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THE PERFORMANCE OF SOME CELL DESIGNS FOR LASER-INDUCED FLUORESCENCE DETECTION IN OPEN-TUBULAR LIQUID CHROMATOGRAPHY*

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SUMMARY

The applicability of fluorescence detection in miniaturized cells using a helium–cadmium laser as the excitation source at 325 nm to open-tubular liquid chromatography has been studied. The investigated cells were fused-silica capillaries of 25 and 10 μm I.D., comprising part of the column or being coupled separately to it, and a sheath flow cell. The volume standard deviation of the various cells was found to be 0.1–2 nl which makes them suitable for the above purpose. The mass detection limits were found to be in the attogram range. However, the concentration detection limits, were found to be one to two orders of magnitude higher than those obtainable with conventional sized fluorescence detectors. This is mainly caused by the instability of the laser.

INTRODUCTION

The advantages of open tubular liquid chromatography (OT-LC) with respect to separation speed and efficiency can be exploited only when the external contribution to peak broadening is kept to the order of a few nanolitres[1,2]. As far as the injection volume is concerned, this is easily achieved by using split injection techniques. However, due to optical problems involved when using conventional light sources, a decrease in the cell volume is not possible without a severe loss in sensitivity. Fluorescence detection can be carried out successfully with laser excitation[3,4] because of the possibility to focus all of the available radiation in extremely small volumes.

In OT-LC the values of the time constants are not critical. From the data of Knox and Gilbert[1], it can be derived that the standard deviations will be over 1 s, thus allowing a minimum time constant (T_{RC}) of 0.3 s. In this respect the situation is comparable to that in conventional high-performance liquid chromatography (HPLC).

In this work we have investigated the limitations of laser fluorescence detectors with respect to extra peak broadening and detection limits. We decided to use a relatively simple and inexpensive laser which might be considered for a more general application in the near future. The study involved the evaluation of three cell designs, *viz.*, on-column detection, fused-silica capillary cells and a sheath flow cell.

MATERIALS AND METHODS

Chemicals

The solvents used were analytical and Uvasol grade methanol from Baker (Phillipsburgh, NJ, U.S.A.) and Merck (Darmstadt, F.R.G.) respectively, and deionized water, filtered through a PSC filter assembly (Barnstead, Boston, MA, U.S.A.). Prior to use, all solvents were degassed by vacuum suction over 0.5-μm acetate filters (Millipore, Bedford, MA, U.S.A.).

The test compounds used were anthracene, fluoranthene, pyrene, fluorescein (Janssen, Beerse, Belgium) and quinine sulphate (BDH, Poole, U.K.). Stock solutions of the polycyclic aromatic hydrocarbons (PAHs) were prepared in methanol, the quinine sulphate was dissolved in 0.1 N sulphuric acid and the fluorescein was dissolved in methanol–1 M sodium hydroxide (1:1). Spectral data for the PAHs were taken from ref. 5.

Apparatus

Fig. 1 shows the general experimental set-up. The system consisted of a Series 4100 constant-flow pump (Varian, Walnut Creek, CA, U.S.A.), a Model 7120 central-port injection valve (Rheodyne, Berkeley, CA, U.S.A.), a home-made splitting device, fused-silica capillaries of different lengths and internal diameters (SGE, Ringwood, Victoria, Australia) and various home-made detection cells, which will be described below.

As a light source, a Model 4110 H-uv helium–cadmium laser (Liconix, Sunnyvale, CA, U.S.A) was employed, operated at 325 nm. At this wavelength the specified minimum power is 2.5 mW. However, after about 1000 h of use, the power had decreased to *ca.* 1.2 mW. Tube replacement became necessary after about 1700 h of operation, due to the formation of so-called colour centres in the brewster win-
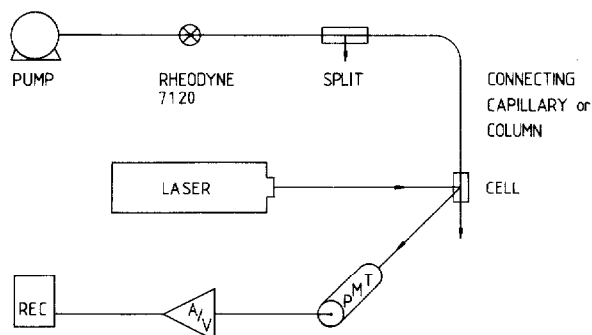

Fig. 1. Diagram of the experimental set-up. A bandpass filter and an appropriate lens were inserted in the laser beam. At the emission side, a lens and a monochromator or a cut-off filter were used. A/V = Current-to-voltage convertor; REC = recorder.

dows, because of the UV absorption by these centres, laser action at the 325-nm line ceases. The line at 442 nm is hardly affected by this effect[6].

Optics

A 325-nm bandpass filter was inserted in the laser beam. In order to focus the beam an appropriate quartz lens was used. The focal length, f, was calculated from the specified full angle divergence, θ, of the laser using the approximate equation

$$f = 2r/\theta \tag{1}$$

in which r is the radius of the illuminated spot[7]. The desired radius can be derived from the size and geometry of the cell used, *e.g.*, for a cylindrical fused-silica cell r should be smaller than the internal radius.

The light emitted under a 90° angle was collected by a fresnel lens, $f = 16$ mm and aperture 0.6, then passed through a 420-nm cut-off filter or a Model 82-410 Ebert monochromator (Jarrell-Ash, Waltham, MA, U.S.A.) and the intensity was measured with a Type 6255 S photomultiplier tube (PMT) (EMI, Hayes, U.K.). The resulting current was converted into a voltage by means of a Diomod 72-W amplifier (Knick, Berlin, F.R.G.) with adjustable low-pass filter and gain.

Cell designs

Three cell designs were investigated: a fused-silica tube which forms the end of the open-tubular column (on-column detection), a separate piece of fused-silica capillary, coupled to the end of the column (coupled cell) and a sheath flow cell.

On-column detection. In this detection mode the laser beam is focused on the end of a fused-silica column, the protective polymer layer of which has been burned off over a short distance. It can be used only if the stationary phase is sufficiently transparant at the excitation and emission wavelengths.

Coupled cells. Coupled cells can be used either when the stationary phase is not sufficiently transparent or when the path length of the light becomes unfavourably short due to the use of columns having small internal diameters. The coupled cells were prepared by cutting an appropriate length of tubing, typically 15 mm, and burning off the polymer layer over the last 5 mm. Connections were made with a 1/32-in. T-type zero dead-volume connector with "FS" vespel adapters (Valco, Houston, TX, U.S.A.). Cells with internal diameters of 25 and 10 μm were investigated.

Sheath flow cell. The principle of the sheath flow cell is that the column effluent, entering a quartz cuvette, is surrounded by a concentric second stream of liquid. Provided that the flow is laminar, no noticeable diffusion will occur over a certain distance. The use of the sheath flow cell was expected to result in a low background signal and background noise, because reflection and scattering at the windows are far removed from the measurement area. On the other hand, the manipulation of this cell was expected to be more complicated than that of a fused-silica cell.

The design of the sheath flow cell, which is well known from flow cytometry[8], was adopted from the cells described by Hershberger et al.[9,10] and John et al.[11]. A view of the cell is shown in Fig. 2. Contrary to the situation in microbore HPLC, in OT-LC the use of a connecting capillary between the column and the cell is not necessary. The column is simply inserted into the flow assembly and the laser beam

is focused through the quartz window onto a point which is typically 3 mm above the column end. The aim of the design was to have a flexible system, in which the outer, shield or sheath entry tube can easily be replaced in order to allow adjustment of the outlet diameter. Also the inner or effluent entry tube can easily be centered in the outer tube. The sheath flow was delivered by a syringe-type LDP 13A constant-flow pump (Labotron, Gelting, F.R.G.).

Splitting device

Splitting devices were constructed using Swagelock 1/16-in. three-way unions. The connection between the valve and the union was made by stainless-steel tubing (1/16 in. O.D., 0.5 mm I.D.). The fused-silica column was inserted all the way through the union and the tubing, as close as possible to the valve rotor. It was sealed at the union using appropriately drilled PTFE "no-hole" ferrules. A capillary restriction was connected to the remaining end of the three-way union. The splitting ratio was varied by adjusting the length and/or the internal diameter of this restriction.

Using splitting ratios of up to 1:1000, the external broadening due to the mixing chamber, present at the top of the column, could easily be minimized. When a splitting device is used the fused-silica columns are effectively operated at constant pressure. The pump delivers a constant flow to the splitting point. This flow is mainly diverted to the restriction and the permeability of the latter and the preset flow-rate determine the inlet pressure of the column.
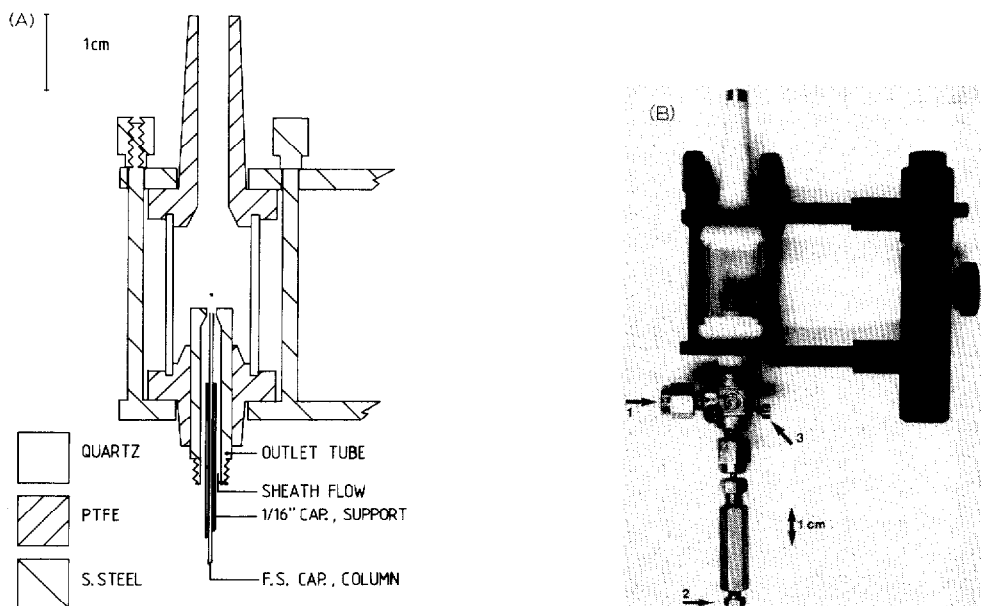

Fig. 2. A, Diagram of the assembly of the sheath flow cell, which is connected to a positioner. Of the flow assembly, only the outlet tube, with a capillary column, is shown. This assembly can easily be removed from the cell assembly by pulling it out of the PTFE holder. B, Picture of the total sheath flow cell. 1 = Sheath flow entry; 2 = column connection (microbore) or column insertion (OT-LC), 3 = screws to adjust the position of the 1/16-in. capillary support.

Columns

As the most stringent requirements on external peak broadening apply to unretained peaks, the columns in our experiments did not contain a stationary phase. The dimensions of the fused-silica columns were 450 cm × 25 μm I.D. and 185 cm × 10 μm I.D., referred to as the 25- and the 10-μm column. Over the investigated range of linear velocities, the columns had volume standard deviations of 5–10 nl and of 0.2–0.5 nl respectively.

Procedure

Prior to the actual experiments, 175 μl of a concentrated fluorescein or pyrene solution were injected. Optical alignment was achieved by adjusting the positions of the parts of the system with home-made positioners in order to obtain the maximum signal. Peak widths were measured at 0.6 of the maximum height. The reported sigma values were calculated as half this peak width.

The performance of the detection modes was tested first by using the 25-μm and then the 10-μm column, the latter providing a more demanding test. The coupled and sheath flow cells were tested by comparing the obtained plate height (*H*) *vs.* linear velocity (*u*) plot with the plot resulting from the use of on-column detection, assuming that this detection mode would not result in any noticeable extra peak broadening. This assumption was checked by comparing the results to the theoretically expected plate heights, which can be calculated using the Golay[12] and Wilke–Chang[13,14] equations. The volume standard deviation of the external peak broadening can then be calculated according to

$$\sigma_v = \pi r_c^2 \sqrt{L(H_{obs} - H_{calc})} \qquad (2)$$

in which r_c is the internal diameter of the column, L is its length and H_{obs} and H_{calc} are the observed and calculated plate heights respectively.

The detection limits, taken as three times the standard deviation of the noise (σ_N), were determined by injecting 175 μl of a solution of the compound of interest and measuring the height of the resulting block in the chromatogram. Noise levels were measured by recording the baseline over 1-min intervals and taking the average of six measurements of the peak-to-peak signal as $4\sigma_N$.

RESULTS AND DISCUSSION

Injection volume

In the set-up shown in Fig. 1 the 25-μm column was installed and the splitting ratio set at 1:360. By partially filling the loop the effective injection volume was varied from 22 to 0.6 nl. The maximum allowable injection volume was determined by measuring the peak width in the on-column detection mode. The results are shown in Table I. It was concluded that an injection volume of 1 nl results in a negligible contribution to the external peak broadening in this system. For practical reasons, the splitting ratio was altered to about 1:800.

With the 10-μm column the maximum allowable injection volume was likewise determined to be 100 pl.

TABLE I

PEAK WIDTH AS A FUNCTION OF THE INJECTION VOLUME ON A CAPILLARY COLUMN

Column: 450 cm × 25 μm, fused silica. Splitting ratio: 1:356. u = 28.5 mm/s. Test compound: fluorescein. Mobile phase: methanol–water (80:20, v/v). On-column detection. Eff. V_{inj} is the effective injection volume, σ_t and σ_v are the peak standard deviations in time and volume units.

Total V_{inj} (μl)	Eff. V_{inj} (nl)	t_R (s)	σ_t (s)	σ_v (nl)
8	22	158	3.7	52
4	11	158	3.5	49
2	5.6	158	3.4	47
1	2.8	158	2.9	41
0.5	1.4	159	2.5	35
0.3	0.8	159	2.2	31
0.2	0.6	159	2.2	31

Performance of the cells

On-column detection. Using the 25-μm column, the theoretical H vs. u curve was calculated for methanol as a mobile phase. Next, the curve was obtained experimentally. The results shown in Fig. 3 and Table II for fluoranthene indicate the good agreement between calculated and observed values. Using eqn. 2 it can be calculated that the difference between the calculated and observed plate height corresponds to an extra volume standard deviation of 2–3 nl over the investigated linear velocity range.

A comparable agreement was found when anthracene was used as the solute and when the mobile phase was changed to methanol–water (80:20, v/v). In Fig. 4 chromatograms are shown for fluoranthene on the 25-μm column, recorded with different time constants.

Similar experiments were carried out with the 10-μm column. The corresponding H vs. u data are shown in Fig. 5 and Table III. The difference between the observed and calculated values corresponds to an extra volume standard deviation of only 0.1–0.2 nl.

From these results it can be concluded that on-column detection fulfils the requirements as far as external broadening is concerned.

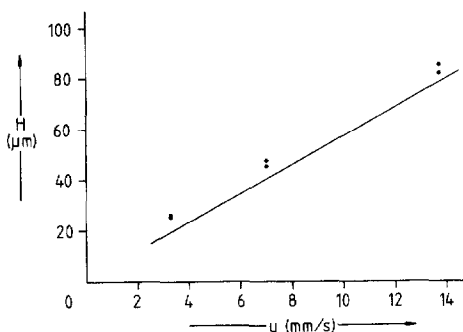*Coupled cells.* A fused-silica cell having an internal diameter of 25 μm, pre-

Fig. 3. Plot of H versus u for fluoranthene on a 450 cm × 25 μm column. Injection volume: 1 nl. Mobile phase: methanol. ●, Observed plate height; ———, calculated plate height. On-column detection mode.

TABLE II

CALCULATED AND OBSERVED PLATE HEIGHTS IN OT-LC FOR SEVERAL DETECTION MODES USING 25-μm I.D. CELLS

Column: 450 cm × 25 μm. Solvent: methanol. Solute: fluoranthene. u_s and u_c are the linear velocities of sheath and column flow, respectively.

u (mm/s)	H (μm)				u_s/u_c for sheath flow cell
	Calculated	Observed			
		On-column detection	Coupled cell	Sheath flow cell	
13.7	79	82	86	127*	0.8
13.7	79	86	90	111*	0.8
7.0	41	46	47	41	1.6
7.0	41	48	46	45	1.6
3.3	19	27	26	23	3.1
3.3	19	27	24	22	3.1

* Caused by too low a flow-rate.

pared as described above, was connected to the 25-μm capillary column. The determined plate heights are shown in Table II. Compared to on-column detection, the use of a coupled cell has no adverse effect on the efficiency of this system, *i.e.*, the average extra deviation was less than 1 nl.

Next a fused-silica cell with an internal diameter of 10 μm was connected to the 10-μm column. As shown in Table III, a significant increase in the plate height was observed compared to on-column detection. The increase amounts to an extra-
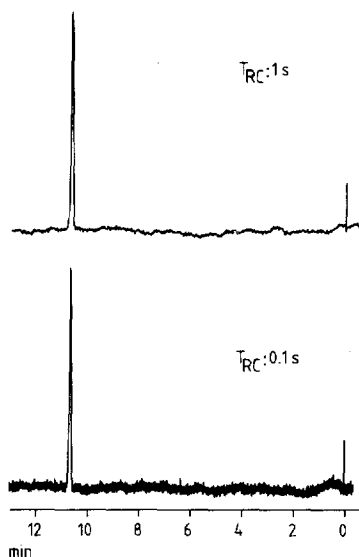

Fig. 4. Chromatograms of 2.5 pg fluoranthene on a 450 cm × 25 μm column. Injection volume: 1 nl. Mobile phase: methanol. Detection limits: for T_{RC} = 1 s, 25 fg (N = 77 000); for T_{RC} = 0.1 s, 75 fg (N = 96 000).

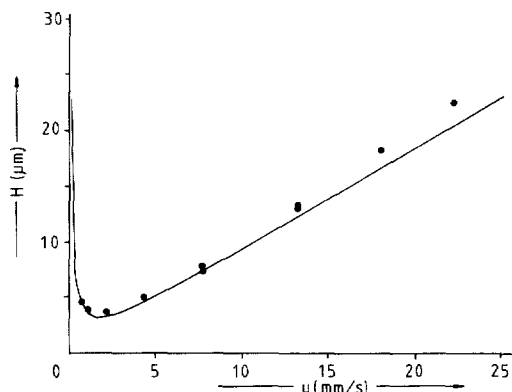Fig. 5. Plot of *H versus u* for fluoranthene on a 185 cm × 10 µm column. Injection volume: 100 pl. Other details as in Fig. 3.

column volume deviation of 0.1–0.3 nl. By applying the exponentially modified gaussian peak model[15], it was found that the additional broadening is that expected of an ideal mixing chamber having a volume of 0.4 nl[16]. It can be assumed that the dead volume in the connector is the cause of this effect. With small internal diameters it becomes increasingly difficult to avoid a significant dead volume in the connector. The wall becomes relatively thick and the capillaries cannot be cut exactly flat and perpendicularly. The fact that effects of the magnitude of 0.4 nl can easily be measured with the present equipment indicates the excellent performance of the system with respect to external broadening.

It can be concluded that 25-µm coupled cells can be used when column standard deviations are greater than 5 nl, and that 10-µm coupled cells give rise to a low

TABLE III

CALCULATED AND OBSERVED PLATE HEIGHTS IN OT-HPLC FOR SEVERAL DETECTION MODES USING 10-µm I.D. CELLS

Column: 185 cm × 10 µm. Solvent: methanol. Solute: fluoranthene. Each value is the average from at least two measurements.

u (mm/s)	H (µm)				u_s/u_c for sheath flow cell
	Calculated	Observed			
		On-column detection	Coupled cell	Sheath flow cell	
22.9	21			60*	0.9
22.9	21	24	27	25	1.8
17.9	16	18	22	18	2.4
13.2	12	13	19	14	3.2
8.8	8	8	11	9	4.8
4.4	4	5	6	5	9.6

* Caused by too low a flow-rate.

but significant extra peak broadening when the column volume standard deviation is less than about 1 nl.

Sheath flow cell. The plate heights determined for fluoranthene as solute and methanol as mobile phase are shown in Tables II and III for the 25- and 10-μm column respectively. These results show that the ratio of the linear velocities of the sheath and column flow should be greater than 1. We presume that this is related to the relatively thick wall of the fused-silica capillaries, and found similar behaviour when 1/16-in. steel tubing with an internal diameter of 0.25 mm was used as the column outlet under microbore conditions. However, after tapering the end of the tube to a needle point, under those conditions an increase of only about 10% was found at a velocity ratio of 0.25[17].

Under OT-LC conditions excellent results are obtained when the sheath flow is large enough. The extra-volume deviations in comparison to on-column detection correspond to only 0.2–1 nl. However, a few drawbacks should be mentioned. It was found that a change in the velocity of the sheath flow often causes a change in the position of the column flow in the cell, resulting in misalignment of the optical system. This effect is probably due to imperfections in the concentricity of the inlet and outer tube at the cell entrance. Because of the small column dimensions, the optical alignment has to be very precise and this leads to a rather laborious procedure. When fused-silica cells are used the alignment is relatively easy, because the position of the liquid is fixed and not influenced by stream patterns. Also, great care has to be taken to cut the end of the column at a right angle. A badly cut tip invariably causes turbulence and contamination of the bulk of the liquid in the cell.

Signal

The optical and electrical paths were followed with the sheath flow cell, and the monochromator replaced by the cut-off filter. For each stage the expected photon, electron or voltage level was calculated. The resulting flow scheme is given in Table IV. Although the numbers are only indicative of the order of magnitude, because of the various uncertainties, it can be concluded that the observed signal corresponds to the expected value. Also it can be seen that the photon collection efficiency in this early design is rather low. In the present system the efficiency is 16%. However, as will be shown, under the present conditions the collection efficiency has no direct effect on the detection limit.

For fluoranthene a linear relationship between the concentration and peak height was obtained over at least four orders of magnitude ($r = 0.9997$, $n = 11$), using the 25-μm column and the sheath flow cell.

Noise and detection limit

Given a certain calibration factor, the detection limit is determined by the noise level. In order to find the predominant source of noise, measurements were carried out for each subsequent element of the system. This was done with PMT cathode voltages between -550 and -800 V, which were shown in preliminary experiments to be the most satisfactory. By operation of the system in the absence of laser excitation and a voltage on the PMT, it was ascertained that the amplifier and recorder noise could be neglected. Next it was verified that the proportional noise in the photometric system (PMT and high voltage supply) was negligible as well. This was

TABLE IV

ENERGY FLOW SCHEME

Test compound: fluoranthene. PMT cathode voltage: -560 V without monochromator. Abbreviations and symbols: Eff = efficiency; P = laser power; T = transmitters; Φ = fluorescence quantum yield; c = concentration; l = length; G = gain.

$P = 1.2$ mW	Laser	$2.0 \cdot 10^{15}$ photons/s
T (tl) = 60%	Filter, lenses and cuvette	$1.2 \cdot 10^{15}$ photons/s
$\Phi = 0.3$ $\varepsilon = 6500$ l/mol/cm $c = 1.37 \cdot 10^{-7}$ M $l = 10^{-2}$ cm	Fluorescence	$7.4 \cdot 10^9$ photons/s
Eff = 8%	Photon capture	$5.9 \cdot 10^8$ photons/s
Eff = 8% $G = 1.6 \cdot 10^4$ 1 A $\cdot$ s = 6.24 $\cdot 10^{18}$ electrons	PMT	$7.5 \cdot 10^{11}$ electrons/s $1.2 \cdot 10^{-7}$ A
$G = 10^{-7}$ A/V	A-V converter	1.2 V
Experimentally found		1.1 V

done with a cell filled with pure solvent. After turning on the laser, the background signal was measured and the total variance of the noise determined. Then the PMT was illuminated with a light-emitting diode fed from a constant-current source and at the same signal level the variance was again determined. From these experiments it could be concluded that the instability of the laser radiation constitutes the predominant contribution to the observed noise.

In Table V the detection limits and signals for several fused-silica cells are compared, including some cells used in microbore scale experiments. It can be concluded that there is no difference in detection limit between cells with diameters of 10–100 μm. Also, the calibration factor decreases when the tube diameter becomes smaller than the laser spot size.

Since in our system the laser is the main contributor to the noise, a decrease in the background signal can be expected to exert a direct influence on the detection

TABLE V

DETECTION LIMITS FOR QUININE SULPHATE USING SEVERAL FUSED-SILICA CELLS

$T_{RC} = 1$ s; $V_{PMT} = 700$ V; em 420 nm cut-off filter. Solvent: methanol–water (60:40, v/v). For other conditions see text.

Cell diameter (μm)	V_{PMT} (V)	$f_{laser\ lens}$ (mm)	Detection limit (10^{-9} mol/l)	Signal (mV)
100	600	100	4	—
50	600	75	3	50
50	700	75	2	—
25	600	50	3	48
25	700	50	2	—
10	600	50	3	12

limit. Fluorescing impurities in the solvents were found to contribute to the background. Also scattered light could be suppressed by inserting and optimizing an aperture in the emitted light path. In this way the detection limits were improved by one order of magnitude. The detection limit for fluoranthene was 1×10^{-9} mol/l. However, no differences were found between the silica cells and the sheath flow cell, and similar results were obtained when the cut-off filter was replaced by the monochromator. It is concluded that at present the use of the sheath flow cell offers no advantage over fused-silica cells.

The laser instability can be compensated for by correcting the signal for laser fluctuations, measured independently. In such a "double beam" instrument the correction can be made by subtracting[18] or, better, ratioing the two photocurrents. Preliminary results with such a system were obtained both with analogue (home-built) and digital (home-modified) circuits. With the latter an approximately eight-fold improvement was typically observed.

The approach of Folestad et al.[19] of collecting the emitted radiation in a direction out of the plane through the laser axis and perpendicular to the capillary did not result in an improvement of the detection limit in our system.

The concentration detection limits, obtained as described above, are about the same as those reported by Guthrie et al.[18]. The data of Folestad et al.[19] correspond to a substantially lower detection limit of about 5×10^{-14} mol/l for fluoranthene. However, this result was obtained with a far more powerful and expensive laser and with the free falling jet cell which at the moment is not compatible with the flow-rates in microbore and OT-LC work.

CONCLUSIONS

From the results obtained we conclude that both the coupled cell and the sheath flow cell can be used as an alternative to on-column detection. Because the volume standard deviations can be well below 1 nl, all three detection modes can meet the requirements for external peak broadening in OT-LC.

The mass detection limits are impressively low. For example, at the concentration detection limit of 10^{-9} mol/l, 0.1 ag of fluoranthene is present in the illuminated volume of a 10-μm cell. A more realistic figure is obtained by considering the amount of fluoranthene present in four volume standard deviations of the chromatographic system when the peak crest is at the concentration detection limit. In the case of the 10-μm column, with a linear velocity of 23 mm/s and on-column detection, 240 ag fluoranthene are present in such a volume.

However, the concentration detection limit itself is more relevant. Our values are higher than those obtained with the best available conventional HPLC fluorimeters, using cells of 3–10 μl. Because of this loss in sensitivity we do not recommend the present system for microbore work. In such cases, when an external peak broadening of 0.5–1 μl is allowed, it is easier to use a conventional detector with a 3-μl cell and about a three-fold make-up flow. For OT-LC a somewhat lower concentration detection limit is allowable, since the first aim of this technique is to generate more plates in a shorter time than is possible with other forms of HPLC.

The applicability of the present system is therefore limited to open-tubular columns and packed capillaries. Since no differences were found in the detection limit

and in the background signal, our first choice would be on-column detection or the use of coupled cells, these being the simplest to use. In view of the extremely small volume deviations obtained, down to 0.1 nl, we may paraphrase Knox and Gilbert[1] by concluding that there are more than excellent prospects for capillary LC.

The laser employed has the advantage of being a relatively inexpensive instrument. On the other hand, the availability of only two lines, 325 and 442 nm, limits the number of fluorophores that can be detected. However, for a large number of fluorimetric detection schemes, pre- or post-column derivatization is used. Many of the customary reagents, such as dansyl chloride, bansyl chloride and *o*-phthalaldehyde, are suitable for excitation at 325 nm, while Gluckman *et al.*[20] showed the applicability of coumarin based reagents with an excitation maximum near 325 nm. We are currently investigating the possibilities of post-column reaction detection on the OT-LC scale[21].

ACKNOWLEDGEMENT

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