

Multi-beam circular dichroism detector for HPLC

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A novel multi-beam circular dichroism (CD) detector for HPLC is described. A unique feature of this detector is the use of a retardation plate to give many quarter-waves in the wavelength region of interest. When a polarizing prism and a thin quartz plate as the retarder are placed in a conventional photodiode array (PDA) detector, the kinetic spectra of an optically active analyte recorded by the instrument contain sinusoidal CD waves in the wavelength axial direction. Extraction of these CD waves superimposed on the absorption spectra was performed with MS Excel. Both the instrumental conditions for the PDA data acquisition and the polynomial data processing were optimized by monitoring the chromatographic elution of camphor. The limit of detection for ajimaline, a chiral alkaloid, was 26 ng injected amount.

Circular dichroism (CD) is defined as the difference in absorbance between left- and right-circularly polarized beams in a chiral medium. In a widely used method, alternating beams with opposite rotational senses are transmitted through the sample cell. Although the sensitivity of a CD spectrometer can be improved by alternating the rotational sense more frequently, the smaller the value of $\Delta\epsilon/\epsilon$ (the anisotropy factor, AF^1) of a general chiral compound, the longer the CD measurement time will be. This causes a problem when a CD spectrometer is used as an HPLC detector. This is because a low volume flow-cell is needed for high resolution, but if the volume is too low, the time that the analyte is in it may be too short for an accurate CD measurement. Only a few studies have used this type of CD detector for the HPLC analysis of microgram quantities of chiral compounds.²⁻⁴

Recently, in order to supplement this defect of the conventional CD spectrometer as an HPLC detector, a double-beam CD detector has been developed. In one such instrument developed by Rosenzweig and Yeung⁵, left- and right-circularly polarized beams created from the double refraction of calcite intersect precisely in the sample cell. Another instrument built in our laboratory is equipped with a split-type flow-cell.⁶ In this type of instrument, two photodiodes are used to offset the noise of the light source so that CD can be measured directly with high sensitivity. In this system, however, individual differences among diodes cause an increase in the background absorption signal, which makes it difficult to maintain the baseline. As a result, it is difficult to measure the CD of analytes with high absorptivities.

This problem with the double-beam detection system was largely attributed to the use of relative difference in absorbance between the two diodes as the CD value. We thought that we could overcome this problem by using more diodes. In this paper the development of a multi-beam HPLC-CD detector, which is excellent in both sensitivity and operability, is presented. This optical system can be easily constructed by placing a polarizing prism and a quartz plate in front of a flow-cell of a conventional photodiode array (PDA) detector.

Experimental

The LC system consisted of a Shimadzu (Kyoto, Japan) LC-10AD pump, a Rheodyne (Cotati, CA, USA) 7125 injector with

a 20 μ L loop, a Tosoh (Tokyo, Japan) CO-8011 column oven and a Shimadzu SPD-M10Avp PDA detector. A Gran-Taylor prism (1 cm square, Sigma Koki, Hidaka, Saitama, Japan) was placed in the compartment for the wavelength calibrating filter in the PDA detector. A quartz plate (5 mm wide, 0.455 mm thick) purchased from Five Lab (Kawasaki, Japan) was placed on the incident light side of the flow-cell, whose principal axis was inclined by 45° from the polarizing axis. The three-dimensional data obtained were converted into ASCII format with the aid of the detector's bundled software, and were processed with a spreadsheet program (Excel, Microsoft Corp., Redmond, WA, USA).

The CD spectra were obtained with a Jasco (Hachioji, Tokyo, Japan) J-500C CD spectrometer and the UV spectra with a Shimadzu UV-2200 spectrophotometer.

Analysis of camphor was performed on a 15 cm $\times$ 4.6 mm id reversed-phase column (Supelco TPR-100, Sigma Aldrich Japan, Tokyo, Japan) maintained at 40 °C. Acetonitrile–water (3 + 1) was used as the mobile phase at a flow rate of 0.6 mL min⁻¹.

Analysis of ajimaline, which is an alkaloid contained in *Rauwolfia serpentina*, was performed on a 25 cm $\times$ 4.6 mm id reversed-phase column (Capcell Pak UG120 Å, Shiseido, Tokyo, Japan) maintained at 40 °C. 0.02 M phosphoric acid–acetonitrile (5 + 3) was used as the mobile phase at a flow rate of 0.6 mL min⁻¹.

Single enantiomers of camphor were purchased from Sigma Aldrich Japan, and ajimaline was from Nacalai tesque (Kyoto, Japan). HPLC-grade acetonitrile and other reagents were purchased from Wako Pure Chemicals (Osaka, Japan).

Results and discussion

Principle of multi-beam CD detection

Holzwarth⁷ suggested the possibility of measuring the CD spectrum by placing a polarizing prism and a multiple-order retardation plate in the spectrophotometer. The principle of this method lies in the opposite rotational senses of the circularly polarized beams created from the adjacent quarter-waves, *i.e.*, the polarization in the incident beam alternates as the wavelength is varied. As a result, the absorption spectrum of an optically active analyte oscillates with wavelength. Although

the sample beam in the spectrophotometer is monochromatic, we applied this principle to the PDA detector where the incident beam is not spectrally diffracted in advance. We assumed that the spectral diffraction does not destroy the CD data which is included in such circularly polarized beams, because CD is derived from light absorption. While in the conventional CD spectrometer the monochromatic circularly polarized beam alternates with time, in the proposed method the circularly polarized beam alternates with wavelength. The experimental design of this multi-beam CD detector is depicted in Fig. 1. A thin plate of quartz as a uniaxial, birefringent crystal, which was vertically cut along the principal crystallographic axis, was used as a multiple-order retarder because of its low cost. It is easy to construct this detector if there is enough space to set a polarizing prism in a conventional PDA detector.

Effect of PDA parameters

In the conventional CD spectrometer, high-frequency modulation of the circularly polarized beam reduces the noise and improves its sensitivity. The same applies to the multi-beam CD detector. The shorter the 1/4 wavelength interval produced from the thin plate is, the better is its detection sensitivity. Since the photodiodes in the general PDA detector are arranged about every 1 nm, there is a limit to the period of the CD wave that is detectable. A quartz plate with 0.455 mm thickness was used to make the 1/4 wavelength interval less than 5 nm. The interval caused by this plate at around 250 nm was 4 nm.

The spectral bandwidth is another factor that influences the resolution of the spectrum. A wide bandwidth reduces the noise but sacrifices the resolution. In the SPD-M10Avp PDA detector, the spectral bandwidth is adjusted by varying the number of diodes used for data acquisition at a specific wavelength. Although the noise levels in the absorption spectrum depended on the stability of the detector, they were usually in the ranges 6×10^{-5} – 10^{-4} absorbance units (AU) when using 1 diode, 2×10^{-5} – 3×10^{-5} AU when using 2 diodes, and 10^{-5} – 3×10^{-5} AU when using 3 diodes, and further increases in the number of diodes did not bring about any improvement. The absorption spectra of (+)-camphor measured by altering the spectral bandwidth are shown in Fig. 2. The spectra were differentially processed to easily find the CD wave. The amplitude of the CD wave decreased as the number of diodes increased. Two diodes, corresponding to a spectral bandwidth of 2 nm, were used for subsequent experiments by considering the ratio of the noise level and the CD amplitude.

Extraction of the CD wave from the absorption spectrum

All data processing was performed by the Savitzky–Golay polynomial algorithm.⁸ In this procedure, the degree of the polynomial used should be decreased and the number of data points used should be increased to reduce the effect of the noise. In this experiment, however, the period of the CD wave obtained was about 10 nm and practicable data was limited to this range. As shown in Fig. 2, high-order differential processing reduces the influence of the original spectrum of the

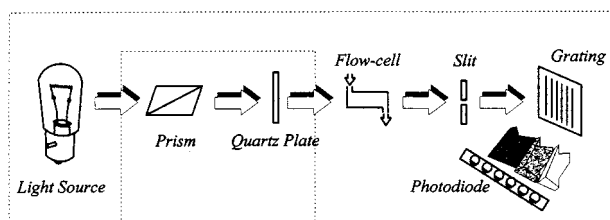


Fig. 1 Schematic diagram of the multi-beam HPLC-CD detector. The part within the dotted line represents a conventional PDA detector.

analyte, but leads to an increase in noise by using a high-degree of polynomial with a limited number of data points. It should be mentioned that the absorbance values at half-waves on this spectrum are independent of the chiral purity of the analyte, and the line passing through these points is indeed the baseline that we were looking for. The CD wave was extracted by subtracting the spectrum of the racemate which was normalized to pass these zero-points. As mentioned above, the higher the degree of the polynomial, the easier is the normalization. The CD chromatograms of camphor with various concentrations and various chiral purities which were drawn up in this manner from their 2nd differential spectrum are shown in Fig. 3. The vertical axes of these chromatograms show the aggregate of the CD values in the range 260–308 nm in order to gain high sensitivity. Differential processing from the 0th to the 3rd gave almost the same chromatograms. One difference between the analysis of a standard and the analysis of an actual sample is that the latter might have some impurities that overlap with the peak of interest. Since differential processing has been very effective in such a case,⁹ 2nd differential spectra were used for all subsequent procedures.

Limits of detection of the multi-beam detector

Although the noise level of this PDA detector is 2×10^{-5} – 3×10^{-5} AU with a spectral bandwidth of 2 nm as mentioned

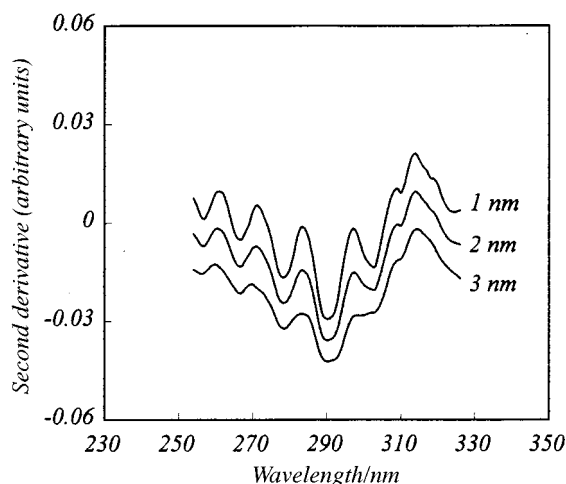


Fig. 2 Second differential spectra of (+)-camphor in a chromatographic cell with various numbers of diodes corresponding to spectral bandwidth: amount injected, 10 µg; spectral bandwidth marked on figure; for chromatographic conditions see Experimental.

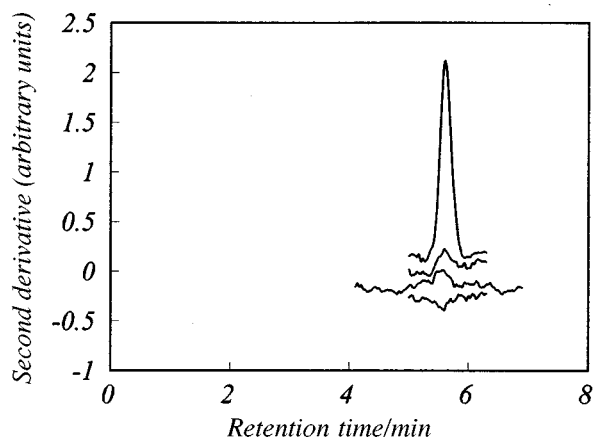


Fig. 3 CD chromatograms of camphor: upper trace, 10 µg of (+)-isomer; second trace, 1 µg of (+)-isomer; third trace, 10 µg of (+)/(-) = 10/9 isomer; bottom trace, 10 µg of (+)/(-) = 9/10 isomer.

above, subsequent data processing reduces it to below 10^{-5} AU. The proposed detection system can theoretically measure the amplitude of the CD wave within about 2×10^{-5} AU. The ratio between this amplitude and the absorption of the analyte is identical to the AF. Generally, the AF of a chiral compound is in the order of 10^{-3} or less. However, the CD wave of such a compound can be detected with data processing if its peak height is more than 0.02 AU. This value can always be obtained with HPLC, so this system is thought to be a universal CD detector for chiral compounds. The limit of detection of this system depends on the ϵ of the analyte.

The CD and UV spectra of ajimaline in acidic medium are shown in Fig. 4. This alkaloid shows both maxima around 250 nm with an AF of about 1/800. Consequently, a CD wave can be detected at this wavelength when the amount that is injected gives a peak height of 16 mAU. Under the present LC conditions, an injection of 0.15 nmol corresponded to this peak height. CD chromatograms of ajimaline obtained by using a spectrum without the quartz plate as a baseline are shown in Fig. 5. We were able to detect 0.08 nmol (26 ng) of ajimaline, which is about half the calculated amount. Moreover, an injection of 0.25 nmol ajimaline resulted in the appearance of a negative peak around 290 nm, where the AF of ajimaline is about $-1/2600$. This suggested that a CD amplitude below 10^{-5} AU could be measured.

In this experiment, the quartz plate used was restricted in thickness for the resolution of the PDA detector. As the measurement accuracy of a conventional CD spectrometer depends on the frequency of the beam modulation, the same may be said of the relationship between the sensitivity of the proposed system and the quarter-wave interval produced by the quartz plate. The application of a thinner quartz plate to the PDA detector with higher resolution will improve the sensitivity of this detection system.

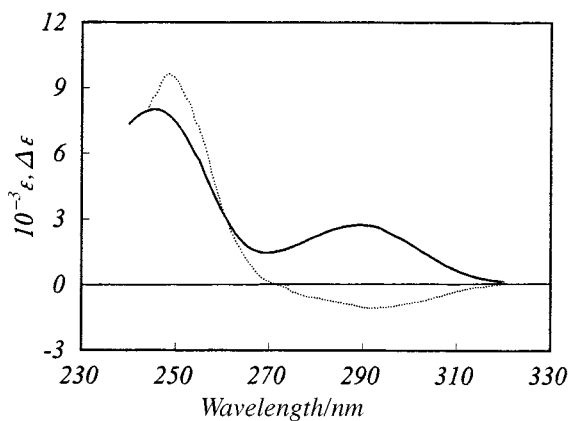


Fig. 4 UV (—) and CD (---) spectra of ajimaline in 0.02 M H_3PO_4 -MeCN (2:1).

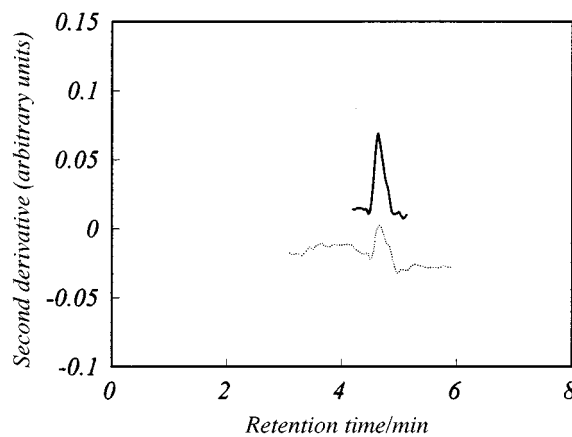


Fig. 5 CD chromatograms of ajimaline at the detection limit. The amounts of analytes are 26 (·····) and 86 ng (—) injected.

Conclusion

A multi-beam CD detector, which can detect a CD wave on the wavelength axial direction, was constructed by inserting a prism and a thin quartz plate into a PDA detector. The CD wave was successfully extracted by differential processing of the absorption spectrum. Although this processing is also useful for removing the influence of impurities on a chromatogram, a high-order differential causes an increase in baseline noise, and further investigations revealed that 2nd differential processing gave the best results. The proposed detection system under the established conditions had not only a sensitivity that was equal to or better than that of a double-beam CD detector, but also a facility comparable to a conventional CD spectrometer to detect the absolute $\Delta\epsilon$. This system was able to detect as little as 26 ng of ajimaline, which is CD-active around 250 nm (ϵ is about 8000 and $\Delta\epsilon$ is about 10).

References

1. P. Salvadori, C. Bertucci and C. Rosini, *Chirality*, 1991, **3**, 376.
2. C. Bertucci, P. Salvadori and L. F. L. Guimaraes, *J. Chromatogr. A*, 1994, **666**, 535.
3. O. Zerbinati, R. Aigotti and P. G. Daniele, *J. Chromatogr. A*, 1994, **671**, 281.
4. K. Takatori, S. Toyama, S. Fujii and M. Kajiwara, *Chem. Pharm. Bull.*, 1995, **43**, 1797.
5. Z. Rosenzweig and E. S. Yeung, *Appl. Spectrosc.*, 1993, **47**, 2017.
6. A. Yamamoto, A. Matsunaga, K. Hayakawa and M. Nishimura, *Biomed. Chromatogr.*, 1997, **11**, 362.
7. G. Holzwarth, *Rev. Sci. Instrum.*, 1965, **36**, 59.
8. A. Savitzky and M. J. E. Golay, *Anal. Chem.*, 1964, **36**, 1627.
9. A. Yamamoto, A. Matsunaga, M. Ohto, E. Mizukami, K. Hayakawa and M. Miyazaki, *Analyst*, 1995, **120**, 337.

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