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A DUAL-PURPOSE ABSORBANCE–FLUORESCENCE DETECTOR FOR HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A detector has been designed for high-speed liquid chromatography that simultaneously responds to sample absorbance and fluorescence in a single flow cell. Besides the advantages offered by different detection systems, the absorbance measurements provide a method for monitoring the fluorescence "inner filter" effect. The volume of the cell is small; the absorbance segment is 16 μl, and included in it is the 8-μl fluorescence segment. The minimum detectable concentration (signal-to-noise ratio = 2) of quinine in 0.1 *N* sulphuric acid is 0.3 ng/ml. The cell performs as well as an absorbance cell as other small-volume cells.

INTRODUCTION

Molecular fluorescence offers a number of attractive characteristics for sample detection in high-pressure liquid chromatography (HPLC). In comparison with other detection techniques fluorescence affords greater sensitivity to sample concentration and less sensitivity to instrument instability and such macroscopic properties as temperature and pressure. In part this may be attributed to the nature of the measured experimental parameter, which in fluorescence detection is a signal superimposed upon a very low background whereas in, for example, optical absorbance measurements the signal is superimposed upon a high and sometimes unstable background. Furthermore, the selectivity of a fluorescence detector is very good because two experimental parameters must be adjusted that are dependent upon the sample molecular characteristics, *i.e.* the excitation and emission energies. The major disadvantage of fluorescence (or phosphorescence) detection is that not all compounds exhibit it under normal HPLC conditions. However, the large number of fluorescent materials, including biochemicals, foods, drugs, dye intermediates, etc. recommend the detection technique and are the subject of several well-known works[1].

As a qualitative analytical tool, fluorescence detection may be used almost without restriction. However, for quantitative analysis caution is emphasized because of the non-linear relation between sample size and fluorescence intensity. The non-linearity arises from the unequal distribution of light in the sample detection region.

The intensity, P, of excitation light leaving the detector is given by the familiar absorbance relation

$$P = P_0\, 10^{-abC} \tag{1}$$

where P_0 is the detector incident light intensity, a is the absorbtivity, b the light path length in the sample, and C is the sample concentration in grams per liter. The intensity of fluorescence, F, is proportional to the light absorbed

$$F = K\, q\, P_0\, (I - 10^{-abC}) \tag{2}$$

where K is an experimental geometry parameter and q is the quantum efficiency. This relation is commonly expressed as the series

$$F = KqP_0 \left[2.303abC - \frac{(2.303abC)^2}{2!} + \frac{(2.303abC)^3}{3!} - \ldots\right] \tag{3}$$

in which all terms but the first are truncated. The consequence of truncation is shown in Fig. 1. The detector response assumed by truncation is given by line a. In fact, the higher-order terms become important as the sample absorbance approaches unity, and the fluorescence intensity asymptotically approaches a limit given by line b.

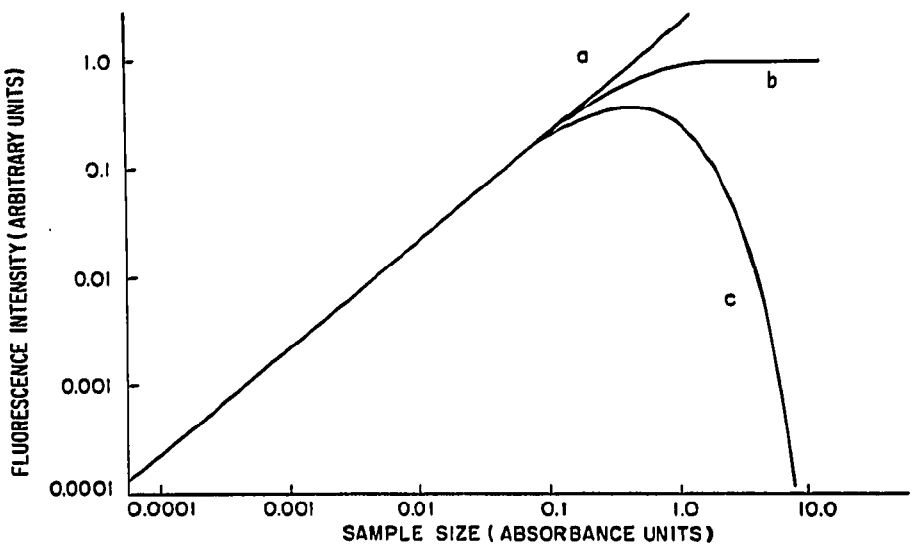

Fig. 1. The calculated fluorescence detector response to sample concentration. K, q, P_0, a, and b are assumed unity.

The presence of absorbing but non-fluorescing components of the eluent in the detector along with the fluorescent sample has the effect of reducing the effective quantum efficiency of the sample and increasing the error of measurement. The error produced by non-fluorescing components is shown in Fig. 2. Error curves are given for

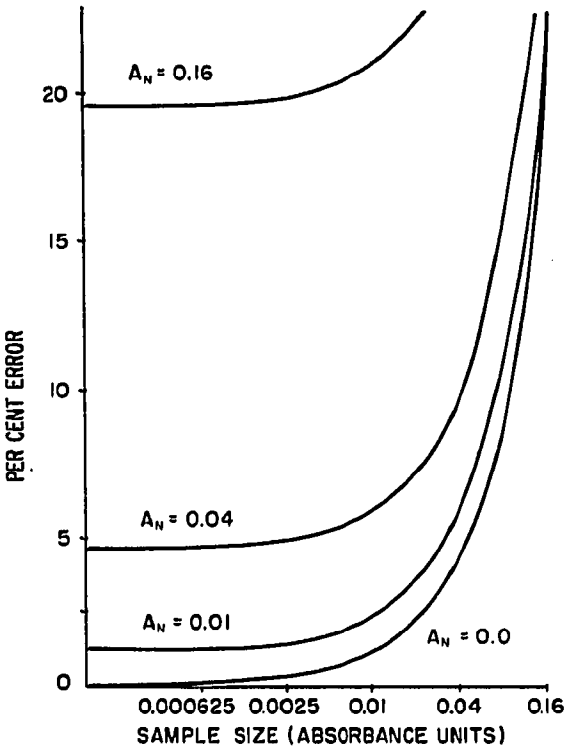

Fig. 2. The per cent error introduced in a concentration determination based upon sample fluorescence without compensation.

four different levels of non-fluorescing component absorbance, A_N. They indicate that absorbance that does not lead to fluorescence limits the accuracy of quantitative measurements regardless of the concentration of fluorescent sample. This is primarily of importance when fluorescent and non-fluorescent components of the sample elute simultaneously, and advantage is being taken of the ability of the detector to respond selectively to the presence of the fluorescent component.

EXPERIMENTAL

Several of the advantages of simultaneous fluorescence and absorbance measurements have been discussed besides the obvious desirability of including two types of detectors in one framework. Several designs were tested with the best shown in Fig. 3. The column effluent flows through a 1-mm I.D. quartz tube (f) that is capped at each end by quartz windows (a). The excitation and absorbance light beam passes coaxially through the tube traversing 20 mm of eluent. The photomultiplier (i) detects only the fluorescence light produced along the central 50% of the tube so that the effect of light scattering at the cell ends is reduced. The total cell volume is 16 μl, and the fluorescence segment is 8 μl. The eluent enters and exits the cell through flow rings (c) at each end of the cell. The efficiency of the cell is maximized by providing the fluorescence chamber (g) with a highly reflective surface.

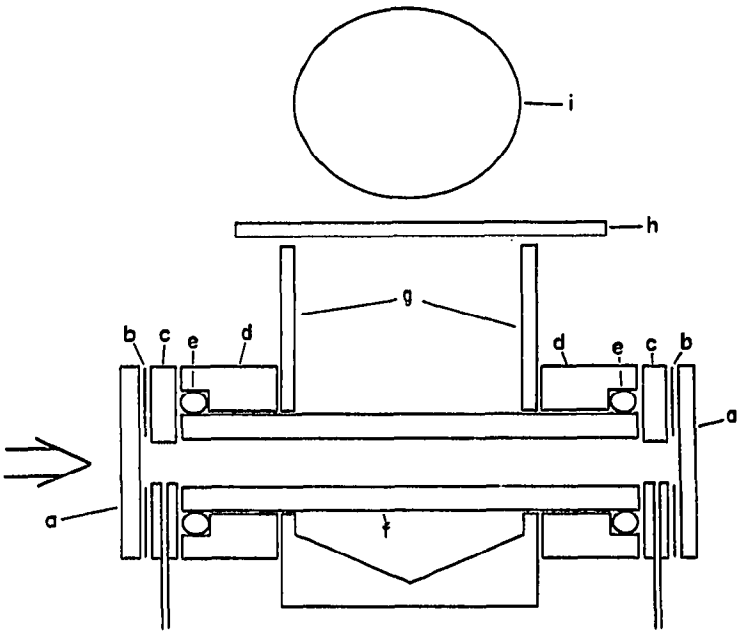

Fig. 3. The flow cell. (a) Quartz window; (b) gasket; (c) flow ring; (d) scatter baffle; (e) O-ring; (f) quartz window; (g) reflective fluorescence chamber; (h) emission filter; (i) photomultiplier.

The flow cell was incorporated into an existing Du Pont Model 835 filter photometer as shown in Fig. 4. For most uses it is found desirable to use a mercury medium-pressure lamp, which provides much more intense radiation than that obtainable from a spectrophotometer. Either interference or absorbance filters are used to isolate the desired excitation lines. The emission energy is selected with Corning absorbance cut-off filters. The fluorescence linear amplifier provides seventeen binary-step ranges with three ranges of background fluorescence suppression. The associated absorbance components and performance have been previously described[2].

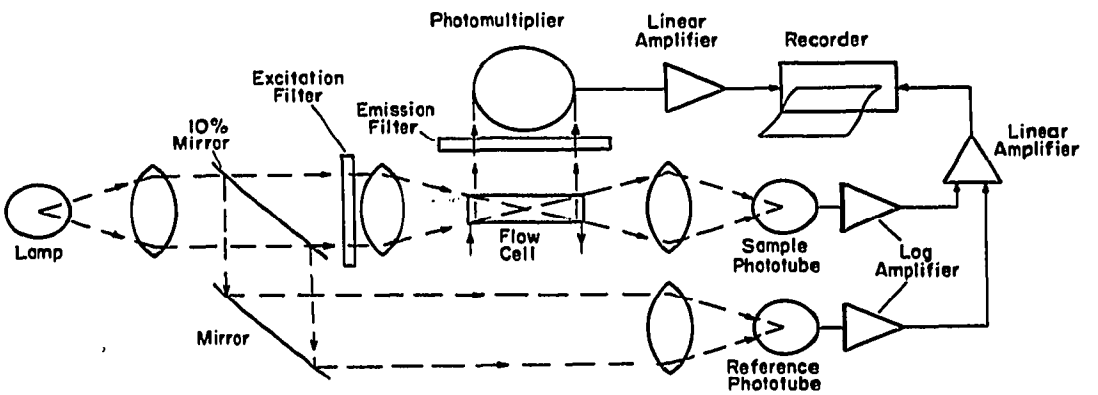

Fig. 4. The absorbance–fluorescence detector.

As with all precision fluorescence cells, the fluorescence segment of the cell volume does not include the sample region where the excitation beam enters the cell in order to reduce the high background signal produced by light scattering at the windows. This subjects the cell to a further perturbation caused by the absorbance of light before it enters the fluorescence-sensitive region and results in an effective variation of incident-light intensity with sample size. In the present cell one fourth of the excitation-light path preceded the fluorescence segment and the incident-light intensity varies by $10^{-A_T/4}$, where A_T is the total cell absorbance. The effect is seen in Fig. 1, line c, where the fluorescence intensity actually decreases at high sample concentrations. This is the so-called "inner filter" effect and may be a hazard in that two sample sizes may correspond to a single fluorescence intensity. However, by simultaneously monitoring the sample absorbance it is easily determined on which side of the response curve the measurement exists and if it is valid. Near the peak of the response curve in Fig. 1 a correction factor may be applied which compensates for absorbance by the fluorescent sample:

$$F_{cor} = F_{obs} \frac{1.15\, A_T}{10^{-A_T/4} - 10^{-3A_T/4}} \tag{4}$$

This correction is effective but must be used with caution because at high concentrations other effects, such as selfquenching, may be involved.

The chromatograms were obtained using a Du Pont Model 841 liquid chromatograph.

RESULTS

The detector performs exceedingly well in that it produces excellent fluorescence response without compromising absorbance performance. The linearity of response was checked with fresh quinine solutions prepared in the column eluent, 0.1 *N* sulphuric acid. A 0.5-m Zipax® column was used to retain the sample peak enough so that it would be separated from the solvent front. It was observed that over several hour's time span during which the calibration was performed, a significant degradation of the dilute solutions occurred as was evidenced by the formation of shoulders on the sample peak.

The linearity data are presented in Fig. 5. They indicate a 1% range of 10^4 for both fluorescence and absorbance with a combined range of 10^6. Of course, the ranges are extended if the error requirements are relaxed, or, in the case of fluorescence, if the correction factor shown in eqn. 4 is applied. It is assumed that the first low absorbance value is due to the inaccuracy of the measurement and not to instrument discrimination. The fluorescence linear range is dependent upon the sample quantum efficiency and would be smaller for less efficient systems.

The fluorescence sensitivity of the detector is illustrated in Fig. 6 in which consecutive 50-pg samples of quinine were injected on the column. The maximum concentration of quinine in the cell was 0.3 ng/ml at the observed signal-to-noise ratio of 2:1. The concentration was determined by recording the chromatographic peaks at high speed and integrating the total peak area and the area at the peak maximum corresponding to an instantaneous cell volume at the recorded flow-rate. The baseline

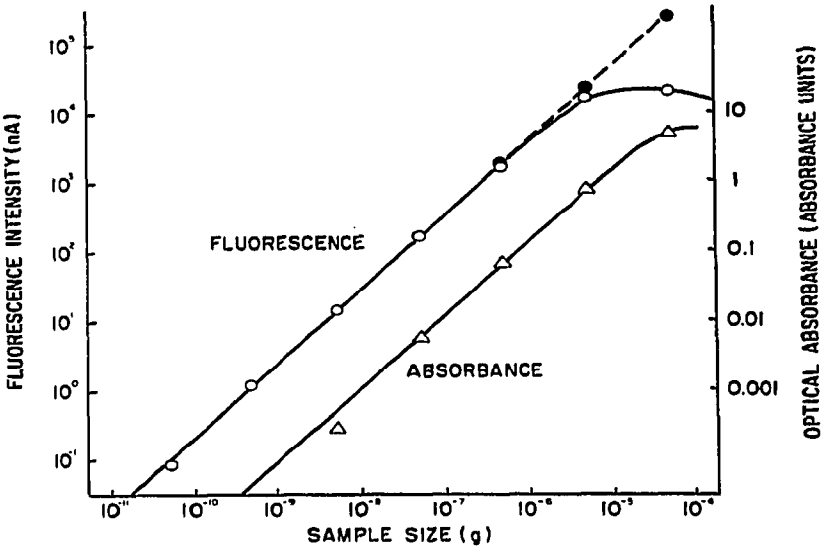

Fig. 5. The measured fluorescence and absorbance response to sample concentration. ○—○, Fluorescence response; ●---●, corrected fluorescence response; △—△, absorbance response.

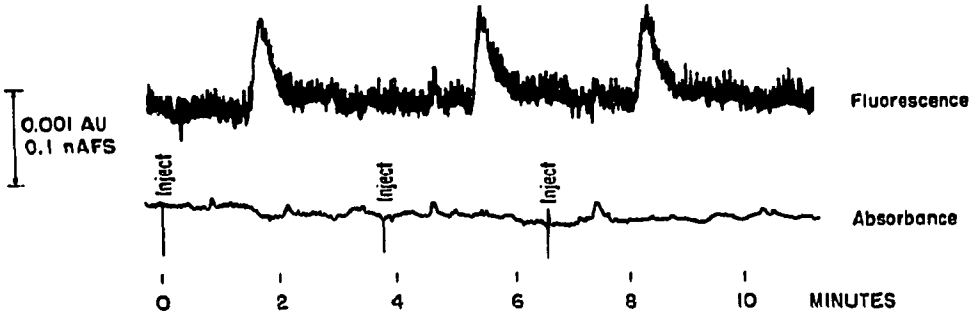

Fig. 6. The fluorescence detection limit. Sample, 50 pg quinine injected in 5 μl; instrument, Du Pont Model 841 liquid chromatograph; column, Zipax® 50 cm × 2.1 mm I.D.; mobile phase, 0.1 *N* sulphuric acid; pressure, 1000 p.s.i.; flow-rate, 1 ml/min. Absorbance photometer: 365 nm, 0.01 AUFS. Fluorescence: 365 nm excitation, Corning 3-72 filter emission, 1 nAFS.

noise is not constant but dependent upon a number of factors including the lamp intensity and type of filters used.

A typical application of the detector would be aflatoxin analysis of food products. In Fig. 7 is shown the chromatogram obtained from a mixture of standards separated with a Zorbax-Sil® 25-cm column. The G_1 and G_2 toxins exhibit much stronger fluorescence than B_1 and B_2 (ref. 3). The low absorbance variation, less than 0.002 absorbance units (AU), attests to the accuracy of the fluorescence intensity measurement. Furthermore, when the fluorescence technique becomes inapplicable because of reduced quantum efficiency, the traditional absorbance measurements may be relied upon as in the case of B_1 and B_2.

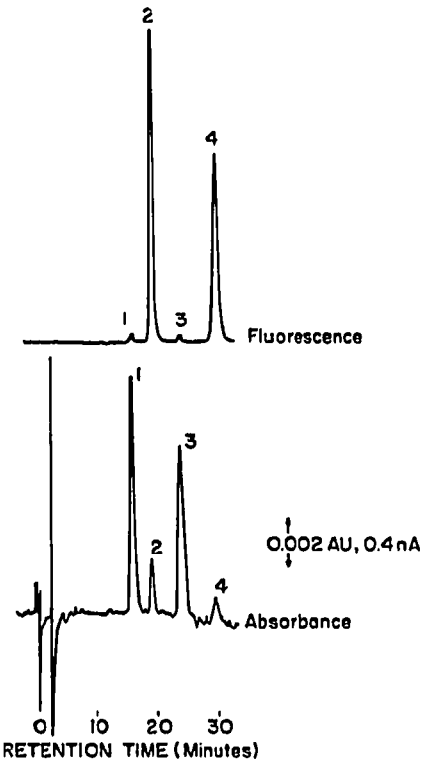

Fig. 7. Simultaneous absorbance and fluorescence detection of aflatoxins. Samples: (1) 12.5 ng aflatoxin B_1; (2) 3.8 ng aflatoxin G_1; (3) 12.5 ng aflatoxin B_2; (4) 3.8 ng aflatoxin G_2. Instrument, Du Pont Model 841 liquid chromatography; column, Zorbax-SIL® 25 cm × 2.1 mm I.D.; mobile phase, methylene chloride (50% H_2O-saturated)–chloroform (50% H_2O-saturated)–methanol (60:40:0.3); pressure, 1500 p.s.i.; flow-rate, 0.5 ml/min. Absorbance photometer: 365 nm ,0.02 AUFS. Fluorescence: 365 nm excitation, Corning 3-72 filter emission, 4 nAFS.

ACKNOWLEDGEMENT

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