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Indirect fluorometric detection in open-tubular capillary column chromatography

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SUMMARY

Reversed-phase open-tubular capillary (OTC) columns are prepared by cross-linking vinyl silicone gums on the inside of fused-silica capillaries. These columns are then provided with a low capacity of anion-exchange sites by dynamic modification with cetyltrimethylammonium bromide. Following anion-exchange chromatography, common anions are detected by indirect fluorescence detection, using micromolar salicylate as the eluent and the visualization agent, and a UV laser as the excitation source. Unmodified reversed-phase OTC columns are used for the separation of various alcohols. These alcohols are detected by indirect fluorescence detection, using 2,7-dichlorofluorescein as the fluorescent reagent and a visible laser as the excitation source. The small dimensions (13–15 μm I.D.) of the OTC columns and good stability of the fluorescence background (dynamic reserve $\geqslant 10^3$) allow femtogram (attomole) amounts of both types of analytes to be detected. The detectable concentrations are in the 30-n*M* range.

INTRODUCTION

The method of indirect detection in liquid chromatography has received a considerable amount of attention in the past few years[1–3]. Essentially, a detectable species is present in the eluent and produces a constant background signal at the detector. When an analyte is eluted from the column, it changes the concentration of that species at the detector and with it the background signal; hence, the analyte is detected indirectly. Schemes for the indirect detection of electrolytes[4], non-electrolytes[5] or both[6] have been devised and used with considerable success. A variety of detectors have been employed, including fluorometric[7–10], polarimetric[11] and electrochemical[12], but the majority of the work has been done with photometric detectors[4–6,13–18].

Another topic receiving considerable interest in chromatography is the use of microcolumns[19,20]. The advantages of using microcolumns are their efficiency for small samples, reduced analyte dilution, reduced flow-rate and high separation efficiency. Of the four types of microcolumns being used — small-bore packed, packed capillary, semi-packed capillary and open-tubular capillary (OTC) — OTC columns

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have the attraction of being able to produce high separation efficiencies in a reasonable time with superior limits of detection (LOD)[21]. Theory suggests that the internal diameter of OTC columns needs to approach that of column packing material (1–10 μm) in order to operate at maximum efficiency[22,23]. Although once difficult to prepare, procedures in which silicone gums are used have somewhat simplified OTC column preparation[24,25].

The coupling of microcolumns with indirect detectors allows sensitive detection of those analytes which yield poor absorption, fluorescence, or electrochemical response. Analytes detected indirectly in microcolumn separations include anions[7,10,13], alcohols[9,14], hydrocarbons[15], and ethylene glycol oligomers[16]. In the examples cited, the photometric detector is the most popular. Because a large, stable background signal is essential for any indirect detection method, restrictions on path length (b) and molar absorptivity (ε) and concentration (C_m) of the background reagent must be adhered to. For example, since absorption detectors typically have noise around 0.0001 a.u.f.s., maintaining a stability of only 1 part in 1000, the background absorbance must be at least 0.1 a.u.f.s. For columns with diameters of 10–100 μm, this fact imposes even stricter constraints on the ε and C_m of the background reagent. Indirect fluorometric detection should not suffer from these limitations to the same degree indirect photometric detection does. When excitation is provided by a laser, reduced b, ε, or C_m can largely be compensated for by increasing the power of the incident radiation to yield ample photons at the detector such that the system remains above the shot-noise limit, *i.e.*, background stability is independent of ε, b and C_m.

In this work, polymer-coated OTC columns of 13–15 μm I.D. were used for the separation of electrolytes and non-electrolytes. When the polymer surface is treated with a quaternary ammonium salt, the capillary effectively functions as an ion-exchange column. The untreated polymer surface allows the capillary to function as a reversed-phase column. Indirect fluorometric detection allows extremely low levels of both types of analytes to be detected.

EXPERIMENTAL

Reagents

Electrolytes. Sodium salicylate, sodium acetate, and high-performance liquid chromatography (HPLC)-grade methanol were supplied by Fisher Scientific (Fairlawn, NJ, U.S.A.). Sodium nitrite was supplied by Mallinckrodt (St. Louis, MO, U.S.A.). The eluent was prepared by dissolving sodium salicylate in water and degassing the solution in an ultrasonic bath under vacuum. Samples were dissolved in the eluent.

Non-electrolytes. The alkanols were supplied by Aldrich (Milwaukee, WI, U.S.A.), Sigma (St. Louis, MO, U.S.A.), or Eastman Kodak (Rochester, NY, U.S.A.). Solutions containing the fluorescent reagent, 2,7-dichlorofluorescein (2,7-DCF, Eastman) were prepared from a 1-m*M* acetic acid buffer in 0–10% acetonitrile (HPLC grade, Eastman). The pH of the final solution was adjusted to a value between 3.0 and 5.0. The eluent was degassed by sparging with nitrogen gas. Alkanol samples were prepared by mixing them with aqueous acetonitrile of the same concentration as the eluent.

In both the electrolyte and non-electrolyte experiments, the water used was purified in a Barnstead Nanopure II system (Barnstead, Division of Sybron, Boston, MA, U.S.A.). All chemicals were of reagent grade and used as supplied unless specified.

Column preparation

The columns were prepared by a procedure adapted from Farbrot *et al.*[24]. A fused-silica capillary column (Polymicro Technologies, Phoenix, AZ, U.S.A.; 50–120 cm × 150 μm O.D. and 13–15 μm I.D.) was purged with helium for 30 min. The column was then filled with a solution of 1–2% (w/v) PS-255 or PS-265 (Petrarch Systems, Bristol, PA, U.S.A.) in pentane, in which the cross-linking agent dicumyl peroxide (Pfaltz and Bauer, Waterbury, CT, U.S.A.) was present at an amount of 1% (w/w) of the polymer. Following removal of the column from the filling device, the ends were cut off and left open. The column was then immersed in a water bath with its ends exposed to air or placed in a gas chromatography (GC) oven and the solvent was evaporated at temperatures of either 35 or 40°C. After evaporation was complete, the column was again flushed with helium for 30 min. The ends were sealed with sodium silicate, and cross-linking was carried out by using a temperature program of 15°C/min to 175°C, then 175°C for 3 min. After cross-linking was complete, the polyimide coating was burned off at one end of the column to facilitate on-column detection.

Chromatographic system

The chromatographic system employed in this work is identical to that described in ref. 10, with the exception that a microsyringe pump (ISCO, Lincoln, NE, U.S.A.; Model μLC-500) was used in the constant-flow mode to propel the eluent through the column. A split-injection ratio of 200 is used. The injector (Rheodyne, Cotati, CA, U.S.A.; model 7520) rotor volume was 0.2 μl for the electrolyte experiments and 0.5 μl in the non-electrolyte experiments. When in use, the ion-exchange column was kept in a water bath held at ambient temperature.

Dynamic modification of the column

The procedure used for dynamic modification of the capillary column is similar to that described earlier[7,10,26]. Here, the column was equilibrated with a solution of 30 m*M* cetyltrimethylammonium bromide (Aldrich) in 95% aqueous methanol. Cetyltrimethylammonium bromide could be removed by washing the column with 50% aqueous methanol, thereby returning it to a reversed-phase column.

Detector

In the electrolyte experiments, 330-nm radiation from an argon-ion laser (Spectra-Physics, Mountain View, CA, U.S.A.; Model 2035) passes through a laser intensity stabilizer (Cambridge Research and Instrumentation, Cambridge, MA, U.S.A.; Model LS100, UV optics installed), where it is stabilized to a power of *ca.* 4 mW. Upon exiting the stabilizer, the radiation is focused onto the column via a 1-cm focal length fused-silica lens. The column normal is at Brewster's angle to the incident radiation. Collection of fluorescence is perpendicular to the plane of incidence and is effected by a 20 × microscope objective. The illuminated region is magnified by

approximately 60 × and forms an image at a spatial filter. Fluorescence from the internal bore of the column passes through the spatial filter, through a cut-off filter (Schott Glass, Duryea, PA, U.S.A.; UV-360) and falls onto the cathode of a photomultiplier tube (Hamamatsu, Bridgewater, NJ, U.S.A.; R928).

The system used for the indirect detection of non-electrolytes is essentially the same as above but with the following exceptions: excitation is provided by the 488-nm line of an argon-ion laser (Laser Ionics, Orlando, FL, U.S.A.; Model 554A). After passing through the stabilizer, the radiation is split, and *ca.* 2 mW is focused onto the capillary by a 0.9-cm focal-length lens; background radiation is rejected via an OG-515 cut-off filter (Schott).

Data were recorded on a strip chart recorder (Houston Instruments, Austin, TX, U.S.A.; Model B5117-5I) or subjected to analog-to-digital conversion (Data Translation, Marlborough, MA, U.S.A.; Model DT2827, 2 Hz) and stored in a personal computer (IBM PC-AT). Data recorded on the strip chart could also be digitized by a video camera and a frame grabber system (Data Translation, Auxiliary Frame Processor; Model DT2858). Digitized data were later smoothed by using a moving-window average.

RESULTS AND DISCUSSION

Electrolytes

The limiting concentration (C_{lim}) of an analyte that can be detected by indirect fluorometry of photometry is generally described by

$$C_{\text{lim}} = C_{\text{m}}/DR \tag{1}$$

where C_{m} is the concentration of the background reagent, D is the dynamic reserve (ratio of the background signal to the noise on the background), and R is the displacement ratio (moles of reagent displaced by moles of analyte). Indirect detection in ion chromatography is possible because as the analyte is eluted, it displaces an equivalent amount of the reagent ion. In this case, R is generally regarded as unity as long as the analyte and reagent ions are of equal valence.

To achieve elution and detection of low concentrations of anions in single-column ion-exchange chromatography, anion-exchange columns of low capacity are required. One way to produce a low-capacity anion-exchange column effectively is by the method of dynamic modification[7,10,26]. Column capacity can be varied by changing modifier concentration or organic content of the modifying solution. Previously, Takeuchi and Yeung[7] modified microbore silica gel columns with quaternary ammonium salts to separate several common inorganic anions with sub-nanogram LOD, using a laser-based double-beam indirect fluorometric detector. Later, by using a similar modification and detection scheme, a reversed-phase OTC column separated anions with picogram LOD[10].

The double-beam design in indirect fluorometric detection has been shown to maintain a dynamic reserve of $5 \cdot 10^3$ until the background concentration falls below 10^{-7} M[8]. The dynamic reserve for indirect photometry is in excess of 10^4 but decreases proportionally with background concentration when absorption falls below 1 a.u.f.s. When capillary columns are used, the lower limit of C_{m} or ε is increased due to

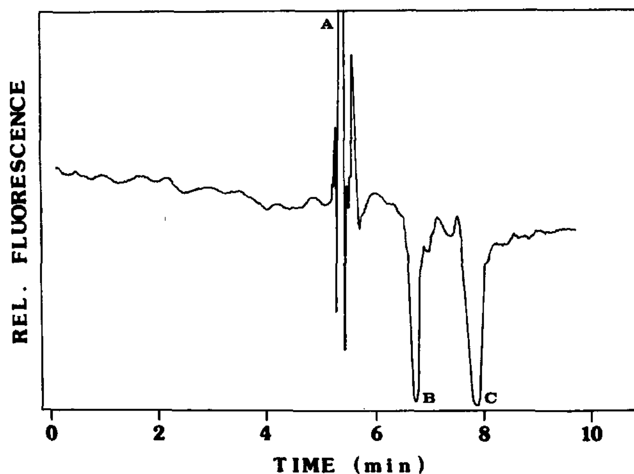

Fig. 1. Anion chromatogram with indirect fluorometric detection. (A) Salicylate (system peak); (B) acetate; and (C) nitrite. The amount of acetate and nitrite injected was 7 fmol each. Flow-rate, 35 nl/min; eluent, $2 \cdot 10^{-6}$ *M* sodium salicylate; column, 120 cm × 15 μm I.D. 2% PS-264.

reduced path-length. If C_m is raised to compensate for b, decreased detectability will result, as shown by eqn. 1.

The high dynamic reserve reported in ref. 7 is due to the use of the double-beam design in conjunction with high-frequency modulation and lock-in detection. This detection scheme is not particularly well suited for use with capillary columns, as discussed in ref. 10. Laser flicker noise, which can be as high as 1% of the total power, can be effectively reduced to levels below 0.02% by use of a laser intensity stabilizer. This approach has been used to produce stable fluorescence backgrounds for indirect fluorometric detection in capillary zone electrophoresis[27,28].

The chromatogram in Fig. 1 shows the separation of acetate from nitrite on a dynamically modified OTC column of 15 μm I.D. Once the column had been modified, it could be used for several days without decrease in performance. Capacity factors for the anions are similar to those reported for a dynamically modified C_8 OTC column of 50 μm I.D.[10]. The displacement ratios for the anions calculated from peak height and peak volumes were found to be 1/18 for acetate and 1/12 for nitrite. Eqn. 1 suggests lowering of C_m to decrease C_{lim}, but further reduction of C_m results in a corresponding reduction in D. Displacement ratios of < 1 have been observed before for separations in which salicylate concentrations of $< 10^{-5}$ *M* were used[7,10]. These low displacement ratios can be attributed to interactions other than ion exchange, occurring at the column surface when low-capacity columns and low reagent concentrations are used.

The baseline in Fig. 1 represents a 0.93-μA current from the phototube and is stable to 1 part in 1000. This is a direct consequence of the intensity-stabilized beam and the use of a 30-point moving-window average for the digitized data. The use of the microscope objective in conjunction with spatial and cut-off filters kept stray radiation levels to below 20% of the total signal when low concentrations of fluorophore were used. This is important, since the displacement ratio is calculated from

only that fraction of the signal due to probe fluorescence. These improvements in the design given in ref. 10 keep the baseline noise at a level of 0.93 nA, which corresponds to 15 fg or 0.3 fmol of nitrite injected. This is *ca.* 70 times less than that reported in ref. 10. Most of this improved mass detectability can be attributed to the reduced column dimensions. The remaining improvement is due to an increase in dynamic reserve and differences in displacement ratio. The concentration detectability for nitrite, which was calculated to be $2.4 \cdot 10^{-8}$ *M*, is slightly better than that reported in ref. 10, due to the increase in dynamic reserve in the present experiments. In separate experiments using 10-μm capillaries, we have achieved baseline noise levels equivalent to 10 fg or 0.2 fmol of nitrate injected.

Non-electrolytes

As opposed to equivalent displacement of the background ions by analyte ions in ion-exchange chromatography, indirect detection of non-electrolytes in reversed-phase chromatography relies on the analyte perturbing the partitioning dynamics of the reagent, which has been equilibrated between the mobile and stationary phases. Thus, the eluted analyte zone contains either an excess or a deficit of the reagent, as compared to the unperturbed reagent concentration. In this case, R is usually $\ll 1$.

Most separations involving indirect photometric or fluorometric detection of non-electrolytes employ an ultraviolet-absorbing, non-ionic reagent[5,9,14–16]. Separations using a reagent which absorbs in the visible region have been reported and are based on a type of complexation between the analyte and probe[17,18]. A simple argon-ion laser, capable of emitting at 488 nm, is a popular, relatively low-cost, and reliable source with excellent beam characteristics. Fluorescent molecules, capable of absorbing at this wavelength, tend to be large, ionizable species which do not chromatograph well in the reversed-phase mode. Ion suppression, as its name implies, is a technique which can improve the chromatographic behavior of ionizable compounds by suppressing their ionization when a buffer is used to control the pH[29]. Maintaining the pH of a 2,7-DCF solution between 3.0 and 5.0, we found that the fluorescent compound could be used as a reagent for indirect detection of non-electrolytes. Although 2,7-DCF has a reduced fluorescence yield in this pH range, its fluorescence was easily visible when micromolar solutions were excited with a 2-mW unfocused beam of 488.0 nm radiation.

The chromatogram in Fig. 2 shows the separation of various alcohols on an OTC column of 13 μm I.D. For the laser power and photomultiplier tube voltage being used, this concentration of 2,7-DCF allowed the system to remain above the shot-noise limit. Unlike ion chromatography, lowering of C_m does not give better detectability for non-electrolytes[9]. The total peak area of the positive peaks does not equal the total peak area of the negative peaks. This could be the result of the ionizable nature of the probe but this has also been observed occasionally for neutral reagents[14]. The hour-to-hour reproducibility of the system was good and could be maintained for several days by allowing the pump to run continuously at a reduced flow-rate. However, when the column was washed with aqueous methanol and later re-equilibrated with the eluent containing the reagent, the retention of the alcohols would remain essentially the same but the system-peak retention would be unpredictable. This suggests that the retention of 2,7-DCF is, in part, due to sites different from those responsible for alcohol retention.

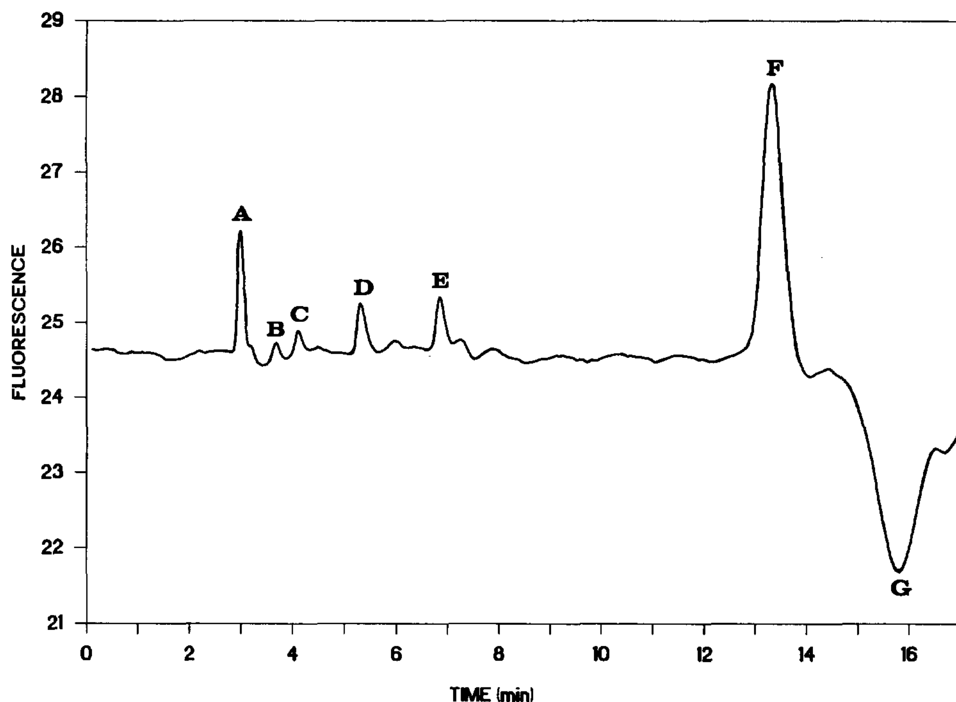

Fig. 2. Chromatogram of none-electrolytes with indirect fluorometric detection. (A) Solvent disturbance due to excess acetonitrile in sample solution; (B) 2-heptanol; (C) 1-heptanol; (D) 2-octanol; (E) 1-octanol; (F) 1-nonanol; and (G) 2,7-DCF (system peak). All alcohols were at 0.01% (v/v), except 1-nonanol which was 0.005%. Eluent, $2 \cdot 10^{-6}$ M 2,7-DCF in 5% acetonitrile containing, 10^{-3} M acetic acid (pH 4.0); flow-rate, 23 nl/min; injection volume, 230 pl; column, 50 cm × 13 μm I.D., 1% PS-255.

The displacement ratio for each of the alcohols in Fig. 2 was found to be: 2-heptanol, 1:3000; 1-heptanol, 1:2400; 2-octanol, 1:850; 1-octanol, 1:870; and 1-nonanol, 1:27. As with other non-electrolyte indirect detection systems, analytes eluted closer to the system peak produced more efficient displacement of the reagent[9]. These ratios are larger than those reported in ref. 9 but comparable to those in ref. 14.

The dynamic reserve in Fig. 2 is calculated to be 1500. As before, this stability is attributed to the use of a laser intensity stabilizer and a moving-window average (10-point). The limit of detection (signal-to-noise ratio, S/N = 2) for the early-eluted alcohols is a few picograms, and 72 fg or 0.5 fmol for 1-nonanol. This figure for 1-nonanol is about four orders of magnitude lower than that reported for 1-nonanol in ref. 14, where packed capillaries (340 μm I.D.) and indirect photometric detection were used. Again, the majority of this improvement is due to the reduction of column dimensions, specifically the internal diameter. For the same detectable concentration, the mass involved is smaller with smaller columns. Naturally, indirect photometry is not applicable to columns with these dimensions.

It is interesting to note that the concentration detectability for 1-nonanol, $3.6 \cdot 10^{-8}$ M, and the concentration detectability of nitrite, $2.4 \cdot 10^{-8}$ M, are nearly equal. This is the result of the displacement ratio for nitrite being unusually small for ion-

exchange chromatography and the displacement ratio for 1-nonanol being unusually large for reversed-phase chromatography.

CONCLUSION

The use of silicone gums simplifies the production of OTC columns. These columns may be dynamically modified to function in the ion-exchange mode or used as they are in the reversed-phase mode. Indirect fluorometric detection of analytes in either mode with OTC columns allows extremely low mass detectability.

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