

Direct Detection of Gradient-eluted Non-labeled Amino Acids using Micro-HPLC with Ultraviolet Thermal Lensing

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Direct detection of gradient-eluted non-labeled amino acids using micro-HPLC with ultraviolet thermal lensing

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Abstract. Thermal lensing (TL) permits ultrasensitive measurements of optical absorption of analytes in very small volumes. Separation-detection conditions of non-labeled amino acids with micro-HPLC/UV absorption detector are optimized, and direct determination of non-labeled amino acids by micro-HPLC/UV-excitation TL detection in a gradient elution is successfully demonstrated. Non-labeled amol-level amino acids is detectable with the TL detection system, which has thousand times better sensitivity than a conventional UV detector.

1. Introduction

Amino acids are not only the building blocks for the synthesis of proteins, peptides and other nitrogenous compounds, but key regulators of fluxes through major metabolic pathway [1]. Some free amino acids in serum are important indicators for metabolic disorders or physiological processes as biomarkers [2]. Therefore, amino acids analyses play important roles in life science, food chemistry, and pharmaceutical development.

Methods for determination of amino acids could be classified into two types: indirect and direct methods with and without derivatization, respectively. Since most amino acids have hydrophobic groups and absorption bands only in a deep ultraviolet (UV) range, the majority of the present methods are with a high performance liquid chromatography (HPLC) and by fluorescence detection after derivatization. Ninhydrin [3] and *o*-phthalaldehyde [4] are the most widely used derivating reagents. However, for non-fluorescent chemicals as amino acids, fluorescence detection requires an extra derivatization step, being time-consuming and potentially detrimental to separation and determination. Moreover, amino acids analyses require laborious sample preparation procedures when assaying biological samples such as blood or urine. Determination of amino acid in a biological matrix without derivatization is advantageous. It reduces laborious procedures and errors introduced by derivatives' instability, side reactions, and reagent interferences. Problems of assaying non-labeled amino acid are both in separation and detection, but it is widely accepted that the most difficulty is not in separation but in detection. Direct and highly sensitive detection methods for amino acids are eagerly required.

Thermal lensing (TL) detection is a promising alternative for ultrasensitive measurements of optical absorption of non-fluorescent chemical species in a small volume [5]. It detects a change in refractive index induced by local heating resulting from optical absorption of a laser beam (excitation beam) focused into the small volume of the sample medium. The light absorption basing TL technique inherits the non-labeled advantage of UV absorption detection. In a side-by-side comparison reported

by R. N. Zare *et al.*, TL shows 140 times better sensitivity than a state-of-art commercial UV-vis detector [6]. TL is well suited for the detection of analytes in short-path-length devices at ultralow cell volumes with no penalty in minimum detectable concentration. Kitamori *et al.* reported a limit of detection at 3σ level of 0.4 molecules in a 7 fL detection volume for a TL detection using an Ar⁺ laser (wavelength, 488 nm) [7]. To detect non-labeled amino acids in solution by TL measurements, excitation with an UV laser is essential. Recently, our group has successfully detected non-labeled amino acids with a home-built UV-excitation TL detection system using an UV-excitation beam at a wavelength ranging from 212 to 220 nm [8].

Thermal lensing detection has many applications in chemical analysis, including its combination with separation techniques such as ion chromatography [9], capillary electrophoresis [10], and isocratic HPLC [11]. However, TL detection has not been routinely used in combination with gradient HPLC, which could provide higher separation performance for a variety of chemical compounds. This is because TL signal is strongly dependent on physical properties of the eluent. During the gradient elution, the eluent composition is changing. Strong local gradients of refractive index caused by incomplete mixing increase the noise level of TL signal, and make detection limits worse [12]. A micro-HPLC system was developed recently, which has a small inner-diameter flow channel. Mobile phases after mixing quickly reach to be homogeneous in the micro-HPLC. It is expected that the micro-HPLC is the best equipment for gradient elution when combined with a TL detection system.

In this study, we combined the UV-excitation TL detection system to the micro-HPLC for separation and ultrasensitive detection of non-labeled amino acids. Separation-detection conditions are optimized of non-labeled amino acids, and direct determination of non-labeled amino acids with micro-HPLC/UV-excitation TL detection system is demonstrated.

1. Experimental

All experiments were conducted with a micro 21 capillary liquid chromatograph (JASCO). A reversed-phase (RP) micro-column, a micro 21 C18S (JASCO, 5 μ m particle size, 0.5 mm inner diameter x 150 mm length), was used. HPLC conditions were as follows: mobile phase, 30 mM phosphate buffer solution (pH 4.5) and acetonitrile; flow rate, 10 μ L/min; sample injection volumes, 1 μ L; column temperature, 20°C. The eluate exiting from the UV detector was connected to the flow cell in the TL detection system.

Figure 1 shows a scheme for the micro-HPLC/UV-excitation TL setup equipped with an UV-excitation beam of the 4-th harmonic of a Ti:sapphire laser (Coherent, Mira 900F). The 4-th harmonic generation system (Inrad, ultrafast harmonic generation system 5-050) produces UV beam by sequential doubling with a BBO (β -barium borate) crystal of the 2-nd harmonic generated with a LBO (lithium triborate) crystal. The excitation beam power is adjusted to 3.7 mW at 214 nm. The excitation beam was intensity-modulated at 100 Hz by a mechanical chopper (NF Electric Instruments, 5844) and was focused on the sample solution in a square capillary cell (Polymicro Technologies, WWP100375, inner side length, 100 μ m) with a convex lens (focal length, 100 mm). Photothermally generated heat in the sample solution was monitored with a diode laser (NEOARK, LDT-6820, wavelength 682 nm) used as the probe beam, which was focused with an objective lens ($\times 40$). The excitation and probe beams were crossed in the solution. A Faraday isolator reduced reflected light of the probe beam. Light intensity at the center of the probe beam after passing through the sample solution was monitored with a photo diode (Hamamatsu, C6386), and TL signal intensity was measured with a lock-in-amplifier (NF Electric Instruments,

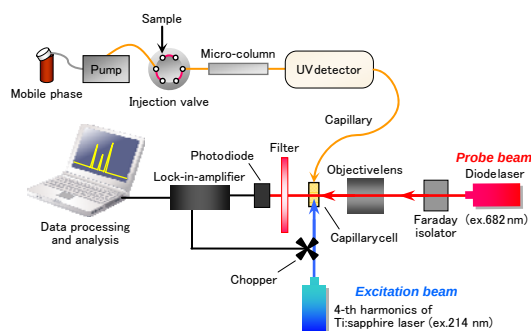


Figure 1. Experimental setup for detection of non-labeled amino acids with the micro-HPLC / UV-excitation TL.

5610B, time constant 300 ms).

UV-vis absorption spectra of the amino acid solutions and some solvents were measured with a UV-vis spectrophotometer (Shimadzu UV-2550).

Amino acids and HPLC grade acetonitrile were purchased from Kishida Regents Chemicals. Stock solutions of the amino acids were prepared in 10 mM HCl aqueous solution. All water used was freshly obtained from the Millipore System.

2. Results and discussion

2.1. Optimum chromatographic conditions

All amino acids absorb at UV wavelengths caused by carboxylic group. Signal-to-noise ratios (S/N) of the amino acids analysed were measured at 190, 210, and 266 nm. Wavelength 210 nm provided the best S/N for the mobile phase used in this study.

Figure 2 shows the relationship between the retention coefficient k and the volume fraction of acetonitrile in eluent. Roughly, the retention times were inversely proportional to the acetonitrile content. This is due to hydrophobic interaction of amino acids and the stationary phase. It was found that with 100% buffer solution, 6 amino acids peaks are allowed to be separated in RP chromatography. However, most amino acid tested among 20 amino acids in this study are not retained in the RP-column due to higher polar characteristics.

Figure 3 shows the RP-chromatogram of a mixture of 6 amino acids each having 0.1 to 30 mM concentrations separated and detected with micro-HPLC/UV detector. The detection wavelength for the UV absorption was 210 nm. A gradient elution scheme was used. Eluent A was the 100% phosphate buffer solution, and eluent B was 20% acetonitrile in the phosphate buffer solution. The 30 minutes binary gradient elution profile was as follows: $t = 0$ min, 0% B; $t = 5$ min, 0% B; $t = 15$ min, 100% B; $t = 30$ min, 100% B. Six non-labeled amino acids were obviously separated within 30 minutes. The limit of detection at 210 nm at 3σ level is 0.3 to 5 μ M for 5 amino acids (except for L-leucine), which corresponds to 0.3 to 5 pmol in the detection volumes of the UV detector (16 nL).

2.2. UV-excitation TL detection for micro-HPLC separation analyses

The UV-excitation TL system was applied to detect micro-HPLC separation optimized as described. The same of analytes was used except for L-leucine,

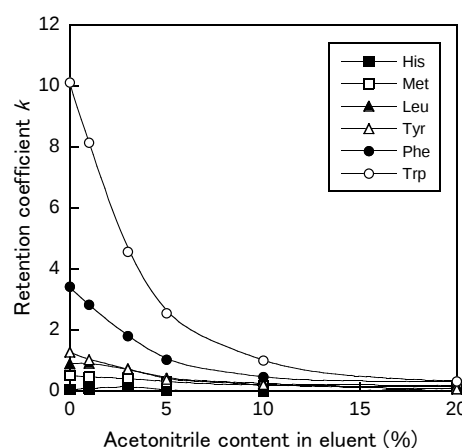


Figure 2. Effects of acetonitrile content in eluent on the retention coefficient of 6 amino acids.

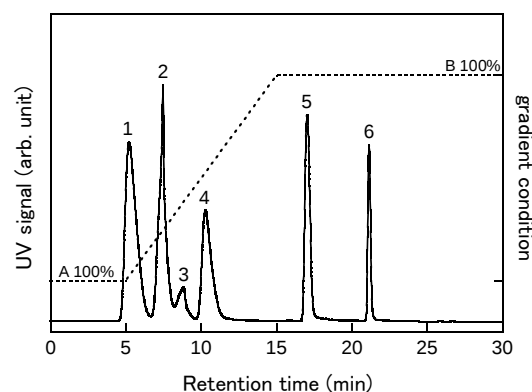


Figure 3. Separation of 6 non-labeled amino acids in a gradient elution using the UV detector. (1) L-histidine, (2) L-methionine, (3) L-leucine, (4) L-tyrosine, (5) L-phenylalanine, and (6) L-tryptophan.

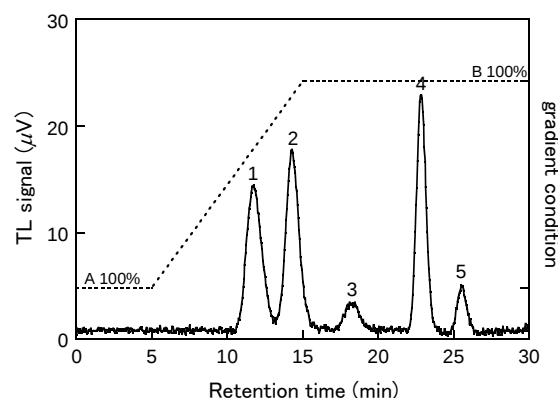


Figure 4. Separation of 5 non-labeled amino acids in a gradient elution using the TL detector. (1) L-histidine, (2) L-methionine, (3) L-tyrosine, (4) L-phenylalanine, and (5) L-tryptophan.

which has one of the lowest water-solubility among the 20 amino acids used.

Figure 4 shows the RP-chromatogram of a mixture of 5 amino acids each having 0.3 to 30 mM concentrations, detected with the UV-excitation TL detection system. The same gradient elution scheme was used as data in Fig. 3. The amino acids were separated and detected successfully. It was found that the difference of retention times for each sample between UV detector and UV-excitation TL detection system was 4 minutes. The time delay well corresponds to the dimension of the square capillary cell and the eluent flux. This is the first time demonstration of direct detection of these non-labeled amino acids with the UV-excitation TL detection and the micro-HPLC separation.

The micro-HPLC calibration curves were made for the peak areas of each of the 3 amino acids (L-histidine, L-phenylalanine, and L-tryptophan) in the concentration range from 0.1 to 30 mM for an isocratic elution with the mobile phase composed of 10% acetonitrile in phosphate buffer solution. Each curve is linear and has correlation coefficients R^2 of 0.999. The limit of detection at 214 nm at 3σ level is 0.2 to 2.6 μM for 3 amino acids, which corresponds to 8.1 to 120 amol in the detection volumes of the UV-excitation TL detection system (44 fL). Namely, the TL detection system has potential to detect non-labeled amino acids in an amol-level, meaning thousand times better sensitivity than the conventional UV detector. Similar performance is expected for a much smaller detection area, which opens a pathway to measure for micro- or nano-HPLC, and capillary electrophoresis, with no penalty in limits of detection.

It is well-known that TL signal is strongly dependent on physical properties of mobile phase such as thermal conductivity, and temperature derivative of the refractive index [13]. Most of organic solvents are advantageous in the TL detection. For example, acetonitrile is about 20 times superior to water as mobile phase. It is noted that hydrophilic interaction chromatography (HILIC) uses a high percentage of organic solvents. Attentions of many chromatographers have been focused on HILIC in these years, because the separation mode of HILIC is useful for highly polar analytes such as amino acids, nucleotides, neurotransmitters, pharmaceutical drugs, and so on. Sensitivity improvement is expected by using HILIC column in the micro-HPLC with UV-excitation TL detection system.

3. Conclusions

For direct determination of non-labeled amino acids, the use of the micro-HPLC equipped with UV-excitation TL detection system is examined. It was found that 5 amino acids were obviously separated within 30 minutes in gradient elution and detected with TL detection. The limit of detection at 214 nm at 3σ level is 0.2 to 2.6 μM for 3 amino acids, which corresponds to 8.1 to 120 amol in the detection cell volumes (44 fL).

With a high performance deep UV laser source, the UV-excitation TL detection system combined with a micro-HPLC becomes a promising method for direct separation analysis of a mixture of non- and less-fluorescent chemicals.

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