*Journal of Chromatography*, 464 (1989) 83–91
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 134

# MICROBORE LIQUID CHROMATOGRAPHY AND REFRACTIVE INDEX GRADIENT DETECTION OF LOW-NANOGRAM AND LOW-ppm QUANTITIES OF CARBOHYDRATES

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(First received July 28th, 1988; revised manuscript received November 24th, 1988)

## SUMMARY

A refractive index gradient detector is presented as a universal detector in the microbore high-performance liquid chromatography analysis of carbohydrates. Simultaneously, low-ng and low-ppm injected quantities of carbohydrates were detected at the 3 × root-mean-square baseline noise level. A typical microbore high-performance liquid chromatography chromatogram separating fructose from sucrose followed by refractive index gradient (RIG) detection is reported. Use of a position sensitive detector (PSD) in the RIG detector design is reported and experimental considerations discussed. Optimization of the PSD-based RIG detector is addressed. Potential for the device in industrial and clinical applications is considered.

## INTRODUCTION

The separation and detection of carbohydrates is an important analytical problem. Analysis of carbohydrates in clinical and industrial settings is commonplace, thus reliable and sensitive analysis techniques are continually being developed and evaluated. The primary approach to carbohydrate analysis for complex samples is high-performance liquid chromatography (HPLC) followed by suitable detection[1]. The separation and detection steps in the analysis are generally independent, and may be examined separately. The state-of-the-art in carbohydrate separations is quite advanced[2–6], especially in the area of ion-exchange chromatography[6–9]. We sought only to demonstrate the refractive index gradient (RIG) detector with a simple microbore normal phase HPLC separation of carbohydrates, although reversed-phase or ion-exchange separations of carbohydrates would have also been appropriate. Thus, we will not emphasize the separation aspect of carbohydrate analysis. The primary problem with the analysis is with detection, especially when coupled with microbore HPLC technology. Detection of carbohydrates following a microbore HPLC separation was the focus of our work.

A variety of carbohydrate detection approaches have been reported, either

0021-9673/89/$03.50 

without derivatization[10–23] or with derivatization[24–33]. Since carbohydrate analysis is hampered due to poor absorbance characteristics, refractive index (RI) detectors have been used for carbohydrate determination by conventional HPLC. Commercially available RI detectors provide at best a 1-$\mu$g detection limit[3]. Recent advances in detector technology have made improved RI detection for conventional[10,11], microbore[12,13], and capillary[14,15] HPLC possible but detection limits of 40–50 ng were optimum. Electrochemical detection approaches have been reported for microbore HPLC with excellent detectabilities in many cases, often in the low-nanogram range for monosaccharides[16–21]. The problem with EC detection appears to be the application of the detector in a wide variety of liquid environments. A promising approach to obtain 10–20 ng detection limits for sugars is microbore HPLC followed by optical activity detection (OAD), where better than $1 \cdot 10^{-6}$° rotation can be detected[22,23]. The drawback to OAD in general practice is the limitation in routinely manufacturing and tuning the detector. Other approaches such as conductivity[24] and chemiluminescence[25] have been reported, but universal applicability of these techniques is somewhat limited as both of these approaches[24,25] involve carbohydrate derivatization reactions prior to detection. Both pre-column and post-column derivatization[26–30] of carbohydrates followed by either UV–VIS absorbance[27–30] or fluorescence[26] detection have been studied in detail. A comparison between RI and absorbance detection of underivatized sugars yielded comparable detection limits for both approaches of 5–10 $\mu$g, which is quite poor[31]. Gas chromatography methods for carbohydrates also generally require pre-derivatization[32,33]. Derivatization reactions are notoriously slow, and are therefore unattractive for many applications where total analysis time must be minimized. Process control in industrial applications and clinical testing are two areas requiring fast turnaround time in carbohydrate analyses. Pre-column derivatization techniques for carbohydrates often have a reaction step of 1–2 h[26–28] and the chromatography must be worked out for the derivatives, with the separation complicated by the common functionality of the derivatization reagent in the carbohydrate reaction product. Post-column derivatization techniques have been developed with the concern for shortening reaction times[30]. Unfortunately, even 20–30-s reaction times[30] may contribute to solute band broadening in microbore HPLC applications. The major advantage of HPLC derivatization techniques for carbohydrates is the excellent detection limits of a few nanograms[26–30].

The most promising HPLC detector for carbohydrate analysis should provide a universal response without derivatization, such as RI, but have the sensitivity obtained in UV–VIS absorbance detection of derivatized samples. Recently, the concept of refractive index gradient (RIG) detection in HPLC has been reported[34–37]. The potential was apparent for a marked improvement in detection sensitivity over conventional RI detection for microbore HPLC. We have designed a RIG detector that provided sub-ng detection limits of polymers in microbore HPLC[36]. The detection was facilitated at a non-absorbing wavelength from a diode laser (780 nm), so the detector is universal. We have recently simplified and improved our RIG detector which now requires only three components: a diode laser, a z-configuration flow cell, and a position-sensitive detector[38–40] (PSD). Previously a PSD was incorporated into a dual-beam absorbance detector[38]. We report here a brief description of the RIG detection principle as pertaining to our RIG detector, and apply the device in the detection of sugars following microbore HPLC. The detection

limit for the sugars was observed to be quite remarkable considering the simplicity of the detector. Detection limits of both low ng and low ppm injected amounts of sugars were obtained and results are presented. These detection limits are three orders of magnitude better than one might anticipate using commercially available RI detectors[3,31]. When injected mass and concentration detection limits are considered collectively, the RIG detector for microbore HPLC is superior to other state-of-the-art RI detectors[10–15].

## EXPERIMENTAL

### *Refractive index gradient detector*

The RIG detector shown with respect to the microbore HPLC system in Fig. 1 is a modified version of our original device[36,37] is composed of three primary components. The single-mode DL 25 diode laser output of 350 $\mu$W at 780 nm (Physitec, Norfolk, MA, U.S.A.) is focused by a 16.85-mm focal length microscope objective (fitted to the diode laser) through a z-configuration flow cell (made in-house) and on to a Hamamatsu (Hamamatsu City, Japan) S1352 with dimensions of 33 mm × 2.5 mm. The PSD sensitivity to beam position is along the long axis. The distance between the flow cell and PSD, labeled $X$ in Fig. 1, was 25 cm, which was found to be near the optimum signal-to-noise ratio (S/N) for detecting the RIG effect with the PSD. The optimum distance of $X$ in Fig. 1 depends upon the relationship of the analytical signal relative to the baseline noise. The optimization procedure will be described with the experimental results. With a single incident beam, the PSD ratio output readily measures angular beam deflections[38,40]. A detailed inspection of the PSD is shown in Fig. 2. The optical and mathematical configuration of the PSD device with respect to RIG detection will be discussed with the experimental results, also. The PSD ratio output is either collected by a Metra Byte (Taunton, MA, U.S.A.) DASH-8 that facilitated the analog-to-digital (A/D) conversion for subsequent data storage and analysis via a Leading Edge (Canton, MA, U.S.A.) Model D, or recorded directly on a D-5000 chart recorder (Houston Instruments, Austin, TX, U.S.A.).

### *Microbore HPLC system*

The microbore HPLC system applied in our studies consisted of an ISCO (Lincoln, NE, U.S.A.) LC-2600 that delivered acetonitrile–water (90:10 v/v) as eluent

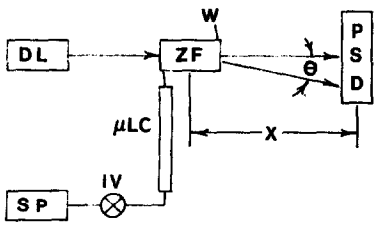


Fig. 1. Microbore HPLC system followed by RIG detection. SP = syringe pump; IV = injection valve; $\mu$LC = microbore HPLC column; DL = diode laser; ZF = z-configuration flow cell; PSD = position sensitive detector; W = waste; $\theta$ = RIG signal (eqns. 2 and 7); $X$ = distance separating ZF from PSD (eqn. 7).

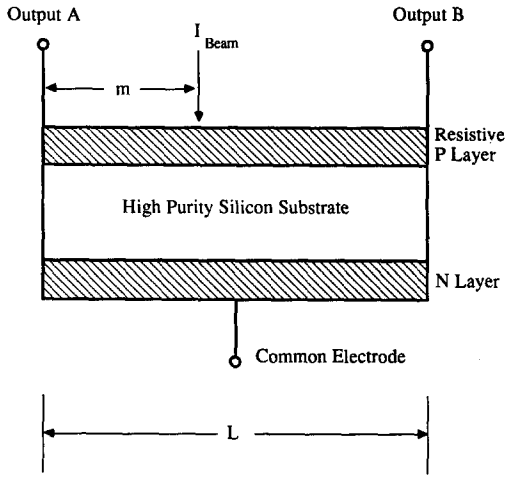


Fig. 2. Single-beam position sensitive detector applied in RIG detection. $I_{Beam}$ (incident probe beam position) separated a distance $m$ from electrode A, where outputs A and B are photocurrents in relation to the common electrode[40].

at 50 μl/min in the normal-phase separation of simple sugars. The eluent was delivered through a Rheodyne (Cotati, CA, U.S.A.), Model 7520 injection valve, fitted with a 0.5-μl loop for subsequent sample introduction to a 250 mm × 1.0 mm I.D., 5 μm particle diameter, Adsorbosphere (Alltech, Deerfield, IL, U.S.A.) silica column. The column effluent was delivered to the z-configuration flow cell, by a short length of 1/16 in. O.D. × 0.007 in. I.D. PTFE tubing, for RIG detection of entering solutes. The z-configuration flow cell is described in detail in a previous report[36], but it is important to note that the flow cell did not contribute significantly to solute band broadening. Due to hydrodynamic considerations, the flow cell has an effective volume of about 1 μl although the total flow cell volume is larger. Band broadening considerations will be discussed in the results.

*Carbohydrates studied*

RIG detection, analogous to conventional RI detection, universally responds to all solutes with an RI different than the eluent. Thus, we limited the demonstration of the RIG detector with microbore HPLC to relatively simple carbohydrates. Reagent grade sucrose (J. T. Baker, Phillipsburg, NJ, U.S.A.) and D-(−)-fructose (Alfa Products, Danvers, MA, U.S.A.) were chromatographically retained and detected by the system (Fig. 1). Linearity of the detected RIG signal as a function of injected solute mass for the separated sugars ranged from about 10 μg down to the detection limit of about 1 ng. Thus, the linearity of the PSD-based RIG measurement is better than we reported previously[36,37], since beam deflection is measured directly via the PSD rather than by an interferometric mechanism.

## RESULTS AND DISCUSSION

*Refractive index gradient effect*

The RIG effect[34–37] is measured as a beam deflection, $\theta$, depending upon

a cross-sectional distance probed, $x$, the RI of the eluent, $n_0$, and magnitude of the RIG, $dn/dx$, existing perpendicular to the probe beam from the diode laser, which for HPLC conditions is given by

$$\theta = \frac{x}{n_0}\frac{dn}{dx} \tag{1}$$

The RIG is related to the concentration gradient $dC/dx$ by the chain rule[34] yielding

$$\theta = \frac{x}{n_0}\frac{dn}{dC}\frac{dC}{dx} \tag{2}$$

where $dn/dC$ is a sensitivity factor[36] for a given solute in a given solvent. The concentration gradient $dC/dx$ has been quantitatively related to the concentration profile of eluting solute using a Gaussian model, and further related to chromatographic principles[34,35].

*Position sensitive detection of RIG effect*

Since one is experimentally observing a beam deflection (eqn. 2), a suitable approach to measure the deflection $\theta$ is required. Initial studies used an interferometric mechanism translating the beam deflection into an intensity measurement at a single photodiode[36–37]. Use of a PSD provides a direct measurement of the beam deflection $\theta$. Understanding the function and application of the continuous PSD (shown in Fig. 2) is essential to optimization of the measurement of $\theta$ of the RIG effect. The RATIO output[38,40] is defined

$$\text{RATIO} = \frac{I_A - I_B}{I_A + I_B} \tag{3}$$

where $I_A$ and $I_B$ are photocurrents measured at electrodes A and B, respectively (Fig. 2). For a single beam

$$\text{RATIO} = 1 - \frac{2m}{L} \tag{4}$$

where $m$ is the position of the incident probe beam on the surface of the PSD relative to electrode A, and $L$ is the length of the photoactive PSD surface (constant). Note that the RATIO output (eqn. 2), is independent of the incident beam intensity. The electronic null condition for the RATIO output occurs when $2m$ equals $L$, *i.e.*, when the beam is centered on the PSD, so the RATIO output would be zero. The electronic null condition will be defined with $m_0$, such that $m_0$ equals $L/2$. A zero RATIO signal is best used as the baseline reading in HPLC, when only eluent is passing through the flow cell. When a deflection, $\theta$, occurs due to the RIG effect, the probe beam is incident upon the PSD at a new position $m'$. The signal $\theta$ (eqn. 2) is derived from the PSD RATIO output, and is observed as a change in probe beam position on the PSD, $\Delta m$, defined by

$$\Delta m = m' - m_0 \tag{5}$$

The beam deflection distance $\Delta m$ relates to $X$ (Fig. 1) and $\theta$ (eqns. 1 and 2) by

$$\Delta m = KX\theta \tag{6}$$

where $K$ is a proportionality constant converting RATIO units to distance[38]. The constant $K$ was calibrated for by precisely moving the PSD[38]. Finally, one may solve for $\theta$ by rearranging eqn. 6

$$\theta = \frac{\Delta m}{KX} \tag{7}$$

It is important to consider the minimum angular deflection that may be measured with the continuous PSD. The minimum angular deflection defines the detection limit for the RIG detector. A minimum angle of about 2 $\mu$rad was routinely measured with the apparatus in Fig. 2 with flowing eluent (operating microbore HPLC system) at a distance $X$ of 25 cm between the flow cell and the PSD. The 2-$\mu$rad angle corresponds to a 0.50-$\mu$m beam displacement, a factor of 14 better than the manufactured specification of a 7-$\mu$m position resolution capability[40]. Readily available low noise electronic components were required to obtain the improved continuous PSD performance. Another report suggests that under ideal conditions a bi-cell PSD[39] may be employed rather than the continuous PSD described by eqns. 3–7. A minimum angular deflection of 50 nrad was reported[39], but the testing system included only a LED light source and the bi-cell PSD. Optics and a chromatography flow cell were not included in the noise study[39]. Yet, the RIG measurement is not presently limited by the choice of PSD since the system (Fig. 1) is not limited by the pointing stability of the light source, but rather subtle fluctuations due to the eluent flow. As the RIG detection technique advances, the bi-cell PSD may be the best choice.

Optimization of the distance $X$ in Fig. 1 and eqns. 6 and 7 was performed empirically. The analytical signal $\theta$ is governed by eqns. 1 and 2, but is independent of the PSD position relative to the flow cell, $X$. Accordingly, the analytical signal is recorded by eqn. 6 as a beam displacement (deflection), $\Delta m$, from the initial baseline position. Thus, the signal increases linearly with $X$ for a given $\theta$. Now, the limiting noise must be considered. While changing $X$, the noise for the system, in terms of $\Delta m$, was observed to remain essentially constant until the beam is no longer imaged within the boundaries of the PSD photoactive surface, leading to increased noise as $X$ is increased beyond the imaging limit. The beam necessarily expands after being focused through the flow cell. Unfortunately, refocusing between the flow cell and the PSD cannot be performed because the signal is sacrificed. Thus, when the signal *and* noise are considered collectively, an optimum S/N was observed at 25 cm $\pm$ 3 cm. Thus, the system was operated at the optimum $X$ of 25 cm.

*Microbore HPLC RIG detection of carbohydrates*

Shown in Fig. 3 is the RIG detection of fructose (6.6 min, 320 ppm and 160 ng injected) and sucrose (9.6 min, 342 ppm and 171 ng injected). The chromatographic data have been plotted in quantitative terms according to eqn. 7. The observed RIG signal is rather unconventional, *i.e.*, derivative of a Gaussian shape[34–37]. Simple integration results in the conventional peak shape[34]. An injection disturbance occurs

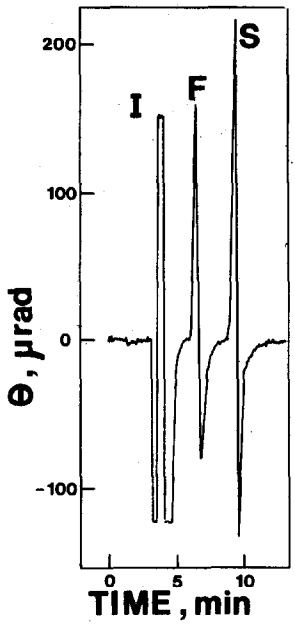


Fig. 3. Microbore HPLC separation and RIG detection of fructose (F) from sucrose (S) with an injection disturbance (I). Quantities injected were 160 ng (320 ppm) fructose and 171 ng (342 ppm) sucrose. Chromatographic conditions given in text.

between 3.0 and 5.2 min due to preparing the sample in pure water. Note that the S/N is quite good for both low injected concentration and low injected mass quantities of the sugars. With the PSD-based RIG detector, solute responses are highly reproducible. For three trials of the separation in Fig. 3 the relative standard deviation of the peak-to-peak RIG signals was 1.8% for fructose and 0.8% for sucrose.

The detection limit was determined from the baseline noise while the microbore HPLC system was functioning with eluent only. Three times the standard deviation of 100-s intervals of baseline noise was used as the detection limit. Several minutes of baseline noise were measured to calculate a reliable detection limit. From eqns. 6 and 7, the deflection distance and angular deflection were measured as 0.50 $\mu$m ($\Delta m$) and 2.0 $\mu$rad ($\theta$), respectively. Extrapolating from the data obtained in Fig. 3, and dilutions from Fig. 3 (not shown for brevity), quite favourable detection limits for the sugars were calculated both for injected concentration and injected mass criteria. For fructose, the detection limits were 3.4 ppm injected concentration and 1.7 ng injected mass. For sucrose, the detection limits were even better at 2.4 ppm injected concentration and 1.2 ng injected mass. The reason sucrose had a better detection limit than fructose is related to the relationship between sensitivity and the detected solute concentration gradient $dC/dx$ (eqn. 2). Band broadening measurements of both solutes revealed that sucrose was less broadened than fructose, necessarily leading to a higher $dC/dx$ at the detector. For fructose the number of theoretical plates, $N$, was $5.1 \cdot 10^3$, and likewise for sucrose $15.1 \cdot 10^3$. Furthermore, these chromatographic efficiency data are quite reasonable for the normal phase separation of sugars. Thus, it is important to realize that chromatographic efficiency, and the detected solute

information, were not sacrificed by the RIG detector, *i.e.*, the flow cell employed. Other studies have supported this idea[36].

After considering the effect of chromatographic dilution, via band broadening, one may calculate the concentration detection limit of the sugars at the detector. For fructose the concentration detection limit at the detector was 68 ppb or $3.6 \cdot 10^{-7}$ *M*, and for sucrose 65 ppb or $1.9 \cdot 10^{-7}$ *M*. Thus, both sugars have about the same detection limit using the ppb concentration at the detector since both have about the same RI and density. The reported RIG detector is superior to previous reports, for RI[10–15] or RIG[34,35] detection, when one considers both the concentration detection limit and the mass detection limit, collectively.

## CONCLUSION

A simple and sensitive approach to carbohydrate detection for microbore HPLC has been presented. Derivatization of the sugars is not required. Simultaneously, detection limits of low-ng and low-ppm injected quantities of sugars were observed. The device is easy to design, tune, and maintain.

## ACKNOWLEDGEMENTS

D.O.H. and R.E.S. thank Alex Hu for suggesting the examination and application of the RIG detector for carbohydrate analysis, and the NSF Center for Process Analytical Chemistry for support of this work (Project Number 86-2).

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