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NEW WAYS TO INCREASE THE SPECIFICITY OF DETECTION IN LIQUID CHROMATOGRAPHY*

J. L. DiCESARE and L. S. ETTRE*
Chromatography Division, The Perkin-Elmer Corporation, Norwalk, CT 06856 (U.S.A.)
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CONTENTS

1. INTRODUCTION

Chromatography is essentially a separation technique in which the compounds of a mixture introduced at the front of the system will elute separately from the column (column chromatography) or occupy spots at different positions (plane chromatography). In order to have a visual record of the separation, various detectors are used which respond to all or a selected group of compounds. Detectors of the first category are universal while those of the second are classified as selective.

Selective detection has two basic advantages. First, it helps in the identification of the individual compounds present in a sample. In addition, in situations where compounds co-elute (*i.e.*, they could not be separated chromatographically), selective detectors may permit the detection of one compound while disregarding the other(s).

The concepts of selective detection have been established in both gas chromatography (GC) and liquid chromatography (LC) for many years and modern chromatography could not exist without it. Three years ago, one of us reviewed the

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philosophy of selective detection, the most widely used selective detectors and their application in both gas and liquid chromatography[1,2]; the interested reader is referred to these papers and the references given therein.

In LC, all presently used detectors, except the refractive index (RI) detector, are more-or-less selective, or can be used as selective detectors. This possibility presents a great potential for liquid chromatography.

The possibilities of using the ultraviolet (UV) spectrophotometric detector for identification purposes are well established[3–5] and we shall not deal with them here. The capabilities of fluorescence spectroscopy for qualitative LC detection are also known[6], both for the analysis of naturally fluorescing compounds, or by preparing the fluorescing derivatives of other compounds[7]. In addition, there are some new techniques which enhance the applicability of the fluorescence detector. The purpose of this paper is to discuss these new techniques; in addition, we shall examine the use of infrared spectroscopy and optical activity as a means of selective detection.

2. EXPERIMENTAL

A Perkin-Elmer Series 2/2 liquid chromatograph equipped with a Rheodyne 7125 injection valve, a Model LC-100 column oven and a Sigma 15 data station were used for the measurements. Detectors included the Model 3000 and 650-10S/LC fluorescence spectrophotometers equipped with 20-μl flow cells, a Model 580 infrared (IR) spectrophotometer equipped with a 50-μl flow cell having a 3-mm path length and calcium fluoride windows, a Model 241 polarimeter equipped with a 33-μl flow cell having a 100-mm path length, a Model LC-75 variable-wavelength UV detector and a Model 25 RI detector. All of these are available from Perkin-Elmer (Norwalk, CT, U.S.A.); the flow cell used in the IR spectrophotometer was from Analabs (North Haven, CT, U.S.A.).

Post-column reactions were achieved by pumping the reacting fluid separately using a single pump (Perkin-Elmer Series 1). This fluid was combined with the column effluent using a tee and mixing was achieved using 4.3 m of tightly coiled tubing of 0.25 mm I.D.

The following columns and mobile phases were used for the investigations:

carbohydrates, apple and orange juice and cola drink: 250 × 8 mm I.D. Shodex S-801/S 10-μm columns (Part No. 0258-8886) using water as the mobile phase at 80°C;

red currant juice: 250 × 4 mm I.D. LiChrosorb NH_2 5-μm column using acetonitrile–water as the mobile phase;

phenol, aniline and polyaromatic hydrocarbons: either a 100 × 4.6 mm I.D. 3-μm (Part No. 0258-1501) or a 125 × 4.6 mm I.D. 5-μm (Part No. 0258-1001) C_{18} bonded-phase column using acetonitrile–water as the mobile phase;

polystyrene, cholesterol, diolein and phosphatidylethanolamine: 250 × 8 mm I.D. Shodex A-802/S (Part No. 0258-8286) and Shodex A-805/S (Part No. 0258-8586) columns using chloroform as the mobile phase.

All the part-numbered columns are available from Perkin-Elmer. The LiChrosorb columns were obtained from E. Merck (Darmstadt, G.F.R.).

Acetonitrile and chloroform, both HPLC grade, were obtained from Fisher

Scientific (Pittsburgh, PA, U.S.A.), while water was purified using a mixed-bed ion-exchange resin and an activated charcoal filter.

Reference chemicals were of the highest purity available and were obtained from Aldrich (Milwaukee, WI, U.S.A.), Sigma (St. Louis, MO, U.S.A.) and Pfaltz and Bauer (Stamford, CT, U.S.A.). Fruit juices and soft drinks used were obtained on the open market and were diluted and filtered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) prior to injection. The polystyrene standard sample was prepared from standards available from Perkin-Elmer (Part No. 0254-0074).

3. RESULTS AND DISCUSSION

3.1. Fluorescence detector

The use of fluorescence spectroscopy for selective detection in liquid chromatography is well established. The selectivity of the technique can be enhanced even further by using complementary techniques. We shall deal here with three: post-column acid–base manipulations, wavelength selection to enhance selectivity and the use of synchronous scanning for qualitative verification.

Unlike many other detection methods, fluorescence has very high sensitivity and, therefore, can be used even in trace analysis[6].

3.1.1. Post-column techniques. Usually, a fluorescing species which has a functional group will be sensitive to the pH of the mobile phase. Thus, by the proper adjustment of the pH, the selectivity of the system to certain compounds can be controlled. However, pH affects chromatography and thus the best method for manipulating this parameter is post-column before the detector. Most recently, Lee *et al.*[8] showed the application of this technique for the analysis of warfarin and its metabolites. Another important and practical example is the analysis of a mixture of phenol and aniline.

Phenol and aniline (and their respective homologues) are frequently found together, *e.g.*, in waste waters or similar samples. In many conventional reversed-phase chromatography systems, they give two very closely eluting peaks (Fig. 1) and often their separation cannot be achieved without affecting the separation of some other components in the chromatogram. However, a selective method for detecting phenol (and some of its derivatives) from aniline (and some of its derivatives) and *vice versa* is the adjustment of the pH just prior to the fluorescence detector:

(a) at high pH (*ca.* 12), phenol is converted to the non-fluorescent phenoxide ion ($C_6H_5O^-$) while aniline is strongly fluorescent at this pH;

(b) at low pH (*ca.* 2), aniline is converted to the non-fluorescent anilinium cation ($C_6H_5NH_3^+$) while phenol is strongly fluorescent at this pH.

Figs. 2 and 3 show the corresponding chromatograms. In Fig. 2, the pH of the column effluent (2 ml/min) was adjusted by the addition of a 1 *M* sodium hydroxide solution at 0.8 ml/min, thereby eliminating the response for phenol, while in the case of Fig. 3, 0.1 % orthophosphoric acid solution at 0.8 ml/min was added to the column effluent, eliminating the response to aniline.

3.1.2. Wavelength selection. Fluorescence is more specific than UV absorption because not all absorbing compounds fluoresce and the appropriate selection of both excitation and emission wavelengths often permits similar compounds to be differentiated[9,10]. In most cases, compromise wavelengths are used to provide appropriate detection for all compounds belonging to a class, but even here, if a wide-range

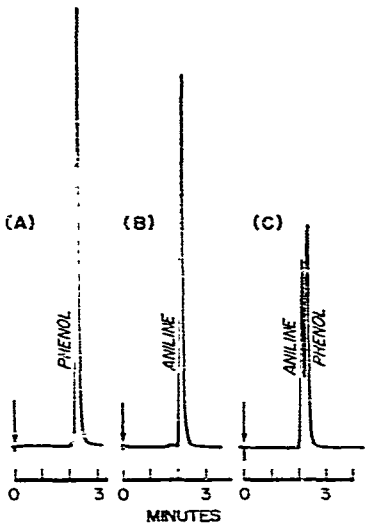
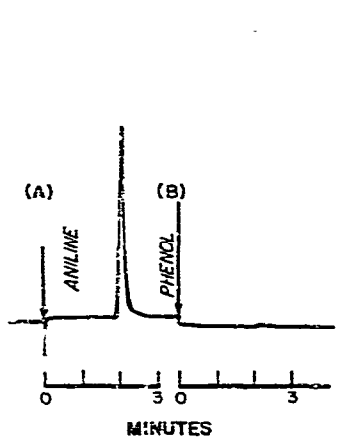


Fig. 1. Analysis of (a) phenol, (b) aniline and (c) their mixture with fluorescence detection. Column: 100 × 4.6 mm I.D., containing C_{18} bonded-phase 3-μm packing. Mobile phase: acetonitrile–water (30:70) at 2 ml/min. Wavelengths: excitation, 274 nm; emission, 325 nm.

mixture is analyzed, it is often advantageous to adjust the wavelengths during the run. For example, using 305-nm excitation and 430-nm emission wavelengths, a wide range of polyaromatic hydrocarbons from naphthalene to coronene can be analyzed with adequate sensitivity. However, as shown by Ogan *et al.*[11], better detectability for



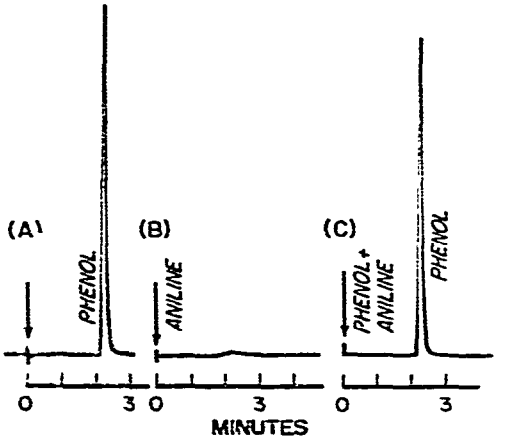


Fig. 2. Analysis of aniline and phenol with fluorescence detection at high pH. Column, mobile phase, flow-rate and wavelengths as in Fig. 1. Column effluent was mixed with a 0.8 ml/min flow of 1 *M* NaOH solution prior to entering the detector cell. (a) Sample contains only aniline; (b) sample contains only phenol.

Fig. 3. Analysis of aniline and phenol with fluorescence detection at low pH. Column, mobile phase, flow-rate and wavelengths as in Fig. 1. Column effluent was mixed with a 0.8 ml/min flow of 0.1% H_3PO_4 solution prior to entering the detector cell. (a) Sample contains only phenol; (b) sample contains only aniline; (c) sample contains a mixture of phenol and aniline.

compounds emerging earlier (naphthalene, acenaphthene, fluorene and phenanthrene) can be achieved by using 280-nm excitation and 340-nm emission wavelengths and later changing to 305/430 nm between the phenanthrene and anthracene peaks. Also, by changing the emission wavelength from 430 to 500 nm after benzo[*ghi*]perylene, a 70-fold increase in response to indeno[1,2,3-*cd*]pyrene could be realized.

By the proper selection of wavelength, not only the response to certain compounds can be enhanced, but also the response to others suppressed. As an example, Fig. 4 shows the analysis of a mixture of five polyaromatic hydrocarbons at four different wavelength combinations. At 275-nm excitation and 335-nm emission wavelengths the response to anthracene and perylene is suppressed while at 365/375 nm only anthracene and at 430/540 nm only perylene shows a response while all others are suppressed. On the other hand, at 265/305 nm, detection can be made specific to fluorene. Earlier Slavin *et al.*[10] have shown that using 338/395 nm detection is almost specific for pyrene, while at 338/450 nm detection is almost specific for coronene.

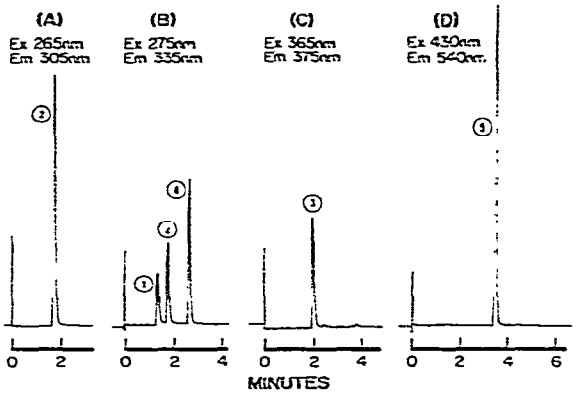


Fig. 4. Analysis of a mixture of five polyaromatic hydrocarbons with fluorescence detection. Column: 100 × 4.6 mm I.D., containing C_{18} bonded-phase 3-μm packing. Mobile phase: acetonitrile–water (65:35) at 3 ml/min. Wavelengths as given. Peaks: 1 = naphthalene; 2 = fluorene; 3 = anthracene; 4 = pyrene; 5 = perylene.

Selection of optimum wavelengths is, naturally, important not only for qualitative purposes, but also to enhance quantitative detection: by increasing the peak height for a given compound, smaller quantities can be detected extending the range of fluorescence detection. Since modern instrumentation permits the automatic change of wavelengths at any given point during an analysis, full utilization of this technique is possible.

3.1.3. Synchronous scanning. In synchronous scanning, the excitation (λ_{ex}) and emission (λ_{em}) wavelengths are varied simultaneously while maintaining a constant step, $\Delta\lambda$, between them:

$$\Delta\lambda = \lambda_{ex} - \lambda_{em} = \text{constant}$$

In this case, the fluorescence of a given compound is restricted to that excited at the wavelengths synchronously trailing the plotted emission. The result of this technique

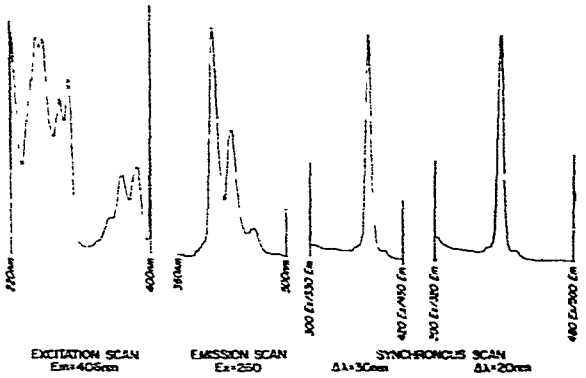


Fig. 5. Fluorescence spectra of benzo[*a*]pyrene obtained by stop-flow technique on the chromatographic peak. Chromatographic conditions: Columns: 125 × 4.6 mm I.D. containing C_{18} bonded-phase 5-μm packing. Mobile phase: acetonitrile–water (90:10) at 1 ml/min. On the scans the start and end wavelengths are indicated. Scanning speed: 120 nm/min.

is generally a much simplified spectrum where the intensity maxima are highly characteristic to a compound and thus, combined with stop-flow techniques, can be used for identification purposes. As an example, Fig. 5 shows the separate excitation and emission scans (keeping constant emission and excitation wavelength, respectively) and then the synchronous scan with two different $\Delta\lambda$ values for benzo[*a*]pyrene.

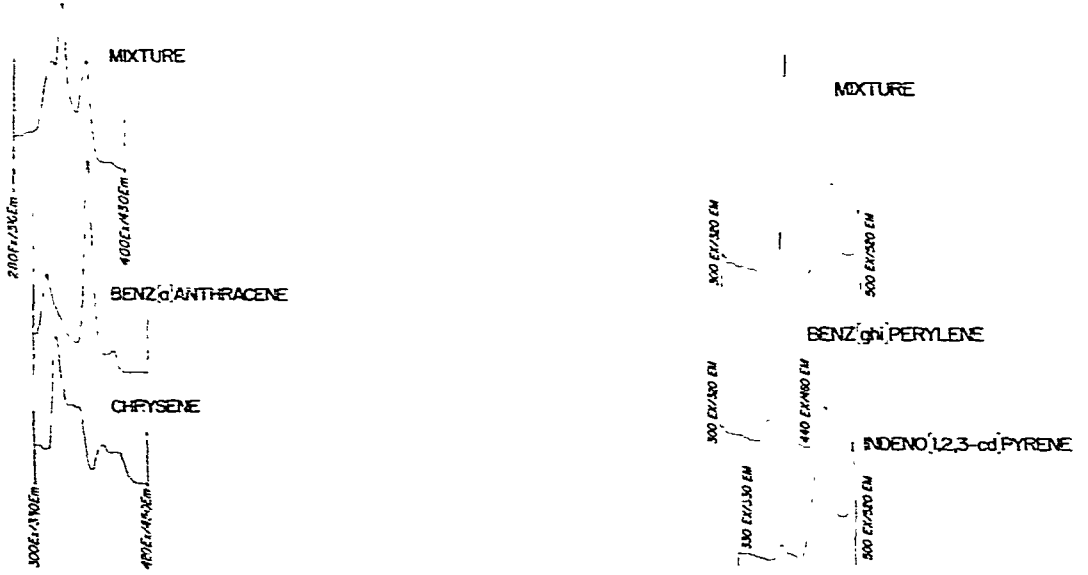


Fig. 6. Synchronous scans of pure chrysene and benz[*a*]anthracene and of their mixture, obtained by stop-flow technique on the chromatographic peak. Column: 125 × 4.6 mm I.D., containing C_{18} bonded-phase 5-μm packing. Mobile phase: acetonitrile–water (90:10) at 1 ml/min. On the scans the start and end wavelengths are indicated. Scanning speed: 120 nm/min; $\Delta\lambda$ = 30 nm.

Fig. 7. Synchronous scans of pure indeno[1,2,3-*cd*]pyrene and benz[*ghi*]perylene and of their mixture, obtained by stop-flow technique on the chromatographic peak. Column: 125 × 4.6 mm I.D., containing C_{18} bonded-phase 5-μm packing. Mobile phase: acetonitrile–water (90:10) at 1 ml/min. On the scans the start and end wavelengths are indicated. Scanning speed: 120 nm/min; $\Delta\lambda$ = 20 nm.

The technique of synchronous scanning has been first described in 1971 by Lloyd[12]. Since that time, a number of papers have dealt with its theory and applications[13–25].

We have applied this technique to two pairs of polyaromatic hydrocarbons which are difficult to separate under various conditions. These are chrysene and benz[*a*]anthracene, and indeno[1,2,3-*cd*]pyrene and benz[*ghi*]perylene. Figs. 6 and 7 show the synchronous scans applied to the pure compounds and then to a peak containing the indicated compound pair. The presence of the two compounds in the respective peak can clearly be identified with help of the synchronous scans.

Synchronous scanning can be utilized with modern liquid chromatography systems with fluorescence detectors which permit stop-flow operation and wavelength scanning: when the peak of interest enters the detector cell, flow is stopped and the proper wavelength scan executed. When this is finished, flow is resumed and separation continued[10,26].

3.2. IR spectrophotometer as an LC detector

IR spectroscopy is one of our most powerful analytical tools and it has been widely used for the investigation of collected fractions, in both LC and GC; the introduction of multiple internal reflection IR spectroscopy[27,28] further enhanced this possibility. Yet, when utilized as an on-the-flow detector in a liquid chromatography system, it suffers from the lack of sensitivity and interference by the strong absorption by most of the mobile phases in use, particularly those in reversed-phase LC.

IR spectroscopy can be used in two ways for on-the-flow monitoring of column effluent: either by obtaining the spectra of each chromatographic peak and comparing these with reference spectra, or by setting the IR detector to a specific frequency characteristic to a given functional group[28]. The previous technique, *i.e.*, stopping the flow and scanning the desired wavelength range[29–31], has gained a new impetus in the last decade by the introduction of Fourier-transform IR spectroscopy[32–35].

Functional group monitoring of the column effluent has been described in the past, particularly for gel permeation[36,37] and non-aqueous reversed-phase liquid chromatography[38–41]. The essential requirements here are to find proper solvents which are transparent to IR radiation at the frequencies selected. In general, chlorocarbons and carbon disulfide fulfill these criteria. Table 1 lists the frequencies characteristic for the major functional groups and the solvents which can be used as the mobile phase[42].

We have adapted a relatively sensitive conventional IR spectrophotometer for use as an on-the-flow LC detector to explore the benefits and limitations of the technique. Here, we want to show a few applications of such a system.

Fig. 8 shows the size-exclusion separation of some polystyrene standards where ring stretching is monitored at 1604 cm^{-1}. The sensitivity of using IR as the detector as calculated from this chromatogram is about 1 μg.

Fig. 9 shows the chromatograms of a sample containing the diglyceride diolein and the phospholipid phosphatidylethanolanine at two different frequencies. Monitoring the ester bond at 1740 cm^{-1}, both compounds are detected along with some impurities, while the chromatogram obtained when monitoring the alcoholic group at

TABLE I

PRINCIPAL FUNCTIONAL GROUP ABSORPTIONS

Compound type	*Functional group*	*Characteristic frequency (cm^{-1})*	*Solvent which is transparent*				
			CH_2Cl_2	$CHCl_3$	CCl_4	CCl_2CCl_2	CS_2
Alkanes	C–H	2850–3000	–	–	+	+	+
Alkenes	C–H	3080–3140	–	–	+	+	+
	C=C	1645–1670	+	+	+	+	+
Esters	C=O	1720–1735	+	+	+	+	+
Ketones	C=O	1665–1745	+	+	+	–	+
Alcohols:	C–H	3400–3600	–	–	+	+	+
Primary	C–O	1050	–	–	–	–	+
Secondary	C–O	1100–1125	–	–	–	–	+
Tertiary	C–O	1150–1200	–	–	–	–	+

3400 cm^{-1} reveals what appears to be a slight response to the phospholipid and a strong response to diolein.

Finally, Fig. 10 shows the application of two spectroscopic detectors, an IR and a UV spectrophotometer in series. The sample contained primarily cholesterol with some impurities present. The UV detector revealed the presence of several minor components while the IR detector, set to monitor the hydroxyl group at 3400 cm^{-1}, showed essentially only one main component.

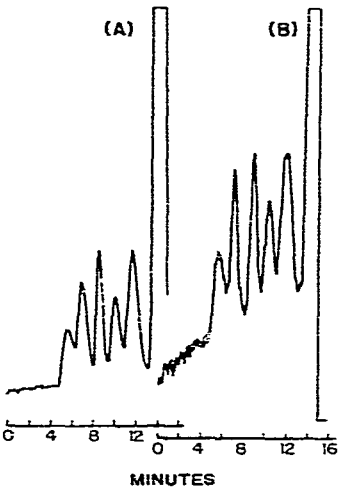


Fig. 8. Analysis of a polystyrene standard mixture using an IR detector. Column: Shodex A-805/S + A-802/S in series, each 250 × 8 mm I.D. Mobile phase: chloroform at 1 ml/min. IR detector at 1604 cm^{-1}; response time for 98% full-scale: 5.8 sec (a) 20 × ordinate expansion, 0.05 a.u.f.s., 90-μg sample. (b) 100 × ordinate expansion, 0.01 a.u.f.s., 20 μg sample. Sample composition: 1,460,000 MW (0.205%), 230,000 MW (0.206%), 19,000 MW (0.209%), 600 MW (0.336%) in chloroform solution.

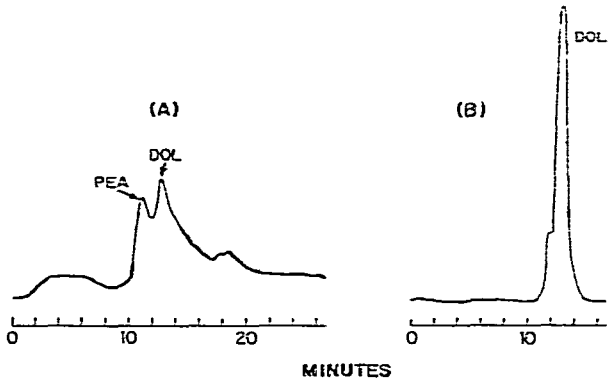


Fig. 9. Analysis of a mixture of diolein (DOL) and phosphatidylethanolamine (PEA) using an IR detector. Column: two Shodex A-802/S columns, each 250 × 8 mm I.D., in series. Mobile phase: chloroform at 1 ml/min. (A) IR detector at 1740 cm^{-1}, 10 × ordinate expansion, 0.1 a.u.f.s., injected sample contained 125 μg of DOL and 250 μg of PEA. (b) IR detector at 3400 cm^{-1}, 2 × ordinate expansion, 0.5 a.u.f.s., injected sample contained 75 μg of DOL and 150 μg of PEA.

3.3. Optical activity measurement for LC detection

The ability to differentiate between optical isomers is important since they occur frequently in nature and often only one of the isomers is active in a chemical or biological sense. However, these isomeric compounds will generally co-elute on most conventional stationary phases available today.

In liquid chromatography, great progress has been made in the development of systems in which a chiral center is present[43–46] either bonded to the stationary phase[47–61] or introduced to the mobile phase[62–73]. However, these phases and techniques are usually specific to certain classes of optical isomers. Therefore, the possibility of using a selective detector which can differentiate between the optical isomers even when no, or only partial, separation is accomplished, is of great interest. Another reason that selective determinations are important is that the compounds of interest are usually present in complex matrices where their separation from the other compounds may represent a problem.

Polarimetry, which has the capability of measuring the direction and degree of rotation of optical isomers, is a well established method in the investigation and

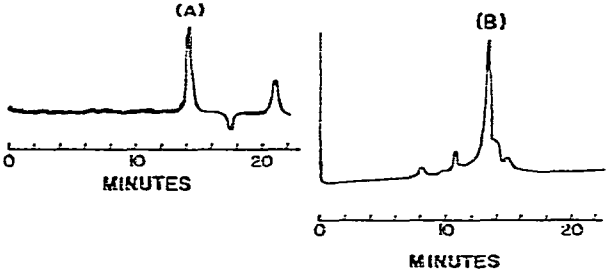


Fig. 10 Analysis of a cholesterol sample, using two spectroscopic detectors (IR + UV) in series. Column: two Shodex A-802/S columns, each 250 × 8 mm I.D., in series. Mobile phase: chloroform at 1 ml/min. Sample: 50 μg of cholesterol. (a) IR detector at 3401 cm^{-1}, 20 × ordinate expansion, 0.05 a.u.f.s. (b) UV detector at 254 nm, 0.08 a.u.f.s.

determination of optically active compounds[74–78]. However, except for a few attempts[79–82], its use as a liquid chromatographic detector has been restricted due to technical difficulties, a lack of sensitivity and the inavailability of appropriate instrumentation.

Recently, Böhme[83] demonstrated the capability of directly connecting a general-purpose polarimeter, modified with a small-volume flow cell, to monitor liquid chromatographic eluates. The polarimeter can also be used in series with other detectors*. Böhme used both a UV spectrophotometer and an RI detector, while in our work, the latter was applied. Using this approach, important detection problems can be solved, thereby extending the applications capabilities of the system.

Fig. 11 shows the separation of a carbohydrate mixture by a group-exchange mechanism, using the polarimeter and the RI detector in series. Three of the carbohydrates, raffinose, sucrose and glucose, rotate plane-polarized light in one direction (+), while fructose and ribose rotate the light in the opposite direction (−), thereby adding a degree of selectivity. The selected wavelength (365 nm) gives increased sensitivity relative to the commonly used sodium D line for all the carbohydrates present in the mixture. As discussed by Böhme[83], the specific rotation and hence detector response are strongly dependent on the wavelength and will increase for these compounds as the wavelength is decreased.

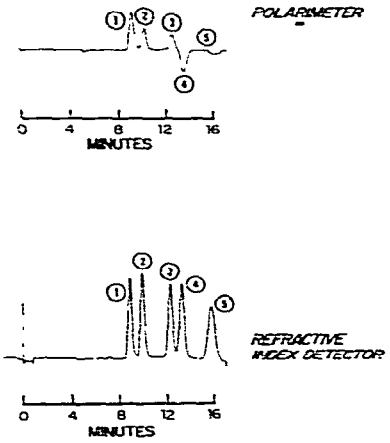


Fig. 11. Analysis of a sugar mixture. Column: two 250 × 8 mm I.D. in series, each containing Shodex S-801/S packing. Column temperature: 80°C. Mobile phase: water at 0.5 ml/min. Polarimeter at 365 nm, ± 0.5° full scale. Peaks: 1 = raffinose; 2 = sucrose; 3 = glucose; 4 = fructose; 5 = ribose.

The determination of sugars in fruit juices or similar soft drinks is an important application of liquid chromatography. In a number of instances, the separations are relatively straightforward; however, even here, one or two of the sugars present may co-elute with some other compounds. Thus, if using UV or refractive index detectors, a composite peak would be recorded. On the other hand, the polarimeter responds to the optically active compounds only and thus is blind to the other sample components which lack optical activity. As an example, Figs. 12–14 show the analysis of

* When the RI detector was used in series with another detector, it was always the last.

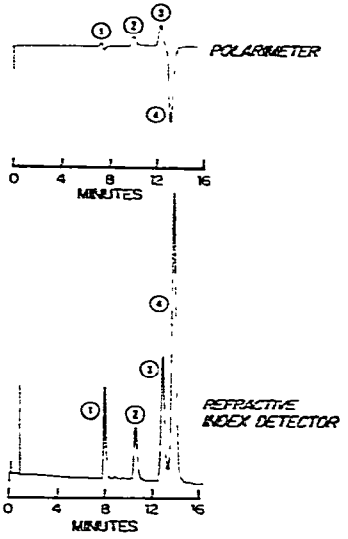


Fig. 12. Analysis of a commercial apple juice. Column, temperature, mobile phase and polarimeter conditions as in Fig. 11. The juice was diluted 10-fold and 40 μl of the diluted solution were injected. Peaks: 1 = ascorbic acid; 2 = sucrose; 3 = glucose; 4 = fructose.

commercial apple and orange juices and a cola drink, respectively. Here the sucrose peak in the RI detector chromatogram may contain at least one additional compound which is likely to give a response similar to that of sucrose. On the other hand, the

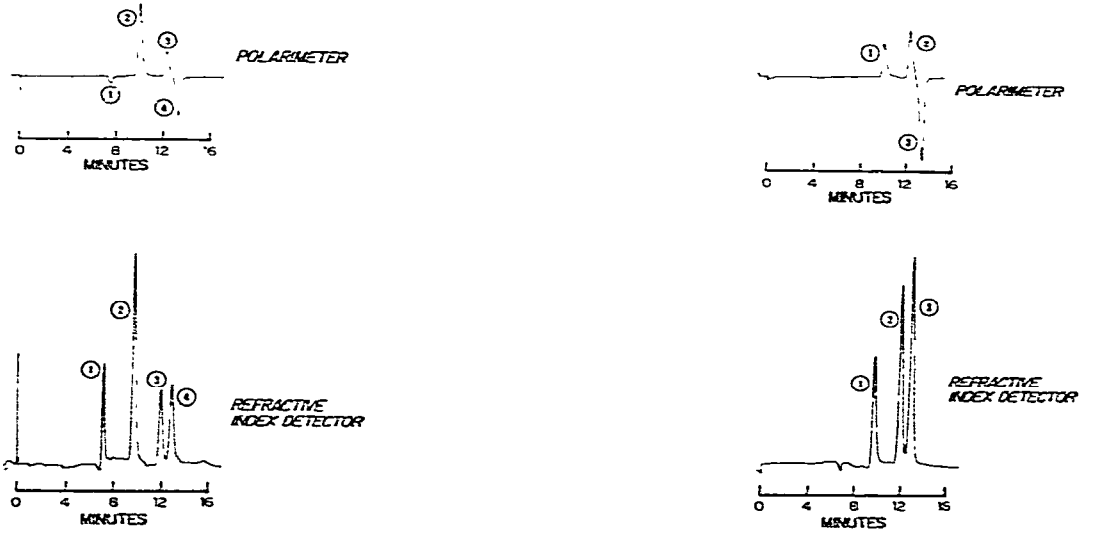


Fig. 13. Analysis of a commercial orange juice. Column, temperature, mobile phase and polarimeter conditions as in Fig. 11. The juice was diluted 10-fold and 75 μl of the diluted solution were injected. Peaks: 1 = ascorbic acid; 2 = sucrose; 3 = glucose; 4 = fructose.

Fig. 14. Analysis of a cola drink. Column, temperature, mobile phase and polarimeter conditions as in Fig. 11. A 3-μl aliquot of the filtered drink was injected without dilution. Peaks: 1 = sucrose; 2 = glucose; 3 = fructose.

possibility that the peak in the chromatogram obtained with the polarimeter corresponds only to sucrose is very likely, thereby increasing the degree of accuracy for this analysis.

In the case of more complex samples, the overlapping is quite clear, as shown in Fig. 15, presenting the chromatograms obtained when analyzing freshly pressed red currant juice[83]. Here, analysis without sample purification or same kind of pre-treatment is only possible when using the polarimeter as the LC detector.

In the chromatograms shown up to now, separation of the individual carbohydrates was adequate. Occasionally, however, one might face a situation where no separation can be observed when using a refractometric or UV detector although, if one carefully investigates the retention times of the individual compounds, it is evident that slight differences occur. If the optical activities of the two compounds are different, however, then it is quite possible that the polarimeter would indicate the presence of both compounds in spite of the apparent overlapping of their peaks on the other detectors. This is illustrated in Fig. 16 which shows the analysis of galactose and fructose both alone and as a mixture. It is clear that the polarimeter permits differentiation of these two carbohydrates, while neither UV nor RI detectors would do so.

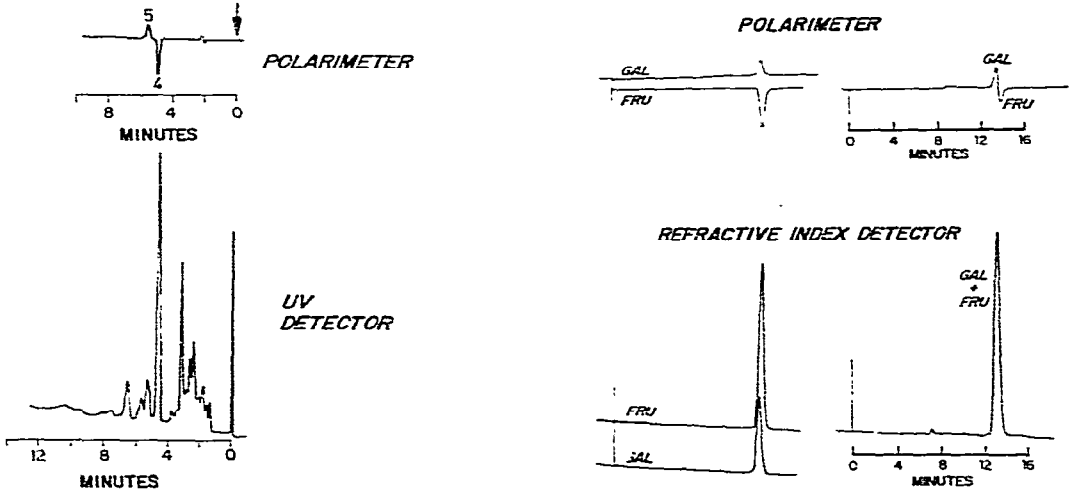


Fig. 15. Analysis of freshly pressed red currant juice[83]. Column: 250 × 4 mm I.D., containing LiChrosorb-NH_2 5-μm packing. Room temperature. Mobile phase: acetonitrile–water (80:20) at 1.5 ml/min. UV detector at 187 nm, 0.32 a.u.f.s. Polarimeter at 365 nm, ± 0.5° full scale. Sample volume: 5 μl. Peaks: 4 = fructose; 5 = glucose.

Fig. 16. Analysis of pure fructose (FRU) and galactose (GAL) and their mixture. Column, temperature, mobile phase and polarimeter conditions as in Fig. 11. Top: chromatograms recorded by the polarimeter. Bottom: chromatograms recorded by a RI detector.

3.3.1. Sensitivity. Among others, the sensitivity of the polarimeter depends on the specific rotation (α) of the compound of interest which in turn depends on the temperature and the wavelength applied. This value can vary in a relatively wide range; Table 2, after Böhme[83], lists specific rotation values of some carbohydrates at four different wavelengths. When comparing compounds of different types, the difference is even greater: *e.g.*, at 589 nm and room temperature, the value of α is +36

TABLE 2

SPECIFIC ROTATION VALUES (α) OF SOME CARBOHYDRATES AT 20°C[83]

Carbohydrate	α *(deg cm³ dm⁻¹ g⁻¹)*			
	365 nm	*436 nm*	*578 nm*	*589 nm*
Sucrose	192.6	127.8	77.8	66.1
Glucose	148.7	97.5	58.6	49.5
Fructose	−271.4	−108.7	−109.8	− 93.0
Galactose	230.3	152.9	81.9	78.3
Raffinose	354.4	235.3	142.7	120.5

for cholic acid and +223 for corticosterone (both in ethanol) or +14.5 for L(+)-alanine and +221 for D(+)-cystine (both in 1 *N* hydrochloric acid).

In general, one can see that the sensitivity of the polarimeter is in the microgram range, somewhat less than that of the RI detector.

3.3.2. Quantitative analysis. By using a concentration-sensitive detector, such as a UV or a refractive index detector, in series with the polarimeter, both the total amount and percentage of each optical isomer can often be determined. This is illustrated using the example of the analysis of penicillamine. D-Penicillamine, a derivative of penicillin, is used in medicine as a metal-chelating agent. The official method for its determination[84] is a volumetric assay where the sample is titrated with a mercury (II) acetate solution in an aqueous medium. Other methods involve a non-aqueous amine titration, a non-aqueous acid titration and a hydroxylamine assay[85]. However, none of these methods can distinguish between the D- or L-isomer.

D- and L-penicillamine cannot be separated chromatographically on a conventional stationary phase. Thus, if using an RI or UV detector, the peak obtained

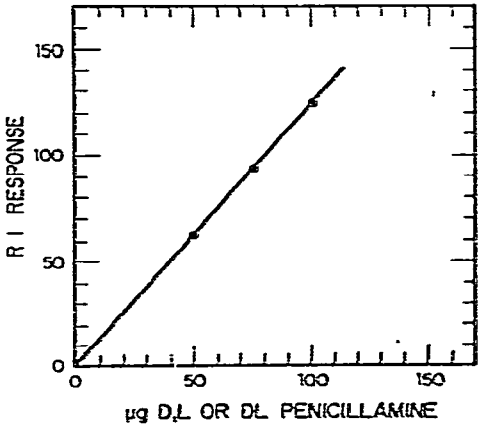


Fig. 17. Calibration curve of penicillamine (D+L) on a refractive index detector.

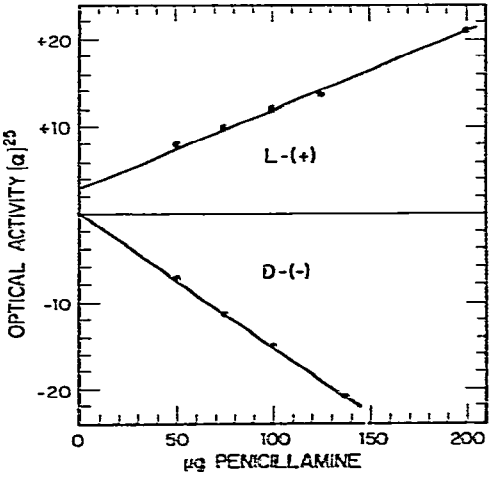


Fig. 18. Calibration curve of D- and L-penicillamine on a polarimeter detector.

corresponds to the total amount of the two optical isomers present. Using standard solutions, a concentration curve as shown in Fig. 17 is obtained. However, by analyzing different amounts of D- and L-penicillamine isomers separately using the polarimetric detector, a plot of optical activity *vs.* concentration for the D(−)- or L(+)-compounds show a linear response but with one plot having a positive slope and the other a negative slope (Fig. 18). From these calibration curves, the total concentration of penicillamine as well as the percentage of each isomer in the peak can be determined.

The following example illustrates the use of these calibration curves. A standard sample consisting of a mixture of 25 μg of L- and 70 μg of D-penicillamine was chromatographed. The peak obtained on the RI detector had an area of 115 units which, according to the calibration plot shown in Fig. 17, corresponds to 93.5 μg of penicillamine.

The polarimeter recorded a negative peak with an area corresponding to an $[\alpha]$ of 7°, equivalent to 44.5 μg of penicillamine (*cf.*, Fig. 18). This means that the mixture consisted of more than 50% D-isomer (hence, the negative peak) and the measured optical activity (or the corresponding amount) represented the difference of the two isomers. Therefore, D- + L- = 93.5 μg and D- − L- = 44.5 μg. From these data we can calculate the amounts of the two isomers as D- = 69 μg and L- = 24.5 μg and the respective deviations from the actual values are −1.4% and −2%.

4. CONCLUSIONS

In addition to the well known use of the fluorescence spectrophotometer to monitor the fluorescence of the sample components or their derivatives, its usefulness as a selective LC detector can be further enhanced either by proper wavelength selection or by additional manipulations such as post-column adjustment of the eluent's pH or simultaneous scanning.

Although their sensitivities are limited, infrared spectrophotometers and polarimeters can be successfully coupled to liquid chromatographs for on-the-flow selective detection of a wide variety of compounds or compound groups. In addition, the polarimeter can also be used for the quantitative analysis of optical isomers even if they are not separated on the column.

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6. SUMMARY

New possibilities of selective detection in liquid chromatography are discussed. In fluorescence spectroscopy, post-column adjustment of the pH of the mobile phase permits selective quenching of the fluorescence of certain compounds making the detection more specific. Detection sensitivity can also be enhanced by wavelength selection. Finally, a special technique permitting the establishment of peak purity and

compound identification is simultaneous scanning of both excitation and emission wavelengths. On-the-flow infrared detection at pre-selected wavelengths is another possibility to enhance selective detection. Finally, analysis of the individual optical isomers can be accomplished by monitoring the optical activity of the compounds emerging from the column.

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* Davankov and co-workers published between 1971 and 1981 a large number of original papers on various aspects of ligand-exchange chromatography used for the separation of optical isomers of amino acids. Here only Parts I[47], V[48] and XII[49] are listed.