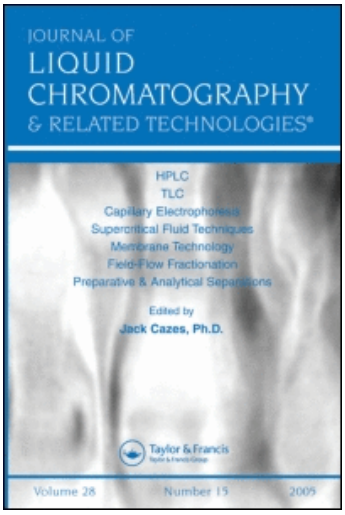

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RECENT ADVANCES IN HPLC OPTICAL DETECTION

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ABSTRACT

HPLC optical detector advances are reviewed in the field of absorbance, fluorescence, phosphorescence, and elemental emission detection. The development of UV-doped silicon photodiodes having excellent response throughout the UV, miniature gas discharge lamps (Hg, Zn, Cd) with high output UV lines, and high throughput UV interference filters allow optical systems with shot noise levels below 10^{-5} au. However, the fundamental noise limitations in such systems are now thermal changes in the optoelectronic components and flow cells. Future absorbance detectors may require thermostatting to achieve the minimal noise and drift performance inherent in the optical design. The emerging development of UV-doped photodiode arrays at reasonable cost offers multichannel absorbance detection and on-the-fly spectral information. Data system design for processing the multichannel data is critical in this field. On-column absorbance detection using packed microbore fused silica columns has been demonstrated, allowing a flow cell volume of <10 nanoliters, compatible with micro HPLC. In a similar development, on column fluorescence detection using an open tubular glass capillary column has been demonstrated in zone electrophoresis. A significant amount of research in fluorescence detection using laser sources is now underway. Sensitivity advantages of approximately 10-fold have been demonstrated due to high laser output and another 10-fold improvement for detecting long radiative lifetime molecules due to pulsed laser/temporal discrimination. Dual photon excited fluorescence, offering unique selectivities, has been demonstrated. This technique requires the high output power of the laser source. Room temperature phosphorescence HPLC detection has been demonstrated, utilizing triplet→triplet energy transfer from a donor solute molecule to a mobile phase acceptor additive (e.g., biacetyl). This energy transfer technique offers the expansion of the emission technique to phosphorescent molecules at nanogram sensitivities. Finally, the development of micro HPLC columns operating at 1-10 μℓ/min offers potential compatibility with element specific GC detectors such as the flame photometric detector (S, P specific).

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I. Introduction

Through a historical quirk, the development of HPLC occurred subsequent to that of GC. Much of the early development of HPLC was thus done by researchers trained in GC. This led to an expectation on the part of these scientists that HPLC detection should provide the sensitivity, selectivity and qualitative information inherent in gas phase detectors such as the structure specific mass spectrometer and the element-specific flame photometric detector.

Researchers in the field of coupling HPLC to the latter detectors have been frustrated by the problems of introducing 1 mℓ/min flow rates of aqueous or aqueous-organic solvents into gas phase detectors. A ray of hope for this field has recently appeared with the development of micro HPLC column technology, providing a reduction in operating flow rates to 1-25 μℓ/min.[1,2]

While academic research has focussed appropriately on the higher risk problem of interfacing gas phase detectors to HPLC, industrial research has focussed on the lower risk task of adapting classical liquid phase optical detection techniques to the specific needs of HPLC. Research has centered on UV-VIS absorbance and emission (fluorescence) detection.

As optical designers began to understand the peculiar needs of HPLC such as microvolume flow cells, low flow sensitivity and ultra-low detector noise, dramatic performance improvements were achieved. In the decade between 1970 and 1980, the noise level of UV absorbance detectors was reduced from ~5×10^{-4} au to ~5×10^{-5} au and typical flow sensitivity was reduced from 2×10^{-3} au per mℓ/min organic solvent to 2×10^{-4} au per mℓ/min. Concomitant with these improvements were a reduction in cell volume from 10 μℓ to 0.5-5 μℓ and a reduction in detector time constant from 1 second to 25-50 milliseconds, allowing optimum performance with "microbore" and "fast" HPLC.

HPLC detectors have also benefitted significantly from advances in peripheral optoelectronic technologies in meeting these performance improvements. Examples are the development and use of small, stable, high intensity discrete line gas discharge UV sources, UV-enhanced silicon photodiodes and UV-

enhanced photodiode arrays in UV absorbance detectors and the use of the laser as an excitation source in fluorescence detection. New experimental techniques in optical detection based on the laser as a source, such as photoacoustic spectroscopy and two-photon fluorescence are now under active study.

Finally, the difficulties encountered by researchers in interfacing mass spectrometers and element-specific gas phase detectors to HPLC have provided a driving force to the development of narrow bore HPLC columns operating at lower flow rates and reducing the degree of the liquid/gas interface problem. 1 mm stainless steel columns ("microbore") run at 25-50 $\mu\ell$/min and 0.3 mm fused silica columns ("narrow bore") run at 2-5 $\mu\ell$/min have thus spurred a new wave of HPLC detector research.[3-5]

Advances in current HPLC optical detection and the development of new optical detection techniques are discussed from the viewpoint of technological advances supporting these developments in the body of this report.

II. Impact of New Technology on HPLC Optical Detectors

A. Light Source

(1) Optical Absorbance Detection

Significant advances in HPLC detector source technology have occurred in both discrete line gas discharge and deuterium continuum sources. The first generation of optical absorbance detectors were fixed wavelength UV detectors which utilized a rather large, low pressure mercury arc lamp to provide 254 nm detection. The use of interference filters to isolate weaker mercury lines above 254 nm (e.g., 365 nm) provided an enhancement to detector selectivity albeit with poor sensitivity. Miniaturization of the mercury lamp soon followed, allowing improved optical design with microvolume flow cells. Finally, the need for lower wavelength UV lines to expand detector universality was met by the development of miniaturized zinc (214 nm) and cadmium (229 nm) gas discharge lamps. The miniature Hg, Zn, and Cd lamps are now widely used in fixed wavelength HPLC detectors. The 214 nm zinc line, in particular, provides

a near-universal wavelength for which relatively strong absorption is observed for ~90% of all organic molecules.

After the introduction of the fixed wavelength 254 nm Hg lamp detectors, a need for a selectable or variable wavelength detector was perceived by the chromatographer. Early versions of this detector were simply standard spectrophotometers, jury-rigged with small volume (10-15 $\mu\ell$) flow cells. The classical UV source, a deuterium lamp, was used in these detectors. However, HPLC needs for higher lamp stability and improved output in the low UV (sub 220 nm) region soon resulted in manufacturers' improvements in the deuterium source. Lamp stability has greatly improved in recent years through better understanding of optimum cathode temperature for operation and control of lamp filament and anode supply voltages.[6] Low UV output has been improved by reduction in absorbance by the lamp envelope.

Current deuterium lamps allow detector operation at low noise levels in both the UV and visible regions of the spectrum. Figure 1 shows the radiant intensity spectral distribution of commercial fused silica envelope deuterium lamps. Output is ~10x lower in the visible (350-700 nm) than the peak of the UV (~220 nm). If the detector noise were shot limited this would yield $\sim\sqrt{10}$ or 3.1x greater noise in the visible than UV. However, most absorbance detectors

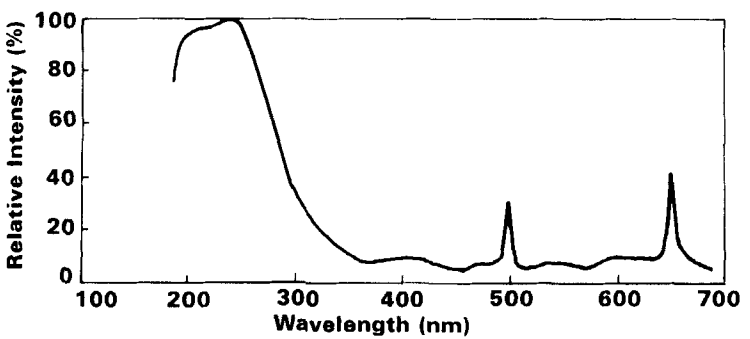

Figure 1. Radiant intensity spectral distribution of deuterium lamp (reprinted with permission from reference 6).

are not shot noise limited (see later section on thermal noise), and since the quantum efficiency of silicon photodiodes increases with wavelength, visible noise is generally within a factor of two of that achieved in the UV.

(2) Emission Detection - The laser as a source

Laser-Induced Fluorescence with Temporal Resolution

The development of the laser offers several features to the HPLC detector designer. Its high intensity allows improved fluorescence sensitivity for the case of shot noise limited detection,[7] and also has allowed the development of two-photon fluorescence excitation, a new optical detection technique. In addition to its high intensity, the pulsed laser allows the chromatographer to utilize temporal resolution in discriminating fluorescence of the analyte versus that of interferences (solvent, flow cell walls, sample matrix) having significantly different emission lifetimes.[9] It is often stated that the coherent output beam of the laser can be focussed down to a diffraction - limited spot, and that the laser therefore offers enhanced compatibility with narrow, microvolume flow cells. It should be noted that standard sources can also be focussed down to a diffraction - limited spot, albeit with more sophisticated focussing optics.

Work to date on laser-induced fluorescence has demonstrated approximately 10-fold sensitivity improvement versus standard deuterium, tungsten or xenon lamp-based fluorometers.[7] This has been disappointing in that the laser output is ~10^4x larger than that available from the typical fluorometer source (watts vs. milliwatts).[8] The use of temporal resolution with pulsed lasers has recently been shown to offer up to 100-fold improvement in detection sensitivity for compounds with long fluorescence lifetimes[9] (see data in Table I). For example, Richardson has demonstrated 0.5 ppt detection of the long-lived fluorescence of pyrene (690 msec lifetime). For the typical 100$\mu\ell$ peak observed in HPLC, this represents detection of 50 femtograms of pyrene. Temporal resolution also provides dramatic selectivity against co-eluting interferences, as seen in Figure 2.

TABLE 1
DETECTION LIMITS OF LASER INDUCED MOLECULAR FLUORESCENCE*

COMPOUND	τ_F^0	LIMF	CONVENTIONAL
NAPHTHALENE	—	1 PPT	—
ANTHRACENE	4 nsec	4	30
FLUORANTHENE	45	1	100
PYRENE	690	0.5	100
TRYPTOPHAN	—	50 (270 exc)	25 (220 exc)
ARGININE/FLURAM	—	10	25
RIBOFLAVIN	—	0.5	5
SOLVENT IMPURITIES	≤ 2	—	—

LIMF data from J. Richardson et al, NBS PUB. 519, 1979.
Conventional data from Varian, Fluorichrom Filter Fluorometer.
Solvent impurity τ_F from Matthews and Lytle, Anal. Chem., 51,583 (1979).

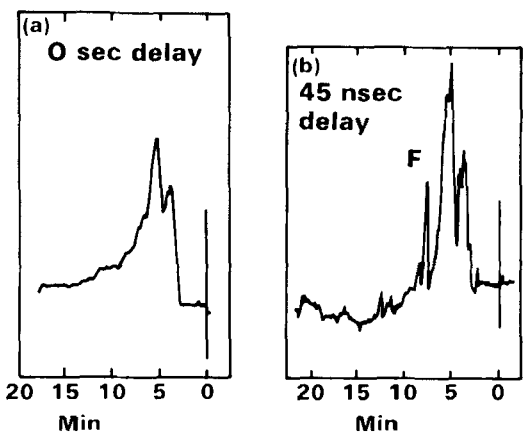

Figure 2. HPLC of coal gasification burn distillate using temporal resolution LIMB. (a) 0 sec delay (b) 45 nsec delay between excitation pulse and emission detection. F=fluoranthene (reprinted with permission from reference 9).

Finally, temporal resolution of fluorescence based on pulsed laser sources offers a potential sensitivity enhancement in post-column reaction detection for HPLC. In this case, one would choose a fluorescence label with a long fluorescence lifetime and excite its fluorescence with an appropriately pulsed laser.

The future utility of laser sources in HPLC fluorescence detection will depend on improvements in laser cost (currently >15K for a tunable dye laser) and reliability. Improved communication between chromatographers and spectroscopists searching for areas of laser applications in analytical chemistry is also required. Laser spectroscopists must stop claiming that the ppt sensitivity achieved with experimental laser based HPLC detectors is 10^3 better than that obtained with conventional HPLC fluorescence detectors. This implies ppb sensitivity of the conventional detectors. Commercially available, conventional HPLC fluorescence detectors provide 10 ppt sensitivity to highly fluorescent molecules.

Two-Photon Excited Fluorescence

The high power of the laser allows the use of two-photon excited fluorescence for HPLC detection. Quantum mechanical selection rules for two-photon excitation differ from those governing single photon excitation, thus expanding the potential selectivity of fluorescence detection. Since one can excite with visible photons yet detect fluorescence in the UV, background due to stray incident light, Rayleigh scatter and Raman scatter is greatly reduced. Matrix interferences are also relatively weak due to the restrictive selection rules of the two-photon effect.

The extinction coefficient for two-photon absorption is relatively small and is proportional to the square of the incident source power at the excitation wavelength. Thus, the technique requires both the high source power of the laser and high source stability. Yueng has demonstrated 100 parts per trillion sensitivity in the HPLC two-photon fluorescence technique using a relatively simple argon ion (Ar^+) laser source.[8] Use of a mode-locked, pulsed laser to

enhance incident power and stability is expected to bring sensitivity to the 1 ppt level. Figure 3 compares single and two-photon fluorescence chromatograms of a coal extract sample and demonstrates the different selectivities of both fluorescence techniques. It should be noted that one can simultaneously monitor single and two-photon excited fluorescence by collecting emitted light on different sides of the flow cell, with appropriate filters.

HPLC-Photoacoustic Detection

Absorption of a photon of light by a molecule produces a high energy electronic "excited" state. The molecule rapidly dissipates this energy, reverting to a lower energy "ground" state by several processes: (1) re-emission of a photon as in fluorescence or phosphorescence; (2) breaking of

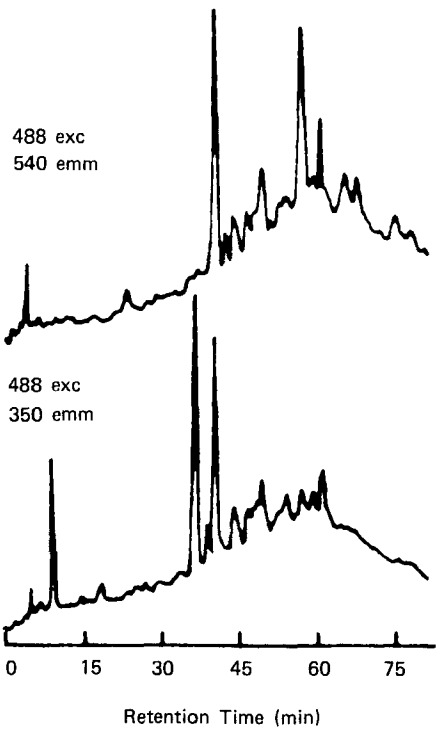

Figure 3. HPLC of PAH in coal liquids. One and two photon fluorescence detection (reprinted with permission from reference 8).

covalent bonds as in photolysis; (3) conversion into thermal energy (heat). The last non-radiative process causes localized heating which results in a pressure fluctuation in the sample, providing the basis for "photoacoustic" detection.

In the photoacoustic detector, the photon source is modulated and the resultant periodic temperature rise in the flow cell due to absorption/heat conversion produces a periodic pressure variation which is detected by a sensitive microphone (typically a ceramic piezoelectric transducer). To obtain optimum signal to noise ratio in photoacoustic detection, one requires a high source power (to increase signal) and vibration-free modulation (to reduce noise). A pulsed laser has thus been utilized as the source in experimental photoacoustic HPLC detectors studied to date.

An HPLC-photoacoustic system[10] based on a pulsed nitrogen laser with output at 337 nm yielded sensitivity for PAH molecules of approximately 100 ppb. Since these molecules are relatively strong fluorescers, one would expect at least a 5-fold sensitivity improvement for highly absorbing, non-fluorescent molecules.

An HPLC-photoacoustic system based on a pulsed argon ion laser[11] with output at 488 nm yielded detectivity down to ~10 ppb for azobenzene dyes. The 10 ppb dye concentration corresponded to an absorbance of 8×10^{-6} au/cm at 488 nm. A comparable state of the art absorbance detector detects down to ~2×10^{-5} au/cm.

In evaluating the potential of photoacoustic detection, one should note that most organic molecules studied in HPLC do not absorb above 300 nm. Thus, the argon ion and nitrogen lasers are not adequate sources for a commercial HPLC detector. State of the art lasers with output in the 200-280 nm region are very expensive and complex.

Finally, photoacoustic response of a sample molecule is solvent-dependent and the detector background signal is highly solvent dependent being a function of the solvent's absorbance and acoustical properties. Thus, gradient compatibility is a potential problem.

Advantages of photoacoustic detection are:

(1) choice of a high modulation frequency eliminates noise due to reciprocating pump pulsations, which have a much lower frequency.

(2) noise limit currently appears to be electronics (phase detection system) limited and might be improved ~10-fold with future development.

(3) signal is not dependent on optical pathlength as in absorbance. As in fluorescence, it is dependent on number of molecules excited and thus is more compatible with microvolume flow cell usage.

Future Absorbance Detector Thermal Stability Requirements

In Table 2, calculations of the photon flux that is optically collectable from miniature, intense gas discharge fixed wavelength UV lamps is compared to that of a deuterium lamp which provided a shot limited noise of 5×10^{-6} au (1 sec τ) on an experimental absorbance detector. These calculations indicate that the shot noise of properly designed fixed wavelength detectors can approach 5×10^{-7} au. However, to achieve the low noise inherent to the use of these intense sources, one must reduce non-shot generated noise components to the 5×10^{-7} au level.

The challenge involved in this task is best grasped by considering thermal noise sources. The temperature coefficients of UV-doped silicon photodiodes are in the range of 10^{-4} to 10^{-3} au per ^{o}C. Diode pairs (for reference and

TABLE 2

COLLECTABLE PHOTON FLUX OF FIXED WAVELENGTH LAMP VS. DEUTERIUM CONTINUUM LAMP

LAMP	λ	PHOTON FLUX RELATIVE TO DEUTERIUM LAMP	ESTIMATED SHOT NOISE*
Hg	254nm	~ 200	3.5×10^{-7} au
Zn	214nm	~ 50	7.1×10^{-7} au
Cd	229	~150	4.1×10^{-7} au

*SHOT NOISE WITH D LAMP IS 5×10^{-6} au

sample paths) can be obtained commercially which are matched in temperature coefficient to within $5x10^{-5}$ au per ^{o}C. Thus, to insure that thermal noise due to a differential temperature change between reference and sample diodes be of the order of $5x10^{-7}$ au will require a differential thermal stability of 10 millidegrees.

The temperature coefficient of mobile phase absorbance increases significantly as one approaches its "end absorption." The end absorbance of acetonitrile is shown in Figure 4. Acetonitrile absorbance temperature coefficient data obtained experimentally on an optical detector for which the incident light beam traversed the flow cell without striking cell walls (minimizing RI generated thermal effects) are presented in Table 3. The data suggest that reduction of solvent absorbance generated thermal noise to ~$5x10^{-7}$ au will require control of the flow cell solvent temperature to 400, 8 and 4 millidegrees at 254, 210 and 195 nm respectively (Table 4).

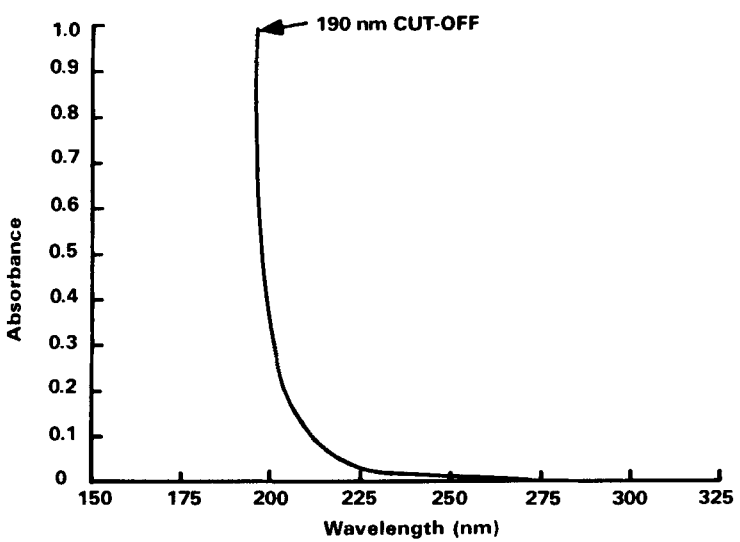

Figure 4. Absorption spectrum of acetonitrile (1 cm optical path).

TABLE 3
MOBILE PHASE TEMPERATURE COEFFICIENTS

— ACETONITRILE, 1 ml/min
— LIGHT BEAM TRAVERSES FLOW CELL WITHOUT STRIKING CELL WALLS

λ	TEMPCO
195 nm	$\leq 1.3 \times 10^{-4}$ au/°C
210 nm	$\leq 6 \times 10^{-5}$ au/°C
254 nm	$\leq 3 \times 10^{-6}$ au/°C

TABLE 4
THERMAL STABILITY REQUIREMENTS OF MOBILE PHASE TO REDUCE THERMAL NOISE BELOW SHOT NOISE

SHOT NOISE	195 nm	210 nm	254 nm
1×10^{-4} au	0.8°C	1.6°C	34°C
1×10^{-5} au	0.08	0.16	3.4
5×10^{-6} au	0.04	0.08	1.7
1×10^{-6} au	0.008	0.016	0.8
5×10^{-7} au	0.004	0.008	0.4

Clearly, in order to take advantage of current and future lamp intensity advances, HPLC detector thermal stability will require significant improvement. The placement of the detector itself in a well-controlled oven may become necessary.

B. Photodetectors

Photodetectors used in early fixed wavelength detectors utilized photoconductive films (e.g., CdS) and were characterized by response times varying from approximately 0.5 sec to several seconds, dependent on the amount of light incident on the film. Early variable wavelength detectors utilized fast response photomultiplier tubes optimized for low UV response. Whereas silicon photodiodes (fast response) initially suffered from poor quantum efficiency below 350 nm, advances in UV-doping semiconductor technology soon

increased photodiode Q.E. to ~2x that of the photomultiplier tube throughout the UV region. The dramatic improvements in silicon photodiode UV response from first through third (current) generations are shown in Figure 5. The improved UV response, lower thermal sensitivity, and very small size of the silicon photodiode have led to its incorporation as a photodetector in current fixed and variable wavelength absorbance detectors.

A silicon photodiode is simply a photosensitive p-n junction. It can thus be manufactured with a very small and precise active area (typically a few mm^2). Of further importance, linear arrays of such photodiodes can be produced on a single chip. The ability of semi-conductor manufacturers to produce linear photodiode arrays compatible in size and active area spacing with both the flat field focal plane of holographic concave gratings and the spectral bandwidth (2-5 nm) requirements of HPLC has led to the development of the diode array absorbance detector, providing multichannel absorbance information and on-the-fly spectra of eluting peaks.

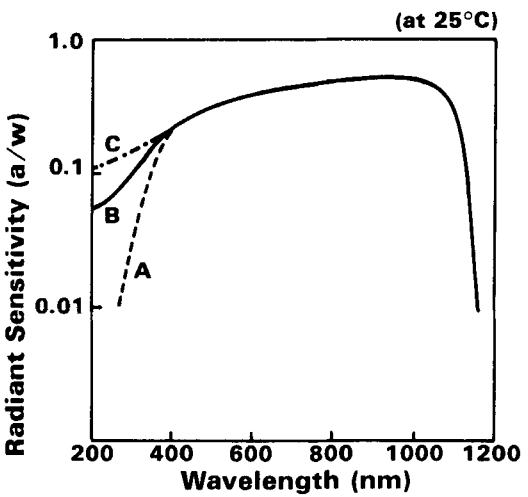

Figure 5. Radiant sensitivity as f(λ) of 3 successive generations of silicon photodiodes. Generation C is state of art 1982.

Time Programmable Diode Array and Absorbance Detectors

The period in which the development of variable wavelength detection in HPLC occurred has coincided fortuitously with developments in optoelectronic technology which have greatly expanded the utility of this detection technique. As discussed above, the development of the silicon photodiode has provided an inexpensive, compact, high performance photodetector. Advances in holographic etch manufacture of diffraction gratings produced inexpensive, low stray light and high efficiency plano and concave gratings. In addition, the development of the microprocessor allowed inexpensive control and hence time programmability of the wavelength drive of the monochromator of the variable wavelength detector.

The time programmable variable wavelength detector has allowed the chromatographer to set each section of the chromatogram to the detection wavelength that is optimum (in terms of sensitivity and/or selectivity) for the peaks eluting in that section. This feature is demonstrated in Figure 6. Time programming also has facilitated the stop-flow scanning technique in which the HPLC pump is stopped during the elution of a peak of interest, trapping the given peak segment in the flow cell, and the monochromator drive is then scanned, yielding a spectrum of that peak section resident in the flow cell.[13]

Diode Array Detection

The absorbance spectra of peak segments passing through a flow cell provide several types of qualitative information:

(1) absorbance ratios - the ratio of absorbance at any two wavelengths is determined by the spectrum of a molecule and is not concentration dependent. Thus for a pure peak, a given absorbance ratio value will be invariant at all points along the peak. Variation in this value indicates the co-elution of another compound which absorbs at least one of the wavelengths chosen in the ratio.

(2) peak confirmation (calculated) - the absolute value of several absorbance ratios can be used as a molecular "fingerprint" to confirm the suspected (based on retention time) identity of a peak.[15]

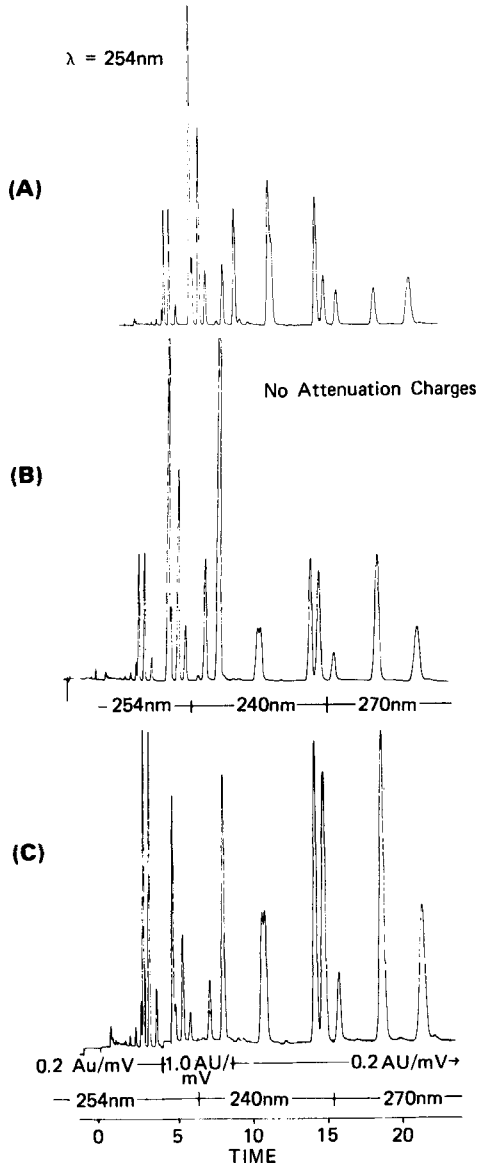

Figure 6. Chromatograms of mixture of PAH's. (a) 254 nm absorbance detection. (b) absorbance wavelength time programmed between 254, 240, 270 nm to optimize detectivity. (c) absorbance wavelength and absorbance range time-programmed (reprinted with permission from reference 12).

(3) peak confirmation (visual) - overlay of the spectrum with that of a known standard (after spectral normalization to a given wavelength) allows a visual confirmation of suspected peak identity.

Spectral information can be obtained with the stop-flow scanning technique available with most current microprocessor-controlled variable wavelength detectors. However, the need to stop the mobile phase flow becomes inconvenient as the number of spectra required per chromatogram increases. In addition, as peak volumes decrease with advances in column technology (e.g., fast LC, microbore LC), the ability to "freeze" a desired peak section in the flow cell becomes critical. Stopping the pump can result in decompression of the pump hydraulic volume and the column internal volume with resultant expansion of the compressible mobile phase through the flow cell, displacing the desired peak segment. This effect can be minimized by the addition of hydraulic elements in the system to seal the expansion volume from the flow cell path, increasing system cost/complexity.

Ideally, one would like to obtain spectral information "on-the-fly" during a chromatogram. This requirement can be met by the use of a linear photodiode array as the photodetector element in the relatively flat field focal plane produced by a concave grating.[15,16] Instead of mechanically rotating a grating so as to serially move successive wavelengths across a fixed slit/single detector element as in scanning a monochromator, one fixes the grating and locates the array so as to intercept the whole spectral region of interest (e.g., the UV). This optical system is called a "polychromator." The polychromator-array system also differs from a monochromator-single photodetector system in that the output of the source (e.g., deuterium lamp) is focussed through the flow cell prior to entering the polychromator and being dispersed by the grating. This configuration is called "reverse optics".

Linear UV-sensitive photodiode arrays are now commercially available with from 2 to 4096 elements. 1024, 512 and 211 element arrays are currently used in commercial HPLC diode array detectors.[17,18] The deluge of multichannel data

produced by the arrays is processed by a microcomputer to provide the chromatographer with wavelength chromatograms, ratio chromatograms and "spectro-chromatograms."[18]

The value of array detectors will grow as these information-rich detectors are applied to HPLC, and user feedback to the detector manufacturers yields improvements in the algorithms by which the spectral data is condensed, interpreted and presented. A certain amount of confusion already exists as to whether the information resident in a solution UV spectrum is useful for peak identification (determine identity of an unknown) or peak confirmation (confirm suspected identity of a peak at a known retention time). This author believes that the array detector will be valuable in providing impurity information based on ratios and peak confirmation based either on comparison of a pattern of several ratios with that of a standard or on the overlay of a peak spectrum and a standard spectrum. Ratio and spectro-chromatograms currently produced by array detection are shown in Figure 7 and 8.

C. Column Technology - HPLC

On-Column Detection Technique

Advances in HPLC column technology have resulted in significant reductions in peak volumes. In "fast HPLC," highly efficient 3 micron packings allow the use of short (4-7.5 cm) columns of standard diameter (4.6 mmID). Typical peak volumes on these columns are 20-100 $\mu\ell$. In microbore HPLC, column diameter is reduced to 1 mm and typical column lengths are 25-50 cm. To date, 5 μ and 10 μ packing technology has been developed for 1 mm columns, yielding typical peak volumes of 10-100 $\mu\ell$. Thus, fast and microbore HPLC techniques have required a reduction in detector flow cell values from 10-15 $\mu\ell$ to 0.5-5 $\mu\ell$, which has been achieved in current absorbance detectors.

In packed capillary HPLC, 1.0-4.6 mmID stainless steel columns are replaced by 0.2-0.3 mmID fused silica columns. These columns are now in the experimental stage of development.[2] Typical peak volumes obtained on 30-100 cm long packed

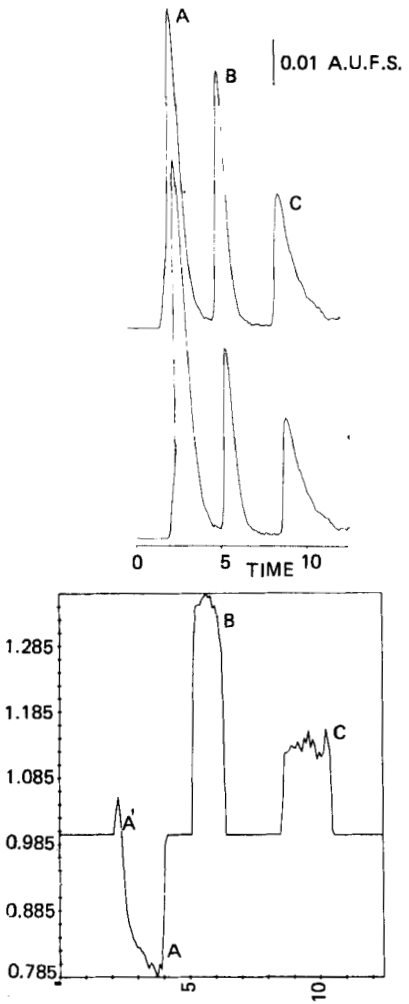

Figure 7. 230/240 nm ratio chromatogram and standard 230, 240 nm chromatograms of N-hydroxythienopyridine sample (reprinted with permission from reference 14).

capillary columns have been of the order of 2-10 $\mu\ell$. Thus, detector flow cell volumes below 200 nℓ are required for packed capillary HPLC. An ingenious solution to this problem has been the use of "on-column detection."[19]

On-column detection has been implemented by inserting the bottom of the optically transparent fused silica capillary column into the light path between

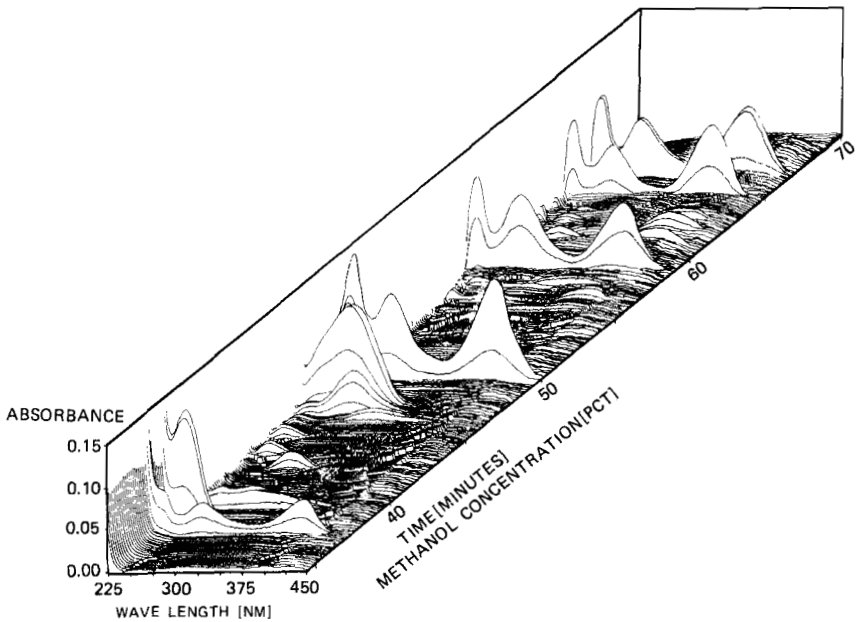

Figure 8. 225-450 nm spectrochromatograms of drug and metabolites in dog bile. (reprinted with permission from reference 14).

the monochromator and photodetector of a standard optical detector (absorbance or fluorescence) as shown in Figure 9. The column itself thus becomes the flow cell, eliminating the need for coupling tubing and providing an extremely small illuminated detection volume. For example, Yang removed 0.2 mm in length of the polyimide cladding at the bottom of a 0.3 mmID fused silica capillary column to form a 0.3x0.2 mm flow cell of 14 nanoliter volume. This produced insignificant band broadening with column generated peak volumes of 2 $\mu\ell$ (see Figure 10).

The disadvantages of the on-column absorbance technique are two-fold. The reduction in the optical path from a standard flow cell length of 5-10 mm to one of 0.3 mm, reduces signal by x15-30. This washes out most of the signal improvement inherent to capillary HPLC's lower dilution of an injected analyte. (100-500 $\mu\ell$ peak values of standard HPLC reduced to 2-10 $\mu\ell$ peak values.) In

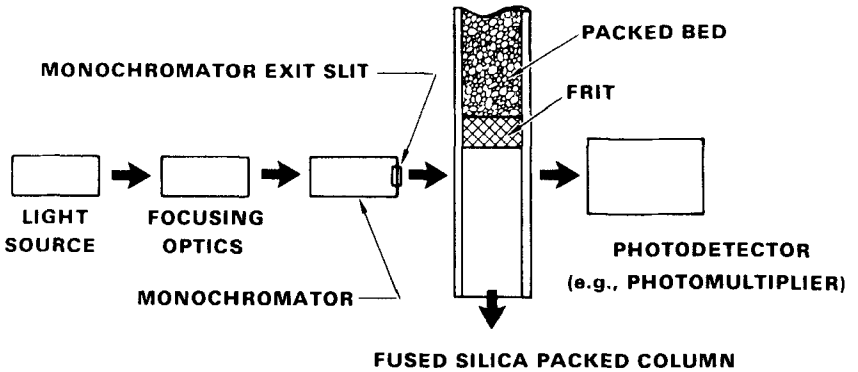

Figure 9. Schematic diagram for on-column absorbance detection in packed capillary micro HPLC.

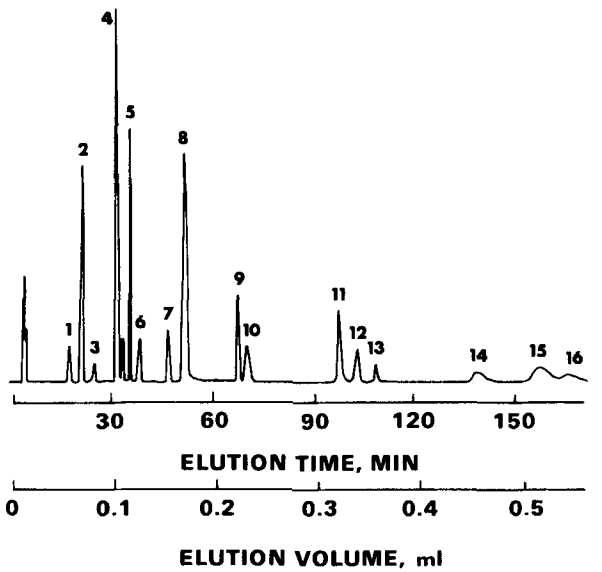

Figure 10. Chromatogram of 16 component PAH mixture separated on 50,000 plate, 45 cm x 330 μID 3 μC18 packed column, used "on-column" absorbance detection. Peak identifications are: 1, naphthalene; 2, acenaphthalene; 3, acenaphthene; 4, fluorene; 5, phenanthrene; 6, anthracene; 7, fluoranthene; 8, pyrene; 9, benzo[a]anthracene; 10, chrysene; 11, benzo[b]fluoranthene; 12, benzo[k]fluoranthene; 13, benzo[a]pyrene; 14, dibenzo[a,h]anthracene; 15, benzo[ghi]perylene, 16, indeno[1,2,3-cd]pyrene.

addition, the focussing optics of the detector into which the "on-column" flow cell was inserted was designed for imaging into larger cells. Thus the on-column flow cell is "over-filled" with incident light. This results in non-optimum light throughput and more importantly in high refractive index and thermal sensitivity of the detection system.* It is now well known in HPLC absorbance detector design,[20] that the incident light beam must traverse the flow cell without striking the cell walls in order to avoid high refractive index and thermal sensitivity.

In Figure 11, we see capillary chromatograms obtained using on-column absorbance and fluorescence detection.[21] Note the long term "wavy" drift of the absorbance detector baseline. This is due to slow variations in ambient temperature and the high thermal sensitivity of the optically overfilled on-column flow cell. The fluorescence on-column technique shows a flat baseline despite the fact that the cell was optically overfilled. The thermal sensitivity of the fluorescence technique is far less than that of absorbance. A further example of on-column fluorescence is shown in Figure 12. In this case, the on-column technique is utilized with a .075 mmID open tubular capillary operated in an electrophoretic mode,[22] yielding sub-$\mu\ell$ peak volumes.

Interfacing Micro HPLC to Optical Gas Phase Detectors

The low flow rates of packed capillary micro HPLC has allowed direct interfacing to the element-specific flame photometric detector[5] (see Figure 13). Initial experimental results demonstrated 100 pg/sec detectivity for phosphorous. For a 10 second micro HPLC peak and a typical phosphorous content in a molecule of ~10% by weight, this translates to detection of 10 ng of a P-

*Although flow sensitivity is also increasd by light striking the cell walls, the absorbance detector's response to a flow fluctuation is directly proportional to the absolute value of that fluctuation. Since the flow rates of capillary HPLC are extremely low (<10 $\mu\ell$/min), the baseline response to a micro-flow fluctuation is too low to be observed.

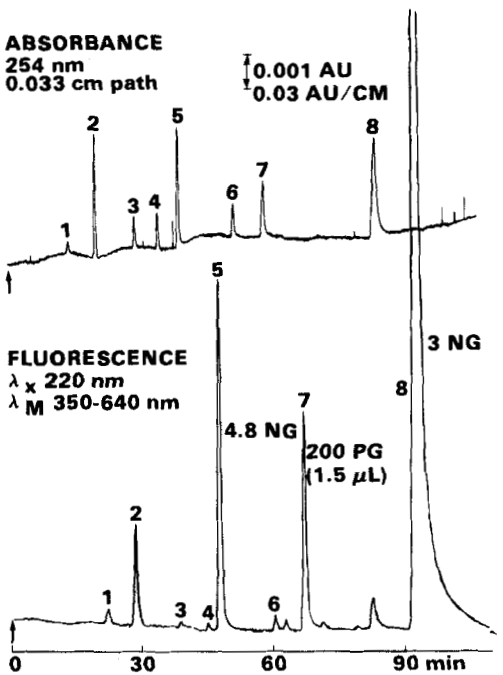

Figure 11. On column absorbance and fluorescence detection of PAH's in micro HPLC. 3 μ C18 reverse phase column, 0.33 mmID x 50 cm. 1.65 μℓ/min 80/20 acetonitrile/water. 250 atm. Absorbance data on Jasco Unidec-III spectrometer. Fluorescence data on Varian Fluorichrom filter fluorometer.

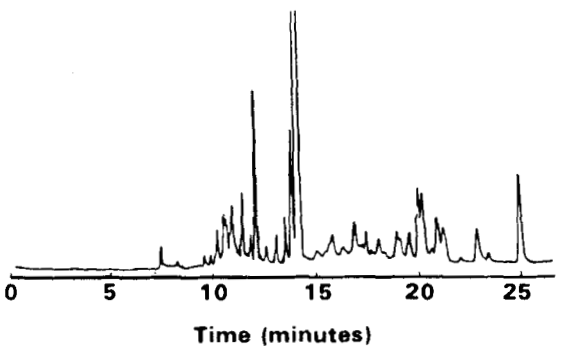

Figure 12. On-column fluorescence detection in glass capillary electrophoresis. .075 mmID x 100 cm column, 30 kilovolts applied voltage. Sample is human urine labelled with fluram tag (reprinted with permission from reference 22).

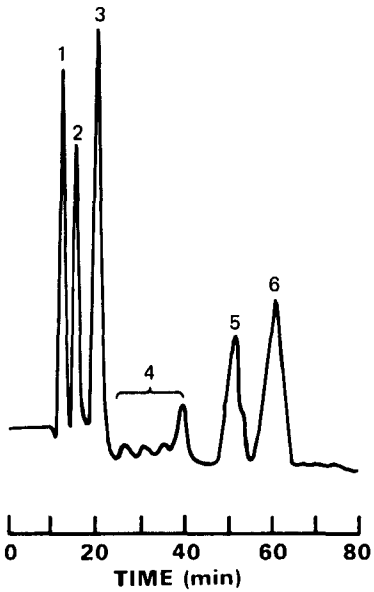

Figure 13. Micro-HPLC-FPD of organophosphorus pesticides. Column-0.07 mmID x 10 meters C18. 1 μℓ/min 42/58 methanol water. Peak identities. 1,4=impurity. 2=80 ng cygon. 3=280 ng DDVP. 5=230 ng malathion. 6=200 ng guthion (reprinted with permission from reference 5).

containing compound. P-specific detection is of great interest to phospholipid research. Although initial work is encouraging, one should note that micro HPLC-FPD success using aqueous buffer mobile phases has not yet been demonstrated. Potential problems in this case would involve precipitation of salts and non-volatile solutes in the detector system. Other potential problems could be flow sensitivity (since FPD is a mass-sensitive detector) necessitating a non-reciprocating or syringe pump to deliver pulseless flow, and gradient elution incompatibility due to dependence of sample response on mobile phase composition.

III. New Optical Detection Techniques in HPLC

Sensitized Room Temperature Phosphorescence

Only 10-20% of organic molecules fluoresce strongly enough ($\epsilon > 10^3$, $Q_F > 0.10$) to dictate use of fluorescence detection in HPLC. The low quantum yields of the

other absorbing organic molecules are typically due to intersystem crossing from the first excited electronic singlet state (the state capable of fluorescence) to an excited triplet state. Whereas singlet state lifetimes are of the order of nanoseconds, triplet state radiative lifetimes are millisecond or longer. Thus, in solution, collisions with solvent and solvent impurities (e.g., O_2) will deactivate the triplet state before it can emit a photon. Thus, with few exceptions, triplet state emission (phosphorescence) of organic molecules is not observed in room temperature solution.

An exception to the above rule is the molecule biacetyl. Strong biacetyl phosphorescence (~10% quantum yield) is observed at room temperature in solution if the solution has been thoroughly deoxygenated ($<10^{-8}$M). This observation has been utilized for the detection of molecules such as polychlorobiphenyls, that are characterized by high intersystem crossing rates. The technique, sensitized room temperature phosphorescence (SRTP), is based on energy transfer from the analyte triplet state to the triplet state of biacetyl which has been added as a dopant to the HPLC mobile phase. The diffusion-controlled energy transfer requires that the analyte donor triplet be $\geq$5 kcal/mole higher in energy than that of the biacetyl acceptor. A standard fluorescence detector is set up to excite the analyte donor at a wavelength at which biacetyl acceptor absorption is negligible (e.g., 300 nm) and to collect biacetyl phosphorescence (520 nm). Biacetyl phosphorescence can thus be used as a direct measure of analyte concentration. Detection of both halonaphthalenes and polychlorinated biphenyls at ~10 ppb levels has been achieved. A typical HPLC-SRTP chromatogram is shown in Figure 14.

Inductively Coupled Plasma (ICP) Detection in HPLC

In an ICP source, an electrodeless argon plasma is formed at atmospheric pressure and is sustained by inductive coupling to a high frequency magnetic field. A liquid stream fed into the plasma by a nebulizer is rapidly vaporized and the vapor is dissociated into free atoms which are excited and ionized by

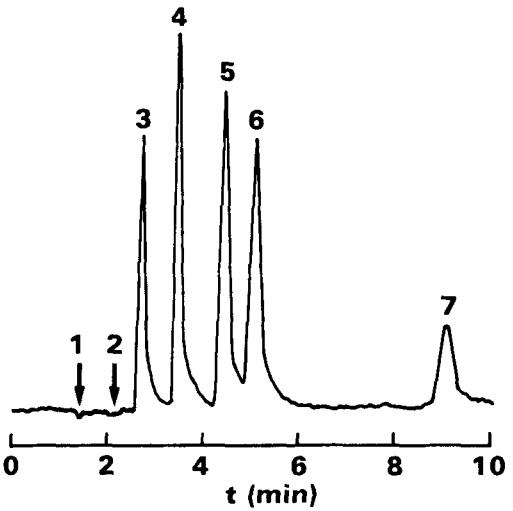

Figure 14. HPLC-SRTP of PCB's by reverse phase. 1 mℓ/min 83.7/16.3 acetonitrile/water. Biacetyl added at 10^{-4}M. λx260 nm. λ_M 552 nm. Peak identities: 1. solvent peak. 2. oxygen. 3. 15 ng biphenyl(B). 4. 23 ng 3-Cℓ-B. 5. 25 ng 3,3'-$C\ell_2$B. 6. 22 ng 3,5-$C\ell_2$ B. 7.39 ng 3,5,3',5'-$C\ell_4$ B.

the 6000-10,000°K plasma temperature. Elemental emission lines emanating from the plasma are collected by a polychromator and detected at suitable wavelengths by photomultiplier tubes appropriately located in the focal plane of the polychromator grating.

ICP, developed as a technique for simultaneous multi-element detection, is widely used for analysis of metals in complex solid and liquid sample matrices. ICP sensitivity for metals is typically ~ppb, and sensitivity to non-metals (e.g., C,P,N) is ~100 ppb. Compatibility with liquid sampling led to interfacing of ICP spectrometers to HPLC in several research laboratories.[24,25]

Results to date demonstrate the advantages of simultaneous multi-element detection:[25] the compatibility of standard HPLC flow rates with pneumatic nebulization into the ICP plasma; plasma stability in aqueous mobile phases; and an element-specific response independent of molecular form and matrix effects.

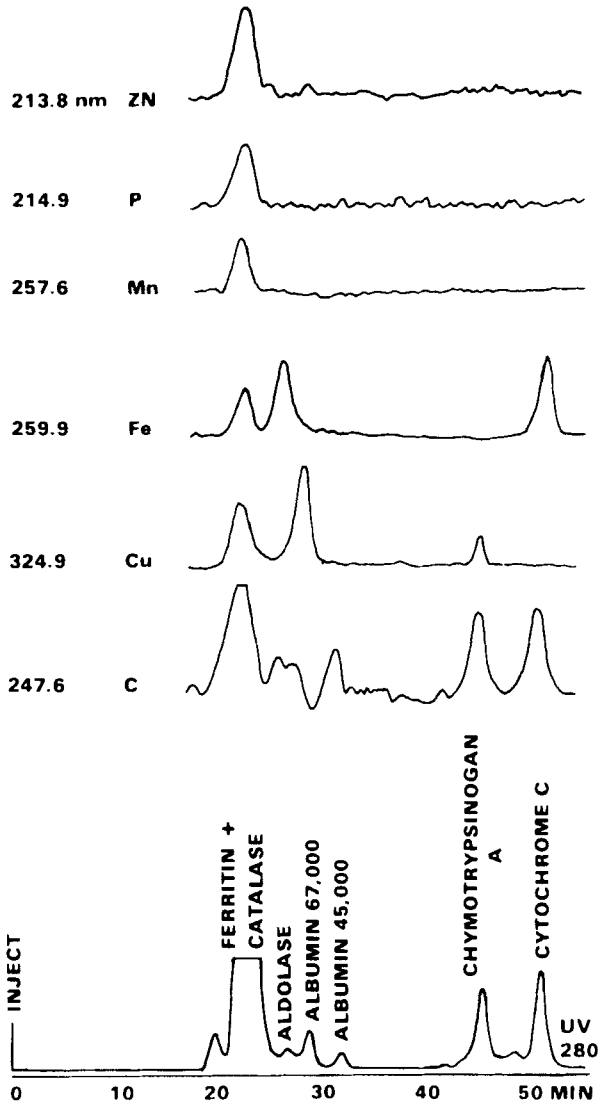

Figure 15. HPLC-ICP of proteins. 100 μg each protein. Steric exclusion separation on two TSK 3000 SW columns, 7.5 mm x 60 cm. 1 mℓ/min 0.9% NaCℓin water (reprinted with permission from reference 25).

TABLE 5
ICP ELEMENTAL LINES FOR HPLC*

NON-METALS		METALS	
C	193.1, *247.9* nm	Fe	*259.9*, 240.5
P	178.3, *214.9*	Zn	*213.9*, 202.6
N	174.3, 411.0	Cu	*324.8*, 224.7
S	180.7, 182.4	Pb	220.4, 217.0
Br	163.4, 157.7	Mn	*257.6*, 294.9
Cl	741.4, 452.6		

Wavelengths in italics are lines used in work to date.

**First wavelength corresponds to strongest line.*

However, work to date also indicates several disadvantages of ICP as an HPLC detector: it is quite expensive (~$100K); observed sensitivity to non-metals of ~ppm[26] is marginal for HPLC significant elements such as phosphorous; and plasma stability problems have been observed with hydrocarbon type organic solvents used in straight phase chromatography.

An HPLC-ICP chromatogram demonstrating the qualitative information inherent to multi-element specific detection is shown in the separation of metal-containing proteins of Figure 15. Note that ICP sensitivity to metals is 100x better than that observed for non-metals. Since non-metals are of greatest significance in HPLC, research aimed at enhanced non-metal ICP detection is required. The use of vacuum UV detection is now under study since the optimum emission lines of most non-metals lie in the 160-195 nm region (see Table 5).

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