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RAPID SCANNING DIODE ARRAY AS A MULTI-WAVELENGTH DETECTOR IN LIQUID CHROMATOGRAPHY

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SUMMARY

A new diode array detector for liquid chromatography is described. The low-volume flow cell of the detector and the real time signal averaging of the spectra allow operation at 0.005 a.u.f.s. with noise levels of 0.0005 a.u. peak to peak. Since the detection is done over a large range of wavelengths, the detector allows identification of various solutes and peak deconvolution. The paper demonstrates how first derivative spectra can be gainfully utilized in a unique way to maximize the information obtained from the detector.

INTRODUCTION

Detector development is one of the areas of greatest activity in high-pressure liquid chromatography (LC). In gas chromatography (GC) the analyst is aided by several sensitive and universal detectors in addition to many selective ones. Unfortunately, the same is not entirely true in LC. The liquid mobile phase, which makes possible many separations unattainable in GC, creates problems in the detection of eluted components. The two most commonly used LC detectors are refractive index (RI) and fixed-wavelength ultraviolet (UV) monitors. The RI detector is fairly universal, but relatively insensitive. The UV detector, while having the capability of being sensitive, is limited to detecting species which absorb at the particular wavelength of the detector, and is limited to mobile phases which are transparent in that region.

A large number of other LC detectors have been developed using a variety of principles, such as dielectric constant[1], electrical conductivity[2], density[3], polarography[4], redisactivity[5], heat of adsorption[6], moving wire flame ionization[7], electrochemical[8,9], spray impact[10], electron capture[11], disk conveyor flame ionization[12], infrared adsorption[13], fluorescence[13], photo-ionization[14], atomic absorption[15], and mass spectrometry (MS)[16–18]. The UV monitor, however, is still one of the most widely used detectors. In general, these detectors operate at one or two wavelengths. This is also true with the variable-wavelength units now available on the market.

The attractiveness of UV detectors can be increased manyfold if the analyst is not limited to one wavelength, and if the the entire spectrum of the solute can be obtained. For modern high-efficient LC this requires a fast scanning spectrometer.

The ability of monitoring complete spectra throughout the chromatogram could greatly facilitate identification of the components, and improve quantitative determination by allowing each solute to be measured at its optimum wavelength. Deconvolution of poorly resolved peaks can also be accomplished.

Several types of rapid scanning spectrometers have been developed in recent years, primarily for the purpose of recording the spectra of transient intermediates in rapid reactions[19]. Several of these instruments can be suitable as LC detectors.

Denton *et al.*[20] have recently reported the use of an oscillating mirror rapid scanning spectrometer as an LC detector, and the advantages of recording complete spectra were clearly demonstrated. However, the noise levels below 250 nm were fairly high (from $\pm$ 0.05 to $\pm$ 0.2 a.u.), and the flow cell was rather large (87 μl). Since single-wavelength LC detectors typically have noise levels several orders of magnitude less than this, the oscillating mirror detector would appear to be unsuitable for many LC investigations. In addition, the price of the spectrometer alone is quite high.

Rapid scanning spectrometers with integrating array detectors, such as vidicons or solid state diode arrays[21,22] have the ability to give improved signal-to-noise ratios (S/N) by virtue of the multiplex advantage. In addition, such spectrometers have no moving parts. Although these devices are several orders of magnitude less sensitive than photomultipliers, overall improvements in S/N can be achieved, especially in UV–visible absorption, where light levels are relatively high and absolute sensitivity is of secondary importance. Applications of linear photodiode arrays in conjunction with LC have been reported[23,24], but quantitative data on noise levels and absorbance sensitivities have not appeared in the literature.

The purpose of this preliminary study was to determine if a solid state array could be easily adapted as a detector for LC, and achieve levels of precision and stability comparable to single-wavelength units. In addition, a novel way of peak deconvolution, obtained by plotting the value of the derivative of the absorbance with respect to the wavelength (*i.e.*, $dA/d\lambda$), at a given wavelength, as a function of time, is described. Throughout the experiments no attempts were made to optimize the chromatographic system.

EXPERIMENTAL

Instrumentation

Diode array spectrometer. Since absorbance sensitivity was of primary importance, the design of the spectrometer was kept relatively simple. A single Reticon (Sunnyvale, Calif., U.S.A.) 256-element photodiode array (RL256EC/17) was used. The control circuits (RC200/204) and signal-processing circuits (CASH-1B-1) were also obtained from Reticon and used without modification. The signal-processing circuits produce a sampled and held box car output which can easily be synchronized and sampled by an on-line minicomputer.

The deuterium lamp light source was powered by a Schoeffel (Westwood, N.J., U.S.A.) LPS 301 power supply, operated at 30 W and connected to a Sola (Elk Grove, Ill., U.S.A.) constant line voltage transformer. Radiation from the lamp is collimated, passed through one side of a flow cell, and imaged onto the entrance slit of the polychrometer. The polychrometer was built in house, and is of a Czerny–Turner

design with a focal length of 100 mm, and a 1200 lines/mm grating blazed for 240 nm (Edmund, Barrington, N.J., U.S.A.). A 110-mm range (about 220–330) is imaged onto the active area of the array (13 mm). An entrance slit width of 0.5 mm was used, giving an overall resolution of approximately 5 nm. If desired, a spectral range larger than 110 nm can be obtained.

The spectrometer is interfaced to a Data General (Southboro, Mass., U.S.A.). Nova II computer with a 16K core memory and three magnetic tape cassette drives. Data are acquired and output through a high-speed (100 KHz) interface with a 12-bit, 8-channel analog-to-digital converter and two 12-bit digital-to-analog converters (ADAC Model 500-DGC-8-DI-APG-A-DMA). Programs were written in BASIC with assembly language subroutines for data acquisition and display.

The control circuits of the diode array were adjusted to produce a spectrum in 2.6 msec with a 11.7-msec delay between each spectrum. During the 11.7-msec period the previously digitized spectrum added to a running sum buffer, allowing spectra to be ensemble averaged at a rate of 70 spectra/sec. Although each spectrum produced by the diode array contains 256 points, only 128 points from alternate diodes were saved on tape. This allows a larger number of spectra to be recorded, at a higher rate, without any significant loss in resolution. The transfer of a 128-point spectrum (double precision words) to the cassette tape requires 1 sec, and limits the maximum data rate of the present system. For most of this work spectra were recorded at an overall rate of one composite spectrum every 2 sec: 1 second to digitize and average 70 spectra and 1 sec to store the averaged spectra on tape. A slower data rate of one spectrum every 3 sec was also used and allowed averaging of 140 spectra. Higher data rates could be achieved with a faster mass storage device such as a disc. A maximum of 332 averaged spectra can be stored on the cassette tapes per experiment. The third cassette is used to hold the BASIC interpreter and programs.

Chromatographic equipment. A Tracor (Austin, Texas, U.S.A.) Model 5000 pneumatic amplifying pump was used in this study. The column used was 250 × 3 mm I.D. stainless steel packed with Partisil 10 (Whatman, Clifton, N.J., U.S.A.). The column output was connected to an 8-μl LDC (Laboratory Data Control, Riviera Beach, Fla., U.S.A.) flow cell. Only one flow cell was used. The second was blocked so that no radiation passed through it. Injections were made with a 10-μl high-pressure syringe (Precision Sampling, Baton Rouge, La., U.S.A.) via an injector fabricated from a Swagelok Union T and a PTFE-lined septum. The pump was operated at pressure ranges of 800–1600 p.s.i. corresponding to flow-rates of 0.53–1.30 ml/min.

Procedure

Prior to the injection of the sample, a spectrum is recorded with the light beam blocked. This spectrum is subtracted from all subsequent spectra to correct for the finite dark current of the diodes. A reference spectrum of the mobile phase is also recorded at the beginning of each experiment and used for the calculation of absorbance for the data spectra. Full-scale absorbance is specified by the operator via a teletype. The chromatogram at a desired single wavelength is displayed in real time on a storage oscilloscope. At the completion of an experiment any one of a number of data processing programs can be used to display absorbance or first derivative spectra at selected times, or a pseudo three-dimensional chromatogram (*i.e.*, time *versus* wavelength *versus* absorbance). Chromatograms at additional single wavelengths can also

be displayed either in absorbance or $dA/d\lambda$ units. Derivative spectra are generated with a 9-point quadratic/cubic least squares smoothing function[25]. All absorbance spectra and chromatograms can be smoothed with a 9-point quadratic/cubic least squares function after acquisition of the entire record, to further reduce noise. The known absorption peaks of a holmium oxide filter were used to calibrate the spectrometer in the wavelength axis.

Reagents

In one study the mobile phase was spectro-analyzed heptane and the solutes were benzene, benzyl chloride, and anisole. These were purchased from various sources and were of reagent grade. The solutes were mixed in a 2:1:1 (benzene–benzyl chloride–anisole) ratio and diluted as needed with the mobile phase. Table I shows the amounts of each solute at the two dilutions used in the experiments; 1 or 2 μl of each mixture were injected.

TABLE I

AMOUNTS OF EACH SOLUTE IN HEPTANE (ng/μl)

Solute	*Dilution*	
	1:250	*1:1500*
Benzene	176	29
Benzyl chloride	110	18
Anisole	100	17

UV-absorbing amino acids were also used as solutes, with distilled dionized water as the mobile phase. Phenylalanine (Phe) and tryptophan (Trp) were purchased from Sigma (St. Louis, Mo., U.S.A.). The standards were made with 0.72 μg/μl of Phe and 4.2 μg/μl of Trp. An 8-μl volume of a 1:1 mixture of the standards was injected.

RESULTS AND DISCUSSION

The system first studied contained the 1:250 mixture of benzene, benzyl chloride, and anisole as solutes, and heptane as the mobile phase. Spectra were recorded every 2 sec. Fig. 1 shows the chromatogram at 254 nm as obtained on the oscilloscope at 0.02 a.u.f.s. Fig. 2 shows a pseudo three-dimensional chromatogram of these components. Full-scale absorbance was 0.05, so that the plot could be fitted on the chart paper of an X–Y recorder. Although the plot shows that the three components are separated, qualitative identification is difficult because of the large differences in absorbance; in fact, in Fig. 2, benzene is barely noticeable. Individual spectra at the retention time of each solute (Figs. 3a–c) allowed a more conclusive identification of the components. The retention order of benzene, benzyl chloride, and anisole was also confirmed by eluting pure solutes. The fine structure of anisole is clearly visible in Fig. 2. The noise in the benzene spectrum (Fig. 3a) should be noted; the scale is 0.005

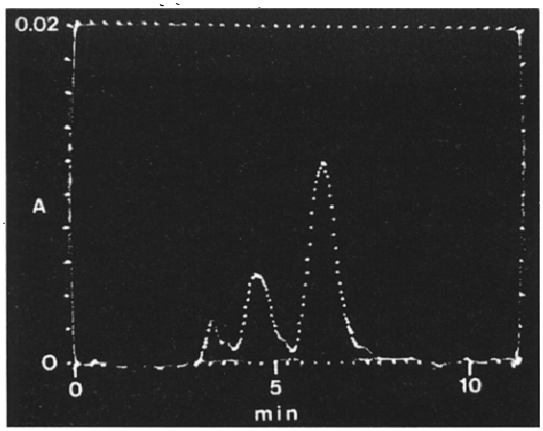


Fig. 1. Smoothed chromatogram of benzene (first peak), benzyl chloride (second peak), and anisole (third peak). Detected at 254 nm. 0.02 a.u.f.s.

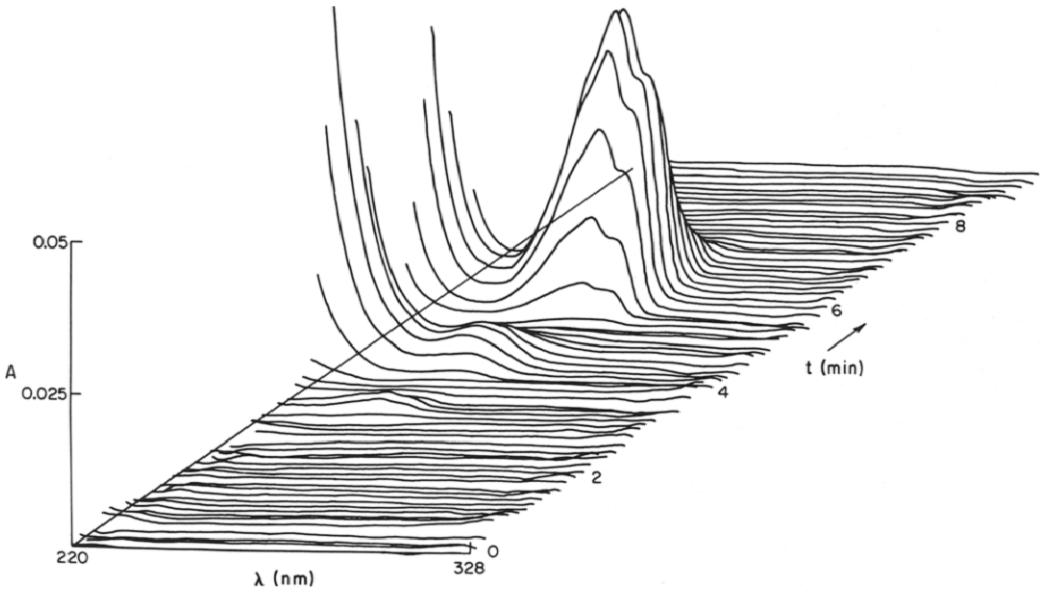
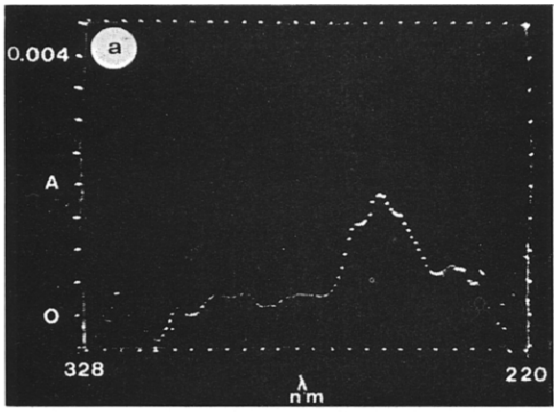
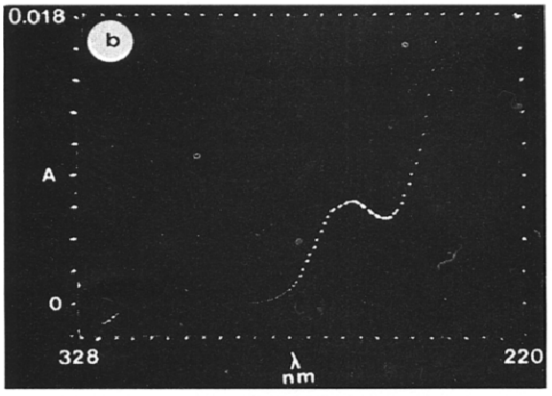


Fig. 2. Three-dimensional representation of the chromatogram in Fig. 1. 0.05 a.u.f.s.

a.u. Nevertheless, the fine structure is still observed. Benzyl chloride and anisole have very high absorbances at low wavelengths, as can be seen in Figs. 2, 3b and 3c.

Absorbance chromatograms at the maximum wavelength of each component (*i.e.*, in the range of 250–280 nm) can be used to optimize the evaluation of peak shape, area, and retention time. However, since the spectra of benzene and benzyl chloride are similar, and both are overlapped by anisole, this approach provides only a small amount of additional information about the system. An increased amount of information can be obtained from the derivative spectra, *i.e.*, $dA/d\lambda$ *versus* λ.





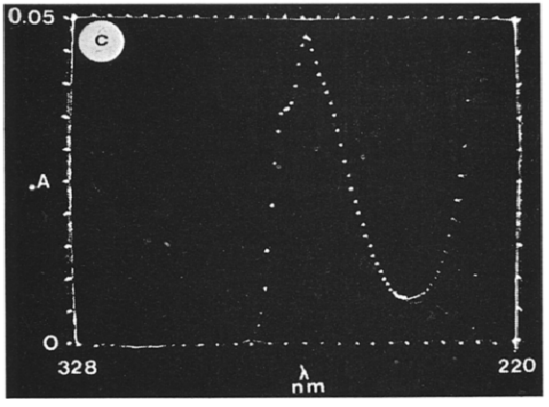


Fig. 3. (a) Spectrum of the benzene peak in Fig. 1. 0.005 a.u.f.s. (b) Spectrum of the benzyl chloride peak in Fig. 1. 0.02 a.u.f.s. (c) Spectrum of the anisole peak in Fig. 1. 0.05 a.u.f.s.

The derivative spectra of each component (Figs. 4a–c) show that each has at least one wavelength where $dA/d\lambda$ passes through zero. Although the derivative plot for benzene is noisy, there is no difficulty in finding the zero point of the peak. By plotting the value of $dA/d\lambda$ *versus* time at these zero crossing wavelengths, each com-

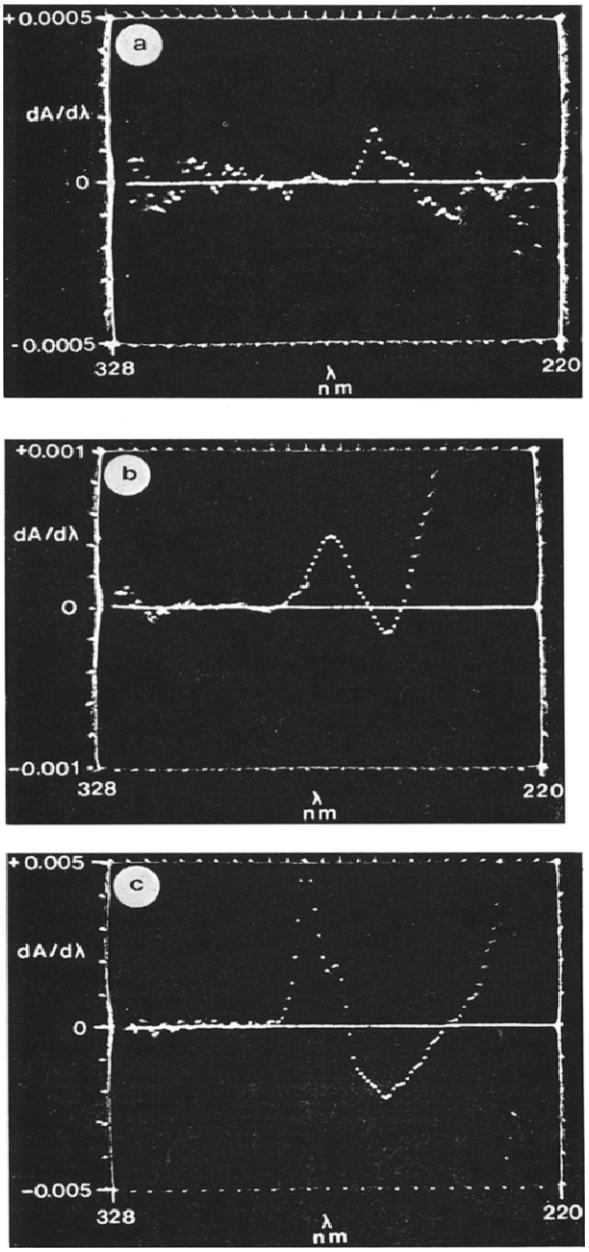


Fig. 4. (a) Derivative spectrum of benzene. 0.001 derivative units full scale (d.u.f.s.). (b) Derivative spectrum of benzyl chloride. 0.002 d.u.f.s. (c) Derivative spectrum of anisole. 0.01 d.u.f.s.

ponent can be effectively eliminated or deconvoluted from the chromatogram. At 273 nm the benzene derivative is zero. A $dA/d\lambda$ chromatogram at this wavelength (Fig. 5a) shows only the benzyl chloride and anisole peaks. At 246 nm (the zero crossing point of anisole) the $dA/d\lambda$ chromatogram (Fig. 5b) shows a small negative peak

for benzene and a positive peak for benzyl chloride; the anisole peak is almost totally eliminated. The benzyl chloride peak can similarly be eliminated by plotting the $dA/d\lambda$ chromatogram at 254 nm (Fig. 5c). To our knowledge, this is the first report of plot-

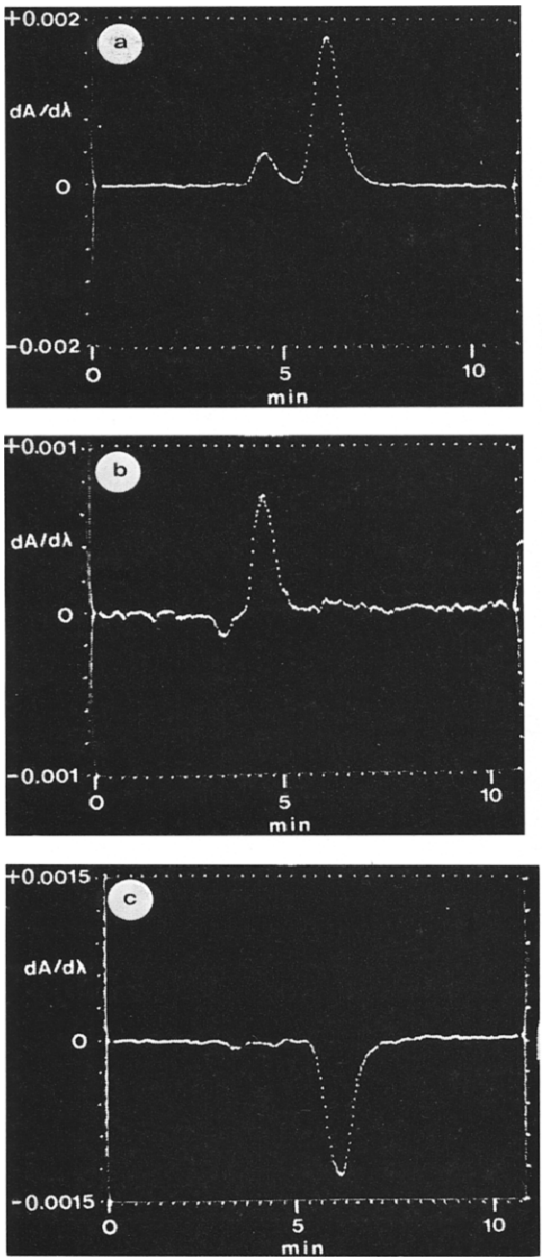


Fig. 5. (a) $dA/d\lambda$ *versus* time at 273 nm. The first peak represents benzyl chloride, the second anisole. (b) $dA/d\lambda$ *versus* time at 246 nm. The first (negative) peak represents benzene, the second (positive) benzyl chloride. (c) $dA/d\lambda$ *versus* time at 254 nm. The negative peak represents anisole.

ting $dA/d\lambda$ values *versus* time at a given wavelength. As seen, and as will be shown, this is a powerful tool which can be of great use in the case of complex chromatograms.

To evaluate the noise level and sensitivity of the system better, data were obtained with the 1:1500 dilution mixture of benzene, benzyl chloride, and anisole at 0.005 a.u.f.s. The chromatogram at 254 nm, recorded in real time, for this mixture (Fig. 6a) shows the benzyl chloride and anisole peaks clearly, but the benzene peak is below the noise level. The peak-to-peak noise in the plot due to the diode array, source flicker, and source drift is less than 0.0005 a.u. The points on the top part of the figure represent negative noise. Once smoothed, the chromatogram improves considerably, *e.g.*, Fig. 6b. The small shoulder on the anisole may be due to an impurity; however, this point was not investigated further. It should be mentioned that the spectrometer was originally designed for other studies and some of the optics used in this work were not enclosed. Thus, the results shown in Fig. 6 are very good indeed. Improvement in the optics should allow for better baseline stability and greater sensitivities.

The system was then tested with a mixture of phenylalanine and tryptophan using water as the mobile phase. The conditions of the experiment were purposely

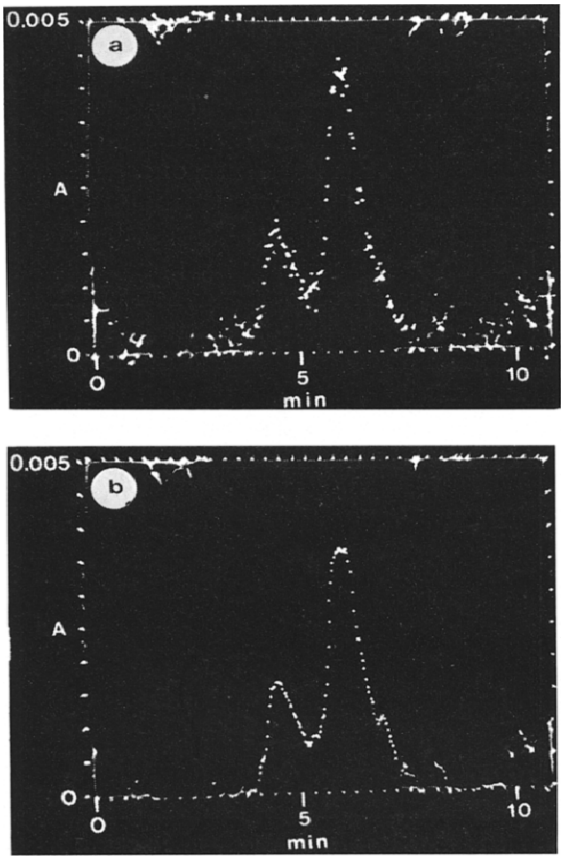


Fig. 6. (a) Real time chromatogram at 254 nm of benzyl chloride and anisole. 0.005 a.u.f.s. (b) Smoothed chromatogram. 0.005 a.u.f.s.

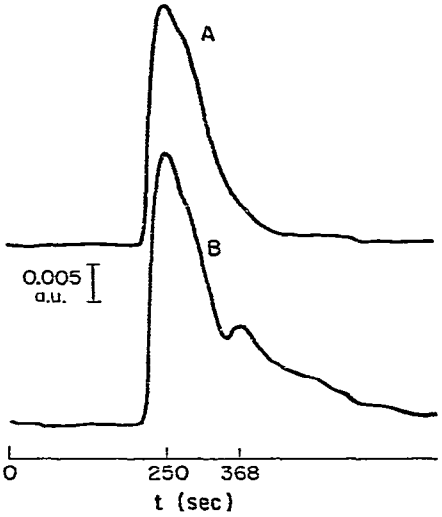


Fig. 7. (B) Chromatogram at 259 nm of a mixture of tryptophan (first peak) and phenylalanine (second peak). Mobile phase, water. 0.05 a.u.f.s. (A) Same chromatogram at 289 nm.

adjusted for an incomplete separation of the components. Spectra were recorded at 3-sec intervals, each composite spectrum being an ensemble average of 140 spectra. A smoothed chromatogram of this mixture at 259 nm and 0.05 a.u.f.s. (Fig. 7A) shows two unresolved peaks at 250 and 368 sec. At 289 nm, the second peak disappears (Fig. 7B). A spectrum at the maximum of each peak was then obtained in order to try to identify the components. The first spectrum (Fig. 8a) at 250 sec can be easily identified as tryptophan, but the second spectrum at 368 sec appears to be a mixture of several spectra. From Fig. 7B, it can be seen that tryptophan is almost completely eluted from the column at 447 sec. The spectrum at this time (Fig. 8b) more conclusively identifies the second peak as phenylalanine. Retention times found for the pure components verified these assignments.

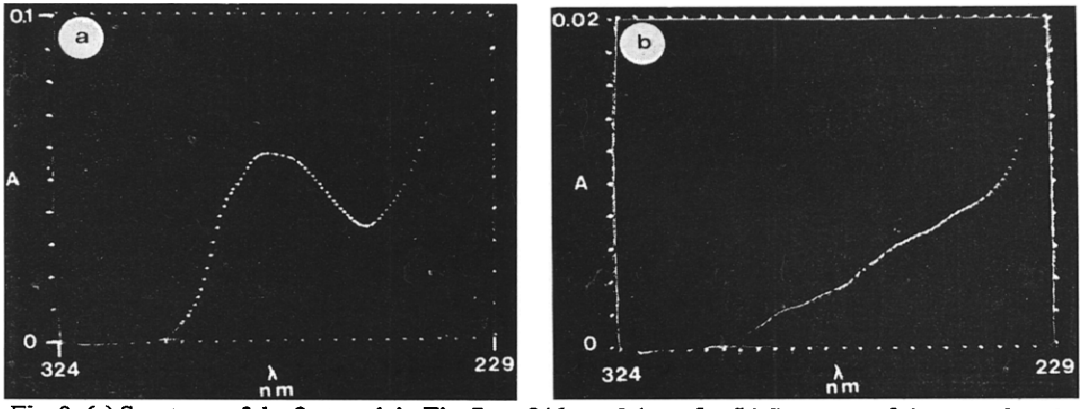


Fig. 8. (a) Spectrum of the first peak in Fig. 7a at 246 sec. 0.1 a.u.f.s. (b) Spectrum of the second peak in Fig. 7a at 447 sec. 0.02 a.u.f.s.

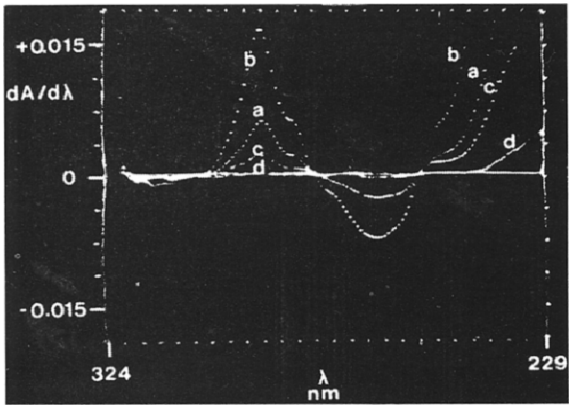


Fig. 9. Derivative spectrum of the Try–Phe mixture. (a) at 224 sec; (b) at 240 sec; (c) at 366 sec; (d) at 450 sec.

Derivative spectra were also generated from these data in an effort to further deconvolute the overlapping peaks. An attractive feature of using derivative spectra in this manner is that spectra of the isolated components are not required. Fig. 9 shows plots of the first derivative spectra at selected times in the chromatogram. Although the oscilloscope photographs are not very clear, spectra at short times have a common zero crossing point at 256 nm. If it is assumed that the leading edge of the first chromatographic peak is due only to tryptophan, then a plot of the value of $dA/d\lambda$ at 256 nm *versus* time should have only one peak namely, phenylalanine. This $dA/d\lambda$ chromatogram (Fig. 10) does show the phenylalanine peak more clearly, and the tryptophan peak has been eliminated. The retention time from the derivative chromatogram is 385 sec. There is, however, an additional small negative peak at 293 sec. This negative peak was at first thought to be noise due to the large-scale expansion. Closer inspection of the original absorbance chromatogram at 259 nm (Fig. 7) revealed a shoulder on the tryptophan peak at this retention time, thus indicating that the negative peak in Fig. 10 is real and is probably due to an impurity. The identity of the peak was not investigated further.

In summary, it is seen that a UV monitor with photodiode array can be used as a detector in LC. The low-volume flow cell, ability to signal average, and elimination of mechanical scanning components, allows operation with the sensitivities needed in modern LC. The availability of spectra rather than absorbance at a single wave-

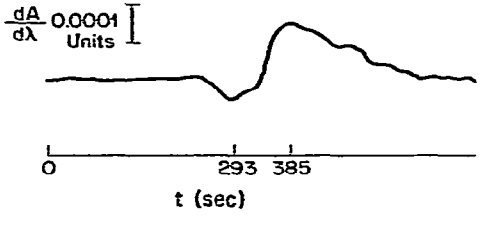


Fig. 10. $dA/d\lambda$ *versus* time at 256 nm.

length allows better identification and quantitation of the solutes. This additional information can also be used for deconvolution of overlapped peaks by plotting absorbance or first derivative absorbance chromatograms at various wavelengths.

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