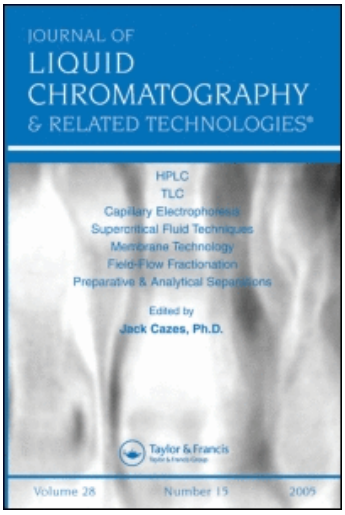

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Differential Indirect Fluorescence Detection for the Reversed-Phase Microcolumn High-Performance Liquid Chromatography

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DIFFERENTIAL INDIRECT FLUORESCENCE DETECTION FOR THE REVERSED-PHASE MICROCOLUMN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Differential indirect fluorescence detection was applied for reversed-phase microcolumn high-performance liquid chromatography of aliphatic alcohols. The differential measurement with single capillary cell and double beams improved dynamic reserve and detectability. The indirect detection system suitable for a visible semiconductor laser was developed with a deep-red dye, 3, 3'-dimethyloxatricarbocyanine chloride. A mass detection limit was in the sub-μg (nmol) range.

INTRODUCTION

Indirect photometric detections provide a universal response and an appropriate sensitivity for underivatized analytes in high-performance liquid chromatography (HPLC) (1). Especially, the laser-based indirect fluorescence detection has been successfully applied for microbore chromatography (2, 3) and capillary electrophoresis (4) because of a small probe volume in the laser irradiation. A mass detectability achieved was in the order of tens amol for the capillary electrophoresis (4). A detectability of the indirect detection strongly depends on a stability of the system such as pressure, temperature and light source. A dynamic reserve of detection electronics must be fully exploited with offsetting a large baseline. A differential measurement can improve the dynamic reserve in the indirect detection because the baseline fluctuations are compensated and the output of the electronics balances at zero (5, 6). A high frequency modulation and a lock-in detection also increase the dynamic reserve (2, 7). Double beam and dual cell arrangements have been employed in the previous reports (2, 6, 7), in which two independent flow systems were required for sample and reference cells. If a single cell and double beam technique is available, it should be advantageous over dual cell arrangements because of its simplicity and stability.

Semiconductor lasers are the low-noise, compact and convenient source of laser light. The power stability of the semiconductor laser improves signal to noise ratio (S/N) straightforwardly in the photometric measurement such as fluorescence (8), polarization (9) and magneto-otical rotation (10) techniques. However, the semiconductor laser emits only at the wavelength longer than the deep-red region. The indirect measurement overcomes this drawback by the selection of visualization reagents. Lehotay and co-workers (11) evaluated the indirect fluorometric detection with a near infrared semiconductor laser.

In this paper we described a differential indirect fluorescence detection technique for an analysis of aliphatic alcohols in reversed-phase microcolumn HPLC. Single capillary cell was used to counteract the experimental system fluctuations and minimize the dead volume. This configuration can be easily constructed with an acousto-optic modulator which generates two laser spots apart small distance with a single laser. Furthermore the indirect detection scheme feasible for a visible semiconductor laser was presented by using a deep-red dye. A visible semiconductor laser is more useful in light beam alignments than a infrared one.

EXPERIMENTAL

A schematic diagram of the differential indirect fluorescence detection system is shown in Fig.1. The microcolumn chromatographic system (JASCO, Familic-100N) consisted of a micro feeder pump (flow rate; 8 ml/min), an injector (Rheodyne, model 7413, 0.5-μl loop) and a teflon tube column (150 mm length x 0.5 mm i. d.) with Fine SIL C18-10 packing. The column was thermostated with a water bath. The mobile phase was degassed acetonitrile-water 80:20 (v/v) with 5×10^{-5} mol/l naphthacene or 1×10^{-5} mol/l 3, 3'-dimethyloxatricarbocyanine chloride (DOTC) (Exciton Chemicals) as the visualization reagent. These fluorophores have a large absorbance at the corresponding laser wavelength and an intense fluorescence. The column was treated with an appreciable amount of eluent to saturate the dyes before measurements.

A light beam of an argon ion laser (Lexel, model 95-4, λ = 476.5 nm, 10 mW) or a semiconductor laser (Toshiba, TOLD 9200, λ = 670 nm, 3 mW) was divided in two modulated beams by using an acousto-optic modulator (AOM) (Hoya, A100). The semiconductor laser was operated with the controller (Meles Griot, DLD003) and not thermostated. The beams passed

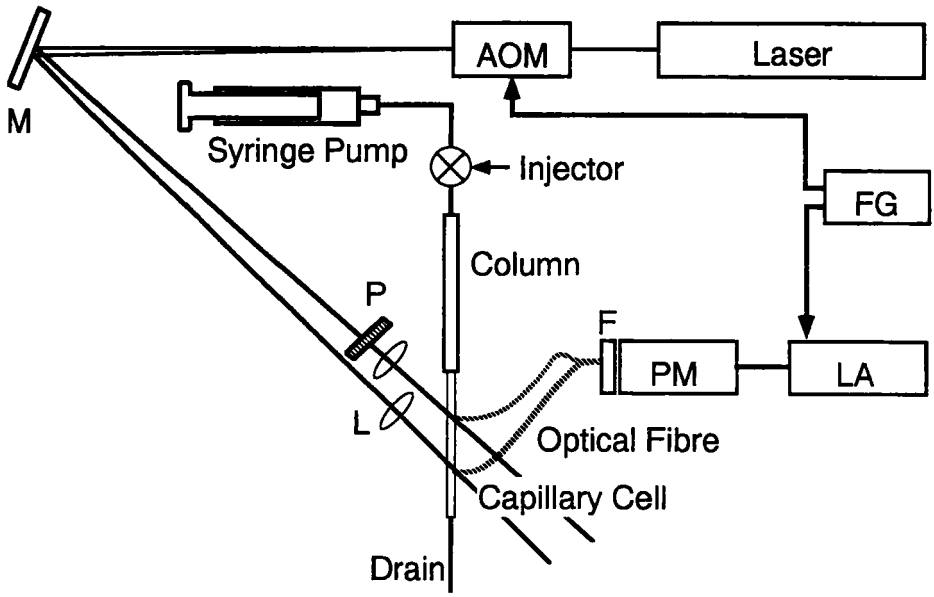

FIGURE 1. Schematic diagram of the differential indirect fluorescence detection system. AOM, acousto-optic modulator; F, optical filter; FG, function generator; L, lens; LA, lock-in amplifier; M, mirror; P, polarizer; PM, photomultiplier.

through a lens (f = 100 mm) and focused on adjacent spots in a quartz capillary cell (100 mm length x 0.4 mm i.d.) connected at the end of the tube column. Fluorescence from two laser spots was collected separately by optical fibers (Mitsubishi Rayon, ESKA 0.5-mm i.d.) and detected with a photomultiplier (Hamamatsu, R1477) through optical filters. A polarizer was used to adjust the laser beam intensity. The dyes, excited wavelengths and optical filters are summarized in Table 1. The fluorescence intensity tended to saturate at the laser power more than 5 mW due to the thermal lens effect of the eluent containing the visualization reagent. Therefore we employed the red-sensitive photomultiplier to compensate for the low power excitation. A

TABLE 1. Summary of Excited and Observed Wavelength for Visualization Reagents

Visualization reagent	Laser	λex.(nm)	Optical filter	λobs.(nm)
Naphthacene	Ar ion laser	476.5	10-nm band pass filter + Glass filter Y51	550
DOTC	Semiconductor laser	670	Jasco CT-10 monochrometor	704

lock-in amplifier (NF Circuit, 5610B) recovered the modulated signal and transmitted the data to a microcomputer (NEC, PC9801RX) for digital integration. A dc (no modulation) measurement was performed without AOM by using a14-bit digital electrometer (Takeda Riken, TR8652).

The principle of the differential measurement is shown in Fig. 2. The zero and first order beams from AOM irradiate the two spots in the capillary cell alternatively. Thus, the fluorescence signals from the two spots show the complementary phase each other. The signal from a paticular analyte delays between the two spots corresponding to the flow of the analyte. As the lock-in amplifier is a phase-sensitive detector, the background compensates each other at zero and the output of the lock-in amplifier provides the first derivative of normal chromatographic peaks. In order to obtain the smooth differential peaks, the time delay was set by the distance of the two spots as about 30 mm. The differential chromatogram is useful in the recognition of peak overlaps (12, 13).

RESULTS AND DISCUSSION

Chromatograms of aliphatic alcohols obtained by the indirect fluorescence detection with the argon ion laser are shown in Fig. 3. The injected sample was an artificial mixture of 1.0 % (w/w) octanol and decanol; the injection amount was 5 μg each. Figure 3-(a) presents a single beam measurement at the modu-

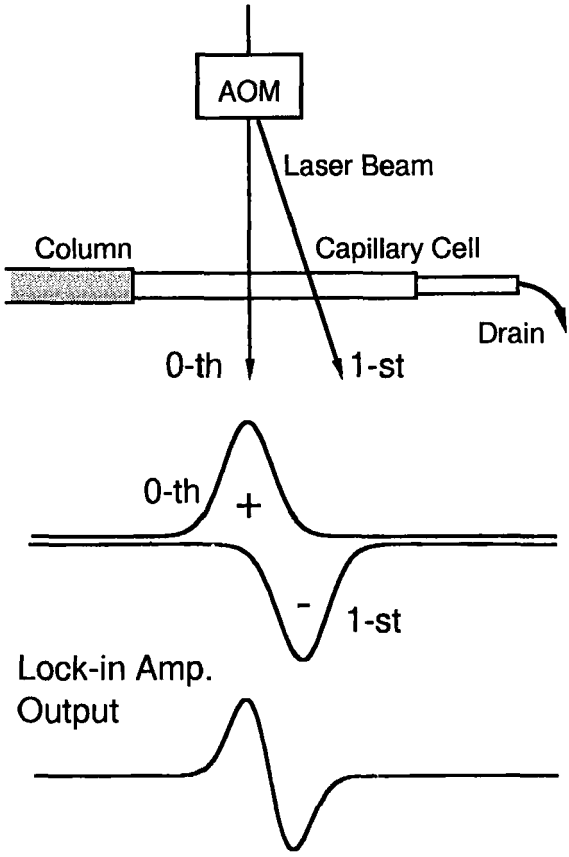

FIGURE 2. Principle of the differential measurement. Notations of 0-th and 1-st represent the diffraction order of the acousto-optic modulator.

lation frequency of 100 kHz and the time constant of 10 s as measured by blocking the first order beam. Indirect peaks of analytes appear positively and a negative system peak followed them. This is the usual reverse-phase indirect pattern (1). The displacement mechanism of the visualization reagent with the analyte is partitioning and solubility enhancements; the presence

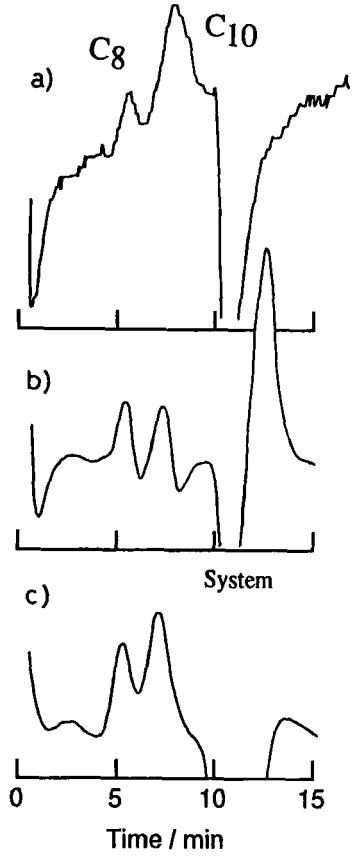

FIGURE 3. Chromatograms of aliphatic alcohols for the indirect fluorescence detection with the Argon ion laser; (a), single beam; (b), double beam; (c); integration of (b). Sample, 1.0 % (w/w) octanol (C_8) and decanol (C_{10}); mobile phase, acetonitrile-water 80:20 (v/v) including 5 x 10^{-5} mol/l naphthacene; modulation frequency, 100 kHz; time constant, 10 s.

of the aliphatic alcohols may induce the partition of the dye from immobile to mobile phase (14). The baseline increase gradually due to laser intensity instabilities and/or column pressure changes. The small noise in the baseline is owing to a quantization error that is a limitation of the resolution of the electronics. The detectability of the single beam measurement with the high-frequency modulation becomes fifteen times better than that of the dc (no modulation) measurement done by the same experimental setup.

Figure 3-(b) shows the double beam differential chromatogram with the same experimental condition with (a). The baseline becomes flat and no noise from the limitation of the electronics resolution appears. A detection limit at S/N = 3 is 0.1 % (w/w) of the alcohols and the mass detectability is in the sub-μg (nmol) range. An analytical curve is linear up to 10 % (w/w); a signal intensity was obtained from the height of the differential peak. This sensitivity is comparable to those of the universal detection for the microbore or capillary chromatography (2, 6). A reduction of the injection amount and a increase of column efficiency can improve the sensitivity because the present detection volume irradiated by the laser is less than 50 nl. An integration of the differential chromatogram makes it normal in appearance, as shown in Fig. 3-(c); the digital integration of the lock-in amplifier output was performed with an adjustment of the baseline. The improvement of S/N was expected because the integration was a sort of averaging procedures (15).

The baseline fluctuation became smaller at higher modulation frequency, as shown in Fig. 4. The noise intensity of the double beam measurement was smaller than that of the single beam one. In both measurements, the noises decrease with increasing the frequency. The major component of the noise is the instability of the system such as pressure and laser intensity fluctuations at lower frequency modulation. On the other hand, at higher frequency region the limitation of the electronics resolution governs the noise especially in the double beam arrangement. A dynamic reserve was evaluated at ca. 2000 from ratio of the

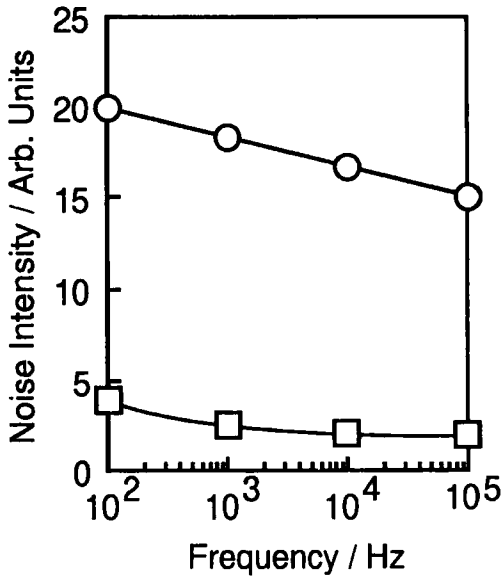

FIGURE 4. Dependence of the chromatographic baseline fluctuation on the modulation frequency. (○), single beam; (□), double beam.

baseline fluctuation and the fluorescence intensity in the single beam experiment.

Figure 5 shows indirect fluorescence chromatograms of aliphatic alcohols with the visible semiconductor laser. The fluorescence from the visualization reagent increased at the chromatographic peaks and the indirect signals were positive, where replacements might occur as the following equation (16):

$$D^+Cl^-(i) + A \rightleftharpoons DA^+ + Cl^-$$

where D^+ and A is the dye cation and the alcohols, respectively. The notation (i) represents the presence on the immobile phase. This mechanism resembles one in the indirect ion-pair chro-

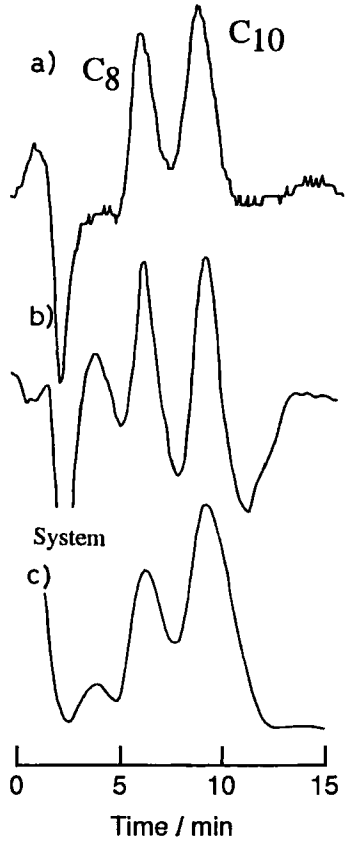

FIGURE 5. Chromatograms of aliphatic alcohols for the indirect fluorescence detection with the visible semiconductor laser; (a), single beam; (b), double beam; (c); integration of (b). Sample, 3.0 % (w/w) octanol (C_8) and decanol (C_{10}); mobile phase, acetonitrile-water 80:20 (v/v) including 1 x 10^{-5} mol/l DOTC; modulation frequency, 100 kHz; time constant, 10 s.

matography and provides the larger transfer (displacement) ratio as compared with the partitioning enhancement and the dilution of the dye by the analyte. The previous study (11) using the semiconductor laser (λ = 796 nm) and the near-infrared dye IR 125 did not present the usual pattern of positive and negative peaks and the transfer mechanism was not discerned clearly, because the fluorophores have the opposite charges; DOTC is positive and IR 125 is negative.

The chromatographic baseline with the semiconductor laser is flat even in the single beam experiment because its output is stabler than gas discharge lasers such as the argon ion laser. The measured power stability of the semiconductor laser is below 0.01 % with the 3-s time constant while the stability of the argon ion laser was reported to be 0.1 - 1 % in the normal operation and 0.04 % with external stabilizer (17). A simple electronic feedback circuit can stabilize the output of the semiconductor laser because direct conversion from electronic energy to light. The double beam differential measurement can eliminate the noise due to the quantization error by using the semiconductor laser, too. The present detection limit with the visible semiconductor laser is in the order of sub-μg (nmol) and ten times as small as that of the previous result with the near infrared one (11). However, the sensitivity could not increase as much as expected from the laser power stability due to the large laser light scattering; the excited and observed wavelengths are close in the semiconductor laser experiment (see Table 1).

In conclusion, the differential measurement with single capillary cell has improved the performance of the indirect fluorescence method applied for the microcolumn HPLC. Additionally, we have investigated the indirect detection system with the deep-red dye feasible for the visible semiconductor laser.

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