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SUBNANOLITER LASER-BASED REFRACTIVE INDEX DETECTOR FOR 0.25-mm I.D. MICROBORE LIQUID CHROMATOGRAPHY

REVERSED-PHASE SEPARATION OF NANOGRAM AMOUNTS OF SUGARS

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SUMMARY

A simple, inexpensive, small-volume refractive index detector has been applied to the separation of nanogram amounts of sugars by capillary column high-performance liquid chromatography.

INTRODUCTION

The incorporation of lasers as light sources in spectroscopic instrumentation has produced spectacular improvements in detection limits. These improvements arise from several unique properties of laser radiation. In particular, the high spatial coherence of the laser beam facilitates study of small volume samples; laser beams may be focused to very small spots without loss of power to produce excellent detection limits[1–3]. Capillary liquid chromatography offers several important advantages compared with conventional chromatographic instrumentation. Particularly important advantages include low solvent consumption and excellent mass sensitivity[4,5]. The latter advantage is of value when small amounts of rare and expensive analytes must be determined.

However, the small volume of capillary columns introduces severe constraints upon the system dead-volume. In particular, the major limitation in capillary liquid chromatographic performance appears to be detector technology[4–6]. It is very difficult to miniaturize conventional detector instrumentation without compromising the detector performance. Subnanoliter detectors have been developed based upon fluorescence, absorbance, light scatter, and electrochemistry[7–16]. However, no universal detector has been developed for capillary chromatography. A universal detector provides an analytical signal for all analytes. The typical example in conventional chro-

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matography is the refractive index (RI) detector. The RI detector is often listed as the second or third most popular detector for conventional liquid chromatography.

Unfortunately, there are no subnanoliter RI detectors available. A very sensitive RI detector has been reported which utilizes a Fabry-Perot interferometer to measure directly the change in optical path length induced by a change in solute concentration[17]. However, the volume of this instrument appears to approach 1 ml, far too large for capillary liquid chromatography. A second, less sensitive, RI detector has been developed which is based upon a miniaturized Fresnel prism and has a volume of 1 μl[18]. The instrument uses a focused laser beam to probe the cuvette; detection limits of $\Delta RI = 2.0 \cdot 10^{-7}$ are reported.

We will describe a subnanoliter RI detector for capillary liquid chromatography. This detector is a miniaturized version of a previously described instrument[19]. A tightly focused laser beam passes slightly off-axis through a fused-silica capillary tube, filled with analyte. The beam profile is distorted upon passage through the tubing, producing a somewhat elliptically shaped beam. This beam profile changes shape with a change in RI of the analyte. A small area photodiode is located at a relatively sharp light–dark boundary on the beam profile. As the RI of the analyte changes, the beam profile changes, producing a change in intensity at the photodiode. The resulting current change produced by the photodiode is linearly related to the RI of the analyte over several orders of magnitude in RI change.

Use of a tightly focused beam and a small I.D. capillary tube can result in a very low detector volume. For example, a 10-μm spot-size laser beam within a 100-μm I.D. capillary defines a cylindrical probe volume of 30 pl. Of course, the dead-volume of the detector is not limited by the probed volume of the laser beam, but instead, is limited by the size of the tubing. For a detection region located immediately downstream from the column end frit, peak spreading is likely to extend down the tube by a length given by the tube radius. A 100-μm diameter cuvette will have a dead volume of approximately 0.4 nl. Smaller diameter capillary tubing will produce a corresponding decrease in detector volume.

EXPERIMENTAL

The laser-based, small-volume, RI detector is similar to one described before[19]. Fig. 1 presents an experimental diagram. A low-power, linearly polarized beam at 632.8 nm is provided by a helium–neon laser (Melles Griot, Irvine, CA, U.S.A.). A linear polarizer is located in the optical train and rotated to maximize the transmitted intensity. A quarter-wave plate is placed after the polarizer and rotated to eliminate any retroreflected light from reaching the laser cavity. These retroreflections primarily originate from the focusing lens and sample cuvette, described below. It also is necessary to tilt each of the polarizing elements slightly to prevent retroreflections from their surfaces from reaching the laser cavity. A microscope cover slip is used to direct ca. 5% of the laser light to a reference photodetector, described below. A piece of frosted glass is located before the reference photodetector. Translation of the frosted glass allows convenient balancing of the intensity of light reaching the signal and reference photodetectors.

The main portion of the beam is focused with a 16-mm focal-length microscope objective into the sample cuvette. The objective is mounted rigidly in space. The
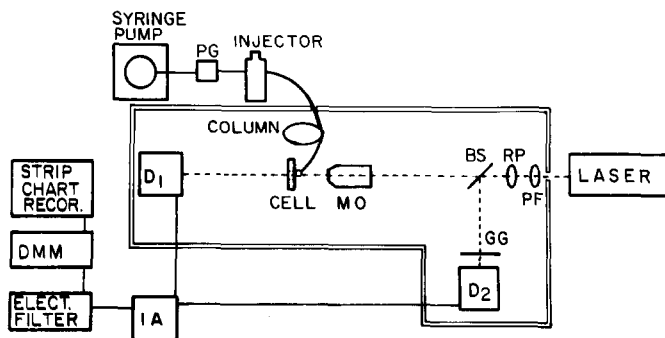
Fig. 1. Experimental diagram of the laser-based, small-volume RI detector. PG = pressure gauge; PF = polarizing filter; RP = quarter-wave retardation plate; BS = beam splitter; MO = microscope objective; D1 and D2 = identical photodiodes; IA = instrumentation amplifier; elect. filter, 1-Hz low pass electronic filter; DMM = digital multimeter. The optical system is enclosed in a box denoted by the double-line figure.

sample cuvette consists of a 100-μm I.D. × 245-μm O.D. fused-silica capillary tube (Polymicro Technologies, Phoenix, AZ, U.S.A.) with a thin polyimide coating. This capillary fits inside a 250-μm I.D. × 400-μm O.D. fused-silica tubing (Polymicro Technologies) which has had the polyimide coating removed. The sample cuvette is tilted slightly from vertical. The larger tubing is the chromatographic column and the smaller tubing holds the column end frit in place. The cuvette is located on a three axis translation stage to provide convenient alignment with respect to the laser beam.

The laser beam is allowed to propogate *ca.* 15 cm to a small area (1 mm^2) photodiode located on a single translation stage. The photodiode may be translated perpendicular to the plane formed by the laser beam and sample cuvette to a region where the laser beam profile undergoes a large change with a change in RI of analyte. The signal and reference photodiodes are identical Model SD 041-11-11-011 silicon photodiodes (Silicon Detector, Newbury Park, CA, U.S.A.). The output of the diode is conditioned with a current-to-voltage converter, consisting of a JFET operational amplifier, LF 351, wired with a 1-MΩ feedback resistor in parallel with a 47 pF capacitor. The outputs of the signal and reference photodiodes are subtracted using a Model AD 524 instrumentation amplifier (Analog Devices, Norwood, MA, U.S.A.). A low pass electronic filter (Ithaco, Ithaca, NY, U.S.A.) with a 1-Hz cut-off is employed to smooth the signal. The chromatogram is displayed on a strip-chart recorder. The injection mark is obtained by shifting the range scale on the recorder coincident with the injection. The injection itself produces no perturbation in the chromatogram.

Alignment

Alignment of the sample cuvette and signal detector with respect to the laser beam is important for achieving good sensitivity and detection limit. First, the vertical position of the cuvette is adjusted so that the laser beam traverses the cuvette a small distance below the chromatographic column end frit. Next, the cuvette is moved along the beam path to locate the sample at the beam waist, about 5 mm from the lens. Last, the cuvette is moved perpendicularly to the beam path so that the center

of the cuvette is about 0.1 mm from the beam axis. This alignment produces an elliptically shaped beam profile at the detector plane. The signal photodiode is placed at the sharp boundary between the main beam and the first adjoining dark fringe.

Chromatographic system

The chromatographic system consists of a Model 314 high-pressure syringe pump (Isco, Lincoln, NE, U.S.A.), a Model 2D430 pressure gauge (Alltech, Chicago, IL, U.S.A.), a 100-μl Model EC14W air actuated injection valve (Valco, Houston, TX, U.S.A.), and a locally prepared reversed-phase capillary liquid chromatography column. The column preparation is similar to that published elsewhere[5,14,20,21]. A 0.6-m long piece of 250-μm I.D. fused silica is used for the column. The polyimide coating is removed from the bottom 5 cm with a cool flame. A short piece of 100-μm I.D. $\times$ 245-μm O.D. fused-silica tubing is inserted in the chromatography column exit and used to hold a thin PTFE frit in place (Fig. 2). The frit is located at the top of the region which has been stripped of polyimide, and the region below is the detector cuvette. A small drop of high temperature epoxy is placed at the bottom of the 250-μm column to hold the 100-μm I.D. tubing in place.

The reversed-phase column is prepared from 5-μm diameter C_{18} spheres (Supelco, Bellefonte, PA, U.S.A.). A slurry of the packing material is prepared in chloroform–methanol (80:20) at a 1:7 (w:v) ratio of stationary phase to slurry solvent. The high-pressure packing pump (Shandon) is filled with methanol. After filling the empty column, the well sonicated slurry is placed in a 700-μl volume reservoir, connected to the column and packed initially at low pressure (1000 p.s.i.). The pressure is slowly raised to 5500 p.s.i. and held at that pressure for *ca.* 2 h. A valve is closed between the column and the pump, and the column pressure is allowed to drop to atmospheric conditions. About ten column volumes of water are next passed through the column to help stabilize the bed[22].
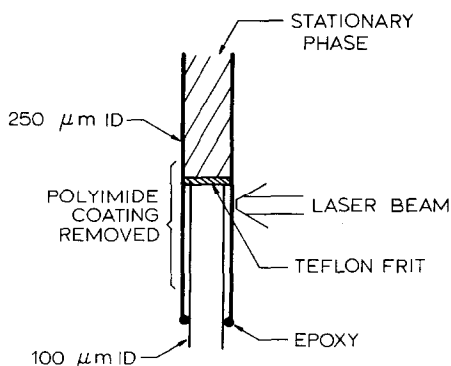

Fig. 2. Detail of cuvette and column exit. The upper tube is a piece of 250-μm I.D. fused-silica tubing which forms the chromatography column. The frit is a thin section of porous Teflon. The polyimide coating of the chromatography column is removed below the frit. A sort section of 245-μm O.D. $\times$ 100-μm I.D. tubing is inserted in the chromatography column and holds the column end frit in place. A small bead of epoxy glue holds the cuvette assembly in place.

Reagents

All chemicals are reagent grade or better. Stock sugar solutions are prepared in water. The chromatographic mobile phase is water with a very small amount of acetonitrile (see below). A typical flow-rates is 1.3 μl/min.

RESULTS AND DISCUSSION

Although not ideal, reversed-phase chromatography on C_{18} columns may be employed for the chromatographic determination of simple sugars. Fig. 3 presents a chromatogram of 667 ng of glucose, 707 ng of sucrose, and 845 ng of raffinose. The chromatographic efficiency is fair, about 4500 plates or 7500 plates/m. Other work utilizing the same detector and column but another separation suggests that detector dead volume does not limit the chromatographic resolution. These sugars are base line resolved; however, an attempt to separate several additional sugars on this column failed. Work is continuing in this laboratory to prepare an NH_2 capillary column for additional separation efficiency of complex mixtures of sugars. Note, the last (inverted) peak in the chromatogram is associated with the solvent used to prepare the solutions and occurs whenever water is injected into the system. Presumably, a small, residual amount of acetonitrile from a previous chromatographic experiment remained in the syringe pump to contaminate the solvent used in this experiment. Injection of pure water yields a small change in RI and a corresponding negative peak in the chromatogram.

A roughly ten-fold dilution of the sugar solution was injected onto the column (Fig. 4) and the sensitivity of the recorder was increased. The detection limit, three standard deviations above the background, is estimated from the peak-to-peak noise in the chromatogram[23]. For the sugars it is about 40–50 ng injected, about half the amount injected in this chromatogram. A 1-s low pass filter was employed in this chromatogram; since the peak elution time is about 30 s, a much longer time constant on the detection electronics could be employed with a corresponding improvement in detection limit.
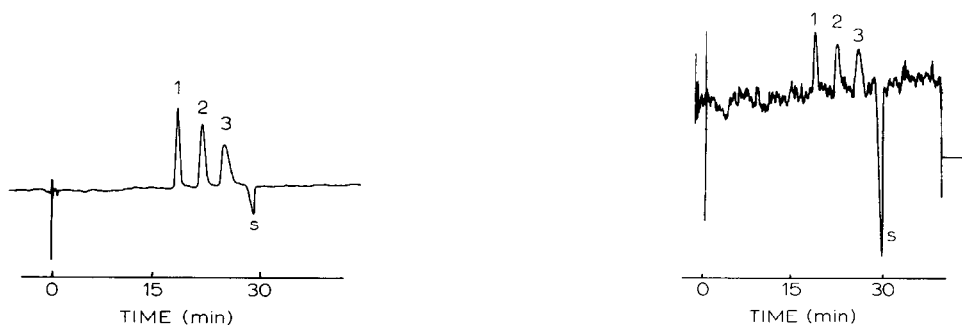

Fig. 3. Chromatogram of sugars. Peaks: 1 = 667 ng of glucose, 2 = 707 ng of sucrose, 3 = 845 ng of raffinose.

Fig. 4. Chromatogram of dilute sugars. Peaks: 1 = 83 ng of glucose, 2 = 88 ng of sucrose, 3 = 106 ng of raffinose. This injected amount is approximately twice the detection limit.

Of course, the sugars which elute from the column are more dilute than the injected sample; it is of interest to seprate the chromatographic performance from the detector performance. Assuming a Gaussian peak and estimating the standard deviation of the peak as 0.42 times the full width at half maximum yields a standard deviation of about 0.8 μl and a peak analyte concentration at the detection limit of about $2 \cdot 10^{-5}$ g/ml. At the detection limit, a RI change of about $2.8 \cdot 10^{-6}$ is produced at the maximum of the sucrose peak. This value is three times larger than the detection limit reported in our earlier work. The larger detection limit probably is a result of the much more narrow cuvette capillary employed in the present work. Narrow capillary columns are more sensitive to both vibrations and temperature changes. The latter effect is due to the small heat capacity of very narrow capillary columns.

Detection limits for the amount of analyte injected into the column fall in the mid-nanogram region for the sugars employed in this separation. However, much less material is present in the detection volume. With a 0.4-nl detection volume, only $8 \cdot 10^{-12}$ g is present at the detection limit whereas $6 \cdot 10^{-13}$ g is present within the 30-pl volume probed by the laser. These very small numbers are a direct result of the small volume detector employed in this experiment. Calibration curves were prepared for the three sugars. The linearity, $r > 0.999$, extended over a factor of 35 in mass injected from the detection limit to about 1.3 μg injected. A negative curvature was observed at higher concentrations. This deviation from linearity appears not to be due to the detector, but instead, due to overloading of the chromatographic column. Future work will explore the ultimate dynamic range of the detector.

It is important to comment upon the simplicity and cost of this instrument. Only one alignment step is critical—positioning of the cuvette perpendicular to the laser beam —and once the desired far-field beam profile is observed, realignment is very rapid. The laser employed is one of the least expensive and most reliable lasers on the market. Any low-power helium–neon laser may be employed, although a polarized laser should give a more stable signal. The optical components individually cost less than US$ 100, and are available from a number of vendors. The cost for the entire detector, minus the optical table, is about US$ 1200, half of the total being the cost of the strip chart recorder!

REFERENCES

1 T. Hirschfeld, *Appl. Opt.*, 15 (1976) 2965.
2 C. Radzewicz, P. Glowczewski and J. Kraninski, *App. Phys.*, 17 (1978) 423.
3 E. K. Gustafson and R. L. Byer, *Opt. Lett.*, 9 (1984) 220.
4 M. Novotny, *Anal. Chem.*, 53 (1981) 1294A.
5 J. C. Gluckman, A. Hirose, V. L. McGuffin and M. Novotny, *Chromatographia*, 17 (1983) 303.

6 H. Poppe, *Anal. Chim. Acta*, 145 (1983) 17.

7 G. J. Diebold and R. N. Zare, *Science*, 196 (1977) 1439.

8 L. W. Hersshberger, J. B. Callis and G. D. Christian, *Anal. Chem.*, 51 (1979) 1444.

9 S. Folestad, L. Johnson, B. Josefsson and B. Galle, *Anal. Chem.*, 54 (1982) 925.

10 N. J. Dovichi, J. C. Martin, J. H. Jett and R. A. Keller, *Science*, 219 (1983) 845.

11 J. W. Jorgenson and E. J. Guthrie, *J. Chromatogr.*, 255 (1983) 335.

12 N. J. Dovichi, T. G. Nolan and W. A. Weimer, *Anal. Chem.*, 56 (1984) 1700.

13 T. G. Nolan, W. A. Weimer and N. J. Dovichi, *Anal. Chem.*, 56 (1984) 1704.

14 T. G. Nolan, D. J. Bornhop and N. J. Dovichi, *J. Chromatogr.*, 384 (1987) 189.

15 F. Zarrin and N. J. Dovichi, *Anal. Chem.*, 57 (1985) 1826.

16 R. L. St. Claire and J. W. Jorgenson, *J. Chromatogr. Sci.*, 23 (1986) 186.

17 S. D. Woodruff and E. S. Yeung, *Anal. Chem.*, 56 (1982) 1174.

18 S. A. Wilson and E. S. Yeung, *Anal. Chem.*, 57 (1985) 2611.

19 D. J. Bornhop and N. J. Dovichi, *Anal. Chem.*, 58 (1986) 504.

20 D. C. Shelly, J. C. Gluckman and M. Novotny, *Anal. Chem.*, 56 (1984) 2990.

21 T. G. Nolan and N. J. Dovichi, in preparation.

22 M. Konishi, Y. Mori and T. Amano, *Anal. Chem.*, 57 (1985) 2235.

23 J. E. Knoll, *J. Chromatogr. Sci.*, 23 (1985) 422.