Far-ultraviolet absorbance detection of sugars and peptides by high-performance liquid chromatography

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ARTICLE INFO

ABSTRACT

A far-ultraviolet (FUV)-absorbance detector with a transmission flow cell was developed and applied to detect absorbance of sugars and peptides by HPLC. The main inherent limitation of FUV-absorbance detection is the strong absorptions of solvents and atmospheric oxygen in the optical system as well as dissolved oxygen in the solvent. High absorptivity of the solvent and oxygen decreases transmission-light intensity in the flow cell and hinders the absorbance measurement. To solve the above drawbacks, the transmission-light intensity in the flow cell was increased by introducing a new optical system and a nitrogen-purging unit to remove the atmospheric oxygen. The optical system has a photodiode for detecting the reference light at a position of the minus-first-order diffracted light. In addition, acetonitrile and water were selected as usable solvents because of their low absorptivity in the FUV region. As a result of these implementations, the detectable wavelength of the FUV-absorbance detector (with a flow cell having an effective optical path length of 0.5 mm) can be extended down to 175 nm. Three sugars (glucose, fructose, and sucrose) were successfully detected with the FUV-absorbance detector. These detection results reveal that the absorption peak of sugar in liquid phase lies at around 178 nm. The detection limit (S/N = 3) in absorbance with a 0.5-mm flow cell at 180 nm was 21 μAU, which corresponds to 33, 60 and 60 μM (198, 360, and 360 pmol) for fructose, glucose, and sucrose, respectively. Also, the peptide Met-enkephalin could be detected with a high sensitivity at 190 nm. The estimated detection limit (S/N = 3) for Met-enkephalin is 29 nM (0.29 pmol), which is eight times lower than that at 220 nm.

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1. Introduction

An ultraviolet (UV)-absorbance detector for high-performance liquid chromatography (HPLC) is widely used in many fields thanks to its ease of use and good stability. However, a UV detector is hardly used to detect substances with weak-to-non-existent UV absorption, such as sugars. Commonly, as a standard method for analysis of sugars, refractive-index (RI) detection has been widely used [1]. However, the sensitivity of RI detection is relatively low, and gradient elution is not possible on the basis of RI detection.

From many spectroscopic studies using vacuum FUV spectrometer, it is well-known that most organic substances show strong absorption due to electronic transition in the FUV region below wavelength of 190 nm [2–5]. A vacuum FUV spectrometer was used to solve the inherent problem (i.e., absorption of atmospheric oxygen and moisture) concerning spectroscopic measurement in the FUV region [6,7]. For example, ethers in vapor phase have strong absorption peaks at around 185 nm [4]. Since sugars contain ether bonds, sugars in aqueous solution are expected to have a strong absorption peak at the same position as that of ethers. Therefore, if the detectable-wavelength region of the absorbance detector could be extended to the wavelength region below 190 nm, it could detect sugars with high sensitivity. However, most spectroscopic studies in the FUV region have been done in vapor phase because it is difficult to obtain FUV spectra in liquid phase.

The main difficulty in obtaining FUV spectra in liquid phase is the strong absorption of a solvent itself [8]. It is difficult to develop the FUV detector for HPLC while the FUV absorbance detector for gas chromatography has been reported [9]. High absorptivity of solvents has prevented acquisition of meaningful data in aqueous solutions. The light absorption of solvents significantly increases as wavelength in the FUV region gets shorter; for example, the extinction coefficient of water at 186 nm is more than 10 times
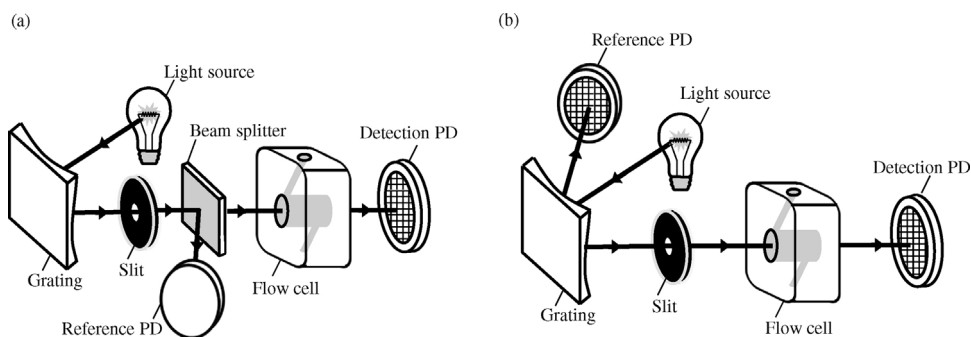
Fig. 1. Schematics of (a) conventional optical system for UV-absorbance detector and (b) new optical system for FUV-absorbance detector.

larger than that at 181 nm [7]. To overcome the above-mentioned difficulties, a new FUV spectrometer, based on attenuated total reflection (ATR) with a nitrogen-purge unit instead of a vacuum unit, has been developed and applied to studies on both water and organic molecules in liquid phase down to wavelength of 140 nm [10–12]. Since an effective optical path length can be reduced to tens of nanometers, which corresponds to a penetration depth for FUV light, absorbance of liquid samples can be detected by avoiding strong background absorption due to solvents. On the other hand, the very short effective optical path length results in reducing absorbance from samples to be analyzed. The sensitivity of a FUV detector with an ATR cell would be four orders of magnitude lower than that of conventional UV absorbance detectors.

In this study, a FUV-absorbance detector with a transmission flow cell used in HPLC was developed and applied for detecting sugars and peptides. To solve the problem concerning low transmission-light intensity in the FUV region, incident-light intensity to the flow cell was increased by implementing a new optical system and selecting usable solvents. In the optical system, a photodiode for detecting reference light was set at a position of minus-first-order diffracted light in order to avoid loss of light intensity at the beam splitter. In addition, a nitrogen-purging unit was implemented in a UV-absorbance detector to remove atmospheric oxygen. Acetonitrile and water were selected as usable solvents because of its lower absorptivity in the FUV region compared with other popular solvents for HPLC [8]. Thanks to the above-described implementations, the detectable wavelength of the FUV-absorbance detector (with a flow cell having effective optical path length of 0.5 mm) can be extended down to 175 nm. Three sugars (glucose, fructose, and sucrose) were successfully detected with this FUV absorbance detector. Peptides were also detected with eight-times higher sensitivity at 190 nm (i.e., 0.29 pmol) than that at 220 nm.

2. Experimental

2.1. Reagents

HPLC-grade acetonitrile was purchased from Wako Pure Chemical Industries (Osaka, Japan). Water was produced by a Milli-Q system (Millipore, MA, USA). Glucose, fructose, and sucrose were purchased from Wako Pure Chemical Industries. Standard peptide mixture (Gly-Tyr, Val-Tyr-Val, Met-enkephaline) was obtained from Sigma Aldrich (MO, USA). Glycine peptides (di- and triglycine) and amino acids with aromatic rings (tyrosine and phenylalanine) were obtained from Peptide Institute (Osaka, Japan). Sodium perchlorate was obtained from Wako Pure Chemical Industries. All analytical samples were diluted with distilled water.

2.2. Design of new FUV absorbance detector for HPLC

A commercial UV-absorbance detector cannot be used for wavelengths below 190 nm because light intensity significantly decreases due to strong absorption of oxygen and solvents. In this study, a UV detector (Hitachi Chromaster 5410, Hitachi High-Tech Science, Japan) was modified to increase light intensity and to extend the detectable wavelength down to the FUV region. First, oxygen was removed from the optical system in the detector by nitrogen purging and from the solvents by degassing because oxygen has strong absorption below wavelength of 200 nm [6]. Second, a new optical system was introduced. In a conventional UV detector, as shown in Fig. 1(a), a beam splitter is placed before a flow cell to divide the light beam into two paths. One path provides detection light for the flow cell, and the other is used as reference light. Absorbance can be calculated from the measured intensities of the detection light and reference light. This optical system can effectively reduce the noise caused by fluctuation of light intensity, because the intensity of the reference light is measured. However, using the beam splitter decreases the intensity of the detection light to less than half. If the optical system without the beam splitter is fabricated, intensities of the detection light will be more than double. To measure intensity of the reference light without using a beam splitter, a new optical system, as shown in Fig. 1(b), was designed. While a photodiode for measuring the reference light was placed at a position of the minus-first-order diffracted light from the grating, the plus-first-order diffracted light was used for the detection light. This optical system can maintain lower noise by increasing the intensity of the detection light.

2.3. HPLC conditions

Chromatographic analyses of samples of sugars and peptides were performed with a Hitachi HPLC system (Hitachi High-Tech Science, Tokyo, Japan) equipped with a pump (L-2130, Hitachi High-Tech Science), an autosampler (L-2200, Hitachi High-Tech Science), and the modified FUV detector described in Section 2.2. At least 30 min of nitrogen purging and degassing were conducted before HPLC measurements.

For all sugar separations, a separation column (NH2P-50 4E, 5 μm, 250 mm × 4.6 mm, Shodex, Tokyo, Japan) with a guard column (NH2P-50G 4A, 5 μm, 10 mm × 4.6 mm, Shodex, Tokyo, Japan) was used with water/acetonitrile (25:75, v/v) at flow rate of 1 mL/min. The injection volume was 6 μL for all the sugar samples (1 mg/mL).

A peptide mixture (Gly-Tyr, Val-Tyr-Val, Met-enkephaline) was separated on a C18 column (218TP54, 5 μm, 250 mm × 4.6 mm, Vydac, SA, USA). Solution A was water/acetonitrile (90:10, v/v) containing 5 mM of sodium perchlorate, while solution B was water/acetonitrile (60:40, v/v) containing 5 mM of sodium