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Laser fluorescence detection for high performance liquid chromatography

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LASER FLUORESCENCE DETECTION FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Iowa State University PH.D. 1980

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Laser fluorescence detection for high performance liquid chromatography

by

Michael Joseph Sepaniak

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For the Major Department

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For the Graduate College

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Ames, Iowa

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CHAPTER I. INTRODUCTION

Statement of the Problem

The determination of organics in environmental and biological samples remains a major problem for the analytical chemist. The effective levels of certain drugs and the toxic levels of some pollutants are in the ppb range. Environmental and biological samples can be extremely complex when scrutinized at these levels. Thus, the challenge becomes one of detecting and resolving ultra-trace, interesting components in a mixture which can contain hundreds and even thousands of potential interferences. It is not surprising that the attention of many chemists has been focused on developing instrumental methods possessing the sensitivity and resolution needed to meet this challenge.

Analytical Approach

Technological or theoretical advances have often increased the resolving power of an existing technique. A typical example is the development of glass capillary columns for gas chromatography (GC). It is now possible to resolve hundreds of components in a single capillary GC separation (1). This is an order of magnitude improvement over GC using conventional packed columns. Similarly, improvements in spectroscopic resolution have resulted from advances in

sample preparation, typified by matrix isolation and Shpol'skii sample preparation techniques (2).

Even when a high resolution technique is suitable for the compounds of interest, the complexity of certain matrices can make the analysis difficult or impossible. Under these conditions, multi-dimensional methods can be employed to increase the differentiating power of the analysis. The large number of hyphenated methods which have appeared in the recent literature attest to the success of this approach to solving complex analytical problems (3). As with the most familiar of these methods, GC-MS, the pre-hyphen technique is usually some form of separation and the post-hyphen technique(s) are usually detectors, most often spectroscopic, which have the characteristics of high sensitivity and high resolving power. When more than one detector is employed, it is desirable that they provide as much exclusive information as possible. The resulting increase in information is often accompanied by an increase in instrument complexity. Furthermore, the information gleaned from a multi-dimensional analysis is usually less easily treated than that obtained from a single technique of exceptional resolving power. Therefore, most multi-dimensional methods require computer automation for instrument control and data manipulation.

The mature development of GC makes it the method of choice for separating volatile organics. Unfortunately, the

majority of organics are not amenable to GC separation. In these cases, the more versatile high performance liquid chromatography (HPLC) is useful. One of the reasons HPLC has lagged behind GC in development has been the need for sensitive detectors comparable to the kathometer, flame ionization and electron capture detectors of GC. For this reason, much of the recent work in HPLC has centered around developing new detection systems. Discussion of various types of HPLC detectors can be found in the literature (4,5). In most instances, the chromatographic efficiency obtained with HPLC is significantly less than that obtained with capillary GC. Thus, the need for detectors with high resolving power is especially great in HPLC.

One detector possessing the desired sensitivity and selectivity is the fluorescence detector (6). Since fluorescence signals are proportional to the light intensity incident on the sample, even greater fluorescence sensitivity is possible when a laser light source is used. Furthermore, when coupled with a suitably designed detector, the spatial and temporal properties of the laser can result in reduced spectral interference and lower background levels.

The main object of the research presented in this dissertation is to demonstrate, with examples in the environmental and biological fields, the excellent sensitivity and selectivity of HPLC with laser fluorometric detection. HPLC

is used for rough separation and as many as three modes of simultaneous detection are employed (one-photon excited fluorescence, two-photon excited fluorescence, and uv absorbance detection). Thus, the methods developed in this work can be termed multi-dimensional. Furthermore, the use of a laser for excitation, a unique optical fiber-flow cell, and nonlinear fluorescence excitation are technological and theoretical advances which have improved the sensitivity and selectivity of fluorescence HPLC detection.

Organization of Dissertation

The remainder of this introductory chapter will be devoted to a review of modern HPLC and a discussion of laser fluorescence detection for HPLC. The theory and practice of HPLC has been thoroughly covered in several monographs on the subject (7,8,9). Therefore, I have elected to present only a brief discussion of HPLC, highlighting those topics most relevant to the research presented in this dissertation. In contrast, laser fluorescence detection for HPLC is a relatively new technique and, as such, deserves a more complete discussion. Theoretical and practical aspects of the subject are presented along with a discussion of two-photon excited fluorescence detection.

Laser fluorescence detection for HPLC is demonstrated with examples in the environmental and biological fields.

The determination of the anti-tumor drugs adriamycin and daunorubicin in body fluids is presented in Chapter II and the characterization of coal-derived liquids is presented in Chapter III. Previous work involving the determination of certain oxadiazole molecules (anti-inflammatory drugs) can be found in my M.S. thesis (10).

Modern High Performance Liquid Chromatography

As a result of their low volatility or thermal instability, about 80% of the compounds known today do not lend themselves to separation by gas chromatography (11). This problem is not encountered when liquid chromatography is used as the separation technique. This fact, along with advances in liquid chromatography equipment, has prompted extensive use of liquid chromatography in recent years (11-13).

One of the most important achievements in HPLC has been the development of column packings that have organic groups chemically bonded to a silica support (14.15). The silica particles are in the form of either pellicular beads or very small uniform sized particles (12). Silica is a high surface area packing composed of a tetrahedral array of SiO_4 molecules. The valency requirements of the silicon atoms at the surface is partially fulfilled by forming silanol (Si-OH) groups. The silanol groups are responsible for the separation properties of silica when it is used by itself as

the stationary phase in liquid-solid absorption chromatography (14).

The surface of the silica can be modified by chemical reaction in several ways, one of which involves converting the silanol groups to chlorides using thionyl chloride then reacting with a suitable Grignard reagent (12).

$$-SiOH + SOCl_2 \rightarrow -SiCl$$

$$-SiCl + RMg \rightarrow -SiR$$

Packings based on siloxane linkages (-Si-O-Si-R) are also very common.

Stationary phases chemically bonded to a silica support were first introduced in 1969 (16). The organic groups (R) vary greatly depending on the desired retention characteristics. If the organic group is a C_1 - C_{18} alkyl group, then the packing will be nonpolar and nonpolar solutes will be retained more strongly than polar solutes. This is referred to as a reverse phase (RP) separation and employs a polar solvent such as methanol as the mobile phase. The C_{18} alkyl group is the most popular RP organic group, but shorter alkyl groups are sometimes used for separations of slightly more polar solutes or when a fast mass transfer between mobile phase and stationary phase is more important than column capacity (17). Packings having bonded alkyl chains terminated with amine ($-NH_2$) or cyano (-CN) groups can be used to separate relatively polar molecules.

The thermal and solvolytic stability of bonded phases are among their biggest advantages (18). In liquid-liquid chromatography, a pre-column is often used to saturate the mobile phase with stationary phase prior to its entry into the separation column. Since bonded phases do not normally dissolve in the mobile phase, they will not bleed away even when gradient elution is employed. This means eluents and gradients can be chosen to optimize separations without solubility considerations (19). In addition, the quick column-eluent equilibration of bonded phase packings can speed-up analysis. This was particularly helpful for the separations presented in Chapter III. In liquid-solid chromatography, it is necessary to activate the column by heating or deactivate it by adding water so as to produce an adsorption surface with a constant activity (11). With bonded phases, the activity of the stationary phase remains essentially constant run after run.

Despite its widespread use, the mechanism of retention is not completely understood for RP-HPLC. Several researchers have explained the retention of lipophilic solutes in terms of solvophobic interactions with the mobile phase and weak dispersive interactions with the stationary phase (20). Alternately, Lochmuller and Wilder have demonstrated how densely packed bonded alkyl chains can resemble a thin liquid layer and likened RP solute partitioning to liquid-liquid partitioning (21).

Obviously, the partitioning forces in RP separations are much weaker than in many other forms of liquid chromatography. This means that rather subtle chemical effects can influence retention (22). One such effect is the suppression of solute ionization through pH adjustment. Maintaining the eluent pH above the pK_a of weakly acidic organic compounds (or maintaining the pOH above the pK_b of weakly basic organic compounds) makes their retention unlikely. This makes pH an additional variant in the separation of complex mixtures, particularly samples of biological origin. Ion suppression effects are utilized in the separation of the anti-tumor drugs in Chapter II.

Ideally, a stationary phase is chosen to match as closely as possible the chemical properties of the solutes to be separated (17). Various types of RP packings have been used in a wide variety of separations. It has been estimated that 75% of all HPLC separations are performed on RP packings (23). Karch, Sebestian, Halasz, and Engelhardt describe and optimize the separation of ten mixtures important in routine work (24). Included in their work were the separations of alcohols and phenols using a C_4 alkyl bonded phase.

Many classes of compounds have been separated using chemically bonded octadecylsilane ($-SiC_{18}H_{37}$), including herbicide residues in cotton seed oil (25), hashish extracts (26), LSD (27), quinones (28), carbamates (29), vitamins and

steroids (12), and sulfa drugs (28). When separating certain aromatic compounds, it is advantageous to use chemically bonded diphenyl ($Si(Ph)_2$). Actinomycins (30) and anti-tussives (28) have been separated with this packing. Recent interest in air and water pollution control has prompted work in the HPLC separation of polyaromatic hydrocarbons (PAH). RP columns have been used extensively for this purpose (11, 26,31-34).

In a few instances, the chromatographic efficiency afforded by HPLC has approached that of capillary GC. Extremely high efficiency HPLC separations of samples, such as the coal derived liquids discussed in chapter 3, have been reported using micro-bore (capillary) columns (35-37). Unfortunately, capillary LC is not nearly as well developed as capillary GC. Reproducibility has not been adequately demonstrated and in many instances the impressive separations which were cited took hours, even days, to complete. This later shortcoming originates in the fact that diffusion coefficients in the mobile phase are about ten thousand times smaller in LC than in GC (38). The resulting slow mass transfer must be compensated by reducing the linear velocity of the mobile phase and using extremely narrow bore columns (1-50μ), if high efficiency is to be maintained. Novotny has circumvented this problem somewhat by packing his micro-bore columns, but the preparation of these columns includes a difficult extrusion step (37).

The object of the research presented in this dissertation does not include exploring capillary LC techniques, but it was mentioned for two reasons. First, capillary LC, when more fully developed, will provide the separatory power needed to solve many complex analytical problems. Second, the laser fluorometric detectors described here meet the low extra-column volume requirements of capillary LC.

Laser Fluorometric Detection for HPLC

General information

While the U.V. absorbance detector is still the most commonly used mode of photometric HPLC detection (8,39), the fluorometric HPLC detector has received a considerable amount of attention in recent years. The carcinogenicity of many PAH is the motivation behind attempts at detecting them in the sub-nanogram range, and the high sensitivity of fluorometric detection of compounds with appreciable fluorescence quantum efficiencies makes it a popular method for PAH analysis (31, 32,40). Fox and Stanley were able to determine the concentrations of several PAH in atmospheric particulate matter in the Baltimore Harbor Tunnel using a commercial fluorometric detector (33).

There are numerous other examples of fluorometric HPLC detection. Biological samples determined with fluorometric HPLC detection include indoles in urine (41), phenols in urine

(42), ergot alkaloids in blood plasma (43), and barbiturates in blood plasma (44). Many samples that are nonfluorescent can be labeled prior to separation. Frei and Lawrence used reagents such as 4-chloro-7-nitrogenzo-2, 1,3-oxadiazole, dansyl-chloride, and dansyl-hydrazine for the labeling and nanogram level detection of carbamates, ureas, organophosphorous compounds, aliphatic amines, aldehydes, ketones, and biphenyls (45).

Several commercial HPLC fluorometric detectors are reviewed in the literature (4,46,47). In addition, Cassidy and Frei describe a detector consisting of a commercially available fluorimeter equipped with a small volume flow cell (48) and several other researchers have adapted spectrofluorimeters to HPLC detection (32,43). Most fluorometric HPLC detectors have the same basic optical arrangement. The exciting radiation from sources such as a mercury vapor lamp, xenon arc lamp, or tungsten lamp is collected then passed through either an optical bandpass filter or excitation monochromator before being focused into a flow cell. Flow cells are often optical quality quartz capillary tubes with the focused excitation beam traversing the capillary tube along its diameter. The fluorescence is collected at right angles to the exciting beam and passed through an optical filter or emission monochromator before being detected by a photomultiplier tube or other photosensitive device.

In fluorescence HPLC detection, background signals are composed primarily of Rayleigh scattering, specular scattering off of highly curved flow cell walls, Raman scattering of the eluent, and eluent impurity fluorescence. Fortunately, molecular fluorescence occurs at a lower energy than the excitation energy and it is possible to at least partially discriminate between fluorescence signals and the various contributions to the background. This is the primary reason for the low detection limits of fluorimetry (49). High sensitivity is just one of the advantages of the fluorometric detector. The detector is also nondestructive, meaning fractions can be collected after separation and subjected to further analysis (33) or series detectors employed in a multidimensional analysis. Another advantage of the fluorometric detector is its relative insensitivity to flow rate and eluent composition (48), which minimizes problems with pumping noise, gradient elution, and flow rate programming.

The most prominent characteristic of the fluorometric HPLC detector is its predictable selectivity (9). The detector is selective for two reasons. First, relatively few of the molecules that absorb radiation detectably fluoresce, and second, those molecules that do fluoresce have both an excitation spectrum and an emission spectrum which can be used to characterize the eluted solute molecules. This advantage in

detection was clearly illustrated by Vaughan, Wheals, and Whitehouse in the detection of PAH (32).

Relevant laser properties

Over the last decade, the laser has evolved into a practical and reliable experimental tool for the analytical researcher. During this period, laser-based spectroscopic techniques have become quite prominent but only recently has the laser been applied to chromatographic detection (50-55). The most notable characteristic of the laser is the high intensity of its output and, since fluorescence signals are proportional to the light intensity incident on the sample, it is only natural that laser fluorimetry has become the most developed laser-based HPLC detector (56).

Simply replacing a conventional light source with a laser does not guarantee improvement in detectability. The chromatographic flow cell must be designed to take advantage of the unique characteristics of the laser. Therefore, it is instructive to discuss those laser properties which are important to fluorescence HPLC detection.

Power Commercially available lasers are currently capable of average powers greater than 10 W and peak powers, in the case of pulsed lasers, greater than a MW. This compares very favorably with the output powers of conventional HPLC light sources. As an example, consider a 100 W mercury arc discrete line source and a 100 W quartz halogen tungsten

lamp continuum source. The former provides 4.5 mW at 254 nm when f/1.0 imaging optics are employed and the latter provides 17.3 mW at 600 nm with the same imaging optics and a typical fluorometric HPLC bandpass of 10 nm (56). In most instances, conventional light sources provide photon fluxes less than those mentioned.

Increasing the photon flux in the sample fluorescence observation area will increase, up to the point of saturation, the fluorescence signal. But this does not guarantee improvements in detectability since noise, which consists primarily of stray radiation, can increase concomitantly. Detection can be further complicated by thermal lensing (57), an effect which results when a large amount of laser light is absorbed. A thermal gradient is formed which tends to defocus or distort the excitation beam. Fortunately, the spatial and temporal properties of the laser can help to minimize the deleterious effects of large photon fluxes. Also, a properly designed flow cell can be helpful in reducing noise and thermal lensing effects. Flow cell design will be discussed later in this chapter.

The ability to adjust excitation and emission wavelengths to minimize interferences is an advantage of fluorimetry. When signals are large, as with laser excitation, it is possible to sacrifice some signal, to reduce interferences, by adjusting wavelengths away from excitation and emission

maximums and by narrowing emission bandwidths. Thus, the high power of lasers can result in improvements in selectivity as well as sensitivity. Selectivity can be further enhanced by monitoring nonlinear fluorescence phenomenon. Nonlinear optical transitions are of very low probability and it is only with high power light sources, such as the laser, that they can be observed.

Collimation Perhaps the most exciting recent development in HPLC has been the exceptional efficiencies obtained with micro-bore and micro-particle (particle diameters $\leq$ 5 microns) columns. The very small peak volumes obtained with these columns can be seriously broadened by large volume detectors. In general, detector broadening is noticeable when detector volumes exceed 25% of the peak volume at the column outlet (58).

The collimated output of the laser, because of the properties of Gaussian beams, can be easily focused into extremely small volumes. Beam diameters of a few mm and divergences of less than a mradian are common for lasers. The Gaussian output of a laser can be focused to a beam waist (w) which is approximated by the product of the divergence of the laser beam and the focal length of the focusing lens. The useful range of collimation of the focused beam, sometimes referred to as the Rayleigh range, is defined by equation 1 where z is

the Rayleigh range and λ is the wavelength of the focused radiation.

$$z = \pi w^2/\lambda \qquad (1)$$

Assuming a wavelength of 500 nm, a beam divergence of 1 mradian, and a focusing lens with a focal length of 4 cm, the Rayleigh range becomes 1 cm (a fairly long fluorescence path length). This corresponds to a detector volume limit of approximately 12 nl, which compares very favorably with the 5-50 µl flow cell volumes found in commercial fluorometric HPLC detectors. A final advantage of the spatially well-defined laser beam comes in minimizing stray radiation. The laser beam is far easier to optically manipulate than the 4π steradians output of conventional light sources, and this can result in less stray light.

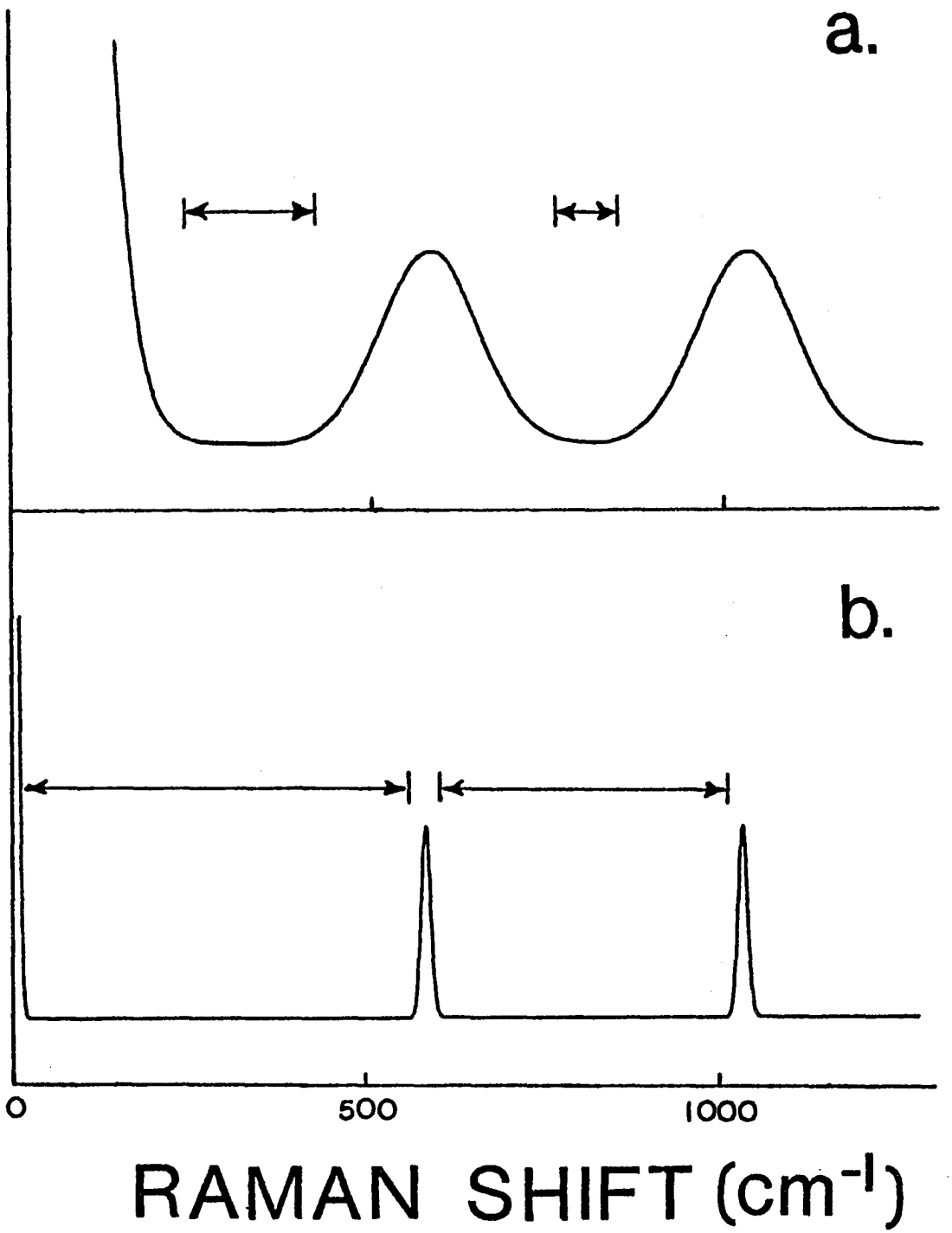
Monochromaticity The output bandwidth of a laser is typically orders of magnitude narrower than liquid phase excitation and emission bandwidths. Thus, the monochromaticity of the laser provides only small improvements in selectivity over conventional light sources. Still, when analyzing extremely complex samples, the sub-angstrom laser output is less likely to produce spectral interferences than the 5-10 nm excitation bandwidths of commercial fluorometric detectors. Obviously, it is possible to limit the excitation bandwidth of commercial instruments to values comparable with

laser output bandwidths, but a severe reduction in photon flux would result.

A major contribution to the background in a fluorescence experiment is Rayleigh and Raman scattering of the solvent. Rayleigh and Raman bands, which have an inherent width of about 1 nm, must be at least as wide as the excitation source bandwidth. A comparison of the spectral distribution of Rayleigh and Raman scattering for a conventional light source and a laser light source is shown in Figure 1. The fluorescence observation "windows" are considerably larger for the laser excitation case and this can result in lower background levels.

It should be noted that most commercial fluorometric HPLC detectors demonstrate detectability on compounds having fluorescence red shifts greater than 3500 cm^{-1} (59,60). Under these conditions, the Rayleigh and Raman background is easily rejected, even using wide bandpass optical emission filters, which transmit a large portion of the fluorescence emission. Because of photodissociation, intersystem crossing at higher excited states, greater contaminant or solvent absorption, and poorer component absorption for transitions to higher excited states, it is often undesirable to excite to an electronic manifold higher than the fluorescing level. Instead, it is necessary to excite directly into the fluorescing level, which is almost always the first excited singlet state of the

Figure 1. Stray light (Rayleigh and Raman scattering) distribution in (a) a broadband source at a 10 nm bandwidth, and (b) a monochromatic source such as a laser. The available "windows" for fluorescence monitoring are larger for case (b)


a.
b.
0
500
1000
RAMAN SHIFT (cm-1)

molecule. In these cases, the Rayleigh and Raman background rejection advantage of the laser is particularly useful.

Polarization Laser outputs are often plane polarized. Rayleigh scattering and many strong Raman transitions are also plane polarized and, therefore, proper orientation of the detector with respect to the laser polarization can provide some measure of rejection of these contributions to the background.

Temporal Fluorescence lifetimes can be used to resolve fluorescent compounds. Additionally, pulsed excitation and synchronous detection can be utilized to discriminate between coincidence photons (Rayleigh and Raman scattering) and fluorescence signals (61). High peak power pulsed lasers, with pulse widths less than typical fluorescence lifetimes, facilitate this discrimination. Pulsed lasers were not employed in the work presented in this dissertation, but improvements in background suppression and component resolution could be expected with their use. Furthermore, the high peak powers and low average powers of pulsed lasers, such as the N_2-pumped or Nd:YAG-pumped dye laser, should result in larger nonlinear fluorescence signals and less thermal lensing. Two-photon excited fluorescence, a nonlinear technique, will be discussed later in this chapter.

Detector design

When interfacing a high performance liquid chromatograph to a fluorometric detector, the important considerations are chromatographic efficiency and fluorescence detectability (62). The implementation of laser excitation places further requirements on detector design. Sensitivity is seldom the limiting factor in fluorescence detection, especially when laser excitation is employed. Rather, background noise levels limit detectability. Noise levels can be reduced if flow cells are designed for rapid laminar flow, minimal stray light collection, and insensitivity to chromatographic related flow irregularities (pump noise, gas bubbles, eluted column packing, etc.).

Chromatographic efficiency can be maintained if flow cell volumes are small with respect to the volume of the chromatographic peak as it exits the column and if there are no stagnant areas in the flow cell, which tend to increase the amount of eluent needed to "wash out" a chromatographic peak. Also, the detector volume from the fluorescence observation area to the exit plumbing should be kept small if detectors are to be connected in series.

The influence of detector design on detectability warrants further comment. Chromatographic baseline noise is a function of the background light level. Improvements in detectability can result from reducing this functionality,

which in the best case should approach the shot noise limit. Pumping noise and other flow irregularities common to HPLC can greatly increase the functionality. In many instances, increases in fluorescence signals, in response to increases in the excitation light intensity, are matched by increases in background light levels. Under these conditions, a high functionality can virtually eliminate improvements in detectability resulting from using a laser light source.

To prevent this from happening, flow cells should be designed to have the following characteristics. To insure minimal thermal lensing, it is best to have rapid flow in the fluorescence observation area. This requirement is completely compatible with the small focusing volumes of laser beams and the low extra-column volume requirements of high efficiency HPLC. Furthermore, fluorescence signals should be preferentially accepted over specular scattering and flow cell fluorescence. Eluent degassing can produce bubbles which collect in the flow cell and contribute significantly to baseline noise levels. Eluents should be purged or sonicated prior to use, but bubble formation is sometimes inevitable, especially when certain gradients are used. To minimize eluent degassing related disturbances, flow cells should be designed to prevent bubble collection. Finally, detectors should provide efficient, convenient, and reproducible imaging with both the excitation and emission optics.

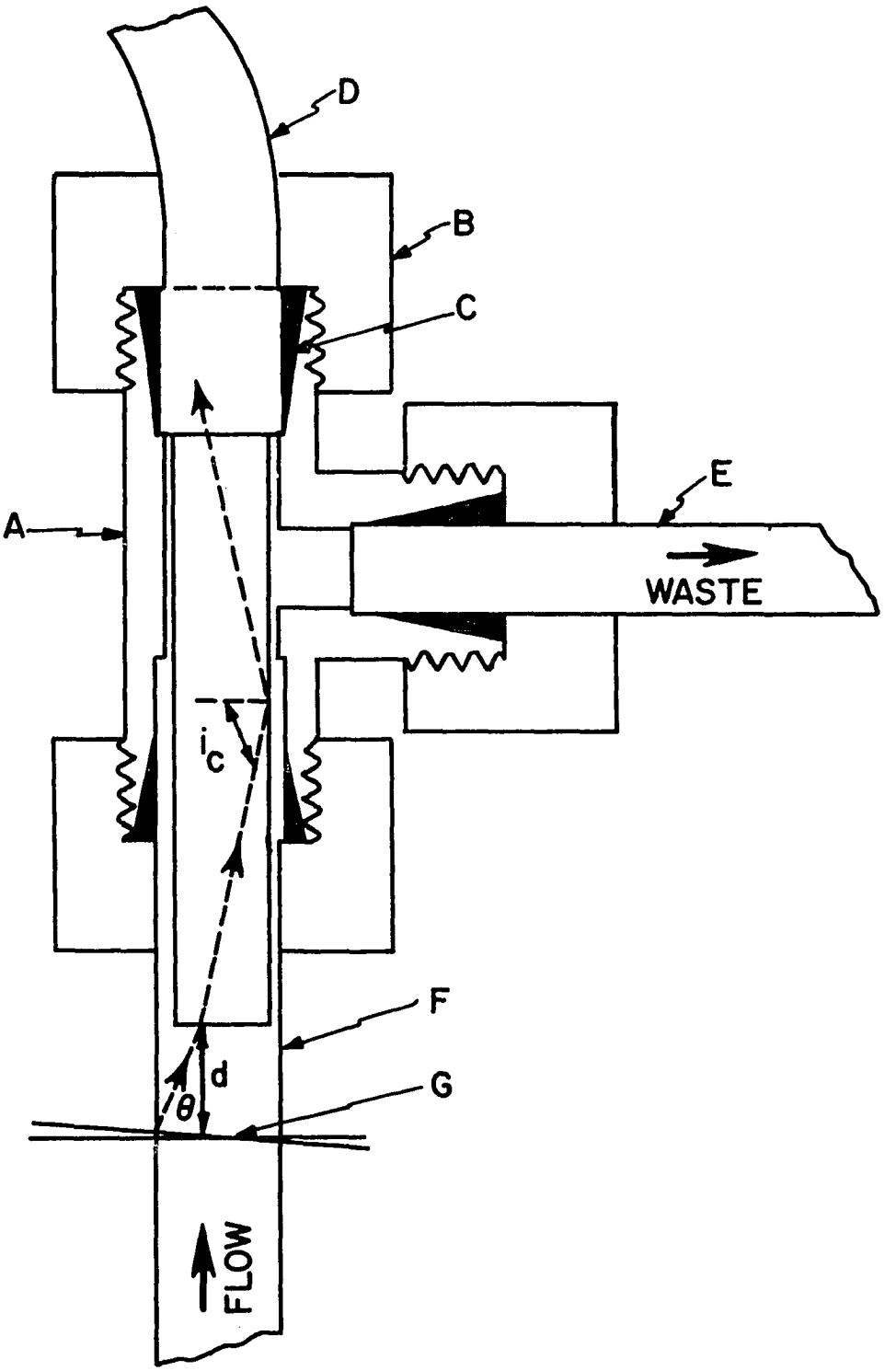
Several HPLC flow cells designed for laser fluorescence detection have been described in the literature (50,52-54). Zare has developed a flow cell which suspends the flowing eluent, in the form of a droplet, between the vertically arranged exit tubing of the column and a solid rod of comparable dimensions (50). The obvious advantage of this design is the absence of flow cell windows, which can contribute significantly to stray light levels. Sub-picogram quantities of certain aflatoxins have been detected using this flow cell. Unfortunately, there are some problems with this design, all relating to the optical integrity of the droplet. Bubbles in the column effluent become trapped in the droplet and produce fluctuations in its shape, until the buoyancy force of the bubble allows it to escape the droplet. The size and shape of the droplet also changes with varying eluent composition, and this produces irreproducible changes in the focusing of the excitation beam and the collection of fluorescence with the emission optics. A final limitation of the flowing droplet detector is that when series detection is employed, it must be the terminal detector.

A capillary tube, with the focused excitation beam traversing its diameter and the fluorescence collected at right angles with a lens, is probably the simplest flow cell. A quartz capillary flow cell was used in my M.S. thesis

research for the two-photon excited fluorescence detection of HPLC separated oxadiazole molecules. While it is an appropriate flow cell for two-photon excited fluorescence detection, it is inadequate for normal fluorescence detection. This is because a simple capillary tube flow cell does not provide any rejection of specular scattering or flow cell fluorescence. Since stray radiation is not a problem in two-photon excited fluorescence detection, for reasons to be discussed in the next section, the capillary tube flow cell can be efficiently used for that type of detection.

A unique adaptation of the capillary tube flow cell was used in the research presented in this dissertation. In this design an optical fiber, with its cladding stripped, was slipped into a capillary tube and held in place with a Swagelok tee. The flow cell is shown in Figure 2 and its construction is described in the experimental section of Chapter II. There are several advantages of this design. The detector can be arranged vertically with the eluent flowing upward around the optical fiber. Any bubbles in the eluent are swept quickly through the detector, rather than being collected. This arrangement, when coupled with a vertically polarized laser, aids in the rejection of Rayleigh and Raman scattering.

Figure 2. Close-up of capillary tube-optical fiber flow cell. A = 1/16" s.s. tee, B = 1/16" s.s. nut, C = graphite ferrule, D = optical fiber (0.99 mm diameter core, 1.5 mm O.D.), E = 1/16" s.s. tubing, F = quartz capillary tube (1.05 mm I.D., 2 mm O.D.), G = focused laser beam


D
B
C
E
A
WASTE
i_c
F
d
θ
G
FLOW

In the limit of low absorption, fluorescence signals are proportional to path length, which is simply the internal diameter (I.D.) of the capillary tube. Unfortunately, detector volume is proportional to the square of the flow cell I.D. and a relatively narrow tubing bore is needed to assure that chromatographic efficiency is maintained. This does not represent a major problem since it is the background noise level, and not the fluorescence signal, that limits detectability in laser fluorescence detection. An I.D. of 1 mm provides excellent detectability and a flow cell volume limit of about 1 µl. A smaller I.D. would provide sub µl flow cell volumes but a decrease in fluorescence signal would result. However, smaller I.D. tubing would also result in faster linear eluent flow and less thermal lensing. This could reduce baseline noise, thereby counteracting the loss in fluorescence signal.

Perhaps the biggest advantage of the capillary tube-optical fiber flow cell is its ability to reject specular scattering and flow cell fluorescence. The path of a light ray emanating from near the flow cell wall is traced in Figure 2. The angle i_c must be greater than the critical angle for the light ray to be transmitted and this will be true if the condition shown in equation 2 is met, where n_f, n_c, and n_e are the indices of refraction of the fiber, fiber

$$\sin \theta \leq (n_f^2 - n_c^2)^{1/2}/n_e \qquad (2)$$

cladding and eluent. θ is smaller for light rays emanating from the center of the flow cell. Hence, the fiber tends to select fluorescence over specular scattering at the flow cell walls. The optical fiber can be placed very close to the focused laser beam, to achieve a good collection efficiency and substantial rejection of specular scattering and flow cell fluorescence.

The path of the excitation source through other types of flow cells is usually magnified and imaged onto the entrance slit of the emission monochromator. Small deviations in the position of the focused beam resulting from refractive index gradients (pressure changes in the pumping cycle and thermal lens effects) are also magnified, and can cause the image to at least partially miss the entrance slit. The detector presented here was found to be insensitive to small changes in the position of the focused laser beam and the light collected with the optical fiber can be simply and permanently aligned with the entrance slit of the emission monochromator.

Two-photon excited fluorescence

In 1931, Maria Gopper-Mayer realized that a molecule could absorb two photons simultaneously to achieve a change in its quantum level (63). The process is usually described

in terms of second order pertubation theory (64,65), but a simple quantum picture of the process will suffice for this treatment. If a molecule is exposed to two photons of frequencies v_1 and v_2 simultaneously, then there is probability that electrons in the molecule will oscillate at the sum of the two frequencies. If the combined frequency (v_1+v_2) corresponds to a transition between quantum levels in the molecule then electronic oscillations in the molecule can cause absorption of the two photons involved and the molecule is left in an excited state (66).

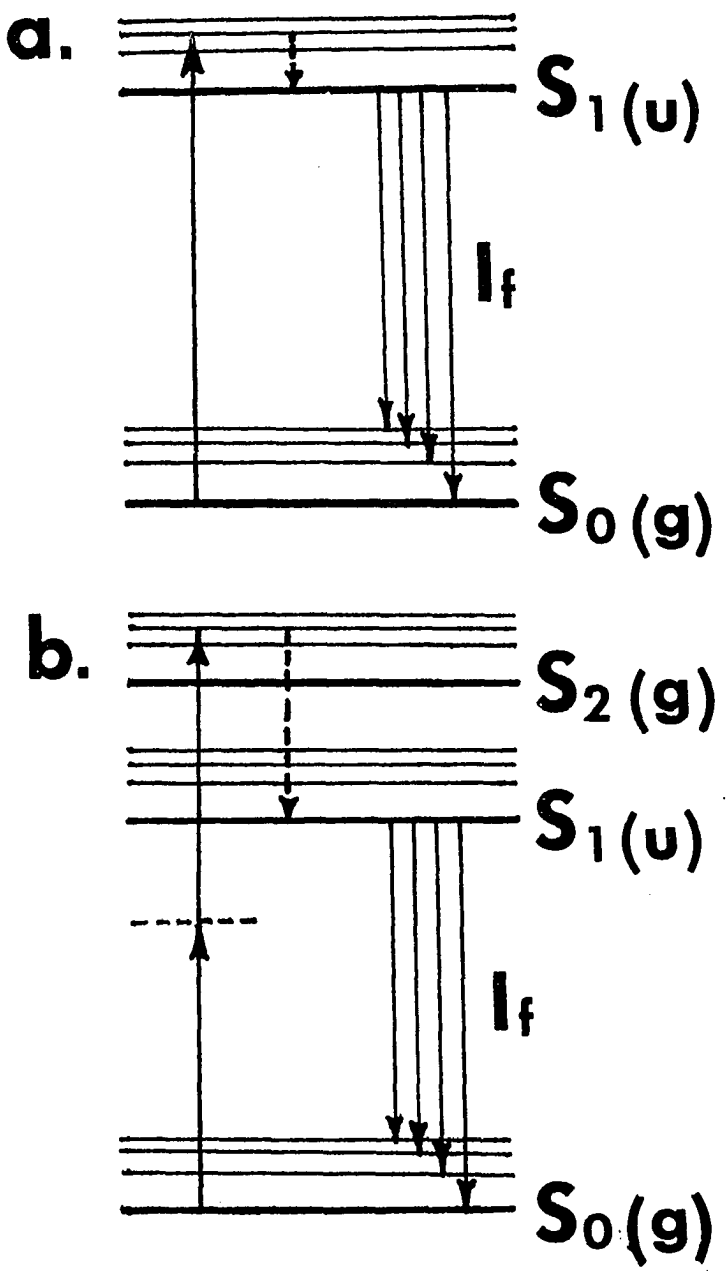
The two-photon process involves some distinctive selection rules and represents a way for spectroscopists to find and describe new molecular states. Quantum theory states that for a transition between an initial state (i) and a final state (f) to be allowed, the transition moment integral

$$M_{if} = \int \psi_i(r)\psi_f^* d\tau \qquad (3)$$

must be nonzero. Consideration of the symmetries for the electric dipole moment operator (r) and the states involved results in the observation that in totally symmetric molecules transitions between states of equal parity are forbidden (67). If the same treatment is made for the two-photon process, it is found that transitions between states of equal parity are allowed, that is, in centrosymmetric

molecules gerade to gerade two-photon transitions are allowed (68). The fact that two-photon absorption involves different selection rules than one-photon absorption results in different one-photon and two-photon absorption spectra. This provides an added dimension for spectral selectivity in HPLC detection. Selectivity is further enhanced by the fact that two-photon excited fluorescence necessarily involves at least three states of the molecule, while normal fluorescence requires only two states. This added restriction results from the fact that direct radiative relaxation from the two-photon state is symmetry forbidden. Thus, an intermediate resonant state of unsymmetric character is needed for appreciable fluorescence to be observed (56). Internal conversion (a nonradiative relaxation process) to or from this intermediate state completes the overall relaxation from the two-photon populated excited state. A comparision of typical one-photon and two-photon excited fluorescence processes is shown in Figure 3. A final type of selectivity, possible with two photon excited fluorescence, is found in its dependence on photon polarizations. Polarization information is lost for one-photon transitions in randomly oriented samples. But polarization information is present for two-photon transitions (66) and, when necessary, this experimental variable can be readily incorporated in the HPLC detector.

Figure 3. Typical one-photon (a) and two-photon (b) excited fluorescence processes. In case (a) excitation (upward arrow) is followed by rapid internal conversion (dashed arrow) to the ground vibronic level of the S_1 state, from which fluorescence (I_f) occurs. Only two electronic states are required (although more can be involved). In case (b) symmetry requirements dictate that an intermediate ungerade resonance state be present. In this example the two-photon state (S_2) decays via internal conversion to the fluorescing state (S_1)


a.
$S_1(u)$
I_f
$S_0(g)$
b.
$S_2(g)$
$S_1(u)$
I_f
$S_0(g)$

The probability of two-photon absorption is small compared to normal absorption processes and quadratically dependent on light intensity (68). The two-photon absorption strength (δ) is defined by the relationship

$$\Delta P = P_1 P_2 CLA^{-1}\delta \qquad (4)$$

where ΔP is the absorbed optical power, P_1 and P_2 are the optical powers of v_1 and v_2, C is the solute concentration, L is the path length, and A is the optical beam cross sectional area (66). Typical values for δ are $\leq 10^{-48}$ cm^4 second $photon^{-1}$ $molecule^{-1}$.

Obviously, the unique selectivity of two-photon excited fluorescence would be of little value if concentrations meaningful in HPLC cannot be detected. Fortunately, the high powers available with lasers coupled with the low background in two-photon excited fluorescence, have resulted in good detectability. The low background results from the fact that fluorescence is observed at wavelengths considerably shorter than those used for excitation. For example, if the 488 nm line of an argon ion laser is used for excitation, then the fluorescence signal might be isolated by wide bandpass filters centered around 300-350 nm. This large blue shift means that specular scattering, Rayleigh scattering, and Raman scattering can be easily rejected. Backgrounds near the dark count of phototubes are observed.

With this in mind, minimum detectable concentrations can be calculated using equation 4. Assuming a normal phototube dark count and a typical fluorescence quantum and optical collection efficiency, a fluorescence signal of 10^5 photons/second is easily detectable. A 4 W continuous laser operating at 500 nm and focused to a 20 μ spot would allow detection of less than 10^{-8} M concentrations of compounds with cross sections of 10^{-50} cm^4 second $molecule^{-1}$ $photon^{-1}$. Assuming a 20-fold chromatographic dilution, a 20 μl injection volume, and a molecular weight of 200, sub-nanogram quantities could be injected and detected. Furthermore, the nonlinear dependence of two-photon excited fluorescence on laser power means that pulsed lasers can lead to even lower detectable quantities. The improvement in detectability with pulsed excitation, ignoring changes in focusing properties and possible changes in background levels, is approximately equal to the peak power divided by the square root of the duty cycle of the laser. Commercially available pulsed lasers, such as the N_2, Nd:YAG, or excimer pumped dye laser, could result in 10^2-10^4 improvement in detectability.

Unique selectivity is not the only advantage of two-photon excited fluorescence detection. The ability to make measurements in optically dense media (69) allows greater freedom in eluent selection. Also, the very small amount of

absorption in this type of detection eliminates the inner filter effect and, thereby, improves linearity. A final advantage is the fact that a simple capillary tube flow cell can be effectively used. This is true for two reasons. First, stray light is spectroscopically easy to reject, and second, because of the inverse area dependence in equation 4, the fluorescence path length is determined by the Rayleigh range (range over which the beam diameter is smallest) and not the physical diameter of the capillary tube. Thus, tight focusing, which reduces the area term in equation 4, and small diameter tubing, which reduces detector volume, are compatible.

The intermediate state in a two-photon transition can be thought of as a combination of all the unsymmetric resonance states of the molecule. This is possible because of the transient nature of the intermediate state, which by the Heisenberg Uncertainty Principle, can have a large energy spread (70). The lifetime of the intermediate state can increase when a resonance state of nearly the same energy exists in the molecule. The probability that the molecule will experience a second photon while in the intermediate state also increases. This is referred to as resonance enhancement. In the extreme case the intermediate state can actually be a resonance state of the molecule. The complex resonance structures of many large organic molecules

can produce low lying energy states which exhibit this effect and, in fact, this was true of the chemical systems studied in Chapters II and III. Just as with normal two-photon excited fluorescence, the resonance enhanced case (or sequential excitation case as it is better termed when the intermediate state is an actual resonance state) has a unique selectivity, low background, and a nonlinear laser power dependence.

Kasha's rule, which states that in condensed phase fluorescence is only observed from the first excited singlet state of the molecule (71), seems, at first glance, to preclude the existence of fluorescence from a higher excited state of the molecule. The key to explaining this contradiction lies in the word "observed". Oftentimes, this is taken to mean that fluorescence does not occur from higher excited states, when, in fact, it should be taken to mean that rapid internal conversion from higher excited states is much more efficient than fluorescence. But fluorescence does occur, and if one could experimentally remove the background, this fluorescence would be observable. By populating the higher excited state through a two-photon process, the background is essentially removed and the relatively inefficient fluorescence process is observed.

The quantum efficiency of a fluorescence process is equal to the ratio of the fluorescence rate constant to the total rate constant for depopulating the excited state in

question. Because of the close proximity of electronic states above the first excited state, and vibrational coupling between these states, internal conversion to the first excited state is by far the most efficient method of depopulating higher excited states. But the rate constant for internal conversion (k_{ic}) has an upper limit equal to typical molecular vibrational frequencies (about 10^{13} $second^{-1}$). Radiative rate constants for highly allowed electronic transitions are in the $10^{8} - 10^{9}$ $second^{-1}$ range. Thus, fluorescence quantum efficiencies for these transitions have a lower limit of about $10^{-4} - 10^{-5}$.

The range of applications of fluorimetry would be extended greatly if molecules with these low fluorescence quantum efficiencies can be measured at concentrations competitive with other spectroscopic techniques. The feasibility of doing this by sequential excited fluorescence (SEF) is explored in Appendix A, where an equation is derived for calculating the magnitude of SEF signals. The calculation is made for HPLC detection using kinetic and experimental parameters similar to those presented in Chapters II and III. A minimum detectable concentration of 3×10^{-8} M is calculated, which, when chromatographic dilution, injection volume, and molecular weight are taken into consideration, is very close to the minimum detectable quantities found for the antitumor drug separation and SEF detection presented in Chapter II.

CHAPTER II. DETERMINATION OF ADRIAMYCIN AND DAUNORUBICIN BY HPLC WITH LASER FLUORESCENCE DETECTION

Introduction

Adriamycin (A_1) and Daunorubicin (D_1) are antibiotic drugs shown to be effective in treating certain malignancies. Sensitive detection of the drugs and their metabolites in body fluids is essential in determining major metabolic pathways and safe clinical dosages (72,73). Total fluorescence spectroscopy is incapable of resolving the drugs, which differ only slightly in structure (see Figure 4). Current methods include HPLC coupled with UV absorbance, visible absorbance, or radioimmunoassay (RIA) detection (73-75). UV absorbance detection has not been selective enough for urine samples (74). While leukemic plasma samples were analyzed without interference at therapeutic levels (20-200 ng/ml plasma) using visible absorbance detection, the procedure includes an undesirable extraction step (75). Furthermore, long term studies may involve drug levels below the detection limits of that method. RIA is capable of quantitating the low drug levels present in urine without interference (74), but the high cost of counting equipment, difficulties associated with handling radioactive tags, and the need to collect and work up chromatographic fractions are all

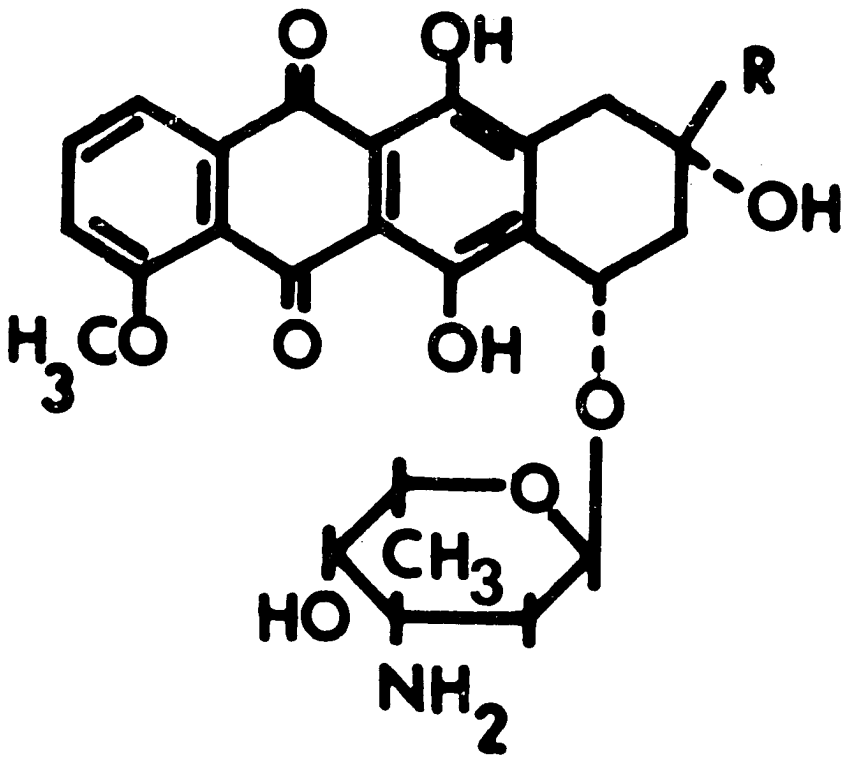


Figure 4. Structural formula; R = $-COCH_2OH$, adriamycin; R = $-COCH_3$, daunorubicin

disadvantages of RIA, which should be avoided if an alternate method is available.

A procedure is presented in this chapter for the RP-HPLC separation of A_1 and D_1 in human urine and blood serum. Filtered samples are injected directly and low picogram quantities are detected fluorometrically, without interference, using the optical fiber-flow cell shown in Figure 2 and an argon ion laser for excitation. Some of the optical properties (signal collection and stray light rejection) of the flow cell are investigated. Finally, two-photon excited fluorescence detection (SEF in this case) of the separated drugs is demonstrated.

Experimental

Chromatography

Separations were performed on a 4.6 mm x 25 cm Alltech 10 μ C_{18} column and samples eluted with a 50% acetonitrile (Burdick & Jackson LC grade) in 0.01 M phosphoric acid solution. The drugs were supplied by Dr. Nicholar Bachur of the Baltimore Cancer Research Center. Twenty μl injections of urine or 5 μl injections of blood serum were made with a Rheodyne fixed looped injector. A flow rate of 1 ml/min was maintained with an LDC minipump.

Detection

Fluorescence detection of the drugs was accomplished with the apparatus shown in Figure 5. Radiation from an argon ion laser (Model 553 from Control Laser Corp., Orlando, Fla.) was focused with a 100 mm focal length lens (a 18 mm focal length lens was used for the two-photon excited fluorescence work) into a 1.0 mm I.D., 2.0 mm O.D. quartz capillary tube (Suprasil grade quartz from Amersil Company, Sayreville, N.J.). Laser powers of 1.2 and 2.8 W were used for fluorescence and two-photon excited fluorescence detection, respectively. The flow cell was positioned with a Model FP-1 fiber optic positioner from Newport Research Corporation. The protective coating and optical cladding was stripped from the end of a 1.0 mm core diameter fused silica optical fiber (Model QSF-1000 from Math Associates, Great Neck, N.Y.). The fiber was slipped into the capillary tube and held in position with a 1/16" stainless steel Swagelok tee, leaving a clearance between the capillary tube and optical fiber of 60 μ. The volume of the flow cell used in this work was approximately 20 μl, but this could be decreased to about 1 μl by simply shortening the capillary tube.

The opposite end of the optical fiber was positioned at the entrance slit of a f/3.9 monochromator (Minichrom 1 from PTR Optics, Waltham, Massachusetts). The monochromator was fitted with 1 mm slits and had a bandpass of 6 nm. Further fluorescence isolation was provided by two Corion BB-6000

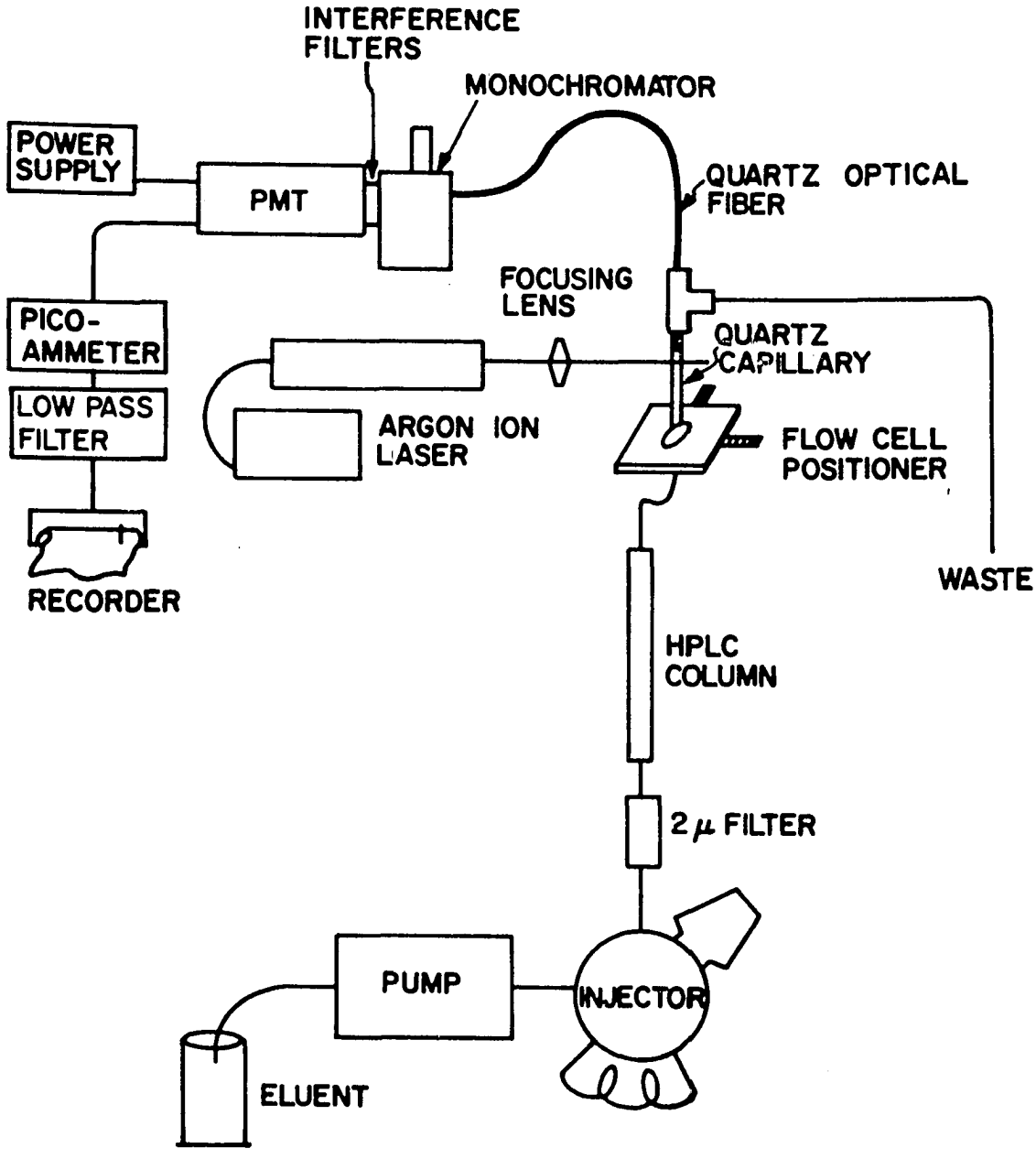


Figure 5. Apparatus used for the laser fluorescence HPLC detection of adriamycin and daunorubicin

broadband interference filters. Signals were measured with either an Amperex 56DVP or 56 TVP photomultiplier tube and photocurrents were fed to a Keithley Model 417 picoammeter. A simple low pass filter, with a two-second time constant, was used to couple the picoammeter output to a strip chart recorder.

Two-photon excited fluorescence detection was demonstrated with the aid of several pieces of equipment not shown in Figure 5. Fluorescence signals were collected with a 1.5" diameter, f/1.0, quartz lens and the high energy fluorescence emission isolated with a filter block containing three Corning 7-54 optical bandpass filters and a 1 cm saturated $CuSO_4$ cell. The transmission bandwidth at half maximum of the filter block was approximately 50 nm centered at 375 nm. Signals were measured with an Amperex 56DVP photomultiplier tube and processed with an Ortec Model 9300 photon counting system. A simple sample and hold circuit was constructed to hold the analog output of the Ortec D/A converter while a new photon count was being accumulated. The output of the sample and hold was fed to a strip chart recorder.

Detector optimization

The one-photon excited fluorescence photocurrent was monitored while a dilute drug solution or pure solvent were alternately circulated through the flow cell. Several parameters were then varied to optimize the ratio of fluorescence

signal to background noise level. The excitation and emission wavelengths were found to be optimum at 488 nm and 590 nm, respectively. The discrete output of the laser limits the excitation tunability. While the 488 nm line of the laser is not at the excitation maximum of the drugs, the high intensity of the laser more than makes up for this shortcoming.

The position of the laser beam relative to the optical fiber (d in Figure 2) was varied from 0.4 mm to 6.0 mm while the background level and the fluorescence signal peak height of an injected standard were monitored. For this study the excitation and emission wavelengths were 488 nm and 540 nm, respectively, and the eluent was a 50% acetonitrile solution. Over the range of (d) mentioned, the background photocurrent decreased by 25% and the fluorescence peak height of the injected standard decreased by 40%. This seems to indicate that, under the conditions mentioned, decreases in Rayleigh and Raman scattering background, accompanying increases in (d), are more significant than increases in specular scattering and flow cell fluorescence background. The latter two contributions to the background should increase because the angle θ shown in Figure 2 and described by equation 2 increases with (d). While 25% and 40% changes in background and signal levels are noticeable, the same changes in the position of the light path through a conventional

fluorometric flow cell would likely produce drastic changes in signal and background levels.

Optical alignment is much more critical to fluorescence sensitivity for two-photon excited fluorescence detection. The primary reason for this is the power density dependence of two-photon absorption (see equation 4). This makes it important to position the focused laser beam waist exactly in the center of the capillary tube flow cell. The focusing lens in this work was placed on a precision translational stage to facilitate this adjustment. The flow cell-collection lens combination used here did not provide any rejection of specular scattering but the large blue shift in two-photon excited fluorescence emission made it possible to spectrally reject specular scattering and other forms of stray light. In fact, even when the laser beam was reflected directly into the fluorescence collection lens, a background level of less than 500 counts per second was obtained.

Procedure

Standard solutions in the range of 3.2 ng/ml to 50 µg/ml were prepared by successive dilutions of a stock 50 µg/ml A_1 and D_1 in methanol (Burdick & Jackson LC grade) solution. A series of spiked urine samples were prepared in the range of 12.5 ng/ml to 0.20 µg/ml by adding 2 ml of various standard solutions to 8 ml of filtered urine. Spiked blood serum

samples were prepared similarly by adding 0.2 ml of various standard solutions to 0.8 ml of pooled blood serum. The prepared solutions were then injected directly using the chromatographic conditions previously mentioned.

Results and Discussion

Chromatography

The chromatographic efficiency and retention of weakly acidic or basic molecules (A_1 and D_1 contain both acidic and basic groups) is strongly influenced by eluent pH (76,77). These molecules partition more strongly in favor of polar RP mobile phases when in their ionic form. If ionization constants are known, the effect of eluent pH on retention is predictable and pH can be varied to improve resolution. But care must be taken not to degrade chromatographic efficiency which can suffer due to the presence of both ionic and neutral forms of the molecule. Peak broadening will be observed if the two forms of the molecule have different distribution coefficients and if the kinetics of the ionization equilibrium reactions are not substantially faster than mass transfer between stationary and mobile phases.

The effect of eluent pH on the chromatographic efficiency and retention of A_1 is demonstrated in Figure 6, where increases in the acidity of the eluent decreased retention and improved efficiency. These observations would have been

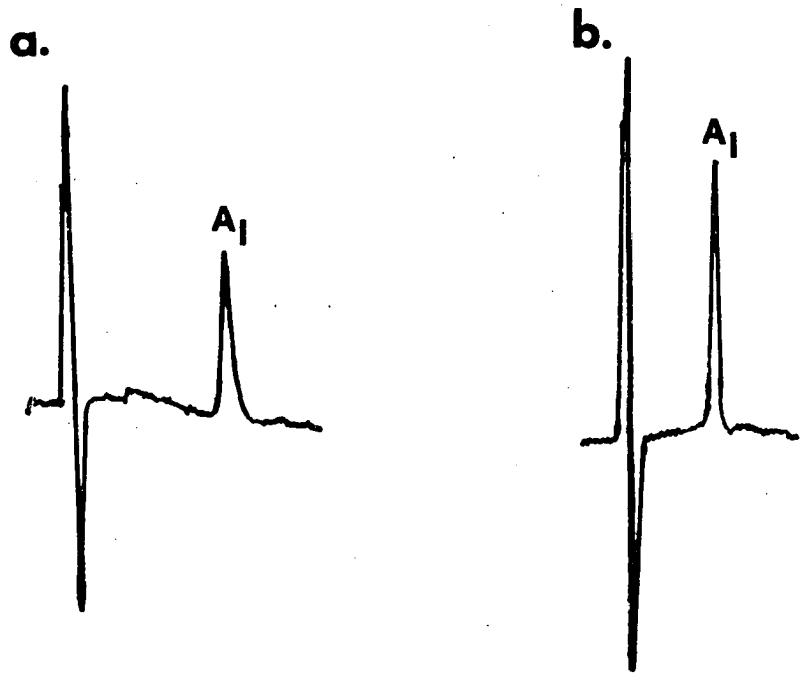


Figure 6. Chromatograms demonstrating the effect of eluent pH on the chromatographic efficiency and retention of A_1; (a) with 0.03 ml concentrated H_3PO_4/100 ml 50% CH_3CN eluent and (b) with 0.10 ml concentrated H_3PO_4/100 ml CH_3CN eluent. The retention times of A_1 were (a) 10.4 minutes and (b) 6 6 minutes and the theoretical plate counts were approximately (a) 1400 and (b) 2000

difficult, if not impossible, to predict, owing to the size and complex functionality of the A_1 molecule.

Detection limits

The detection limits of A_1 and D_1 in urine, based on a S/N of 3, are 10 pg and 15 pg, respectively. The detection limits in blood serum are about a factor of two poorer, apparently due to metabolic reactions which degrade the drugs. For this reason, spiked drug solutions were injected immediately after preparation.

The observed chromatographic baseline noise is a function of the background level when eluent is flowing. Improvement in detectability can result from reducing this functionality which is strongly dependent on pumping and other flow irregularities. A reciprocating piston pump was used for this work, without pulse dampening, and improvements could be expected with a more sophisticated pumping system.

An alternate approach to decreasing the baseline noise is to minimize the background light level. During the optimization process described in the experimental section, the various contributions to the background were investigated. It was found that the flow cell design and the stray light rejection of the monochromator combine to make the background contributions of Rayleigh and specular scattering negligible. The contribution of eluent Raman scattering is minor in this case mainly because the largest Raman shift for the eluent

is still considerably smaller than the 3540 cm^{-1} red shift between excitation and emission wavelengths used in the work. The majority of the $6x10^{-9}$A photocurrent background resulted from eluent impurity fluorescence. LC grade eluents were used in order to minimize this contribution to the background, but further purification is recommended for extremely low level work.

Linearity

Calibration plots drawn for the A_1 and D_1 standards exhibited linearity up to the 50 µg/ml stock solution (see Figure 7). Linear regression constants of 1.000 were obtained for both drugs indicating that the inner filter effect, which often produces nonlinear calibration plots in fluorometric analysis (4), is not a significant problem. This is not surprising considering the design of the detector and its insensitivity to small changes in the relative positions of the flow cell and the focused laser beam. Calibration plots were also drawn for the spiked urine and blood serum solutions yielding linear regression constants of $\geq$ 0.997 in all cases.

Selectivity

The excellent selectivity of this procedure is demonstrated in Figures 8A and 8B where the chromatograms of spiked urine and blood serum samples reveal almost no extraneous

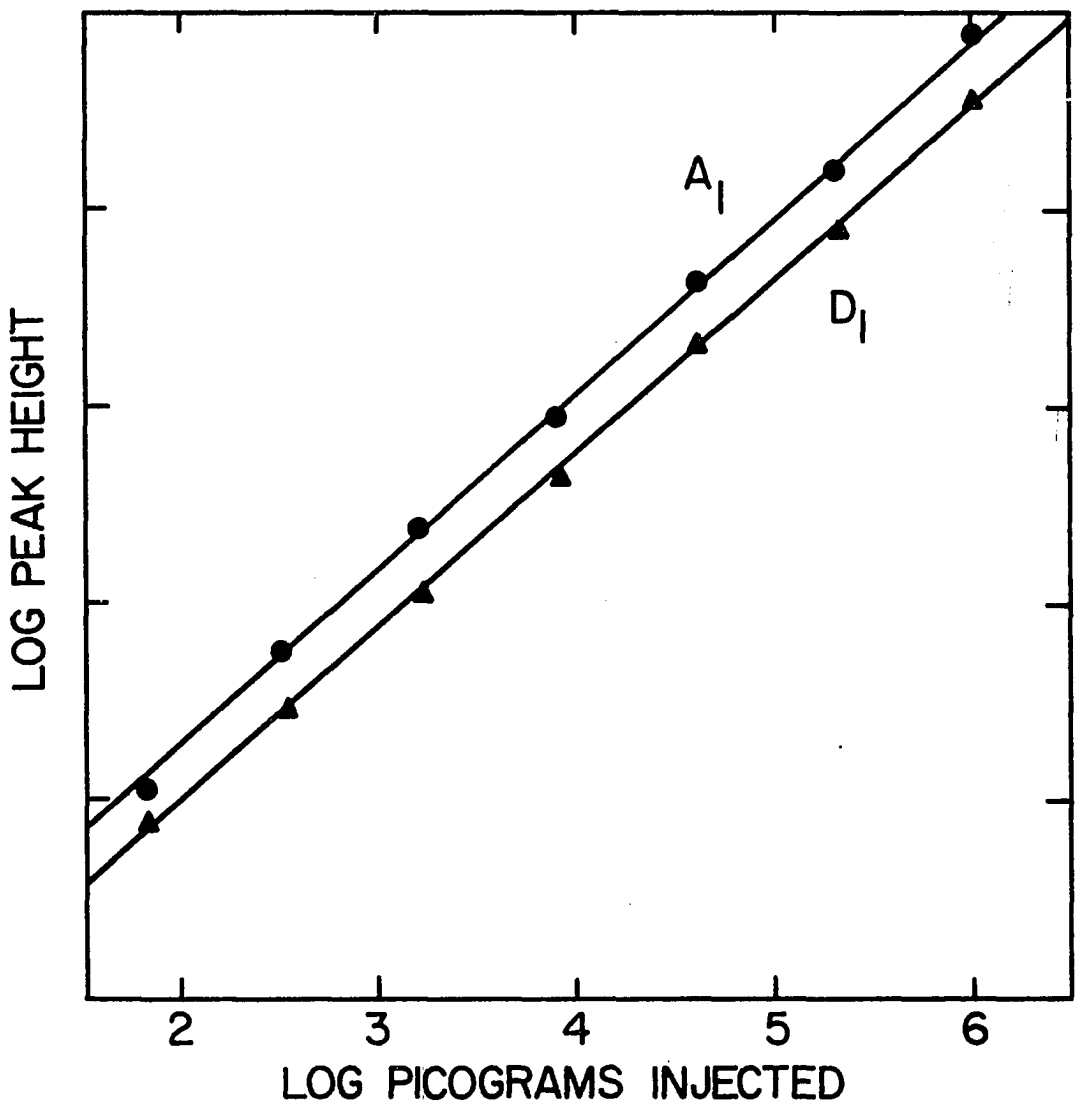


Figure 7. Calibration plots for A_1 and D_1 standards; linear regression constants of 1.000 were obtained for both drugs

peaks when injected directly. The selectivity observed is mainly due to the uniqueness of visible fluorescence. When uv excitation is used, biological samples can be expected to show some interferences. When this happens, selectivity can be improved by narrowing the slits and adjusting excitation and emission wavelengths to minimize interferences. The monochromatic and intense output of the laser is more amenable to these measures than conventional fluorometric light sources, which require larger slit widths to compensate for their lower intensity. This and other advantages of laser excitation and the optical fiber flow cell are discussed in Chapter I.

The same selectivity can be expected for other biologically important determinations in body fluids. The determination of porphyrins in urine is important in the diagnosis of liver diseases (78). Porphyrins are highly fluorescent and can be excited with the visible output lines of the argon ion laser. Likewise, the determination of riboflavin in urine, important in nutritional studies, can be performed with the detector used in this work. Moderately intense argon ion laser lines at 350 and 360 nm make this detector also applicable to the determination of Dns-amino acid derivatives in body fluids.

Figure 8A. Typical chromatograms; (a) A_1 and D_1 standards in methanol (94 pg of each injected), (b) urine blank, and (c) spiked sample (63 pg of A_1 and D_1 injected). Chromatographic and detection conditions are given in the experimental section

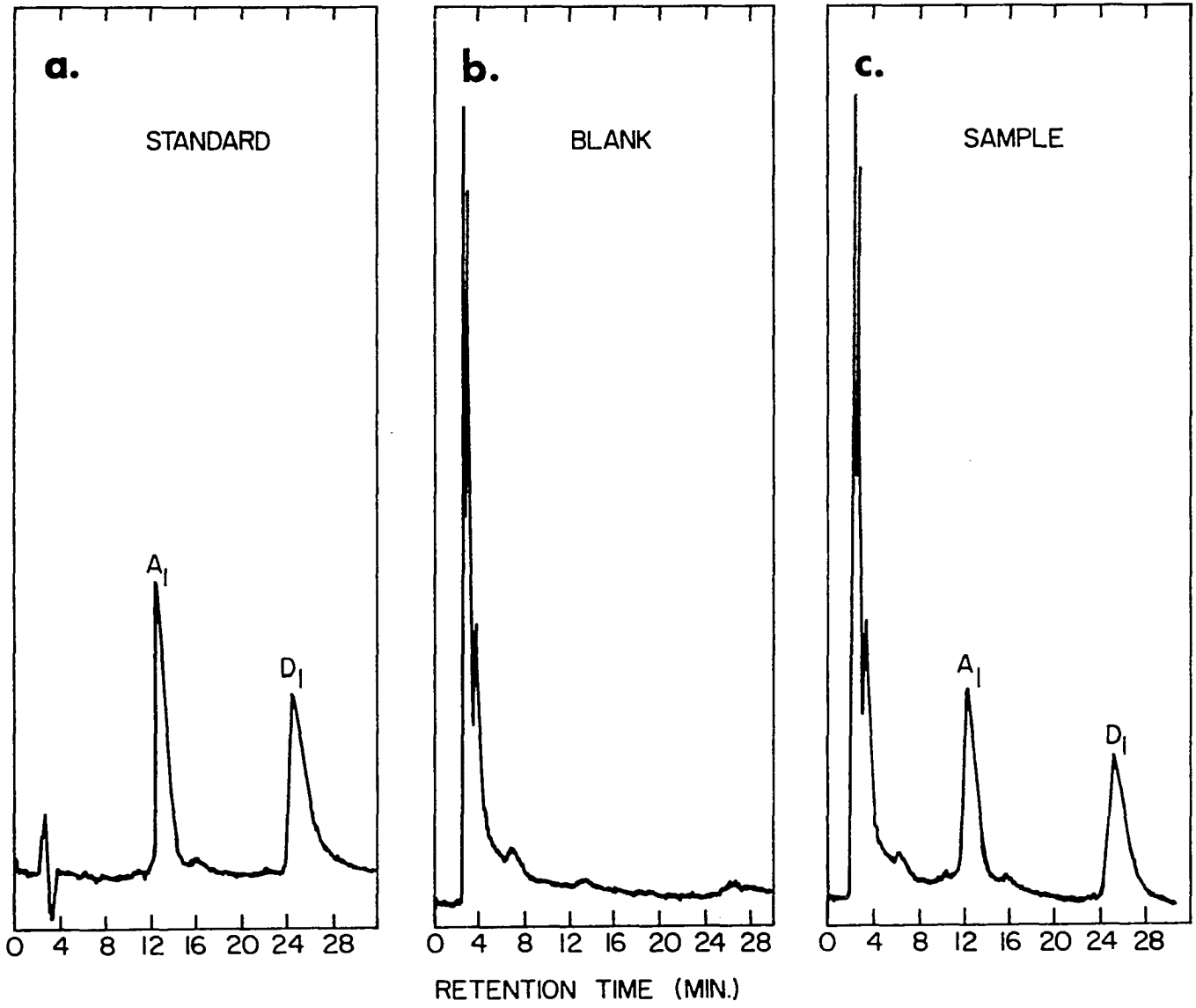
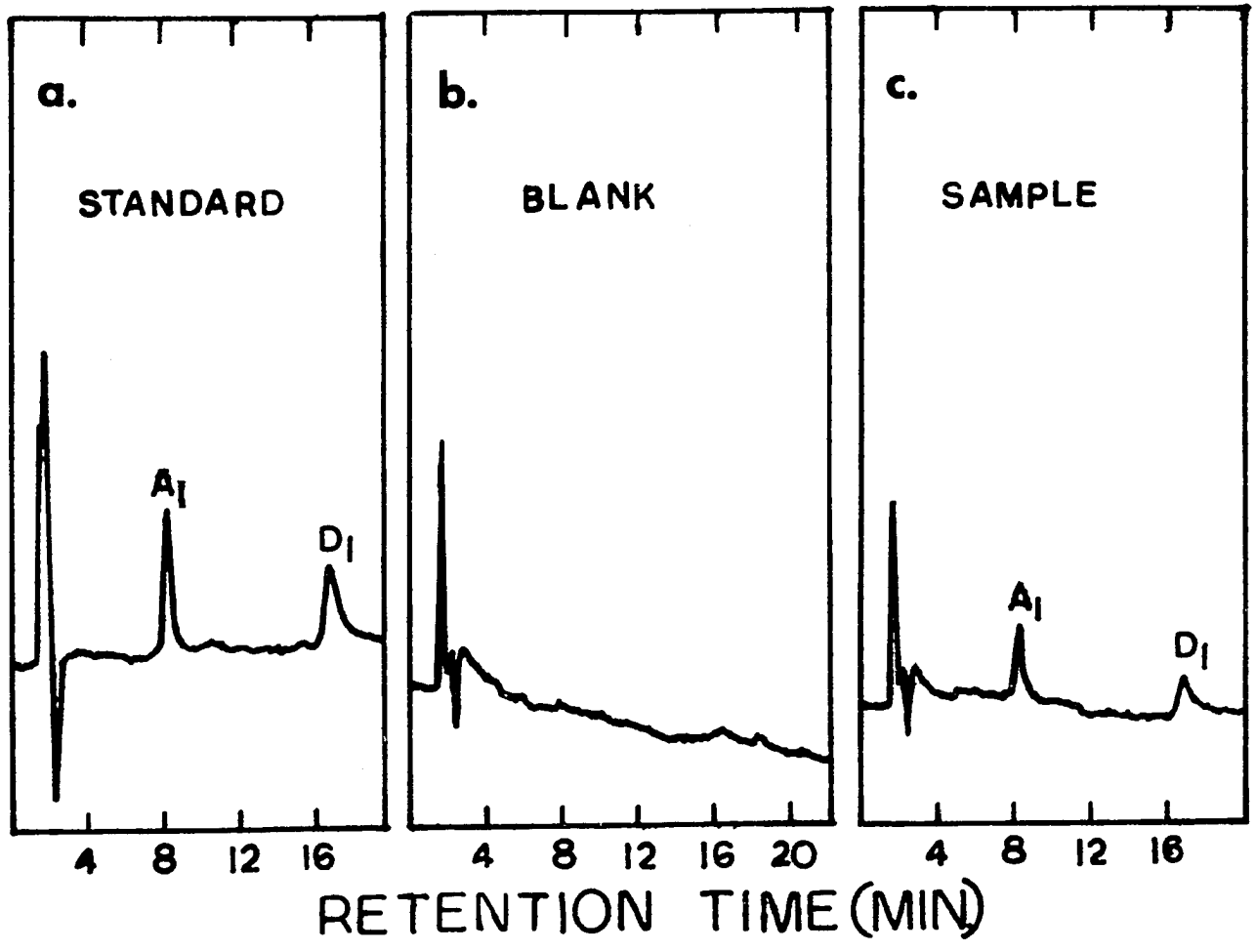

a.
STANDARD
A1
D1
0 4 8 12 16 20 24 28
b.
BLANK
0 4 8 12 16 20 24 28
c.
SAMPLE
A1
D1
0 4 8 12 16 20 24 28
RETENTION TIME (MIN.)

Figure 8B. Typical chromatograms; (a) A_1 and D_1 standards in methanol (82 pg of each injected), (b) blood serum blank, and (c) spiked blood serum sample (164 pg of A_1 and D_1 injected). Chromatographic conditions are given in the experimental section. The levels of the drugs in blood serum decrease with time as evidenced by the fact that the sample drug response is less than that of the standard


a.
STANDARD
A1
D1
4
8
12
16
b.
BLANK
4
8
12
16
20
c.
SAMPLE
A1
D1
4
8
12
16
RETENTION TIME (MIN)

Two-photon excited fluorescence detection

Two-photon excited fluorescence detection of the separated drugs is shown in Figure 9. Fluorescence emission was collected in the near uv (350-400 nm), while the excitation wavelength was at 488 nm, the same as for the one-photon excited fluorescence detection reported earlier. Thus, this two-photon absorption process is a sequential process involving an intermediate resonance state, which is the same state populated in the one-photon absorption process. This permits simultaneous monitoring of both the one-photon excited fluorescence and SEF signals.

The SEF detection limits for A_1 and D_1 (low ng range) are approximately three orders of magnitude higher than those for one-photon excited fluorescence detection. Therefore, it is better to determine the drugs by the more sensitive one-photon excited fluorescence technique, except in those cases where the unique selectivity of SEF is needed to resolve the drugs. Because the one-photon excited fluorescence is monitored in the red region of the spectrum, where very few molecules fluoresce, it is unlikely that this situation will arise. But there are many biologically important molecules with poor one-photon excited fluorescence quantum efficiencies, which could theoretically produce SEF signals similar to those obtained for A_1 and D_1. This fact, plus the possible improvements in detectability resulting from using

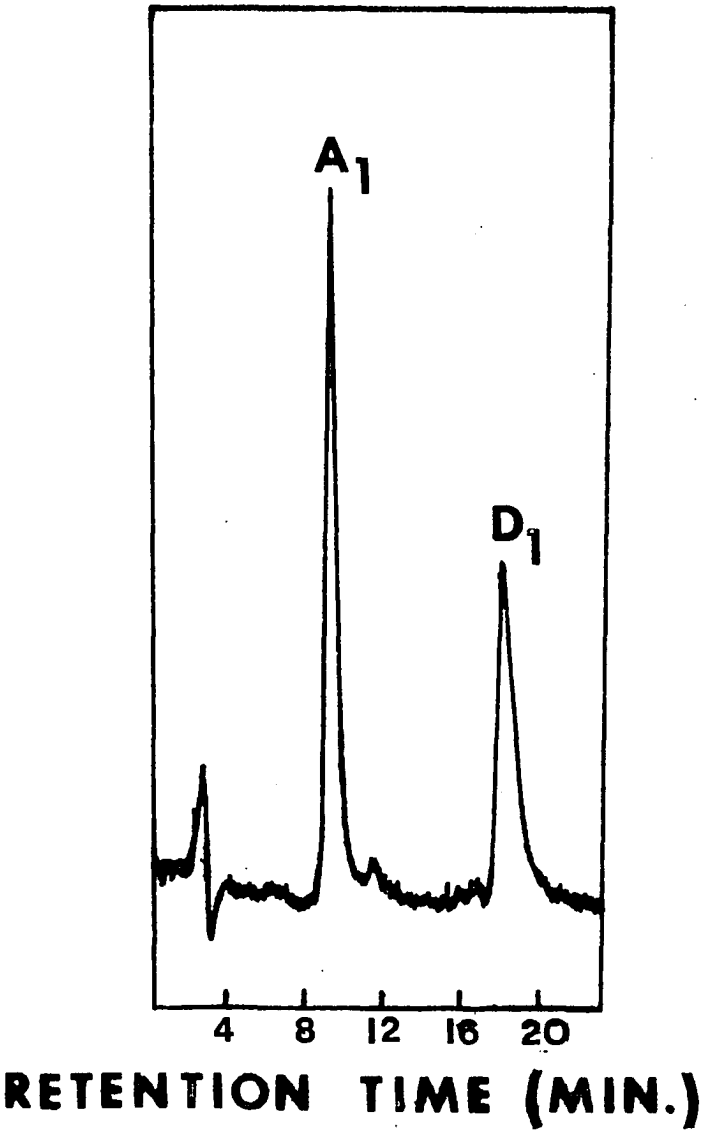


Figure 9. Two-photon excited fluorescence detection chromatogram of A_1 and D_1 (41 ng of each injected); chromatographic and detection conditions are given in the text

high power pulsed lasers, may lead to biological applications for SEF.

CHAPTER III. CHARACTERIZATION OF COAL-DERIVED ASPHALTENES

Introduction

The current economic and political situation has resulted in an intense interest in our nation's vast supply of coal as an increasingly important source of energy. In recent years, natural gas and oil have accounted for 75% of our energy consumption, despite the fact that coal accounts for 75% of our fossil-fuel resources (79). This situation results from the fact that oil is a much cleaner fuel and is more convenient to mine and refine. Effective utilization of our coal resources will likely depend on developing the technology needed to economically convert coal to gas and oil on a large scale.

There are two primary goals in coal liquefaction. First, hydrogen must be added to the coal. The ratio of hydrogen atoms to carbon atoms in coal is about 0.8 while the ratio is about 1.7 in oil (79). Second, the impurities in coal must be removed or significantly reduced since these impurities find their way into the environment during the combustion of the coal product. Obviously, proper evaluation of a coal liquefaction process, in terms of the goals just mentioned, will require characterization of the liquefied product. The information gleened from this characterization is important to the optimization of processing conditions, product quality

control, predicting the impact the coal liquefaction product will have on the environment, and should also aid in coal liquefaction reaction studies.

The three most common direct coal liquefaction processes are solvent refining, catalytic hydrogenation, and stage pyrolysis (80). Solvent refining involves slurrying the ground coal in an initial solvent oil at elevated temperatures and in a hydrogen environment. After the coal dissolves, the ash is separated by filtration or centrifugation. In catalytic hydrogenation, the coal is reacted with hydrogen at high temperatures and pressures in the presence of a catalyst. Staged pyrolysis is the simplest of the processes and involves heating the coal in the absence of air. The heating causes the coal to decompose into a tar and gas and leaves behind a portion of the coal as a solid residue. All of these processes, as well as hybrid processes, are currently being investigated by various organizations.

Sulfur (both organic and inorganic), nitrogen, and metals are the three major classes of elemental impurities in coal (81). Liquefaction processes yield products which contain some of these coal impurities, as well as undesirable high molecular weight, highly aromatic, char-like materials (81). Coal liquefaction products include a fraction called the asphaltenes which are operationally defined as the pentane insoluble, benzene soluble components of the coal liquefaction

products. The asphaltenes have been postulated as intermediates in the conversion of coal to oil and contain a high concentration of the coal impurities (82). Therefore, asphaltene characterization is vital to an understanding of the mechanism by which coal is converted to oil.

Current methodologies for the characterization of a coal-derived sample usually involve measuring properties of the bulk sample. Proton NMR spectroscopy has been used to quantitate the hydroxyl groups in coal-derived asphaltene samples (83) and C_{13} NMR spectroscopy has been used to place aromatic carbon atoms into bridgehead, protonated, or substituted categories (84). Ether oxygen, basic nitrogen, and nonbasic nitrogen content in coal-derived asphaltenes have been determined using a combination of elemental analysis, IR spectroscopy, proton NMR spectroscopy, and chemical derivation (85). Mass spectroscopy and gel permeation chromatography have been used to obtain molecular weight profiles of coal liquids (86-89).

A typical coal-derived asphaltene sample can contain hundreds of components, mostly in the 200-800 molecular weight range. Considering the complexity of these samples, it is logical to use some form of chromatographic separation in conjunction with the analytical methodologies mentioned in the previous paragraph. Preparative scale liquid chromatography has been used to fractionate coal derived samples for

this purpose (89-91), but very few examples of high resolution chromatography with selective, on-line detection have been reported (86,91). This is true in spite of the obvious potential to investigate the composition of coal-derived samples in greater detail with high resolution chromatographic techniques. Capillary GC is the method of choice for separating extremely complex volatile mixtures. Unfortunately, most of the asphaltene components are nonvolatile and, therefore, not amenable to GC separation. While HPLC is useful for separating nonvolatile mixtures, it has a few limitations when applied to the separation of coal-derived asphaltenes.

Although HPLC chromatographic efficiency is significantly better than that of preparative scale liquid chromatography, it is still only possible to partially resolve the components in a mixture as complex as the asphaltene sample. Furthermore, the ability to collect fractions for further chemical analysis is severely curtailed when one switches from preparative scale liquid chromatography to HPLC and this makes the HPLC detector extremely important. If universal detection is employed for the asphaltene separation, the lack of chromatographic resolution will result in very undistinctive chromatograms. In effect, one would have traded measuring specific properties of the bulk or fractionated sample, such as the total basic nitrogen content, for

measuring a nondescriptive property, such as refractive index, of the partially separated asphaltene mixture. It is more desirable to employ selective detection, preferably multi-dimensional, in order to obtain chromatograms which better characterize the asphaltene sample. Mass spectroscopy has been used for the detection of the HPLC separated hexane soluble fraction of coal-derived liquids (91). The mass spectroscopy detector would be ideal, since it provides structural information in addition to selective detection, if it were not for problems with interfacing it to a liquid chromatograph (92). Laser fluorescence HPLC detection should provide the selectivity needed to produce distinctive asphaltene chromatograms and the inherent sensitivity of this type of detection should permit small injection quantities. Column plugging is a problem when injecting a "dirty" sample such as coal-derived asphaltenes; therefore, decreasing the quantity of sample injected tends to prolong the useful life of the column.

Multi-dimensional detection of HPLC separated coal-derived asphaltenes is presented in this chapter. Chromatograms from uv absorbance, laser fluorometric, and laser two-photon excited fluorometric detectors are used to classify asphaltene samples in terms of the liquefaction process and the processing conditions used in their production. ARTHUR, a pattern recognition program from the chemometrics

laboratory at Washington State University, was used to aid in this classification. The three detectors are shown to provide exclusive spectroscopic information which is helpful in classifying the asphaltene samples. A unique pre-column sample isolation technique is demonstrated as a means of increasing column life and improving chromatographic reproducibility.

Experimental

Asphaltene preparation

A total of four solvent refined coal samples were obtained from two sources. Dr. T. F. Yen, Chemical Engineering Department, University of Southern California, provided samples of Catalytic Inc., Wilsonville, Alabama - solvent refined coal and PAMCO, Fort Lewis, Washington - solvent refined coal. Analytical, molecular weight, and NMR methods have been used to study these samples (90). Two samples of solvent refined coal from the Synthoil process were obtained from the Department of Energy, Pittsburgh Energy Technology Center, Pittsburgh, Pennsylvania. The two Synthoil samples differ in the processing conditions used for their production. Synthoil 1 was produced in a 400 pound per day unit at 2500 psi, 450°C, and a feed coal to oil ratio of 35/65 by weight. Synthoil 2 was produced in a 1000 pound per day unit at 2000 psi, 450°C, and a feed coal to unfiltered coal solvent ratio of 29/71 by weight.

The asphaltene fractions of the four solvent refined coals were obtained by the procedure of Schweighardt and Thames (93). Briefly, this involves several pentane washings of the coal samples, then the asphaltene components are extracted with several portions of benzene. The combined benzene extracts were rotary evaporated to a small volume, then freeze-dried to remove the remaining benzene solvent. Asphaltene solutions for injection were made by dissolving 2 mg of asphaltene per ml of tetrahydrofuran. The tetrahydrofuran was spiked with an appropriate amount of the internal standards 1,4 diphenyl oxadiazole (PPD) and NBD-Cl derivatized dimethyl amine (DMA-NBD).

Detection

The asphaltene samples were characterized with the apparatus shown in Figure 10. The chromatographic effluent was passed first through a Chromatronix Model 260 uv absorbance detector (operating at 254 nm) and then through the optical fiber-flow cell described in the previous chapters. Laser fluorescence and laser two-photon excited fluorescence signals were monitored using the same conditions and equipment described in Chapter II with the following exceptions and additions. A laser power of 2.5 W at 488 nm was focused into the flow cell with an 18 mm focal length lens. Fluorescence emission from the asphaltene samples was

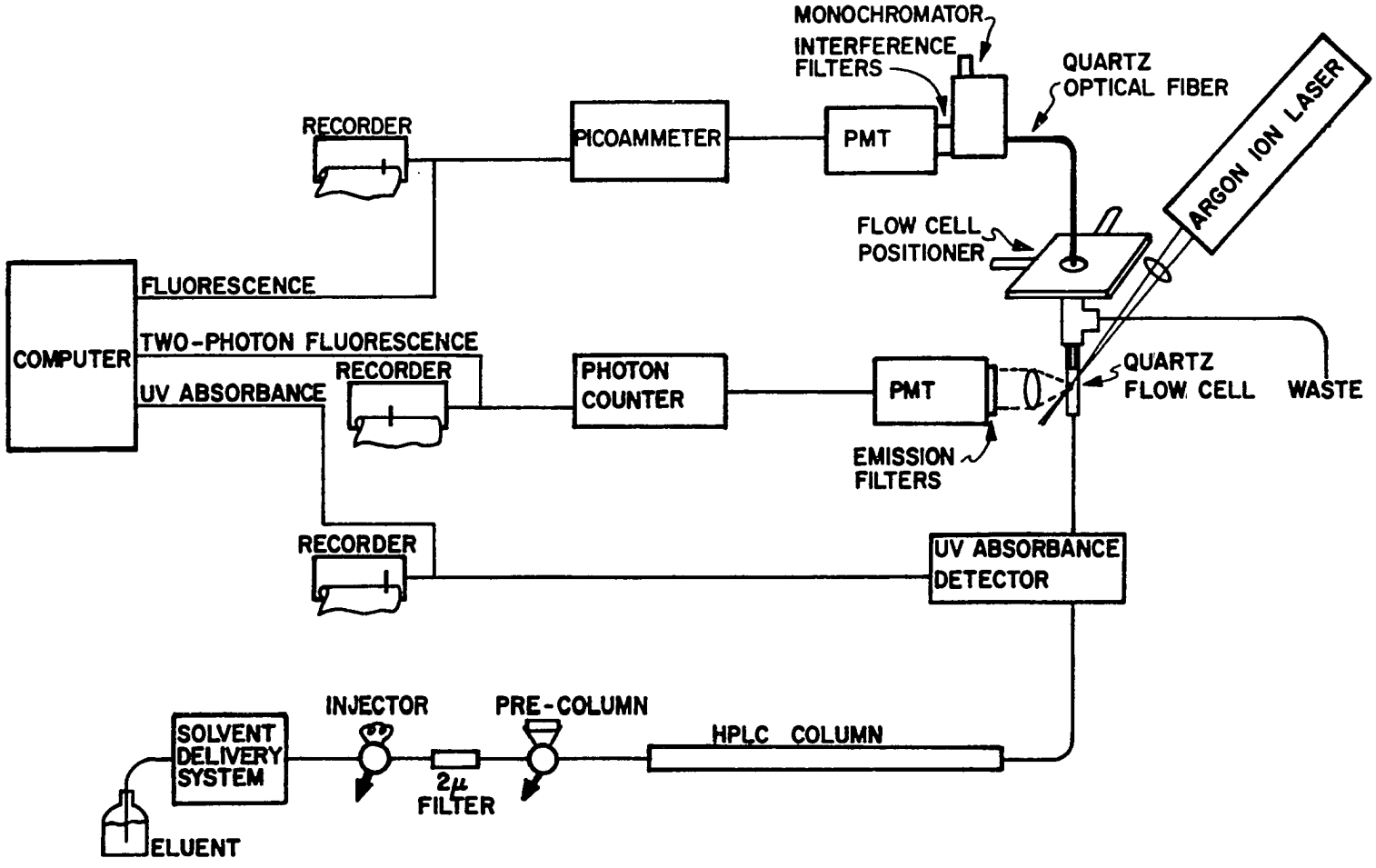


Figure 10. Apparatus used for the characterization of coal-derived asphaltenes

isolated at 540 nm with the Minichrom 1 (bandpass of 6 nm) and two Corion SS-5400 interference filters.

The outputs from the three detectors were buffered to a full scale output of one volt, then fed to stripchart recorders and a Digital Equipment Corporation PDP 11/10 computer equipped with a laboratory peripheral system. The analog detector signals were digitized, averaged over three second intervals, then stored on floppy disks for future processing. The fortran program used to collect this data (COALN) appears in Appendix B. The laser power was also monitored and stored for later normalization, but the good laser stability during the course of this experiment made normalization unnecessary.

Chromatography

Separations were performed on 4.6 mm x 25 cm Alltech C_{18} columns and samples eluted with a 50% to 100% acetonitrile in distilled water gradient. The solvent delivery system used for this gradient was composed of two LDC minipumps and a stirred 50 ml mixing chamber. The mixing chamber was filled with a 50% acetonitrile solution and one of the pumps was used to deliver this solution to the column at a flow rate of 1.0 ml/minute. When the volume in the mixing chamber reached 40 ml, the sample was injected and when the volume reached 35 ml, the second pump was turned on to deliver acetonitrile

(Burdick and Jackson LC grade) to the mixing chamber at a flow rate of 1.0 ml/minute. Using these conditions, the mixing chamber composition 25, 50, and 75 minutes after injection was 72, 86, and 93% acetonitrile in distilled water, respectively. Under ideal conditions (constant column backpressure) this solvent delivery system provides a reproducible gradient. An average relative standard deviation in retention time of 2.2% was obtained when several polynuclear aromatic compounds were separated in a test of the gradient system. Unfortunately, column backpressure was not constant during the asphaltene separations. This was due to changes in column permeability resulting from injecting the "dirty" asphaltene samples and large changes in the eluent viscosity during the gradient used for the asphaltene separation. The pumps used for this work were unable to maintain a constant flow rate during the column backpressure changes and chromatographic reproducibility suffered as a consequence.

Two Rheodyne fixed-loop injectors were used to introduce the asphaltene sample into the analytical column (see Figure 10). The second injector had a 2.4 mm x 4.5 cm pre-column (filled with Waters Associates Corasil C_{18}, 37-44 μ, packing) placed where the sample loop is normally connected. This pre-column was switched off-stream four minutes after 20 μl of the asphaltene sample solution was injected with the first

injector, thereby trapping components in the sample which would be difficult or impossible to elute from the analytical column. The packing and dimensions used for the pre-column are important. If the capacity of the pre-column is exceeded, severe band broadening is observed. In contrast, it is desirable to use a small pre-column so that the majority of the asphaltene components are introduced into the analytical column as a sharp plug. The pre-columns were repacked after each injection. The effect of the pre-column on uv absorbance and laser fluorescence chromatograms can be seen by comparing the chromatograms in Figure 11 where the pre-column was left on-stream, with chromatograms appearing later in this chapter.

The analytical columns were reconditioned between injections by passing 20 ml each of tetrahydrofuran, acetonitrile, and the initial eluent through the column. Shortly into the acetonitrile reconditioning, the baseline signal would reach its pre-injection value, indicating that the previously injected asphaltene sample had been effectively eluted from the column. The useful life of the analytical columns was roughly 8-10 injections. Because of the cost factor, relatively inexpensive, untested columns were used. These columns were changed whenever the column backpressure reached about twice its initial value or when column settling produced void spaces at the column inlet which destroyed

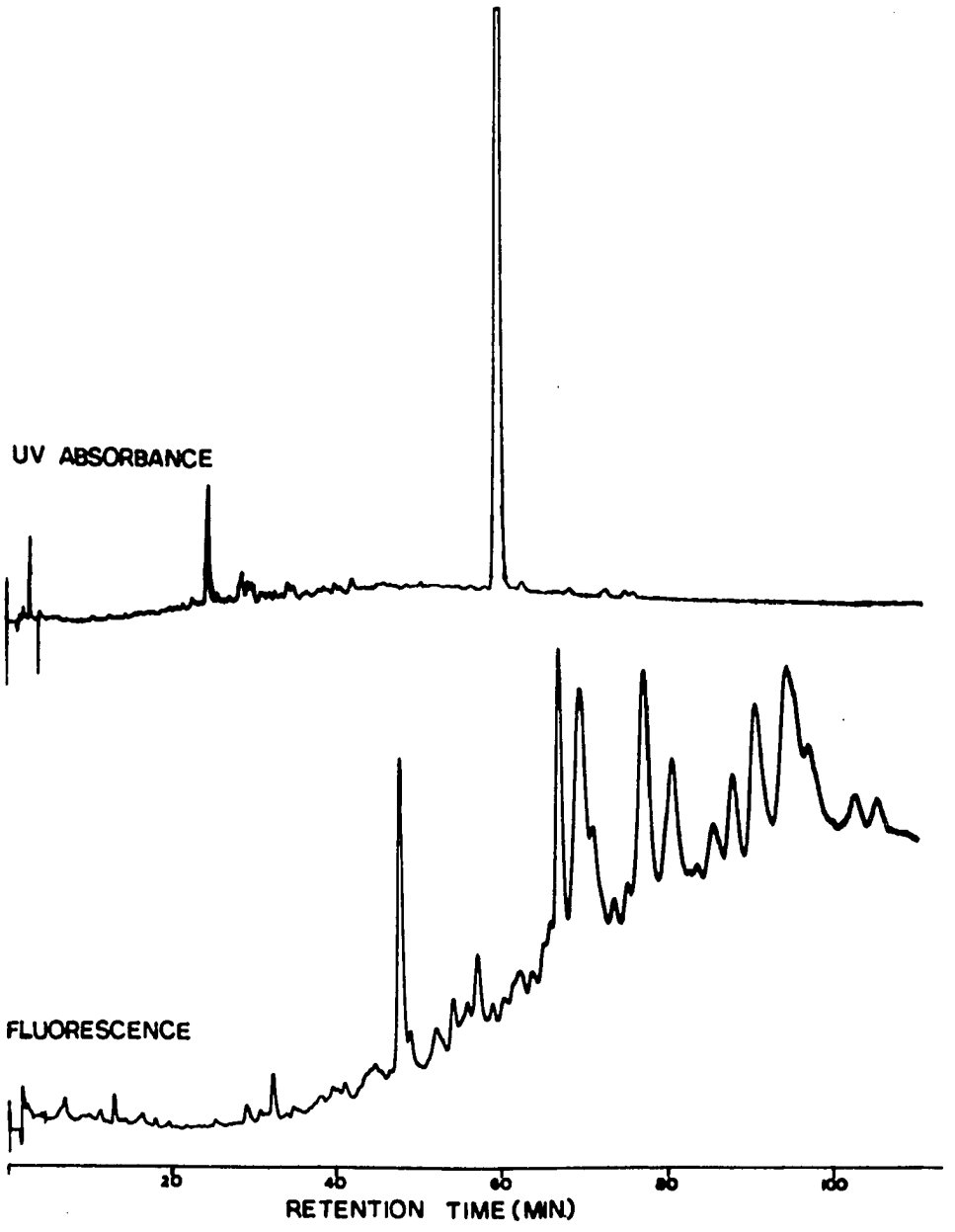


Figure 11. Laser fluorescence and uv absorbance chromatograms of an asphaltene sample when pre-column is left on-stream

chromatographic efficiency. The technique of filling these void spaces with pellecular packing is effective only when the compounds being separated do not overload or are unretained by the pellecular packing. Unfortunately, the wide range of compounds in the asphaltene sample make it impossible to completely satisfy either of these conditions.

Data treatment

Each of the four asphaltene samples were injected 7 or 8 times yielding a series of 75 minute digitized chromatograms containing 1500 data points per detector. The digitized chromatograms were treated with a series of fortran programs (listed in Appendix B) which were designed to normalize and smooth the chromatograms. Next, peaks in these chromatograms were correlated within the injections of a particular sample and the peaks that correlated well were used as features for pattern recognition with ARTHUR. The exact procedure used to treat the digitized chromatograms is presented in the following paragraphs.

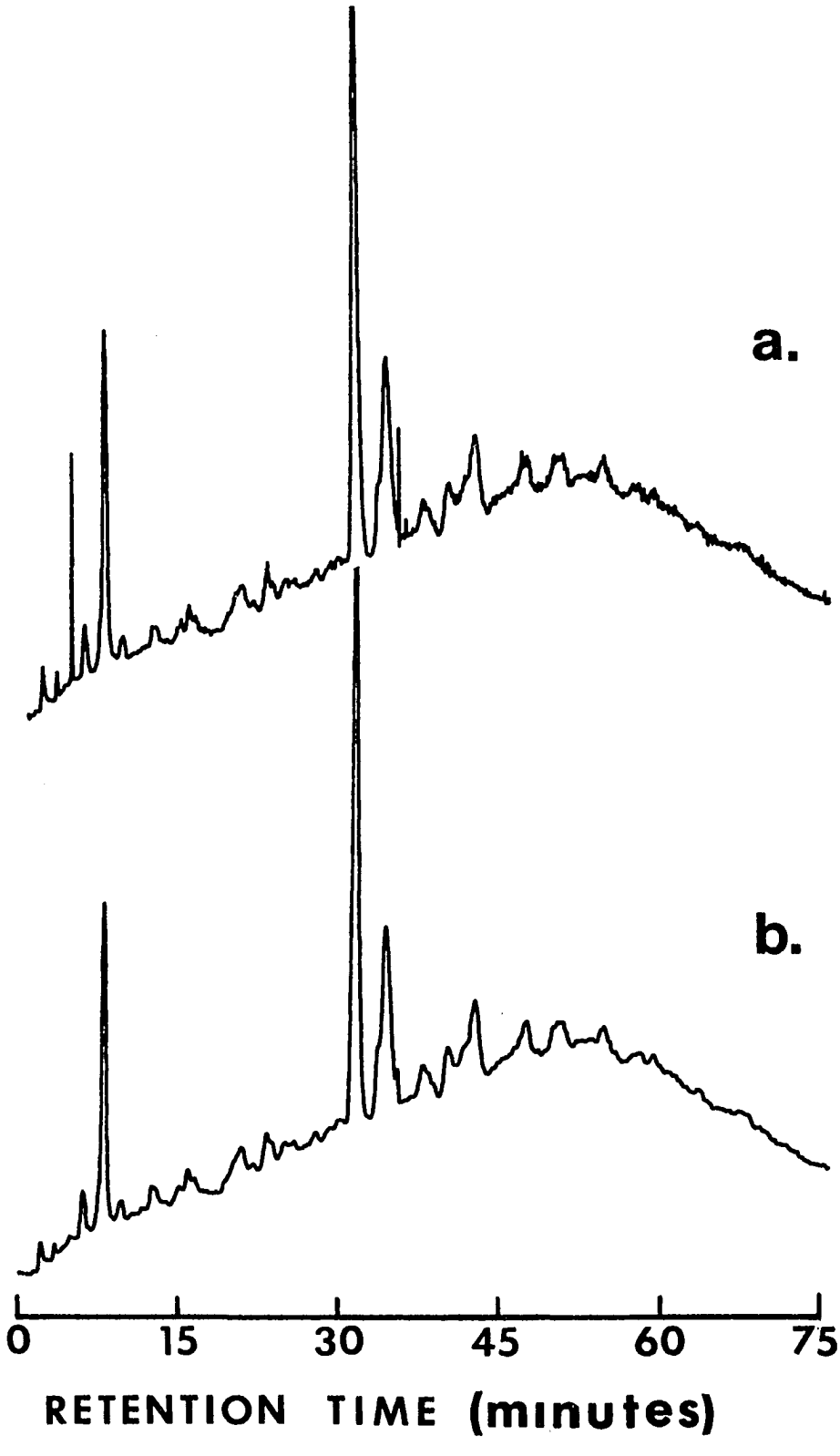
Glitches which occasionally appeared in the chromatograms were removed with GLITCH. The laser fluorescence and laser two-photon excited fluorescence chromatograms were normalized to the peak heights of the internal standards and all of the chromatograms had their background levels subtracted with NORMAL. The internal standards, DMA-NBD for

the laser fluorometric detector and PPD for the laser two-photon excited fluorometric detector, appear at about 4 and 9 minutes in their respective chromatograms. PPD also appears in the uv absorbance chromatograms but interferences make its peak height measurement difficult and so the uv absorbance chromatograms were not normalized.

Signal to noise ratios for laser two-photon excited fluorescence chromatograms were considerably lower than for laser fluorescence chromatograms and this made it advantageous to perform some type of computer data smoothing on the former chromatograms. This was accomplished with SMOOTH, a program which contained a 7 point, third degree polynomial, data smoothing algorithm (94). The effect of GLITCH and SMOOTH on the digitized chromatograms is demonstrated in Figure 12. All of the chromatograms shown in this chapter were plotted from the digitized chromatographic data using a plotting routine which scales the chromatograms to the same X and Y coordinate range. Thus, only relative comparisons between chromatograms should be made and, when possible, the internal standard peaks should be used in the comparisons.

A poor detector to computer connection created a large amount of noise in about half of the laser fluorescence chromatograms. Since this did not effect the strip chart chromatograms, they were used to generate 1500 data point, digitized chromatograms. This was done by measuring maximums

Figure 12. Two-photon excited fluorescence chromatograms before (a) and after (b) data treatment with GLITCH and SMOOTH. The glitches at about 6 and 38 minutes and the baseline noise have been significantly reduced without adversely effecting chromatographic efficiency


a.
b.
0
15
30
45
60
75
RETENTION TIME (minutes)

and minimums in the strip chart chromatograms, then using FIXPTS to fit straight lines between these maximums and minimums, creating chromatograms such as the one plotted in Figure 13.

It was decided the most complete description of the chromatograms for pattern recognition with ARTHUR would involve identifying the signal levels and retention times of maximums and minimums in the digitized chromatograms. This was done with MINMAX, which identified minimums and maximums based on the criterion that they were surrounded by a consistent, significant upslope and downslope. RETIME was used to normalize the retention times of the maximums and minimums to the retention time of the major peak in the laser two-photon excited fluorescence chromatogram of that particular injection. The maximum and minimum data for a particular sample and detector was then assembled into a large file containing 7 or 8 records, one for each injection of that sample. This was done with REFILE.

The ARTHUR program has a limited data storage capability. Faced with this restriction, it was decided to initially use only the heights of well-correlated peaks (maximums from MINMAX) as features for pattern recognition. The program CORLAT was used to correlate intrasample peak data. CORLAT places similar peaks into a given category if the retention time and peak height differences of the peaks

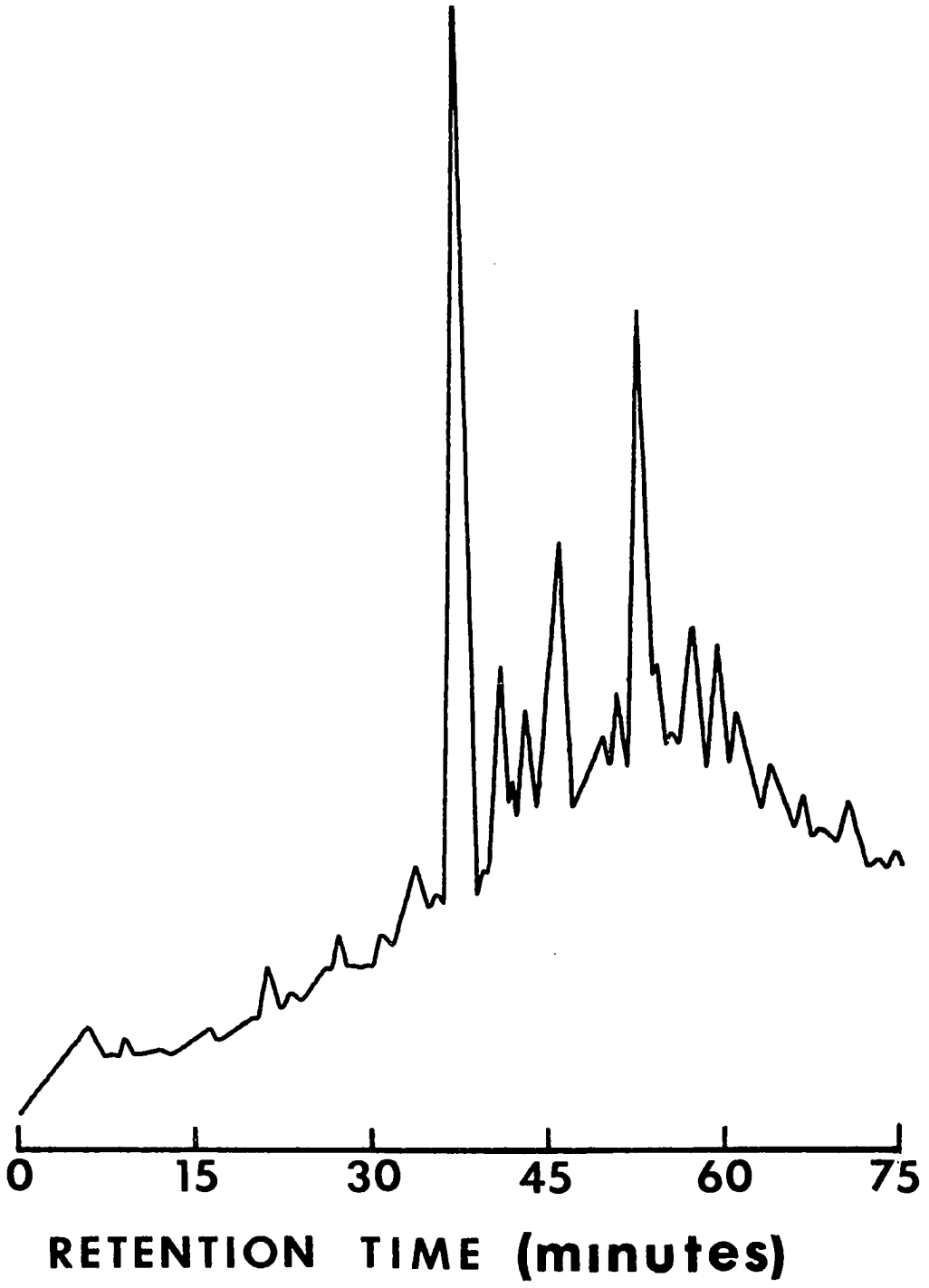


Figure 13. Laser fluorescence chromatogram after graphical treatment and computer data filling with FIXPTS

are within entered tolerances for these differences. The category carries an average retention time and peak height which is adjusted whenever a new peak is placed into the category. Upon completion, CORLAT prints the final average retention times and peak heights of the categories as well as the number of peaks which fell into each of the categories. When all of the files had been correlated, intersample peak retention time correlations were performed manually. All categories from CORLAT which had a population of three or more peaks were used for these intersample correlations. A total of 38, 38, and 41 peak retention time categories were obtained for the laser two-photon excited fluorescence, laser fluorescence, and uv absorbance detectors, respectively. These categories were stored on floppy disk with the program KFILES and called by the program FEATUR.

FEATUR scans the peak retention times of a given chromatogram and looks for matches with the intersample correlated categories stored in the KFILES file for that particular detector. If it happens that no peak in the chromatogram is within an entered retention time window of a category retention time in the KFILES file, then a default value is entered for that category. An n-dimensional data vector, composed of either a peak height value or default value for each category in the KFILES file, is created for each chromatogram.

$PKHT_1$, $PKHT_2$, DEFAULT, $PKHT_4$, ... $PKHT_n$

When more than one peak fits into the retention time window of a category, the peak with the closest fit is used. The programs described in this section are summarized in Table 3 in Appendix B. Appendix B also contains printouts of the more important computer programs.

Pattern recognition

FEATUR was used to create 31 data vectors for each detector, which included 8 data vectors from each of the Synthoil 1, Synthoil 2, and Catalytic Inc. samples and 7 data vectors from the PAMCO sample. The (n) peak heights which comprise the n-dimensional data vector are usually referred to as "features" when pattern recognition programs, such as in ARTHUR, are used to classify the data vectors.

ARTHUR consists of a collection of data manipulation and pattern recognition programs designed to aid in the analysis of complex data sets. The ARTHUR subroutines utilized in this work include CORREL, WEIGHT, BACLAS, SICLAS, MULTI, and KNN. CORREL calculates the covariances between features in the data vector and WEIGHT weights the features based on their intersample to intrasample variance ratios. The last four routines are pattern recognition programs designed to classify the data vectors using various statistical algorithms (95). The ARTHUR program was compiled and run on

the Lawrence Berkeley Laboratory CDC 7600 computer. Access to this computer was made at the Iowa State University, Physics Department, remote terminal. A total of seven runs were made with ARTHUR. The first three runs were made using data vectors which contained all of the features from the intersample correlation of each detector. The output of WEIGHT was used to reduce the data vectors for each detector to include only the 6 or 7 most important features. ARTHUR was then run three more times using these reduced data vectors. Finally, the 6 or 7 features from the three detectors were combined into one data vector of 20 features which was used for the last computer run.

Results and Discussion

Reproducibility

It has become common practice to demonstrate the separatory power of new HPLC column technology, such as capillary columns, with impressive (albeit time consuming) chromatograms of coal-derived samples. However, the step from demonstration to reproducible chromatography is often formidable when "dirty" samples are being injected. In addition, utilizing selective detection for the purpose of simplifying complex chromatograms does not solve the chromatographic problems associated with injecting "dirty" samples, namely, column contamination and short column life (96).

A good way to minimize column contamination and increase column life is to employ very sensitive detectors so that small injection quantities can be tolerated. The need for sensitive detection is especially great for the separation of coal-derived asphaltenes since the injected amount is spread over hundreds of components. Injection quantities of 40 μg were used for the asphaltene separations presented here. The results of the previous chapter indicate that smaller injected samples could be detected with the laser fluorometric detector; unfortunately, the uv absorbance and laser two-photon excited fluorometric detectors are not as sensitive. Depending on the application, the analytical information needed may be present in the laser fluorescence chromatogram, in which case improvements in reproducibility, beyond those reported here, could be observed by injecting submicrogram quantities of the asphaltene samples.

The use of small pre-columns to protect expensive analytical columns from particulate matter and contaminants is quite common. These columns are typically filled with pellecular-type packings and replaced or repacked after several injections. Unfortunately, a simple pre-column arrangement does not provide the protection needed for repetitive asphaltene injections. This is because the complexity of the asphaltene sample and the wide gradient needed to obtain a reasonable separation results in many

difficult to elute compounds breaking through the less retentive pre-column. The pre-column switching technique described earlier helped to minimize problems with pre-column breakthrough. The majority of the compounds in the sample that are eluted from the pre-column before it is switched off-stream are eluted from the analytical column before the gradient has reached its upper limit of 100% acetonitrile. This can be seen by comparing the general shape of the chromatograms in Figure 11 with those appearing later in this chapter. The effectiveness of the pre-column switching technique at isolating elutable compounds in the asphatene sample was further reinforced by the ease with which the analytical columns were reconditioned between injections and by the absence of carry-over from previous injections.

Obviously, pre-column switching would be undesirable if it altered the composition of the sample reaching the analytical column to the point where it no longer resembles the original sample. Experiments performed with the analytical column removed revealed that about 80-90% of the asphaltene components (as measured with laser fluorescence and uv absorbance detectors), which are eluted from the pre-column with 15 ml of 50% acetonitrile in water, are eluted with the first 4 ml portion. Thus, it was felt that the integrity of the asphaltene sample was maintained in spite of the pre-column switching procedure.

Figures 14 and 15 contain chromatograms from triplicate injections of asphaltene samples employing laser two-photon excited fluorescence and laser fluorescence detection, respectively. Two observations can be made from these chromatograms. First, the column to column chromatogram reproducibility is poorer than the injection to injection chromatogram reproducibility. This is more evident in Figure 15. Second, the peaks which appear late in the chromatograms are less reproducible. This is reasonable since the late eluting components tend to be clipped when the pre-column is switched off-stream. Slight changes in the switching time can have a large effect on the amount of these late eluting components introduced into the analytical column. It should be stated that while burn spots on the fluorescence flow cell walls occasionally produced changes in the chromatograms, it was the chromatography, and not the detection, which limited reproducibility.

Detector comparison

Figure 16 illustrates how the three detectors used in this work provide exclusive information. This is important when chromatographic efficiency is not sufficient to provide baseline separation of the components in the injected sample. The first observation to be made from the chromatograms in Figure 16 is that the largest uv absorbance, laser two-photon excited fluorescence, and laser fluorescence peaks come

Figure 14. Chromatograms from triplicate injections of the Catalytic Inc. asphaltene sample illustrating the reproducibility of laser two-photon excited fluorescence detection. Chromatograms (a) and (b) were obtained using the same Alltech C_{18} column while a different Alltech C_{18} column was used for (c). Chromatographic and detection conditions are given in the Experimental section

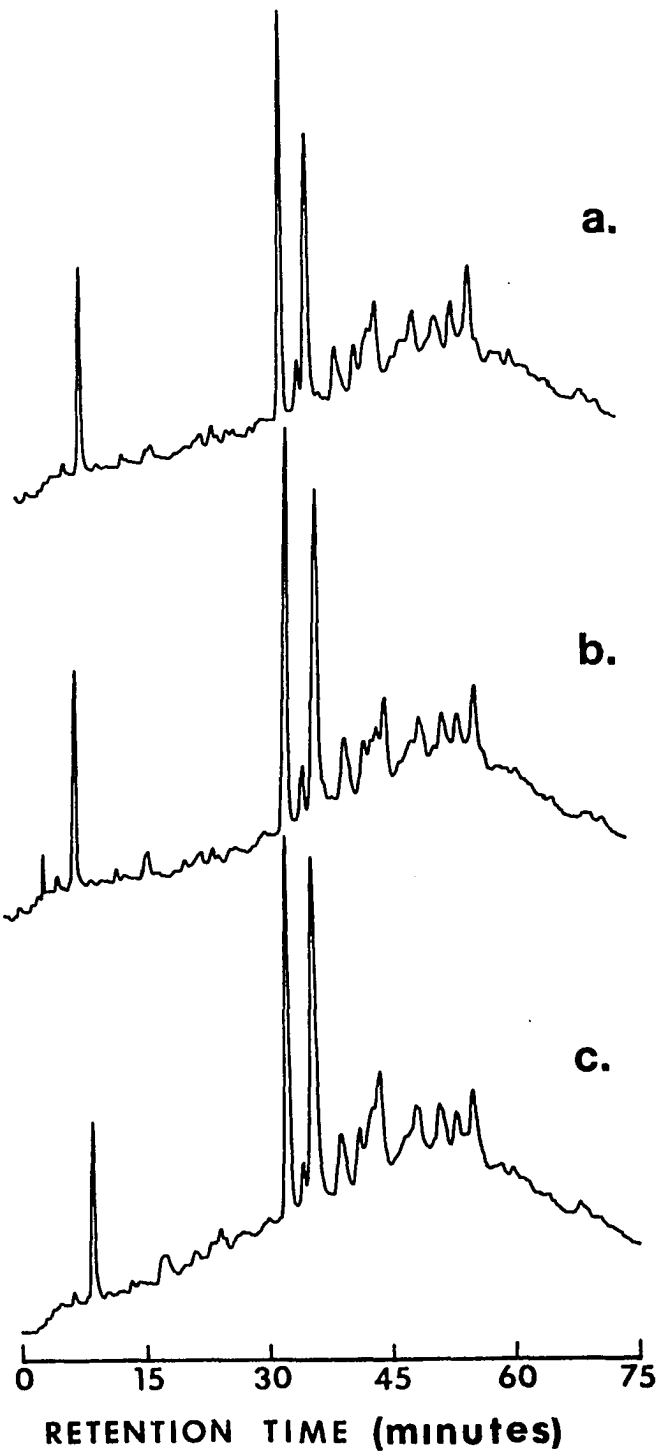

a.
b.
c.
0
15
30
45
60
75
RETENTION TIME (minutes)

Figure 15. Chromatograms from triplicate injections of the PAMCO asphaltene sample illustrating the reproducibility of laser fluorescence detection. Chromatograms (a) and (b) were obtained using the same Alltech C_{18} column while a different Alltech C_{18} column was used for (c). Chromatographic and detection conditions are given in the Experimental section

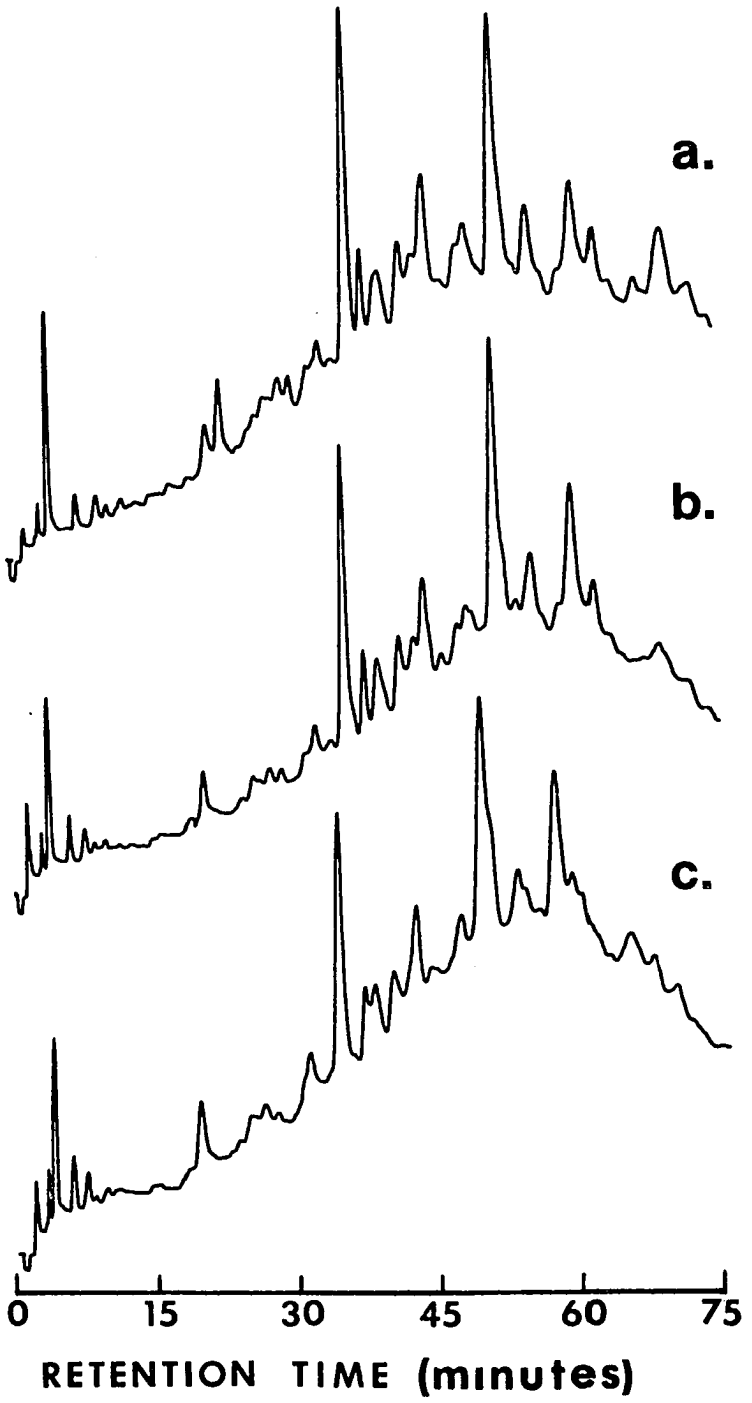
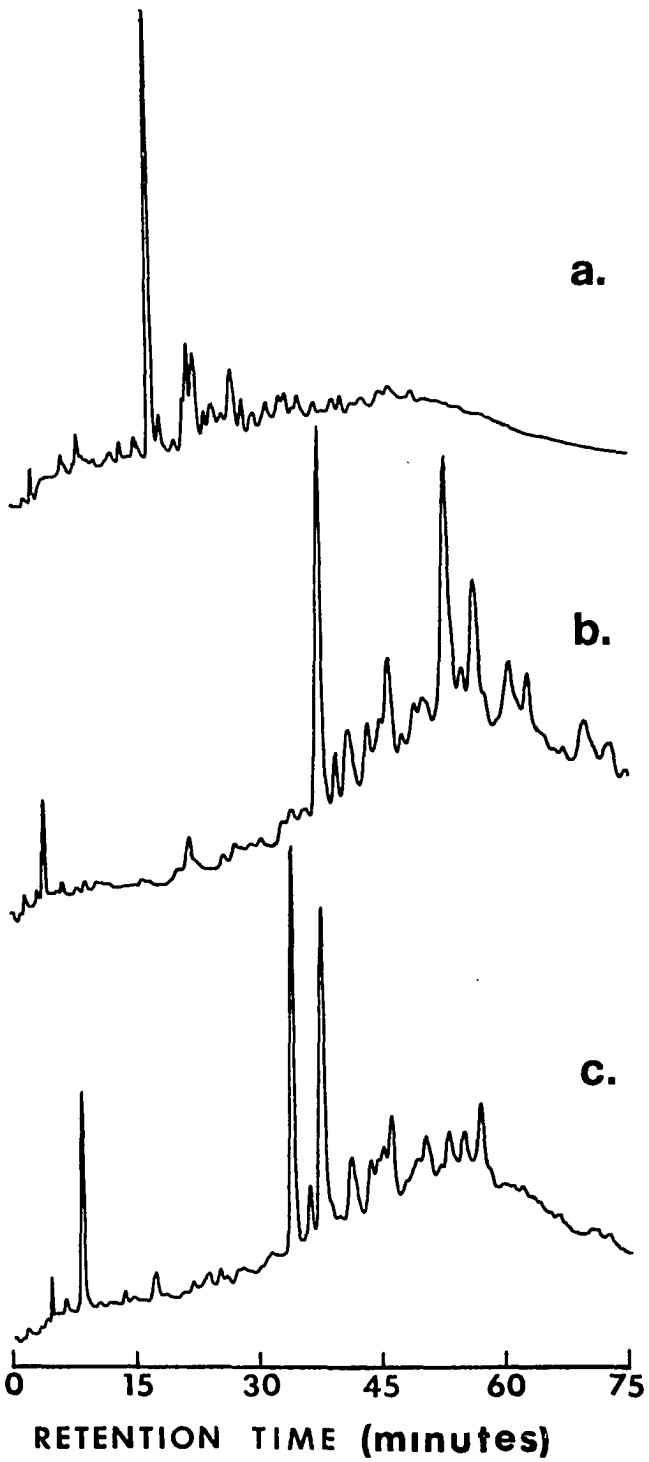

a.
b.
c.
0
15
30
45
60
75
RETENTION TIME (minutes)

Figure 16. Detector comparison for uv absorbance (a), laser fluorescence (b), and laser two-photon excited fluorescence (c) detection; chromatographic and detection conditions for this Catalytic Inc. asphaltene separation are given in the Experimental section


a.
b.
c.
0
15
30
45
60
75
RETENTION TIME (minutes)

early, midway, and late in the separation, respectively. This is consistent with the expected order of elution. It is the smaller compounds in the asphaltene sample that are eluted early and these compounds do not have the extended resonance structures needed to absorb the 488 nm laser radiation. Their lowest excited states, which are in the uv, can be populated by absorbing the 254 nm radiation from the uv absorbance detector or by absorbing two photons of the 488 nm laser radiation. For example, the major peak in the uv absorbance chromatogram, which can be used to distinguish the Synthoil samples from the two other samples, is also present in the laser two-photon excited fluorescence chromatogram, but absent in the laser fluorescence chromatogram. Actually, very few peaks appear early in the laser two-photon excited fluorescence chromatogram, probably because it is the SEF process that produces the largest two-photon excited fluorescence signals and the SEF process requires the same highly conjugated π-electron systems that are needed for laser fluorescence signals. Strong visible and uv absorbing states are likely present in the late eluting compounds. The fact that the uv absorbance response falls off midway in the separation and the laser two-photon excited fluorescence response falls off late in the separation is presumably due to a lower concentration of the late eluting compounds. The laser fluorescence response does not fall off because of greater sensitivity.

The peaks appearing between 33 and 43 minutes in the chromatograms in Figure 16 best illustrate the differences in the three detector responses. The ratio of the peak heights of the two largest peaks in the laser two-photon excited fluorescence chromatogram is 1.2, while peaks appearing at the same retention times in the laser fluorescence chromatogram have a ratio of 0.05. The asymmetry of the peaks appearing at 43 minutes in the two fluorescence chromatograms indicates the presence of two unresolved compounds, the later eluting one giving a much smaller response. The two compounds give similar responses in the uv absorbance chromatogram and appear as a nearly resolved pair of peaks.

Sample comparison

Figures 17-22 contain 12 chromatograms representing the four samples and three detectors used in this work. Comparisons of specific peaks or general patterns of peaks in these chromatograms can be used to classify the four asphaltene samples. Several consistent, distinctive features of the chromatograms will be discussed.

Peaks occurring after 50 minutes, using any of the detectors, have a large intrasample variance and, therefore, are less desirable sample features. As expected, the two Synthoil samples are the most difficult to distinguish. Figures 20 and 22 show that the Synthoil 2 sample gives

Figure 17. Sample chromatograms using laser two-photon excited fluorescence detection; chromatographic and detection conditions are given in the Experimental section. Additional sample chromatograms using this detector appear in the next figure

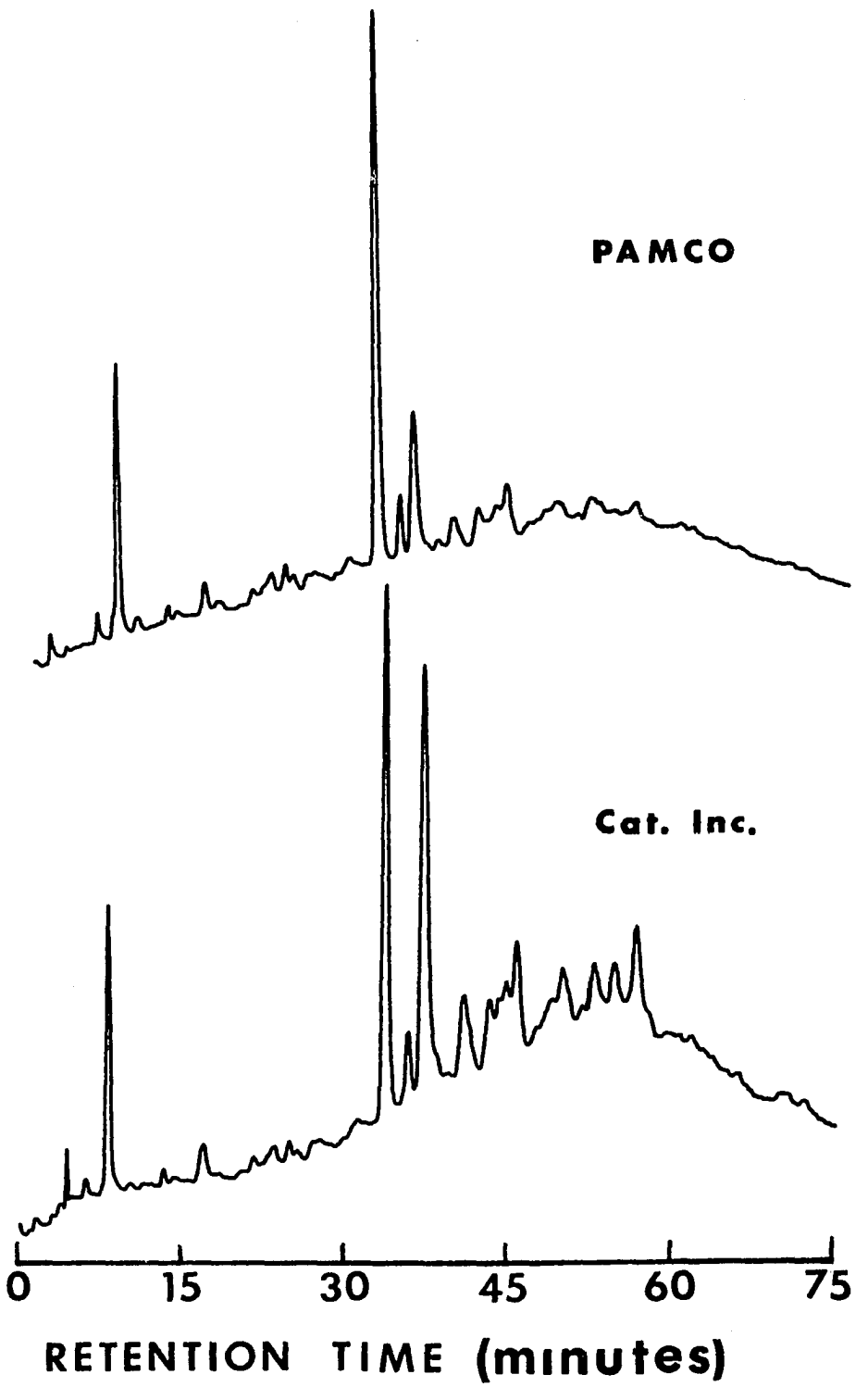

PAMCO
Cat. Inc.
0
15
30
45
60
75
RETENTION TIME (minutes)

Figure 18. Sample chromatograms using laser two-photon excited fluorescence detection; chromatographic and detection conditions are given in the Experimental section. Additional sample chromatograms using this detector appear in the previous figure

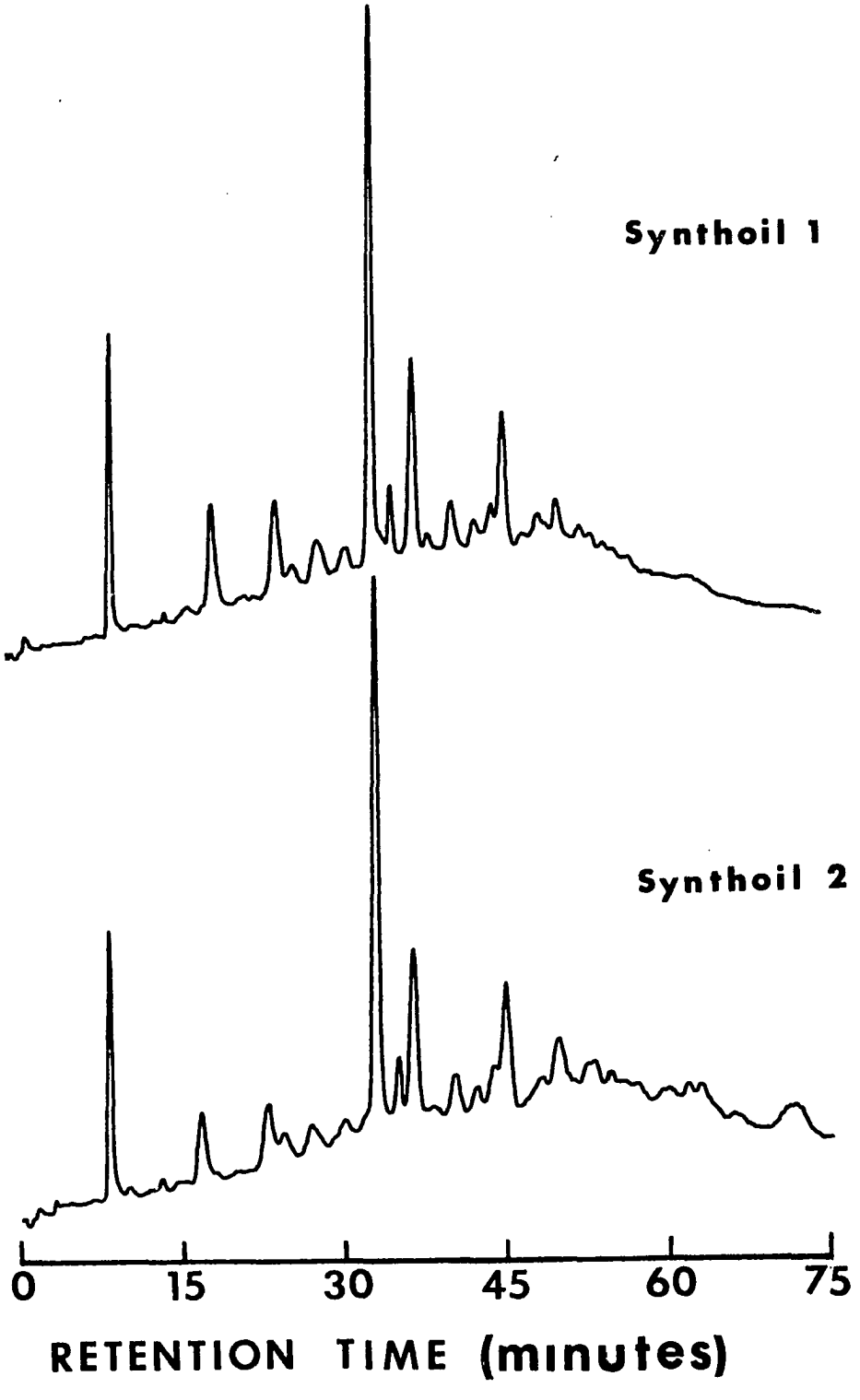

Synthoil 1
Synthoil 2
0
15
30
45
60
75
RETENTION TIME (minutes)

Figure 19. Sample chromatograms using laser fluorescence detection; chromatographic and detection conditions are given in the Experimental section. Additional sample chromatograms using this detector appear in the next figure

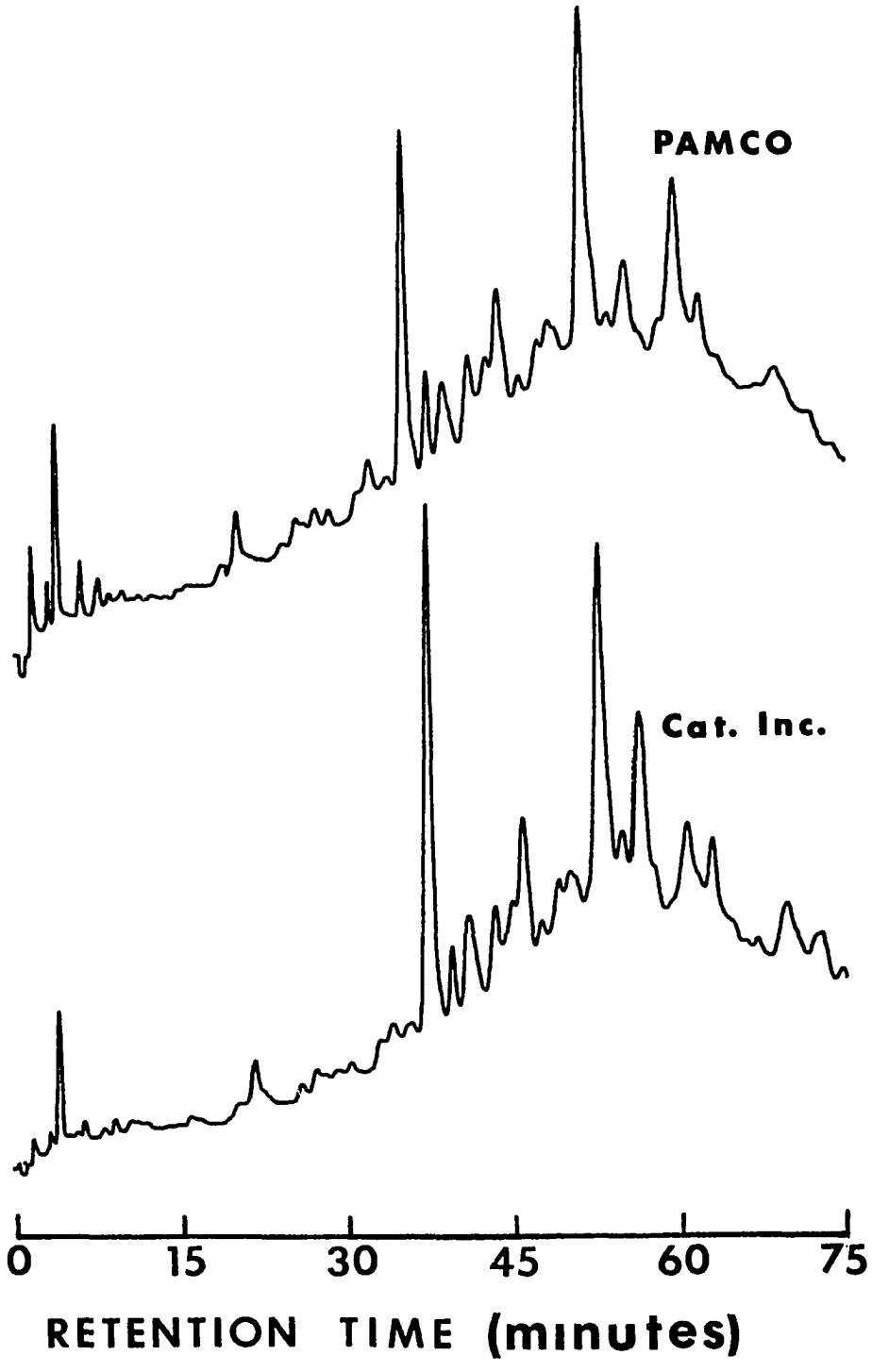

PAMCO
Cat. Inc.
0
15
30
45
60
75
RETENTION TIME (minutes)

Figure 20. Sample chromatograms using laser fluorescence detection; chromatographic and detection conditions are given in the Experimental section. Additional sample chromatograms using this detector appear in the previous figure

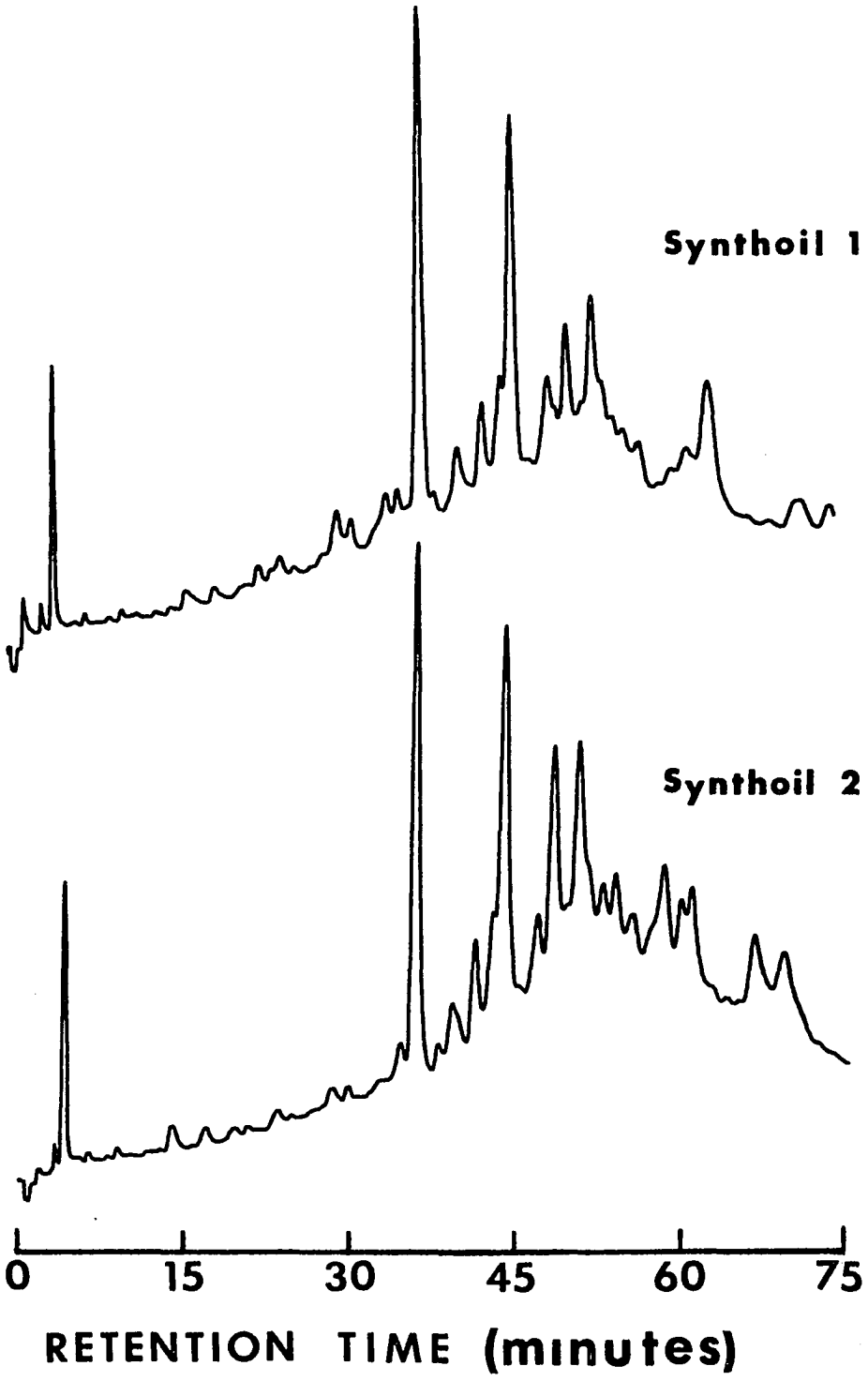

Synthoil 1
Synthoil 2
0
15
30
45
60
75
RETENTION TIME (minutes)

Figure 21. Sample chromatograms using uv absorbance detection; chromatographic and detection conditions are given in the Experimental section. Additional chromatograms using this detector appear in the next figure

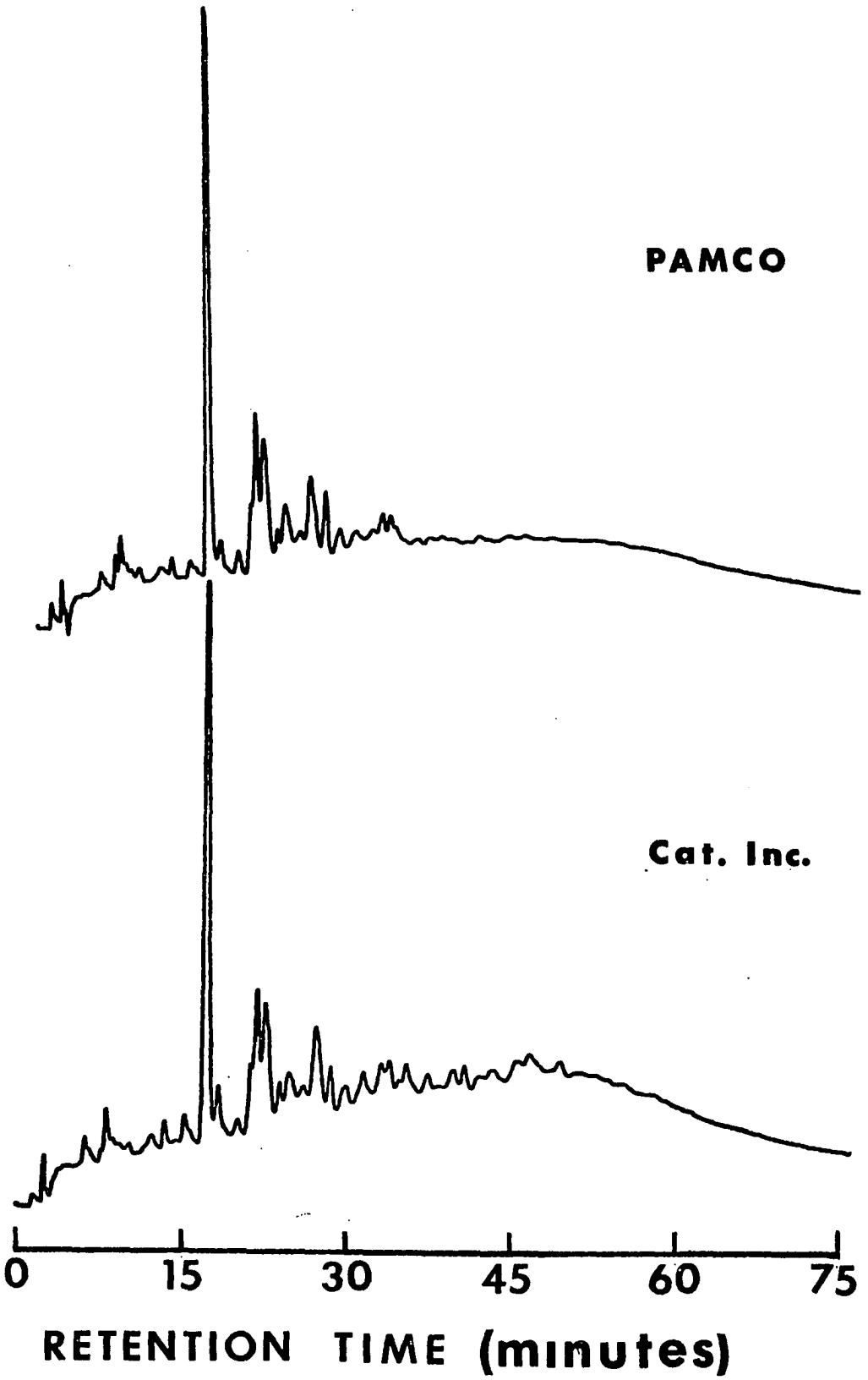
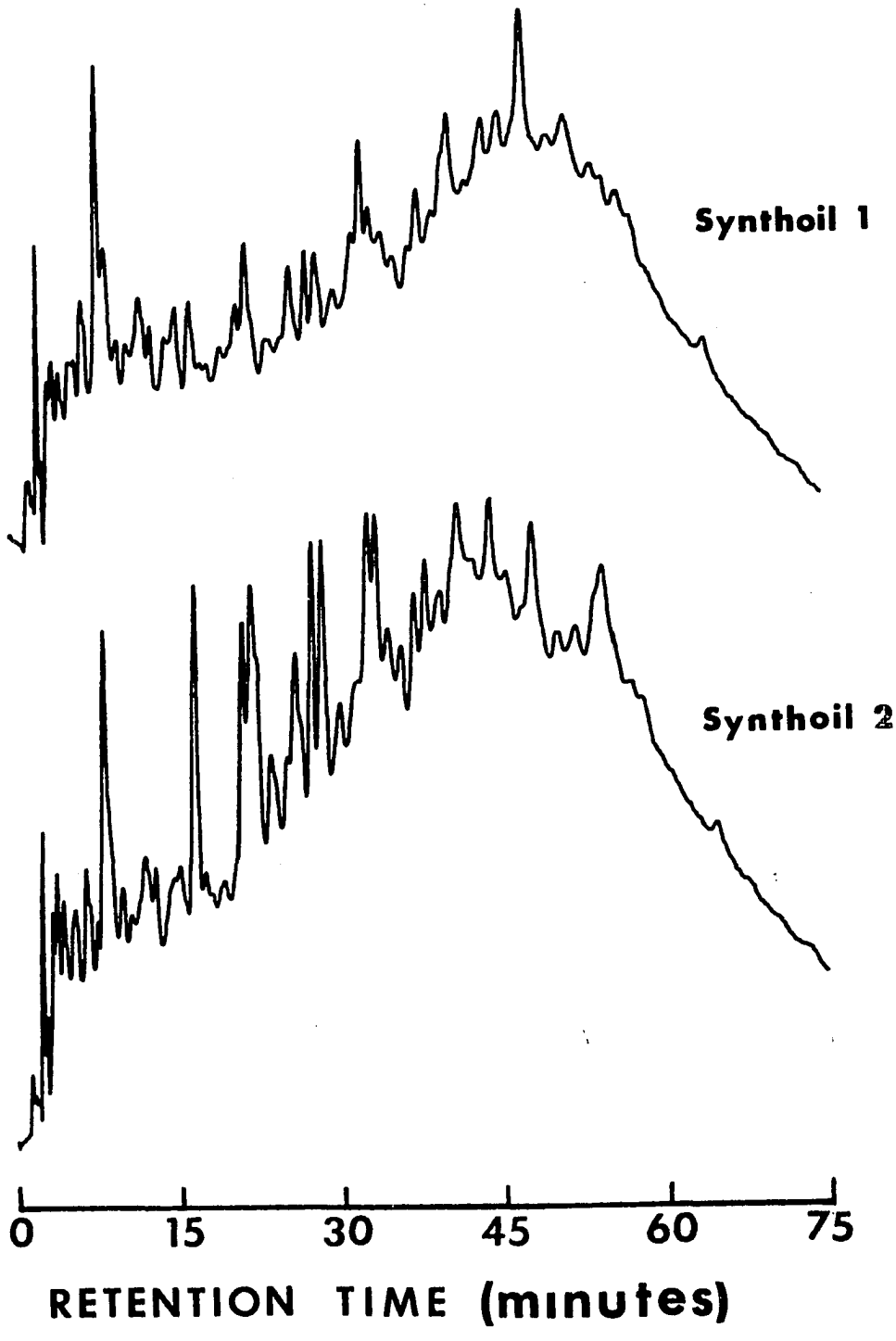

PAMCO
Cat. Inc.
0
15
30
45
60
75
RETENTION TIME (minutes)

Figure 22. Sample chromatograms using uv absorbance detection; chromatographic and detection conditions are given in the Experimental section. Additional chromatograms using this detector appear in the previous figure


Synthoil 1
Synthoil 2
0
15
30
45
60
75
RETENTION TIME (minutes)

slightly larger early eluting peaks than the Synthoil 1 sample. Figure 18 shows a minor peak at about 41 minutes that is larger for the Synthoil 1 sample. Unfortunately, the slope sensitivity and consistency parameters used in MINMAX for this work did not allow this small peak to be recognized. The PAMCO and Catalytic Inc. samples can be easily distinguished from each other using the major peaks in the laser two-photon excited fluorescence chromatograms (see Figure 17), and from the Synthoil samples using a major peak at about 17 minutes in the uv absorbance chromatograms. The PAMCO and Catalytic Inc. samples can also be distinguished from each other using the uv absorbance chromatograms in Figure 21. These chromatograms show larger peaks in the 30 to 50 minute range for the Catalytic Inc. sample. Close inspection of the chromatograms would likely reveal other unique features. Since the chromatograms of a sample vary slightly from injection to injection (see Figures 14 and 15), ARTHUR should be used to add statistical validity to the sample classifications.

Pattern recognition

Pattern recognition techniques are especially well suited to predicting properties of a sample, which are not directly measurable, but are related to certain analytical measurements that can be made on the sample (97). The exact

relationship between the measurements and a property need not be known. The identity, quality, and origin of a sample are a few typical properties.

This work has focused on predicting the identity of coal-derived asphaltene samples, in particular, distinguishing between samples that differ only in the conditions used to produce the coal liquids. The identity of the asphaltene samples might well be predicted using gross measurements, such as the average molecular weight of the compounds in the sample, the ratio of aliphatic to aromatic carbon atoms in the sample, or even the simple appearance of the sample. But the prediction of more sophisticated properties will require measurements more intimately related to the composition of the sample. For example, the environmental impact of a coal liquid might well be strongly dependent on the thiophene-like sulfur content of the asphaltene fraction of that coal liquid. Predictions concerning this property would likely require measurements such as those made in this work.

The components in oil spill samples have been separated by GC and the resulting chromatograms analyzed with pattern recognition techniques (98). But the chromatographic reproducibility and resolution problems associated with separating the asphaltene samples makes feature assignment difficult and feature measurements less reproducible. The question of how to best represent the asphaltene chromatograms for pattern

recognition is not easily answered, unlike the work in reference 98 where the chromatograms were described by features which were simply the peak heights of well-resolved compounds in the sample. In this work the peak heights of mostly unresolved peaks were used as features in describing the chromatograms. The peak height measurements were made from the initial background level and not from the peak base, hoping that the former would carry some information about the broad background under the peak. The broad background might be useful in identifying the asphaltene samples. Feature assignment was a problem because changes in the retention time of a given peak resulted in it being assigned as a different feature for different injections. This problem was minimized by using only well-correlated peaks.

The results of pattern recognition with ARTHUR are summarized in Table 1, where the success percentages for classifying the 31 data vectors in the training set are given for several pattern recognition routines. The BACLAS (99) and SICLAS (100) programs were nearly 100% correct at classifying the data vectors, even when the reduced data vectors were used. The MULTI program (101) classifies on the basis of a discriminant function which is generated by making a maximum of 2001 training passes through the training set vectors. The classification success percentages with MULTI were much less for the reduced data vectors than for the

Table 1. Results of pattern recognition with ARTHUR

Detector	# of Features	Sample	Success at classification (%) SICLAS	BACLAS	KNN	MULTI	
2-PH	38	Synthoil 1	75	100	63	100	(9 passes)
		Synthoil 2	100	100	38	100	
		PAMCO	100	100	71	100	
		Cat. Inc.	88	100	0	100	
2-PH	6	Synthoil 1	63	88	25	50	(2001 passes)
		Synthoil 2	88	100	50	100	
		PAMCO	57	86	57	100	
		Cat. Inc.	88	100	75	100	
1-PH	38	Synthoil 1	63	100	63	100	(17 passes)
		Synthoil 2	88	100	0	100	
		PAMCO	100	100	43	100	
		Cat. Inc.	100	100	63	100	
1-PH	7	Synthoil 1	88	88	88	75	(2001 passes)
		Synthoil 2	88	88	63	100	
		PAMCO	86	100	43	100	
		Cat. Inc.	100	100	100	100	
uv abs.	41	Synthoil 1	88	100	63	100	(7 passes)
		Synthoil 2	100	100	100	100	
		PAMCO	100	100	71	100	
		Cat. Inc.	100	100	38	100	

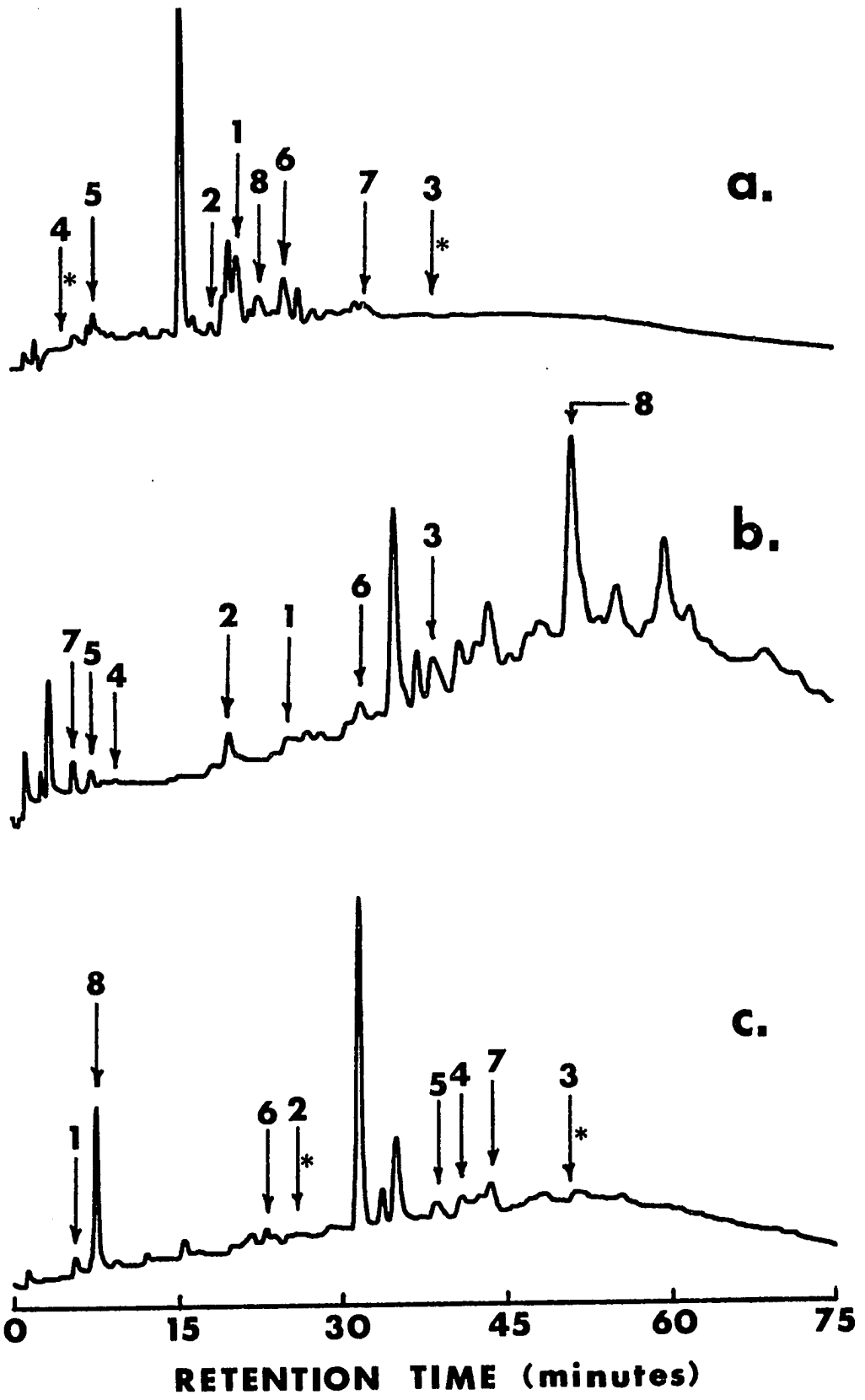
Table 1. (Continued)

Detector	# of Features	Sample	Success at classivication (%) SICLAS	BACLAS	KNN	MULTI	
uv abs.	7	Synthoil 1	100	100	88	100	(15 passes)
		Synthoil 2	100	100	100	100	
		PAMCO	100	100	100	100	
		Cat. Inc.	63	100	75	100	
combination	20	Synthoil 1	100	100	100	100	(9 passes)
		Synthoil 2	100	100	75	100	
		PAMCO	100	100	100	100	
		Cat. Inc.	100	100	100	100	

original data vectors. The K-nearest neighbor classification program (102) was the least successful of the programs. In general, the data vectors composed of 6 laser two-photon excited fluorescence chromatogram features, 7 laser fluorescence chromatogram features, and 7 uv absorbance chromatogram features were the most successfully classified. This indicates that each of the detectors provides information, useful in identifying the asphaltene samples, which is not present with the other detectors. It is somewhat surprising that the classification success percentage was only slightly poorer for the Synthoil samples and, furthermore, the Synthoil samples were misclassified as PAMCO and Catalytic Inc. samples almost as often as with each other.

As discussed in the Experimental section of this chapter, the large data vectors were reduced in dimensionality with the aid of WEIGHT. The 8 features for each detector with the largest intersample to intrasample variance ratios are shown in Figure 23. These features are the most significant in distinguishing the four samples, but not necessarily the most significant in distinguishing a particular sample pair. It should be mentioned that the peak in a chromatogram which is assigned as a particular feature can change when some of the features are dropped. This is because the program used to assign peaks as features (FEATUR) looks for

Figure 23. Chromatograms of PAMCO sample showing 8 most significant features, as determined by WEIGHT. The uv absorbance (a), laser fluorescence (b), and laser two-photon excited fluorescence (c) chromatograms have some unique and some common features. Features with * did not have a measurable peak for this injection of this sample.


a.
4
*
5
2
1
8
6
7
3
*
b.
8
3
6
2
1
7
5
4
c.
8
1
6
2
*
5
4
7
3
*
0
15
30
45
60
75
RETENTION TIME (minutes)

the best peak-feature retention time fit when making assignments.

It is clear from Figure 23 that the three detectors yield both unique and common significant features. There are a few observations of particular interest to be made from Figure 23. First, the 6th most significant feature in the laser fluorescence chromatogram appears at the same retention time as the major peak in the laser two-photon excited fluorescence chromatogram, but the latter peak is not a significant feature. This seems to indicate that the peaks result from different compounds and this is reinforced by the presence of two poorly resolved peaks in the uv absorbance chromatogram at that retention time. Second, the 8th most significant feature in the laser two-photon excited fluorescence chromatogram is the internal standard peak. This is probably due to the importance of the early broad background as a descriptive feature. It should be stated that the baseline noise in these chromatograms is insignificant on their corresponding scales and legitimate ($S/N > 3$) "peaks" are present in the baseline.

Finally, the chromatograms in Figure 23 illustrate that the most significant features need not be major peaks. This would seem to contradict the statements made in the previous section concerning the discriminating value of certain major peaks in the uv absorbance and laser two-photon excited

fluorescence chromatograms. This contradiction can be explained by the fact that the significant features appearing in Figure 23 were chosen based on their overall discriminating value and not their ability to distinguish between a given sample pair. A case in point is the major peak in the uv absorbance chromatogram. It was mentioned that this peak is useful in distinguishing the Synthoil samples from the other two samples. Close inspection of the printout from WEIGHT revealed that the weighting factors for this feature (intersample to intrasample variance ratios) were about 50% greater for Catalytic Inc.-Synthoil combinations than for Catalytic Inc.-PAMCO or Synthoil 1-Synthoil 2 combinations. The overall weighting factor for this feature was only good enough for it to be ranked 21st among the original 41 features. The fact that this feature was not used in the reduced data vector may have contributed to the fact that three Catalytic Inc. reduced data vectors were misclassified by SICLAS as Synthoil reduced data vectors, while no such misclassifications were made for the 41-dimension data vectors.

This work has demonstrated that coal-derived asphaltene samples can be reproducibily separated by HPLC and distinctively detected with a uv absorbance and two laser fluorometric detectors. It has been shown that the resulting multi-dimensional chromatograms provide exclusive information

that make it possible to identify the asphaltene fractions of coal liquids that differ only in the conditions used to produce them. The ability to perform this identification with ARTHUR, a collection of pattern recognition programs, was also demonstrated. The compounds in the asphaltene samples which gave rise to major peaks and/or significant features were not identified. The identification of these compounds or, at least, the injection of model compounds to better understand what types of compounds could contribute to the observed peaks, would seem to be a logical continuation of this work.

CHAPTER IV. CONCLUSIONS

The motivation for the work presented in this dissertation might best be reflected in the recently published five-year outlook report of the National Science Foundation (103). In this report a need was expressed for analytical methodologies capable of determining drugs, toxic and carcinogenic agents, and pesticide residues at ppb or even ppt levels. Efforts aimed at meeting analysis challenges of this type using HPLC with laser fluorescence detection were explored here and, in summary, the following conclusions are offered.

The spectral advantages of laser excitation for fluorescence HPLC detection, discussed in Chapter I, were realized in the low picogram level determination of the antitumor drugs adriamycin and daunorubicin in body fluids. A unique optical fiber-flow cell was shown to maintain chromatographic efficiency (a result of low volume and laminar flow) while providing good fluorescence collection efficiency and stray light rejection. The inherent selectivity of visible fluorescence resulted in chromatograms that were free from interferences. Also, sequentially excited fluorescence, one of the many nonlinear optical techniques made possible by the high output power of lasers, was demonstrated as a fluorescence technique which provides its own unique selectivity.

As drugs and toxins are shown to produce their effects at lower levels, the applications of laser fluorescence HPLC detection for substances in biological matrices will likely increase. Undoubtedly, steady advancements in laser technology (better, more reliable, and more cost effective laser systems) will accelerate the increase in applications for laser fluorescence HPLC detection.

The complexity and nature of coal-derived asphaltene samples has made their analysis extremely difficult. The sensitivity of laser fluorescence HPLC detection has permitted small injection quantities. This fact plus a unique pre-column arrangement contributed to reasonably reproducible asphaltene chromatograms. While the chromatographic efficiency of HPLC is not sufficient for baseline resolution of all the compounds, it was shown that multi-dimensional detection can be used to better characterize asphaltene samples. Laser fluorescence, laser two-photon excited fluorescence, and uv absorbance detection was used for this purpose. The asphaltene chromatograms from these detectors were shown to be destinctive and provide exclusive information which can be used to classify asphaltene samples with pattern recognition techniques.

REFERENCES

1. Cram, S. P.; Yang, F. J. Industrial Res./Dev. April 1978, 89.

2. Wehry, E. L.; Mamantov, G. Anal. Chem. 1979, 51, 643A.

3. Hirschfeld, T. Anal. Chem. 1980, 52, 297A.

4. Baker, D. R.; Williams, R. C.; Steichen, J. C. J. Chromatogr. Sci. 1974, 12, 499.

5. Polesuk, J.; Howery, D. G. J. Chromatogr. Sci. 1973, 11, 226.

6. Wise, S. A.; May, W, E, Research/Development October 1977, 54.

7. Byrne, S. H. "Modern Practice of Liquid Chromatography"; Kirkland, J. J., Ed.; Wiley Interscience: New York, 1971.

8. Done, J. N.; Knox, J. H.; Loheac, J. "Applications of High Speed Liquid Chromatography"; J. Wiley and Sons: New York, 1974.

9. Perry, S. G.; Amos, R.; Brewer, P. I. "Practical Liquid Chromatography"; Plenum Press: New York, 1972.

10. Sepaniak, M. J. M.S. Thesis, Iowa State University, Ames, Iowa, 1977.

11. Kirkland, J. J. Anal. Chem. 1971, 43, 36A.

12. Locke, D. C. J. Chromatogr. Sci. 1973, 11, 120.

13. Done, J. N.; Kennedy, G. J.; Knox, J. H. Nature 1972, 237, 77.

14. Pryde, A. J. Chromatogr. Sci. 1974, 12, 486.

15. Grushka, E. "Bonded Stationary Phases in Chromatography"; Ann Arbor Science Publishers, Inc.; Ann Arbor, Michigan, 1974.

16. Halasz, I.; Sebastian, I. Angew. Chem. 1969, 81, 464.

17. Gurken, M. Amer. Lab. 1977, 9, 29.

18. Karch, K.; Sebastian, I.; Halasz, I. J. Chromatogr. 1976, 122, 3.

19. Leitch, R. E.; DeStefano, J. J. J. Chromatogr. Sci. 1973, 11, 105.

20. Horvath, C.; Melander, W. J. Chromatogr. Sci. 1977, 15, 393.

21. Lochmuller, C. H.; Wilder, D. R. J. Chromatogr. Sci. 1979, 17, 574.

22. Cooke, N. Altex Chromatogram 1978, 1, 3.

23. Bakalyar, S. R.; McIlwrick, R. Industrial Research February 1978, C4.

24. Karch, K.; Sebestian, I.; Halasz, I.; Engelhardt, H. J. Chromatogr. 1976, 122, 171.

25. Chromatography Notes; Waters Associates: Milford, Mass.; 3, 4.

26. Schmitt, J.; Henry, R.; Williams, R.; Dieckman, J. J. Chromatogr. Sci. 1971, 9, 645.

27. Jane, I.; Wheals, B. J. Chromatogr. 1973, 84, 181.

28. Waters Associates: Milford, Mass.; Publication No. DS72-015.

29. Williams, R. Dupont Liquid Chromatography Methods Bulletin 820M13, October 1972.

30. Rzeszotarski, W.; Mauger, A. J. Chromatogr. 1973, 86, 246.

31. Vaughan, C. G.; Wheals, B. B.; Whitehouse, M. J. J. Chromatogr. 1973, 78, 203.

32. Vaughan, C. G.; Wheals, B. B.; Whitehouse, M. J. J. Chromatogr. 1975, 106, 109.

33. Fox, M. A.; Stanley, S. W. Anal. Chem. 1976, 48, 992.

34. Krstulovic, A.; Rosie, D.; Brown, P. Anal. Chem. 1976, 48, 1383.

35. Scott, R. P. 15th Midwest Regional Meeting of the American Chemical Society, St. Louis, MO., October 1979; American Chemical Society: Washington, D.C.; Symposium 4.

36. Ishii, D.; Tsuda, T.; Hibi, K.; Takeuchi, T.; Nakanishi, T. J. High Res. Chromatogr. & Chromatogr. Comm. 1979, 2, 371.

37. Tsuda, T.; Novotny, M. Anal. Chem. 1978, 50, 271.

38. Hibi, K.; Ishii, D.; Fujishima, I.; Takeuchi, T.; Nakanishi, T. J. High Res. Chromatogr. & Chromatogr. Comm. 1978, 1, 21.

39. Krejci, M.; Pospisilova, N. J. J. Chromatogr. 1972, 73, 105.

40. Pellizzari, E. D.; Sparachino, C. M. Anal. Chem. 1973, 45, 378.

41. Chilcote, D. D.; Mrochek, J. E. Clin. Chem. 1972, 18, 778.

42. Cassidy, R. M.; LeGay, D. S.; Frei, R. W. J. Chromatogr. Sci. 1974, 12, 85.

43. Perchalski, R. J.; Winefordner, J. D.; Wilder, B. J. Anal. Chem. 1975, 47, 1993.

44. Dunges, W.; Naundorf, G.; Seiler N. J. Chromatogr. Sci. 1974, 12, 655.

45. Frei, R. W.; Lawrence, J. F. J. Chromatogr. 1973, 83, 32.

46. Veening, G. J. Chem. Educ. 1970, 47, A749.

47. Steichen, J. C. J. Chromatogr. 1975, 104, 39.

48. Cassidy, R. M.; Frei, R. W. J. Chromatogr. 1972, 72, 293.

49. Giering, L. P.; Horning, A. W. American Lab. November 1977, 113.

50. Diebold, G. J.; Zare, R. N. Science 1977, 196, 1439.

51. Imasaka, T.; Zare, R. N. Anal. Chem. 1979, 51, 2082.

52. Sepaniak, M. J.; Yeung. E S. Anal. Chem. 1977. 49, 1554.

53. Sepaniak, M. J.; Yeung, E. S. J. Chromatogr. 1980, 190, 377.

54. Hershberger, L. W.; Callis, J. B.; Christian, G. D. Anal. Chem. 1979, 51, 1444.

55. Yeung, E. S. In "Lasers and Chemical Analysis"; Heiftje, G.; Lytle, F. E.; Travis, Eds.; Humana Press: Clifton, N.J., 1979.

56. Sepaniak, M. J.; Yeung, E. S. Anal. Chem., in press.

57. Harris, J. M.; Dovichi, N. J. Anal. Chem. 1980, 52, 695A.

58. Johnson, E. L.; Stevenson, R. "Basic Liquid Chromatography"; Varian Associates, Inc.: Palo Alto, CA., 1978; Chapter 11.

59. Schoeffel Model FS-970 Fluorescence Liquid Chromatography Detector Product Literature; Kratos, Inc., Schoeffel Instrument Division: Westwood, N.J.

60. Varian Fluorichrom Fluorescence Liquid Chromatography Detector Product Literature; Varian Instrument Division: Palo Alto, CA.

61. Harris J. M.; Chrisman, R. W.; Lytle, F. E.; Tobias, R. S. Anal. Chem. 1976, 48, 1937.

62. Porro, T. J. Trends in Fluor. January 1978, 1, 7.

63. Gopper-Mayer, M. Ann. Phys. 1931, 9, 273.

64. Mahr, H. In "Quantum Electronics"; Robin H.; Tang, C., Eds.; Academic Press: New York, 1975; Vol. 1, Chapter 4.

65. Kleiman, D. Phys. Rev. 1962, 125, 87.

66. McClain, W. M. Acc. Chem. Res. 1974, 7, 199.

67. Drago, R. S. "Physical Methods in Inorganic Chemistry"; Reinhold Publishing Corp.: New York, 1975; p. 148.

68. Swofford, R. L. Ph.D. Dissertation, University of California, Berkeley, CA., 1973, p. 9.

69. Wirth, M. J.; Lytle, F. E. Anal. Chem. 1977, 49, 2054.

70. Wirth, M. J.; Lytle, F. E. In "New Applications of Lasers to Chemistry"; Hieftje, G. M., Ed.; American Chemical Society: Washington, D.C., 1978; Chapter 3.

71. Kasha, M. Disc. Faraday Soc. 1950, 9, 14.

72. Finkel, J. M.; Knapp, K. T.; Mulligan, L. T. Cancer Chemother. Rep. 1969, 53, 159.

73. Eksborg, S. J. Chromatogr. 1978, 149, 225.

74. Langone, J. J.; Van Vunakis, H.; Bachur, N. R. Biochem. Med. 1975, 12, 283.

75. Eksborg, S.; Ehrsson, H.; Anderson, B.; Beran, M. J. Chromatogr. 1978, 153, 211.

76. Horvath, C.; Melander, W.; Molnar, I. Anal. Chem. 1977, 49, 142.

77. Palalikit, D.; Block, J. H. Anal. Chem. 1980, 52, 624.

78. Slavin, W.; Rhys Williams, A. T.; Adams, R. P. J. Chromatogr. 1977, 134, 121.

79. Cochran, N. P. Scientific American 1976, 234, 24.

80. Burke, D. P. Chem. Week 1974, 38, 115.

81. Battelle Energy Program Report, "Liquefaction and Chemical Refining of Coal" July 1974, Columbus, Ohio.

82. Yoshida, R.; Maekawa, Y.; Ishii, T.; Takeya, G. Fuel 1976, 55, 337.

83. Schweighardt, F. K.; Retcofsky, H. L.; Friedman, S.; Hough, M. Anal. Chem. 1978, 50, 368.

84. Yoshida, T.; Maekawa, Y.; Uchino, H.; Yokoyama, S. Anal. Chem. 1980, 52, 817.

85. Schwager, I.; Yen, T. F. Anal. Chem. 1979, 51, 569.

86. Brown, R. S.; Hausler, D W.; Taylor, L. T. Anal. Chem. 1980, 52, 1511.

87. Bockreth, B. C.; Schroeder, K. T ; Steffgen, F. W. Anal. Chem. 1979, 51, 1168.

88. Anbar, M.; St. John, G. A. Fuel 1978, 57, 105.

89. Dooley, J. E.; Thompson, C J.; Scheppele, S. E. In "Analytical Methods for Coal and Coal Products"; Karr, C., Ed.; Academic Press: New York, 1978, Chapter 16.

90. Schwager, I.; Yen, T. F. Fuel 1979, 58, 219.

91. Dark, W. A.; McFadden, W. H.; Bradford, D. L. J. Chromatogr. Sci. 1977, 15, 454.

92. Arpino, P. R.; Guiochon, G. Anal. Chem. 1979, 51, 683A.

93. Schweighardt, F. K.; Thames B. M. Anal. Chem. 1978, 50, 1381.

94. Milne, W. E. "Numerical Calculus"; Princeton University Press: Princeton, N.J., 1949; Chapter 9.

95. Harper, A. M.; Duewar, D. L.; Kowalski, B. R.; Fasching, J. L. In "Chemometrics: Theory and Applications"; Kowalski, B. R., Ed.; American Chemical Society Symposium Series No. 52, American Chemical Society: Washington, D.C., 1977; Chapter 2.

96. Lawrence, J. F. Anal. Chem. 1980, 52, 1123A.

97. Kowalski, B. R. Anal. Chem. 1975, 47, 1152A.

98. Clark, H. A.; Jurs, P. C. Anal. Chem. 1979, 51, 618.

99. Duewer, D. L. "Documentation for ARTHUR Version 1-8-75"; Chemometrics Society Report No. 2, 1975.

100. Wold, S. J. Pattern Recognition 1976, 8, 127.

101. Nilsson, N. J. "Learning Machines"; McGraw Hill: New York, 1965.

102. Cover, T. M.; Hart, P. E. IEEE Transactions on Information Theory 1967, IT-13, 21.

103. Borman, S. A. Anal. Chem. 1980, 52, 1121A.

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APPENDIX A

Equations relating the number of higher excited state fluorescence photons to various experimental parameters and electronic transition rate constants, will be derived. In Figure 24, the processes competing for population and depopulation of excited electronic states are depicted. The rate of depopulation of electronic level (n) is given by

$$k_{d_n} = k_{f_r} + k_{x_n} + k_{ic_n} \qquad \text{(A-1)}$$

where k_f, k_x, and k_{ic} are the individual rate constants for fluorescence, intersystem crossing, and internal conversion, respectively. When n is greater than one, k_{ic} usually dominates the other two terms.

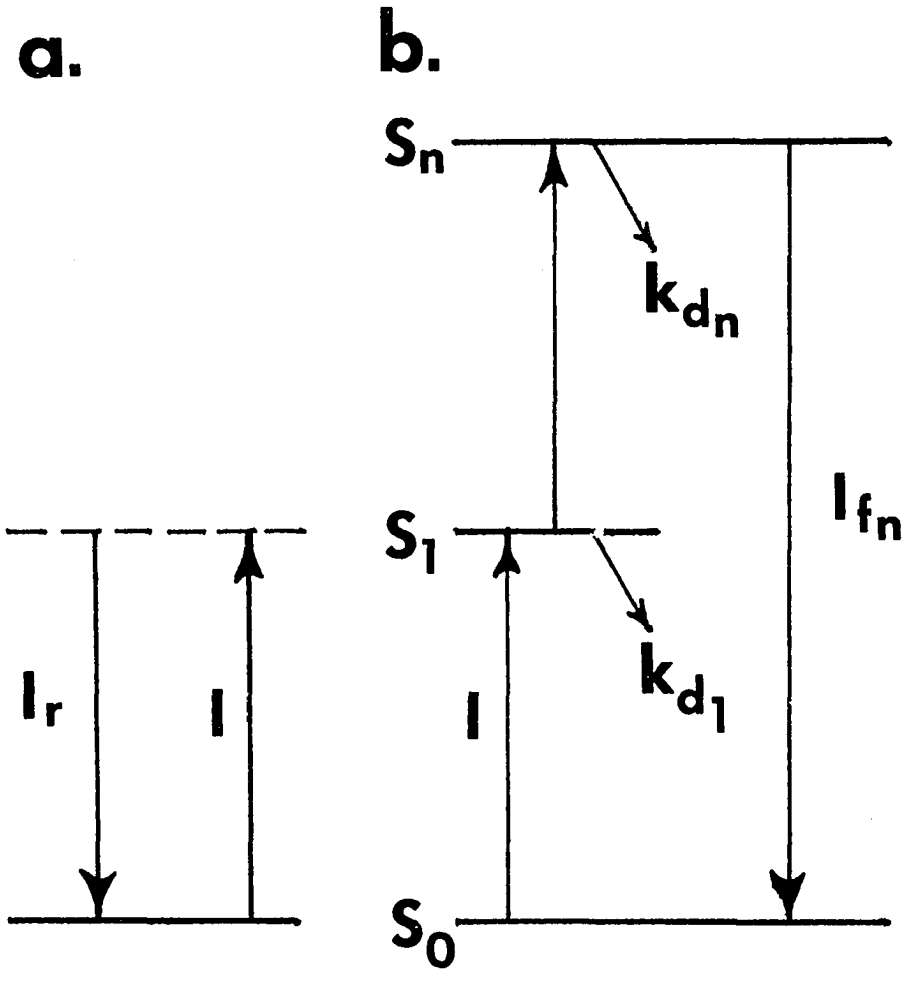
The steady state rate expressions for the populations of the electronic levels in Figure 24 are

$$dN_1/dt = P_1N_0 - k_{d1}N_1 - P_nN_1 - P_1N_1 + P_nN_n + k_{ic_n}N_n = 0 \qquad \text{(A-2)}$$

$$dN_n/dt = P_nN_1 - P_nN_n - k_{d_n}N_n = 0 \qquad \text{(A-3)}$$

where N_n is the population of level (n). The terms in equation A-2 from left to right are absorption from S_0 to S_1, decay from S_1, absorption from S_1 to S_n, stimulated emission from S_1 to S_0, stimulated emission from S_n to S_1, and relaxation from S_n to S_1. The populating rate for the

Figure 24. Rayleigh and Raman scattering (I_r) of the solvent (a) and sequential excited fluorescence (I_{f_n}) of a solute (b). (I) is the laser intensity and k_{d_n} is the total rate constant for depopulation of S_n


a.
b.
S_n
k_{d_n}
I_{f_n}
S_1
k_{d_1}
I_r
I
I
S_0

transition in question (P_n) is given by

$$P_n = \sigma I/h\nu \qquad (A-4)$$

where σ is the transition cross section and (I) is the intensity of the populating radiation. If a single laser, lasing at one wavelength, is used for both the S_0 to S_1 and S_1 to S_n transitions, and if the transitions are assumed to be equally probable (same σ), then P_1 and P_n are equal.

Letting $k_{d_n} = k_{ic_n}$ and $P_1 = P_n = P$, equations A-2 and A-3 can be combined to yield:

$$N_n = P^2 N_0/(k_{d_1} k_{ic_n} + k_{ic_n} P + k_{d_1} P + P^2) \qquad (A-5)$$

The total number of higher excited state fluorescence photons (N_{n_f}) is given by

$$N_{n_f} = N_n k_{f_n} = VC_n k_{f_n} \qquad (A-6)$$

where V is the illuminated volume and C_n is the concentration of molecules in that volume which are in electronic level (n).

The equations derived in this appendix can be used to estimate minimum detectable concentrations by the technique of sequential excited fluorescence. Experimental parameters similar to those used for the work presented in Chapters II and III and typical values for strongly allowed electronic rate constants and absorption cross sections appear in

Table 2. When these parameter values are used for calculations, a minimum detectable concentration of 3×10^{-8} M is

Table 2. Numerical values for parameters

Parameter	Value
I	7×10^{5} W/cm^2
k_{f_n}	10^{9} sec^{-1}
k_{d_i}	10^{9} sec^{-1}
k_{ic_n}	10^{13} sec^{-1}
σ_n	2×10^{-19} cm^2
$h\nu$	4×10^{-19} J
V	4×10^{-7} cm^3 (4×10^{-6} cm^2 x 0.1 cm)
N_{n_f} (minimum needed to detect)	10^{5}

obtained. This corresponds to an injection quantity of 6 ng of the antitumor drug adriamycin (using the chromatographic and detection conditions used for its determination in Chapter II and assuming a 20-fold chromatographic dilution).

APPENDIX B

This Appendix contains printouts of the computer programs used to collect and treat data from the laser two-photon excited fluorescence, laser fluorescence, and uv absorbance detection of HPLC separated coal-derived asphaltenes. The programs, which are described in the Experimental section of Chapter III, are also summarized in Table 3, which follows.

Table 3. Summary of the computer programs used to treat asphaltene chromatographic data

Program	Function	Parameter values used
GLITCH	Remove glitches in the chromatograms (see Figure 12)	GC = 20
NORMAL	Normalize signals to internal standard peak height and subtract background level	BKGRD & PKHT values from stripchart chromatograms and computer data
SMOOTH	Reduce baseline noise	
FIXPTS	Recreate digital chromatographic data from graphical measurements on stripchart chromatograms	
MINMAX	Find minimums and maximums in digitized chromatograms	ISC = 4 SS = 1.5
RETIME	Normalize retention times	RNF values from two-photon fluorescence stripchart chromatograms

Table 3. (Continued)

Program	Function	Parameter values used
CORLAT	Correlate intrasample peak heights	RTW = 50 SW = 1.5
KFILES	Store intersample, manually correlated, peak retention times (for use by FEATUR)	
FEATUR	Place peak heights into categories based on peak retention times	IRTW = 25 NP(L) values are the category retention times from intersample correlation

```
      COALN

C     PROGRAM FOR AVERAGING, DISPLAYING, AND STORING CHROMATOGRA-
C     PHIC DATA OBTAINED ON THREE CHANNELS (CHAN. 1 =
C     ONE-PHOTON FLUORESCENCE DATA, CHAN. 2 = UV ABS. DATA,
C     AND CHAN. 3 = TWO-PHOTON FLUORESCENCE DATA) FOR HPLC SEPA-
C     RATION OF COAL ASPHALTENE FRACTIONS. LASER POWER
C     IS MONITORED ON CHAN. 4.
      DIMENSION FLUA(1500),UVA(1500),TWOPA(1500),POWER(1500)
      EXTERNAL IADC
100   N=0
      FLUO=0
200   ICMF=0
      CALL SETR(4,0,50.,ICMF)
      CALL LWAIT(ICMF,0)
      IFLU=IADC(1)
      FLUO=FLUO+FLOAT(IFLU)/60.
C     DATA PTS ARE TAKEN EVERY 50MS. AND AVERAGED OVER 60 PTS.
      N=N+1
      IF(N .LT. 60) GO TO 200
      CALL LED(FLUO,'F7.2')
      ISTART=IADC(0)
      IF(ISTART .LT. 1000) GO TO 100
      WRITE(5,300)
300   FORMAT('  FILE#?...10<=IFILE<=99')
      READ(5,400)IFILE
400   FORMAT(I2)
      DEFINE FILE IFILE(1,12006,U,NREC)
      WRITE(5,500)
500   FORMAT('  ENTER # DATA POINTS TO BE TAKEN')
      READ(5,600)MPTS
600   FORMAT(I4)
      DO 900 M=1,MPTS
700   N=0
      FLUA(M)=0
      UVA(M)=0
      TWOPA(M)=0
```

```
      POWER(M)=0
800   ICMF=0
      CALL SETR(4,0,50.,ICMF)
      CALL LWAIT(ICMF,0)
      IFLU=IADC(1)
      IUV=IADC(2)
      ITWOP=IADC(3)
      IPOWER=IADC(4)
      FLUA(M)=FLUA(M)+FLOAT(IFLU)/60.
      UVA(M)=UVA(M)+FLOAT(IUV)/60.
      TWOPA(M)=TWOPA(M)+FLOAT(ITWOP)/60.
      POWER(M)=POWER(M)+FLOAT(IPOWER)/60.
      N=N+1
      IF(N .LT. 60) GO TO 800
900   CALL LED(FLUA(M),'F7.2')
      WRITE(IFILE'1)MPTS,(FLUA(M),UVA(M),TWOPA(M),POWER(M),M=1,MPTS)
      STOP
      END
```



```
      NORMAL
C     PROGRAM TO NORMALIZE AND REMOVE BACKGROUND FROM ASPHALTENE
C     CHROMATOGRAPHIC DATA
      DIMENSION FLUA(1500),UVA(1500),TWOPA(1500),POWER(1500),DATA(1500)
      WRITE(5,100)
100   FORMAT('  ENTER FILE#...10<=IFILE<=99')
      READ(5,200)IFILE
200   FORMAT(I2)
      DEFINE FILE IFILE(1,12006,U,NREC)
      READ(IFILE'1)NPTS,(FLUA(N),UVA(N),TWOPA(N),POWER(N),N=1,NPTS)
      WRITE(5,300)
300   FORMAT('  ENTER 0 FOR UV, 1 FOR 1-PHOTON, OR 2 FOR 2-PHOTON')
      READ(5,400)INUM
400   FORMAT(I1)
      IF(INUM-1)500,600,700
500   DO 550 N=1,NPTS
```

```
550     DATA(N)=UVA(N)
        GO TO 800
600     DO 650 N=1,NPTS
650     DATA(N)=FLUA(N)
        GO TO 800
700     DO 750 N=1,NPTS
750     DATA(N)=TWOPA(N)
800     WRITE(5,900)
900     FORMAT('  ENTER # DATA POINTS')
        READ(5,1000)NPTS
1000    FORMAT(I4)
        WRITE(5,1100)
1100    FORMAT('  ENTER BKGRD LEVEL AND INT STD PK HT')
        READ(5,1200)BKGRD,PKHT
1200    FORMAT(2F8.4)
        DO 1300 N=1,NPTS
1300    DATA(N)=(DATA(N)-BKGRD)/PKHT
        IF(INUM-1)1400,1500,1600
1400    DO 1450 N=1,NPTS
1450    UVA(N)=DATA(N)
        GO TO 1700
1500    DO 1550 N=1,NPTS
1550    FLUA(N)=DATA(N)
        GO TO 1700
1600    DO 1650 N=1,NPTS
1650    TWOPA(N)=DATA(N)
1700    WRITE(IFILE'1)NPTS,(FLUA(N),UVA(N),TWOPA(N),POWER(N),N=1,NPTS)
        STOP
        END
```

GLITCH

```
C       PROGRAM TO REMOVE GLITCHES FROM ASPHALTENE CHROMATOGRAMS
        DIMENSION FLUA(1500),UVA(1500),TWOPA(1500),POWER(1500),DATA(1500)
        WRITE(5,100)
```

```
100     FORMAT('  ENTER FILE#...10<=IFILE<=99')
        READ(5,200)IFILE
200     FORMAT(I2)
        DEFINE FILE IFILE(1,12006,U,NREC)
        READ(IFILE'1)NPTS,(FLUA(N),UVA(N),TWOPA(N),POWER(N),N=1,NPTS)
        WRITE(5,300)
300     FORMAT('  ENTER 0 FOR UV, 1 FOR 1-PHOTON, OR 2 FOR 2-PHOTON')
        READ(5,400)INUM
400     FORMAT(I1)
        IF(INUM-1)500,600,700
500     DO 550 N=1,NPTS
550     DATA(N)=UVA(N)
        GO TO 800
600     DO 650 N=1,NPTS
650     DATA(N)=FLUA(N)
        GO TO 800
700     DO 750 N=1,NPTS
750     DATA(N)=TWOPA(N)
800     WRITE(5,900)
900     FORMAT('  ENTER # DATA POINTS')
        READ(5,1000)NPTS
1000    FORMAT(I4)
        WRITE(5,1100)
1100    FORMAT('  ENTER GLITCH CONSTANT')
        READ(5,1200)GC
1200    FORMAT(F4.1)
        N=1
1300    CONTINUE
        X=DATA(N)
        N=N+1
        Y=DATA(N)
1400    IF(Y-X-GC)1500,1500,1600
1500    IF(X-Y-GC)2000,2000,1900
1600    X=DATA(N)
        N=N+1
        Y=DATA(N)
        IF(X-Y-GC)1700,1700,1800
```

```
1700    IF(N .LT. NPTS)GO TO 1400
        GO TO 2500
1800    N=N-2
        X=DATA(N)
        N=N+1
        DATA(N)=(X+Y)/2.
        N=N+1
2000    IF(N .LT. NPTS)GO TO 1300
        GO TO 2500
1900    X=DATA(N)
        N=N+1
        Y=DATA(N)
        IF(Y-X-GC)2100,2100,1800
2100    IF(N .LT. NPTS)GO TO 1500
2500    IF(INUM-1)3000,4000,5000
3000    DO 3500 N=1,NPTS
3500    UVA(N)=DATA(N)
        GO TO 6000
4000    DO 4500 N=1,NPTS
4500    FLUA(N)=DATA(N)
        GO TO 6000
5000    DO 5500 N=1,NPTS
5500    TWOPA(N)=DATA(N)
6000    WRITE(IFILE'1)NPTS,(FLUA(N),UVA(N),TWOPA(N),POWER(N),N=1,NPTS)
        STOP
        END
```

SMOOTH

```
C       PROGRAM TO SMOOTH ASPHATENE CHROMATOGRAPHIC DATA
        DIMENSION FLUA(1500),UVA(1500),TWOPA(1500),POWER(1500),DATA(1500)
        WRITE(5,100)
100     FORMAT('  ENTER FILE#...10<=IFILE<=99')
        READ(5,200)IFILE
200     FORMAT(I2)
        DEFINE FILE IFILE(1,12006,U,NREC)
```

```
      READ(IFILE'1)NPTS,(FLUA(N),UVA(N),TWOPA(N),POWER(N),N=1,NPTS)
      WRITE(5,300)
300   FORMAT('  ENTER 0 FOR UV, 1 FOR 1-PHOTON, OR 2 FOR 2-PHOTON')
      READ(5,400)INUM
400   FORMAT(I1)
      IF(INUM-1)500,600,700
500   DO 550 N=1,NPTS
550   DATA(N)=UVA(N)
      GO TO 800
600   DO 650 N=1,NPTS
650   DATA(N)=FLUA(N)
      GO TO 800
700   DO 750 N=1,NPTS
750   DATA(N)=TWOPA(N)
800   WRITE(5,900)
900   FORMAT('  ENTER # DATA POINTS')
      READ(5,1000)NPTS
1000  FORMAT(I4)
C     A THIRD DEGREE POLYNOMIAL LEAST SQUARES SMOOTH WILL BE APPLIED
C     7 POINT SMOOTH FACTORS (-2,3,6,7,6,3,-2)
      Y0=DATA(1)
      Y1=DATA(2)
      Y2=DATA(3)
      Y3=DATA(4)
      Y4=DATA(5)
      Y5=DATA(6)
      Y6=DATA(7)
      N=4
1300  CONTINUE
      DATA(N)=(-2*Y0+3*Y1+6*Y2+7*Y3+6*Y4+3*Y5+-2*Y6)/21
      Y0=Y1
      Y1=Y2
      Y2=Y3
      Y3=Y4
      Y4=Y5
      Y5=Y6
      Y6=DATA(N+4)
```

```
      N=N+1
      IF(N .LT. (NPTS-3))GO TO 1300
      IF(INUM-1)1400,1500,1600
1400  DO 1450 N=1,NPTS
1450  UVA(N)=DATA(N)
      GO TO 1700
1500  DO 1550 N=1,NPTS
1550  FLUA(N)=DATA(N)
      GO TO 1700
1600  DO 1650 N=1,NPTS
1650  TWOPA(N)=DATA(N)
1700  WRITE(IFILE'1)NPTS,(FLUA(N),UVA(N),TWOPA(N),POWER(N),N=1,NPTS)
      STOP
      END
```

```
      FIXPTS

      DIMENSION RT(99),NRT(99),SIG(99),DATA(1500)
      WRITE(5,3)
3     FORMAT(' ENTER FILE#...10<=IFILE<=99')
      READ(5,5)IFILE
5     FORMAT(I2)
      DEFINE FILE IFILE(1,3002,U,NREC)
      WRITE(5,7)
7     FORMAT(' ENTER # OF POINTS...10<=NPTS<=99')
      READ(5,10)NPTS
10    FORMAT(I2)
      NPTS=NPTS+1
      RT(1)=0
      SIG(1)=0
      WRITE(5,13)
13    FORMAT(' ENTER EACH PAIR OF POINTS, SEPARATED BY ONE BLANK,
     1 WITH NO SIGNS, AS...3.56 0.08')
      DO 100 I=2,NPTS
          READ(5,15)RT(I),SIG(I)
15        FORMAT(F4.2,F5.2)
```

```
100         CONTINUE
110       WRITE(5,120)
120       FORMAT(' DO YOU WANT TO CHANGE ANY VALUES(YES=1,NO=0)??')
          READ(5,140)IANS
140       FORMAT(I2)
          IF(IANS.EQ.0)GO TO 190
              WRITE(5,150)
150           FORMAT(' ENTER # OF POINT TO BE CHANGED, IN I2 FORMAT.')
              READ(5,160)IPT
160           FORMAT(I2)
              WRITE(5,170)
170           FORMAT(' ENTER THE NEW PAIR OF VALUES, IN THE ORIGINAL
     1    FORMAT.')
              READ(5,180)RT(IPT+1),SIG(IPT+1)
180           FORMAT(F4.2,F5.2)
              WRITE(5,185)RT(IPT+1),SIG(IPT+1)
185           FORMAT(' NEW VALUES ARE:',F4.2,F6.2)
              GO TO 110
190       CONTINUE
          WRITE(5,23)
23        FORMAT(' ENTER SIGNAL NORMALIZING FACTOR, INCLUDING A
     1    DECIMAL POINT.')
          READ(5,24)FACTOR
24        FORMAT(F10.4)
          DO 200 J=1,NPTS
              NRT(J)=IFIX(RT(J)*(1500/RT(NPTS)))
              SIG(J)=SIG(J)*FACTOR
200           CONTINUE
          N=1
          DATA(1)=0
          DO 300 K=2,NPTS
              SLOPE=(SIG(K)-SIG(K-1))/(NRT(K)-NRT(K-1))
40            IF(N.EQ.NRT(K))GO TO 290
                  DATA(N+1)=DATA(N)+SLOPE
                  N=N+1
                  GO TO 40
```

```
290          DATA(N)=SIG(K)
300          CONTINUE
         NREC=1
         WRITE(IFILE'1)1500,(DATA(L),L=1,1500)
         STOP
         END



         MINMAX

C        PROGRAM TO LOCATE MAX. AND MIN. IN ASPHALTENE CHROMATOGRAMS
         DIMENSION FLUA(1500),UVA(1500),TWOPA(1500),POWER(1500)
         DIMENSION DATA(1500),NA(150),A(150),NB(150),B(150)
         WRITE(5,100)
100      FORMAT('  ENTER FILE#...10<=IFILE<=99')
         READ(5,200)IFILE
200      FORMAT(I2)
         DEFINE FILE IFILE(1,12006,U,NREC)
         READ(IFILE'1)NPTS,(FLUA(N),UVA(N),TWOPA(N),POWER(N),N=1,NPTS)
         WRITE(5,300)
300      FORMAT('  ENTER 0 FOR UV, 1 FOR 1-PHOTON, OR 2 FOR 2-PHOTON')
         READ(5,400)INUM
400      FORMAT(I1)
         WRITE(5,450)
450      FORMAT('  ENTER FILE # ...1<=JFILE<=99')
         READ(5,475)JFILE
475      FORMAT(I2)
         DEFINE FILE JFILE(1,600,U,NREC)
         IF(INUM-1)500,600,700
500      DO 550 N=1,NPTS
550      DATA(N)=UVA(N)
         GO TO 800
600      DO 650 N=1,NPTS
650      DATA(N)=FLUA(N)
         GO TO 800
700      DO 750 N=1,NPTS
```

```
750     DATA(N)=TWOPA(N)
800     WRITE(5,900)
900     FORMAT('  ENTER # DATA POINTS')
        READ(5,1000)NPTS
1000    FORMAT(I4)
        WRITE(5,1100)
1100    FORMAT('  ENTER SLOPE SENS. AND SLOPE CONSIST.')
        READ(5,1200)SS,ISC
1200    FORMAT(F4.1,I3)
        M=1
        N=1
        ICN=0
        IUM=1
        IDM=1
        IUF=0
        IDF=0
        IDS=1
        IUS=1
1300    CONTINUE
        X=DATA(N)
        N=N+1
        Y=DATA(N)
        IF(X-Y)2000,4000,3000
2000    IF(Y-X-SS)5000,5000,2100
2100    IF(IDS .EQ. 0)GO TO 2200
        ICN=0
        IDS=0
        IUS=1
2200    IF(IUF .EQ. 1)GO TO 4000
        IF(ICN .GT. ISC)GO TO 2350
        ICN=ICN+1
        GO TO 4000
2350    IUF=1
        IUM=N
        IF(IDF .EQ. 0)GO TO 4000
C       LOCATE VALLEY
        N=IDM
```

```
      A(M)=DATA(N)
      NA(M)=N
2400  N=N+1
      B(M)=DATA(N)
      NB(M)=N
      IF(B(M)-A(M))2500,2600,2600
2500  A(M)=B(M)
      NA(M)=NB(M)
2600  IF(N .LT. IUM)GO TO 2400
      NA(M)=-NA(M)
      WRITE(5,2700)M,NA(M),A(M)
2700  FORMAT(1X,I10,I10,F16.4,'    VALLEY')
      M=M+1
      IDF=0
      GO TO 4000
3000  IF(X-Y--SS)5000,5000,3100
3100  IF(IUS .EQ. 0)GO TO 3200
      ICN=0
      IUS=0
      IDS=1
3200  IF(IDF .EQ. 1)GO TO 4000
      IF(ICN .GT. ISC)GO TO 3350
      ICN=ICN+1
      GO TO 4000
3350  IDF=1
      IDM=N
      IF(IUF .EQ. 0)GO TO 4000
C     LOCATE PEAK
      N=IUM
      A(M)=DATA(N)
      NA(M)=N
3400  N=N+1
      B(M)=DATA(N)
      NB(M)=N
      IF(A(M)-B(M))3500,3600,3600
3500  A(M)=B(M)
      NA(M)=NB(M)
```

```
3600    IF(N .LT. IDM)GO TO 3400
        WRITE(5,3700)M,NA(M),A(M)
3700    FORMAT(1X,I10,I10,F16.4,'   PEAK')
        M=M+1
        IUF=0
        GO TO 4000
5000    ICN=0
4000    IF(N .LT. NPTS)GO TO 1300
        MPTS=M-1
        WRITE(JFILE'1)MPTS,(NA(M),A(M),M=1,MPTS)
        STOP
        END
```

RETIME

```
C       PROGRAM TO READ IN JFILE AND NORMALIZE RETENTION TIMES
        DIMENSION NA(150),A(150)
        WRITE(5,100)
100     FORMAT('  ENTER FILE # ...1<=JFILE<=99')
        READ(5,200)JFILE
200     FORMAT(I2)
        DEFINE FILE JFILE(1,600,U,NREC)
        READ(JFILE'1)MPTS,(NA(M),A(M),M=1,MPTS)
        WRITE(5,300)
300     FORMAT('  ENTER RETENTION TIME NORMALIZATION FACTOR')
        READ(5,400)RNF
400     FORMAT(F4.3)
        DO 500 M=1,MPTS
500     NA(M)=RNF*NA(M)
        WRITE(5,600)(M,NA(M),A(M),M=1,MPTS)
600     FORMAT(1X,2I10,F16.4)
        WRITE(JFILE'1)MPTS,(NA(M),A(M),M=1,MPTS)
        STOP
        END
```

```
      BIGFIL
C     PROGRAM TO COMBINE SIMILAR FILES AND THEN CHECK THE RESULTING
C     ENLARGED FILE
      DIMENSION NA(1500),A(1500)
50    WRITE(5,100)
100   FORMAT(' ENTER LFILE # AND RECORD #')
      READ(5,200)LFILE,NREB
200   FORMAT(2I2)
      DEFINE FILE LFILE(10,452,U,NREA)
      NREA=1
300   IF(NREA .EQ. NREB)GO TO 400
      READ(LFILE'NREA)MPTS,(NA(M),A(M),M=1,MPTS)
      NREA=NREA-1
350   WRITE(LFILE'NREA)MPTS,(NA(M),A(M),M=1,MPTS)
      IF(NREA .LT. 11)GO TO 300
      GO TO 499
400   WRITE(5,410)
410   FORMAT(' ENTER JFILE #')
      READ(5,420)JFILE
420   FORMAT(I2)
      DEFINE FILE JFILE(1,452,U,NREC)
      READ(JFILE'1)MPTS,(NA(M),A(M),M=1,MPTS)
      GO TO 350
499   WRITE(5,500)
500   FORMAT(' ENTER A (1) IF YOU WISH TO CHECK LFILE')
      READ(5,600)ICHECK
600   FORMAT(I1)
      IF(ICHECK .EQ. 1)GO TO 601
      GO TO 1000
601   WRITE(5,700)
700   FORMAT(' ENTER # OF RECORDS TO BE CHECKED')
      READ(5,200)NREAMX
      NREA=1
800   IF(NREA .GT. NREAMX)GO TO 1000
      READ(LFILE'NREA)MPTS,(NA(M),A(M),M=1,MPTS)
      WRITE(5,900) (M,NA(M),A(M),M=1,MPTS)
```

```
900     FORMAT(1X,2I10,F16.4)
        GO TO 800
1000    STOP
        END


        CORLAT

C       PROGRAM TO CORRELATE ASPHALTENE PEAK DATA
C       READ PEAKS FROM ALL INJECTIONS OF A GIVEN SAMPLE
        DIMENSION NA(150),A(150),NP(150),AP(150),IX(150)
        DIMENSION NNA(500),AA(500),LP(5)
        WRITE(5,100)
100     FORMAT(' ENTER LFILE # AND # OF RECORDS')
        READ(5,200)LFILE,NREB
200     FORMAT(2I3)
        DEFINE FILE LFILE(10,452,U,NREA)
        N=1
        NREA=1
250     READ(LFILE'NREA)MPTS,(NA(M),A(M),M=1,MPTS)
        DO 300 M=1,MPTS
        IF(NA(M) .LT. 0)GO TO 300
        NNA(N)=NA(M)
        AA(N)=A(M)
        N=N+1
300     CONTINUE
        IF(NREA .LT. (NREB+1))GO TO 250
699     WRITE(5,700)
700     FORMAT(' ENTER RTW AND SW')
        READ(5,800)IRTW,SW
800     FORMAT(I5,F5.3)
        DO 900 L=1,150
900     IX(L)=1
        L=1
        NF=N-1
        N=1
950     NP(L)=NNA(N)
```

```
      AP(L)=AA(N)
      LF=L
1000  N=N+1
      IF(N .GT. NF)GO TO 1725
      I=1
      L=1
      DO 1100 L=1,LF
      IDIF=NP(L)-NNA(N)
      IDIF=IABS(IDIF)
      IF(IDIF .GT. IRTW)GO TO 1100
      RDIF=1.-AA(N)/AP(L)
      RDIF=ABS(RDIF)
D     WRITE(5,10)RDIF,SW
D10   FORMAT(2F12.4)
      IF(RDIF .GT. SW)GO TO 1100
D     WRITE(5,20)
D20   FORMAT(' HI!')
      LP(I)=L
      I=I+1
1100  CONTINUE
      IF(I-2)950,1200,1300
1200  L=LP(1)
      GO TO 1700
1300  L=LP(1)
      IDIFA=NP(L)-NNA(N)
      IDIFA=IABS(IDIFA)
      L=LP(2)
      IDIFB=NP(L)-NNA(N)
      IDIFB=IABS(IDIFB)
      IF(IDIFA-IDIFB)1500,1500,1700
1500  L=LP(1)
1700  NP(L)=(IX(L)*NP(L)+NNA(N))/(IX(L)+1)
      AP(L)=(IX(L)*AP(L)+AA(N))/(IX(L)+1)
      IX(L)=IX(L)+1
      GO TO 1000
1725  LPTS=LF
      WRITE(5,1750)(L,NP(L),AP(L),L=1,LPTS)
```

```
1750    FORMAT(1X,2I10,F16.4)
        WRITE(5,1800)(L,IX(L),L=1,LPTS)
1800    FORMAT(2I10)
        WRITE(5,1750)(N,NNA(N),AA(N),N=1,NF)
        WRITE(5,1900)
1900    FORMAT(' ENTER MFILE #...DONT USE A LFILE #')
        READ(5,2000)MFILE
2000    FORMAT(I2)
        DEFINE FILE MFILE(1,452,U,NREC)
        WRITE(MFILE'1)LPTS,(NP(L),AP(L),L=1,LPTS)
        STOP
        END

        FEATUR
C       PROGRAM TO PLACE PEAK DATA INTO FEATURE CATEGORIES
        DIMENSION NA(100),A(100),NNA(100),AA(100)
        DIMENSION AP(100),NP(100),NCLOSE(100)
        WRITE(5,100)
100     FORMAT(' ENTER KFILE#')
        READ(5,200)KFILE
200     FORMAT(I3)
        DEFINE FILE KFILE(1,452,U,NREK)
        READ(KFILE'1)LPTS,(NP(L),L=1,LPTS)
        WRITE(5,300)
300     FORMAT(' ENTER IRTW')
        READ(5,200)IRTW
        WRITE(5,400)
400     FORMAT(' ENTER LFILE# AND # OF RECORDS')
        READ(5,500)LFILE,NREA
500     FORMAT(2I3)
        DEFINE FILE LFILE(10,452,U,NREL)
        WRITE(5,600)
600     FORMAT(' ENTER MFILE#')
        READ(5,200)MFILE
        DEFINE FILE MFILE(10,512,U,NREM)
        WRITE(5,650)
```

```
650     FORMAT(' ENTER DEFAULT VALUE')
        READ(5,675)DEFALT
675     FORMAT(F6.2)
        NREM=1
        NREL=1
700     N=1
        IF(NREL.GT.NREA)GO TO 4000
        READ(LFILE'NREL)MPTS,(NA(M),A(M),M=1,MPTS)
D       WRITE(5,200)MPTS
D       WRITE(5,200)NREL
        DO 800 M=1,MPTS
        IF(NA(M).LT.0)GO TO 800
        NNA(N)=NA(M)
        AA(N)=A(M)
        N=N+1
800     CONTINUE
        NPTS=N-1
D       WRITE(5,200)NPTS
C
C
        L=0
2000    L=L+1
        IF(L.GT.LPTS)GO TO 3000
        LP=L
        DO 2100 N=1,NPTS
2100    NCLOSE(N)=0
2110    N=1
2125    IF(NCLOSE(N).EQ.0)GO TO 2150
        N=N+1
        GO TO 2125
2150    IDIFA=NP(LP)-NNA(N)
        IDIFA=IABS(IDIFA)
        NBEST=N
2175    N=N+1
        IF(NCLOSE(N) EQ.1)GO TO 2175
        IDIFB=NP(LP)-NNA(N)
        IDIFB=IABS(IDIFB)
        IF(IDIFB.GT.IDIFA)GO TO 2200
```

```
      NBEST=N
      IDIFA=IDIFB
      IF(N.LT.NPTS)GO TO 2175
2200  CONTINUE
      IF(IDIFA.LT.IRTW)GO TO 2300
      AP(LP)=DEFALT
      L=LP
D     WRITE(5,2250)L,AP(L)
D2250 FORMAT(' L IS ',I5,' AP(L) IS ',F10.2)
      GO TO 2000
C
C
2300  DO 2400 L=1,LPTS
      IF(L.EQ.LP)GO TO 2400
      IDIFB=NP(L)-NNA(NBEST)
      IDIFB=IABS(IDIFB)
      IF(IDIFB.LT.IDIFA)GO TO 2500
2400  CONTINUE
      AP(LP)=AA(NBEST)
      L=LP
D     WRITE(5,2250)L,AP(L)
      GO TO 2000
2500  IDIFA=IDIFB
      LL=L
      DO 2600 N=1,NPTS
      IF(N.EQ.NBEST)GO TO 2600
      IDIFB=NP(LL)-NNA(N)
      IDIFB=IABS(IDIFB)
      IF(IDIFB.LT.IDIFA)GO TO 2700
2600  CONTINUE
      NCLOSE(NBEST)=1
D     WRITE(5,200)N
      GO TO 2110
2700  IDIFA=IDIFB
      NN=N
      DO 2800 L=1,LPTS
      IF(L.EQ.LL)GO TO 2800
```

```
      IDIFB=NP(L)-NNA(NN)
      IDIFB=IABS(IDIFB)
      IF(IDIFB.LT.IDIFA)GO TO 2500
2800  CONTINUE
      GO TO 2400
3000  CONTINUE
      WRITE(MFILE'NREM)LPTS,(AP(L),L=1,LPTS)
D     WRITE(5,200)NREM
      GO TO 700
4000  STOP
      END
```