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High-performance liquid chromatography applications of optical rotation detection with compensation for scattering and absorbance at the laser wavelength

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Abstract

Use of instrumentation developed to enable simultaneous monitoring of optical rotation (OR) and transmittance allows OR measurements to be made in the presence of high levels of absorbance, scattering or other effects that change the intensity of the plane-polarised light at the photodiode detector. This extends the application of OR detection to areas where it was previously difficult. Examples of the application of high-performance liquid chromatography (HPLC) with the improved OR detector include (i) the analytical scale separation of fructose and sucrose and (ii) the semi-preparative separation of enantiomers of warfarin and Trögers base. A signal-to-noise improvement of up to 150% is found when comparing signals with and without correction for transmittance changes. The improved OR detector has been used in series with a UV detector and the system shown to be suitable for on-line measurement of peak purity in separations using a chiral column under overload conditions.

Keywords: Optical rotation; Scattering; Absorbance; Enantiomer separation; Detection, LC; Chiroptical detector; Sucrose; Fructose; Warfarin; Trögers base

1. Introduction

Quantification and identification of single enantiomers have become increasingly important, as many pharmaceutical and agrochemical compounds, as well as some used in the food industry, are chiral. It is well known that enantiomers often have different biological activities; one enantiomer may have no biological effect, existing as a 50% impurity in a racemic mixture with the active enantiomer. Alternatively one enantiomer may enhance, negate or otherwise alter the effects of the other.

Enantiomers have identical physical properties in an achiral environment except that they rotate plane-polarised light in opposite directions but to an equal extent. This is used in chiroptical detectors [1,2]; polarimetry measures OR, the angle through which the plane-polarised light is rotated by an optically-active species, and circular dichroism (CD) measures

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differential absorbance (ΔA), the difference in absorbance of left and right circularly polarised light.

HPLC methods are among the most versatile and widely used techniques for the analysis of chiral compounds [3,4] and the collection of single enantiomers from racemic or enantiomerically enriched samples [5–7]. Using chiroptical detectors in HPLC provides certain advantages over conventional detection techniques such as UV absorbance; in particular, they have an inherent selectivity towards chiral molecules, and the complete separation of the achiral components of a mixture is not necessary. Achiral method development is simplified, as the position of the chiral compounds can be easily followed.

OR detection is very important when chiral molecules lack a strongly UV absorbing chromophore, as shown in recent work analysing a novel macrolide [8]. The use of OR detection in the analysis of the aminoglycoside antibiotic gentamycin avoided the time-consuming derivatisation procedures required for absorbance or fluorescence detection [9].

The coupling of UV absorbance and chiroptical detectors in HPLC produces a powerful technique for the determination of enantiomeric purity of a sample [10–15]. It can be shown that the enantiomeric purity is directly proportional to the chiroptical signal, either OR or CD, divided by the UV absorbance signal (OR/abs or ΔA/abs) [16]. This dual detector approach allows enantiomeric purity determination without the use of chiral HPLC where a suitable standard of known enantiomeric composition is available for calibration purposes [12,16], and there is no suggestion of self-association [17].

This technique was recently used in high throughput screening where rapid and efficient analysis is crucial. HPLC–UV–CD has been used to monitor the enantioselectivity of the enzyme- or metal-catalysed reduction of acetophenone to (*R*)- or (*S*)-1-phenylethanol [18]. If the analyte lacks a UV chromophore, HPLC–OR can be coupled to a refractive index detector and the enantiomeric purity can be calculated in a similar way [19].

Semi-preparative chiral HPLC can be used to prepare samples of single enantiomers from racemic or enantiomerically enriched mixtures; this is important as it may avoid a potentially complicated asymmetric synthesis. During semi-preparative HPLC the concentrations of analyte are often so high that peak overlapping is observed. Evaluation of the enantiomeric purity of carvone samples, at elevated concentrations, has been aided by using computer software that deconvolutes the peaks and determines the ratio OR/abs [20]. It was suggested that this system would be useful for on-line analysis of enantiomeric composition.

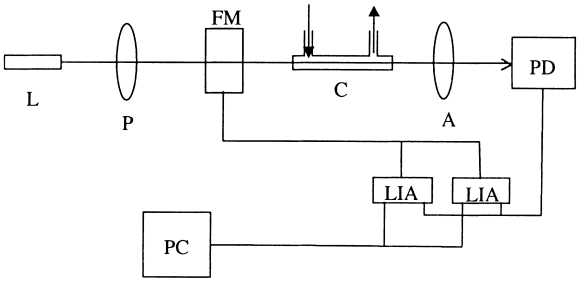


Fig. 1. Schematic diagram of the improved OR detector: L, laser; P, polariser; FM, Faraday modulator; C, sample cell; A, analyser; PD, photodiode detector; LIA, lock-in amplifier; PC, computer.

We have developed an improved OR detector, which measures the OR of chiral compounds in the presence of species that cause scattering or absorbance of the plane-polarised light. A schematic diagram of the detector is seen in Fig. 1. A detailed description of the theory behind the detector is given elsewhere [21]. In this paper the advantages of the improved OR detector are demonstrated by analysing a solution of sucrose in the presence of polystyrene spheres, to cause scattering of the laser light, and in the presence of Tinolux, a dye that absorbs at the laser wavelength. The detector is then used with analytical HPLC to separate a mixture of fructose and sucrose. Samples of racemic warfarin and Trögers base are shown to be separated into their single enantiomers, on a semi-preparative scale, by monitoring the ratio OR/abs.

2. Experimental

2.1. Apparatus

HPLC analysis was performed with an HP 1090 liquid chromatography instrument (Agilent Technologies, Stockport, UK) with standard HP 1090

solvent delivery system, autosampler and auto injector. A 100-μl injection loop was fitted for the analytical scale separations and a 5-ml loop was used for the semi-preparative analyses. A Knauer 6-port/3-channel injection valve and a 7-port/1-channel switching valve (Polymer Labs, Shropshire, UK) were used in series to collect samples of the single enantiomers. UV absorbance detection was carried out with a Spectroflow 757 absorbance detector (ABI Analytical, Ramsey, NJ, USA) with an 8-mm path length cell.

The improved OR detector used either a 10-mW semiconductor diode laser at 635 nm (Laser 2000, Kettering, UK) or a 35-mW laser at 820 nm (Spindler and Hoyer, Göttingen, Germany). A schematic diagram is shown in Fig. 1 and further details are given elsewhere [21]. A Spectrosil 5-mm path length cell was used for the scattering and absorbance experiments, and a jacketed HPLC cell, 10 mm path length, was used for HPLC analyses. A PC-based digital lock-in amplifier was used to measure the amplitudes at the two frequencies of interest, 1*f* and 2*f*, and Excel was used to process the data.

2.2. Materials and reagents

Polystyrene spheres (Nanosphere™ Size Standards, 204±6 nm diameter, 1% solids content) were purchased from Duke Scientific (Palo Alto, CA, USA). The Tinolux dye (sulfonated aluminium phthalocyanine) was a gift from Zeneca Specialties. Fructose (99+%), warfarin (98+%) and Trögers base (98%) were from Aldrich (Gillingham, UK) and sucrose (99+%) from Sigma (Poole, UK). The structures of these compounds are given in Fig. 2. Diethylamine (98+%) was from Lancaster Synthesis (Morecambe, UK) and glacial acetic acid and all solvents, which were HPLC grade, were purchased from Fisher Scientific (Loughborough, UK). The water was doubly deionised.

2.3. HPLC conditions

A Hypersil ODS2 (octadecyl silica) column, 250×4.6 mm with 5-μm particles, was supplied by Hichrom (Berkshire, UK) and a Daicel Chiralcel OD (cellulose tris(3,5-dimethylphenylcarbamide)) column, 250×4.6 mm, with 5-μm particles, from J.T. Baker (Middlesex, UK). Specific HPLC conditions are given in the Figure legends.

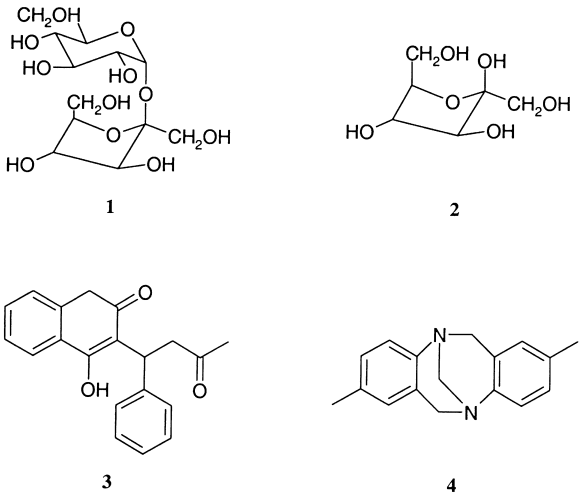


Fig. 2. Structures of compounds used in this work. **1** Sucrose. **2** Fructose. **3** Warfarin, 3-(α-acetonylbenzyl)-4-hydroxycoumarin. **4** Trögers base, 2,8-dimethyl-6H,12H-(5,11)-methanodibenzo[b,f]-[1,5]diazocine.

2.4. Scattering and absorbance experiments

For each experiment 1 ml of 0.04 g cm^{-3} sucrose solution was placed in the cell, which was thermostatted at 25°C. The cell was not removed during the experiments, and solutions were replaced using a needle and syringe.

In the scattering experiment, 100 μl of polystyrene spheres were added to 1 ml of 0.04-g cm^{-3} sucrose solution. A 100-μl volume of this solution was added to the sucrose in the cell and agitated. This process was repeated approximately every 10 min, until the cell was full. The 1*f* and 2*f* signals were recorded at 820 nm for these experiments.

In the absorbance experiment 0.3 mg of Tinolux dye was dissolved in 1 ml of 0.04 g cm^{-3} sucrose solution. A 100-μl volume of this solution was added to the sucrose in the cell and the contents agitated. Further 100-μl aliquots were added at approximately 10 min intervals, until the cell was

full. 1*f* and 2*f* signals were recorded, using a laser wavelength of 635 nm.

3. Theory

In the detector, the root mean square (RMS) amplitude of the AC component of the signal at the frequency of the Faraday modulator is measured at the photodiode detector. The expression for the amplitude, which we term 1*f*, follows from previous work [22–24]:

$$1f \approx \sqrt{2}\,\theta_o\,\phi_o\,\alpha \quad (1)$$

where θ_o is the peak to peak modulation angle, ϕ_o is the intensity of the plane-polarised light at the photodiode detector when polariser and analyser are parallel, and α is the OR due to the chiral sample. This and other equations are applicable when the angles θ_o and α are both small, as in the case of our experiments, and sin θ_o and sin α are equal to θ_o and α to a high level of approximation. The RMS amplitude of the AC component of the signal at twice the frequency of the Faraday modulator arriving at the photodiode, termed 2*f*, is also recorded:

$$2f \approx (\sqrt{2}/4)\,\theta_o^2\,\phi_o \quad (2)$$

From Eq. (1) it can be seen that the 1*f* signal is proportional to the OR of the chiral sample. Whereas this was previously used to calculate the OR, such an approach is problematic because the 1*f* signal is also proportional to the transmitted light intensity at the photodiode detector. This means that any change in ϕ_o will cause an apparent change in the OR of the sample. Such changes are possible if the chiral sample has species present that cause absorbance, scattering or depolarisation of the plane-polarised light, and could adversely affect results obtained using previous designs of OR detectors [2].

The problems of the apparent OR changes are solved in the improved detector, as the ratio of 1*f* to 2*f* signals (1*f*/2*f*) is used to calculate the OR. A combination of Eqs. 1 and 2 shows that 1*f*/2*f* is proportional to only the OR of the sample, and is independent of the light intensity at the photodiode detector:

$$1f/2f \approx 4\alpha/\theta_o \quad (3)$$

4. Results and discussion

4.1. Scattering and absorbing samples

The aim of these experiments was to show the benefits of the improved detector when analysing samples that caused scattering and absorbance of the plane-polarised light.

Addition of polystyrene spheres to sucrose solution produced a sample that scattered the laser light. The results are shown in Fig. 3. As more polystyrene was added, the solution became more turbid and the transmitted light intensity reaching the photodiode detector (2*f*) decreased. Before any polystyrene was added the OR based on the 1*f* and 1*f*/2*f* signals had the same value, 65 mdeg. As the sucrose solution became more turbid, 1*f* decreased, as expected from Eq. (1). This change must have been due to the decrease in transmitted light intensity because there were no changes of conformation or concentration to affect the OR. By the end of the experiment the 2*f* signal had fallen to 18% of the original value, and the OR based on the 1*f* signal was 12 mdeg. The OR based on the 1*f*/2*f* signal remained constant at 65 mdeg throughout the experiment, and was seen to be independent of the transmitted light intensity, as expected from Eq. (3).

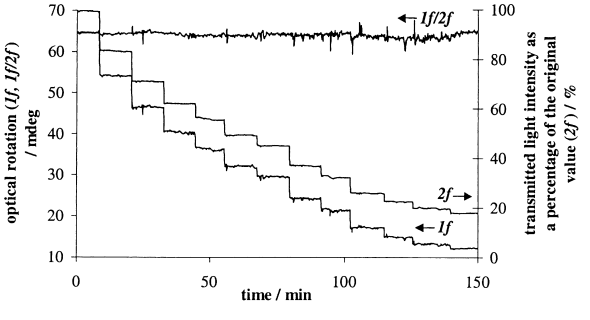


Fig. 3. The OR (at 820 nm) based on 1*f* and 1*f*/2*f* signals and the transmitted light intensity (2*f*) as a function of the amount of scattering material present, polystyrene spheres added to a solution of 0.04 g cm^{-3} sucrose at approximately 10-min intervals.

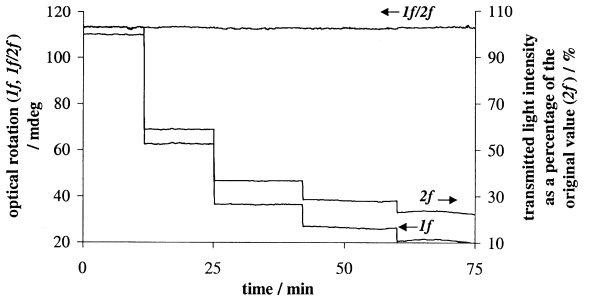


Fig. 4. The OR (at 635 nm) based on $1f$ and $1f/2f$ signals and the transmitted light intensity ($2f$) as a function of the amount of absorbing material present, Tinolux dye added to a solution of 0.04 g cm^{-3} sucrose at approximately 15 min intervals.

Similar results were found in the absorbance experiment shown in Fig. 4. The Tinolux dye was added to the sucrose solution at approximately 15 min intervals. This dye absorbed light at the laser wavelength (635 nm) and the $2f$ signal decreased as more dye was added. The OR based on the $1f$ signal followed the same pattern, whereas the OR based on the $1f/2f$ signal again remained constant at 113 mdeg. The difference in initial OR values between the scattering and absorbance experiments was due to the different laser wavelengths used.

4.2. Analytical scale HPLC

OR detection is particularly useful for analysis of sugar molecules, as they do not possess a UV chromophore. Fig. 5 shows the $1f$, $2f$ and $1f/2f$ signals for a HPLC separation of fructose and sucrose. Fructose was the first to elute, indicated by the negative peak (specific rotation of -92.4 deg g^{-1} cm^3 dm^{-1}) and sucrose was the second, positive peak (specific rotation of 66.5 deg g^{-1} cm^3 dm^{-1}). The traces of OR based on $1f$ and $1f/2f$ were virtually super-imposable upon each other but the $1f$ signal was noisier, both in the long term and the short term. Table 1 shows a comparison of the signal (S) and noise (N) values for the OR based on the $1f$ and $1f/2f$ signals. The results show that there was a S/N improvement of approximately 150% for both fructose and sucrose when the $1f/2f$ signal was used instead of the $1f$ signal to calculate the OR.

There were two substantial decreases in the $2f$ signal during the run, corresponding to the elution of the analytes. A possible reason for this is that the laser beam was not striking the photodiode centrally, and when the compounds passed through the cell they caused a lensing effect so that the light intensity at the detector changed. Alternatively, if the laser beam was in part hitting the sides of the cell as it

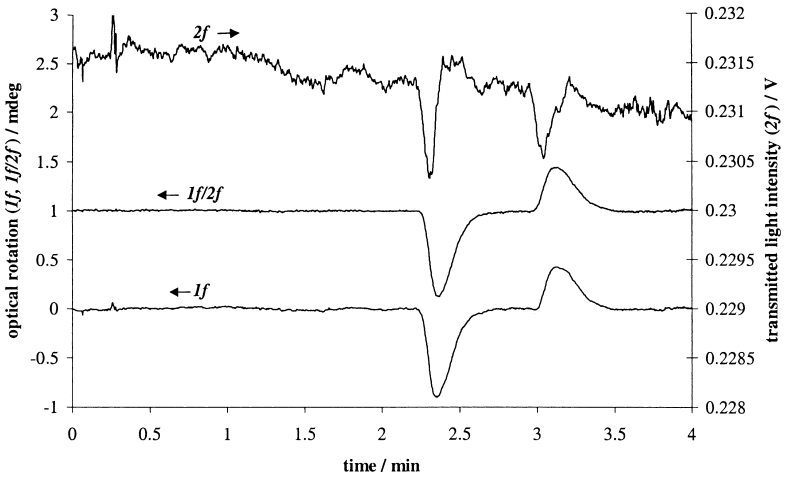


Fig. 5. Separation of 20 μl of 1% w/v fructose and 1% w/v sucrose solution. The traces shown are the transmitted light intensity ($2f$), the OR based on the $1f/2f$ signal and the OR based on the $1f$ signal. HPLC conditions: ODS2 column (250×4.6 mm, 5 μm particles) with a 100% water mobile phase at a flow rate of 1 ml min^{-1}. OR detection was at 635 nm.

Table 1
Signal-to-noise (S/N) analysis for the fructose and sucrose separation shown in Fig. 5[a]

	OR based on $1f/\mu$deg	OR based on $1f/2f/\mu$deg
Fructose peak height	−889	−875
Sucrose peak height	433	444
Noise	12	5
Fructose S/N	74	175
Sucrose S/N	36	89

[a] The signal values are based on peak heights, and the noise values are obtained from the standard deviation of the baseline between 1 and 2 min.

passed through, such lensing could cause changes in the depolarisation of the plane-polarised light.

4.3. HPLC under overload conditions

4.3.1. Warfarin

Warfarin was chosen for initial analysis by HPLC–UV–OR, because under semi-preparative conditions it gave good chromatography, with baseline resolution between the two enantiomer peaks. Warfarin has a reasonably high specific rotation (150 deg g^{-1} cm^3 dm^{-1}), so there are no sensitivity problems.

Fig. 6 shows the separation of racemic warfarin into its single enantiomers under semi-preparative conditions. The OR based on $1f$ and $1f/2f$ and the absorbance traces are shown. The advantages of the $1f/2f$ signal can be seen. Firstly, the $1f/2f$ signal was less noisy than the $1f$ signal. Secondly, it was more stable, as it returned to the baseline after the elution of the enantiomers. The S/N improvement on using the $1f/2f$ instead of $1f$ signal to calculate the OR is 100%.

As previously mentioned, the ratio OR/abs is directly proportional to the enantiomeric purity. If chiral HPLC is used to separate enantiomers, OR/abs can be used to monitor the enantiomeric purity and determine when to collect the eluent to produce samples of pure enantiomers. The OR/abs trace for the warfarin separation is shown in Fig. 7. The level parts of the trace, with equal magnitude but opposite signs, are when the pure enantiomers are eluting. Two switching valves were used to direct the eluent and collect samples of the pure enantiomers based upon these level sections of OR/abs, showing the benefits of on-line analysis of enantiomeric composition.

4.3.2. Trögers base

Trögers base was also analysed under overload conditions. It has a high specific rotation (287 deg

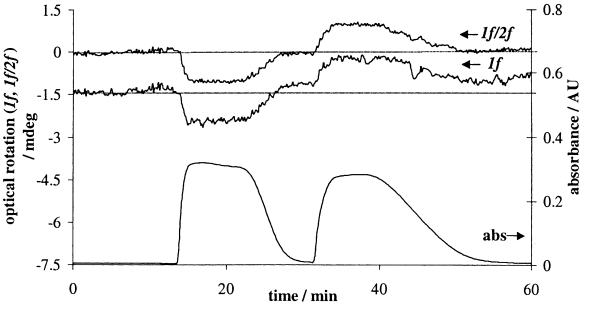


Fig. 6. Separation of a 5-ml injection of 9 mM racemic warfarin. The traces shown are OR based on $1f/2f$, OR based on $1f$ and absorbance (abs). HPLC conditions: Chiralcel OD column (250× 4.6 mm, 5 μm particles) with a hexane–propan-2-ol–acetic acid (80: 19.9: 0.1) mobile phase at a flow rate of 1 ml min^{-1}. OR detection was at 635 nm and absorbance detection at 330 nm.

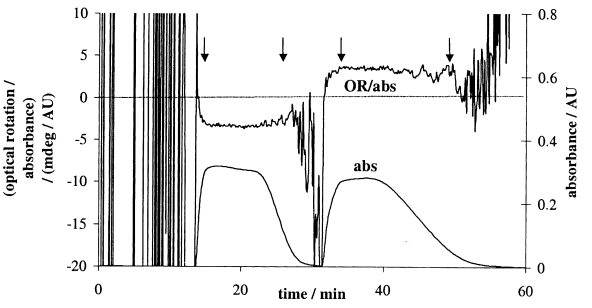
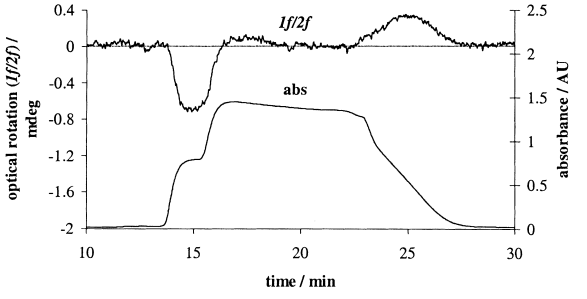


Fig. 7. Separation of enantiomers during a 5-ml injection of 9 mM racemic warfarin. The traces shown are OR divided by absorbance (OR/abs) and absorbance (abs). The OR/abs levels off when the single enantiomers are eluting and arrows indicate positions where valves were switched. HPLC conditions as in Fig. 6.

g^{-1} cm^3 dm^{-1}), but shows poorer chromatography than warfarin at high concentrations. Fig. 8a shows the OR based on 1*f*/2*f* and the absorbance trace for the separation of enantiomers in the racemic mixture. The peaks in the absorbance trace were overlapping, and thus could not be used to determine when the single enantiomers were eluting. Likewise the OR trace could not be used on its own, so the OR/abs signal must be used and this can be seen in Fig. 8b. The equal but opposite signals at 14–15 min and 26–27 min (OR/abs −1.0 and +1.0 mdeg/AU respectively) indicated the elution of the single enantiomers, which were collected using the switching valves. The co-eluting central portion of the trace between 15 and 26 min was recycled back on to the column, using the switching valves, for further separation.



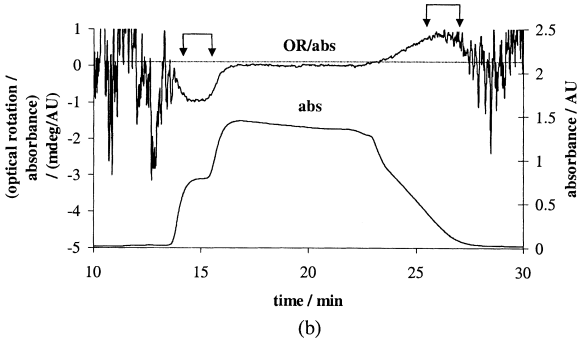


Fig. 8. Separation of a 5-ml injection of 0.25 m*M* racemic Trögers base. HPLC conditions: Chiralcel OD column (250×4.6 mm, 5 μm particles) with a hexane–propan-2-ol–diethylamine (90: 9.9: 0.1) mobile phase at a flow rate of 0.5 ml min^{-1}. OR detection was at 635 nm and absorbance detection at 250 nm. (a) The OR based on the 1*f*/2*f* signal and absorbance (abs) traces. (b) The OR/abs and abs traces. Regions of single enantiomer elution are indicated.

5. Conclusions

An improved OR detector has been described for measuring OR in the presence of species which cause scattering and absorbance at the laser wavelength, and the benefits of this have been demonstrated. Advantages include a *S*/*N* increase of up to 150% when comparing the OR based on 1*f*/2*f* and the OR based on 1*f*. Applications of the OR detector with analytical and semi-preparative HPLC have been given. Using the OR detector in series with a UV absorbance detector, samples of optically pure enantiomers have been collected by on-line monitoring of the OR/abs ratio and the use of switching valves.

Other potential applications of the OR detector are in monitoring solid-phase synthesis reactions of chiral molecules, without separation of the solid-phase particles, and on-line monitoring of enantioselective reactions in bioreactors. Work to investigate these processes is in progress. The detector could potentially be used in series with a refractive index detector or evaporative light scattering detector to observe the enantiomeric purity of compounds without UV chromophores.

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