

《Original Paper》

HPLC / fluorescence detected circular dichroism detection by three-dimensional fluorescence detector

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Abstract

An HPLC detection method based on fluorescence-detected circular dichroism (FDCD) is described. The proposed detector consists of a polarizing prism and a retardation plate fitted on the excitation light side of a conventional three-dimensional fluorescence detector and uses a modulated beam in the wavelength axial direction. The FDCD wave is superimposed on the excitation spectrum measured at a fixed emission wavelength. When estrone with a large anisotropy factor, $\Delta\epsilon/\epsilon$, was used as a probe analyte, the difference between the two excitation spectra obtained by reversing the direction of the optical axis of the retardation plate showed a wave-like curve whose phase is consistent with that of the plate. We suggest that FDCD can be measured by using the optical system of three-dimensional fluorescence detector, which obtains a spectrum by the grating with high rotational speed. However, in the detection of an enantiomer with a low anisotropy factor, such as catechin, the amplitude of the artifact wave was comparable with that of the FDCD wave and obstructed the FDCD measurement. This wave was thought to be induced by a lack of uniformity of the rotation of the grating. As both the artifact

and FDCD waves varied sinusoidally with wavelength, the FDCD wave with a short period was successfully extracted by using the differential properties of a trigonometric function.

INTRODUCTION

Chiroptical analysis is important because the physiological and therapeutic effects of many chemical species depend on their enantiomeric forms. Circular dichroism (CD) is a popular method for the determination of optically active compounds. A commercially available CD detector that was specifically designed for high-performance liquid chromatography (HPLC) showed good agreement for the determination of enantiomeric purity at levels less than 99% with the enantio-selective HPLC (Hadley and Jonas, 2000). However, it is unlikely that the CD detector can provide the accuracy and sensitivity of the direct resolution techniques. Various modifications have been made to CD detector to improve their accuracy and sensitivity. One approach is to exploit the superior optical properties of a laser. A laser beam has high spectral and polarization purities and is highly collimated. Lasers can provide higher beam power than conventional light sources, which require a polarizing prism, a dif-

fraction grating and collimators. Thus, a laser-based CD detector can provide a higher signal-to-noise ratio (Yeung et al., 1985 and 1993). Recently, CD in the vacuum ultraviolet region has been detected with synchrotron radiation (Orry et al., 2001).

Another approach stems from the fact that CD phenomenon is based on the light absorption. Traditional technique is fluorescence-detected circular dichroism (FDCD), which is based on the assumption that the excitation profile of the chiral fluorophore has the same profile as the absorption spectrum (Turner et al., 1974 and 1976, Geng and McGown, 1994). Besides this, thermal lens CD (Tran et al., 1989 and 1990) and thermal grating CD (Nunes and Tong, 1993), are based on the unequal thermal radiation from the chiral compound excited by circularly polarized beams. Recent technique, which is used in dissociative photoionization mass spectrometry, is based on the difference in fragmentation of a chiral compounds irradiated with circularly polarized beams (Mackie et al., 2002). Although several additional reports on the detectors based on thermal lens CD (Xu and Tran, 1990) and FDCD (Yeung et al., 1986 and 1989) for HPLC and capillary electrophoresis have described the potential of these techniques, their special optical systems are difficult to set up.

We previously reported that a conventional fluorescence spectrophotometer can be used to measure FDCD (Yamamoto et al., 2002). This technique is based on the principle of Holzwarth (1965) that a multiple-order retardation plate can produce a modulated beam in the wavelength axial direction. An HPLC/CD detector based on this principle can be easily constructed by using a optical system of a conventional photodiode array detector (Yamamoto et al., 1999 and 2001). In this paper, we tried to apply this technique to a three-dimensional fluorescence detector for HPLC. FDCD method is presently used for a few fields, such as analyses of the tertiary structure of a protein (Park et al., 1996) and the conformation of a natural product based on the exciton CD chi-

rality method (Nishida et al., 1997, Nehira et al., 1999). Development of an easy-to-use HPLC/FDCD detection should result in the discovery of many more applications for FDCD in analytical chemistry.

EXPERIMENTAL

Instrumental setup

The HPLC/FDCD detection system was constructed by using the optical system of an Agilent Technologies (Waldbronn, Germany) 1100 FLD three-dimensional fluorescence detector. A Gran-Taylor prism (1 cm square, Sigma Koki, Hidaka, Saitama, Japan) was placed between the excitation grating and the flow-cell in the fluorescence detector. A quartz retardation plate (0.371 mm or 0.425 mm thick) purchased from Five Lab (Kawasaki, Japan) was placed on the excitation light side of the flow-cell, whose principal axis was inclined by 45 degrees from the polarizing axis (Fig.1). The width of the excitation slit (slit 1 in Fig.1) was reduced to about 0.7 mm (normal; 2 mm) by handicraft. The three-dimensional data obtained were processed with a spreadsheet program (Excel, Microsoft, Redmond, WA, USA). For all data, noise was smoothed by the Edwards' two-dimensional data processing technique (Edwards 1982).

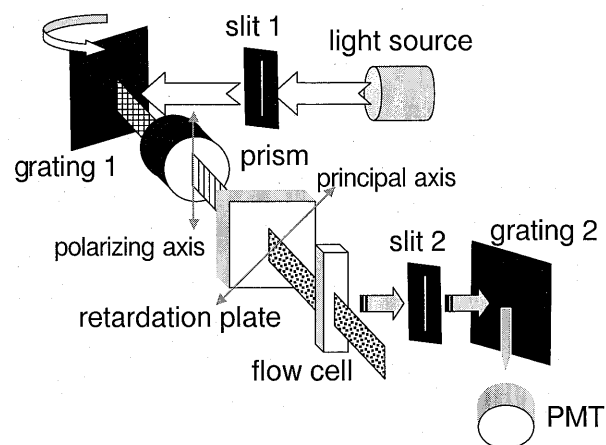


Fig. 1. FDCD optical system in the present work.

Performance test of the FDCD detection system

In order to test the performance of the proposed FDCD system, the HPLC system, which consisted of a Shimadzu (Kyoto, Japan) LC-10AD

pump, a Shodex (Tokyo, Japan) degassing unit, a Rheodyne (Cotati, CA, USA) 7125 injector, a Tosoh (Tokyo, Japan) CO-8011 column oven, has been used.

Single enantiomers of catechin were obtained from Sigma Aldrich Japan (Tokyo, Japan). HPLC-grade acetonitrile, n-hexane, ethanol and other reagents were purchased from Wako Pure Chemicals (Osaka, Japan). Water was purified with a Toray (Mishima, Japan) LV-05 ultrapure water system.

Analysis of estrone was performed on a 25 cm×4.6 mm i.d. reversed-phase column (Capcell Pak UG120, Shiseido, Tokyo, Japan) maintained at 40 °C. Acetonitrile-water (2+1) was used as the mobile phase at a flow rate of 0.8 mL/min. Emission wavelength was set at 345 nm and excitation spectra in the range of 280–320 nm were recorded. Analysis of catechin was performed on the same manner. Acetonitrile-water-formic acid (8+91.5+0.5) was used as the mobile phase at a flow rate of 1.0 mL/min. Emission wavelength was set at 340 nm and excitation spectra in the range of 255–305 nm were recorded. In all data acquisition modes, grating 1 in Fig. 1 is in a state of rotation and grating 2 is fixed.

RESULTS AND DISCUSSION

Improvement of the spectral bandwidth

The spectral bandwidth of the 1100 FLD detector is fixed at 20 nm. In the FDCD method, which uses a modulated beam in the wavelength direction, the 1/4 wavelength interval is dependent upon the spectral bandwidth of the instrument. If the interval is wider than the CD spectral band of an analyte, the proposed method can not accurately detect the FDCD wave. The spectral bandwidth of the detector may be improved by reducing the width of the excitation slit. Estrone, which is a follicular hormone with a large FDCD value around 300 nm (Yamamoto et al., 2002), was measured with different slit widths (2.0, 0.7 and 0.4 mm). The difference spectrum between the two excitation spectra was obtained by reversing the plane of the birefringence of a 0.371

mm-thick quartz plate, whose 1/4 wavelength interval is about 10 nm at 300 nm. None of difference spectra showed any remarkable FDCD waves, and so differential processing was employed. Because the FDCD wave varies sinusoidally, and a sinusoidal curve is emphasized by the differential processing. Differential processing was performed by the Savitzky-Golay polynomial algorithm (1964). Second differential excitation spectra obtained with 2.0 and 0.7 mm slit width are shown in Fig. 2. The ordinate for the difference spectra was magnified 2-fold. Only the

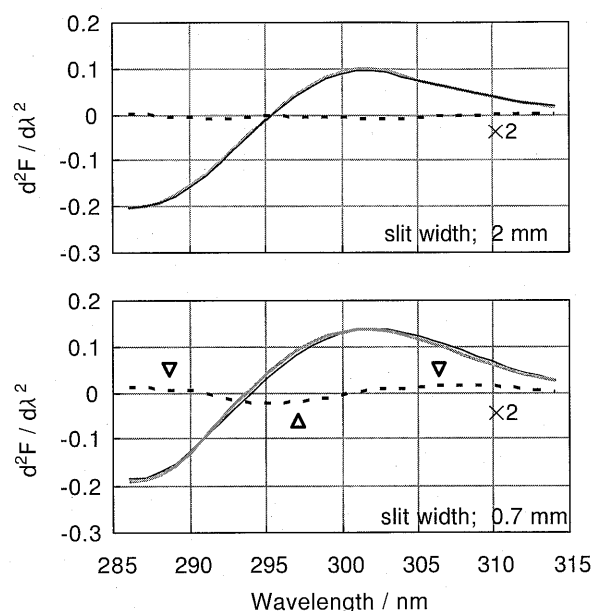


Fig. 2. Difference spectra (broken lines) between two second differential excitation spectra of estrone (solid lines) at an emission wavelength of 345 nm in a chromatographic cell using a 2 mm slit (top) and a 0.7 mm slit (bottom). The two spectra in each panel were obtained by reversing the direction of the optical axis of the retardation plate. The amount of sample injected was 2.5 µg. Triangles indicate $\lambda/4$ of the plate with 0.371 mm thickness.

narrower slit, i.e., 0.7 mm, gave a FDCD wave whose phase was consistent with that of the quartz plate. This means that the narrowing of the slit width was effective to improve the spectral bandwidth. However, the optical resolving power can not exceed the theoretical resolving power of the optics at the narrowest slit width. No improvement in the amplitude of the FDCD wave at 0.4 mm slit width was observed, but this slit lowered the fluorescence emission intensi-

ties. A 0.7 mm slit width was used in the following experiments. The spectral bandwidth seemed to be improved by about 10 nm.

Appearance of artifact wave

Catechin is a polyphenol that is typical of the polyphenols contained in tea leaves. To determine the FDCD wave of catechin, high-order differential processing is required due to the low anisotropy factor of catechin (see Fig. 4 in Yamamoto et al., 2002). Sinusoidal patterns such as FDCD wave are embossed as the order of differentiation increased. Successive 6th differential excitation spectra of (+)-catechin at the peak top are shown in Fig. 3. These waves showed different phase from the FDCD wave, and were thought to be induced by a lack of uniformity of the rotation of the excitation grating. If it is assumed that the periodic repetition of this lack of uniformity yields an artifact wave possessing a regular pattern in the excitation spectrum, a crossing point of successive differential spectra indicates a real value (points enclosed with circles in Fig. 3). The real differential spectrum of the enantiomer would be formed from an aggregation of the crossing points extracted from many spectral data. The differential spectra of catechin enantiomers obtained in such a way differed apparently from each other (Fig. 4) and the difference between them was exactly the FDCD wave, because its frequency and phase were in agreement with those of the retardation plate. However, the difficulty of extracting the FDCD wave by the proposed procedure has prevented

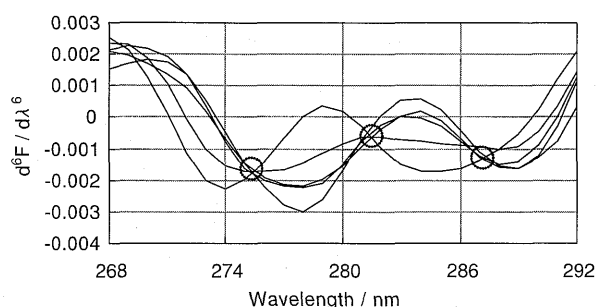


Fig. 3. Successive excitation spectra of (+)-catechin at an emission wavelength of 340 nm in a chromatographic cell at intervals of 0.6 seconds. The amount of sample injected was 250 ng.

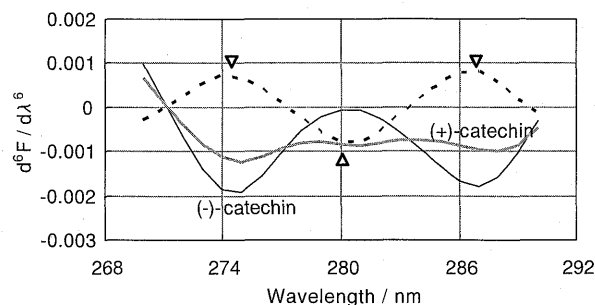


Fig. 4. FDCD wave for catechin obtained by a retardation plate with 0.371 mm thickness. Broken line is the difference spectrum between catechin enantiomers. The quarter wavelengths of the plate are marked with triangles.

this method from being used for routine analyses.

Extraction of FDCD wave of catechin

As mentioned above, both the FDCD and artifact waves vary sinusoidally with wavelength. These waves are approximated by the following equation when the amplitude and the wavelength are plotted along the vertical and horizontal axes, respectively.

$$y = \sin(x/a) \quad (1)$$

where a is a parameter that is proportional to the period of the wave. When $a = 1$, the period is correspond to 2π nm. Consequently, a of both the FDCD and artifact waves are more than 1 under these instrumental conditions. The i th derivative of equation (1) gives

$$d^i y / dx^i = (1/a)^i \sin(x/a + i\pi/4) \quad (2)$$

Thus, in a spectrum composed of two sinusoidal curves with different frequencies, the long-term wave can be eliminated by increasing the order of differential processing. To obtain the FDCD wave by high-order differentiation, the period of the FDCD wave must be shorter than that of the artifact wave. Although the period of the artifact wave was around 14 nm, the spectral bandwidth of the 1100 FLD detector was not able to be narrowed less than 10 nm. A quartz plate with 0.425 mm thickness whose $1/4$ wavelength interval is 12 nm around 280 nm was used to determine the FDCD wave of catechin. The difference between 6th differential excitation spectra obtained for the peaks of catechin enantiomers is shown in Fig. 5.

The artifact wave with shorter period has some adverse effects on the differential spectra. However, the FDCD wave whose phase agreed with that of the 1/4 wavelength was obtained by using the average values of the data. Although an increase in the amplitude of the FDCD wave was expected because the parameter a in equation (2) was smaller than that in Fig. 4, the amplitude was almost the same. No increase in the amplitude appeared to be due to the fact that the spectrum bandwidth is nearly equivalent to the 1/4 wavelength interval.

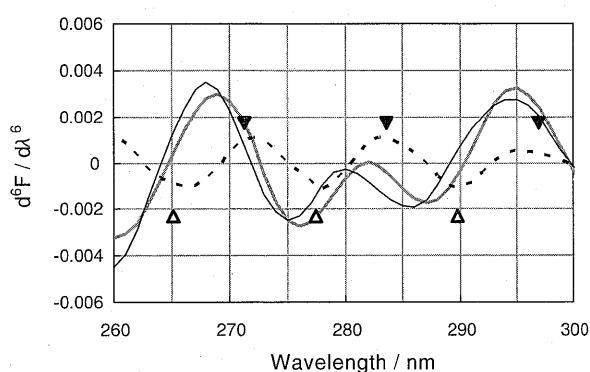


Fig. 5. Difference spectra between the 6th differential excitation spectra of catechin enantiomers. The quarter wavelengths of the plate with 0.425 mm thickness are marked with triangles. Sample identifications are as in Fig.3.

FDCD chromatograms were prepared by using the differential values at the 1/4 wavelengths (Fig. 6). The vertical axis in Fig. 6 is the difference between the sum of the differential values at 271, 283 and 296 nm, which correspond to the closed triangles in Fig. 5, and those at 265, 277 and 290 nm, which correspond to the open triangles in Fig. 5, of each excitation spectrum. To clarify the peak shapes, all chromatograms were smoothed by a polynomial fitting method

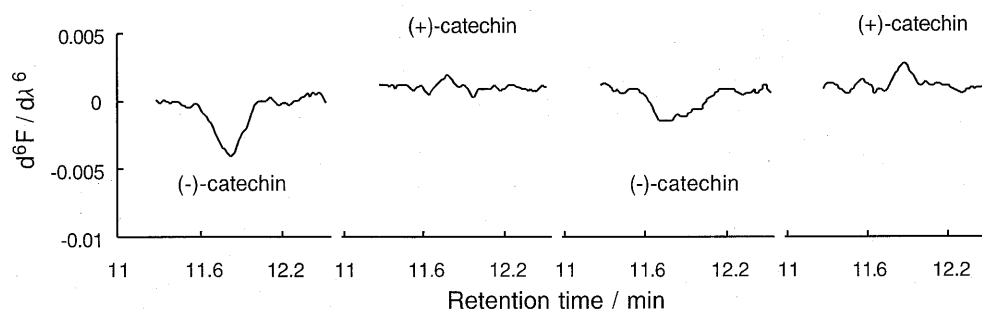


Fig. 6. HPLC/FDCD chromatograms of catechin enantiomers.

(Savitzky and Golay, 1964). The catechin peaks were inverted by altering their enantiomeric composition, but the reproducibility of the peak intensities was unsatisfactory due to the presence of a short-term artifact wave. Work on the development of an effective extraction technique of FDCD wave whose period is longer than that of the artifact wave is in progress.

CONCLUSIONS

FDCD detection utilizing a modulated beam in the wavelength axial direction was examined by a three-dimensional fluorescence detector. The FDCD wave was detectable in our optical system, in which the spectrum is obtained by a grating spinning at a high rotational speed (4,000 rpm). However, the lack of uniformity of this rotation yielded an artifact wave on the excitation spectrum, which complicated the detection of the FDCD wave of an enantiomer with low anisotropy factor. The ability to remove the artifact wave from the excitation spectrum by using the differential properties of trigonometrical function was limited by the spectral bandwidth of the detector. Improvement of the rotational accuracy of the grating and the spectral bandwidth of the detector would make it possible to detect FDCD with high accuracy.

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