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A FLUORESCENCE DETECTOR FOR HIGH-SPEED LIQUID CHROMATOGRAPHY*

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SUMMARY

A simple, inexpensive and efficient detector for monitoring high-speed liquid chromatographic columns has been developed. The detector consists of a commercially available fluorimeter equipped with a small volume flow-through cell. The short-term noise obtained with this cell corresponds to 2 p.p.b. of quinine sulfate or $1.5 \cdot 10^{-11}$ g for a 7.5 μl cell volume. Variations in solvent velocity do not affect the baseline. The detector response is linear over a wide range for both peak height and peak area. Injections of 3 ng amounts of a fluorescent compound have been detected consistently.

INTRODUCTION

During the last 3–4 years, a large proportion of the studies concerned with high-speed liquid chromatography (HSLC) has been directed toward the development of new detection systems. Excellent discussions of various commercial detectors have appeared elsewhere[1–4]. Fluorescence detectors have been available commercially for some time[3], but there has been a definite lag in their application to the monitoring of HSLC columns. Fluorescence detectors are not as universal as other HSLC-detectors such as refractive index and UV detectors. This, coupled with the cost of such detectors, could be responsible for this lack of published data. However, a detector that shows some degree of selectivity can be useful for certain analytical problems. This work describes the conversion of a Turner fluorimeter into an HSLC detector and outlines some of the advantages associated with fluorescence detection. A flow-through cell is available for the Turner fluorimeter but is unsuitable for HSLC[5].

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EXPERIMENTAL

Fluorescence detector

The detector used in this work consisted of a Turner III fluorimeter* equipped with a thin-layer chromatogram scanning door (Model 110-700). Fig. 1 shows a schematic diagram of the optics of the Turner fluorimeter. Radiation from the light source passes through the primary filter and onto the flow-through cell via the right-hand side of the hole in the door of the fluorimeter. Fig. 2 shows the position of the flow-through cell. The fluoresence produced in the cell is collected at the left-hand side of the hole by a leucite light-pipe, which directs it to a secondary filter placed in front of the phototube.

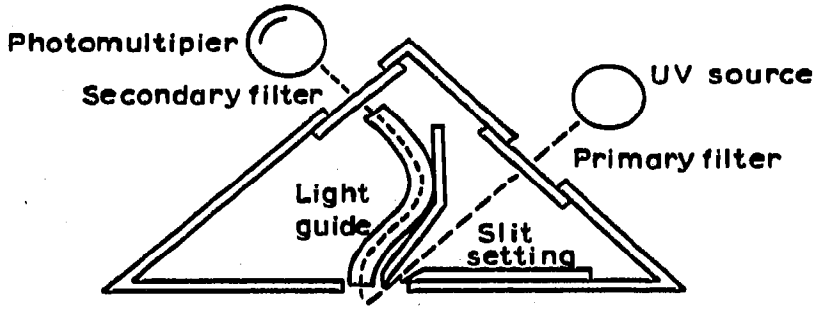

Fig. 1. Optical arrangement of detector.

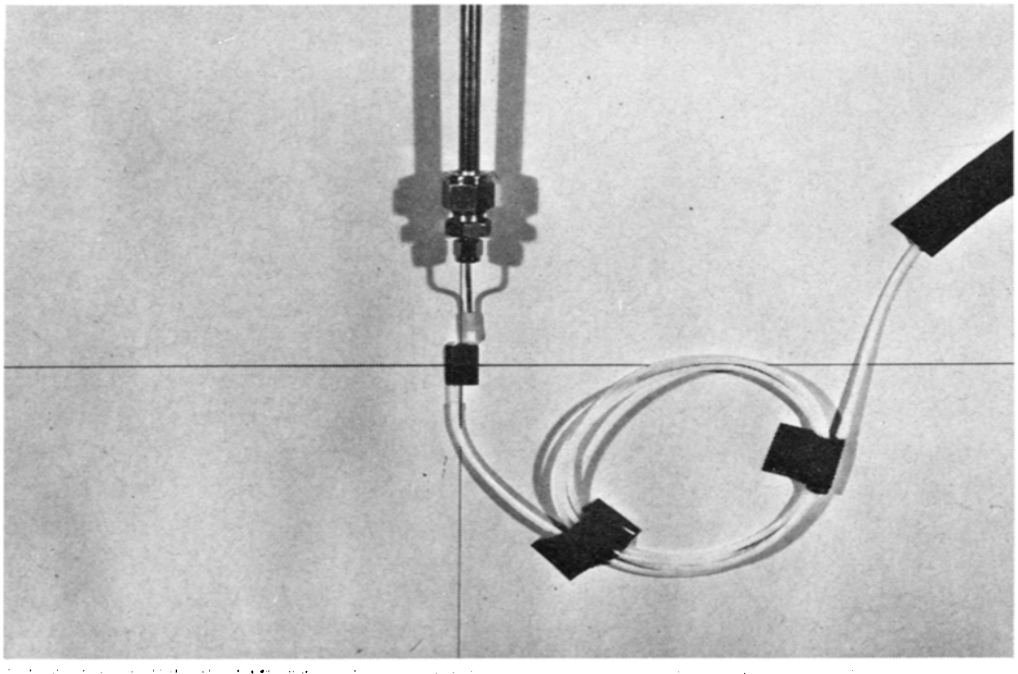

Fig. 2. Flow-through cell.

The connecting tubing was covered with several layers of black cloth to eliminate stray radiation. Fig. 3 shows the location of the detector in the chromatographic system.

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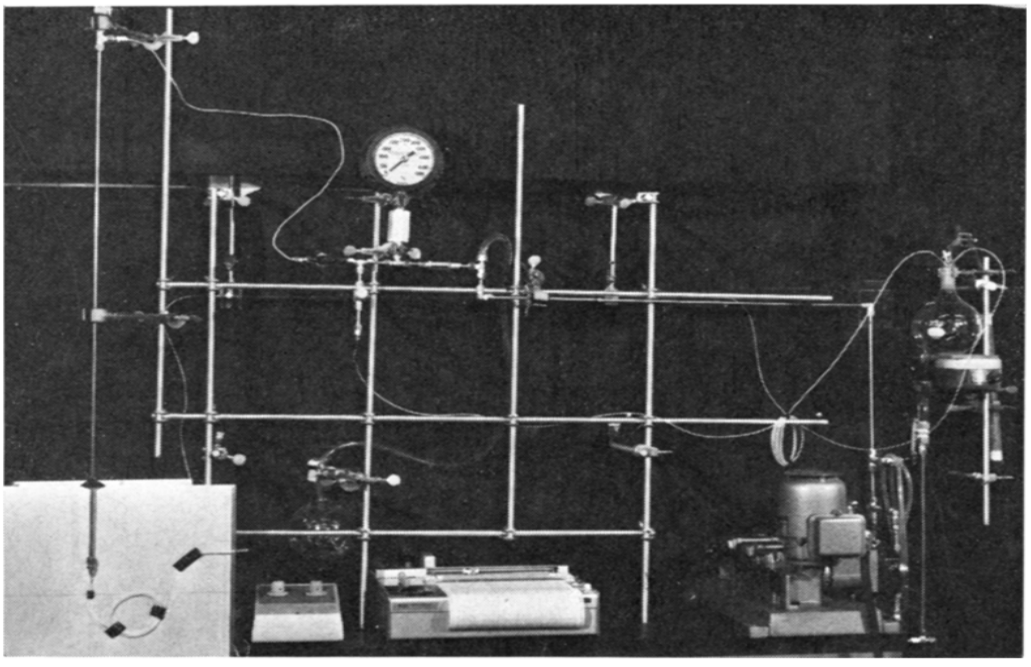

Fig. 3. High-speed liquid chromatograph.

Detector cell

The flow-through cell was made by drawing out soft glass tubing so that the final diameter was about 1/16 in. O.D. This tubing was bent into the desired shape and then connected to the 1/16 in. O.D. steel capillary tubing, extending from the chromatographic column, by means of a short piece of 1/16 in. I.D. PTFE tubing. Care was taken to ensure that the ends of the steel and glass tubing were in close contact. The cell exit was fastened to 1/16 in. I.D. PTFE tubing that directed the eluent into a flow-measuring device. As it was found that the PTFE tubing acted as a light-pipe and produced high and irregular backgrounds, about 2 ft. of this tubing was fastened to the detector in the form of a coil (Fig. 2) and covered with black cloth to avoid this effect.

Chromatographic system

The apparatus which was constructed for this work is shown in Fig. 3. The design of this equipment is based on that described elsewhere[6]. The injection port and safety relief valve have been described elsewhere[7]. Samples were injected directly on top of the column with a long-needle (10 cm) 10 μl Unimetric syringe*. The pump was a three-head diaphram pump (Type S4, Orlita K. G., Giessen, G.F.R.), which is capable of operating at pressures up to 350 atm. The detector output was connected to a Autolab 6300 digital integrator**. The signal from the integrator was amplified with a Beckman 73490 scale expander*** and then displayed on a Servogor strip-chart recorder§.

* 1010-T, Unimetrics Universal Corp., Anaheim, Calif. 92801.
** Vidar Autolab, Mountain View, Calif. 94040.
*** Beckman Instruments, Inc., Fullerton, Calif. 92634.
§ Carl Zeiss, Oberkochen, G.F.R.

The columns were made from 2.4 mm I.D. seamless stainless-steel* and were packed with uncoated Zipax®**. Two column lengths, 100 cm and 2.5 cm, were used. The short column served to study the band broadening caused by sample injection, cell flow patterns and the response time of the detector.

A UV detector*** was used for comparison purposes. The solvents were either spectranalyzed grade or re-distilled reagent grade.

RESULTS AND DISCUSSION

Detector characteristics

After an initial warm-up period of *ca.* 30 min, the detector exhibited a very stable baseline, as shown in Fig. 4. A similar baseline was obtained over much longer periods. The average peak to peak noise corresponds to 2 p.p.b. (parts per 10^9) of quinine sulfate in a 7.5 μl cell. This sensitivity compares favorably with that reported for a commercial fluorescence detector§, which has a noise level equivalent to 1 p.p.b. of quinine sulfate for a 10 μl cell.

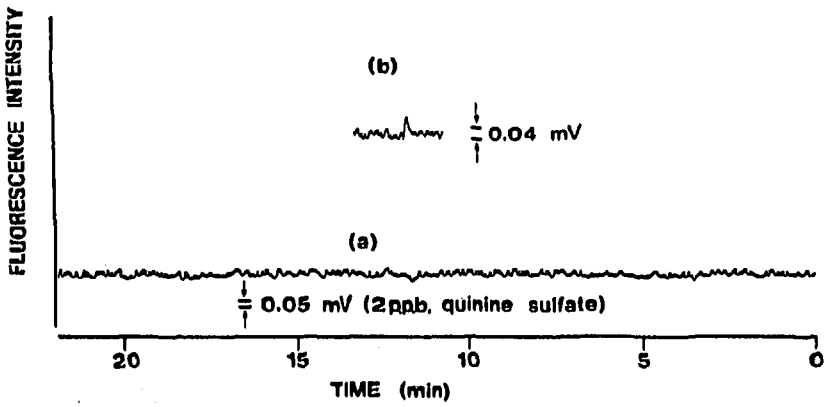

Fig. 4. (a) Background noise. Solvent, *n*-hexane. (b) Chromatographic peak corresponding to 3 ng of NBD ($k' = 0.18$). Solvent, *n*-hexane–acetone (8:2); flow-rate, 1.1 ml min^{-1}; 100 cm column.

The baseline shows no dependence on the flow-rate. The use of only one or two heads of the pump or a complete stop of flow did not have any visible effect on the baseline. This is an obvious advantage over refractive index and UV detectors, as simple and inexpensive pumps can be used without sophisticated damping devices. The response of the detector is also not affected by changes in solvent composition and consequently baseline stability will not be a problem for gradient elution techniques.

Samples containing 3 ng of 4-methylamino-7-nitrobenzo-2,1,3-oxadiazole (NBD) could be detected consistently.

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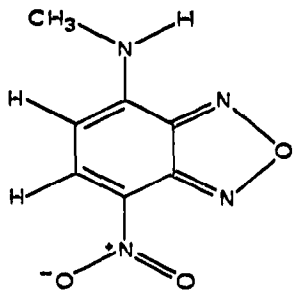
* American Instrument Co., Inc., Silver Spring, Md.
** E. I. du Pont de Nemours, Wilmington, Del.
*** Pharmacia Fine Chemicals Inc., Piscataway, N.J. 08854.
§ Laboratory Data Control, Inc., Riviera Beach, Fla. 33404.



NBD

A typical peak obtained for this sample is shown in Fig. 4. NBD is used for the fluorigenic labelling of pesticides[8]. The fluorescence intensity of NBD is nine times higher than that for quinine sulfate, as measured with an Aminco-Bowman spectrophotofluorimeter*. Consequently, the peak shown in Fig. 4 corresponds to the injection of 27 ng of quinine sulfate. However, it is assumed that the detection limit for NBD could be further reduced by using an excitation source that will produce more intense emission at the excitation wavelength for NBD (465 nm for 20% acetone in *n*-hexane). The light source used in this work was a general-purpose mercury lamp, which has a major emission at 360 nm as well as longer wavelength lines at 405, 436 and 546 nm. Such a lamp is ideally suited for quinine sulfate ($\lambda_{excitation}$ = 352 nm). As the dilution factor for a sample passing through an HSLC column is of the order of 5–100 (ref. 9), the detection limit (twice the noise level) for the injection of quinine sulfate should be 0.2–4.0 ng.

In Fig. 5 it is shown that the chromatographic peaks produced by this detector are symetrical and reproducible. The response of the detector has a range of linearity that is greater than a factor of 340 for both peak area and height measurements (Fig. 6). This range can probably be extended to much higher concentrations, as the fluorimeter was set to maximum sensitivity in this work. The average relative devi-

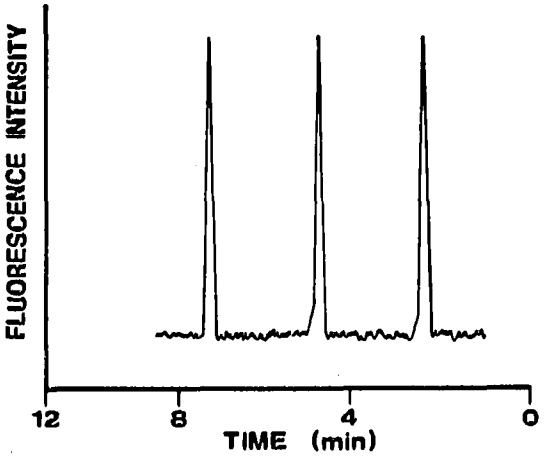


Fig. 5. Reproducibility of chromatographic peaks. NBD, 64 ng; flow-rate; 0.5 ml min^{-1}; 100 cm column.

* American Instrument Co., Inc., Silver Spring, Md.

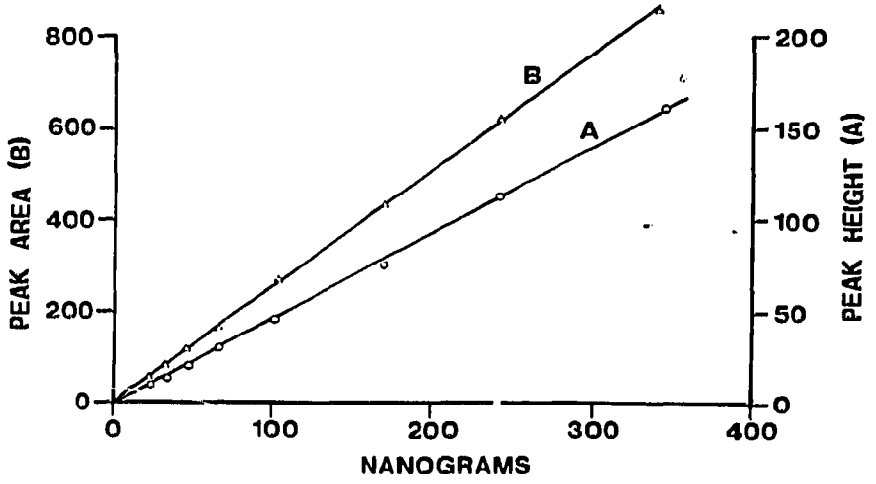


Fig. 6. Calibration curves. Sample, NBD; solvent, *n*-hexane–acetone (8:2); flow-rate, 0.5 ml min^{-1}; 100 cm column.

ations from the mean for the points shown in Fig. 5 (each point corresponds to the average of at least three samples) were 1.8% and 1.5% for peak area and peak height measurements, respectively.

Cell characteristics

The detector cells are made by drawing out glass tubing, and consequently very thin cell walls are obtained. This is an advantage with respect to light transmission and the production of different cell shapes. The physical stability of the cell, which is also important, seems to be entirely satisfactory. As both ends of the cell are fastened to PTFE tubing, there is some freedom of movement, which facilitates installation and removal and prevents breakage of the cell. At excitation wavelengths below 350 nm the cell should be constructed of quartz.

Because of this procedure used for making the cells, duplication of cell volumes is difficult, but this is not critical provided that the same cell is used for a particular set of experiments. As these cells are inexpensive and simple to make, it is simple to increase the cell volume for an enhancement sensitivity (larger cross-section) provided that the loss of resolution is not serious. Some studies have shown that an appreciable increase in cell volume can be tolerated with surprisingly little loss in resolution[9,10]. In the present work, it was found that an increase in the cell volume from 7.5 to 20 μl produced no noticable increase in band width for a 100 cm column.

The shape of the cell was found to be an important factor with respect to maximum efficiency. Fig. 7 shows the shapes of two cells that were studied. A number of other shapes were investigated but they gave no significant advantage. It was found that the use of the straight cell resulted in a 12% increase in band width compared with the bent tube (Fig. 7). This increase can be attributed to poor mixing in the straight cell. The small effluent stream from the steel capillary tube has a much faster velocity than the liquid in the larger glass cell and a considerable distance is required for complete mixing. The processes occurring in the two different cells are shown schematically in Fig. 5. A single bend is apparently sufficient to improve the radial mass transfer.

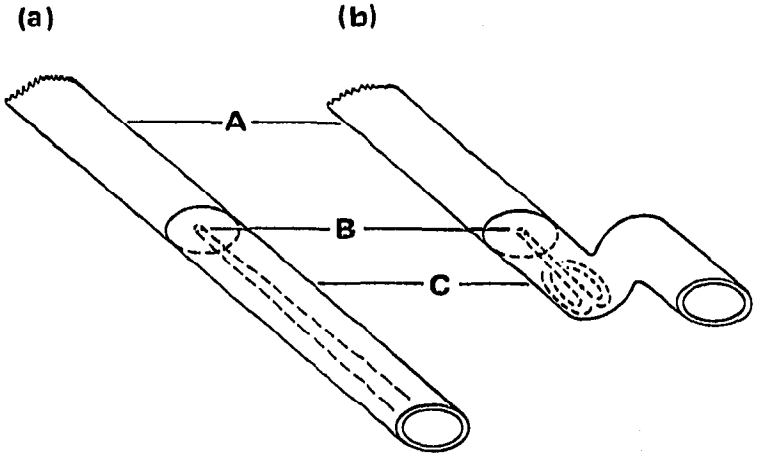


Fig. 7. Cell flow patterns. A, steel capillary tubing; B, exit from A; C, glass cell. Flow patterns are approximated by dashed lines.

The time variance of a chromatographic peak, σ^2_t, is given by the following equation:

$$\sigma_t^2 = (\sigma_c)^2_t + (\sigma_i)^2_t + (\sigma_d)^2_t \quad (1)$$

where $(\sigma_c)^2_t$ is the time variance due to processes occurring in the column, $(\sigma_i)^2_t$ is the time variance caused by sample injection and $(\sigma_d)^2_t$ is the time variance from processes occurring in the cell of the detector.

It is important that the contribution to band broadening by $(\sigma_{it})^2_t$ and $(\sigma_d)^2_t$ should be small compared with $(\sigma_c)^2_t$. An estimation of $(\sigma_d)^2_t$ was obtained by a procedure similar to that used by KIRKLAND[10]. By studying band broadening for a 100 cm and a 2.5 cm column and assuming that there is a linear relationship between $[(\sigma_c)^2_t + (\sigma_d)^2_t]$ and column length, it was found that $(\sigma_d)^2_t$ was 0.5 sec^2 for a flow-rate of 0.6 cm sec^{-1}. This value was corrected for $(\sigma_i)^2_t$. If a 5% increase in band width due to the detector is acceptable, then the minimum band width can be found as follows. The band width, W_t, caused by processes in the column is

$$W_t = 4(\sigma_c)_t \quad (2)$$

For a 5% increase in W_t due to $(\sigma_d)_t$

$$(W_t + 0.05\ W_t)^2 = 16(\sigma_c)^2_t + 16(\sigma_d)^2_t \quad (3)$$

Substitution of the experimentally determined value of $(\sigma_d)^2_t$ and eqn. 2 into eqn. 3 gives

$$17.6\ (\sigma_c)^2_t = 16\ (\sigma_c)^2_t + 8$$

$$(\sigma_c)_t = 5 \text{ sec} \quad (4)$$

$$W_t = 9 \text{ sec}$$

This means that the contribution to W_t by $(\sigma_d)_t$ for any peak with W_t smaller than 9 sec at a flow-rate of 0.6 cm sec^{-1} will be greater than 5% of W_t. However, the minimum W_t at this flow-rate (*i.e.*, $K' = 0$) is 26 sec. Consequently, the increase in W_t is negligible.

The value obtained for $(\sigma_d)^2_t$ corresponds to 0.2 cm^2 for $(\sigma_d)^2$. This agrees with previous results obtained under similar experimental conditions with a UV detector[10].

As a final check of the importance of $(\sigma_d)^2_t$, the cell was placed between the outlet of a 2.5 cm column and the inlet of a commercial UV detector. No increase in band broadening compared with that for the UV detector alone could be observed.

The above results suggest that moderate increases in cell volume, which will result in increased sensitivity, can be tolerated without serious loss in resolution.

Detector response

The disadvantage of this detector is the slow response time. On sudden application of a light signal, the detector required 5.5 sec to reach a constant maximum value. The time required for the response to decrease to the baseline value was 7.5 sec.

The effect of this slow response was noticed as an unusually rapid loss of efficiency or resolution when the solvent velocity, μ, was increased. The HETP *versus* μ curve (Fig. 8) has a sudden upward trend above 1.7 cm sec^{-1}. Such behavior is unusual and can be attributed only to the sluggish response of the detector. As W_t at this point is 10 sec, this is the minimum allowable peak width for maximum resolution at any velocity. A comparison of peak areas for the same sample at different velocities revealed that, after correcting for different residence times in the cell, the areas were identical until the peak width fell below 10 sec.

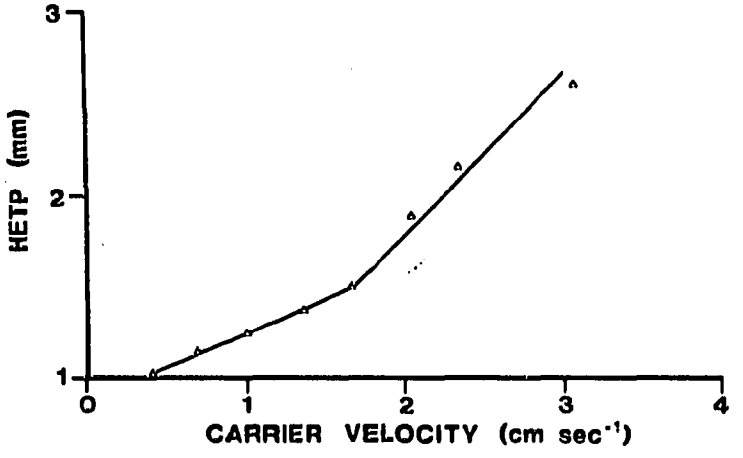


Fig. 8. Relationship between HETP and carrier velocity.

In the above investigation of band broadening processes, it was necessary to determine $(\sigma_i)^2_t$. A linear relationship was found between $(\sigma_i)^2_t$ and injection volume (1–10 μl) for the 2.5 cm column. The value of $(\sigma_i)^2_t$ for a solvent velocity of 0.6 cm sec^{-1} was 0.5 sec^2 per microliter injected. This means that the extra column variance $(\sigma_i)^2_t$ due to the injection process is as important (if not more so) than that due to the detector, $(\sigma_d)^2_t$. This agrees with results found elsewhere[10].

CONCLUSIONS

The detector described in this paper offers an inexpensive and selective means for monitoring high-performance liquid chromatographic columns. The Turner fluori-

meter equipped with the chromatogram scanning door is common to many laboratories and conversion to HSLC can be accomplished quickly and simply. The degree of selectivity and sensitivity can be modified by the appropriate choice of primary and secondary filters and light source. For certain analytical problems the enhanced selectivity of this detector, compared with UV and refractive index detectors, could be advantageous, especially when used in conjunction with one of these detectors. A particularly promising area of application is HSLC in conjunction with fluorigenic labelling procedures[11]. Such techniques would permit the expansion of the range of applicability of fluorescence detectors without serious losses in selectivity. The complete insensitivity of fluorescence detectors to flow patterns could permit the construction of low-cost HSLC instruments with simple pumping systems.

The response time of the detector is a disadvantage for faster solvent velocities. This is not a serious drawback, however, as velocities up to 2 cm·sec^{-1} can be used. The possibility of decreasing the response time is currently under investigation.

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REFERENCES

1 H. VEENING, *J. Chem. Educ.*, 47 (1970) A549.
2 H. VEENING, *J. Chem. Educ.*, 47 (1970) A675.
3 H. VEENING, *J. Chem. Educ.*, 47 (1970) A749.
4 S. H. BYRNE, JR., in J. J. KIRKLAND (Editor), *Modern Practice of Liquid Chromatography*, Wiley-Interscience, New York, 1971, Ch. 3.
5 K. J. BOMBAUGH, in J. J. KIRKLAND (Editor), *Modern Practice of Liquid Chromatography*, Wiley-Interscience, New York, 1971, Ch. 10.
6 R. VIVILECCHIA, M. THIEBAUD AND R. W. FREI, *J. Chromatogr. Sci.*, 10 (1972) 411.
7 R. M. CASSIDY AND R. W. FREI, *Anal. Chem.*, in press.
8 J. F. LAWRENCE AND R. W. FREI, *Anal. Chem.*, in press.
9 S. R. BAKALYAR, *Amer. Lab.*, 3 (6) (1971) 29.
10 J. J. KIRKLAND, *Anal. Chem.*, 40 (1968) 391.
11 R. W. FREI AND J. F. LAWRENCE, *J. Chromatogr.*, 61 (1971) 174.