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DOUBLE-BEAM LASER-EXCITED INDIRECT FLUOROMETRIC DETECTION OF NON-ELECTROLYTES IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Double-beam laser-excited indirect fluorometric detection of organic non-electrolytes is demonstrated in high-performance liquid chromatography. Anthracene is added as a visualization reagent in the mobile phase to maintain a background signal. When the analyte perturbs partitioning of the visualization reagent between the mobile and the stationary phases, it can be detected indirectly. A mass detectability of 50 ng of decanol is obtained (signal-to-noise ratio of 2) by using a microbore column.

INTRODUCTION

Indirect photometric detection has been widely applied in ion-pair chromatography[1–14]. In these cases, an UV-absorbing ion is added to the mobile phase to visualize non-UV-absorbing ionic species. If the added ion has opposite charge to that of the analyte, effectively "on-column" derivatization occurs. If the added ion has the same charge as that of the analyte, then competing equilibria exist between the two and the counter ions either in the mobile or the stationary phase.

On the other hand, several papers have dealt with indirect detection of non-electrolytes[15–21]. Gnanasambandan and Freiser used methylene blue in the eluent and achieved indirect detection of aliphatic alcohols[15,17], ketones[15] and monosaccharides[18] with an UV–VIS detector. In this case, complexation between the analytes and methylene blue is involved. The distribution of methylene blue between the stationary and the mobile phases is thus altered by the analyte, which leads to a signal. Parkin[19] added an UV-absorbing species such as benzamide and *n*-propyl-*p*-aminobenzoate in the mobile phase for indirect photometric detection of alkanols and esters. The non-UV-absorbing species are visualized because they perturb the partitioning characteristics of the UV-absorbing species during their elution. Banerjee[21] described another approach for indirect detection using an eluent saturated with toluene. When the analyte enters, it increases the solubility of toluene and thus the analyte is monitored by the increased concentration of toluene.

In general, when the analyte transfers visualization reagent molecules from the

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stationary phase to the mobile phase and *vice versa*, it can be indirectly detected due to the change in the background signal of the eluent. Mechanistic interpretations of these induced peaks were given by Stranahan and Deming[22]. Do we need a transfer of such reagent molecules for indirect detection? If the analyte gives no detection response and does not affect the equilibration of a visualization reagent, the analyte only dilutes the visualization solution by its volume fraction. For simplicity, we can assume that 100 μg (10^{-4} ml in volume) of an analyte with molecular weight = 100 are injected on a conventional high-performance liquid chromatographic (HPLC) column, that the peak volume is 1 ml and that the peak shape is rectangular. The volume fractional concentration of the analyte is then 10^{-4} and its molar concentration is 10^{-3} *M*. If we use a detector with a dynamic reserve larger than 10^4, which is defined as the ratio between the background signal and its noise level, we can detect the analyte without any interaction (or displacement) between the analyte and the visualization reagent. The typical dynamic reserve of an UV–VIS detector is around $5 \cdot 10^3$, which means that it cannot detect the analyte mentioned above. Refractive index detectors and optical activity detectors[20] have larger dynamic reserves, being 10^6 and $2.5 \cdot 10^7$, respectively. This is the reason why these two detectors work well as a universal detector.

On the other hand, if interaction (or displacement) between the analyte and the visualization reagent occurs, it becomes possible to detect the analyte mentioned above with the same detector at a much lower concentration. Assuming that one analyte molecule transfers one visualization reagent molecule, which is common in the ion-exchange mode, the background concentration changes by 1 m*M* in the above example. Changes of 1 m*M* can easily be recognized by most of the detectors used for HPLC. This is why indirect photometry[23] has been successful for ion chromatography applications. The concentration detectability at the detector is simply given by the concentration of the visualization reagent divided by the dynamic reserve. Unfortunately, the detection is eventually limited by the decrease in dynamic reserve at low dopant concentrations and changes in the separation process itself at low dopant concentrations.

In fluorometry, the dynamic reserve depends on the stability of the light source (flicker noise). The stability of lasers is usually 1–0.1%. A double-beam arrangement with high-frequency modulation has been shown to increase the dynamic reserve and has been applied to indirect fluorometric detection of anions[24], in which a dynamic reserve of $5 \cdot 10^3$ has been achieved. In addition, lasers allow better focusing into small volumes, which is necessary for micro-scale HPLC, *e.g.*, to enhance mass sensitivity.

This paper will describe double-beam laser-excited indirect fluorometric detection of neutral species in reversed-phase chromatography. Anthracene is used as a fluorescence agent.

EXPERIMENTAL

Acetonitrile and distilled water were HPLC-grade. The other reagents used were reagent grade. The chromatographic system comprised a reciprocating pump (Mini Pump; LDC/Milton Roy, Riviera Beach, FL, U.S.A.) or a syringe pump (Model 314; ISCO, Lincoln, NE, U.S.A.), a sample injector (Model 7520; Rheodyne,

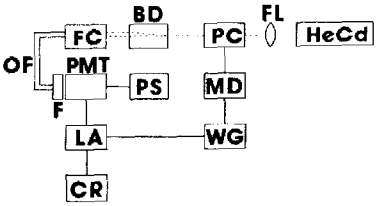


Fig. 1. Block diagram of the experimental arrangement for double-beam laser-excited fluorescence detection. HeCd = Helium–cadmium laser (325 nm); FL = focusing lens, focal length 50 cm; PC = Pockels cell; MD = modulation driver; WG = wave-form generator; BD = calcite beam displacer; FC = flow cells; F = filters; OF = optical fibers; PMT = photomultiplier tube; PS = power supply; LA = lock-in amplifier; CR = chart recorder.

Cotati, CA, U.S.A.) with a 1-μl injection volume and a conventional ODS column (VAL-U-PAK, 250 mm × 4.6 mm, 5 μm; Regis, Morton Grove, IL, U.S.A.) or a microbore ODS column (Adsorbosphere C_{18} HS, 250 mm × 1 mm, 5 μm; Alltech, Deerfield, IL, U.S.A.). Preliminary studies have been performed by using a mini pump, the conventional column and a fixed-wavelength (254 nm) UV detector (Model 260; Chromatronix, Berkeley, CA, U.S.A.).

The laser fluoremetric detection system used was similar to one reported previously[24]. A block diagram of the experimental arrangement for double-beam fluorescence detection is shown in Fig. 1. The 325-nm UV beam of a helium–cadmium laser (Model 4240NB; Liconix, Sunnyvale, CA, U.S.A.) was used as an excitation source. The polarization of the beam was modulated at 100 kHz by an electrooptic modulator (Model 3030; Lasermetric, Teaneck, NJ, U.S.A.) and the polarization-modulated beam was split into two beams by a calcite beam displacer (MBDA10; Karl Lambrecht Corp., Chicago, IL, U.S.A.). The modulator was driven by a high-frequency modulator driver (Model 25; Conoptics, Danbury, CT, U.S.A.) and unipolar positive square waves (0 to +1.0 V) from a wave-form generator (Model 162; Wavetek, San Diego, CA, U.S.A.) were in turn applied to the modulator driver. Each signal from the sample and the reference cell was collected by separate optical fibers (Superguide 600, 1.5 m × 0.6 mm core × 0.8 mm O.D.; Fiberguide Industries, Sterling, NJ, U.S.A.) to a common photomultiplier tube (1P28; RCA, Harrison, NJ, U.S.A.) after passing two filters (4-96 and 0-52; Corning Glass, Corning, NY, U.S.A.). The photomultiplier tube output was directed through an amplifier (461A; Hewlett-Packard, Palo Alto, CA, U.S.A.) to a lock-in amplifier with a 1-s time constant (Model HR-8; EG & G, PAR, Princeton, NJ, U.S.A.). Two parallel quartz tubes were used as the flow cells and were aligned vertically. A sample flow cell was connected to the outlet union of the column via stainless-steel tubing (35 mm × 0.30 mm I.D. × 0.55 mm O.D.; Hakkoshoji, Tokyo, Japan), a 1/16-in. zero-dead-volume union (Valco; Houston, TX, U.S.A.) and PTFE tubing (100 mm × 0.19 mm I.D. × 1/16 in. O.D.; Gasukuro Kogyo, Tokyo, Japan). Both ends of the above stainless-steel tubing were covered with PTFE tubing (0.5 mm I.D. × 1 mm O.D. or 0.5 mm I.D. × 1/16 in. O.D.) so that they could be tightened. The same eluent was supplied to a reference flow cell by gravity with a flow-rate of *ca.* 0.3 ml/min. Both eluents flowed upward in the cell and optical fibers were inserted into the flow cells from the top of the quartz tubes. Laser beams impinged on the quartz tubes at right

angles and their positions were adjusted in order to minimize the dead volume. The optical fibers were placed close to the beams and the two signals balanced by moving the optical fibers up and down to change the collection efficiencies.

RESULTS AND DISCUSSION

In indirect fluorometry, the dynamic reserve, the concentration of the visualization reagent and the displacement ratio all play important roles in the sensitivity that can be achieved. The displacement ratio is defined in this work as the number of visualization reagent molecules which are transferred by one analyte molecule. It can be calculated from a peak height and a peak volume. The change of concentration of the reagent added is calculated from the peak height, while the analyte concentration is calculated from the peak volume using the following equation

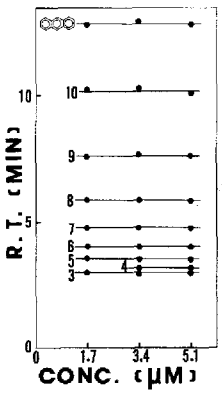
$$C_{max} = \sqrt{\frac{8}{\pi}} \cdot \frac{C_0 V_i}{V_w} \tag{1}$$

where C_{max} is the concentration of the analyte at top of the peak, C_0 is the concentration of the analyte in the sample solution, V_i is the injection volume and V_w is the peak volume.

In order to improve the sensitivity in indirect fluorometry, we should decrease the background noise as much as possible. This can be achieved by decreasing the background signal, which in turn decreases with the concentration of the visualization reagent. As long as shot noise can be neglected, sensitivity is improved by using a lower concentration of the visualization reagent, if the dynamic reserve can be maintained independent of the background level. However, when the retention time of the analyte increases or the displacement ratio decrease in proportion to the concentration of the fluorescence agent, no improvement in concentration sensitivity of the analyte can be expected by changing the concentration of the additive.

The effects of the concentration of anthracene on the retention time and peak heights of the analytes (constant injected amount) are shown in Figs. 2 and 3, respectively. The retention times of alkanols and the system peak are independent of the concentration of the reagent added in the region between 1.7 and 5.1 μM, while the peak heights of these solutes are almost proportional to the concentration of the visualization reagent. The latter result indicates that the displacement ratio changes in proportion to the concentration of anthracene. In other words, no improvement in concentration sensitivity can be expected by changing the concentration of anthracene, as mentioned above. This is in contrast to ion chromatography[24], where the displacement ratio is always unity. Any concentration of anthracene can then be selected if the shot noise can be neglected and the signal does not saturate the photomultiplier tube. The fact that at these levels the anthracene concentration does not affect retention is also different from observations in ion-pair chromatography[4].

The amount of each analyte injected in Figs. 2 and 3 is *ca.* 100 μg. The retention times increase with increasing carbon number of the analyte, as predicted from the polarities of the analytes. Alkanols with carbon numbers of 4–10 give positive peaks when the composition of the eluent is acetonitrile–water (70:30), while *n*-propanol



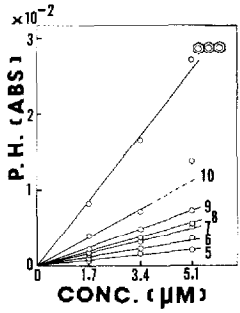


Fig. 2. Retention time (R.T.) *versus* concentration of visualization reagent. Columns: ODS, 250 mm × 4.6 mm. Mobile phase: acetonitrile–water (70:30) including anthracene; flow-rate, 1.0 ml/min. Sample: 1 μl, 12.5% of each. The numbers refers to the carbon numbers of the *n*-alkanols.

Fig. 3. Peak height (P.H.) *versus* concentration of visualization reagent. Operating conditions as in Fig. 2.

gives a negative peak under these conditions. This indicates that *n*-propanol transfers anthracene from the mobile phase to the stationary phase, while the other alkanols transfer anthracene from the stationary phase to the mobile phase. The displacement ratio is the smallest for *n*-butanol and it increases with increasing carbon number for the analytes eluting before the system peak. When a 50% acetonitrile solution is used as a mobile phase, *n*-propanol and *n*-butanol give negative peaks and the former gives a higher peak than the latter. In addition, retention times can be altered by changing the relative compositions of acetonitrile and water similar to common reversed-phase chromatography. The intensity of the signal also depends on the eluent composition. The peak areas of the analytes generally increase with increasing fraction of water.

The way to improve mass sensitivity is to use micro-scale HPLC columns. A laser is naturally suitable for small-volume detection. The dimensions of the flow cell used in this work are ideal for microbore HPLC. There is the possibility to improve mass sensitivity by a factor of 20 (the ratio of the cross-sectional areas of microbore HPLC and conventional HPLC columns). Fig. 4 demonstrates indirect fluorometric detection of alkanols with a microbore column, in which *ca.* 5 μg of each analyte dissolved in the mobile phase are injected. The limit of detection for *n*-decanol is 50 ng at a signal-to-noise ratio of 2. This points to an important feature of indirect fluorescence detection *versus* indirect absorption detection for the same system. The dynamic reserves for fluorescence and for absorption vary differently with the magnitude of the background signal. While the former remains at $5 \cdot 10^3$ until the concentration of the visualization reagent falls below 10^{-7} M[24], the latter is proportional to the concentration when the background absorption is below 1.0 absorbance unit (a.u.). This is a direct consequence of the higher sensitivity of fluorescence detection. So, at low visualization reagent concentrations, needed to avoid interfering with the separation processes itself, or at short optical pathlengths, which is characteristic of microcolumn LC, indirect fluorescence provides better detectabilities.

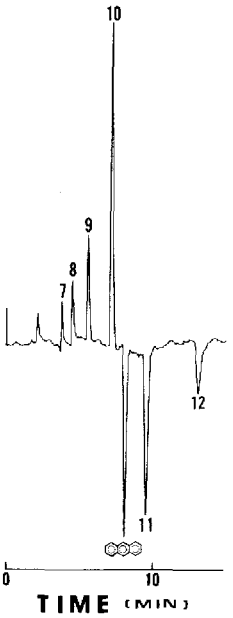


Fig. 4. Indirect fluorometric detection of alkanols. Column: ODS, 250 mm × 1 mm. Mobile phase; acetonitrile–water (80:20) including $3.3 \cdot 10^{-5}$ *M* anthracene; flow-rate, 50 µl/min. Sample: 1 µl, 0.6% of each. The numbers refer to the carbon numbers of the *n*-alkanols.

For the system here, larger signals are observed for analytes eluting close to the system peak. The same phenomenon was observed previously[19]. It is expected that the displacement ratio increases with increasing carbon chain length of the analyte based on its polarity. However, drastic increases in peak height for the peaks eluting close to the system peak cannot be explained by the polarity of the analyte. It should also be noted that undecanol gives a larger signal than dodecanol. Stranahan and Deming[22] theoretically predicted that an analyte coeluting with the system peak gives a large differential peak because the analyte continues to transfer the additive reagent as the analyte proceeds down the column. Thus, it can be predicted that analytes eluting closer to the system peak give larger signals because both bands coexist over a longer column bed as they proceed down the column. The minimum length of the column bed required for the separation of the two bands is correlated with the resolution. The resolution is proportional to the square root of the theoretical plate number. Thus, the column bed length on which both bands overlap totally or partly is inversely proportional to the square of the resolution. If the effect of changes in the distribution coefficient of the visualization reagent due to the analyte is neglected, the peak areas are expected to be correlated with the reciprocal of the square of the resolution. We find this relationship to hold quite well, except that dodecanol gives a much higher signal than expected. The variations in the distribution coefficient of anthracene are probably the reason for the errors. A much more precise simulation will be required for the estimation of the signal intensity. Another observation is that the sum of the areas of positive peaks coincides with that of negative peaks. When the analytes dissolved in a solution of acetonitrile–water (70:30) in-

cluding $3.3 \cdot 10^{-5}$ *M* anthracene were injected under the same conditions as in Fig. 4, a very large negative peak appeared at the solvent front, and this contributes to the size of the system peak. Displacement ratios calculated from the results in Fig. 4 are $1.2 \cdot 10^{-4}$ for *n*-nonanol, $4.8 \cdot 10^{-4}$ for *n*-decanol, $3.3 \cdot 10^{-4}$ for *n*-undecanol and $1.9 \cdot 10^{-4}$ for *n*-dodecanol. In other words, $8.5 \cdot 10^{3}$ *n*-nonanol, $2.1 \cdot 10^{3}$ *n*-decanol, $3.0 \cdot 10^{3}$ *n*-undecanol or $5.4 \cdot 10^{3}$ *n*-dodecanol molecules transfer one anthracene molecule. These peak heights are proportional to the amount of the analyte up to 3 μg for the microbore column, which indicates that this method gives good quantitative information.

Fig. 5 demonstrates indirect fluorometric detection of other organic species using a conventional ODS column. Carbon tetrachloride gives a much larger negative peak than expected, which is due to fluorescence quenching. The intensity of the system peak is not affected by quenching, however. The limit of detection for carbon tetrachloride is 17 ng when a microbore column is used. The use of quenching thus allows increased sensitivity in indirect fluorometric detection, at the expense of a non-linear response and the lack of universality.

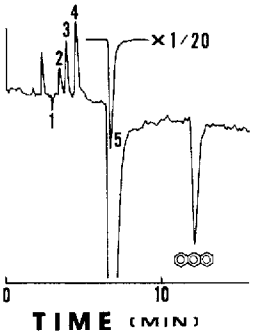


Fig. 5. Indirect fluorometric detection of organic solvents. Column: ODS, 250 mm × 4.6 mm. Mobile phase; acetonitrile–water (70:30) including 3.3×10^{-5} *M* anthracene; flow-rate, 1.0 ml/min. Sample: 1 μl, 20% of each. Peaks: 1 = *n*-propanol; 2 = tetrahydrofuran; 3 = dichloromethane; 4 = chloroform; 5 = carbon tetrachloride.

CONCLUSION

Double-beam laser-excited indirect fluorometric detection of neutral organic compounds is demonstrated. Mass sensitivity is improved by the use of a microbore column. The selection of the visualization reagent and the chromatographic conditions is important because analytes eluting closer to the system peak give higher signals. Quenching of the fluorescence due to the analyte has the potential for improving sensitivity.

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