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SIGNAL ENHANCEMENT IN ON-COLUMN FLUOROMETRIC DETECTION IN OPEN-TUBULAR CAPILLARY LIQUID CHROMATOGRAPHY

TOYOHIDE TAKEUCHI and EDWARD S. YEUNG*
Ames Laboratory-USDOE and Department of Chemistry, Iowa State University, Ames, IA 50011 (U.S.A.)
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SUMMARY

Signal enhancement in on-column fluorometric detection in open-tubular capillary liquid chromatography is reported. Signal enhancement due to the environment effect of the stationary phase was estimated by using equations derived here. The enhancement factor was about 3 for anthracene on the *n*-octylmethylpolysiloxane stationary phase.

INTRODUCTION

It is predicted that optimum open-tubular capillary liquid chromatographic (LC) columns can generate in excess of 10^6 theoretical plates[1,2]. The internal volume of such a column is around or less than 10 nl. Therefore, injection and detection volumes should be substantially reduced compared with conventional LC so that they do not degrade column efficiency. Injection of small volumes of sample can be readily achieved by split injection techniques, while detection has been a more difficult problem in open-tubular capillary LC. Post-column UV detection with a miniaturized flow cell[3,4] or a conventional flow cell with make-up flow[5], on-column UV[6] or fluorescence detection[7–10], on-column electrochemical detection with a carbon fiber[11], post-column mass spectrometric detection[12] and thermal lens detection[10] have been investigated. On-column detection as proposed by Yang[6,13], in which the capillary column itself is used as a flow cell, is superior to post-column detection because of negligible extra-column band broadening.

Fluorescence detection can be carried out successfully with a laser source because of its high intensity and better focusing property. Laser-induced fluorometric detection in micro-scale HPLC has been reviewed by Folestad *et al.*[14] and four types of detection cell designs, which involve sheath flow, optical fiber-capillary tube, free falling jet and fused-silica capillary flow cells, have been discussed. Van Vliet and Poppe discussed cell designs for laser-induced fluorescence detection in open-tubular capillary LC[15].

Guthrie and Jorgenson[7] pointed out that on-column detection provides increased sensitivity by a factor of $(1 + k)$ compared with post-column detection because the stationary phase focuses analytes in a partitioning zone. Further, it can be

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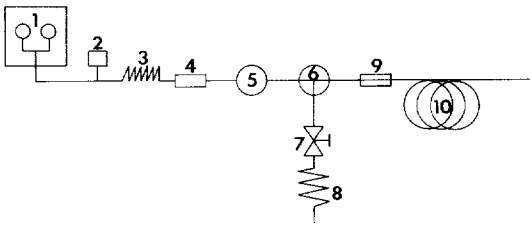


Fig. 1. Diagram of the chromatographic system. 1 = pump; 2 = pressure gauge; 3 = pulse dampener; 4 = line filter; 5 = injector; 6 = T-joint; 7 = two-way valve; 8 = restrictor; 9 = 1/16 in. × 1/32 in. reducing union; 10 = capillary column.

expected that analytes partitioned in the stationary phase have different fluorescence properties, thus affecting quantitation and selectivity due to the difference in environment. This paper will estimate signal enhancement by on-column fluorometric detection in open-tubular capillary LC as a result of these effects.

EXPERIMENTAL

Apparatus

Fig. 1 shows a diagram of the chromatographic system employed in this work. A Model 750 solvent delivery system (Micromeretics, Norcross, CA, U.S.A.) was used as a pump and was operated in the constant-pressure mode. A Li-Chroma-Damp III pulse dampener (Handy & Harman Tube, Norristown, PA, U.S.A.) was connected between the pump and the injector to reduce pulsations. A line filter SSI 05-0105 (0.5 μm, supplied by Alltech, Deerfield, IL, U.S.A.) was connected before the injector. A Rheodyne Model 7520 (1 μl, Cotati, CA, U.S.A.) was employed as a sample injector and the split ratio was regulated by an SSI two-way valve (Alltech) and stainless-steel tubing of 2 m × 0.1 mm I.D. (Japan Spectroscopic, Tokyo, Japan). An SB-OCTYL-50 (*n*-octylmethylpolysiloxane, 50% *n*-octyl groups) open-tubular fused-silica capillary column (10 m × 50 μm I.D. and 0.5 μm film thickness) was commercially available from Lee Scientific (Salt Lake City, UT, U.S.A.). The capillary column was directly connected to the sample injector through a T-joint (0.75 mm bore; Valco, Houston, TX, U.S.A.). The 325-nm UV beam of a HeCd laser (Model 4240NB; Liconix, Sunnyvale, CA, U.S.A.) was used as an excitation source. The polyimide coating of one end of the capillary column was removed carefully with a razor for on-column detection. The output from the photomultiplier tube (PMT) (1P-28; RCA, Harrison, NJ, U.S.A.) after passing two filters (4-96 and 0-52; Corning Glass, Corning, NY, U.S.A.) was directed to a picoammeter (414S; Keithley, Cleveland, OH, U.S.A.). A Series 5000 (Fisher Recordall, Austin, TX, U.S.A.) was used as a chart recorder. Fluorescence was collected at 90° to the excitation beam and the horizontal intense light reflecting from the cell was prevented from reaching the PMT by an aperture. For post-column detection, fused-silica capillary tubing with 55 μm I.D. × 0.24 mm O.D. (SGE, Victoria, Australia) was connected to the column with PTFE tubing. The flow cells were fixed with a double stick tape on the aluminium block. The laser beam was larger than the column diameter.

Reagents

Acetonitrile and distilled water were HPLC-grade and were obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.) and Aldrich (Milwaukee, WI, U.S.A.), respectively. Other reagent used in this work were reagent-grade and were obtained from Fisher Scientific.

RESULTS AND DISCUSSION

When the beam diameter of the incident light is larger than the flow cell diameter (or column diameter), fluorescence intensity (F) can be expressed by the following equation.

$$F = \alpha\varepsilon\Delta VCI\varphi \tag{1}$$

where α is a constant, ε is the collection efficiency, ΔV is the detection volume, C is the concentration of an analyte, I is the light intensity and φ is a factor accounting for the quantum efficiency, selectivity, absorptivity, energy transfer, etc. Here, collection efficiency is assumed not to be constant. When the analyte is retained on the stationary phase with a capacity factor of k', a fraction $k'/(1 + k')$ of the analyte is partitioned in the stationary phase and a fraction $1/(1 + k')$ of the analyte is present in the mobile phase. In the case of on-column detection, fluorescence intensity due to the analyte in the stationary phase zone can be expressed by:

$$F_{osi} = \alpha_o\varepsilon_o\Delta V_{os}\frac{k'_i a_i}{(1 + k'_i)\Delta V_{os}} I_{os}\varphi_{osi} \tag{2}$$

where subscript o denotes on-column detection, s denotes the stationary phase, i denotes the analyte component, and a_i is the amount of the analyte i to be detected. Eqn. 2 can be simplified to eqn. 3.

$$F_{osi} = \frac{\alpha_o\varepsilon_o k'_i a_i I_{os}\varphi_{osi}}{1 + k'_i} \tag{3}$$

Fluorescence intensity due to the analyte in the mobile phase zone can be represented by:

$$F_{omi} = \alpha_o\varepsilon_o\Delta V_{om}\frac{a_i}{(1 + k'_i)\Delta V_{om}} I_{om}\varphi_{omi} \tag{4}$$

$$F_{omi} = \frac{\alpha_o\varepsilon_o a_i I_{om}\varphi_{omi}}{1 + k'_i} \tag{5}$$

where subscript m denotes the mobile phase.

On the other hand, fluorescence intensity for post-column detection (p) is presented as follows because the analyte is diluted by a factor of $(1 + k'_i)$.

$$F_{pi} = \alpha_p\varepsilon_p\Delta V_p\frac{a_i}{(1 + k'_i)\Delta V_p} I_p\varphi_{pi} \tag{6}$$

$$F_{pi} = \frac{\alpha_p \varepsilon_p a_i I_p \varphi_{pi}}{1 + k'_i} \tag{7}$$

From Eqns. 3, 5, and 7, the ratio of peak height for on-column detection (h_{oi}) to that for post-column detection (h_{pi}) can be derived.

$$\frac{h_{oi}}{h_{pi}} = \frac{F_{osi} + F_{omi}}{F_{pi}} = \frac{\alpha_o \varepsilon_o}{\alpha_p \varepsilon_p} \left(k'_i \frac{I_{os} \varphi_{osi}}{I_p \varphi_{pi}} + \frac{I_{om} \varphi_{omi}}{I_p \varphi_{pi}} \right) \tag{8}$$

As for the analyte (j) which is not retarded on the stationary phase ($k' = 0$), eqn. 8 becomes:

$$\frac{h_{oj}}{h_{pj}} = \frac{\alpha_o \varepsilon_o I_{om} \varphi_{omj}}{\alpha_p \varepsilon_p I_p \varphi_{pj}} \tag{9}$$

If we can assume that φ_{om} is equal to φ_p (same environment) and that I_{os} is equal to I_{om} (uniform beam intensity), eqns. 8 and 9 become as follows, respectively.

$$\frac{h_{oi}}{h_{pi}} = \frac{\alpha_o \varepsilon_o I_{om}}{\alpha_p \varepsilon_p I_p} \left(k'_i \frac{\varphi_{osi}}{\varphi_{pi}} + 1 \right) \tag{8a}$$

$$\frac{h_{oj}}{h_{pj}} = \frac{\alpha_o \varepsilon_o I_{om}}{\alpha_p \varepsilon_p I_p} \tag{9a}$$

Substitution of eqns. 9a and 8a and rearrangement yields:

$$\frac{\varphi_{osi}}{\varphi_{pi}} = \left(\frac{h_{oi} h_{pj}}{h_{oj} h_{pi}} - 1 \right) \Big/ k'_i \tag{10}$$

Eqn. 10 indicates that we can estimate the signal enhancement $\varphi_{osi}/\varphi_{pi}$ due to the environmental effect of the stationary phase by measuring peak heights of both non-retained and retained solutes. When the enhancement factor $\varphi_{osi}/\varphi_{pi}$ is unity, enhancement of signal of retained solutes for on-column detection still takes place by a factor of $(1 + k'_i)$ due to focusing by the stationary phase.

On the other hand, when the beam diameter is much smaller than the flow cell diameter (or column diameter), fluorescence intensity can be described as follows:

$$F = \alpha \varepsilon L C I \varphi \tag{11}$$

where L is the light path length. Note that α in eqn. 11 has different units from α in eqn. 1. In the case of on-column detection, fluorescence intensity due to the analyte in the stationary phase zone can be expressed as follows.

$$F_{osi} = \alpha_o \varepsilon_o 2 d_f \frac{k'_i a_i}{(1 + k'_i) \Delta V_{os}} I_{os} \varphi_{osi} \tag{12}$$

$$F_{osi} = \frac{\alpha_o \varepsilon_o k'_i a_i I_{os} \varphi_{osi}}{(1 + k'_i)\pi r \Delta l} \tag{13}$$

where Δl is longitudinal length of the column involved in detection, r is the column radius, and d_f is film thickness of the stationary phase. Because r is generally much larger than d_f in open-tubular capillary LC, ΔV_{os} is approximately equal to $2\pi r d_f \Delta l$. Fluorescence intensity due to the analyte in the mobile phase zone can be expressed in the same way. ΔV_{om} is given by $\pi(r - d_f)^2 \Delta l$, and is approximately equal to $\pi r^2 \Delta l$ because r is much larger than d_f.

$$F_{omi} = \alpha_o \varepsilon_o 2r \frac{a_i}{(1 + k'_i)\Delta V_{om}} I_{om} \varphi_{omi} \tag{14}$$

$$F_{omi} = \frac{2\alpha_o \varepsilon_o a_i I_{om} \varphi_{omi}}{(1 + k'_i)\pi r \Delta l} \tag{15}$$

In the case of post-column detection, fluorescence intensity can be expressed by the following equation.

$$F_{pi} = \alpha_p \varepsilon_p 2r' \frac{a_i}{(1 + k'_i)\Delta V_p} I_p \varphi_{pi} \tag{16}$$

$$F_{pi} = \frac{2\alpha_p \varepsilon_p a_i I_p \varphi_{pi}}{(1 + k'_i)\pi r' \Delta l} \tag{17}$$

where r' is the radius of the flow cell. From eqns. 13, 15 and 17, the peak height ratio can be represented by:

$$\frac{h_{oi}}{h_{pi}} = \frac{\alpha_o \varepsilon_o}{\alpha_p \varepsilon_p} \left(\frac{k'_i r' I_{os} \varphi_{osi}}{2r I_p \varphi_{pi}} + \frac{r' I_{om} \varphi_{omi}}{r I_p \varphi_{pi}} \right) \tag{18}$$

The enhancement factor can be expressed in the same way.

$$\frac{\varphi_{osi}}{\varphi_{pi}} = 2\left(\frac{h_{oi} h_{pj}}{h_{oj} h_{pi}} - 1 \right) \Big/ k'_i \tag{19}$$

Fig. 2 shows separations of salicylic acid and anthracene with post-column and on-column detection. Salicylic acid is considered to be non-retained under these conditions and the capacity factor of anthracene is 0.51. The relative peak height is 0.46 for post-column detection, while it is 1.21 for on-column detection. If signal enhancement of anthracene takes place only due to the focusing effect by the stationary phase for on-column detection, the ratio of the relative peak heights should be 1.51. Actually, the value is 2.61, which indicates that signal enhancement due to the environmental effect by the stationary phase takes place.

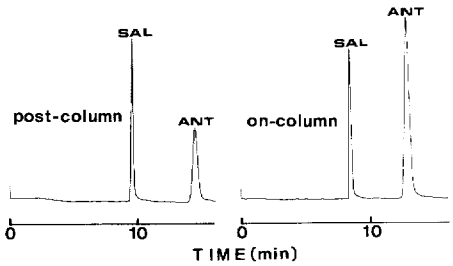


Fig. 2. Fluorometric detection of salicylic acid and anthracene. Column: SB-OCTYL-50, 10 m × 50 μm I.D. Mobile phase: acetonitrile–water (50:50). ΔP: 2.2 mPa. Sample: $5 \cdot 10^{-3}$ M salicylic acid (SAL) and $2 \cdot 10^{-3}$ M anthracene (ANT), *ca.* 20 nl were injected.

Table I shows the signal enhancement factor ($\varphi_{osi}/\varphi_{pi}$) calculated by using eqn. 10. The enhancement factor for anthracene is 3.16 with acetonitrile–water (50:50) as the mobile phase. Table I also shows enhancement factors when the eluents contain a fixed concentration of anthracene. The enhancement factor decreases with increasing concentration of anthracene in the eluent. The decrease is probably due to mutual interaction of the anthracene molecules on the stationary phase, partially quenching the fluorescence. The results shown in Table I indicate that we should use eluents which contain low concentrations of fluorescing species in indirect fluorometric detection, an application which will be discussed elsewhere.

Fig. 3 illustrates the peak area of anthracene as a function of the capacity factor. The mobile phase composition was adjusted to vary the capacity factor. Lines A and B show the cases for post-column detection and for on-column detection with enhancement only due to the focusing effect by the stationary phase. Observed peak areas are larger than those calculated for both cases, depicting the environmental effect.

The theoretical plate height (H) changes substantially with k' in open-tubular capillary LC, while it does not change very much in packed column LC. Assuming that the contribution of the longitudinal diffusion and the mass transfer resistance in the stationary phase on H can be neglected, the Golay equation[16] becomes:

$$H = \frac{(11k'^2 + 6k' + 1)r_c^2 u}{96(1 + k')^2 D_m} \tag{20}$$

TABLE I

ESTIMATION OF THE ENHANCEMENT FACTOR

Operating conditions as in Fig. 2 except for the mobile phase which was acetonitrile–water (50:50) containing anthracene. Subscripts ANT and SAL denote anthracene and salicylic acid, respectively.

Concentration of anthracene in the eluent (M)	$\frac{h_{o,\,ANT}}{h_{o,\,SAL}} = A$	$\frac{h_{p,\,ANT}}{h_{p,\,SAL}} = B$	A/B	$\frac{d_{os,\,ANT}}{d_{p,\,ANT}}$
0	1.21	0.463	2.61	3.16
$1 \cdot 10^{-5}$	0.821	0.444	1.85	1.66
$3 \cdot 10^{-5}$	0.761	0.410	1.86	1.68
$1 \cdot 10^{-4}$	0.402	0.276	1.46	0.90

where r_c is the column radius, u is linear velocity and D_m is the diffusion coefficient of a solute in the mobile phase. Without any signal enhancement, peak height (h) is inversely proportional to the square root of H as shown by the next two equations.

$$h \propto \frac{1}{\sqrt{H(1 + k')}} \tag{21}$$

$$h \propto \sqrt{\frac{D_m}{11k'^2 + 6k' + 1}} \tag{22}$$

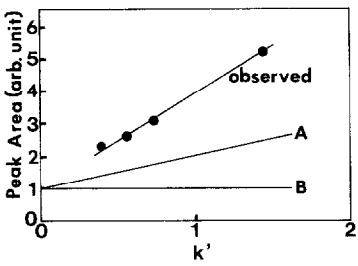
Eqn. 22 shows the variation of peak height with the capacity factor for post-column detection in open-tubular capillary LC. Assuming that D_m is constant and that signal enhancement only takes place due to the focusing effect by the stationary phase, h for on-column detection is determined by combining eqns. 8a and 22.

$$h \propto \frac{1 + k'}{\sqrt{11k'^2 + 6k' + 1}} \tag{23}$$

On the other hand, if we assume H is constant regardless of k', *e.g.* for packed column LC, variation of peak height with k' can be described by

$$h \propto \frac{1}{1 + k'} \tag{24}$$

Fig. 4 illustrates variations of peak height with k' for on-column detection in open-tubular capillary LC and for post-column detection in open-tubular and packed column LC. The relative peak height converges to $(1/11)^{\frac{1}{2}}$ ($= 0.302$) as k' goes to infinity



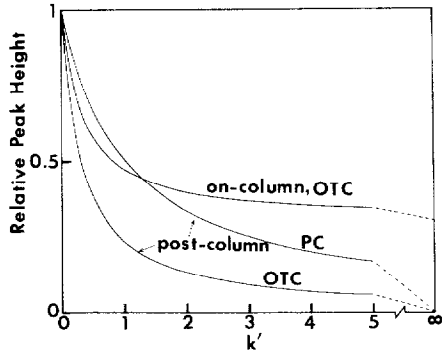


Fig. 3. Peak area of anthracene (signal × time) as a function of k' (different mobile phase compositions). Line A: on-column detection (assuming that enhancement takes place only due to the focusing effect); line B: post-column detection.

Fig. 4. Relative peak height *versus* k' for on-column detection (eqn. 23) and post-column detection. PC, packed column LC (eqn. 24); OTC, open-tubular column LC (eqn. 22).

in the case of on-column detection in open-tubular capillary LC (eqn. 23), while relative peak heights for the other cases converge to zero. On-column detection will thus be advantageous for improving sensitivity of solutes eluting later.

CONCLUSION

Signal enhancement due to the environmental effect of the stationary phase is reported and can be estimated for on-column detection in open-tubular capillary LC by using equations derived here. The fluorescence signal obtained by on-column detection was larger than that calculated by assuming that enhancement is only due to the focusing effect by the stationary phase. The environmental effect observed here is not necessarily only due to the increased fluorescence quantum yield of anthracene. There might also be a shift in the excitation spectrum leading to more effective laser excitation at 325 nm. However, it is unlikely that the broad absorption feature of anthracene near 325 nm increases by a factor of 3 when it is immobilized on the stationary phase. On-column detection will improve detectability of analytes eluting later compared with post-column detection.

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