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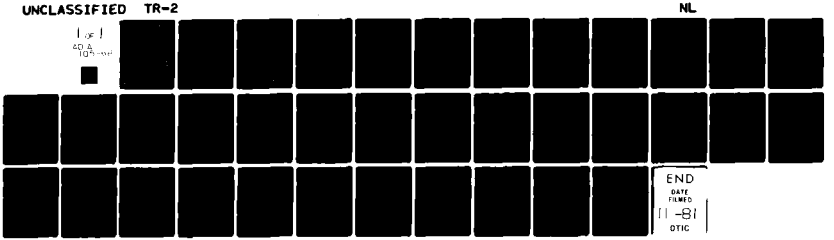

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High Performance Liquid Chromatography/Video Fluorometry: Part I. Instrumentation

by

Michael P. Fogarty, Dennis C. Shelly and Isiah M. Warner

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Department of Chemistry
Texas A&M University
College Station, Texas 77843

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A rapid scanning, two-dimensional fluorometer has been used to obtain Emission-Excitation Matrices (EEMs) of the effluent of an HPLC. These spectra, obtained "on the fly," provide qualitative as well as quantitative information about unknown samples. An in-depth discussion of the instrumentation developed as well as data processing alternatives is presented.

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High Performance Liquid Chromatography/Video Fluorometry:

Part I. Instrumentation

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Key Words:

Video Fluorometry
Fluorescence Detector for HPLC
HPLC/Video Fluorometry
Data Reduction
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Summary

A rapid scanning, two-dimensional fluorometer has been used to obtain Emission-Excitation Matrices (EEMs) of the effluent of an HPLC. These spectra, obtained "on the fly," provide qualitative as well as quantitative information about unknown samples. An in-depth discussion of the instrumentation developed as well as data processing alternatives is presented.

Introduction

Current detectors for HPLC have certain limitations in the ability to completely characterize the column effluent during the separation of a complex sample. These limitations result, chiefly, from a lack of selectivity in the detectors used. One such example is the refractive index (RI) detector which monitors the change in a bulk property of the effluent, the refractive index. Even absorption and fluorescence detectors which use a single monitoring wavelength are typically more selective and sensitive than RI detectors. Still, the multicomponent capabilities of these devices are severely limited. More selective detection schemes have been developed. These employ atomic absorption and electrochemical techniques to selectively analyze components [1-3]. Advanced, scanning absorption and fluorescence detectors have improved multicomponent capabilities. Yet, scan times are usually greater than the residence time of a component in the flow cell. A simple solution to this dilema has been through the use of stop-flow scanning. However, this can often lead to complications in the analysis, particularly since diffusion caused by halting the flow will degrade the chromatographic resolution. Recently an absorption detector with scan times at least an order of magnitude faster than chromatographic elution, has been developed [4]. The usefulness of this instrument for multicomponent analysis was demonstrated by its dual wavelength monitoring, scanning and ratio recording features.

The application of new technologies has extended the multicomponent detection capabilities of HPLC detectors. Extensive work has been done on interfacing HPLC directly to a mass spectrometer [5,6].

Talmi [7], and McDowell and Pardue [8] have reported the use of a vidicon as a multicomponent absorption detector for HPLC. Fluorescence detection with an intensified vidicon multichannel analyzer system was described by Jadamec and co-workers [9] in an ambitious analysis of fluorescent components in petroleum oils. A similar instrument, the rapid scanning (video) fluorometer [10-12] has been studied as a detector for HPLC [13,14]. This instrument provides multiple excitation and emission spectra in a single scan, at data rates sufficiently fast to monitor changes in the chromatographic stream without stopping flow.

The video fluorometer (VF) has also been used on other time dependent systems, such as the kinetics of cell growth [15] and photochemical reactions [16]. Powerful algorithms have been advanced for both the qualitative as well as quantitative evaluation of the Emission-Excitation Matrix (EEM) [17-22]. These features provide the rationale for the use of the video fluorometer as a powerful detector for HPLC. This combination has the potential to identify and quantify almost all fluorescent compounds in a very complex sample.

Experimental

Chemicals

All polynuclear aromatic hydrocarbons used were obtained at 98+% purity from various suppliers and used without further purification. The solvents used were glass distilled acetonitrile and cyclohexane (Burdick and Jackson Labs Inc., Muskegon, Michigan). Type III reagent grade water was obtained through a Millipore filtration system (Milipore Corp., Bedford, Mass.).

Chromatography

All high resolution, analytical chromatography was done in reverse phase mode on an ultrasphere ODS column (250 X 4.6 mm, 5 μm particle size, Altex Scientific Inc., Berkeley, California). The mobile phase used was an acetonitrile/water mixture in a programmed linear gradient varying from 65 to 90% over a 40 min. period at a flow rate of 1 mL/min.

Instrumentation

An Altex model 312 MP chromatograph was used (model 110A pumps) with a dual beam absorption detector (model 153) tuned to 254 nm as the analytical wavelength. Detector signal was recorded on a Hewlett-Packard (Avondale, Pennsylvania) model 3390A recording integrator.

Fluorescence was measured using a video fluorometer designed and constructed at Texas A&M University [12]. This instrument has been improved by including a thermostatic housing for the silicon intensified vidicon (EG&G Princeton Applied Research, Princeton, New Jersey). This housing required that the original spectrograph, a UFS 200, be replaced by a HR320 spectrograph (Instruments S.A. Inc., Metuchen, New Jersey). This combination gives enhanced emission resolution with greater detector background stability. A 20 μL flow-cell was obtained from Precision Cells Inc. (Hicksville, New York, model 8830). The flow cavity has a square bore (1 mm^2), which reduces the amount of scattered light in the Emission-Excitation Matrix (EEM). Since data was acquired for four frames (2.08 s.), the cell volume would turn over 1.5 times during one data set acquisition.

Data Processing

All data storage and manipulation were performed on a Hewlett-Packard (Fort Collins, Colorado) model 9845T desktop computer. This 16 bit computer contains 184 kbytes of user available random access memory while maintaining over 200 kbytes of read only memory providing all system functions and two color interactive graphics. Mass storage is provided by tape cartridge (two drives of 200 kbytes each) or floppy disk (two drives of 0.5 Mbytes each). Due to shorter access times and higher capacities, the floppy disks were used exclusively in this study. Programs were written in a combination of BASIC and assembly language to achieve maximum speed.

Results and Discussion

Since HPLC has become such a popular technique in recent years, a great deal of research has centered on the development of detectors that are either very specific or very general. One of the reasons for this approach is the lack of exact reproducibility in retention times. With "real world" samples reproducible and reliable retention values usually require significant sample preparation to avoid distortion and interference. Many advanced detection techniques are only useable in HPLC after considerable modifications to the outlet stream have been made [1-3,5,6]. As an HPLC detector, the video fluorometer provides two dimensions of qualitative information for a chromatographic effluent. This large amount of information can be obtained without extensive instrumental modifications to the HPLC, requiring only a conventional, commercially available flow cell.

The video fluorometer is able to obtain a spectrum (EEM) that provides emission information over 300 nm in both excitation and

emission wavelengths. This data set can be acquired as a 2500 data point matrix in 0.5 s. Data acquired in this manner helps to reduce the spectral overlap inherent in most UV/visible spectroscopic techniques. Since many fluorescent patterns are unique, the EEM provides a superior means of identifying the composition of a chromatographic peak.

In the particular instrumental arrangement used (Figure 1) several considerations had to be made. This arrangement seeks to operate three microprocessors simultaneously and in conjunction (Figure 2). This is difficult since each of the three (OMA-2 1215 console, 312-MP HPLC controller, and HP9845T computer) is programmable in a different language, and the degree to which each can be programmed varies. Earlier versions of the video fluorometer passed data between the OMA-2 and a processing computer over a 9600 baud serial line in ASCII format. A single data set required 45 s to pass from the OMA-2 to the computer, and only after temporary storage on the floppy disk of the OMA-2 1215 console. A major improvement has been made by introducing a 16 bit parallel interface between the 1215 console and the HP9845T. Since data is now transferred in the internal binary format of both machines, and occurs directly from the live memory of the 1215 console, a full 2500 point set can be transferred in approximately 1 s.

A major goal in this design is to insure that spectra are obtained at identical times in consecutive HPLC runs. This means that one processor must be chosen as the master controller. The 312 MP HPLC controller is the obvious choice since it most closely controls the chromatography itself. Yet, it is the least accessible due to its

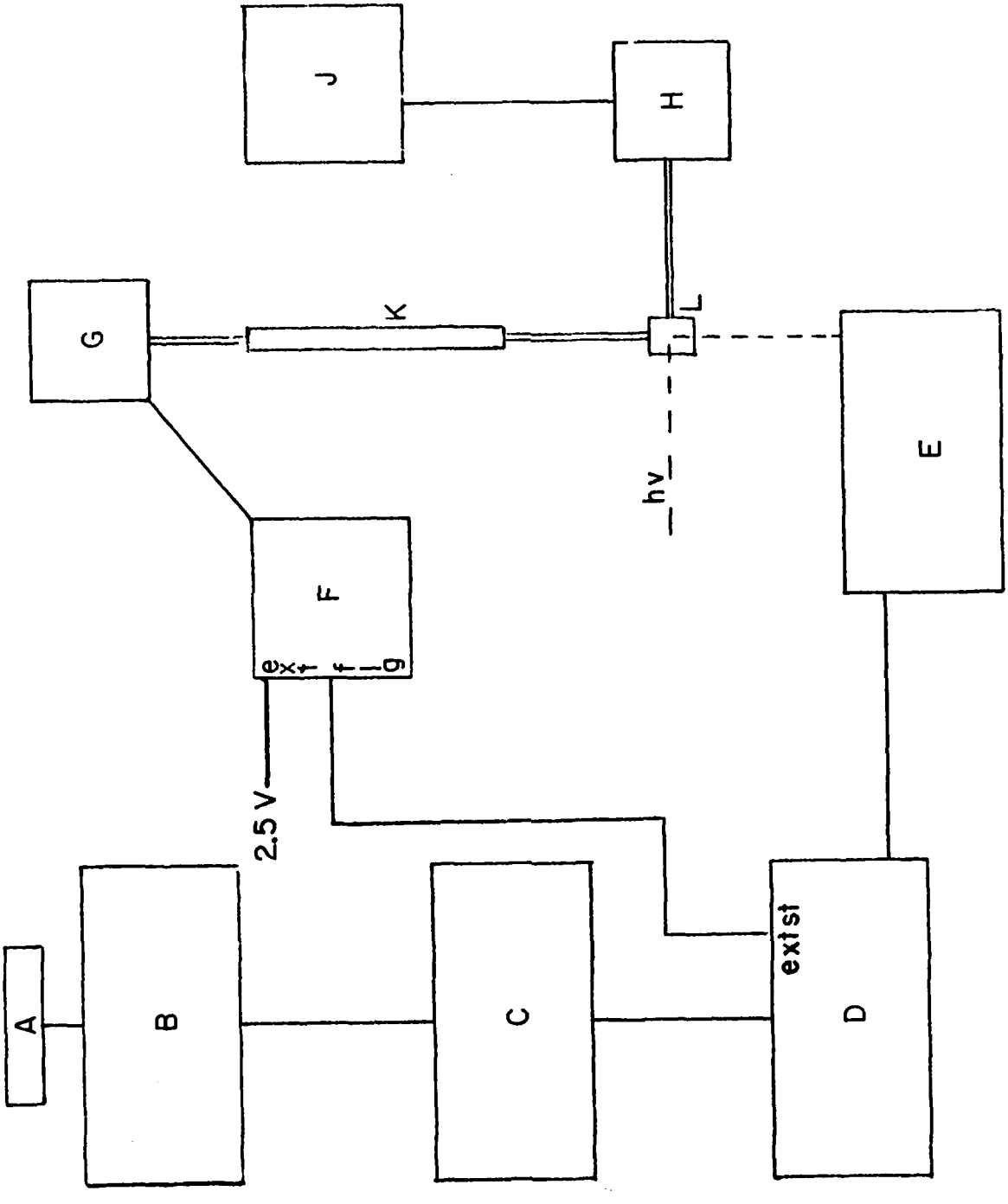
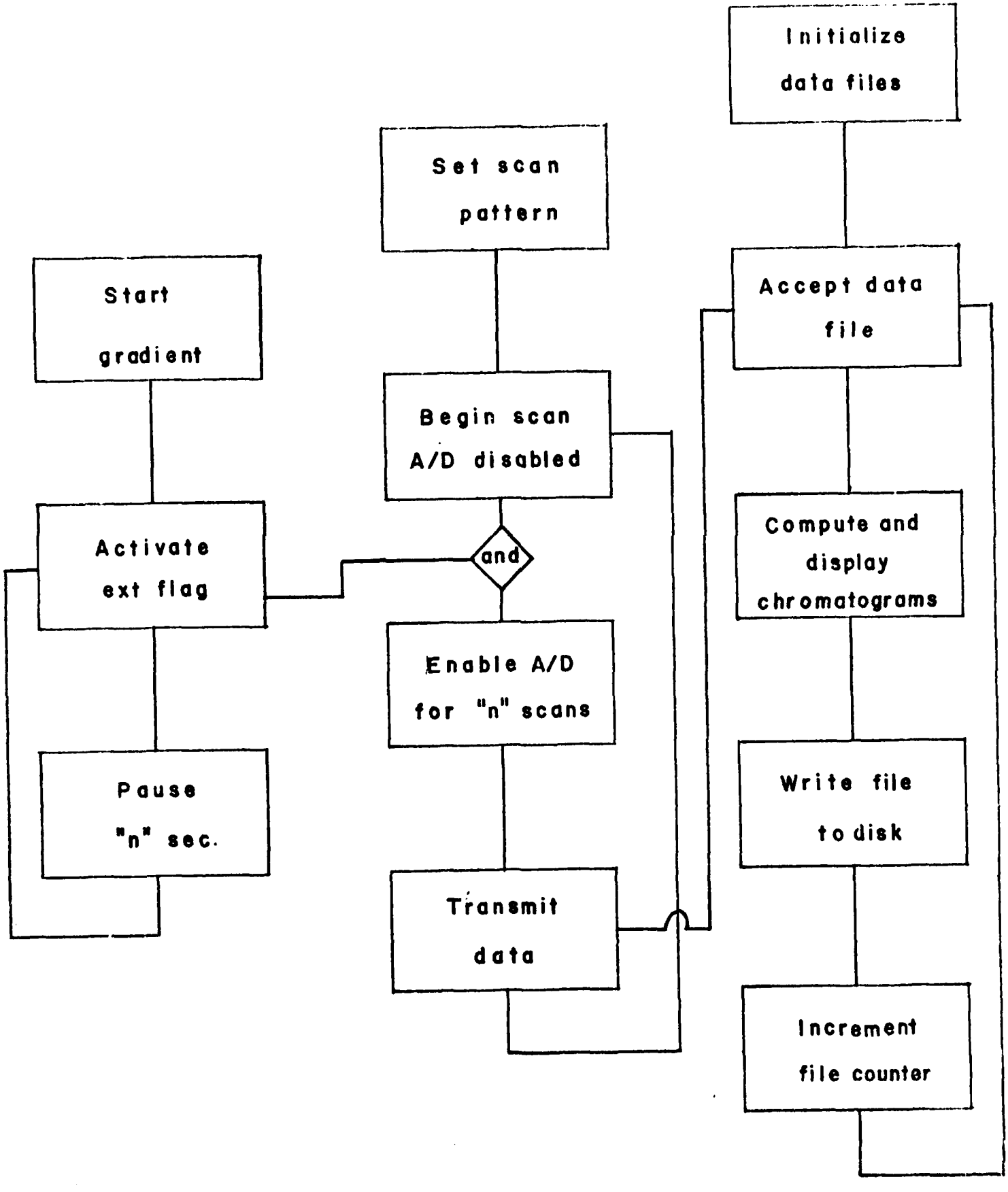


Figure 1

Figure 2


Initialize data files
Set scan pattern
Start gradient
Accept data file
Begin scan A/D disabled
Activate ext flag
and
Compute and display chromatograms
Enable A/D for "n" scans
Pause "n" sec.
Write file to disk
Transmit data
Increment file counter

extremely limited programability, and lack of input/output capabilities. The 312 MP can trigger an external flag (Figure 2), a contact closure switch, on command. It is this feature that allows this instrument to control the data acquisition of the entire system. A 2.5 V signal source is applied to one input of an external flag. At regular intervals in the run the flag is turned on passing the 2.5 V signal for 1 s. The resulting, regular pulse train is then used to synchronize data acquisition by the video fluorometer. All of this is happening while the 312 MP continues to control the chromatographic gradient and flow rate.

The OMA-2 system serves as the intermediary in this data link. It comprises two units, the 1216 detector controller and 1215 console. The 1216 controls all operation of the vidicon detector including digitization of the detector signal. Since the 1216 has limited memory, it dumps data to the 1215 console over a 4 Mbaud serial line. The 1215 is a full microcomputer running an operating sytem that allows vidicon control through simple literal commands. The 1215 is programmable, but only in the sense that certain keyboard functions can be accessed and performed in sequence. For HPLC/VF operation,the 1215 executes a continual loop (Figure 2). First the detector is directed to begin scanning but not to digitize the incoming signal until a pulse is received from the 312 MP HPLC controller. This pulse triggers the acquisition of data for four frames with a pre-selected scan pattern. When data acquisition is completed the program directs the 1215 to transfer the data from live memory to the HP9845T over the parallel interface. Once the transfer is completed the program loops back to the beginning. Suprisingly,

this cycle is the rate limiting step in the entire operation. A complete cycle requires 20.4 s. Considerable time is lost in accessing programs and functions from floppy disk as needed rather than storing them permanently in memory. This is necessary due to the small memory capacity of the 1215, and is thus incorporated by Princeton Applied Research into their design of the OMA-2.

The final link in the HPLC/VF system is the HP9845T minicomputer. Although it is the most powerful processor of the three; its main function in normal HPLC/VF operation is as a mass storage unit. The HP9845T can acquire a data set from the OMA-2 1215 console and store it on floppy disk at the rate of 1 data set every 6 s. Faster rates are possible using overlap processing, but are unnecessary due to the slow cycle rate of the OMA-2. Even so a considerable amount of time is left for the HP9845 to do some preliminary data manipulations between acquisitions (Figure 2).

As an example of the processing power of the HPLC/VF interface we chose to examine an 18 component mixture of polynuclear aromatic hydrocarbons. For this study the absorption detector and video fluorometer were run in tandem to allow a comparison of results (Figure 1). The absorption detector chromatogram is shown in Figure 3d. Based on repetitive injections of less complex standard mixtures, peaks were assigned to 17 of the 18 components in the mixture (Table I). Without some form of a chromatogram equivalent to the absorption trace it is difficult to locate spectra of interest in the mass of HPLC/VF data. An earlier solution to this problem [14] was to monitor the total fluorescence signal (analogous to total ion current in GC/MS) and the fluorescence at two individual coordinates in the EEM. The

Figure 3 a-c

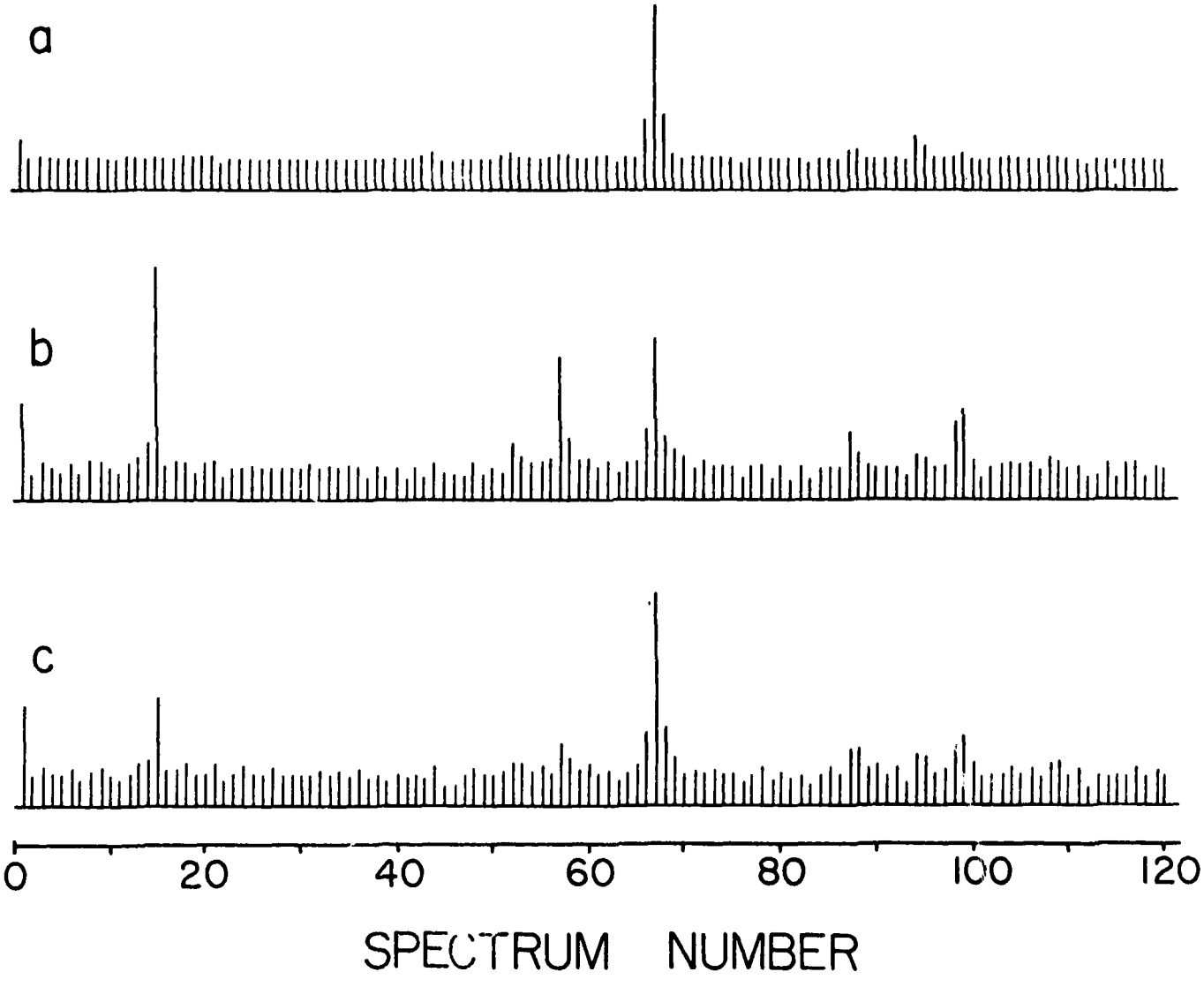


Figure 3d

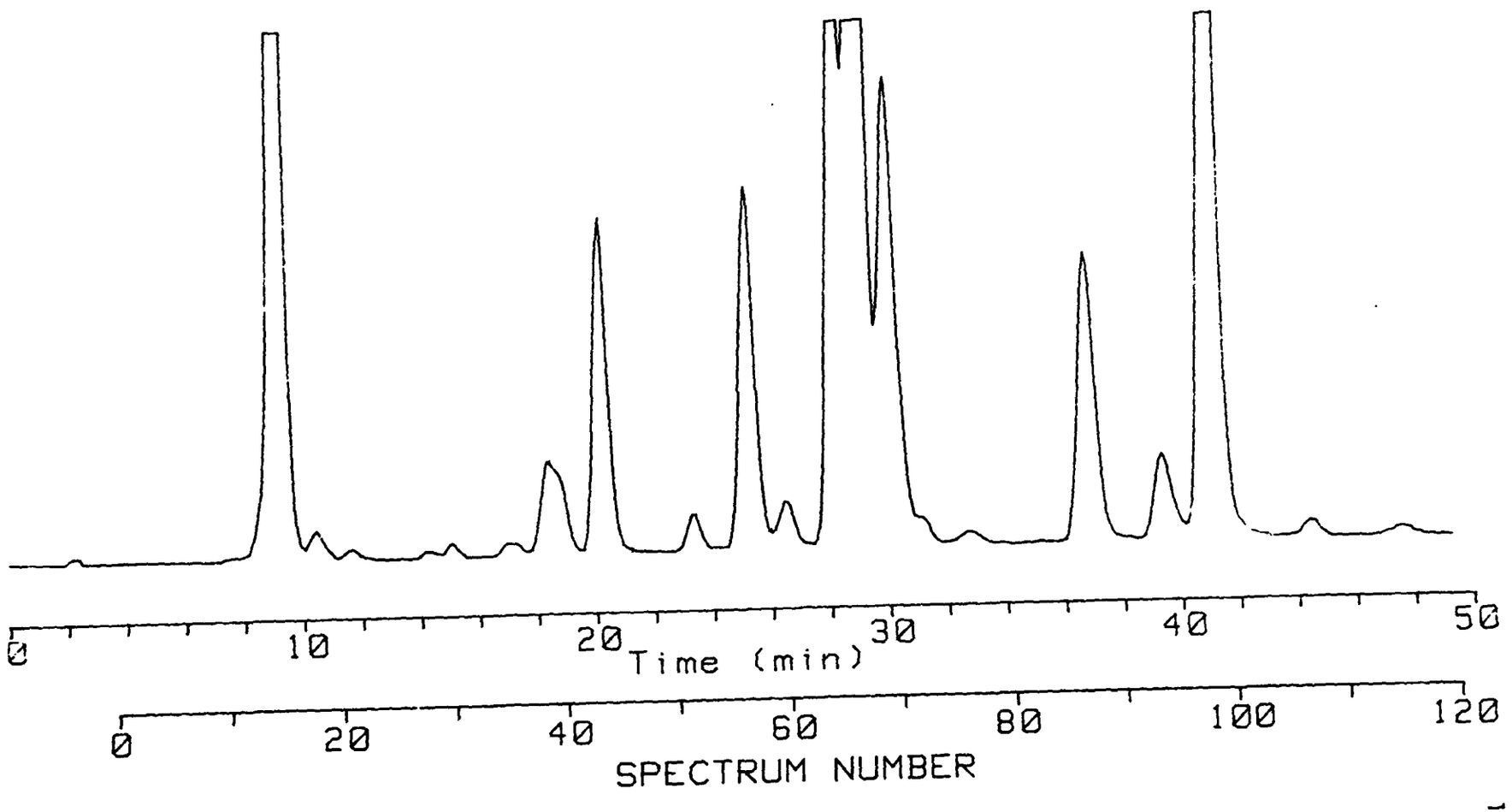


Table 1

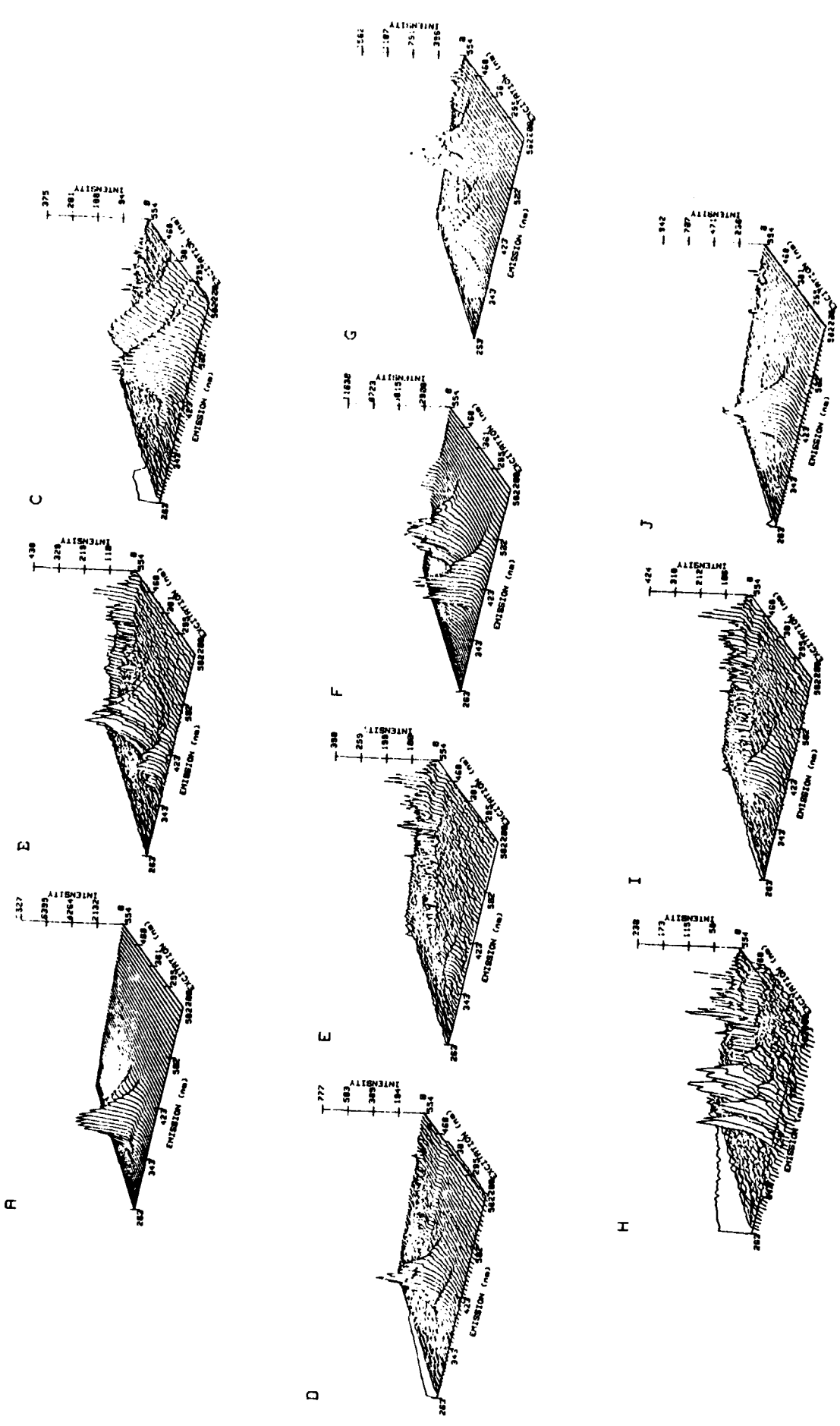
Comparison of Retention Times Established by Absorption and Video Fluorometric Detection

Compound	R_T(min) 254 nm	R_T(min) HPLC/VF
Carbazole	9.09	9.3
9,10-Dimethyl-1,2-Benzanthracene	18.35	Not Found
Fluorene	17.07	Not Found
Dibenzothiophene	28.95	29.1
Phenanthrene	~18.70	Not Found
Anthracene	20.15	20.3
Fluoranthene	23.31	23.4
Pyrene	25.16	24.9
Triphenylene	26.45	Not Found
p-Terphenyl	28.18	28.3
9,10-Dimethylanthracene	28.95	29.1
Chrysene	44.42	44.7
1,2-Benzanthracene	29.96	29.5
Benzo[a]pyrene	39.32	39.0
Perylene	36.72	36.7
Benzo[e]pyrene	40.98	40.5
1,2,3,4-Dibenzanthracene	36.72	36.7
Pentacene	Not Found	Not Found

latter technique is fairly restrictive for unknown samples, but extremely selective to particular components. A more general approach would be to monitor all fluorescence resulting from a particular excitation wavelength. This signal is an approximation of the signal one might obtain using an absorption detector tuned to a particular wavelength. Since each row of the EEM represents an emission spectrum acquired at a particular excitation wavelength, the sum of all elements in a row of the EEM reflects such a signal. The total fluorescence chromatogram along with traces derived from summing rows corresponding to excitation at 385 nm and 314 nm (effective band width of 7 nm) can be compared to the conventional absorption trace in Figure 3. Since sampling occurs at 22 s intervals, some detail is lost in peak shape, yet there is still excellent peak-to-peak correlation. This summing procedure does suffer from some disadvantages. The peak height is not indicative of signal strength for that component. For a given peak maximum, broad, structureless features furnish far more nonzero pixels than do well resolved, sharp, structured features, and thus are more highly weighted. For this reason, these structured emissions may be hidden.

Figure 4 provides a display of 10 of the 120 spectra obtained during the chromatography on the 18 component standard mixture. Each spectrum was acquired at or near a chromatographic peak maximum. Two of these spectra (F,G) are multicomponent. Still, spectroscopically, the components are well resolved. Table I is a comparison of retention times derived from the absorption detector and the VF. Again, these values compare favorably. In a single experiment retention times were established for 13 of the 18 components in the

Figure 4



sample. Of the five species not observed, one was not found by either method of detection. The remaining four have some combination of low quantum yield, low end excitation bands (λ_{ex} < 300 nm), and low end emission bands (λ_{em} < 300 nm). This particular spectral region is the area of lowest sensitivity in VF due to low excitation intensities and declining detector sensitivity. An example of this is the chromatographic peak for anthracene. In absorption detection using 254 nm as the analytical wavelength, a large peak is observed since anthracene has a very strong absorption band at 252 nm. With VF detection, little excitation intensity is available at 254 nm. Thus, virtually all of the observable signal for the total fluorescence chromatogram comes from the first excited band of anthracene. Since this band is a weaker absorber by a factor of 50 no peak is observed in the fluorescence trace (Figure 3) although an observable spectrum is present (Figure 4B).

Another concern in handling two-dimensional fluorescence data is to derive the contribution of each component to the total signal at any point in time. This becomes extremely difficult when components coelute and overlap spectrally. An algorithm has been developed for the deconvolution of data sets in which the relative contributions of each component to a series of matrices varies [22]. This algorithm is based on the premise that a multicomponent EEM is simply a linear combination of a set of single component EEMs (Equation 1). In this terminology

$$M_j = \sum_{i=1}^{n} \alpha_{ij}' N_i \qquad (1)$$

where M_j is a mixture matrix, N_i a single component matrix, and α_{ij}' a relative concentration dependent term. These α_{ij}' can be

estimated by dividing two data sets containing the same components and forming a ratio matrix (Equation 2). The α_{ij}' for the basis

$$R_j = M_j/M_0 = \sum_{i=1}^{n} \alpha_{ij}' N_i \Big/ \sum_{i=1}^{n} N_i \qquad (2)$$

matrix, M_0, are assumed to be one. If an area exists in which a component is a sole emitter, then Equation 2 becomes simply a plateau of height α_{ij}'. The linear system described by Equation 1 has a solution provided one has "n" linearly independent sets of α_{ij}'. Thus, one must find a suitable means of altering the relative contributions of each component independently. Several methods have been suggested [22,23], but few are simple, and fast enough to be of use. An unresolved chromatographic peak, however, provides the necessary conditions to alter these concentrations independently *provided each component has either a displaced maxima or* different peak width relative to the remaining components. This is best represented diagramatically (Figure 5). Each component has a different slope as one moves along the unresolved chromatographic peak. Therefore the concentrations of each of the three components is varying independently of the remaining two.

In the standard mixture (Figures3 and 4) all components are fairly well resolved by both the chromatography and the spectroscopy. Still, the peak at 29 min. (spectra 65-70) shows a complex mixture of three components. Three of the EEMs in this series are shown in Figure 6. Since the three components are well separated spectroscopically, this is not a rigorous test of the deconvolution technique. This fact is easily seen in Figure 7 where spectrum 67 has been divided into spectra 66 and 68. Three well separated and relatively flat

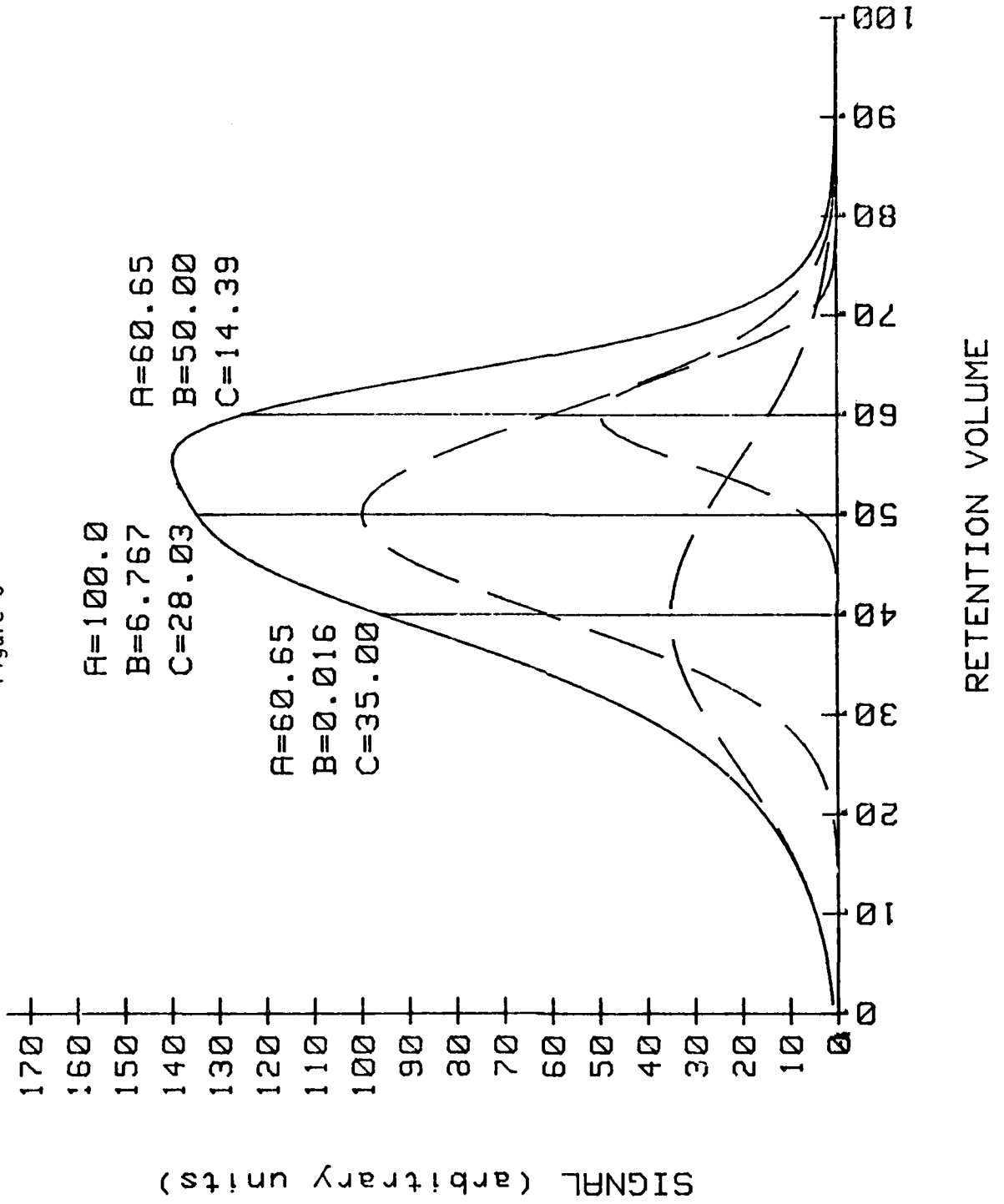

A=100.0
B=6.767
C=28.03
A=60.65
B=0.016
C=35.00
A=60.65
B=50.00
C=14.39
SIGNAL (arbitrary units)
RETENTION VOLUME

Figure 5

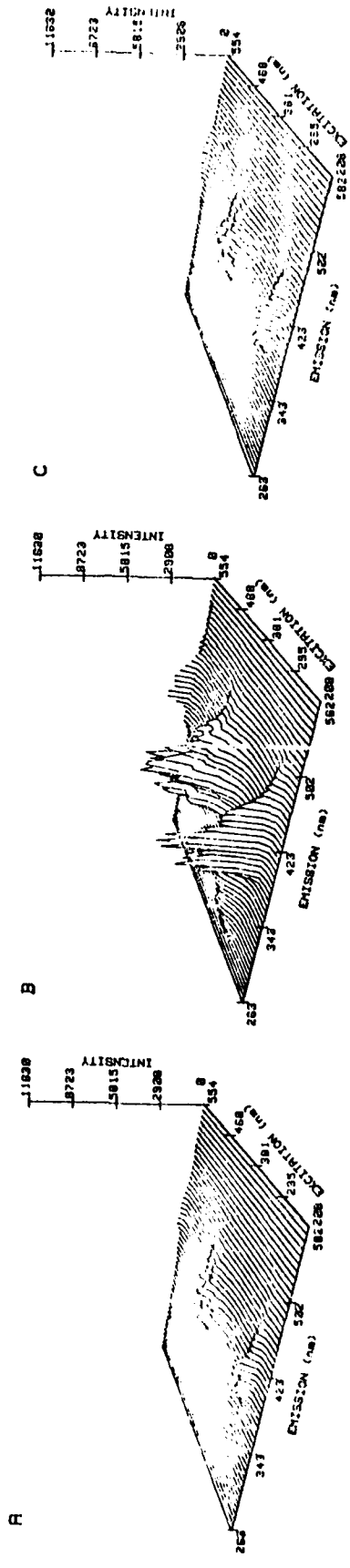
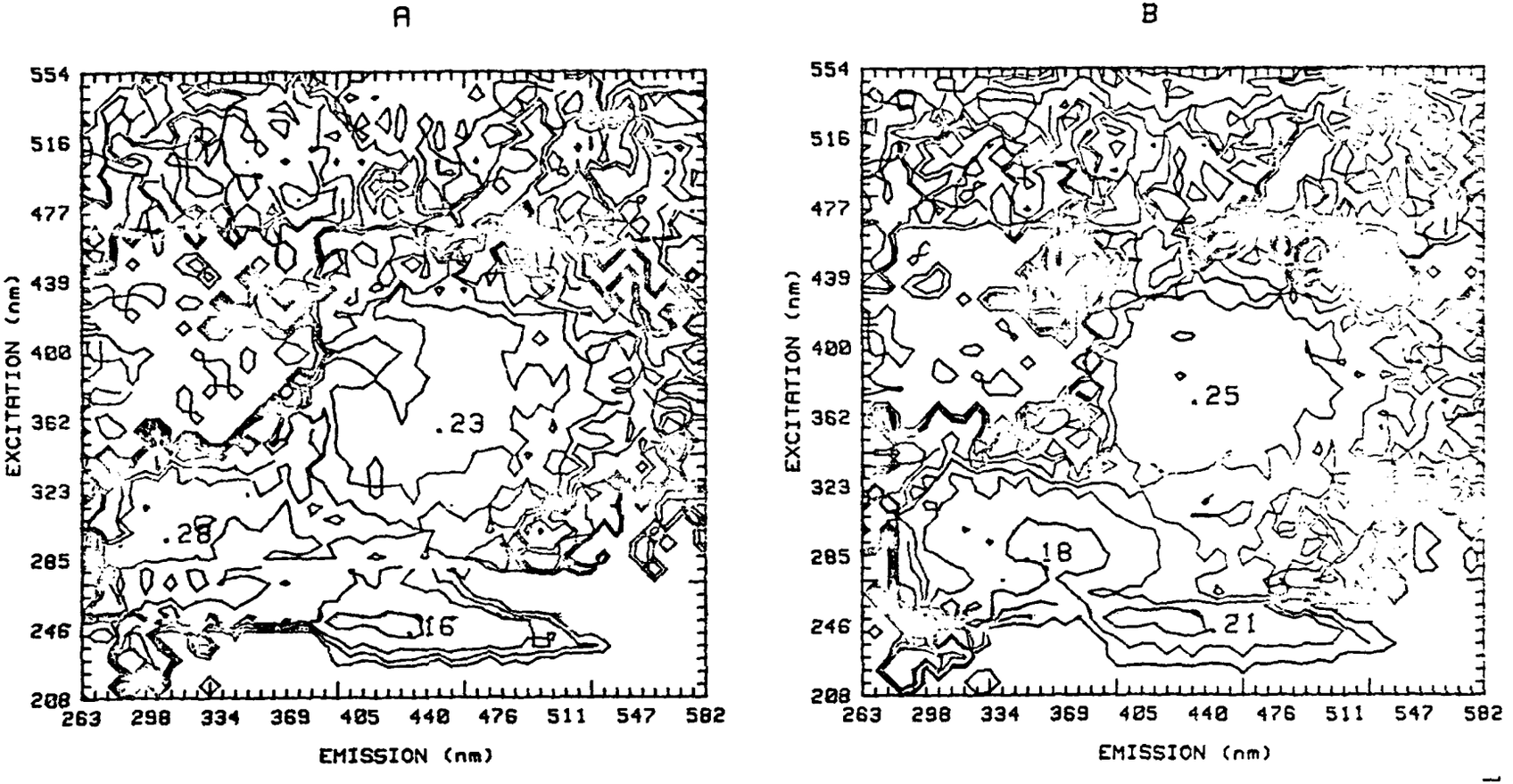


Figure 6

Figure 7


A
B
554
516
477
439
400
362
323
285
246
208
EXCITATION (nm)
263 298 334 369 405 440 476 511 547 582
EMISSION (nm)
.23
.28
16
.25
18
21

plateaus are visible. Still, one unusual feature is apparent. Two horizontal grid lines cross the matrix, one in the spectral area of interest. This is due to an instrumental artifact in the detector that caused steps to appear in these data sets. The deconvolution derived (Figure 8) shows the three isolated components. The result for dibenzothiophene(Figure 8b) is excellent, however a large notch is left in the emission profile of 1,2-benzanthracene (Figure 8c). This missing portion seems to have been added to the middle emission peak in 9,10-dimethylanthracene (Figure 8a). While these spectra are not perfect, they do show the methods' power to enhance spectral features that might otherwise go unnoticed. Yet this sample also shows the limitations resulting from distortions in the acquired data.

A simplified method for displaying the entire data collected during a HPLC/VF run is demonstrated in Figure 9. These plots represent either the total emission (as a function of excitation wavelength) or the total excitation (as a function of emission wavelength) versus time. In this fashion all 300,000 data points collected during the chromatography can be represented equally in a small, simple, easy to view manner. The matrix in Figure 9A represents the change in excitation profile with time (Time-Excitation Matrix, TEXM). This is a more difficult data set to derive information from since many compounds contain multiple excitation bands, increasing the spectral overlap probability. The matrix in Figure 9B represents the change in emission profile with time (Time-Emission Matrix, TEMM). For some isolated components (e.g. carbzaole) the emission patterns are easily recognizable. A benefit from this form of data representation is that the product of the TEMM with the

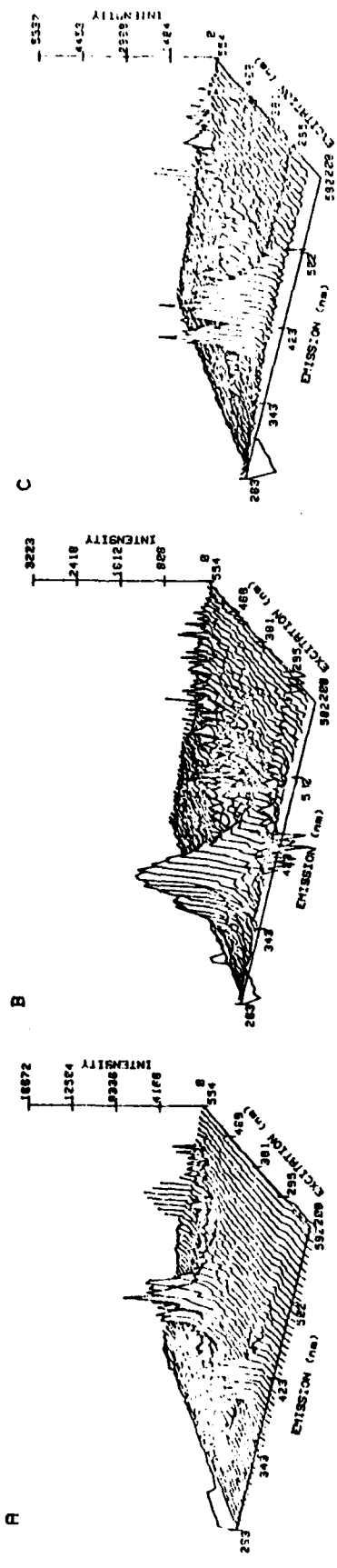
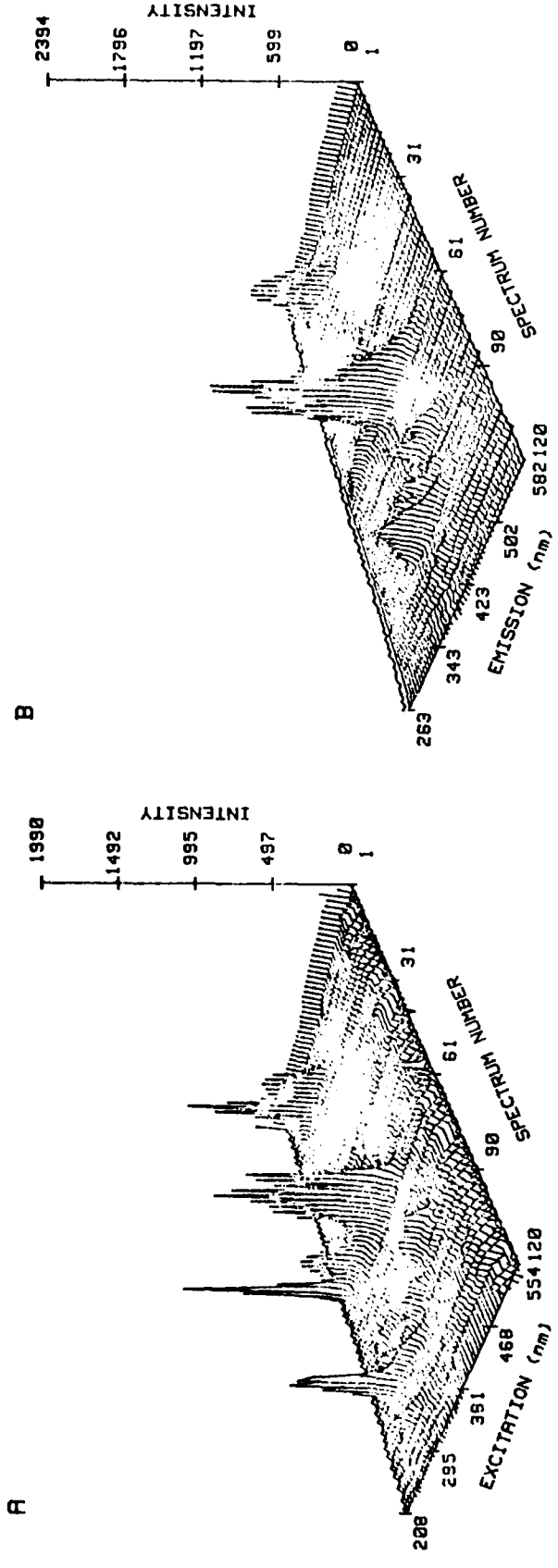


Figure 8

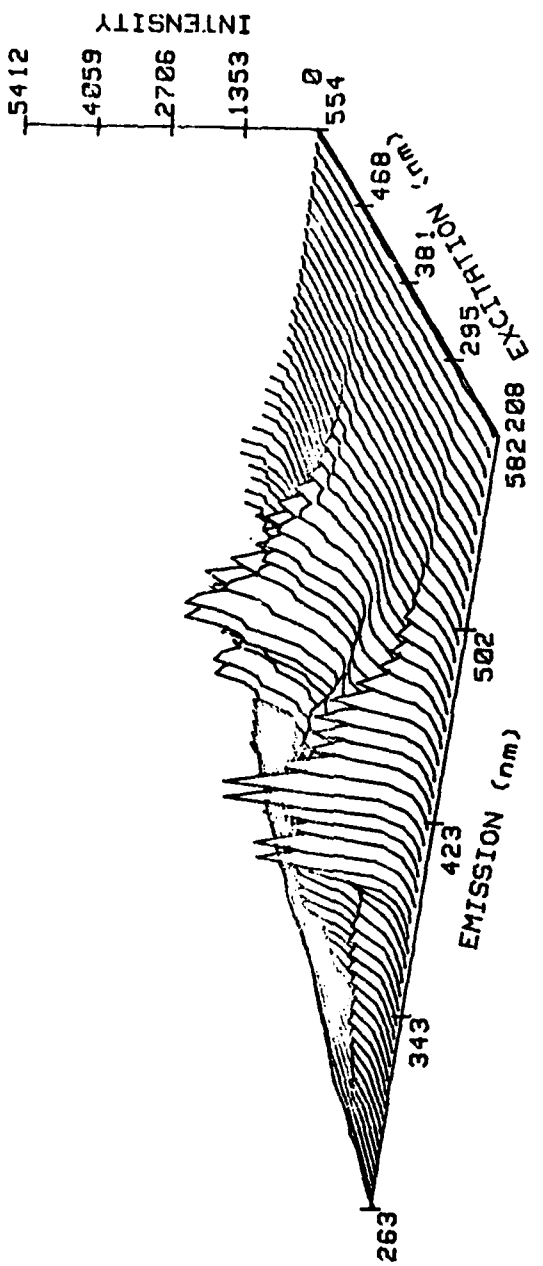
Figure 9


B
INTENSITY
2394
1796
1197
599
0
1
31
61
90
SPECTRUM NUMBER
263
343
423
502
582120
EMISSION (nm)
A
INTENSITY
1990
1492
995
497
0
1
31
61
90
SPECTRUM NUMBER
208
295
381
468
554120
EXCITATION (nm)

transpose of the TEXM yields a representation of the original sample EEM (figure 10). This can be compared with an original spectrum acquired before the chromatography to verify the efficiency and completeness of the chromatography.

Conclusions

This paper has dealt, primarily, with the instrumental and processing aspects of HPLC/VF analysis. It must be noted, however, that significant advantages exist for qualitative and quantitative analysis. Several powerful algorithms [18-21] have been developed for the quantitative evaluation of multicomponent EEMs. However, these were designed to handle a single EEM, not a series changing in time. Several solutions to this problem are possible, each with distinct advantages and disadvantages. The ideal arrangement would be to correlate an unknown, and standard EEM obtained at identical positions in the chromatography. In the present situation, retention times have a reproducibility of $\pm$ 15 s from run to run. Therefore it is extremely difficult to be certain that the unknown and standards have been acquired in identical positions along the chromatographic peak. This could result in large errors particularly if sampling occurred on the upslope or downslope of the peak. Another option is to integrate the fluorescence over a chromatographic peak by summing EEMs. This would eliminate uncertainties due to peak shifts as long as no change in resolution occurs. Conventional algorithms [18-21] can then be applied. This approach will become more accurate at higher sampling rates. In most real world samples, however, it is virtually impossible to judge the beginning and end of a peak.


INTENSITY
5412
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2706
1353
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381
295
EXCITATION (nm)
582208
502
423
EMISSION (nm)
343
263

Figure 10

Therefore, integration leads to a reconstruction of the original sample matrix, negating any advantage gained in performing the chromatography. Finally, a linear chromatogram can be formed by deconvoluting all overlapping peaks, thus eliminating interferences. This approach is particularly attractive due to the ease of computing peak areas and the abundance of software. On the other hand, the required amount of processing makes this approach prohibitive.

As we have shown HPLC/VF shows significant promise as a tool for fluorescent mixture analysis. This tool will not be strictly limited to the analysis of naturally fluorescent compounds, since many post column modification techniques can be used to label selected compound classes with fluorescent "tags". Likewise, it may be possible to analyze for specific compounds which act as quenchers by monitoring the influence of column effluent on the emission of a select group of fluorophores [24]. The major advantage will continue to be the acquisition of full spectra in real time. With the continued development of a dedicated and integrated software package to enhance acquisition and data processing speed, the HPLC/VF will provide an impressive new approach to fluorescence detection in HPLC.

Acknowledgements

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Figure Captions

Figure 1. HPLC/VF interface; solid lines represent electrical connections, hollow lines represent flow pathways and dashed lines represent optical paths. The HP9845T desktop computer (B) with 1 Mbyte of floppy disk storage (A) is connected to the PAR 1215 console (C) by a 16 bit parallel data line. The 1215 communicates with the 1216 detector over a 4 Mbaud serial line. The controller oversees all operations of the SIT vidicon, installed in a thermostatically controlled housing (E). The Altex 312 MP HPLC controller (F) controls all flow rates of the 110A pumps (G) as well as triggering data acquisition through an EXT Flag. Column effluent goes from the pumps (G) through the column (K) and into the Precision Cells flow cell (L) for fluorometric analysis and then to the model 135 UV detector (H). The UV detector output is recorded by the HP3390A recording integrator (J).

Figure 2. Program interplay among the three microprocessors. All vertical columns represent programs within a single processor (HPLC on the left, OMA-2 in the center, HP9845T on the right). Cross connections represent events in one processor that interact with another.

Figure 3. Linear chromatograms derived from reverse phase chromatography of an 18 component PNA mixture. a) fluorescence detection corresponding to EEM row 25 (385 nm) b) fluorescence detection corresponding to row 35 (314 nm) c) total fluorescence signal d) conventional 254 nm absorption detection. The two x-axis scales relate sampling by the VF to real chromatographic time.

Figure 4. Ten EEMs collected during HPLC/VF on an 18 component PNA standard A) Spectrum 15; carbazole B) Spectrum 44; anthracene C)Spectrum 52; fluoranthene D)Spectrum 56; pyrene E) Spectrum 65; p-terphenyl F) Spectrum 67; dibenzothiophene, 1,2-benzanthracene, 9,10-Dimethyl-anthracene G) Spectrum 87; 1,2,3,4-dibenzanthracene, perylene H) Spectrum 93; benzo[a]pyrene I) Spectrum 97; benzo[e]pyrene J) Spectrum 108; chrysene.

Figure 5. Representation of the concentration gradient provided by an unresolved chromatographic peak for each of three hypothetical components separated in time or differing in peak width.

Figure 6. EEMs of the unresolved chromatographic peak at 29 min. containing the three components dibenzothiophene, 1,2-benzanthracene and 9,10-dimethylanthracene A) Spectrum 66 B) Spectrum 67 C) Spectrum 68

Figure 7. Ratio plots derived from the unresolved chromatographic peak at 29 min. A) Spectrum 66/Spectrum 67 B) Spectrum 68/ Spectrum 67. Contour heights are written within the plateau boundaries.

Figure 8. Ratio deconvolutions derived from Spectrum 67 A) 9,10-dimethylanthracene B) dibenzothiophene C) 1,2-benz-anthracene.

Figure 9. Time-luminescence matrices derived from the chromatography of the 18 component PNA mixture A) Time-Excitation Matrix (TEXM) B) Time-Emission Matrix (TEMM).

Figure 10. Reconstructed mixture EEM formed from the product of the transpose of the Time-Excitation Matrix with the Time-Emission Matrix.

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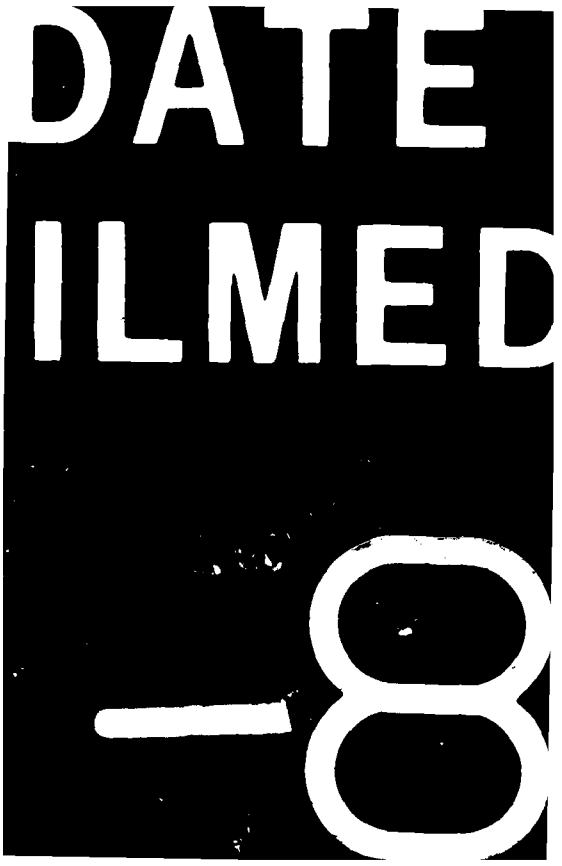
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