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# Refractive-index detection by interferometric backscatter in packed-capillary high-performance liquid chromatography

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**Abstract**

A simple, inexpensive, micro-volume refractive index detector has been applied to packed capillary high-performance liquid chromatography. Nanoliter detector volumes produced by a unique optical train, based on interferometric backscatter, allows for universal solute detection at the picogram level. System utility is demonstrated by reversed-phase separation of a test mixture containing ppm levels of NaCl, phenol, ethyl benzene and toluene.

*Keywords:* Refractive index detection; Detection, LC; Interferometric backscatter; Aromatic compounds; Sodium chloride

## 1. Introduction

The refractive index (RI) detector is the workhorse of detectors in conventional liquid chromatography separations because it is a concentration sensitive, bulk property, non-destructive sensor. Yet, RI measurement schemes for universal detection have found little acceptance in capillary chromatography [1]. The lack of a commercial device and limited use in capillary HPLC stems not from opulence, for a real need exists for a detector that provides an analytical signal for all analytes. While attempts have been made to measure RI changes in small volumes [2–10], limitations inherent to miniaturizing bulk property detection schemes to nanoliter volumes without a significant loss in performance have persisted.

Packed capillary chromatography offers several advantages compared to conventional separation instrumentation. As previously detailed [11–13], these advantages include improved mass detection limits and low solvent consumption. However, capillary-scale HPLC and nanoscale liquid chromatography (LC) [12,13] impose severe constraints on the detection system, requiring high sensitivity and low dead volumes (nanoliters or smaller).

With the incorporation of the laser into spectroscopic instrumentation, spectacular improvements in detection limits have been realized [14,15]. These improvements arise from the properties of laser radiation. One such property is the high spatial coherence which allows the beam to be focused to very small spots without loss of power [16–18]. In the case of interferometry, the monochromatic characteristics of laser light allows the determination of very small phase changes resulting in high

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sensitivity [19,20]. Lasers have been employed in the attempt to construct micro-volume RI detectors that performed with various levels of success [6–9]. The most sensitive devices demonstrated have been based on some form of interferometry as described below.

Refractometric interference spectrometry can be used to measure organic pollutants in water as demonstrated by Gaugliz and co-workers [3,4]. This technique uses the change in optical path length resulting from the absorption of a solute onto a film that swells reversibly. Path length sensitivity and flow cell volume constraints limit this method to schemes larger than capillary techniques. In the work by Woodruff and Yeung [2], a Fabry–Perot interferometer provided excellent sensitivity, but is expensive to configure and due to path length sensitivity, can only provide detection in low microliter volumes. Bornhop and Dovichi [5] demonstrated the use of a laser-based, off-axis on-capillary technique that can probe nanoliter volumes and has been applied to detect nanogram quantities of sugars in micro-LC separations [5]. This technique was found to be tedious to align. Bruno and co-workers further developed the forward scatter, off-axis technique [7]. They showed how improved performance is possible by employing an RI-matching fluid to surround the capillary tube, active thermal control of the flow cell assembly and position sensitive detection. These improvements facilitated the use of the RI detector for capillary electrophoresis (CE), yet the device still requires off-axis alignment and is limited by the need to modify the capillary tube by removing the polymer coating to aid in index matching.

Another technique for detecting changes in $n$ in capillary tubes uses a holographic grating to produce two beam interference in a forward scatter configuration [21]. Krattiger and co-workers were able to separate and detect metal ions by CE [21] using this holographic grating, a capillary that is encapsulated in an index matching glue and a photodiode array wired to produce position sensitive detection. Indeed a meritorious advance in universal detection in capillary dimensions. Yet there are several drawbacks to the holographic technique, as in the forward scatter configuration of the past [5,7,9,21]. The major drawback of grazing angle forward scatter optical configurations is the existence of a fundamental path length dependence which ultimately limits the level of miniaturization.

Other on-column RI detection methods have been investigated including the concentration gradient method which probes the on-axis optical perturbation produced by a transient solute band [8,10]. Although somewhat insensitive to thermal noise, the concentration gradient detector seems most suitable to capillary isoelectric focusing schemes and is, therefore, somewhat limited in its application.

We have recently shown that on-capillary RI analysis can be performed using interferometric backscatter [22–24]. Micro-interferometric backscatter detection (MIBD), previously termed laser interferometric backscatter (LIB), employs a simple optical train, produces high sensitivity RI measurements in small diameter capillaries with minimal path length sensitivity [23] and requires no modification of the capillary tube. Here we will describe the use of a MIBD system for RI detection in packed capillary liquid chromatography. In MIBD, an unfocused, linearly polarized He–Ne laser beam impinges on a tube of capillary dimensions producing a fan of scattered radiation, spatially contained, in 360° and perpendicular to the long axis of the tube. When viewed on a flat surface, a series of light and dark spots (high contrast interference fringes) are seen. The central fringe pattern at nearly zero degrees in backscattering angle is similar in appearance to that produced by single slit diffraction. The position of these maxima and minima can be employed in the measure of fluid bulk properties. Detector response is linear and changes in n can be measured to about two parts in $10^7$.

Use of an unfocused beam and an unmodified 75 mm I.D. capillary can result in the low detector volume required for capillary LC. For example, a 0.4 mm spot size laser beam impinging on the capillary defines a cylindrical probe volume of 1.2 nl. By using a very short section of tubing for the transfer line and detector flow cell, the post column dead volume is minimized and has been estimated to be approximately 0.44 nl [6]. We show that micro-LC–MIBD can be used to detect solutes at the ppm level, with mass detection limits in the picogram range. Further, we demonstrate that the measurement of $\Delta n$ in small volumes at high sensitivity, a formidable task, can be accomplished with the micro-interferometric backscatter detector; an easily configured, inexpensive device that has a folded optical train for a small foot print.

## 2. Experimental

### 2.1. Detection System

The basic optical configuration for MIBD has been described in detail elsewhere [22–24] and is depicted in Fig. 1. A low power linearly polarized beam at 632.8 nm is provided by a 4 mW helium–neon laser (Melles Griot, CA, USA). The beam traverses a microscope cover slip (which directs ca. 5% of the laser light to a reference photodetector), is directed onto a mirror and then impinges on the capillary tube. 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.

Fig. 2 is a generalized block diagram for the entire separation and detection system. The separation column is terminated as described below with a 75 μm capillary (Polymicro Technologies, Phoenix, AZ, USA) that has a 12–15 μm polyimide protective coating and is mounted on a flow cell assembly [24] with active thermal control. The complete flow cell assembly is tilted at an angle which is convenient for placement of the signal photodetector and normally does not exceed a 7° angle. The backscatter radiation is allowed to propagate ca. 60 cm to a small area (1.5 $mm^2$) photodiode mounted in a fixture which also holds a 632.8 nm interference filter (Optical Coating Tech., Southampton, MA, USA) and a 250 μm precision air slit (Melles Griot). The assembly is mounted on a high precision translation stage. Translation of the detector allows for easy selection of one of the central fringes. These fringes have been found to shift spatially with an RI change from the analyte [22]. The signal and reference photodetectors are identical Model PIN2DI silicon photodiodes (UDT Sensor, Hawthorne, CA, USA). The output of the diode is conditioned with a current-to-voltage converter, consisting of a JFET operational amplifier TLO82, wired with a 1 MΩ feedback resistor in parallel with a 50 pF capacitor. The outputs of the signal and reference photodetectors are subtracted and smoothed using a Model TM 503 differential amplifier (Tektronix, Beaverton, OR, USA). Chromatograms are displayed on a strip-chart recorder. Injection is marked by changing the range of the recorder.

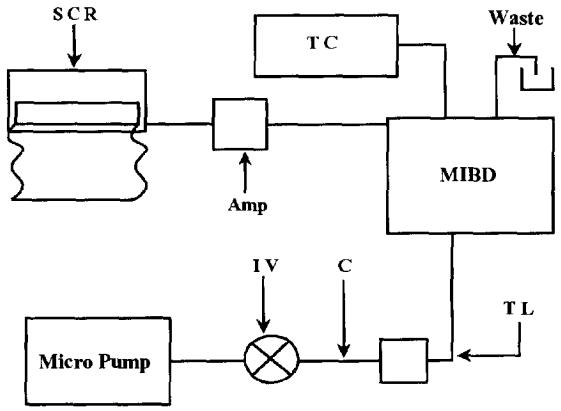


Fig. 2. Block diagram for separation system. P, micro-gradient pump; C, separation column; IV, manual injector configured with a 100 nl loop; TL, transfer line and detection flow cell; Amp, differential amplifier; SCR, strip chart recorder. MIBD is the RI detection system configured as described in Section 2.

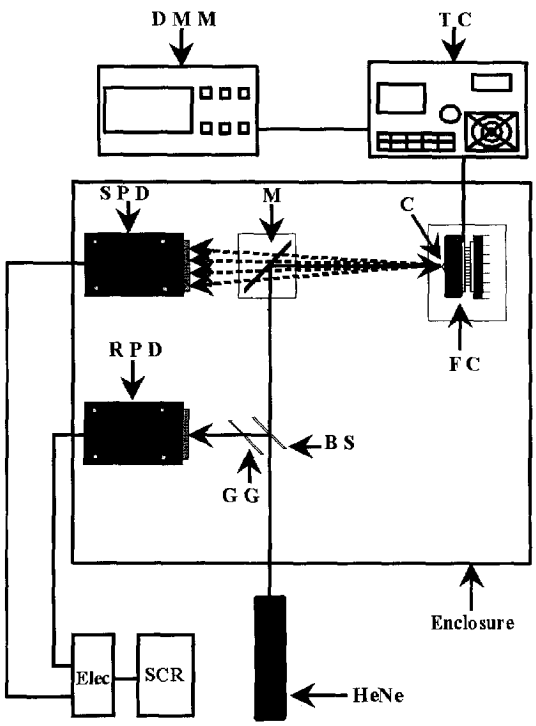


Fig. 1. Micro-Interferometric Backscatter Detector optical train. He–Ne, helium–neon laser; BS, beam splitter; GG, ground glass; Rpd and Spd, reference and sample photodetectors respectively; Amp, differential amplifier; M, mirror; C, detection capillary; FC, flowcell assembly; DMM, digital multimeter; TC, Peltier temperature controller; SCR, strip-chart recorder.

All optical components and detectors are rigidly mounted on a 4 foot by 4 foot (1 foot=30.5 cm) optical bread board (Newport Research, Irvine, CA, USA). Manual micrometer driven translation stages are used to provide reproducible translation of the photodetector, capillary tube and optical components. Excluding the pump and injector, the entire experiment in enclosed in a Plexiglas box.

The flow cell described in detail elsewhere [24] is thermostated with active control using a Peltier thermoelectric cooling chip (Melcore, Trenton, NJ, USA) controlled by a power supply (Alpha Omega Instruments, Power Puncher P-37, Johnston, RI, USA) wired in feedback from a calibrated thermocouple. Thermal stability is found to be better than $1.0 \cdot 10^{-2}$ °C over a 30 min period.

*2.1.1. Alignment*

High sensitivity is easily achieved in MIBD by simply insuring that the laser beam illuminates the entire capillary in a region where the coating has not been scratched or marred. Upon obtaining a back-scatter interference pattern, the flow cell assembly is tilted to direct the beam onto the photodetector assembly. Once a signal is obtained the detector assembly is translated across two full fringes allowing the identification of the minima. The detector is then positioned so that a small voltage output is observed, thus placing the sensor at the edge of the sloping intensity gradient of the working fringe which has a Gaussian intensity distribution.

*2.2. Separation System*

A syringe pump, Model MicroPro (Eldex Labs., Napa, CA, USA), is operated in the flow-rate control mode providing either isocratic or gradient flow to the column. The samples are introduced using a manual nanoliter (100 nl loop) injection valve (Valco, Houston, TX, USA). Separations are performed on a 25 cm Zorbax RX $C_8$ packed capillary column with the inlet end attached directly to the injector and the outlet end connected to a 75 μm I.D. detection capillary that is mounted on the flow cell housing.

*2.2.1. Column Preparation*

Reversed-phase micro-LC columns are prepared using 25 cm lengths of fused-silica capillary tubing with an I.D. of 250 μm and an O.D. of 450 μm (Polymicro Technologies). Ceramic frit bed supports are prepared as described elsewhere [25]. The columns are slurry-packed with 5 μm diameter $C_8$ bonded phase packing (Zorbax Rx-$C_8$, Mac Mod Analytical, Chadds Ford, PA, USA) using acetonitrile as the slurry solvent and packing solvent. Connection between the separation column and the detection capillary is made using a double-ended ferrule, capillary butt connector to reduce extra-column broadening and to allow the detection capillary to be easily changed.

*2.3. Chemicals*

All chemicals are reagent grade or better. HPLC grade acetonitrile and DDI water are used as the solvent or the blank solution. Solvent compositions are detailed in the appropriate figure captions. Calibration standards are prepared in the solvent used for chromatography and is normally a mixture of acetonitrile–water (65:35).

## 3. Results and discussion

The principal of operation for the RI measurement has been described in detail elsewhere [22–24] and involves the measurement of optical path length changes within the tube as n changes for materials contained therein. Upon solute introduction, the position of the fringes shift. We use a simple approach to measuring this shift by placing the slit-photodetector assembly on the edge of a fringe and integrating the intensity change as the solute RI changes. The shift is found to be proportional to analyte concentration and is linear over three decades [23,24]. This simple intensity-based detection method is inexpensive and easy to implement, yet will ultimately limit the dynamic operation range. Consequently, we are investigating the use of array detection techniques to improve detector performance and further simplify beam–detector alignment. It should be noted that the fringes show smooth monotonic intensity variation with coated tubes as supplied by the manufacturer.

Isocratic, reversed-phase chromatography was per-

formed on a test mixture to evaluate the performance of MIBD with capillary LC. Fig. 3 presents the chromatogram of a four component mixture containing 0.58 ppm of NaCl, 8.0 ppm of phenol, 1.4 ppm of toluene and 8.0 ppm of ethyl benzene. All major components are baseline resolved. While the chromatographic efficiency could be improved, performance is good, about 5184 plates or 20 736 plates/meter. There is some band broadening that results from having a short distance between the column outlet and probe region. NaCl gives a differential response as expected due to a refractive index of the components being both larger and smaller than the solvent. This solute band can be used as the column dead volume marker and shows the universal response of RI detection. A roughly 10 fold dilution of the test mixture 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 [26]. For the solutes, it ranges from about 5.8 ng for NaCl to about 16.7 ng for ethyl benzene. Since no active filtering was employed and the elution time for the solutes exceeds 12 min, a low pass filter could be used to improve *S/N*.

Of course, the solutes which elute from the column are more dilute than in the injected solution. Detector performance and chromatographic performance can be separated by assuming the solutes elute as Gaussian peaks. Estimating a standard deviation of the peak as 0.42 times the full width at half maximum yields a standard deviation of about 0.8 μl. Therefore the measured analyte concentration for ethyl benzene at the detection limit is about $2.05 \cdot 10^{-5}$ g/ml or about 20 ppb. Ignoring dilution from chromatographic performance (at the maximum for the toluene peak), the RI change at the detection limit is about $8 \cdot 10^{-7}$. This value is about seven times larger than the detection limit reported earlier [24] and comparable to those found in our initial work [23]. The larger value can be attributed to uncontrolled temperature changes due to an unthermostated column and transfer line and the small heat capacity of the very narrow capillary. Proper rede-

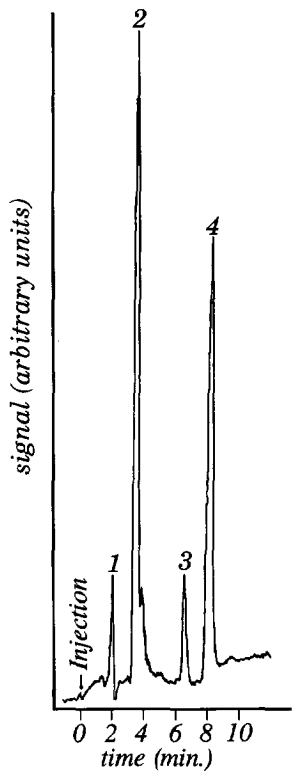


Fig. 3. Chromatogram of test mixture. Peaks: 1=58 ng of NaCl, 2=800 ng of phenol, 3=140 ng toluene, 4=800 ng of ethyl benzene. Isocratic conditions using acetonitrile–water (65:35) at a flow-rate of 3.0 μl/min produced a pressure of about 2000 p.s.i. (1 p.s.i.=6894.76 Pa).

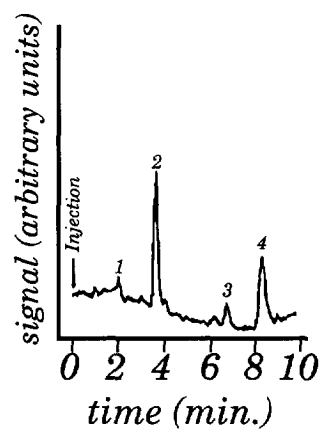


Fig. 4. Chromatogram of ten fold dilution of test mixture. Peaks: 1=98 pmol of NaCl, 2=841 pmol of phenol, 3=152 pmol of toluene, 4=750 pmol of ethylbenzene. Isocratic conditions using acetonitrile–water (65:35) at a flow-rate of 3.0 μl/min produced a pressure of about 2000 p.s.i.

Table 1
Detection limits for MIBD with μ-HPLC

| Test solute | Detection limits[a,b] | Detector volume DL[a,c] | Limit of Detection ($\Delta n$)[a] |
|---|---|---|---|
| NaCl | 2.9 ng/49 pmol | 29 pg | $7.6 \cdot 10^{-7}$ [d] |
| Phenol | 8.6 ng/91.5 pmol | 87 pg | $8.7 \cdot 10^{-7}$ |
| Toluene | 8.4 ng/91.3 pmol | 84 pg | $8.2 \cdot 10^{-7}$ |
| Ethylbenzene | 16.7 ng/157 pmol | 167 pg | $1.67 \cdot 10^{-6}$ |

[a] Determined at $3\sigma$.
[b] Amount injected on-column.
[c] Amount found in the detection volume of approximately 1 nl at the detection limits.
[d] Estimated from table of densities CRC Handbook.

sign of the system will probably eliminate these performance losses.

Analyte injected detection limits fall in the nanogram or picomolar range (Table 1). However, much less material is present in the detection volume. With a conservative estimate for the detection volume of 1.2 nl, there is only 29–167 pg of solute present. Within this detection volume there is less than $5 \cdot 10^{-13}$ mol of NaCl and less than $1 \cdot 10^{-12}$ mol of phenol or toluene present. Detector response is linear over a dynamic range of about 2.5 decades, for each solute, with a linearity of $r > 0.998$. Above this value, either a differential response is seen because the peak maximum shifts past the center of the slit or there is chromatographic solute overloading leading to non-linearity.

Table 2 presents system performance characteristics such as retention time reproducibility and the deviation in peak height for duplicate injections. Retention time reproducibility varies from 0.093% to about 0.43%, while peak height reproducibility is on the order of about 1%. System performance is good, but needs to be improved. Not shown in the table is the value for the long term drift of the system. We have determined this value to be about $2.1 \cdot 10^{-5}$ $\Delta n/h$. This relatively large value results from having poor or no active thermal control on much of the transfer line and separation column. By further enclosing the optical train and by redesigning the flow cell assembly for better temperature control, drift will be reduced significantly.

A comment regarding the practical operation of the MIBD with capillary HPLC is in order. The major limitation with the current system is the need to physically re-align the photodetector assembly when changing mobile phases or detection capillaries. Since the absolute interference pattern is dependent on the absolute RI of the fluid in the capillary, it is necessary to fine tune the alignment when the mobile phase has been changed. This simple alignment process, of moving the slit-photodetector assembly to the edge of a fringe, is necessitated in the current design because we use a single photodetector and will be circumvented with array detection. When the mobile phase remains constant, we find alignment unnecessary for a period of days. Finally, positioning and alignment of a new capillary normally takes about 30 min, involves making one butt-connection, cleaning the capillary with methanol, finding a high contrast beam profile and positioning the photodetector on the leading edge of the fringe.

Table 2
Performance characteristics of MIBD with μ-HPLC

| Test solute | Retention time reproducibility[a] | Peak height reproducibility[a] |
|---|---|---|
| NaCl | 1.997±0.0085 min (0.43%) | 51.92±0.49 mm (0.94%) |
| Phenol | 4.325±0.0106 min (0.25%) | 176.3 ±1.6 mm (0.91%) |
| Toluene | 7.167±0.0105 min (0.15%) | 23.47±0.082 mm (0.35%) |
| Ethylbenzene | 8.642±0.0083 min (0.09%) | 119.5 ±1.2 mm (1.0%) |

[a] Calculated for five replicate injections.

In summary, it was shown that the backscatter micro-interferometric detector can be used for universal detection in capillary HPLC. The optical configuration is simple, facilitates the use of unmodified capillaries and can be constructed inexpensively (less the optical bench, the total cost is about US$ 1000). MIBD is sensitive to small changes in refractive index (picogram quantities can be detected currently) allowing ppm quantities to be quantified. Employing solid state lasers and recently reported attachment techniques [27,28], it should be possible to configure the entire detector onto a single chip. This is important, considering that the current trend toward analysis system miniaturization [29,30] seems limited by the availability of a ultra-small detection system. Current investigations in our laboratories at Texas Tech include the further application of MIBD for RI detection in μ-HPLC, its miniaturization to the size of a single chip and its use as a polarimeter of capillary dimensions [31].

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