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USE OF FLUORESCENCE DETECTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The use of fluorescence detection in liquid chromatography is now recognized as a powerful method which not only complements conventional UV absorption methods, but in many instances achieves sensitivity and specificity advantages that make it the optimum method of detection. The effect of optical and spectroscopic parameters on measurement sensitivity is illustrated in a number of applications involving UV-absorbing naturally fluorescent compounds as well as non-absorbing compounds for which fluorescence derivatives can be made.

A fluorescence detector specially designed for high-performance liquid chromatography was used in these studies. The optical design of this detector is described, and examples of its sensitivity at the picogram level are presented.

INTRODUCTION

General purpose detectors, also known as universal, non-specific, or non-selective detectors, are very useful to the analyst since they are capable of detecting all components of a sample mixture. Classical examples of universal detectors include refractive index and heat of adsorption detectors[1]. While these detectors are entirely satisfactory for analysis at part per million (ppm) concentration levels, at lower sample concentrations one quickly finds that slight changes in flow-rate, temperature, and solvent composition often drastically affect baseline stability and noise and ultimately limit precision and detectivity.

Substance-specific or selective detectors that measure a property exhibited by the sample component of interest and not exhibited by the solvent or other interfering species are inherently more sensitive and are generally less influenced by changes in solvent conditions. With the current emphasis on determination of materials at part per billion (ppb)** and lower levels, general purpose detection must give way to the higher sensitivity substance specific detection.

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** Throughout this article, the American billion (10^9) is meant.

Several investigators have already reported on the use of fluorescence detection in conjunction with high-performance liquid chromatography (HPLC)[2–16], and a few instruments have been developed for this application. The inherent high sensitivity of the technique coupled with the large amount of information readily available from thin-layer chromatography and clinical analysis literature[17,18] are two major factors for the increased use of this technique in HPLC. Also, the fact that many organic compounds either display native fluorescence or can be made fluorescent by derivatization has contributed to the popularity of fluorescence detection. Several areas of HPLC applications involving both native and derivatized fluorescent compounds illustrate the usefulness and power of the fluorescence method of detection.

EXPERIMENTAL

Fluorescence detector

An experimental filter fluorometer designed for use with HPLC was employed in this work. Fig. 1 shows a diagram of this detector. A miniature tungsten–halogen lamp, with a filament selected to match the flow cell dimensions, provides the excitation radiation. Fused silica lenses collect energy from the source and focus it on the flow cell. A combination of glass and/or interference filters isolates the spectral bandwidth of interest for sample excitation. The flow cell is designed to withstand an operating pressure of 500 p.s.i. The flowing liquid contacts only stainless steel, PTFE, or quartz in its passage through the cell. The total cell volume is 25 μl while the liquid volume actually illuminated is about 12.5 μl.

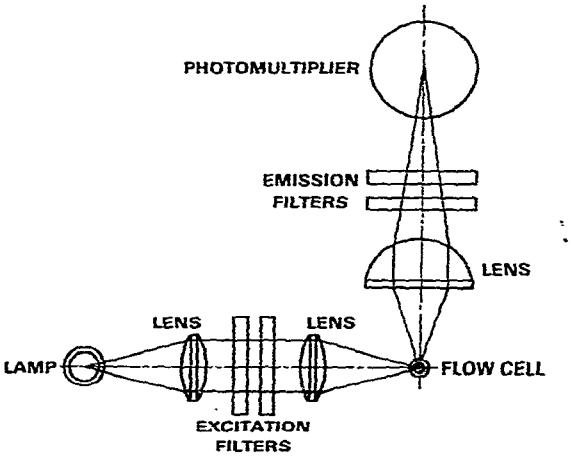

Fig. 1. Optical diagram of filter fluorometer.

An aspheric lens collects emission from the flow cell at 90° relative to the excitation beam. This geometry minimizes background radiation due to cellwall fluorescence and scattering. Again, a combination of glass and/or interference filters isolates the spectral bandwidth of interest. A photomultiplier tube detects the emitted radiation. A highly regulated constant current supply is used to operate the lamp. Also, a well regulated high voltage supply provides stable gain characteristics in the

photomultiplier tube. The detector output is compatible with conventional 1 mV full span recorders.

Other equipment and materials

The liquid chromatograph used in this work was a Varian Model 8520. A variable wavelength spectrophotometric detector (Varichrom; Varian) was connected in series with the fluorometer to provide simultaneous fluorescence and absorbance chromatograms. A short length of low dead-volume tubing was used to minimize band broadening between the detectors. All solvents used were spectroquality, obtained from Burdick & Jackson (Muskegon, Mich., U.S.A.). All other chemicals were reagent grade unless otherwise stated. More specific details as to columns, filters, and solvents are shown in the legends of the relevant figures. The filter "CS" notation refers to Corning Glass color standard numbers. Interference filters had 10 nm bandwidths.

APPLICATIONS OF FLUOROMETRIC DETECTION IN HPLC

Polynuclear aromatics

Polynuclear aromatics (PNAs) are being intensively studied in a wide varie y of matrices. This interest is due to the carcinogenicity of these materials and their widespread presence as contaminants in air, water, auto exhaust, and cigarette smoke. Polynuclear aromatic compounds display native fluorescence due to their extensive π bonding. Specificity for compounds such as fluoranthene, perylene, and benz[*a*]pyrene in the presence of naphthalene, acenaphthalene, pyrene, and chrysene is achieved by exciting at wavelengths in the 360 nm region. Fig. 2 shows a chromatogram of several PNAs with simultaneous UV and fluorescence detection. The UV detector was set at 254 nm and 0.01 a.u.f.s. Glass filters were selected to provide excitation at 360 nm, and light emitted at wavelengths longer than 450 nm was monitored by the photometer. The analysis was performed on an octadecyl bonded phase column using the reversed-phase mode. Note that only three components are detected by fluorescence, *i.e.*, anthracene, fluoranthene, and perylene since the other components are not excited at 360 nm. This chromatogram also shows that the fluorescence detector is not subject to severe baseline changes during the course of a gradient analysis.

Fig. 3 shows chromatograms obtained on a solution containing only fluoranthene, perylene, and benz[*a*]pyrene. The amounts injected were 3.6, 2.3, and 1.6 ng respectively. Note that the components are barely discernable from the baseline noise of the UV detector. Fig. 4 shows the same solution one order of magnitude more dilute than the previous figure. Now the peaks are no longer detected by the UV absorption but are easily measured by fluorescence. Minimum detectable concentrations (MD) and quantities (MDQ) were determined for these components and are shown in Table I.

PNAs may be found in various sources of water. An experiment was designed to investigate the presence of PNAs in tap water in the authors' laboratory. A 300-ml volume of this water was passed through a short column of octadecyl packing to extract the non-polar components. This short column was then placed at the head of the analytical column and the extracted organics eluted by the acetonitrile/water eluent. Fig. 5 shows the chromatogram obtained. The fluorescence detector shows the presence of many constituents. The identity of these materials has not been con-

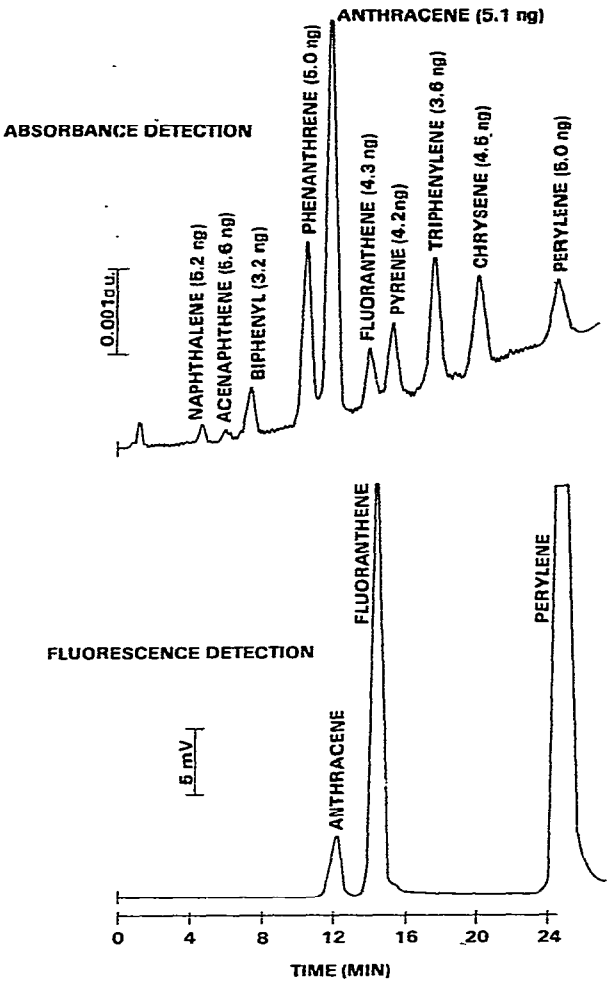

Fig. 2. Chromatographic separation of polynuclear aromatics illustrating the selectivity of fluorescence detection. Column: MicroPak-CH-10, 25 cm × 2.2 mm; solvent A: water; solvent B: acetonitrile; gradient program: 50% B, + 1% B/min; flow-rate: 1 ml/min; detector wavelength: λ_{exc} = 360 nm (CS7-54, CS7-60), $\lambda_{em} > 450$ nm (CS4-76, CS3-72), $\lambda_{abs} = 254$ nm.

firmed but they do elute within the fluoranthene–benz[*a*]pyrene region. Assuming a response similar to that of fluoranthene, one calculates concentrations of these materials to be well below the ppb level.

Aflatoxins

In recent years much attention[19–25] has been devoted to the determination of trace levels of aflatoxins. These materials (toxic metabolites of the fungus *Aspergillus flavus* Link *ex* Fries) are of great interest because of their extreme carcinogenicity and toxicity. Peanuts and peanut meal are routinely analyzed for these substances. Other grains such as corn are also being studied to determine their aflatoxin content.

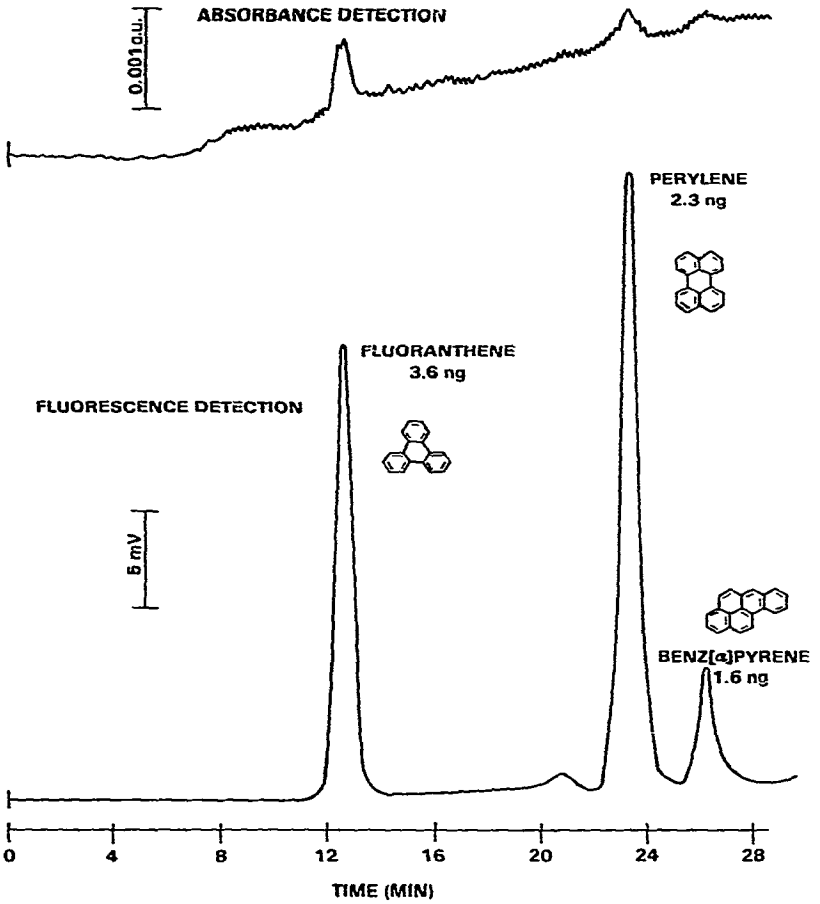

Fig. 3. Chromatographic separation of fluoranthene, perylene and benz[*a*]pyrene showing relative response of fluorescence and absorption. Same conditions as in Fig. 2.

The structures of the four major aflatoxins are shown in Fig. 6. These compounds have very interesting fluorescence behavior. Robertson *et al.*[25] have shown that the emission wavelength and fluorescence yields of aflatoxins are quite dependent upon the solvent. All of the aflatoxins have long wavelength absorption maxima near 362 nm so this was chosen as the excitation wavelength. The emission wavelengths are different for the two classes. The emission maxima of B_1 and B_2 are in the 410–430 nm region, depending upon the solvent system used. Glass filters collecting the emitted light above 450 nm were used for the emission. Fig. 7 shows a chromatogram of the four aflatoxins obtained on a bonded phase CN column. Since the quantum yields of B_1 and B_2 are quite low in non-polar solvents and their emission maxima occur below the transmission region of the emission filters, the fluorometer shows no response to them. Calculated MDQ values for G_1 and G_2 are 40 and 30 pg respectively. The fluorescence detector thus complements the UV detector for the G aflatoxins which are generally found at much lower concentrations than the B aflatoxins.

Fig. 8 shows a chromatographic separation of the four aflatoxins by reversed-phase techniques on a bonded phase CH column. Since the same filter system was

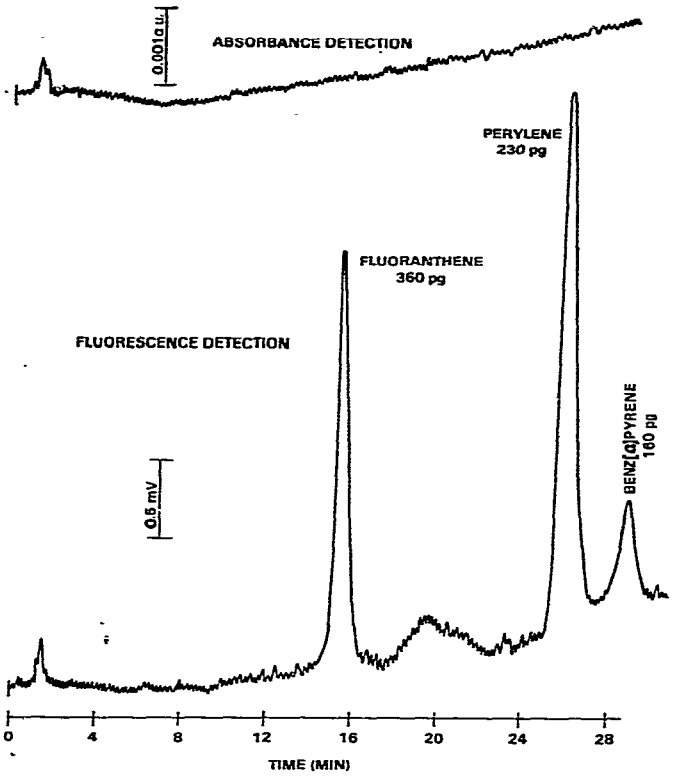

Fig. 4. Absorption and fluorescence chromatograms of subnanogram quantities of compounds shown in Fig. 3. Same conditions as Fig. 2.

TABLE I

MINIMUM DETECTABLE CONCENTRATIONS AND QUANTITIES

Compound	*MDC (pM)**	*MDQ (fmoles)***
Fluoranthene	44	25
Perylene	15	12
Benz[*a*]pyrene	56	44

* Calculated as moles of solute per liter of eluent multiplied by 10^{12}. MDC is minimum concentration in the sample cell necessary to yield a signal of two times the peak-to-peak noise.

** Minimum quantity injected on column that will give peak detector signal equal to two times the noise. Calculated as moles multiplied by 10^{15}.

used as that in the bonded phase CN separation, the results indicate that the more polar solvent has increased the quantum yield of B_1 and B_2 and shifted the emission maxima to longer wavelengths. The chromatogram shown represents the injection of 40 ng B_1, 20 ng G_1, 40 pg G_2, and 30 pg B_2. Thus the higher polarity solvent improves the quantum yield of B_1 and B_2. It also enhances the fluorescence of the saturated G_2 but reduces that of the unsaturated G_1. Calculated MDQ values for G_1 and G_2 in this system are 300 pg and 1 pg respectively, in comparison to the equivalent sensitivities obtained in the normal phase separation. This example clearly points out the need for

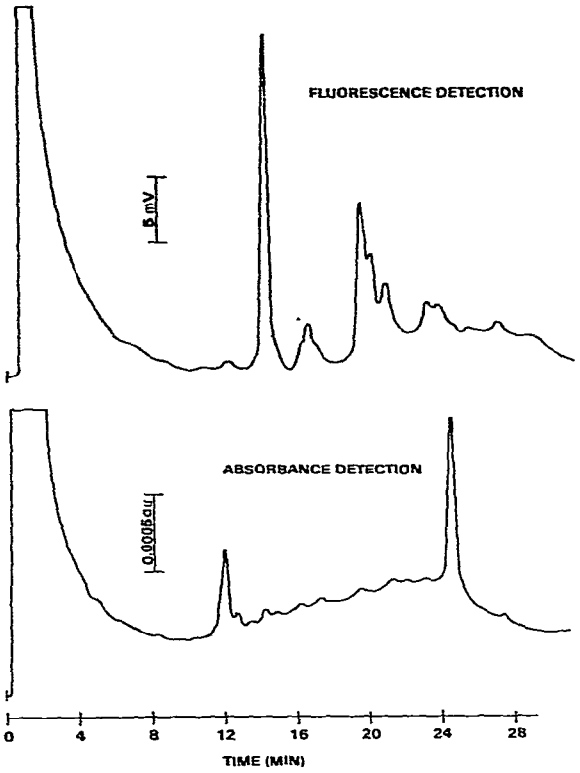

Fig. 5. Chromatograms of tap water extract illustrating the presence of fluorescent organic contaminants. Same conditions as Fig. 2 except gradient program: 50% B for 8 min, then 3% B/min to 100% B.

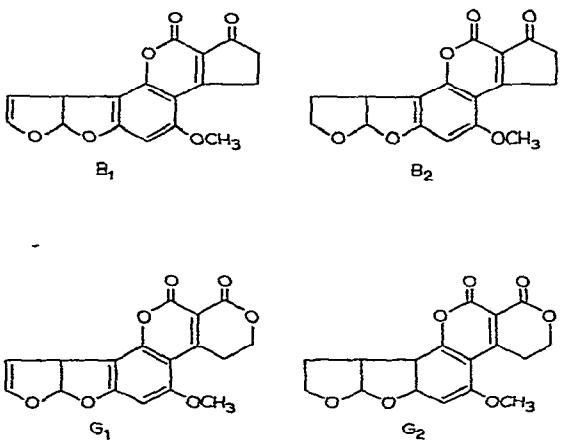

Fig. 6. Structures of the aflatoxins.

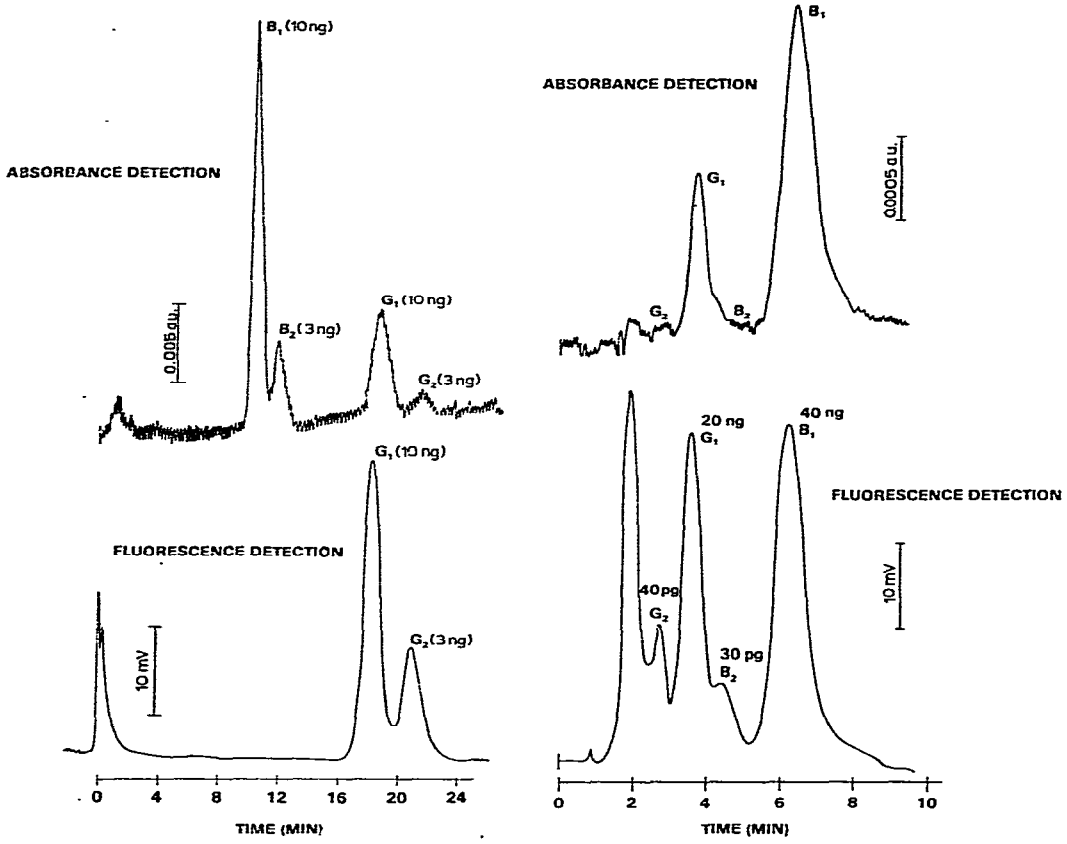

Fig. 7. Separation of aflatoxins B_1, B_2, G_1, and G_2 by normal-phase HPLC. Column: MicroPak-CN-10, 25 cm × 2.2 mm; mobile phase: hexane–tetrahydrofuran–isopropanol–water (70:26.7:3.0:0.3); flow-rate: 1 ml/min; detector wavelengths: λ_{exc} = 360 nm (CS7-54, CS-7-60), λ_{em} > 450 nm (CS4-76, CS3-72), λ_{abs} = 362 nm.

Fig. 8. Separation of aflatoxins B_1, B_2, G_1, and G_2 by reversed-phase HPLC. Column: MicroPak-CH-10, 25 cm × 2.2 mm; mobile phase: isopropanol–ethanol–water (1.25:23.75:75); flow-rate: 1 ml/min; detector wavelengths: λ_{exc} = 360 nm (CS7-54, CS7-60), λ_{em} > 450 nm (CS4-76, CS3-72), and λ_{abs} = 362 nm.

understanding the effect of the mobile phase on the fluorescence behavior of the sample under investigation.

LSD

Forensic analysts are often concerned with detection of N,N-diethyl-D-lysergamide (LSD) and many other drugs of abuse. This material displays native fluorescence when excited at 330 nm. Fig. 9 shows results obtained on analysis of an illicit sample of LSD. A 330-nm interference filter was used for excitation and wavelengths longer than 420 nm were used for emission. The reversed-phase separation was performed on a bonded-phase column in the ion suppression mode. Note that the major peak observed in the UV detector is not LSD. MDQ for LSD with the fluorescence detector is estimated at 5 pg.

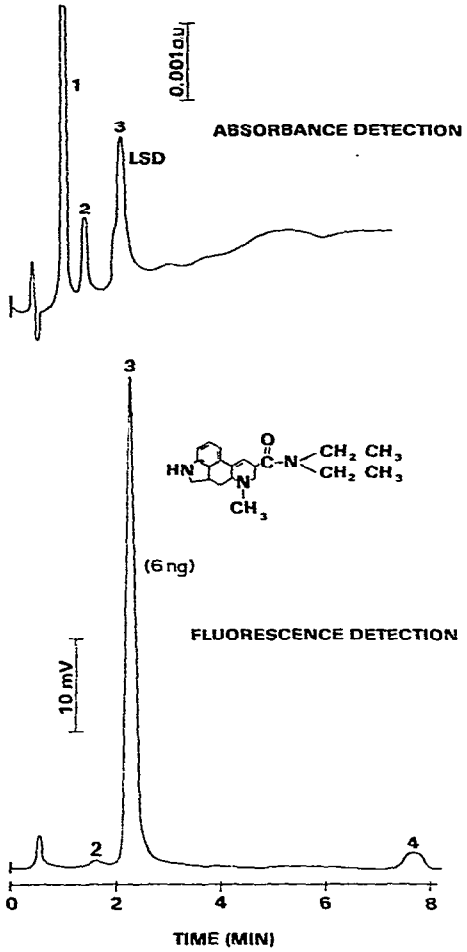

Fig. 9. Separation of LSD. Column: MicroPak-MCH-10, 25 cm × 2.2 mm; Mobile Phase: acetonitrile–0.1 *M* aqueous $(NH_4)_2CO_3$ (50:50); flow-rate: 1 ml/min; detector wavelengths: $\lambda_{exc} = 330$ nm (interference filter, 8 mm bandwidth), $\lambda_{em} > 420$ nm (CS4-76, CS3-71) $\lambda_{abs} = 254$ nm.

Derivatization

Derivatization adds another dimension to fluorescence detection since it allows many naturally non-fluorescent compounds to be detected. While many reagents for preparing fluorescent derivatives have been reported in the literature, three have become very popular (Dns chloride, *o*-phthaldehyde, and fluorescamine). Of these, only Dns-Cl is useful for compounds other than primary amines. This material will react with compounds such as phenols, secondary amines, and under proper conditions, alcohols.

Derivatization may be accomplished either pre- or post-column. Post-column derivatization is the technique used in amino acid analyzers. The technique allows the chromatographer to use the physical properties of the solute to achieve the separation. In pre-column derivatization the material is derivatized prior to the chromato-

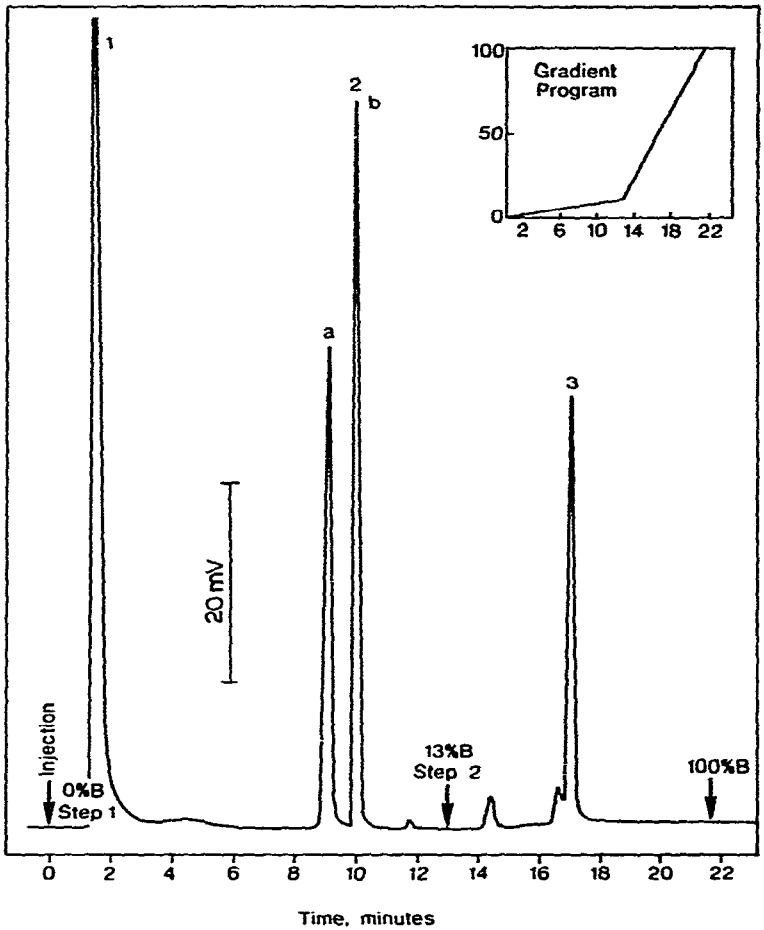

Fig. 10. Chromatogram of Dns Δ^9-tetrahydrocannabinol (1), mono- and di-Dns cannabidiol (2a, b), and Dns 11-hydroxy-Δ^8-tetrahydrocannabinol (3). Column: MicroPak-Si-10, 25 cm × 2.2 mm; flow-rate: 2 ml/min; solvent A: hexanes; solvent B: dichloromethane–methanol (98:2); Gradient: 1% B per min for 13 min, then 10% B per min to 100% B and hold; detector wavelengths: λ_{exc} = 360 nm (CS7-54, CS7-60), λ_{em} > 450 nm (CS4-76, CS3-72).

graphic separation. It has often been assumed that derivatization with a large bulky group tends to make the chromatographic separation more difficult. However, our experiments have shown that in many instances it may lead to enhanced selectivity.

Trace detection of cannabinols and their metabolic products in physiological fluids is of interest in drug research. Fig. 10 shows the chromatographic separation of a mixture of Dns derivatized cannabinols: Δ^9-tetrahydrocannabinol, cannabidiol, and 11-hydroxy-Δ^8-tetrahydrocannabinol. A 10-μm silica column and a gradient system as indicated in the figure were used for this separation. Eluted peaks represent 100 ng quantities of each of the cannabinols. Under ideal conditions minimum detectable quantities appear to be below 5 pg.

Low level detection of amino acids is another area of current interest. Many

fluorometric methods have been developed for their determination. Fig. 11 shows the chromatographic separation of 10 Dns-amino acids on a bonded-phase NH_2 column. The filter system was the same as that used for the Dns cannabinoids. The separation was achieved using gradient elution. Strong eluents are necessary to elute the amino acid derivatives from the column because of the remaining underivatized carboxylic acid groups. MDQ values calculated in terms of free amino acids are given

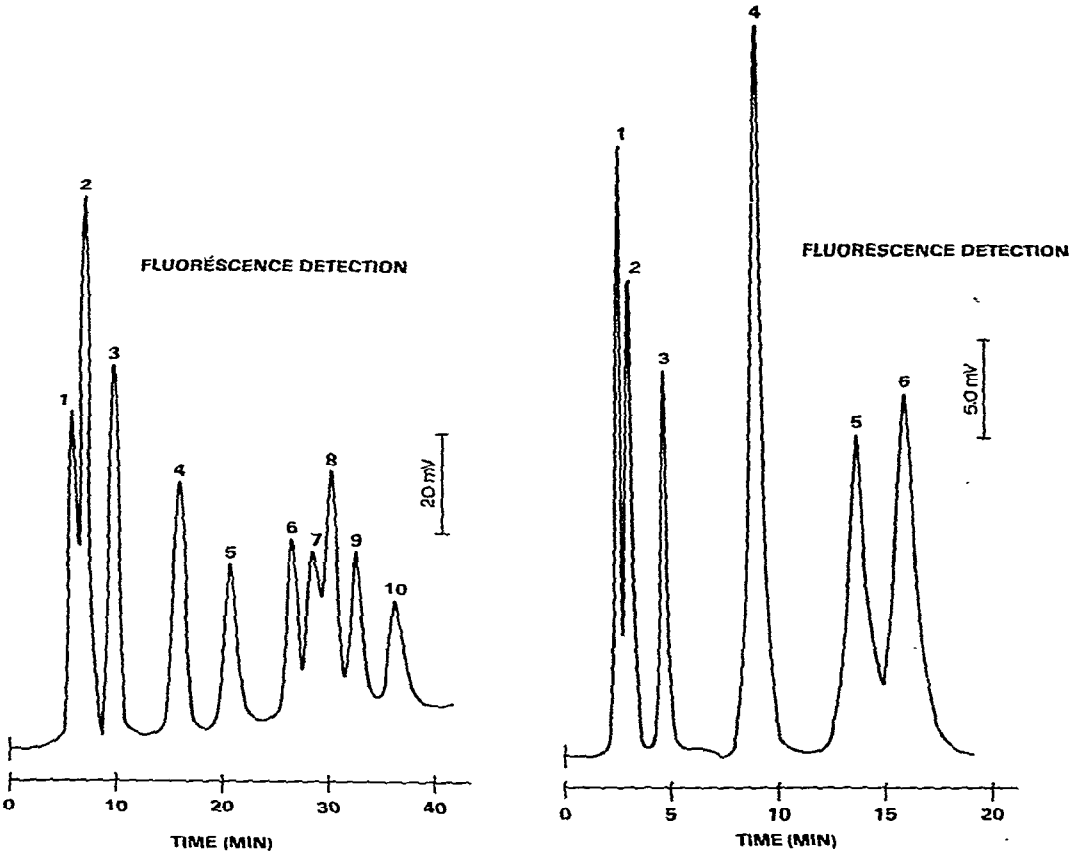

Fig. 11. Separation of selected Dns-amino acid derivatives by normal-phase HPLC. Column: MicroPak-NH_2-10, 25 cm × 2.2 mm; flow-rate: 1 ml/min, solvent A: dichloromethane–acetic acid (99:1) solvent B: acetonitrile–acetic acid (90:10); Gradient: 10% B + 1% B/min for 20 min, then + 3% B/min to 70% B; detector wavelengths: λ_{exc} = 360 nm (CS7-54, CS7-60), λ_{em} > 450 nm (CS4-76, CS3-72). Peaks: 1 = valine (2.0 ng); 2 = leucine (5.8 ng); 3 = alanine (5.5 ng); 4 = glycine (3.9 ng); 5 = hydroxyproline (4.5 ng); 6 = threonine (3.4 ng); 7 = glutamic acid (2.7 ng); 8 = serine (4.2 ng); 9 = asparagine (3.7 ng); 10 = aspartic acid (3.2 ng).

Fig. 12. Separation of selected Dns-amino acid derivatives by reversed-phase ion-pairing techniques. Column: MicroPak-MCH-10, 25 cm × 2.2 mm; flow-rate: 0.5 ml/min, mobile phase: methanol–water (30:70); mixture is buffered and contains 0.01 M $(CH_3)_4NCl$ as counter ion source, see text for more details; detector wavelengths: λ_{exc} = 360 nm (CS7-54, CS7-60), λ_{em} > 450 nm (CS4-76, CS3-72). Peaks: 1 = hydroxyproline (5.7 ng); 2 = serine (5.1 ng); 3 = alanine (4.8 ng); 4 = valine (5.3 ng); 5 = leucine (3.2 ng); 6 = tryptophan (3.7 ng).

TABLE II

MININUM DETECTABLE QUANTITIES OF AMINO ACIDS SEPARATED AS Dns DERIVATIVES

Compound	*MDQ (fmoles)*
Valine	44
Leucine	70
Alanine	113
Glycine	117
Hydroxyproline	200
Threonine	104
Glutamic acid	43
Serine	66
Asparagine	83
Aspartic acid	116

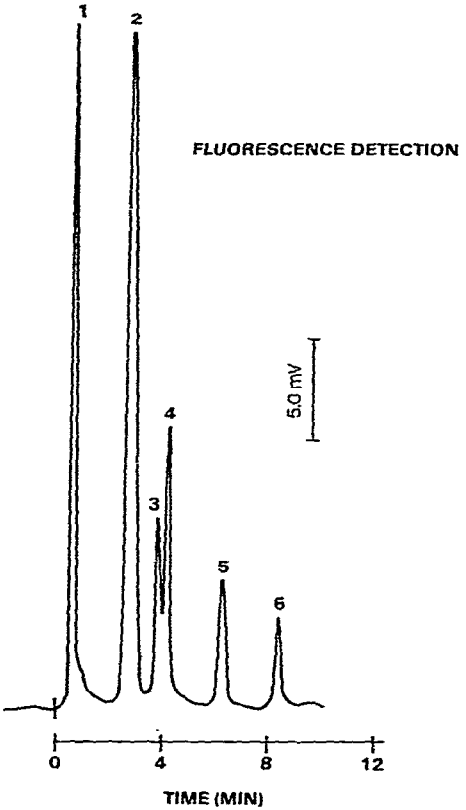

Fig. 13. Separation of Dns polyamine derivatives by reversed-phase HPLC. Column: MicroPak-CH-10, 25 cm × 2.2 mm; flow-rate: 1 ml/min; solvent A: water; solvent B: acetonitrile; gradient: 50% B for 4 min, then + 5% B/min to 100% B; detector wavelengths: $\lambda_{exc} = 360$ nm (CS7-54, DC7-60), $\lambda_{em} > 450$ nm (CS4-76, CS3-72). Peaks: 1 = reagent decomposition product; 2 = propylamine (10 ng); 3 = putrescine (4.2 ng); 4 = cadaverine (4.9 ng); 5 = spermidine (4.1 ng); 6 = spermine (4.0 ng).

in Table II. Improved selectivity for many of the neutral amino acids such as valine and leucine can be obtained if reversed-phase ion pair techniques are employed. Fig. 12 shows such a separation on a reversed-phase column. The solvent system was methanol–water (30:70). The water was adjusted to pH 7.0 with 0.01 *M* sodium acetate and acetic acid. The counter ion was tetramethylammonium ion at the 0.01 *M* level. Note that valine and leucine, which differ by a CH_2 group, are easily resolved ($k' = 8.3$ and 13.2 respectively).

Polyamines such as cadaverine, putrescine, spermidine and spermine are of interest in cancer studies[5] since abnormally high levels of these amines are often found in the urine of cancer patients. These amines are difficult to detect at low levels unless a chromophore is added by derivatization. Fig. 13 shows a chromatogram of the Dns derivatives obtained using reversed-phase chromatography on a bonded-phase CH column. The solvent system was acetonitrile–water and gradient elution was employed. MDQ values are calculated to be in the pg range.

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