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New detection schemes in liquid chromatography*

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Abstract: A micropolarimeter interfaced to a liquid chromatograph is shown to be suitable for selective monitoring of the optically-active components in complex samples. When an optically-active eluent is used, indirect determination of even optically-inactive materials is possible, down to the level of 10 ng of an injected component. If a second chromatogram is obtained using the racemic analogue of the optically-active eluent, quantitation can be achieved without standards and without prior analyte identification. This concept is also applicable to the refractive index detector, the absorption detector and the conductivity detector in the special case of ion chromatography, and the ultrasonic detector in gas chromatography.

Keywords: *Optical activity; liquid chromatographic detectors; ion chromatography; quantitation without standards; indirect detection.*

Introduction

Chromatography involves two parallel problems — separation and detection. Except for the case of preparative and qualitative applications, quantitation of the components is the ultimate goal. For complex samples such as those of pharmaceutical and biomedical interest, detector selectivity is highly desirable, so that interferences can be minimized. With the proper type of selectivity, quantitation is possible even when complete physical separation is not achieved in the chromatographic column.

An interesting molecular property is optical activity, which is indicative of chirality in the molecular structure. Since many biochemical processes are based on the specific stereochemistry at reactive sites, optical activity is normally associated with biological activity. It is therefore important to develop detectors in liquid chromatography (LC) based on polarimetry. Such a detector has already been demonstrated [1], but recent advances in microbore LC columns provide incentives for further refinement and miniaturization of the instrumentation involved.

For the initial survey and characterization of complex samples, however, detector selectivity is not desirable, since certain components may then be missed by the detector. The only universal detector routinely used in LC at present is the refractive index (RI)

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detector. The poor sensitivity and the relatively large cell volumes of commercial RI detectors make it worthwhile to seek alternatives for universal detection.

An attractive approach is to use a detector to monitor some physical property of the eluent, rather than that of the analyte. When the analyte is eluted, a decrease in detector response is observed so that universal detection can be accomplished. The critical point is that one must be able to detect a small change in addition to the large signal produced by the eluent, which implies that not all detectors can be used in this indirect mode. In polarimetry, however, the large rotation produced by an optically active eluent can be cancelled out by the analyser in the instrument. It is thus ideal for operation in the indirect mode as a universal detector.

A further use of universal detectors is the possibility of providing quantitative information without standards and thus without analyte identification [2]. In the determination of impurities in truly unknown samples, if one knows in advance the number of components present and the amounts of each, then it is not necessary to test for each of the six million or so known compounds. The quantitative information can be used to limit the scope of the qualitative analysis, and total accountability is possible. In synthetic chemistry, even after the components have been identified, it may not be possible to obtain enough of the pure material to establish an analytical calibration curve for quantitation. Also, in complex samples, it may not be possible to achieve complete separation of the components. Even so, an unresolved chromatogram can be used for characterization if quantitation is achieved without identification.

In this paper, applications of polarimetry will be demonstrated for selective detection, for universal detection, and for quantitation without standards. Other LC detection schemes relevant to the last-mentioned concept will also be illustrated.

Theory

The principles of quantitation without standards have been previously described [2–4]. Briefly, if the detector responds to the eluent and to the analyte, the peak area, S_1, observed in a given chromatogram, is related to this particular physical property of the eluent, to the physical property of the analyte, and to the concentration of the analyte. If then the same sample is eluted in a second eluent for which this physical property is different from that of the first eluent, a different peak area, S_2, will be observed. The two measured areas are then defined by two independent mathematical equations, which can be solved to determine the two unknowns, *viz.* the concentration of the analyte and its physical property. To provide useful sensitivities, the two eluents must show very different responses in the detector. This implies that not all detectors can be adapted for this quantitation scheme. Specifically, the detector must be able to maintain the same stable baseline when different eluents are used.

To apply this absolute quantitation scheme, the correct mathematical relationship must be derived to relate the peak areas to the corresponding concentrations. For the polarimeter, the detector response (peak area) can be shown to be [5]:

$$S_1K_1 = V_x\{[\alpha_x]\rho_x - [\alpha_1]\rho_1\}, \tag{1}$$

where K_1 is a constant depending on the size of the injection loop and the eluent flow rate, V_x is the volume fractional concentration of the analyte, $[\alpha]$ is the specific rotation, ρ is the density, and subscripts x and 1 refer to the analyte and the eluent, respectively.

Here, $[\alpha]_x\rho_x$ can be considered as a single unknown, being the special molecular physical property of x relevant to detector response in polarimetry.

For the refractive index detector, the correct expression is [2]:

$$S_1K_1 = V_x\left(\frac{n_x^2 - 1}{n_x^2 + 2} - \frac{n_1^2 - 1}{n_1^2 + 2}\right), \tag{2}$$

where n is the refractive index.

For the absorption detector, it has been shown that [4]:

$$S_1K_1 = V_x[\epsilon_x/v_x - \epsilon_1/v_1] \tag{3}$$

where ϵ is the molar absorptivity and v is the molar volume. Equation (3) is different from the standard form of Beer's Law, which uses concentrations in moles per litre. The absorption detector is not very useful in general because it cannot be used for eluents with a high molar absorptivity due to loss of sensitivity. However, in conjunction with ion chromatography [6], the eluting ion is the relevant species and not the bulk eluent (e.g. water). The eluting ion is already at a low concentration typically, so that reasonable sensitivity based on the replacement of these eluting ions can be achieved [4]. Because it is a charge replacement process,

$$S_1K_1 = N_x[\epsilon_x/m_x - \epsilon_1/m_1], \tag{4}$$

where N is the concentration in normality and m is the charge number of the species.

New instrumentation for conductivity detection provides a reasonably stable baseline even when an eluting ion with high equivalent conductance, λ, is used. So, the same quantitation scheme can be applied to ion chromatography using the relationship [7]:

$$S_1K_1 = N_x[\lambda_x - \lambda_1]. \tag{5}$$

Finally, preliminary results in the author's laboratory have shown that the same concept is applicable to gas chromatography using the ultrasonic detector, with a detection limit of 1 pg of material injected.

Experimental

The principles underlying the micropolarimeter have been previously described [1]. Several modifications have been introduced to improve the original design. Instead of an argon ion laser, it has been found that a 5 mW HeNe laser (Spectra Physics, Mountain View, CA, Model 134) is sufficient for operation. Instead of the air-based Faraday cells used for modulation, a liquid cell filled with acetonitrile is used, and this has essentially the same dimensions as the original sample cell. The increased molecular number density in liquids allows the use of fewer turns in the solenoid (400 turns) and a smaller modulation current (0.1 A). In this way, higher modulation frequencies can be used, and the square-wave generator (Wavetek, San Diego, CA, Model 162) can be connected directly to the solenoid without an extra current driver. To interface with microbore columns with 1 mm i.d., the cell volume must be reduced to 1 μl. This is possible using an aluminium disk 1 cm thick and 2.5 cm in diameter, and drilling through the centre a hole

0.34 mm i.d. [5]. Chromatographic plumbing from the column is connected directly to this aluminium disk. The laser beam diameter is smaller than the cell i.d. when a 33 cm f.l lens is used: further reduction in the cell volume should be possible if the machining process can be improved.

All materials and solvents used are reagent grade chemicals without further purification. The microbore chromatographic system consists of a syringe pump (ISCO, Lincoln, NB, Model 314), a 250 × 1.0 mm i.d. 5-μm C_{18} or a 250 × 1.0 mm i.d. 10-μm silica column (Alltech, Deerfield, IL), with a 0.5-μl sample loop coupled to an internal loop injection valve (Rheodyne, Berkeley, CA, Model 7410).

The analytical scale chromatographic system consists of a reciprocating pump (Milton Roy, Riviera Beach, FL, Model 196-0066) and an external injection loop on a conventional valve (Rheodyne, Berkeley, CA, Model 7010). The columns used (all 4.6 mm i.d.) are a 250 mm 10-μm C_{18}-column (Anspec, Warrensville, IL), a 250 mm 5-μm silica column (Anspec, Warrensville, IL), a 300 mm 5-μm PLgel (100Å) size-exclusion column (Anspec, Warrensville, IL), or a 250 mm 15-μm ion chromatographic column (Vydac, Hesperia, CA). All peak areas are determined by a computer (Digital Equipment, Maynard, MA, Model PDP 11/10 with LPS-11 laboratory interface) connected to the detector output. The standard detectors are a UV-absorbance detector at 254 nm (Rainin, Woburn, MA, model 153-00) and a RI detector (Waters Associates, Milford, MA, model R401).

The crude oil sample studied was a North Slope crude (Tank 6000X1) from Mobil Oil Corporation, Ferndale Refinery, Ferndale, WA, USA.

Results and Discussion

Laser-based micropolarimetry

The use of the laser-based micropolarimeter for biomedical analysis has been described previously [8, 9]. For example, the direct injection of 100 μl of human urine into a heavy-metal ion-exchange column with a pre-column allowed the determination of five naturally occurring sugars in addition to glucose [8]. A simple extraction followed by chromatography using 50 μl of human blood plasma allowed quantitation of free and various esterified cholesterols without interference from other lipids [9]. Even complex samples such as extracts from coals [10] have been studied with polarimetry to obtain chiral profiles that can potentially be used for characterization. In all these cases, the special selectivity of this detector is the key, so that complete physical separation is not necessary for quantitation.

The applicability of the miniaturized version of the detector was tested in a reverse-phase elution of fructose using water as the eluent. A detection limit of 10 ng of injected material was found (S/N = 3) [5], and the peak did not show any band-broadening beyond the specified efficiency of the column used (N = 25,000 plates) at a k' of 2.1.

Indirect polarimetry

The concept of indirect polarimetric detection is illustrated in Fig. 1. The eluent used is optically active (+)-limonene ($[\alpha]_D$ = 106°). When each of the three optically inactive species elutes from the column, a corresponding volume of the eluent in the detector cell is displaced, so that a lower optical rotation is observed. Unless a compound has a specific rotation identical to that of the eluent, a peak will be observed. This is then a universal detection scheme. The limit of detection is found to be the same as that using

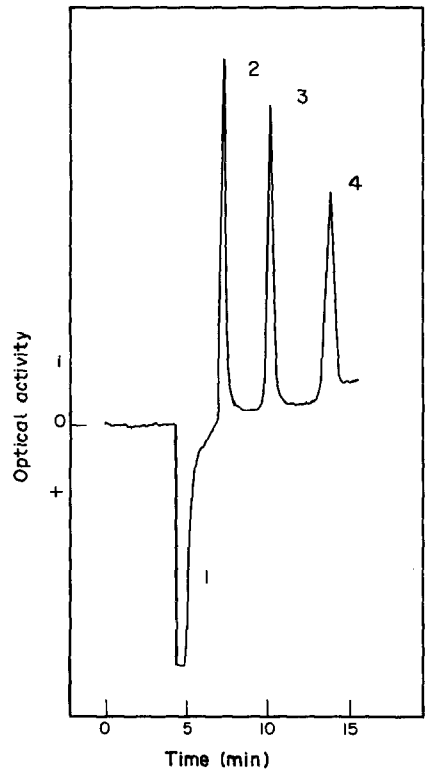

Figure 1
Determination of optically inactive compounds by indirect polarimetry and normal-phase chromatography. Eluent: (+)-limonene; 1, solvent peak; 2, dioctyl phthalate; 3, dibutyl phthalate; 4, diethyl phthalate.

optically inactive eluents for species with $[\alpha] = 100°$, i.e. 10 ng of injected material ($S/N = 3$) [5]. The reason that the sensitivity is maintained despite the high optical rotation produced by the eluent is that the analyser is set to effectively suppress all the background. The additional noise caused by thermal fluctuations in the cell, which does not contribute to noise using optically inactive eluents, is apparently still below the 10^{-5} degree level. In this mode of operation, a 'system' peak appears due to the displacement of the eluent when the solute is retained on the column (peak 1, Fig. 1). For weak eluents, this appears at the void volume and does not interfere with the retained peaks. Naturally, the use of microbore LC is essential here due to the cost of chiral solvents.

Quantitation without standards

The concept of quantitation without standards can be tested using the polarimeter as discussed in the theory section above. The two eluents used are (-)-2-methyl-1-butanol and (±)-2-methyl-1-butanol, each modified with an equal volume of acetonitrile. Table 1 shows the results of the solution to the two simultaneous equations for each chromatographic peak given by equation (1) compared to the true values. The results are within experimental error. In fact, if an eluent with a higher specific rotation $[\alpha]$ is used, the precision should be even better.

For unresolved chromatograms, such as those from crude oils, one has to have correlated chromatographic behaviour in the two eluents. For reversed-phase LC, the author has found that acetonitrile and dimethylformamide–H_2O (96.5:3.5 v/v) give almost identical retention behaviours for a series of *n*-alkanes. The two chromatograms of a crude oil sample can thus be paired according to 1-s time segments after injection, so

Table 1
Quantitation of sample components (in v/v) using polarimetric detection

	(ℓ)-2-Octanol	$C_{10}H_{22}$	$C_{14}H_{30}$	$C_{16}H_{34}$
True C_x ($\times 10^{-2}$)	4.00	8.00	8.00	8.00
Calculated C_x ($\times 10^{-2}$)*	3.93 ± 0.38	7.74 ± 0.34	7.83 ± 0.36	8.36 ± 0.27
True $[\alpha_x]^{20}_{590}$†	−9°	0	0	0
Calculated $[\alpha_x]^{27}_{488}$‡	−8.0 ± 2.8	0.07 ± 0.2	0.01 ± 0.2	0.01 ± 0.1

* Calculated according to equation (1).
† $[\alpha]^{20}_{590}$ = specific rotation at 20°C at 590 nm.
‡ $[\alpha]^{27}_{488}$ = specific rotation at 27°C at 488 nm.

that equation (2) can be used. The resulting solution of the sets of simultaneous equations can then give a concentration chromatogram as shown in Fig. 2a. Figure 2b is obtained with a UV-detector in series (254 nm), and clearly gives an incorrect impression of the real distribution of components when compared to Fig. 2a. The RI information can also be obtained, as shown in Fig. 2c, for qualitative information. These results are far easier to obtain and more reliable than the alternative of fractionation and weighing, since the latter procedure can be influenced by the loss of the more volatile components.

For normal-phase LC, the author has found that *n*-pentane and iso-octane are good choices for the two eluents. The concentration chromatogram of the same sample of crude oil is shown in Fig. 3. The RI values for the three main features at 4.5, 5.5 and 6.7 min are found to correspond to those of alkanes, aromatics and heterocycles. It should

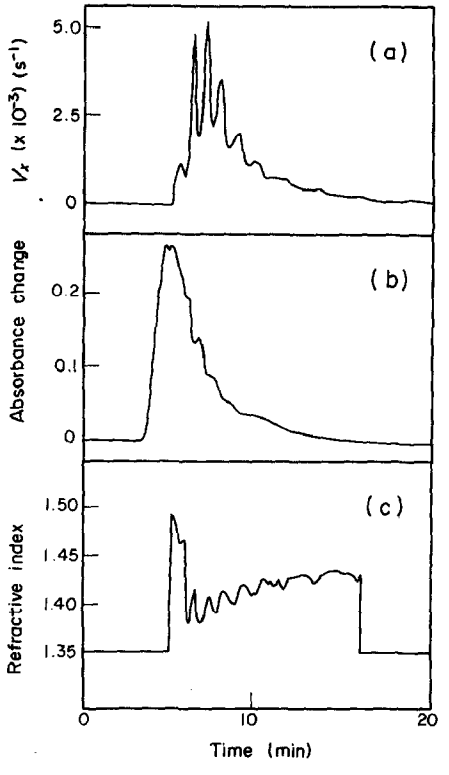

Figure 2
Absolute quantitation of crude oil components by refractive index detection and reversed-phase liquid chromatography. (a) Volume eluted for each 1-s interval; (b) absorbance of components; and (c) calculated RI of components.

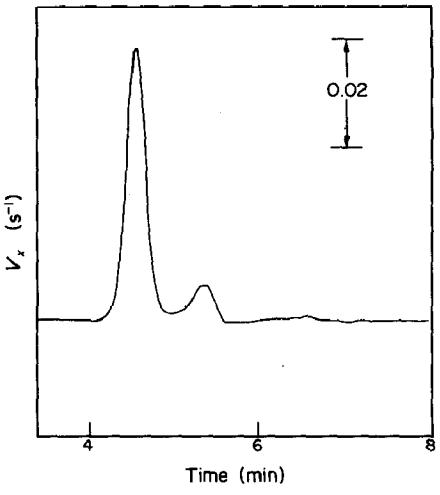

Figure 3
Absolute quantitation of crude oil components by refractive index detection and normal-phase liquid chromatography. The ordinate represents volume of analyte x eluted for each 1-s interval (V_x).

be noted that the first two features provide a convenient way to calculate the aliphatic/aromatic ratio, often used for fossil fuel characterization.

For size-exclusion chromatography, one can use toluene and chloroform as the two eluents. Figure 4a shows the resulting absolute volumes eluted at each point. Since molecular volume is implicit in the retention time, one can use this and Fig. 4a to determine the molar quantity of material eluted at each point, as shown in Fig. 4b. Similar applications in pharmaceutical and biomedical analysis should be possible.

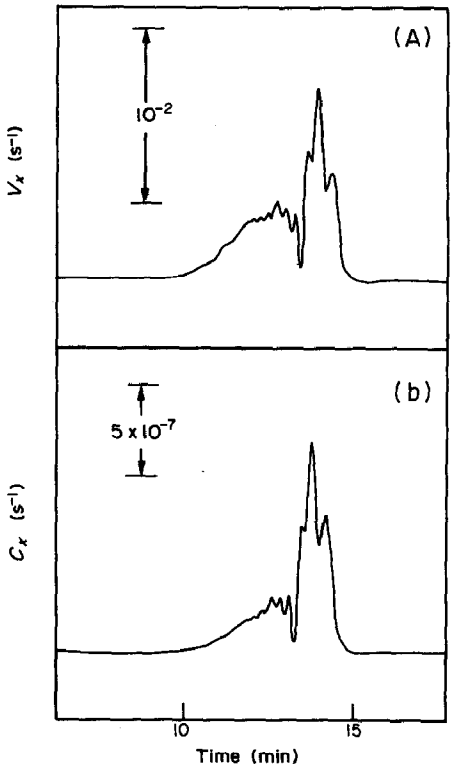

Figure 4
Absolute quantitation of crude oil components by refractive index detection and size-exclusion chromatography. (a) Volume eluted for each 1-s interval; and (b) moles of material eluted for each 1-s interval.

Quantitation in ion chromatography can be performed using equation (4) and the absorption detector [4]. Figure 5 shows the analyte ions eluted first with phthalate and then with citrate ions. By applying equation (4), the results in Table 2 can be obtained. If a conductivity detector is used instead, equation (5) can be applied [7]. The results in Table 2 are obtained using benzoate and thiosulphate as the two eluting ions. Both types of detector show good agreement with the true values. The absorbance detector is slightly more sensitive, while the conductivity detector provides more useful qualitative information.

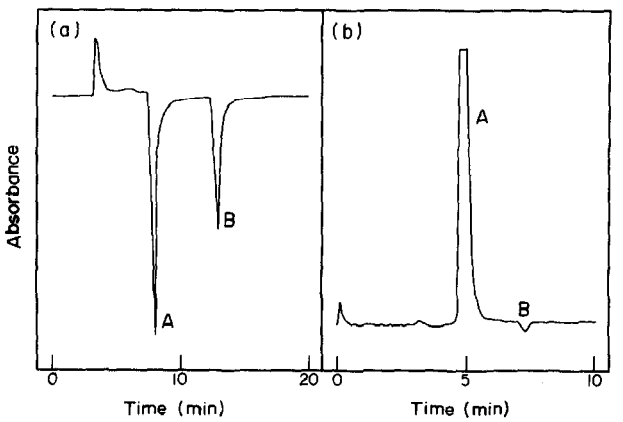

Figure 5
Absolute quantitation of ions by the absorbance detector and anion-exchange chromatography. (a) Elution by highly absorbing ion (phthalate) (0.08 AUFS); (b) elution by slightly absorbing ion (citrate) (0.005 AUFS); A, IO_3^-; and B, Br^-.

Table 2
Absolute quantitation in ion chromatography

	IO_3^-*	Br^-*	Trichloroacetate†	SO_4^{2-}†
True C_x ($\times 10^{-3}$N)	1.14	0.62	2.0	4.0
Calculated C_x ($\times 10^{-3}$N)	1.07	0.64	2.0	4.1

* Absorbance detector.
† Conductivity detector.

Conclusion

In summary, recent developments in instrumentation and detection concepts can bring important new information to pharmaceutical and biomedical analysis. The detector principles reported here are certainly worthwhile techniques to consider in every type of biomedical application.

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