than the retention volume of the peak need be considered. Points less than the retention volume can be determined from their reflections about the peak center. The lower scale in this figure shows the position of the sampling point in units of standard deviation of the peak from the center. The left hand y axis in this figure shows the reproducibility as approximated by

the relative range. A relative range value of 0% corresponds to no variation in the amount transferred. The right hand y axis show the transfer efficiency. This is the percent fraction of the total peak volume which is transferred.

The effect of the sampling point acts in conjunction with the retention reproducibility of the peak. The irreproducibility in the amount of the peak transferred is due to small variations in the retention time moving the peak relative to the time based sampling point. If the peak is sampled at the maximum, where the slope of the peak is a minimum, the effect of changes in the retention time is minimized. This is because the resulting changes in the point at which the peak is sampled lead to only small changes in the actual amount transferred. If, on the other hand, the peak is sampled on the steep slope, the same small changes in retention result in large changes in the amount transferred, and therefore high irreproducibility. In fact, at the inflection point of the Gaussian peak the variation will be the greatest. In terms of analytical utility, however, the relative reproducibility is of greater interest. The relative reproducibility is approximated by the relative range. Although the variation in the amount transferred is a maximum at the inflection point on the

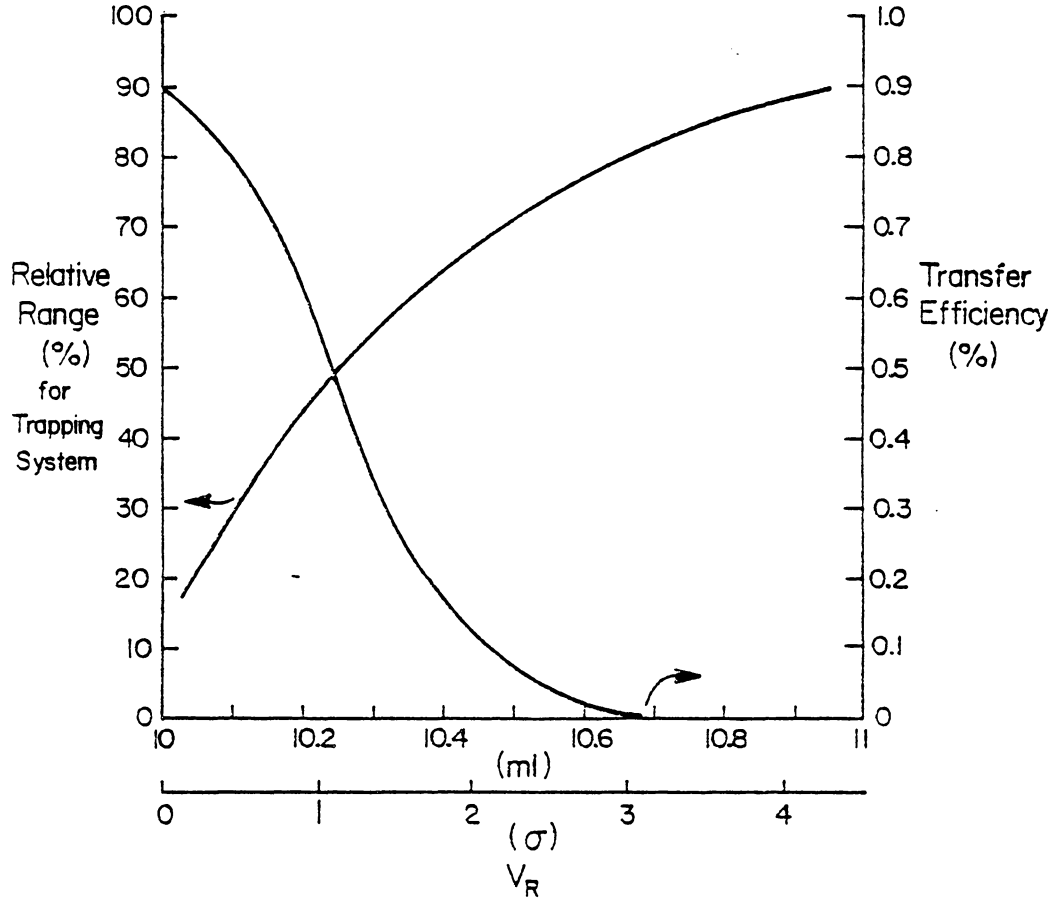


Figure 80. Effect of Sampling Point on Quantitation for Trapping System

slope of the peak, relative to the amount transferred, the farther off peak center the peak is sampled, the larger the relative range.

In terms of transfer efficiency, as would be expected, if the peak is sampled at the apex, where the concentration is the greatest, for a fixed sample volume, the maximum fraction of the total peak volume is transferred. Therefore the transfer efficiency follows the Gaussian concentration distribution from a maximum at the peak center, assymptotically approaching zero as the distance from the peak center at which the peak is sampled increases.

The retention precision, or the reproducibility of the peak retention time, is the primary source of variation in the multidimensional trapping transfer process. The effect of this variable on reproducibility is shown in Figure 81 . In this graph, the x axis shows the retention precision of the initial separation. This quantity is expressed as a percentage of the retention time. Typically, modern HPLC equipment operates with retention precisions of approximately 0.3%. As can be seen, as the retention precision increases (poorer precision), so does the variation in the transferred amount as determined by the relative range. This effect is not independent of the sampling point. Although the magnitude of the effect will

decrease as the peak is sampled closer to the maximum, the trend will remain the same. Retention variations do not affect the average amount of sample transferred. Therefore, the retention precision does not affect the transfer efficiency.

Several variables affect the width of the sampled peak including flowrate, column efficiency and retention volume. For a fixed retention precision, as the peak width increases, the slopes of the peak become less and, therefore, variation in retention will lead to less variation in the amount transferred. Broader peaks can be sampled more reproducibly. Conditions of these variables which lead to narrower peaks will also cause poorer reproducibility. This is illustrated in Figures 82, 83 and 84 . Figure 82 shows the effect of flow rate on reproducibility and transfer efficiency. As the flow rate increases, the peak becomes narrower and the retention variations lead to larger variations in the amount sampled. As the flow rate increases, the peak only becomes narrower in time, but not in volume. The concentration at any point, therefore is unchanged. Thus the transfer efficiency is unaffected. This statement is actually an approximation since it disregards any effect of flow rate on column efficiency or band broadening.

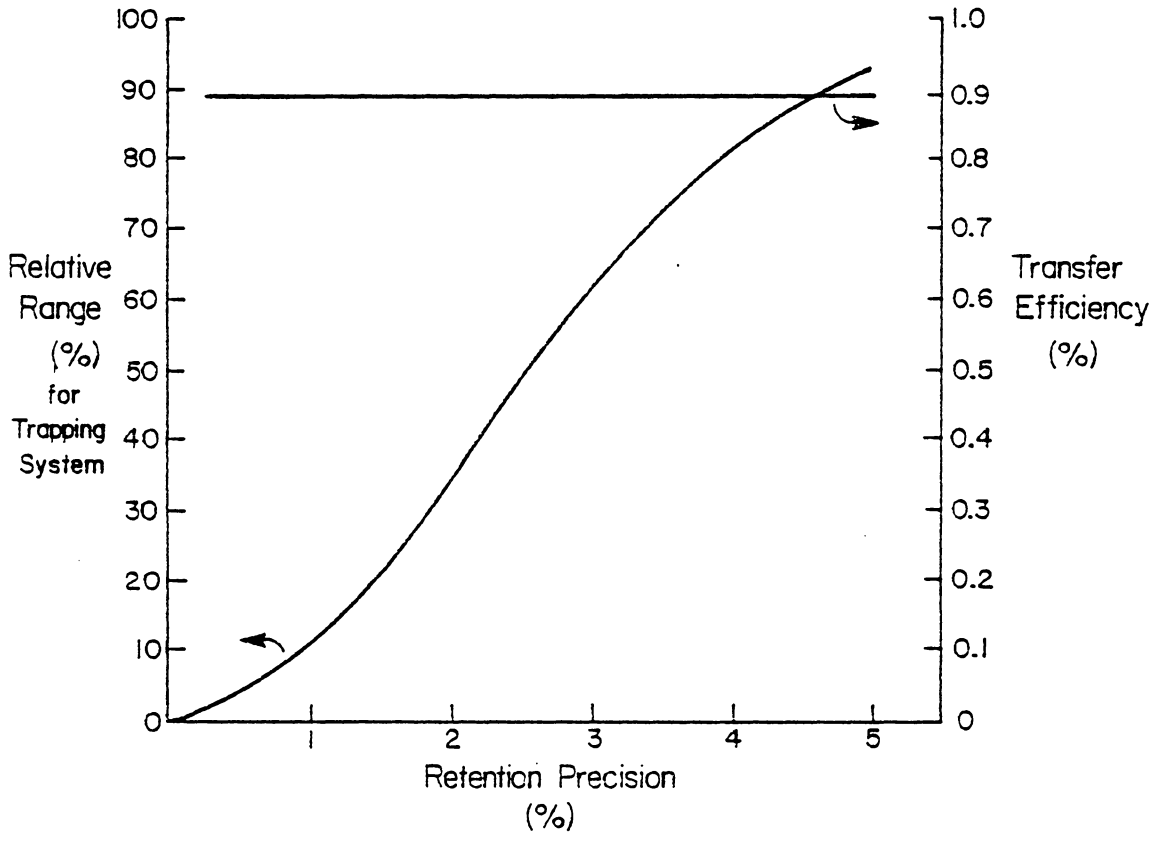


Figure 81. Effect of Retention Precision on Quantitation for Trapping System

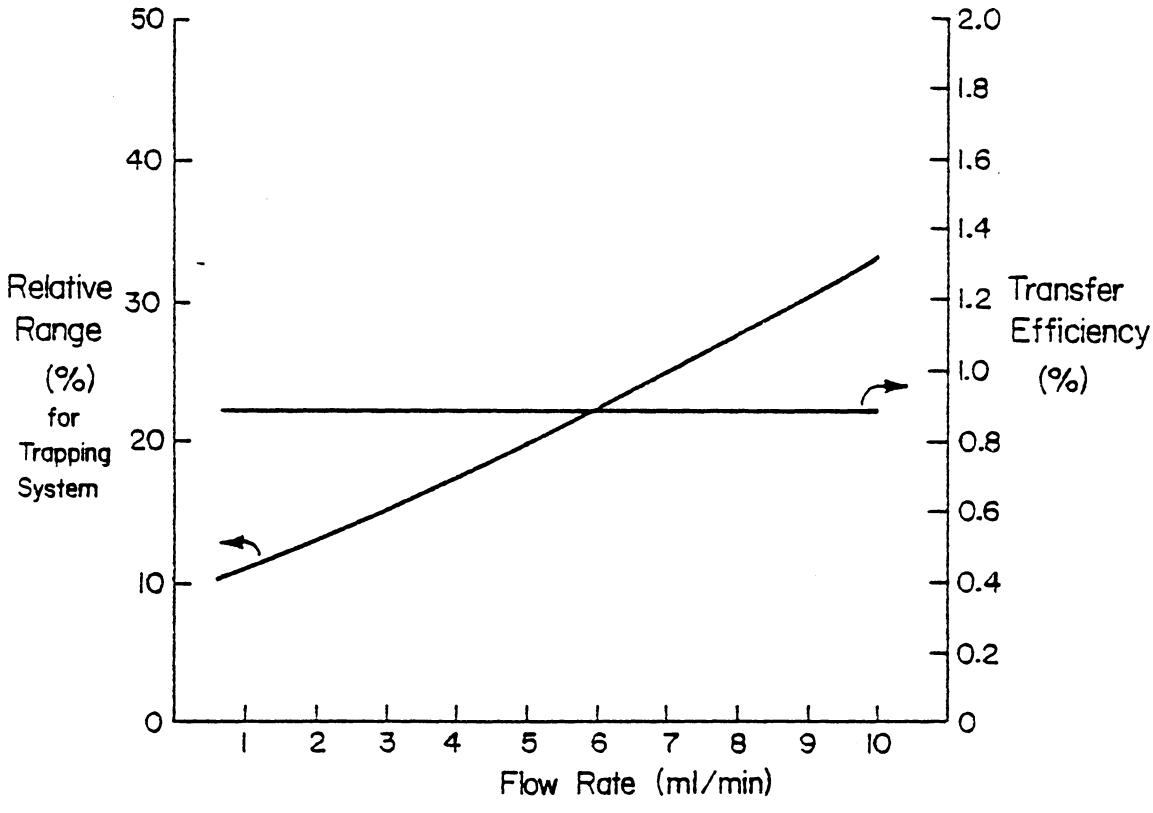


Figure 82. Effect of Flow Rate on Quantitation for Trapping System

Figure 83 shows the effect of the column efficiency expressed as the number of theoretical plates, on reproducibility and transfer efficiency. As the number of theoretical plates increases, the peaks become narrower in terms of volume and, at a fixed flow rate, also time. The relative range therefore also increases. Less efficient separations yield broader peaks and can be sampled more reproducibly. Unlike the flow rate, increases in column efficiency narrow the peak width in terms of volume and increase the concentration at any point on the Gausian distribution. Therefore, increasing column efficiency also increases transfer efficiency.

The effect of retention volume on reproducibility and transfer efficiency is identical to that of column efficiency. As the retention volume increases, and the peak becomes broader in both time and volume, the relative range and the transfer efficiency decrease. This effect is shown in Figure 84 . From these curves, it can be seen that later eluting peaks can be sampled more reproducibly, but at a given position on the Gaussian distribution, the concentration of the sampled volume is lower and therefore the transfer efficiency is lower.

The final group of variables which have a major effect on the quantitation of a trapping system affect the overall

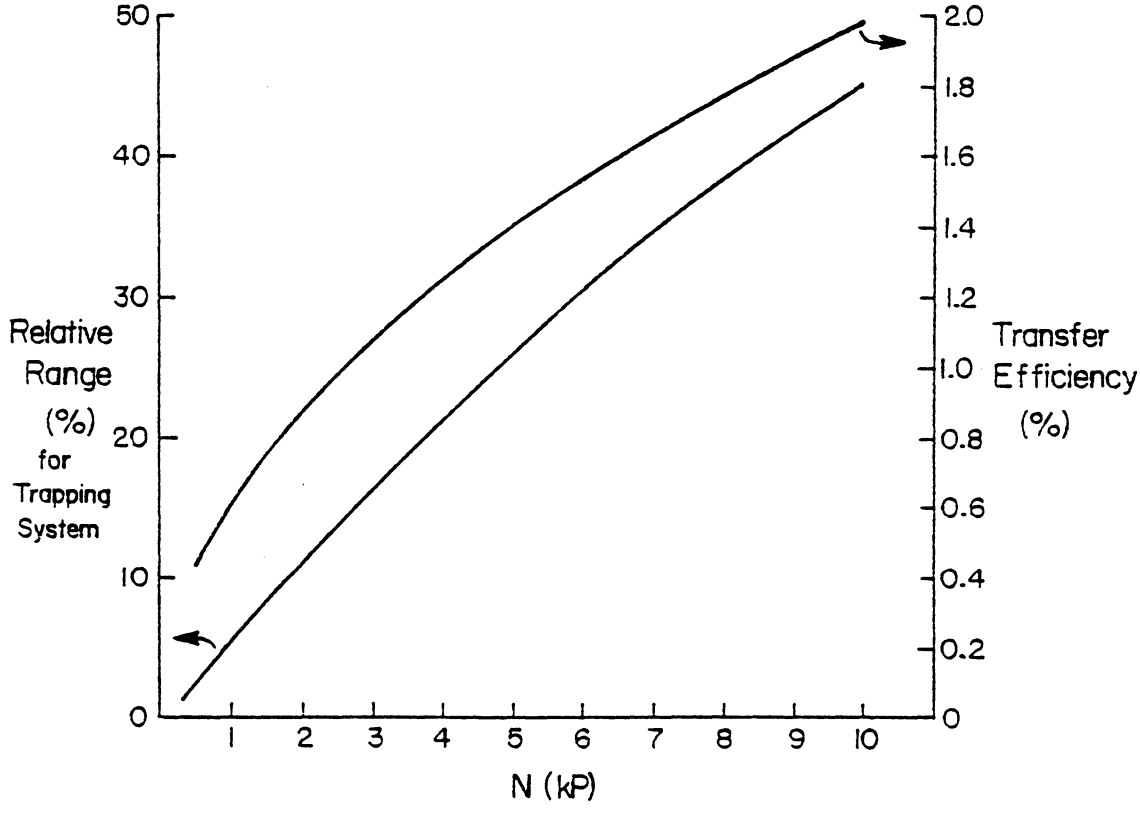


Figure 83. Effect of Column Efficiency on Quantitation for Trapping System

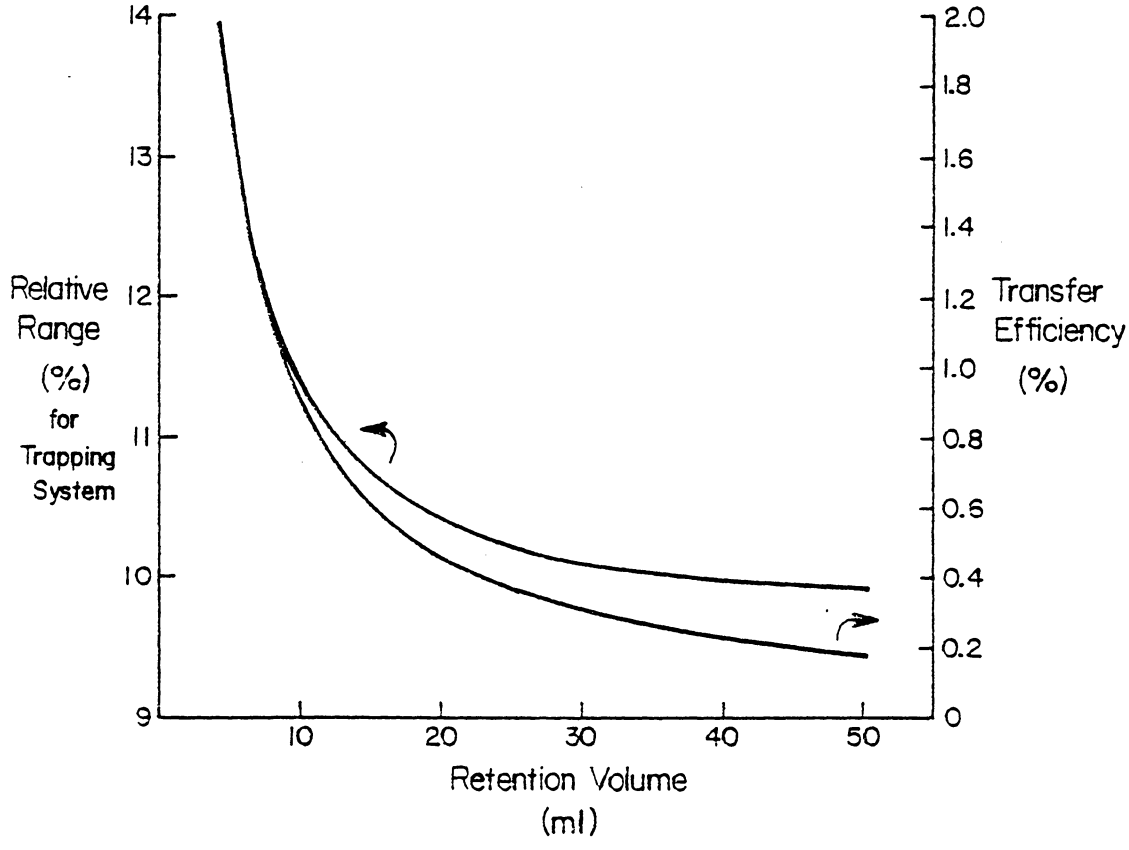


Figure 84. Effect of Retention Volume on Quantitation for Trapping System

amount of the analyte transferred. These include the initial sample concentration and volume and the transfer volume. If it is assumed that the initial amount of analyte injected onto the primary column does not exceed the capacity of that column, then the initial sample concentration and volume do not affect the transfer efficiency or reproducibility. These two factors do, obviously, affect the total amount of sample present and therefore transferred in the multidimensional chromatographic system. The transfer volume, on the other hand, does affect the transfer efficiency. If the sampled volume is greater, then, by definition, a larger portion of the total peak volume present is transferred and therefore, the transfer efficiency is higher. This effect is illustrated in Figure 85 . If it is assumed the transfer volume is still small with respect to the total peak volume, and therefore the assumptions used in deriving the theoretical trapping model are valid, then the reproducibility is not affected by the transfer volume.

Of the variables discussed above, for a particular analysis, some are more accessible than others. Experimentally, the analytical system can be easily optimized with respect to flow rate, sampling point, initial sample volume and concentration, and transfer volume.

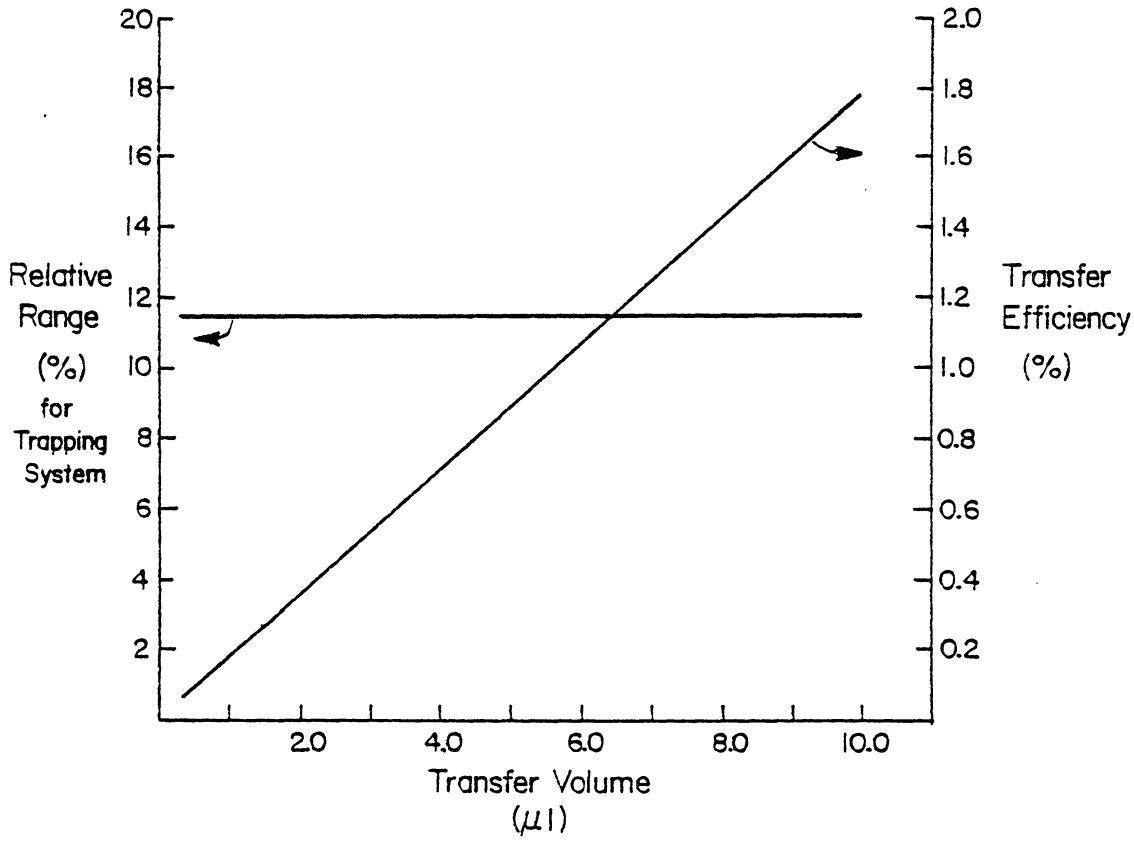


Figure 85. Effect of Transfer Volume on Quantitation for Trapping System

Retention volume can be adjusted, but not without affecting the separation. Column efficiency can be affected by choosing a column of specific efficiency or to a lesser extent by adjusting column temperature, mobile phase and flow rate. Finally, retention precision is fixed for a specific analytical instrument. This, of course, should be optimized, but little other adjustment is possible.

In summary, the following conclusions can be made concerning quantitation in trapping systems. Most favorable reproducibility is obtained by sampling the peak maximum, using low flow rates, low efficiency columns and long retention times. Maximum transfer efficiency is obtained by sampling the peak maximum, using high efficiency columns, long retention times, and large transfer volumes. Obviously, some compromise must be made between reproducibity and transfer efficiency. The variables which need to be compromised have minor effects compared to the effect of the sampling point. It should also be noted that the effects of the column efficiency and retention time may have to be compromised in terms of resolution and quantitation. Although optimum reproducibility is obtained with broad peaks, this may severely interfere with resolution.

5.3.1.2 Experimental Verification

The equations which have been developed to predict the quantitation factors, reproducibility and transfer efficiency, for an on-line mutidimensional chromatographic trapping system have been verified experimentally using the LC/GC analysis of the PAH's, fluoranthene, pyrene and benzo(a)pyrene as a model. Conditions for these analyses have been discussed previously. As stated in the last section, the two major variables which affect quantitation are the position of the sampling point and the flow rate of the initial fractionating step. Therefore, these variables have been evaluated for the model separation.

The following data represent the effect of the quantitation due only to the transfer process. These results were abstracted from the experimental results through the application of equation 49 in Chapter III.

In the evaluation of the the effect of flow rate on relative range, 10 replicate analyses were run for each analyte at each flow rate. The samples analyzed contained the PAH's listed in Table 6 in the concentration of 200 ug/ml each. The sampling point for each analysis was the retention time of the analyte determined at the flow rate in question. The effect of flow rate on relative range is shown in Figure 86. The solid lines correspond to the

predicted values, and the points represent experimental data. The uppermost curve is that of fluoranthene, below that pyrene and the bottom curve, benzo(a)pyrene. Although there is some variation of the points around the curves, the trends are valid. As defined, the relative range should only approximate the maximum values obtained from relative standard deviation measurements. In examining the several high values, it should be noted that the maximum deviation is less than 0.4%. In addition to qualifying the predictions concerning flow rate, these data also verify the predicted effect of retention time. As can be seen, the relative range is lower for the later eluting compounds.

For evaluating the effect of the sampling point on reproducibility and transfer efficiency, fluoranthene and pyrene were run together in sets of five replicates for each sampling point. Benzo(a)pyrene was run in separate sets of five replicates.

Figures 87 and 88 show the effect of sampling point on reproducibility for fluoranthene and pyrene and benzo(a)pyrene respectively. In both of these figures, the x axis represents the sampling point in terms of elution volume from the injection point. The minima in the curves correspond to the peak maxima. In all cases the agreement is excellent. Again it should be mentioned that the

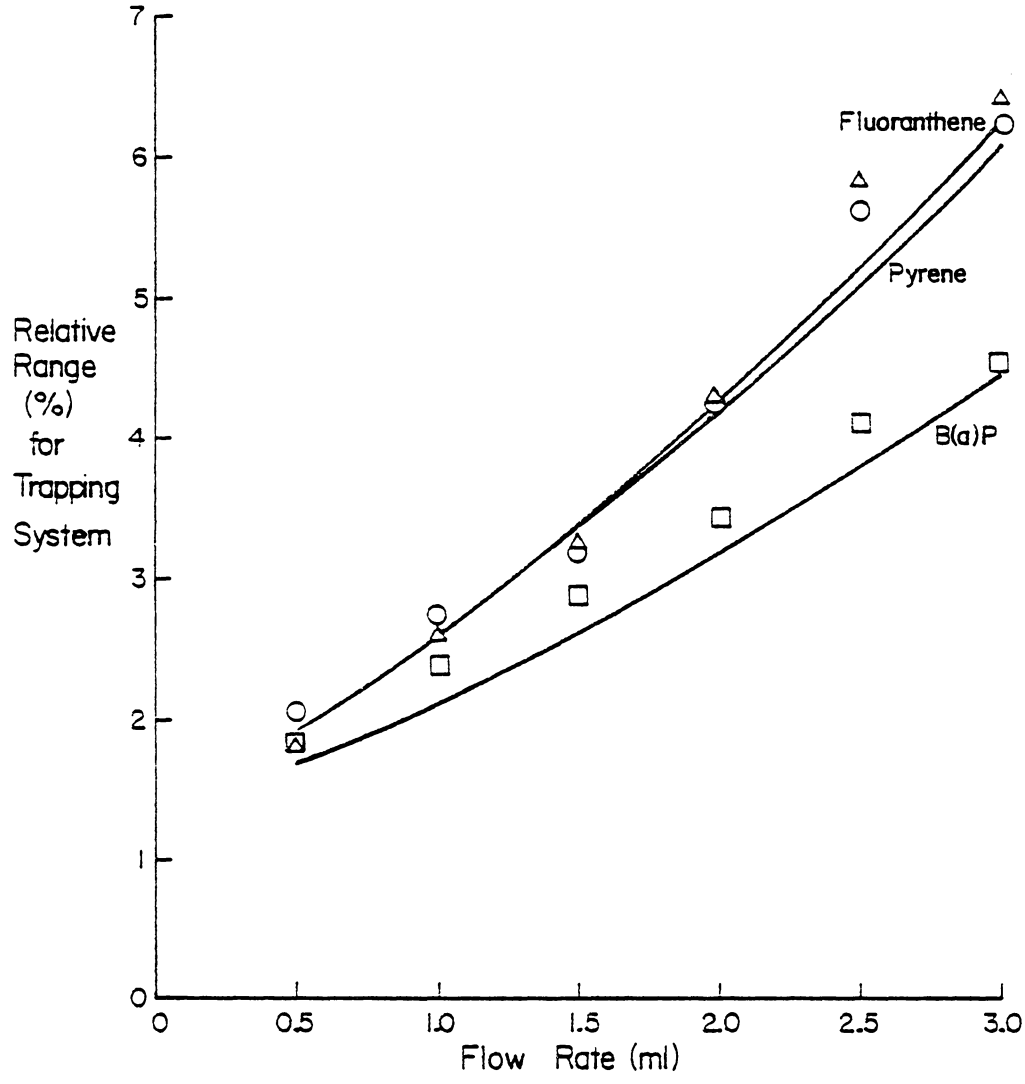


Figure 86. Experimental Evaluation: Effect of Flow Rate on Relative Range for Trapping System

relative range represents a maximum expectation value for the experimental relative standard deviation. For sampling points distant from the peak retention volume, the experimental points begin to deviate steadily and significantly. This is due to the samples approaching the detection limits of the analytical method. This factor is not accounted for by the theoretical model. This is not a serious drawback, however, since it only further emphasizes the importance of sampling the peaks at the peak maximum.

Three important characteristics should be noted in these data. First, is the high rate at which the relative range increases as the peak is sampled at points farther from the peak maxima. In all three cases, for the contribution of the transfer process to the overall relative reproduciblity to be less than 10%, the peak must be sampled within 0.1 minute of the peak maxima. Second, if the peak is sampled at the maximum, the relative range can be as low as 1%. Finally, in the analysis of fluoranthene and pyrene, although these peaks nearly co-elute, their retention times differ by 0.1 minutes. This difference can not be seen in the LC detector response, but unless the peaks are sampled separately, optimum reproducibility cannot be obtained for both.

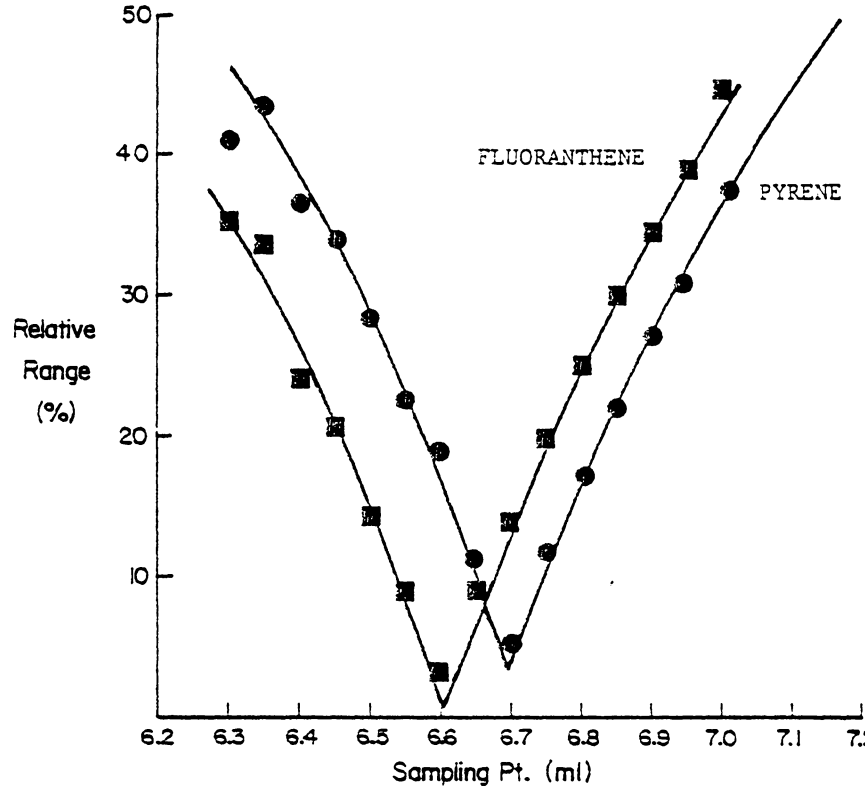


Figure 87. Experimental Evaluation: Effect of Sampling Point on Reproducibility for Trapping System: I

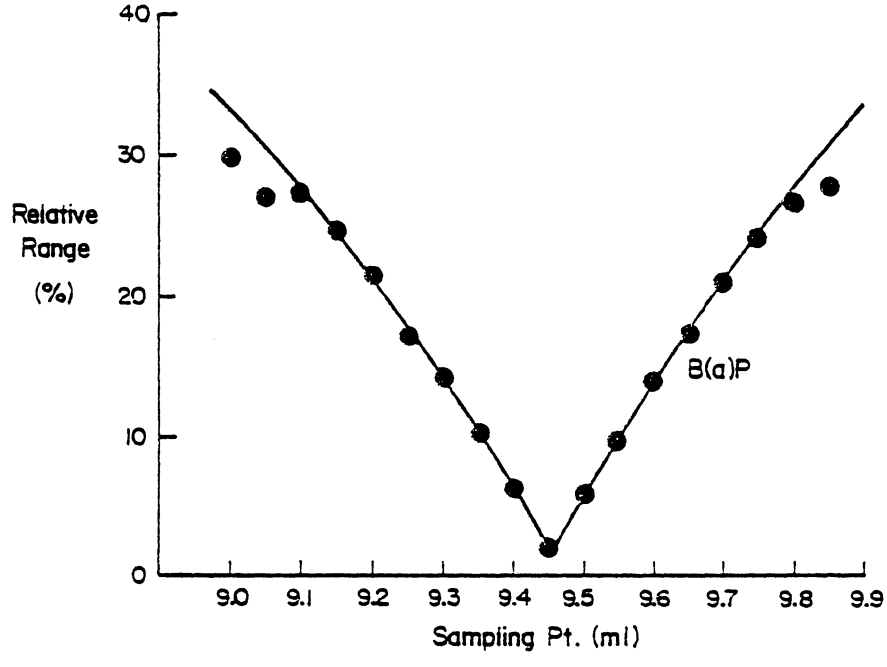


Figure 88. Experimental Evaluation: Effect of Sampling Point on Reproducibility for Trapping System: II

Figures 89 and 90 show the effects of the sampling point on the transfer efficiency for the three model components. The y axis represents the amount of the component transferred. This directly accessible experimental value is used rather than the percentage of the total peak transferred. The amount transferred can easily be converted to the transfer efficiency by dividing by the total amount of the analyte present, which is 100 g, and multiplying by 100. In all three cases, the agreement between the theoretically derived values and the experimental values is very good. It will be observed immediately that these curves represent the elution profiles of the original separation. In the case of fluoranthene and pyrene, for optimum transfer efficiency, each peak must be sampled separately.

The question of the effect of the transfer process on accuracy has not yet been addressed in this discussion. The theoretical considerations, developed in Chapter III, predict that the accuracy of a determination is independent of the mutidimensional chromatographic transfer process, so long as accurate calibration procedures are used. Experimentally, this statement is verified by the results obtained in the analysis of PAH's in SRM 1580 shale oil, SRC-II solvent refined coal and Wilmington Crude Oil presented in the applications section of this discussion.

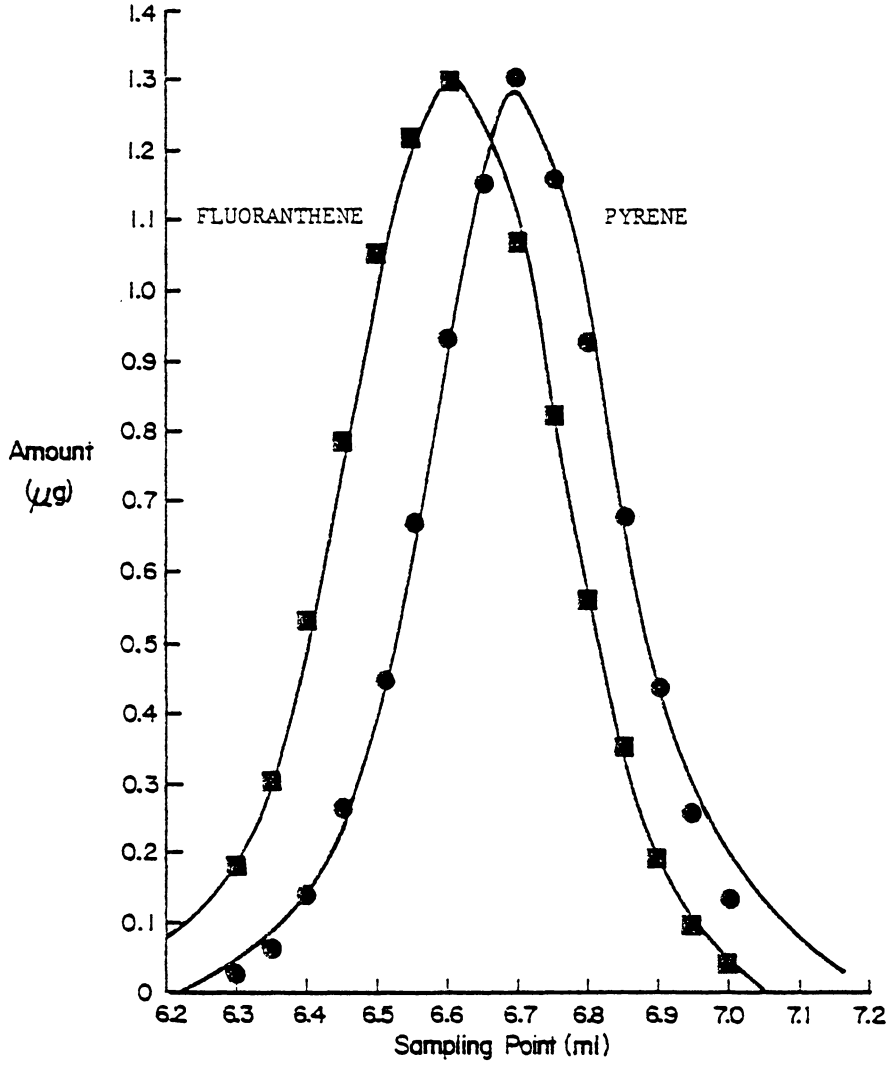


Figure 89. Experimental Evaluation: Effect of Sampling Point of Transfer Efficiency for Trapping System: I

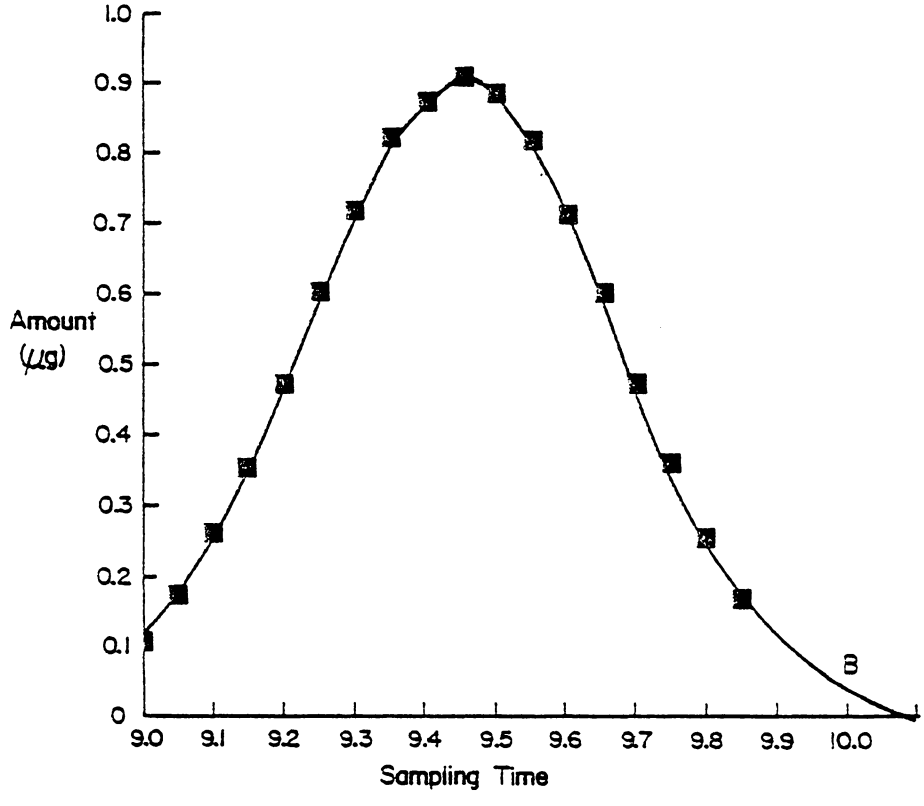


Figure 90. Experimental Evaluation: Effect of Sampling Point of Transfer Efficiency for Trapping System: II

In summary, the theoretical equations predicting the effect of the operation of an on-line multidimensional chromatographic trapping system on reproducibility and transfer efficiency have been verified through the evaluation of an experimental model. Two important conclusions can be drawn from this evaluation. First, for optimum reproducibility, low flow rates should be used. Practically, excessively low flow rates lead to long analysis times, so a compromise must be made. Second, for optimum reproducibility and transfer efficiency (and therefore detectability), it is critical that the analytes be sampled at the peak maxima.

5.3.2 The Switching System

5.3.2.1 Effects of Operating Variables

As in the case of the trapping system, quantitation in the on-line multidimensional switching system can be affected either by the characteristics of the sampled peak or by the characteristics of the sampling process. In the switching system, operating variables of the separation that generates the sampled peak which can have an effect on the quantitation include the flow rate, the retention volume, the retention reproducibility and the column efficiency. The primary characteristic of the transfer process which can

affect the quantitation is the size of the portion of the peak which is transferred. This is determined by the width of the sampling window.

Table 14 shows the conditions for the hypothetical separation which will be used to evaluate the effects of operating variables on the reproduciblity and transfer efficiency of the switching system.

In the following discussion, the effects of experimental variables on quantitation in the switching system are evaluated from equations 35 and 38 in Chapter III.

In the previous discussion of the trapping system, the individual variables were isolated and evaluated separately. As was stated, several act in conjunction, such as the sampling point and the retention precision, but to an approximation they could be evaluated separately. In the switching system, this is not the case, particularly in the evaluation of the effects of flow rate, retention volume and column efficiency. The magnitude of these effects is highly dependent on the size of the sampling window used. In the following, each of these parameters is evaluated for a series of sampling windows. The effect of these operating variables increases as the sampling window decreases. In the limit of very small sampling windows, the quantitation

TABLE 14

Hypothetical Conditions for Switching System

Retention Volume	10 ml
Flow Rate	1 ml/min
Theoretical Plates	5000
Device Precision	0.5 seconds
Retention Precision	1 %
Sample Volume	10 µl
Sample Concentration	10 µg/ml

factors approach those for the trapping system. For large sampling windows, on the other hand, where the switching events occur outside of the peak elution profile, the quantitation is relatively independent of the operating variable. The sampling windows are assumed to be symmetrical about the peak center and are denoted in units of standard deviations. For example, a sampling window of ± one standard deviations indicates the volume eluting between one standard deviation before the peak center to one standard deviation after the peak center is transferred.

The effect of the flow rate on the contribution of the transfer process to the overall reproducibility is shown in Figure 91. As the flow rate increases, the ability of the switching device to reproducibly be actuated in the same relative position is decreased. This effect is similar to that seen for the trapping system. In the case of the switching system, however, even for narrow sampling windows ($\pm 0.5\sigma$), the effect of flow on relative range is small compared to the effect for the trapping system. For large sampling windows, the flow rate shows very little effect on the relative range.

The effects of retention volume and column efficiency is shown in Figures 92 and 93, respectively. The trends in these two figures are identical. As the width of the peak

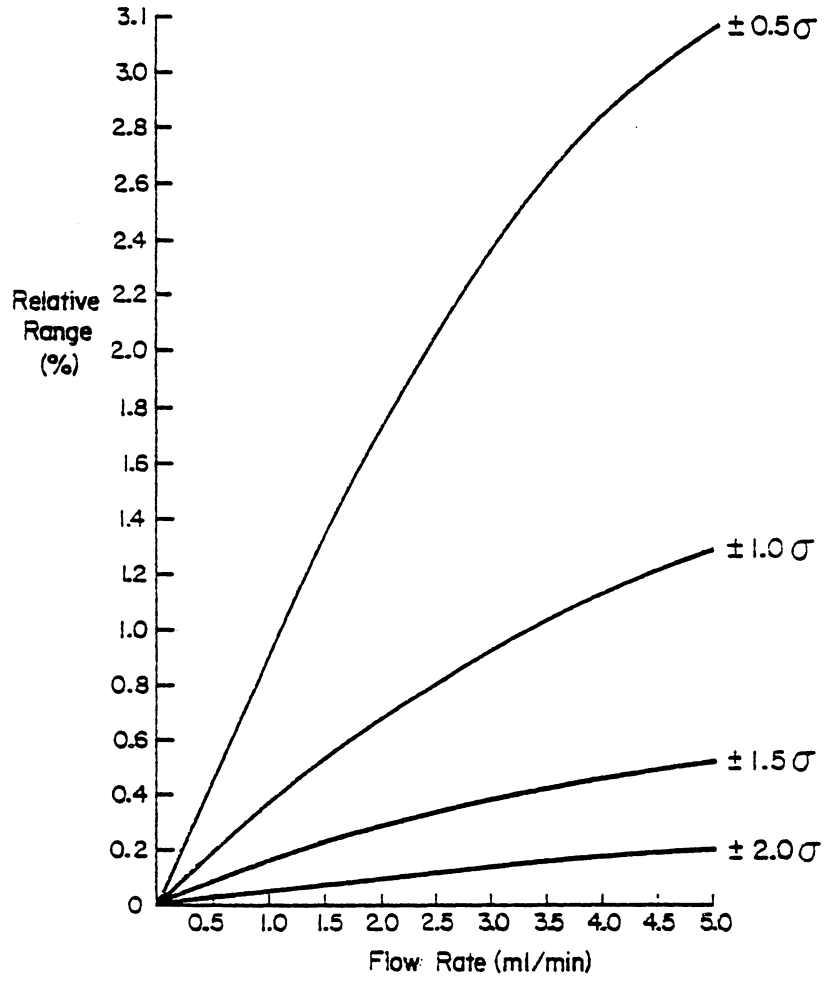


Figure 91. Effect of Flow Rate on Reproducibility for Switching System

decreases, the slope of the peak increases and any variation in the position of the peak relative to the sampling window causes a lack of reproducibility in the transfer process. Thus, low efficiency columns and long retention times optimize reproducibility. Again, for larger sampling windows, this effect is decreased in magnitude.

The above variables are described in conjunction with the sampling window given in units of ± standard deviation. The transfer efficiency of a switching technique depends only on the sampling window size. Therefore, given the same sampling window, none of these variables affects the transfer efficiency.

The effect of the size of the sampling window on both reproducibility and transfer efficiency can be evaluated separately by holding other variables constant. For the system described in Table 14, Figure 94 shows the effect of the sampling window on the reproducibility. As was shown in the previous figures, as the width of the sampling window increases, the relative range decreases. For sampling windows wider than ± three standard deviations, essentially all the peak is transferred and any variation in the switching events will be of negligible consequence.

Figure 95 shows the effect of the sampling window size on transfer efficiency. The result, as expected, is that

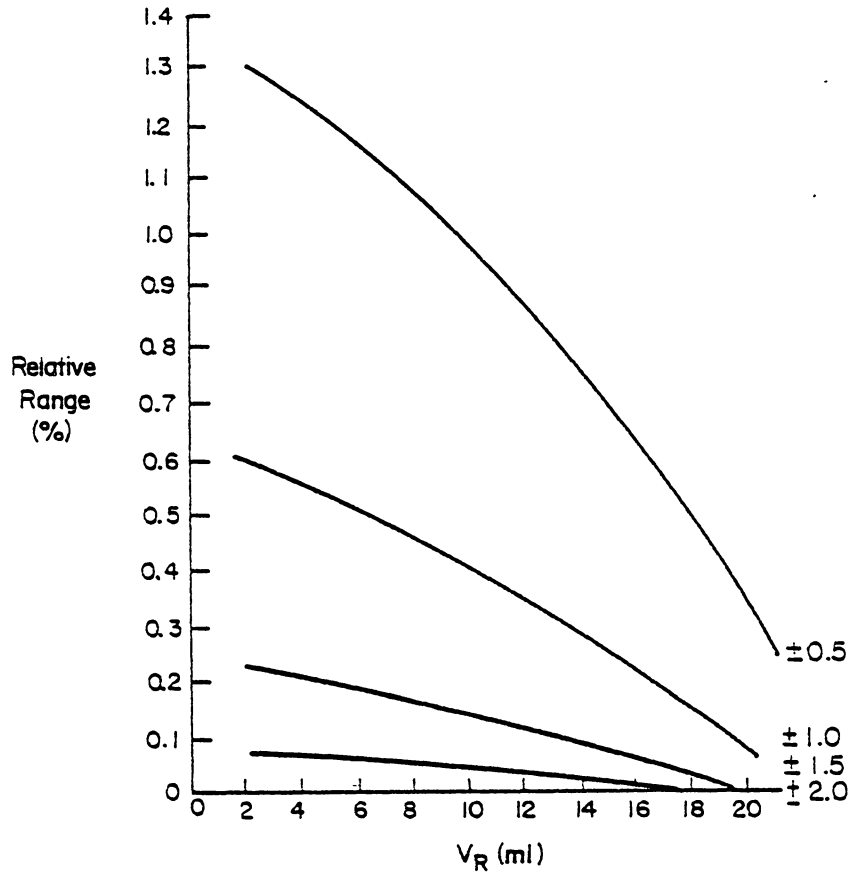


Figure 92. Effect of Retention Volume on Reproducibility for Switching System

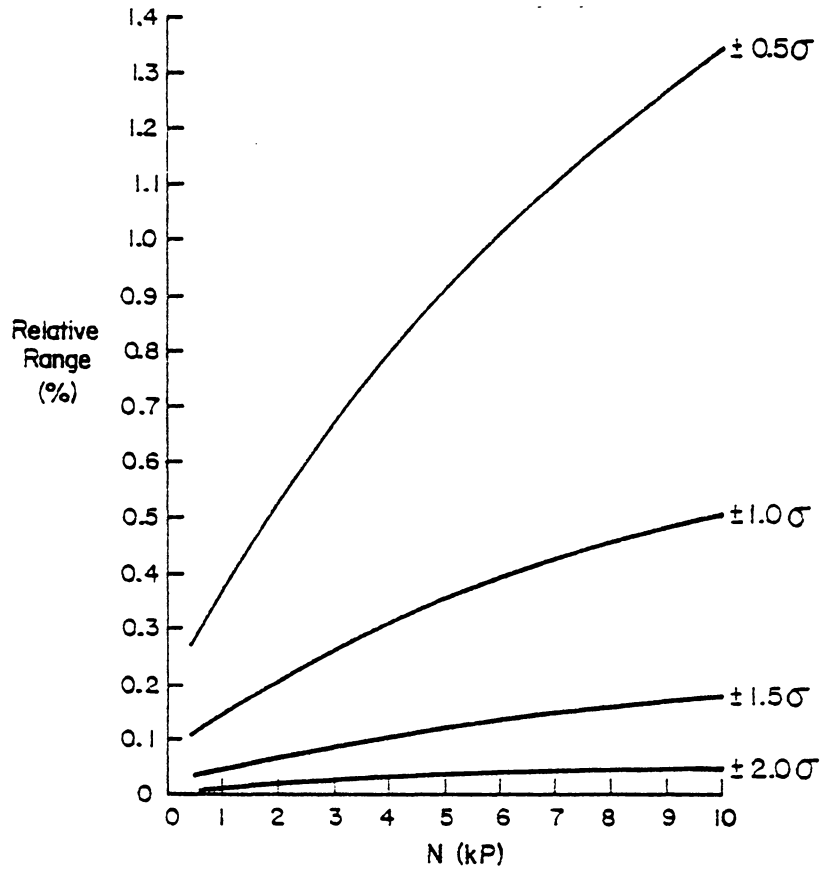


Figure 93. Effect of Column Efficiency on Reproducibility for Switching System

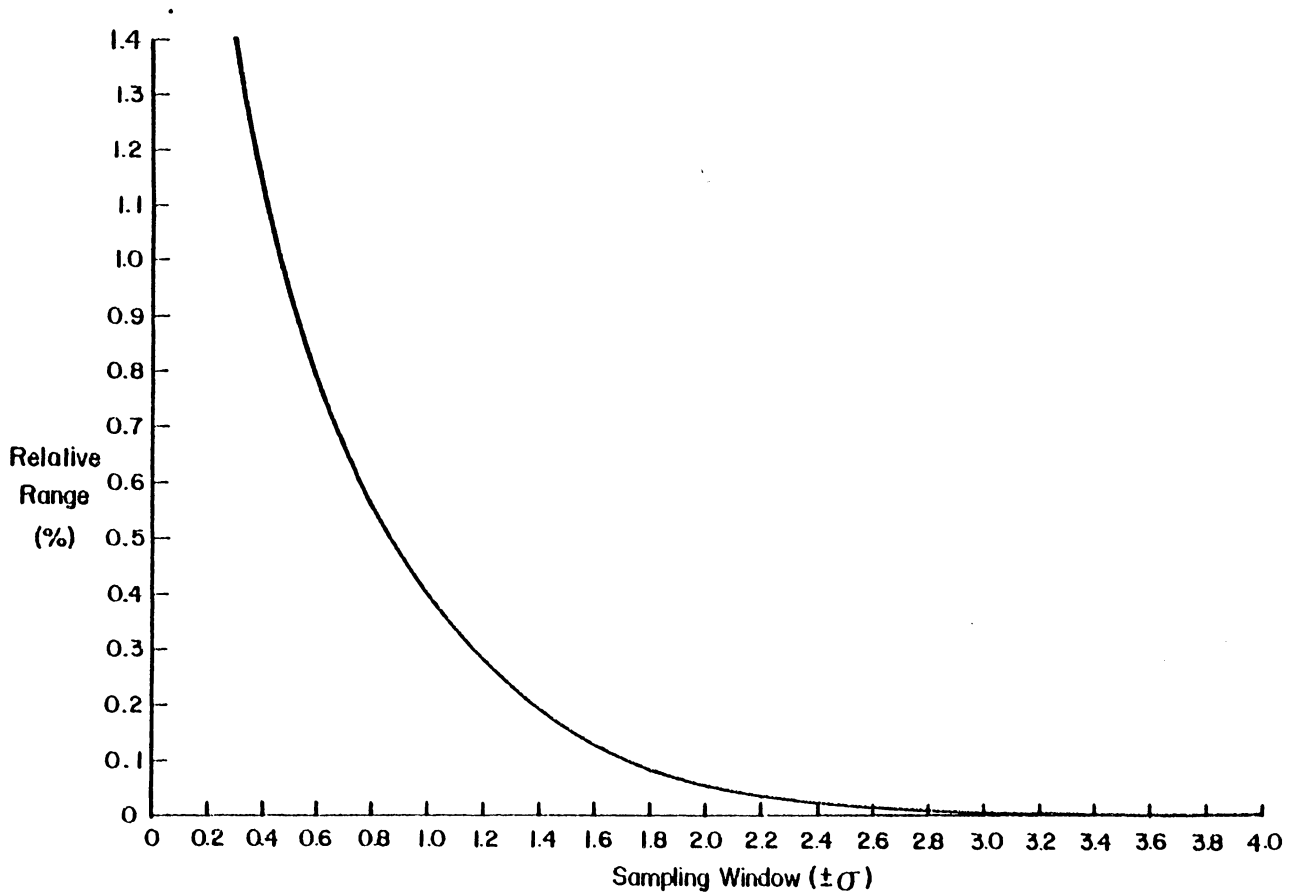


Figure 94. Effect of Sampling Window on Reproducibility for Switching System

the larger the sampling window, the greater portion of the analyte peak is transferred. For sampling windows greater than three standard deviations, essentially all of the peak is transferred.

The conclusions which can be drawn from the above evaluations are that for optimum reproducibility, peaks at large retention time, with low flow rates and low efficiency columns should be transferred using broad sampling windows. Optimum transfer efficiency is obtained through the use of broad sampling windows. In typical switching analyses, there will be little conflict between optimum quantitation and optimum resolution, because, if broad sampling windows are used, the effects of column efficiency, flow rate and retention time can be ignored in terms of quantitation and used to optimize resolution.

5.3.2.2 Experimental Verification

The validity of the theoretical model describing the quantitation of on-line multidimensional chromatographic switching systems was evaluated using the LC/LC analysis of DOPA as an example. The coupling of two ion pair chromatographic separations to effect this analysis was described in detail in the applications section of this chapter.

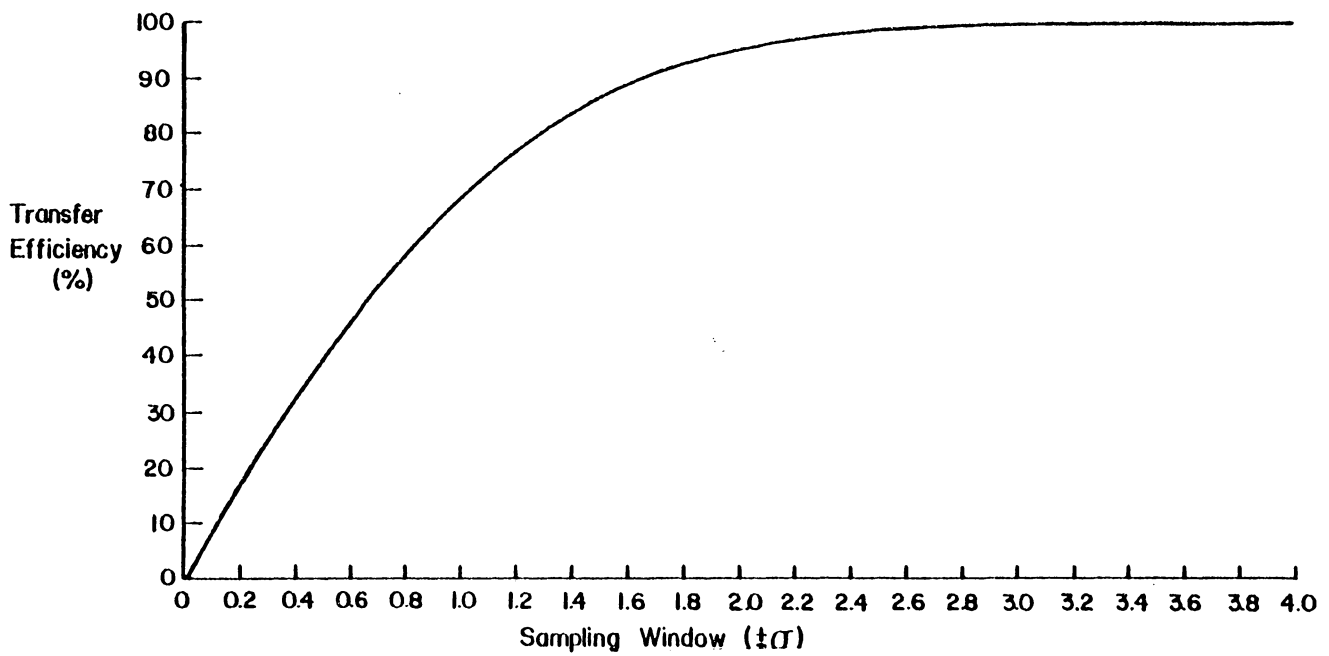


Figure 95. Effect of Sampling Window on Transfer Efficiency for Switching System

In the following studies, analyses were run in sets of ten replicates at each set of experimental conditions. The DOPA sample analyzed was at a concentration of 10 μg/ml dissolved in the LC mobile phase.

As has been pointed out in the previous section, the most critical experimental variable affecting both reproducibility and transfer efficiency is the size of the sampling window. In all other respects, the operating variables affect the quantitation in the switching system in a minor way and in analogous ways to the trapping system. In order to verify the theoretical model concerning the unique aspects of the switching system, therefore, the effect of sampling window on both reproducibility and trapping efficiency was the major evaluation performed using the analysis of DOPA as a model.

Figure 96 shows the experimental results of this evaluation. The x axis in this graph represents the sampling window size as in previous figures and is given in two scales: time and the corresponding number of standard deviations. The sampling windows are symmetric about the peak center. The solid lines represent theoretically calculated results and the points represent the experimental results. As in the experimental evaluation of the trapping system, the quantitation here describes only that component

due to the transfer process. In an analytical system such as this, there is also some contribution to the lack of reproducibility due to the separation process. The data agree well with the predicted results. There is some variation of the data from the predicted values, but this can be accounted for by the experimental error and the fact that the theoretical model, as defined predicts only the maximum values expected.

The only readily accessible experimental variable which affects the quantitation, other than the sampling window size is the flow rate of the primary fractionation system. Figure 97 shows the effect of this variable on the relative range. The three solid curves show the theoretical values for three sampling window widths. The points show the experimental values at approximately these sampling windows. The agreement is good for low flow rates and broad sampling windows. For the narrower sampling windows, the agreement becomes progressively worse with increasing flow rate. These deviations can be accounted for by the fact that at higher flow rates, the sampling windows can be kept at only approximately the same width. It may be possible that the effect of flow rate on the column efficiency also contributes to this deviation. This factor is not taken into account in the theoretical model.

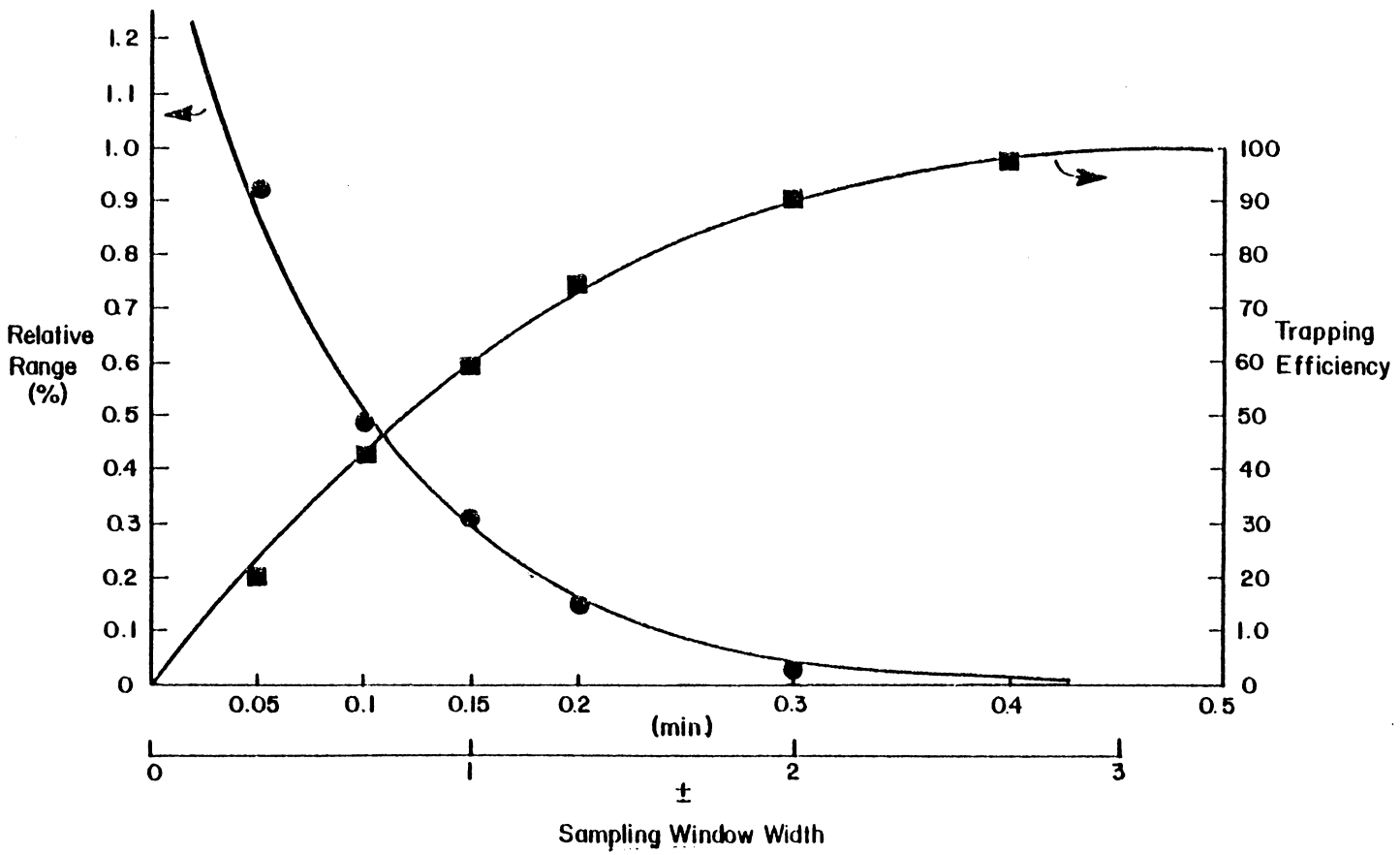


Figure 96. Experimental Evaluation: Effect of Sampling Window on Quantitation for Switching System

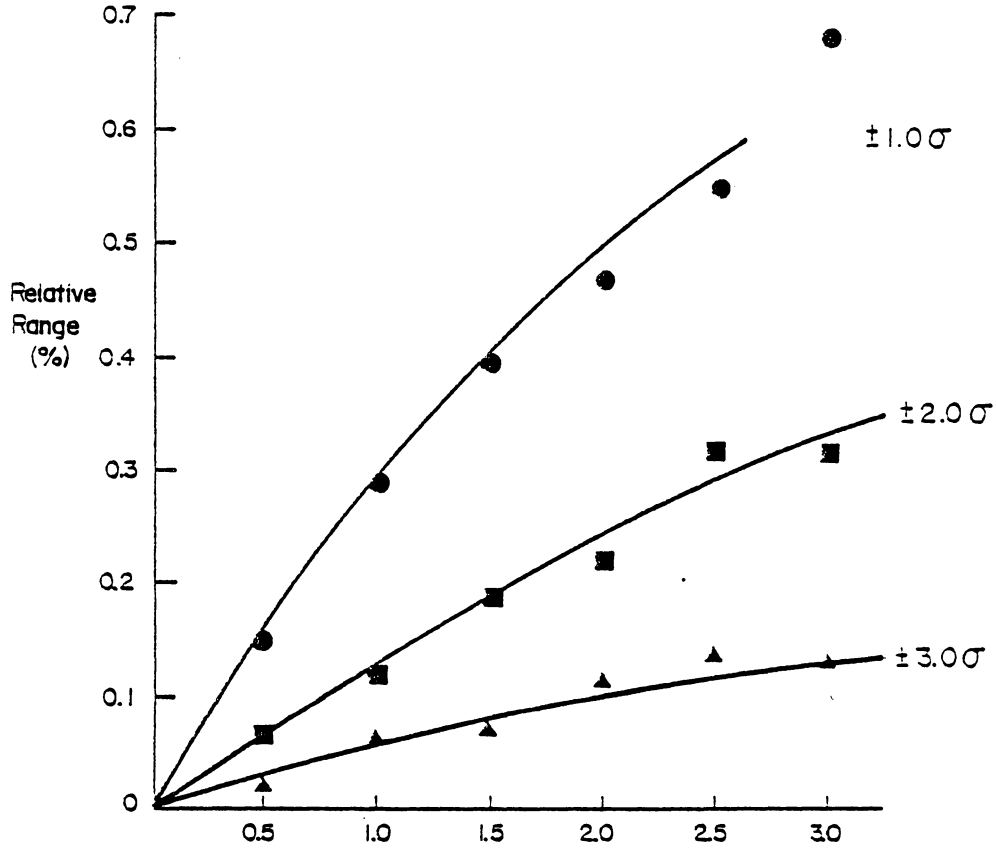


Figure 97. Experimental Evaluation: Effect of Flow Rate on Relative Range for Switching System

Despite these deviations, the data agree favorably with the theoretical predictions. The evaluation points out the importance of using as low a flow rate as is practically possible for optimum reproducibility.

5.3.2.3 Summary: Quantitation

From the these evaluations, it can be stated that the experimental data verifies the theoretically predicted trends for both trapping and switching systems in on-line multidimensional chromatography. In accounting for the variations between theory and experiment, it must be considered that the model is relatively simplistic and makes a number of assumptions concerning the nature of chromatographic processes which are not universally true. It is assumed that the chromatographic elution profile can be described by a Gaussian distribution, which is only true in nearly ideal cases. The presence of adsorption, column overload and column voids can lead to peak assymetry. It is further assumed the total peak volume is independent of flow rate. The dependence of band broadening affects on flow rate, however, is well known. Finally, as noted in the derivation of the theoretical equations in Chapter III, mathematical assumptions and approximations were made in the derivation concerning the integration of the Gaussian

function. In spite of the these approximations, the experimental data support the theoretical predictions. For the most part the agreement is good, but in all cases the trends predicted are verified by the data.

Chapter VI

CONCLUSIONS

On-line multidimensional chromatography has been shown to be useful in a variety of applications including automated sample preparation and the separation of complex samples through the exploitation of chromatographic selectivity. The optimization of resolution through the adjustment of chromatographic selectivity is a viable alternative to the use of increased column efficiency. Systems and techniques of on-line multidimensional chromatography have been described which are both applicable and accessible to the solution of problems currently encountered in a range of analytical fields.

The characteristics of two on-line multidimensional chromatographic transfer systems, trapping and switching, have been delineated. Trapping systems have been shown to be highly selective, but exhibit poor detectablility and reproducibility compared to switching systems. Switching systems have been shown to be less selective, but yield better detection limits and reproduciblity.

The effects of operating variables in the quantitation of these systems have been modelled from a theoretical standpoint and evaluated experimentally. For the trapping

system the most influential variable is the position of the sampling point relative to the sampled peak maximum. Optimum reproduciblity and transfer efficiency is obtained if the analyte peak is sampled at the maximum. The use of low flow rates, low efficiency columns and long retention times favor reproducibility. The use of high efficiency columns and short retention times yield optimum transfer efficiency. For the switching system, the most critical variable is the size of the sampling window used. Optimum reproducibility and transfer efficiency is obtained if a broad sampling window is used, transferring the entire peak volume.

The use of two instrumental systems, LC/LC and LC/GC, has been demonstrated. Both systems have the advantages compared with a single dimensional separation technique of easy automation, high selectivity and versatility and flexibility in application to sample handling. LC/GC system also has the advantage, when used with capillary GC, of high chromatgraphic resolution through high efficiency. The LC/GC system has the disadvantages of poor reproducibility and detection limits. The coupling of LC and GC in this system is subject to some restrictions. In particular, buffers may not be used as LC mobile phases and aqueous mobile phases should be avoided. The LC/LC system has the

restriction that miscible mobile phases must be used in the two separation steps.

Using these two systems, six major applications were developed, resulting in a range of success in terms of usefulness and applicability.

The application of both LC/LC and LC/GC to the analysis of hydrocarbon groups was highly successful. Two analytical techniques were developed in this application which provide significant improvement in resolution and quantitation to previous methods.

The application of LC/LC to the analysis of theophylline and caffeine in biological fluids and LC/GC to the analysis of pesticides in butter, both demonstrated useful systems in automated sample preparation and analysis. The use of these systems is capable of improved sample through-put and reproducibility compared to conventional analyses.

The application of LC/GC to the analysis of PAH's in fuel related samples demonstrated a useful technique in the analysis of pollutants in environmental and industrial samples. This application, however, shows limitations in terms of detectability.

The application of LC/LC to the analysis of catecholamines in urine was useful in protecting the

sensitive electrochemical detector from poisoning by polar sample components. This procedure, however, provided limited resolution with respect to techniques utilizing off-line sample preparation methods.

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ON-LINE MULTIDIMENSIONAL HPLC: DEVELOPMENT, THEORY AND APPLICATIONS

by

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(ABSTRACT)

Two on-line multidimensional HPLC systems are described; one coupling two liquid chromatographic columns (LC/LC), and one coupling liquid and gas chromatography (LC/GC).

Theoretical equations relating the reproducibility, accuracy and transfer efficiency to system operating variables such as flow rate, retention time, column efficiency and transfer volume have been developed. These effects are explained and verified using experimental systems.

Three major applications are shown for each of the systems. For the LC/LC systems, these include; the analysis of caffeine and theophylline in biological fluids; the analysis of hydrocarbon group types in fuels and oils and the analysis of catecholamines in urine with electrochemical detection. For the LC/GC system, the applications include; the analysis of pesticides in butter, the analysis of hydrocarbon group types in fuels and the analysis of polycyclic aromatic hydrocarbons in petroleum related samples.