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COMPARISON OF SOME HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC FLUORESCENCE DETECTOR FLOW CELLS

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SUMMARY

Open-tubular cells as well as tubular and thin-layer cells packed with retentive and non-retentive packings were evaluated for use in a standard filter fluorimetric high-performance liquid chromatographic detector with respect to band broadening and signal-to-noise ratio. The best performance was obtained with a 0.5-mm diameter open-tubular flow-cell.

INTRODUCTION

The increasing number of sample-limited analyses in the life sciences area is the driving force in the trend towards low-dispersion separation techniques for improvement of the minimum detectable quantity. One of the most promising techniques is high-performance liquid chromatography (HPLC) with microbore columns (0.3 mm I.D. fused-silica columns, packed with 3-μm particle size reversed-phase silica) with fluorescence detection.

Since the cross-sectional area of these fused-silica columns is two orders of magnitude smaller than that of conventional (4.6 mm I.D.) columns, a conventional fluorimeter cannot be used without matching its flow cell to the column.

The adaptation of a standard fluorescence detector to perform microbore chromatography is the subject of this paper.

THEORY

Bandbroadening

According to systems theory, the variances of the independent sub-systems can be added to give the total system variance, σ_t^2:

$$\sigma_t^2 = \sigma_{t_c}^2 + \sigma_{t_{OD}}^2 + \sigma_{t_{DD}}^2 + \sigma_{t_{TL}}^2 \quad (1)$$

where: σ_t^2 = total variance, $\sigma_{t_c}^2$ = variance due to column, $\sigma_{t_{OD}}^2$ = variance due to "optical peak broadening" in detector cell, $\sigma_{t_{DD}}^2$ = variance due to dispersion in detector cell, $\sigma_{t_{TL}}^2$ = residual variance.

The "optical peak broadening" is caused by the non-negligible size of the illuminated part of the detector cell relative to the peak volume. The dispersion in the detector cell is caused by longitudinal mixing. Three cases can be discriminated:

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Cell packed with column material (PC). The peak broadening due to dispersion in the detector should be negligible for packed cells[1], in particular for on-column detection[2].

$$\sigma_t^2 = \sigma_{t_c}^2 + \sigma_{t_{OD}}^2 + \sigma_{t_{TL}}^2 = \left(\frac{1 + k'}{F}\right)^2 \left(\frac{V_c^2}{N_c} + \frac{V_D^2}{\psi^2}\right) + \sigma_{t_{TL}}^2 \quad (2)$$

where: F = flow-rate, k' = capacity factor, V_c = column volume, V_D = detector cell volume (illuminated), N_c = theoretical plate number of column, ψ^2 = shape factor (= 12–36, depending on the homogeneity of illumination and fluorescence collection).

Open tubular cell (OTC).

$$\sigma_t^2 = \sigma_{t_c}^2 + \sigma_{t_{OD}}^2 + \sigma_{t_{DD}}^2 + \sigma_{t_{TL}}^2 = \quad (3)$$

$$= \left(\frac{1 + k'}{F}\right)^2 \left(\frac{V_c^2}{N_c} + \frac{V_D^2}{\psi^2 (1 + k')^2} + \frac{V_D^2 F}{\chi D_m L_D (1 + k')^2}\right) + \sigma_{t_{TL}}^2$$

where: D_m = diffusion coefficient of analyte, L_D = length of total detector cell, χ = constant $\approx$ 75 for long cells, for the short OTC used here $\chi = \alpha F^{1/6}$ in which α = constant[3].

At fixed cell size $\sigma_{t_{OTC}}^2 < \sigma_{t_{PC}}^2$ if

$$(1 + k')^2 > 1 + \frac{\psi^2 F}{\chi D_m L_D} \quad (4)$$

Thus, only at low flow-rates and high k' values does an OTC give less broadening than a PC. At fixed light spot size, $V_{D_{PC}} \approx 0.65\ V_{D_{OTC}}$ so $\sigma_{t_{OTC}}^2 < \sigma_{t_{PC}}^2$ if

$$0.4\,(1 + k')^2 > 1 + \frac{\psi^2 F}{\chi D_m L_D} \quad (5)$$

This theory also suggests that a longer OTC with the same volume would be more efficient (*e.g.*, a spiral cell). However, this should not be done at the expense of noise. A fundamentally better design than the spiral cell is the capillary bundle or multi-hole tube cell with a 170°-angle distributor cone on top and bottom, giving lower pressure and flow-rate at the same volume and dwell time. Capillary resistance (diameter) inhomogeneity may be a problem.

Cell packed with non-retentive material.

$$\sigma_t^2 = \sigma_{t_c}^2 + \sigma_{t_{OD}}^2 + \sigma_{t_{TL}}^2 = \left(\frac{1}{F}\right)^2 \left(\frac{V_c^2 (1 + k')^2}{N_c} + \frac{V_D^2}{\psi^2}\right) + \sigma_{t_{TL}}^2 \quad (6)$$

This type of cell causes at most as much bandbroadening as an OTC or PC.

Signal-to-noise ratio

The fluorescence signal, S, is (at low absorbances) proportional to the number of fluorescing sample molecules in the lightbeam and to the solvent background fluorescence, both of which increase with flow cell volume and excitation intensity. A third component of the signal is the flow cell material itself:

$$S = \{(k_1c_1 + k_2c_2)V_D + k_3M\}I_0 \quad (7)$$

where: c_1 = concentration of the sample molecules in the flow cell, c_2 = concentration of the solvent, k_1,k_2,k_3 = constants containing absorptivity, effective path length and quantum efficiency of sample, solvent, and flow cell material, respectively, M = illuminated mass of the flow cell material, I_0 = number of photons per second entering the flow cell.

The noise in high-sensitivity fluorescence detection appears to be caused in practice by background fluorescence and scattered light.

For microbore columns with the same efficiency as large-bore columns, the peakwidths in time units are the same, so the sample component concentration in the detector increases with decreasing column diameter, while the solvent concentration is unaffected. To decrease band broadening, the volume of the flow cell will be decreased approximately in proportion to the increase in analyte concentration. This may lead to an improvement in signal-to-noise ratio until scatter becomes the dominant source of noise.

If the column and flow cell dimensions are decreased to the point where only a part of the excitation beam is captured in the cell, no gain in signal-to-noise ratio (but rather a loss, if residual noise sources are dominant) can be expected.

EXPERIMENTAL

A Model LC 5500 liquid chromatograph (Varian) was used for all experiments; the split-flow mode[2] was employed with 0.32 mm I.D. fused-silica columns. Stainless-steel columns of 150 and 175 mm length and 1.0 mm I.D. and 1/16 in. O.D. and a 130 × 0.32 mm I.D. fused-silica column were packed at Varian with 5-μm and 3.μm Micropak SP C_{18} octadecyl silica (Varian). The Fluorichrom (Varian) is a double-filter fluorimeter, equipped with a 50-W deuterium lamp and measures fluorescence emission in a 90° angle from the exitation beam. Its focal spot is about 3 mm^2 at the flow cell. The Zn lamp used for Table I was a Pen-Ray Z-800 (Ultra-Violet Products, San Gabriel, CA, U.S.A.). A 220I excitation filter (λ_{max} = 221 nm, bandwidth 16 nm) and a 7-60 emission filter (330–380 nm, Varian) were used, except for Fig. 5, where a 305 FG 01-25 (305 nm) cutoff filter and a 011 FG 09-25 (340 nm) wide bandpass filter (both from Andover, Lawrence, MA, U.S.A.) were used on the emission side, and in Fig. 2 where a 254I excitation filter (λ_{max} = 255 nm, bandwidth 12 nm) and a 4-76 (380–550 nm) emission filter (both from Varian) were used.

The standard Fluorichrom flow cell is a quartz tube of 8 × 2 mm I.D., and 3 mm O.D. The bed of the packed flow cell was supported by the standard on the flow cell entrance and exit which is contained in a PTFE fitting. As packings in the standard cell Micropak SP C_{18}-5 (Varian), Micropak SP CN-5 (Varian) and Macherey-Nagel 30-μm silica (Duren, F.R.G.) were used. The thin-layer cell consisted of

TABLE I

EXTRA-COLUMN BANDBROADENING

Cell	*Packing*	$\sigma^2_{v,ex}$ (μl^2)*	$\sigma^2_{t,ex}$ (s^2)	H_∞ (μm)
Standard	–	–	–	–
Standard	SP C_{18}-5	8–14	10 –1.8	105
Standard	SP NH_2-5	12–19	17 –1.7	45
0.5 mm OTC	–	2– 6	3.2–0.5	50
0.5 μl UV	–	3.5	4.5–0.35	42

* Extra-column variance expressed in volume units.

the stainless-steel half of a BAS TL-5A (Bioanalytical Systems, West Lafayette, IN, U.S.A.) electrochemical detector cell as the back of the cell, containing cell entrance and exit, two 0.005-in. thick PTFE spacers (BAS) and a 19-mm diameter 1-mm thick Suprasil window (Esco, Oak Ridge, NJ, U.S.A.) in a Kel-F frame, clamped to the stainless-steel back half of the cell with four screws. Macherey-Nagel 10-μm silica was used in this cell and supported by 1/16 in. and 1/4 in. diameter, 0.5-μm porosity

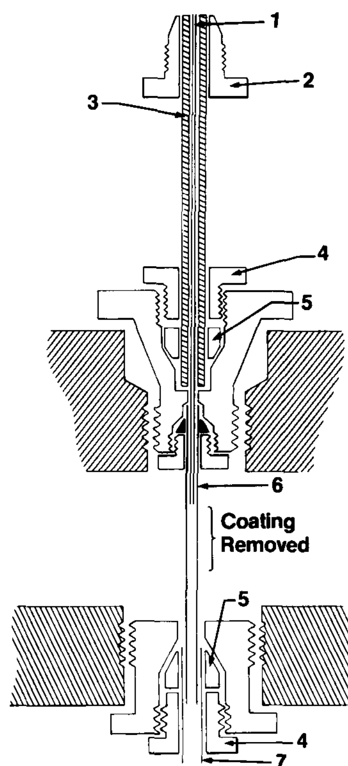


Fig. 1. Open-tubular cell for microbore chromatography. (1) 75 μm I.D., 0.010 in. O.D. fused-silica tubing; (2) 1/16 in. fingertight nut; (3) 0.010 in. I.D., 1/16 in O.D. PTFE tubing; (4) 1/16 in. stainless-steel nut; (5) 1/16 in. Vespel ferrule; (6) 0.53 mm I.D., 0.026 in. O.D. fused-silica tube; (7) 0.035 in. I.D., 1/16 in. O.D. PTFE tubing.

screenfits (Mott Metallurgical, Farmington, CT, U.S.A.) in the cell entrance and exit fittings, respectively.

This cell, when placed in the Fluorichrom cell compartment at 40–50° angle to the excitation beam, gave optimum signal-to-noise ratio.

The OTC is shown schematically in Fig. 1. The 75-μm I.D. fused-silica capillary was obtained from Scientific Glass Engineering (Austin, TX, U.S.A.), the 0.53-mm fused-silica cell tubing from Polymicro Technologies (Phoenix, AZ, U.S.A.), the 0.010 in. I.D. PTFE tubing from Zeus (Raritan, NJ, U.S.A.) and 1/16 in. fingertight nut from Upchurch (Oak Harbor, WA, U.S.A.). The other parts of the OTC are either standard or custom-made Varian parts.

To assess extra-column bandbroadening on-column concentration was effected by using as a sample solvent the mobile phase, diluted with 50% water. Injection duration times were kept negligibly short relative to retention times.

A set of fluorescent compounds analogous to those for UV detection[4] was tested and gave the same results with a microbore UV detector as the UV absorbance test set. In an equivalent concentration range on a 150 × 4.6 mm I.D. Micropak SP C_{18}-3 column they showed a small but correctable deviation from the ideal[4]. The test compounds (*m*-cresol, benzonitrile, indole, and 2-naphthol) were obtained from Chem Service (West Chester, PA, U.S.A.). The mobile phase for extra-column variance tests was an aqueous solution of 25 or 28% acetonitrile–0.1% acetic acid–0.1% triethylamine. Acetonitrile and acetic acid were obtained from Burdick & Jackson (Muskegon, MI, U.S.A.), triethylamine, naphthalene and anthracene from Aldrich (Milwaukee, WI, U.S.A.) and trifluoroacetic acid from Pierce (Rockford, IL, U.S.A.). The fluorescence detector was used at maximum sensitivity to prevent possible overloading of the column by benzonitrile.

The resulting peaks were almost symmetrical, as indicated in the close agreement between the second moment data and those based on the assumption of a Gaussian band (see Fig. 3).

RESULTS AND DISCUSSION

Table I shows the extra-column variance, $\sigma^2_{t,ex}$ (*i.e.* the total variance without the variance due to the column) at 50–200 μl/min flow-rate and the theoretical plate height, extrapolated to infinite capacity factor, H_∞, for a standard OTC, a standard cell packed with retentive and non-retentive material, a capillary OTC, and a microbore UV-absorbance cell. The bandbroadening with the standard OTC is too excessive to allow accurate measurement when a 150 × 1 mm microbore column is used. Even with this old, less-than-high performance test column the increase in H_∞ by optical peakbroadening in a standard cell packed with retentive material is striking. There appears to be no advantage to packing a cell with retentive material (Micropak SP C_{18}-5) relative to non-retentive packing (Micropak SP NH_2-5), except in tests to study dispersion due to injection[5] or in cases of enhancement of selectivity[1] (*e.g.* for aflatoxins in peanut butter). Although packing the detector cell decreases the peakbroadening, it also decreases the signal-to-noise ratio, such that a standard cell packed with non-retentive material shows slightly worse detection limits than a 0.5-mm I.D. OTC (*cf.* Fig. 2)

The signal-to-noise ratios of several types and sizes of fluorescence detector

TABLE II

MINIMUM DETECTABLE CONCENTRATION (MDC)

Lamp	*Cell*	*Packing*	*Sample**	*Peakwidth at 50% height (μl)*	*Conc. (ppb)*	*S/N***	*MDC**** *(ppt)*
Deuterium	Standard	–	a	25	1.6	110	30
			b	20	25	470	110
Deuterium	Standard	SP C_{18}-5	a	30–40	1	7–9	220–380
			b	30	17	40	800
Deuterium	Standard	SP NH_2-5	a	20	2	5–10	400–800
Deuterium	0.53 mm	–	a	22	1.8	23	150
Deuterium	0.53 mm + Cap.	–	a	17	3.1	30	200
	0.53 mm + Cap.	–	b	10	50	220	450
Zinc	0.53 mm + Cap.	–	b	12	42	51	1650
Deuterium	0.32 mm	–	ax 100	134	30	18	3300
Deuterium	0.32 mm	SP C_{18}-5	ax 10	2.6	153	38	8100

* Samples: a = 40 pg anthracene, 254I excitation filter, 4-76 emission filter; b = 500 pg naphthalene, 220I excitation filter, 7-60 emission filter.

** S/N = signal-to-noise ratio (peak height divided by the average peak-to-peak baseline noise over the time interval of a peak).

*** MDC = concentration calculated to give a signal twice the noise.

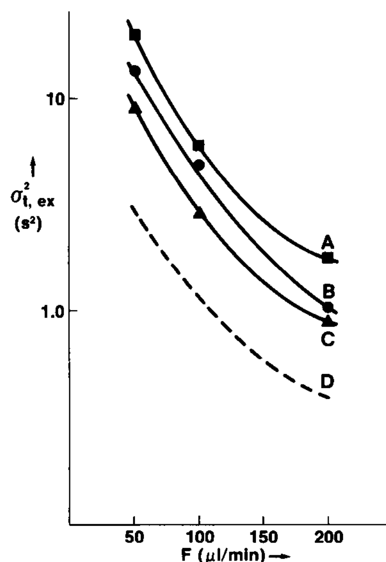
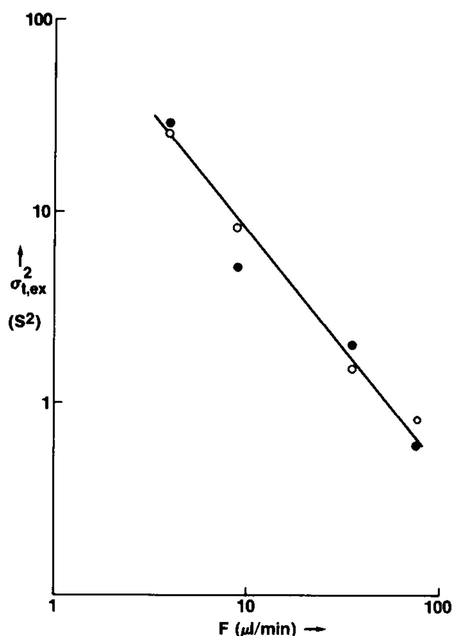


Fig. 2. Extra-column variance *vs.* flow-rate for three types of fluorescence detector flow cells. (A) Standard (2 mm I.D.) flow cell, packed with 30-μm silica, MDC 600 ppt; (B) Thin-layer cell, packed with 10-μm silica, MDC 800 ppt; (C) 0.53 mm I.D. open-tubular flow cell; MDC 200 ppt; (D) Extra-column variance not due to the detector. For details, see Experimental section. The MDC was determined for anthracene.



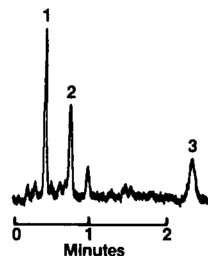


Fig. 3. Dependence of the variance of the extra-column bandbroadening on eluent flow-rate with a 0.53 mm I.D. open-tubular flow cell. (○) Calculation based on the assumption that the peakwidth at 13.5% height is 4σ; (●) according to second moment. See Experimental section for further details.

Fig. 4. Separation of sub-picomole quantities of dipeptides. Column, 75 × 1 mm Micropak SP C_{18}-3; sample, 1 μl, containing 0.4 pmole Trp-Gly, 0.3 pmole Trp-Tyr, and 0.3 pmole Trp-Phe (in order of elution); mobile phase, aqueous solution of 22% acetonitrile–0.2% trifluoroacetic acid; flow-rate, 200 μl/min; pressure, 310 bar; detector, Fluorichrom 0.53 mm OTC, 213–229 nm excitation filter, 330–380 nm emission filter.

cells were measured (see Table II). The decrease in minimum detectable concentration (MDC) for OTCs in Table II is less than the decrease in the illuminated volume, which may be caused by the different cell wall thickness and material and inhomogeneity of the focussed excitation beam (due to chromatic aberration). Note that the 16-nm half-width of the exitation filter transmission band allows a larger photon flux of the deuterium lamp to reach the cell than a single-line Zn-lamp procedures.

Table II also shows that packing a standard 2-mm I.D. cell decreases the signal-to-noise ratio by a factor of 10, while packing a 0.32-mm OTC decreases this ratio only be a factor of 2.5, suggesting the use of a thin-layer fluorescence (TLF) cell, capturing most of the incident lightbeam without the concomitant increase in cell volume. Therefore, a TLF cell 14 mm long, 5 mm wide, and 0.25 mm thick, was assembled and included in this comparison. The results are shown in Fig. 2.

It can be concluded that the TLF cell is equivalent in MDC and in bandbroadening characteristics to the packed cell but is worse with respect to both of these parameters when compared to the 0.53-mm OTC. Although further optimization of cell parameters (and elimination of scatter from the cell back panel) may greatly improve performance of the TLF cell, the ease of construction (see Fig. 1) of fused-silica capillary OTCs makes them the obvious choice for microbore chromatography with this standard detector. The bandbroadening of the cell of Fig. 1 was measured over a wide range of flow-rates and is shown in Fig. 3. Demonstrations of its per-

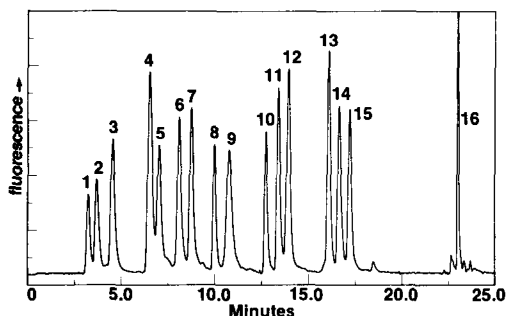


Fig. 5. Separation of 9-fluorenylmethyl chloroformate (FMOH) derivatives of amino acids. Sample 1: 1, Ser; 2, Asp; 3, Glu; 4, Arg; 5, Thr; 6, Gly; 7, Ala; 8, Tyr; 9, Pro; 10, Met; 11, FMOH; 12, Val; 13, Phe; 14, Ile; 15, Leu; 16, Lys (60 pmole each). Column, 130 × 0.32 mm Micropak SP C_{18}-3; detector, Fluorichrom, 220 I exitation filter, 011FG09-25 and 305FG01-25 emission filters. Injection at 2 min into the gradient; pressure, 350–400 bar; split-flow operation[2]. Mobile phases: (A) 1.4 ml acetic acid–0.65 ml triethylamine–10 ml 1 *M* sodium hydroxide in 1000 ml water (pH 4.6); (B) 200 ml methanol–3 ml acetic acid–1 ml triethylamine in 800 ml water (with 1 *M* sodium hydroxide to pH 4.5); (C) acetonitrile. Solvent and flow-rate program:

Time (min)	*A (%)*	*B (%)*	*C (%)*	*Flow-rate (ml/min)*
0	63	30	7	1.4
10	63	10	27	1.4
20	63	0	37	1.8
23	45	0	55	1.8
24	45	0	55	2.5
26	45	0	55	2.5
27	63	30	7	2.0

formance with 75 × 1 mm and 130 × 0.32 mm microbore columns in isocratic and ternary gradient operation are given in Figs. 4 and 5, respectively.

The minimum detectable quantity for 9-fluorenylmethyl chloroformate amino acid derivatives[6] is *ca.* 10-fmole (signal-to-noise ratio = 2), slightly worse then the 2-fmole detection limit shown with the same flow cell[7] for *o*-phthalaldehyde derivatives of primary amino acids.

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