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CROSSED-BEAM THERMAL-LENS DETECTION FOR 0.25-mm DIAMETER MICROBORE LIQUID CHROMATOGRAPHY

SEPARATION OF 2,4-DINITROPHENYLHYDRAZONES

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SUMMARY

The crossed-beam thermal-lens detector is applied to capillary liquid chromatography for the separation of picomole amounts of 2,4-dinitrophenylhydrazones. A relatively inexpensive instrument is described, which utilizes a 3-mW helium–cadmium pump laser and a low-power helium–neon probe laser. Detection limits of 120 fmol of acetone (injected) are obtained.

INTRODUCTION

The incorporation of lasers as light sources in optical methods of analysis has produced spectacular improvements in detection limits. These improvements arise from several unique properties of laser light. In particular, the high spatial coherence of continuous wave laser beams facilitates the study of small-volume analytes. For example, tightly focused laser beams have been used to probe subnanoliter to subpicoliter volumes by fluorescence[1–3], light scatter[4–6], and absorbance[7–9]. Unfortunately, the limited wavelength selection available from inexpensive lasers and the limited spectral information produced by electronic transitions of fluid-phase molecules results in limited qualitative information about the analyte. The nature of the analyte may be discerned in favorable cases by chromogenic reaction. However, highly specific chromogenic reactions are not available for most analytes. Instead, chromatographic separation and retention are used to provide qualitative information about the analyte.

Capillary liquid chromatography is a powerful method for the separation of complex mixtures. Two primary advantages of capillary chromatography compared with conventional chromatography are decreased solvent consumption and improved mass detection limits. The latter property is important when analyzing small amounts

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of rare or expensive analytes. However, to obtain good performance from capillary chromatography, it is necessary to reduce the dead-volume to a minimum. Unfortunately, only a limited number of detector technologies exist for capillary chromatography[10–12]; it is often difficult to miniaturize conventional detectors to the required low volume without concomitant loss of sensitivity. It is not surprising that the combination of capillary liquid chromatography with laser-based detection is attractive[11]. The high sensitivity and low probed volume of the laser techniques compliments the high separation efficiency and low dead volume requirements of capillary liquid chromatography.

In this paper, we describe a low-volume, high-sensitivity absorbance detector based upon the crossed-beam thermal-lens for 0.25-mm I.D. slurry-packed capillary liquid chromatography. The detector has been utilized before for amino acid determination with a commercial 1-mm I.D. chromatography column[12]. Detection limits of subpicomole quantities of amino acids injected into the column were reported. Presumably, combination of this detector with capillary liquid chromatography should produce improved detection limits. In the crossed-beam thermal-lens technique, two laser beam are crossed within a weakly absorbing sample[7–9]. Absorbance of light from a tightly focused and modulated pump beam produces a time-varying temperature rise within the sample. The resulting refractive index perturbation within the sample acts to defocus periodically the probe beam. A simple, small-area photodiode, centered in the probe beam profile, is used to measure the beam defocusing. A lock-in amplifier acts as a phase-sensitive amplifier to demodulate the crossed-beam thermal-lens signal. This thermo-optical technique has a property important for small-volume absorbance determinations: the signal is generated *only* at the intersection region of the two beams and is independent of path-length. Small-volume samples may be probed simply by using tightly focused laser beams.

Like other thermo-optical techniques, the sensitivity of the crossed-beam thermal-lens increases linearly with pump-laser power[13]. Furthermore, the sensitivity is inversely proportional to the pump beam spot size; as the pump beam is more tightly focused, both the sensitivity and spatial resolution of the technique improve. It is not surprising that the crossed-beam thermal-lens produces excellent mass detection sensitivity. For example, detection limits corresponding to 120 iron atoms as the 1,10-phenanthroline complex have been observed with the crossed-beam thermal-lens in a 0.2-pl probe volume[9]. These detection limits were achieved with a modest power pump laser, 100 mW at 514.5 nm, and a water–methanol solvent.

EXPERIMENTAL

The chromatographic system is constructed from a Model 314 high-pressure syringe pump (Isco, Lincoln, NE, U.S.A.), a 60-nl Model EC14W air-actuated injection valve (Valco, Houston, TX, U.S.A.), and a 40 cm × 0.25 mm I.D. fused-silica capillary column. The chromatographic stationary phase consist of 5-μm diameter C_{18} particles (Supelco, Bellefonte, PA, U.S.A.). The column is packed with a chloroform–methanol (80:20) slurry at 5000 p.s.i.[14–17]. The mobile phase flow-rate is 2 μl/min. The detector cuvette is an 80-μm I.D. square-bore glass-capillary tube (Wale, Hellertown, PA, U.S.A.) which has been glued into the end of the column and holds the column end-frit in place.

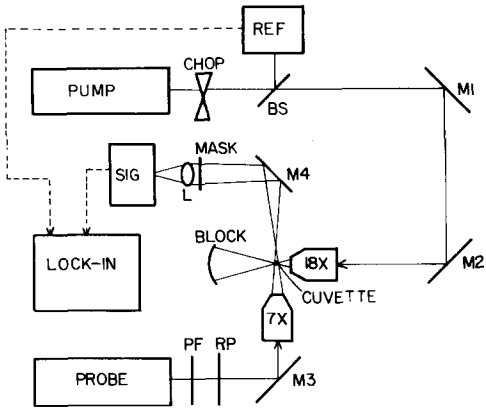

Fig. 1. Block diagram of the crossed-beam thermal lens detector. PUMP, helium–cadminum pump laser; CHOP, mechanical chopper; BS, beam splitter; M1–M4, mirrors; 18 ×, microscope objective; CUVETTE, 80-μm square-bore capillary tube; BLOCK, beam block, PROBE, helium–neon probe laser; PF, polarization filter; RP, quarter-wave retardation plate; 7 ×, microscope objective; MASK, mask formed by two razor blades; L, one inch focal lenth lens; SIG, signal detector; REF, reference detector; LOCK-IN, lock-in amplifier.

The block diagram of the crossed-beam thermal-lens instrument is presented in Fig. 1. The optical system is constructed on a Model KST-48 4 ft. × 8 ft. optical table (Newport, Fountain Valley, CA, U.S.A.). The pump laser beam is provided by a Liconix (Sunnyvale, CA, U.S.A.) model 4210B helium–cadmium laser, which delivers to the sample a linearly polarized, 3-mW beam at modulated in a symmetric square wave by a variable-frequency chopper at 128 Hz. A microscope cover slip is used to divert a small portion of the laser beam to a reference detector, described below. The transmitted beam is reflected from two mirrors and focused into the detector cuvette with a 10-mm focal length (18 ×) microscope objective. After transmission of the pump beam through the sample, it is absorbed by a metal beam stop. The probe beam is provided by a 5-mW He–Ne helium–neon laser (Melles Griot, Irvine, CA, U.S.A.) at 632.8 nm. A dichroic polarization filter and a quarter-wave plate are used to reduce retroreflected light reaching the probe laser cavity. This retroreflected light can produce a significant amount of intensity noise in the probe beam. After passage through the polarization components of the optical train, the probe beam is reflected from one mirror and focused before the sample with a stationary 22-mm focal length microscope objective. The transmitted probe beam is reflected from a mirror and centered upon a 1-mm slit constructed from two razor blades. The slit is oriented in the plane of the two laser beams and acts as a spatial filter to average spatial noise in the probe beam profile while simultaneously acting as a limiting aperture for the thermal-lens signal. The transmitted light is collected with a large diameter lens and focused onto a photodiode. Any defocusing of the probe beam produced by the heated sample is translated into a change in optical power at the detector.

It is important to note that all optical components are held with massive fixtures and precision micrometer-driven translation stages. In our instrument, the

probe-beam path is fixed in space. The sample cuvette is glued to a piece of aluminum stock so that the cuvette windows are perpendicular to the pump- and probe-beam paths. The cuvette is positioned with a three-axis translation stage and located past the probe beam waist so that the cuvette does not vignette the beam. The pump-beam lens, mounted on a three-axis translation stage, and the two mirrors allow great freedom in the location of the pump beam waist. Typically, the pump beam is focused in the center of the cuvette. Small adjustments in the pump beam position are performed while a high-concentration peak is eluted from the chromatographic column. With high efficiency columns and quickly eluted peaks, this adjustment requires nimble fingers.

The reference and signal detectors are identical 1-mm^2 silicon photodiodes (Silicon Detector, Newbury Park, CA, U.S.A.). The photodiode signal is conditioned with a current-to-voltage converter constructed from a FL 351 JFET operational amplifier wired with a 1-MΩ feedback resistor in parallel with a 470-pf capacitor. The crossed-beam thermal-lens signal is demodulated with a Model 393 lock-in amplifier (Ithaco, Ithaca, NY, U.S.A.) operating in the amplitude mode and with a 4-s time constant. The amplitude mode eliminates any phase noise produced by temperature or flow-rate fluctuations. The chromatograms are displayed on a strip-chart recorder.

Reagents

2,4-Dinitrophenylhydrazine was from Aldrich, as were the six ketones: acetone, methyl ethyl ketone, cyclohexanone, *tert.*-butyl methyl ketone, adamantanone, and 4-methyl benzophenone. Acetonitrile was from Alltech; all other chemicals were reagent grade or better. The chromatographic mobile phase was acetonitrile–water (3:1). 2,4-Dinitrophenylhydrazone crystals were prepared following a standard recipe[18,19]. The samples were recrystalized from ethanol and dried. Sample solutions were prepared in acetonitrile.

RESULTS AND DISCUSSION

Flowing samples resent as particular challenge to thermo-optical instruments[20–23]. Flow acts to translate heat downstream from the pump beam, decreasing the strength of the thermo-optical element. However, some of the decrease in signal may be counteracted by translating the pump beam upstream from the probe beam[22]; heat flows downstream to the probe laser, increasing the thermal-lens signal. Furthermore, flow acts as a heat-loss mechanism, in addition to thermal diffusion. The thermal-lens signal exhibits a faster approach to steady state in flowing samples compared to static samples; higher modulation frequencies may be employed in flowing systems to decrease low frequency noise in the measurement. However, to optimize the instrument, low linear flow-rates are desirable.

In this manuscript we present preliminary results of the crossed-beam thermal-lens for detection in packed 0.25-mm I.D. capillary chromatography columns. In particular, separation of 2,4-dinitrophenylhydrazine derivatives of ketones was chosen as a model. This derivatization reagent is commonly employed for identification of carbonyl compounds and forms dark-red derivatives. These derivatives have modest molar absorptivity at the helium–cadmium laser 442-nm line, 2500–5000

$l\ mol^{-1}\ cm^{-1}$. The absorbance maximum of these derivatives is located around 360 nm where the molar absorptivity is about 20000 $l\ mol^{-1}\ cm^{-1}$.

We employ a helium–cadmium laser operating at 442 nm. This laser is relatively inexpensive and relyable and is quite simple to operate. However, the 442-nm wavelength is not ideally suited for this analysis; the 325-nm line would better match the absorbance maximum. On the other hand, the reduced power available at 325 nm coupled with greater solvent absorbance should limit the crossed-beam thermal-lens performance.

For the separation of 2,4-dinitrophenylhydrazones, conventional chromatographic instrumentation has previously been used[18]. Little modification of this separation scheme is required for capillary liquid chromatography. Fig. 2 presents a chromatogram of a mixture of six derivatized ketones. The concentration of each ketone was $5.0 \cdot 10^{-5}$ *M*. At an injection volume of 60 nl, only 3 pmol of each ketone was injected. For acetone, 180 pg was injected. The variation in response for these compounds appears to reflect variations in molar absorptivity at the 442-nm helium–cadmium laser wavelength. Detection limits, three standard deviations above background[24], were near 120 fmol for acetone and 600 fmol for adamantanone, injected on-column. Of course, much less material is present within the detection volume: assuming a 2-pl probe volume, at the detection limit *ca.* $3 \cdot 10^{-19}$ mol or $1.5 \cdot 10^{-17}$ g of acetone is present at the peak maximum. Clearly, the crossed-beam thermal-lens is well designed for absorbance detection in small volume chromatography. Actually, the detector volume is much greater than the probed volume in this instrument. It appears that open tubular liquid chromatography will be required to utilize fully the small volume detection capabilities of the crossed-beam thermal-lens.

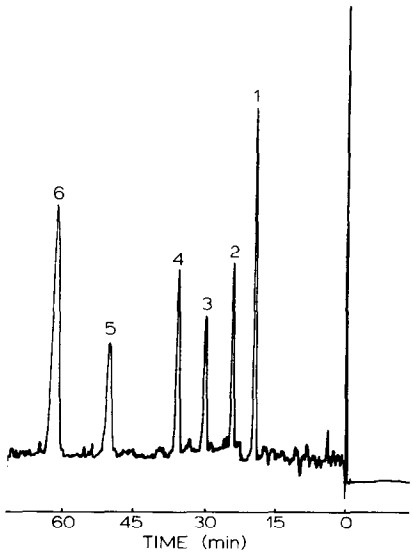

Fig. 2. Chromatogram of 2,4-dinitrophenylhydrazine derivatives of six ketones. Peaks: 1 = acetone, 2 = methyl ethyl ketone, 3 = cyclohexanone, 4 = *tert.*-butyl methyl ketone, 5 = adamantanone derivative, 6 = 4-methylbenzophenone. An amount of 3 pmol of each compound are injected. Note that the injection mark is obtained by momentarily blocking the probe beam; no transient signal is observed coincident with the injection.

Other thermo-optical techniques have been applied to liquid chromatographic detection[12,25–33]. For example, absorbance detection limits of 500 fg of rug dye were reported with a 1-mm I.D. chromatographic column using the coaxial pump-probe thermal lens[33]. However, that analysis required a high-power argon ion laser, 1.25 W, as the pump beam and tested an analyte with very high absorptivity. The high cost of the argon ion laser required in the pump-probe thermal lens will undoubtedly discourage frequent application. More importantly, the relatively large detection volume of that instrument (8 μl) precludes application for 0.25-mm I.D. capillary liquid chromatography.

It is worth emphasizing that very low-power lasers were employed in our instrument; the pump and probe lasers produced 3 and 5 mW, respectively. Since the sensitivity of thermo-optical techniques scales linearly with pump laser power, the low-pump laser power employed in our crossed-beam instrument produces a factor of 400 lower sensitivity than would be produced by a pump laser of the same power as employed in the coaxial pump-probe thermal lens instrument. Of course, detection limits for the two instruments are similar. The improvement in performance of the crossed-beam thermal lens over the coaxial pump-probe thermal-lens is based upon the path length independence property of the crossed-beam instrument. No penalty is paid for small path-length measurements.

Furthermore, it should be noted that the crossed-beam instrument employs relatively inexpensive lasers. The crossed-beam thermal-lens detector could be duplicated minus the optical table, for less than US$ 6000, comparable to the cost of the remainder of the chromatographic system. Although some optical experience is required for the initial alignment of the system, alignment after a change of column takes only *ca.* 5 min.

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