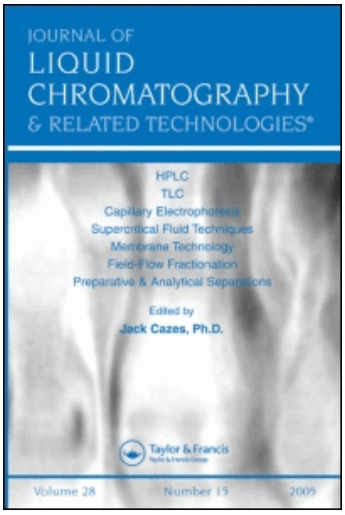

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PULSED UV LASER-INDUCED FLUORESCENCE DETECTION OF NATIVE PEPTIDES AND PROTEINS IN CAPILLARY ELECTROPHORESIS

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ABSTRACT

A pulsed UV laser operating at 248 nm was used for the laser-induced fluorescence (LIF) detection of native tryptophan-containing compounds in capillary electrophoresis. The limit of detection (LOD) of tryptophan was found to be 3.3×10^{-9} M (S/N=2). The LODs of the model proteins conalbumin and bovine serum albumin were found to be 1.3×10^{-9} and 4×10^{-9} M (S/N=2), respectively. These results were at least two orders of magnitude more sensitive when compared to UV absorption at 214 nm.

INTRODUCTION

Capillary electrophoresis (CE) is a rapid and efficient separation technique for small and large molecules (1). Because a nanoliter or less of the sample's volume is usually injected in a typical CE experiment, on-column detection is often used to avoid extracolumn band broadening. UV absorption is by far the most widely used on-column detection mode because of its applicability and simplicity, but its sensitivity is limited due to the short detector optical pathlength. Higher sensitivity can be obtained by using electrochemical or fluorescence detection. In particular, laser-induced fluorescence (LIF) is one of the most sensitive detection modes available today. With pre-column derivatization, LIF offers highly sensitive detection for various biological molecules such as amino acids, small peptides, and carbohydrates. However, this procedure is generally not desirable for proteins because of the formation of multi-labeled products (2). In addition, it is difficult to label analytes that have a low concentration. The biological activity of proteins can also be lost during the labeling process. Alternatively, proteins can be accurately detected via the intrinsic fluorescence of tryptophan, tyrosine, and phenylalanine units (3). Swaile and Sepaniak (4) first demonstrated the feasibility of detecting native proteins in CE

using LIF. In their work, excitation of native proteins was provided by an argon ion laser (514 nm) that was frequency doubled to 257 nm. Recently, Lee and Yeung (5) obtained higher sensitivity in a similar study with a CW laser operating at 275 nm. This improvement in sensitivity is mainly attributed to the fact that 275 nm is the optimum excitation wavelength for detecting tryptophan and tyrosine units. The results obtained by Lee and Yeung were very impressive, but the implementation of their LIF system in most laboratories can be a problem due to the high cost and large size of the CW UV laser. In this study, our primary objective was to evaluate the feasibility of applying a relatively economical, compact, pulsed UV laser operating at 248 nm in CE for the LIF detection of native tryptophan-containing peptides and proteins.

EXPERIMENTAL

Separation with absorption detection was carried out with a Beckman P/ACE CE system. For LIF detection, a home-built CE system was used (Figure 1). Excitation was provided by a 12 mW, pulsed UV laser operating at 248 nm (#GX-500, Potomac Photonics, Lanham, MD). After passing through the line filter centered at 250 nm

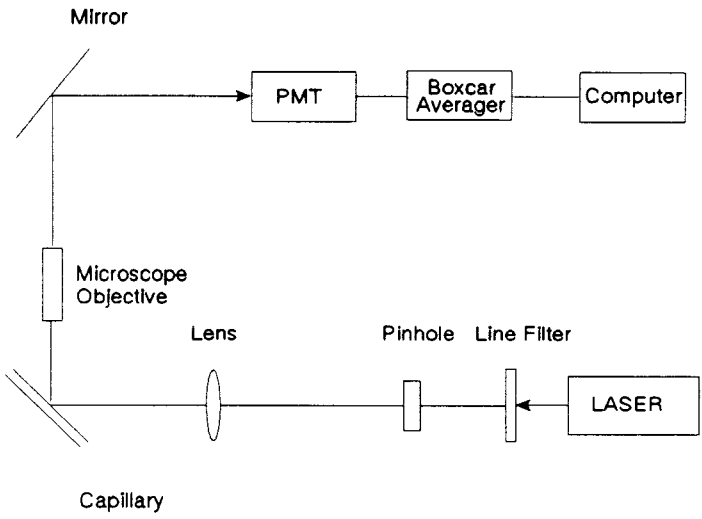

Figure 1. A sketch of the LIF CE system.

(#250-S-1D, Acton Research Corp., Acton, MA) and a pinhole, the laser beam was focused onto a capillary with a fused-silica, bi-convex lens (#SBX016, Newport, Irvine, CA). The capillary was mounted on an X-Y translational stage for precise movement. The typical laser pulse repetition rate was 2000 Hz, and about 2 mW of laser power was used for excitation. Fluorescence was collected at 90° from the excitation beam with a conventional 20X microscope objective. The collected emission was then directed by a mirror to a photomultiplier tube (PMT, #70680, Oriel, Stratford, CT) for detection. The PMT was equipped

with a filter holder that allowed a quick change of the desired filters. Emission filters UG1 or UG11 (Melles Griot, Irvine, CA) were used for tryptophan-containing compounds. To correct for the laser power pulse-to-pulse fluctuation, the current output of the PMT was fed into a boxcar integrator (#4100, EG&G, Princeton, NJ) for signal averaging. Typically, 100 samples were averaged in most of our experiments. The averaged signals were digitized, displayed, and then stored in an IBM PS/2 computer via a Beckman 406 analog interface.

All separations were performed with 75 μm capillaries which were obtained from Polymicro Technologies Inc. (Phoenix, AZ). A detection window was created by burning off a small portion of the capillary coating. The positive power supply for electrophoresis was obtained from Glassman High Voltage Inc. (Whitehouse Station, NJ). Tryptophan, tyrosine, and phenylalanine were obtained from Aldrich (Milwaukee, WI). Bovine serum albumin (BSA) and conalbumin were obtained from Boehringer Mannheim (Indianapolis, IN) and Sigma (St. Louis, MO), respectively. Dipeptides were obtained from United States Biochemical (Cleveland, OH). All other chemicals were reagent-grade and were obtained from Sigma. Gravity injection was performed by raising the injection end about 10 cm for 15 s. Limits of detection

(LOD) were determined using peak heights. Detailed running conditions are listed in the figure captions.

RESULTS AND DISCUSSION

The sensitivity of a fluorescence detector can be evaluated by the detection of an injected fluorescence dye such as sodium fluorescein. Although the optimum excitation wavelength of fluorescein is 488 nm, we achieved a reasonable detection for this dye using a pulsed UV laser at 248 nm as shown in Figure 2. The limit of detection (LOD) was estimated to be $1x10^{-11}$ M (S/N=2). This was about one order of magnitude higher than that typically obtained in conventional LIF detection using an argon laser.

The native fluorescence of peptides and proteins, when excited by UV light between 230-300 nm, is determined by the three aromatic amino acids, tryptophan, tyrosine, and phenylalanine, while tryptophan is the dominant factor (3). Figures 3 and 4 are electropherograms of these three amino acids. When it is compared to tryptophan under the indicated experimental conditions, the fluorescence intensity of tyrosine is about 100 times less, while that of phenylalanine is negligible. The extremely weak fluorescence signal of phenylalanine is due to its small

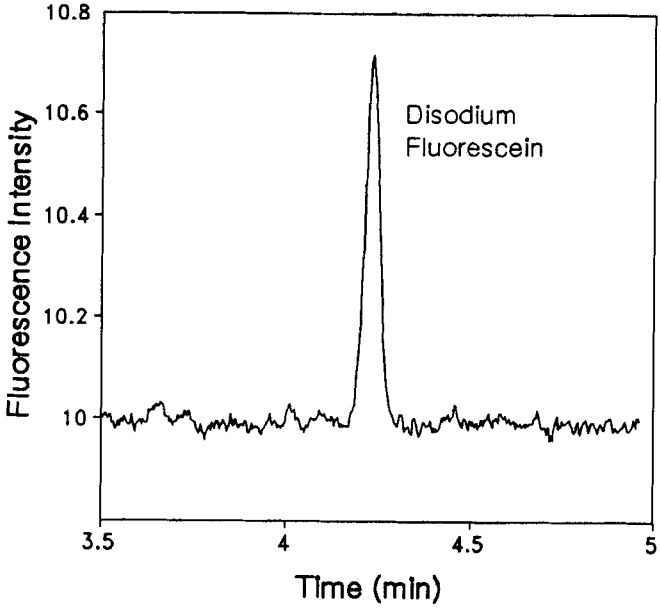

Figure 2. Electropherogram of sodium fluorescein with LIF detection. Buffer: 5 mM sodium phosphate (pH 10). Capillary: 75 μm i.d. x 70 cm total length, 60 cm effective length. Applied voltage: 25 KV. Sample: $1x10^{-10}$ M fluorescein. Injection: 15 s, gravity.

absorption and its small quantum yield. Using tyrosine as the internal standard, a linear calibration curve for tryptophan (peak height) was obtained between $1x10^{-8}$ and $1x10^{-5}$ M with R = 0.9989. From Figure 3, the LOD of tryptophan was estimated to be $3.3x10^{-9}$ M (S/N=2).

Figure 5 shows the electropherogram for the separation of the dipeptides, Gly-Trp, Trp-Ile, and Trp-Trp. The fluorescence signals of these molecules were not proportional to the number of tryptophan units

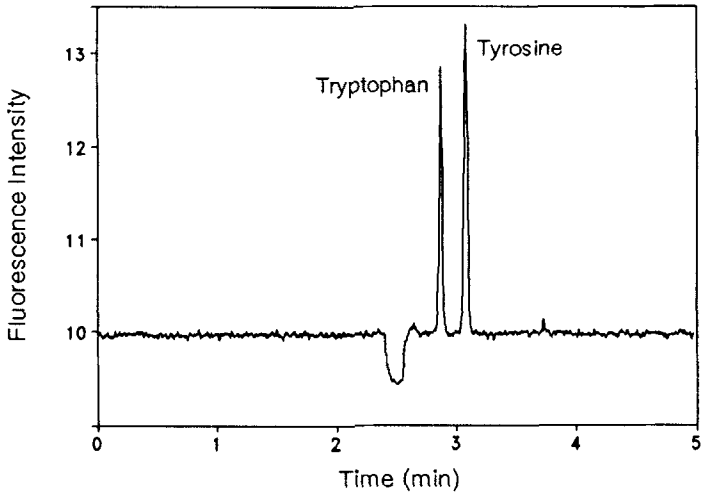

Figure 3. Electropherogram of tryptophan and tyrosine with LIF detection. Buffer: 5 mM sodium borate (pH 9.1). Capillary: 75 μm i.d. x 70 cm total length, 60 cm effective length. Applied voltage: 25 KV. Samples: $5x10^{-8}$ tryptophan and $5x10^{-6}$ M tyrosine. Injection: 15 s, gravity.

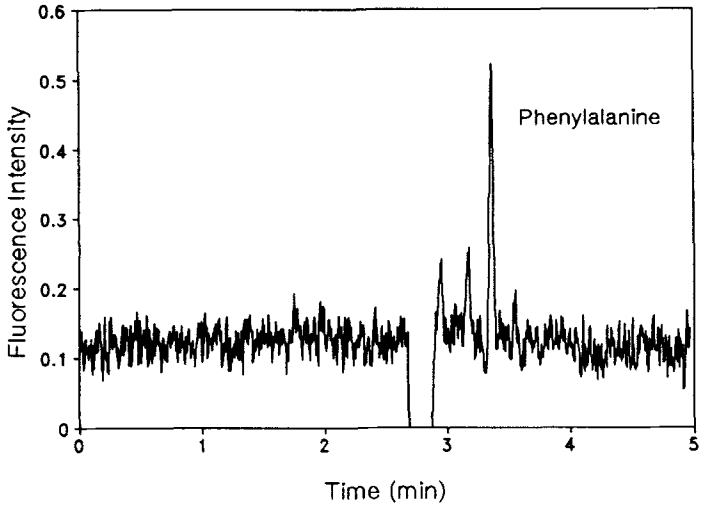

Figure 4. Electropherogram of phenylalanine with LIF detection. Same running conditions as in Fig. 3. Sample: $1x10^{-4}$ M phenylalanine.

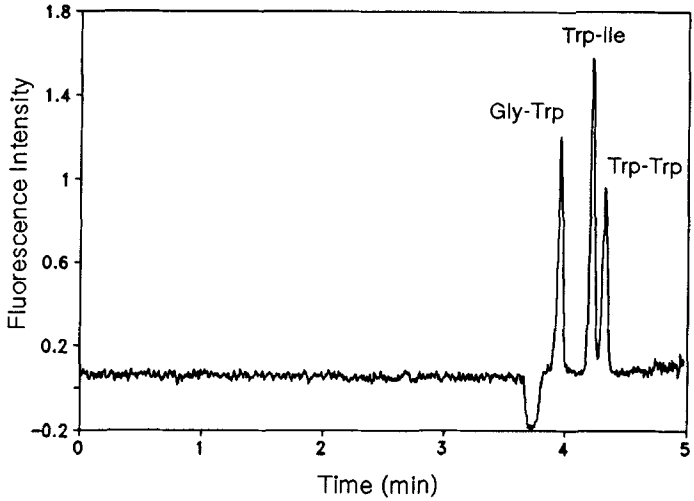

Figure 5. Electropherogram of dipeptides with LIF detection. Buffer: 10 mM sodium phosphate (pH 7.0). Capillary: 75 μm i.d. x 65 cm total length, 55 cm effective length. Applied voltage: 23 KV. Samples: 2.1×10^{-7} M Gly-Trp, 8.1×10^{-8} M Trp-Ile, and 6.9×10^{-8} M Trp-Trp. Injection: 15 s, gravity.

found within the molecules. We measured the molar absorptivities of these dipeptides at 248 nm and found that their values were, in this case, proportional to the number of tryptophan units. The differences in fluorescence of these tryptophan derivatives were probably due to their different fluorescence quantum yields (6).

Most CE separations to date use UV absorption detection, which has wide application but is not very sensitive. The optical pathlength can be increased by using a wide i.d. capillary to maximize sensitivity

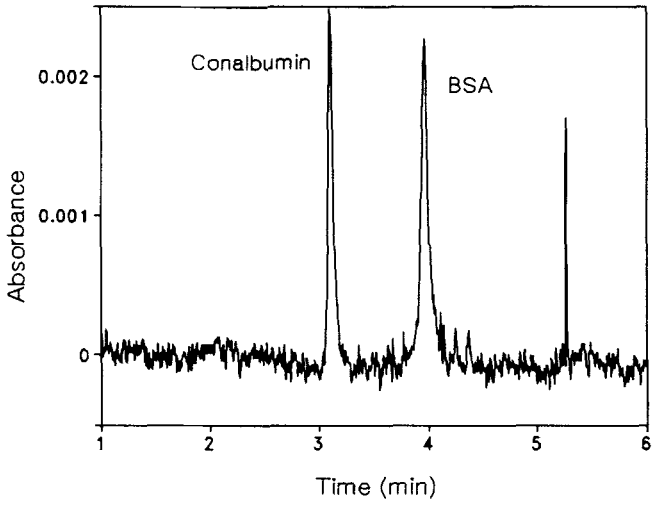

Figure 6. Electropherogram of proteins with UV absorption detection at 214 nm. Capillary: 75 μm i.d. x 57 cm total length. Applied voltage: 22 KV. Samples: 1.3×10^{-6} M conalbumin, 1.5×10^{-6} M BSA. Injection: 3 s. pressure.

but this will also degrade separation efficiency due to Joule's heating. Figures 6 and 7 are the electropherograms for the separation of native conalbumin and BSA with UV absorption (214 nm) and LIF detection, respectively. It is clear from the figures that native fluorescence is at least 100 times more sensitive than the absorption detection. With LIF, the LODs of conalbumin and BSA were found to be 1.3×10^{-9} and 4×10^{-9} M, respectively. These results were not linearly related to the number of tryptophan within the molecules of these proteins, although conalbumin and BSA contain 15 (7) and 2 tryptophan units (8), respec-

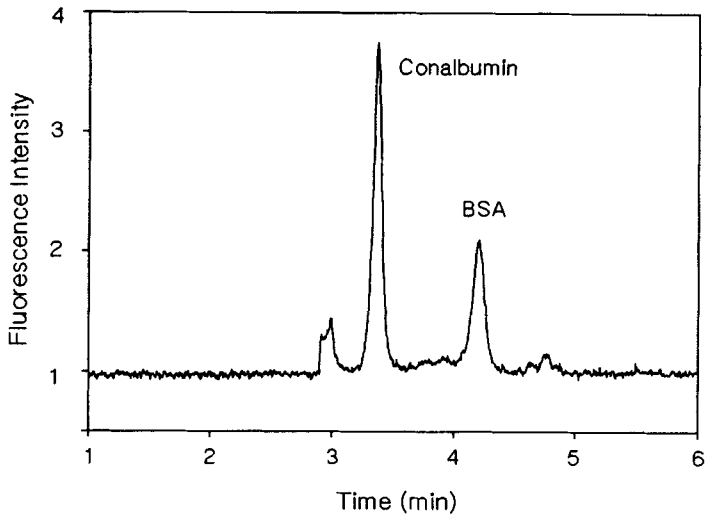

Figure 7. Electropherogram of proteins with LIF detection. Same running conditions as in Fig. 3. Sample: 2.5×10^{-8} M conalbumin, 3.0×10^{-8} M BSA.

tively. This observation has also been previously reported (5). The nonlinearity in detection may be due to several reasons such as the structure of proteins (9), different excitation and fluorescence properties between tryptophan and proteins (10), the number of exposed tryptophan units and the micro-environment of proteins (11), and a sharper tryptophan peak in comparison with those of proteins. The LODs of proteins obtained in this work are about 13 (conalbumin) and 20 (BSA) times higher than those obtained using a 275 nm CW laser (5), although the LOD of tryptophan found in this study is comparable to that

reported in reference 5. This difference is probably due to the fact that at 275 nm, the excitation of proteins is more efficient than the excitation of tryptophan. In addition, the emission of tyrosine (20 units for both proteins) may become significant at 275 nm; this may indirectly increase the tryptophan emission via energy transfer (11). Nevertheless, the LIF detection system described here should provide adequate sensitivity for many applications. In addition to peptides and proteins, we are also investigating the feasibility of applying our LIF CE system for the analysis of other molecules such as polycyclic aromatic hydrocarbons and their DNA adducts (12,13).

Because of the large divergence of the laser beam profile, we found that focusing the pulsed laser could be difficult for capillaries with an i.d. smaller than 75 μm. In this case, it may be necessary to collimate the laser beam before focusing. As mentioned above, a boxcar integrator was used in this work to correct for the laser power pulse-to-pulse fluctuation. Alternatively, this procedure can be performed using high-speed data acquisition with computer smoothing. However, a boxcar integrator will also allow one to perform gated detection (14).

CONCLUSIONS

This study demonstrates that it is feasible to use a pulsed UV laser operating at 248 nm for the LIF detection of native tryptophan-containing peptides and proteins in capillary electrophoresis. Although the wavelength (248 nm) is not optimum for the excitation of tryptophan molecules, native conalbumin and BSA were detected in the nm range. This represents an improvement in sensitivity that is at least two orders of magnitude higher than absorption detection at 214 nm. Because of the large divergence of the beam profile, collimating the laser beam may be required for effective focusing onto capillaries of less than 75 μm i.d. Finally, adequate sensitivity, compactness, and the low cost of this pulsed laser make it attractive for many applications.

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