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# Capillary zone electrophoresis with time-resolved fluorescence detection using a diode-pumped solid-state laser

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ABSTRACT

A capillary electrophoresis system with time-resolved fluorescence detection was developed using a diode-pumped solid-state laser. The laser provides 2.5-ns pulses at 349 nm with kHz repetition rates, and the Gaussian-shaped beam can easily be focused to the dimensions of a capillary. The short pulse width makes it possible to achieve nanosecond resolution and time-filtered detection. With this approach, the total signal from one injection can be collected and digitally processed to yield simultaneously "two-channel" data. Instrument capability is demonstrated by resolving short-lived fluorescent interferences in biological sample matrixes from long-lived fluorophores.

## INTRODUCTION

Due to high separation efficiency and low sample volume requirements, capillary zone electrophoresis (CZE) has become an established analytical tool in the decade since its introduction [1]. Among the many detection methods used in CZE, laser-induced fluorescence (LIF) has achieved some of the best detection levels. As examples, Yeung *et al.* [2] recently demonstrated a value of 3 p*M* for fluorescein, Wu and Dovichi [3] reported a value of 1.3 p*M* fluorescein isothiocyanate-labeled arginine, and Chen *et al.* [4] constructed a linear calibration curve down to 64 p*M* tetramethylrhodamine isothiocyanate. Additionally, the excellent detectivity of LIF has been used to study the chemical content of individual cells [5].

Attaining low detection limits in capillaries with diameter of 100 $\mu$m and smaller presents some unique challenges. It is necessary to not only maximize the collection of the desired signal, but to concomitantly minimize unwanted radiation. Scatter from capillary walls is potentially the largest source of interference. In CZE instruments with LIF detection, the wavelength selection optics are most often comprised of spectral filters to maximize the fluorescence collection efficiency. While highly efficient, spectral filters are often incapable of fully rejecting light at the laser wavelength. As a result, much of the optical design of LIF detectors in CZE systems is geared toward discrimination against scatter. To enhance the performance of spectral filters, a wide variety of spatial filtering or isolation techniques have been used. Sheath-flow cuvettes [3,4] can be used for post-capillary detection and serve to distance the walls of the detection cell from the analyte. Yeung *et al.* [2] place the capillary at 20° with respect to the laser beam to reduce specular reflections. The capillary can also be placed at Brewster's angle to reduce scatter produced at the air–capillary

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interface [6]. Judicious choice of capillary inner and outer diameters may also be important [7].

In addition to spectral and spatial filtering, it has been observed that temporal resolution could provide a third approach to the reduction of background signal in CZE [3,8]. Indeed, time-resolved fluorescence detection has been applied to HPLC for over a decade [9–17]. This strategy has been used to discriminate against background signals [9–12,15–17]. Additionally, the lifetime of the eluent has been employed as an aid to identify peaks [10,12–15]. All but one of the instruments were constructed with either a nitrogen laser or a nitrogen-pumped dye laser. Ref. 17 used a phase fluorometer based on a xenon arc lamp.

A significant difficulty in adapting temporal resolution of CZE has been the lack of pulsed lasers that are both easily focused and user-friendly. None of the sources used in the liquid chromatography studies fit these criteria. Recently a prototype diode-pumped, frequency-tripled, Q-switched neodymium doped yttrium lithium fluoride (Nd:YLF) laser has been developed [18]. This device produces 2.5-ns pulses at 349 nm. The Gaussian-shaped beam ($TEM_{00}$) can easily be focused to the dimensions of a capillary. The solid-state technology makes operation as simple as turning a key, and the diode provides thousands of hours of use without maintenance. The repetition rate of the laser can be adjusted from 200 Hz to 3 KHz with a maximum average power of 1.9 mW occurring at 2 kHz.

A second limitation to the liquid chromatographic detectors was the universal use of a gated integrator, be it a boxcar integrator of sampling oscilloscope. A much more flexible approach would employ a transient recorder, since it permits software data processing [19]. Examples of possible processing software modules are those emulating oscilloscope presentation, boxcar integration, and photon counting. Additionally, the same data can be reprocessed using an alternative emulation if the first choice does not provide the needed information. An ideal match to the above mentioned ND:YLF laser is a commercially available digital oscilloscope capable of capturing waveforms at 1 Gsample/s and averaging at repetition rates as high as 8 kHz.

In this paper we demonstrate the use of time-resolved fluorescence detection for CZE using the Nd:YLF laser–digital oscilloscope combination. Two thousand fluorescence decays are averaged with the oscilloscope and transferred, via an IEEE-488 bus, to a personal computer. Software data processing permits the construction of two electropherograms from two independently adjustable integration apertures (temporal filters). Performance of the instrument is demonstrated by injection of several biochemically related samples where short-lived interferences are separated from long-lived analytes.

## EXPERIMENTAL

### *Materials*

4-Methoxy-$\beta$-naphthylamine (4-MBNA) and *l*-isoleucine were obtained from Sigma (St. Louis, MO, USA). 2-Dimethylaminonaphthalene-5-sulfonyl chloride (2,5-Dns-Cl) was obtained from Molecular Probes (Eugene, OR, USA) and used without purification. The supernatant (10 000 *g*) from *E. coli* strain BL21 homogenate was donated by Cheng Chang Wang (Purdue University). Human urine was obtained from a healthy male donor. The *E. coli* supernatant and urine were diluted as needed in the electrophoresis buffer.

The isoleucine was derivatized with 2,5-Dns-Cl using conditions similar to those by Tapuhi *et al.* [20] for the dansylation of amino acids. The 2,5-Dns-Cl solution was prepared to 0.5 m*M* in acetonitrile. Isoleucine was dissolved in 40 m*M* $LiCO_3$ adjusted to pH 9.5 with HCl. The isoleucine concentration was 5 m*M*. An aliquot of 200 $\mu$l of the 2,5-Dns-Cl solution was added to 200 $\mu$l of the isoleucine solution. The reaction mixture was agitated for 2 min and incubated for 1 h protected from room light. No reaction terminator was used. The sample contained detectable amounts of 2 fluorescent impurities, believed to be 2,5-Dns-OH and 2,5-Dns-$NH_2$ [20]. The large excess of amino acid used in the derivatization was found to reduce the formation of the 2,5-Dns-OH impurity.

*CZE instrumentation*

The laser, optics and capillary were placed in a plexiglass box painted flat black on the interior. The door of the box was interlocked to the high-voltage power supply. The box protected the collection optics from room light and protected the user from the high voltage. The highly compact ($30 \times 30 \times 14$ cm) laser was placed in a lower compartment of the plexiglass box below the optics with the laser power supply placed outside the box. The laser repetition rate was set to 2 kHz. The laser is described in the Introduction section and in ref. 18. Fig. 1 shows a block diagram of the instrument. Ultraviolet mirrors were used to direct the beam to a quartz 1.6-mm focal length plano convex lens. The lens focused the laser beam into the capillary. Fluorescence was collected 90° from the excitation beam using a 20× (0.40 numerical aperture) microscope objective. Sample fluorescence was isolated from scatter using an iris, a Corion (Holliston, MA, USA) 450-nm bandpass 40-nm bandwidth interference filter and a Schott (Duryea, PA, USA) KV 399-nm cut-on filter.

The fluorescence signal was detected with a Burle (Lancaster, PA, USA) 931A photomultiplier wired for fast response [21] and biased at −1200 V. A trigger signal was generated by using a quartz window to direct *ca.* 4% of the laser beam to a photodiode. The photomultiplier anode current was monitored by an Analytek (Sunnyvale, CA, USA) Series 2000B digital oscilloscope operated at 1 Gsample/s. Two thousand fluorescence decay waveforms of 200 points each were averaged by the oscilloscope. The averaged waveform was transferred to an IBM PS/2 via an IEEE-488 interface card. A program was written in Microsoft Quick-C to collect and process each averaged waveform.

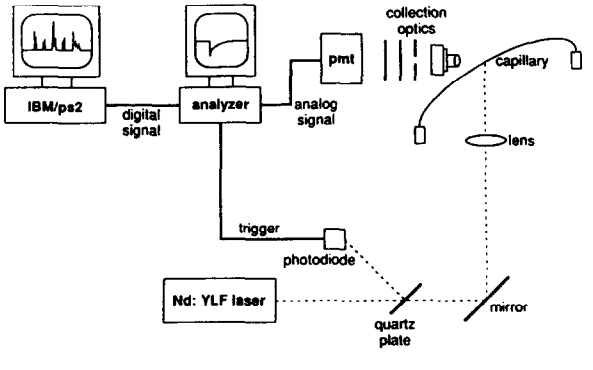


Fig. 1. Block diagram of CZE instrument. Collection optics are described in text. pmt = Photomultiplier tube.

The digitized points from each averaged waveform within the given user-specified integration apertures were summed. The successive sums were saved in an output file along with the elapsed time to create two electropherograms. The successive sums were also plotted to the screen in real time in order to view the peaks as they eluted.

Potential across the capillary was generated using a Glassman (Whitehouse Station, NJ, USA) Series EL high-voltage power supply. The power supply was modified to include the interlock. Polyimide-coated fused-silica capillaries (360 μm O.D. × 75 μm I.D.) were purchased from Polymicro Technologies (Phoenix, AZ, USA). Capillaries were cleaned prior to use by the procedure described by Yeung *et al.* [2].

*CZE conditions*

A 60-cm capillary was used (50 cm, injection end to detector.) The electrophoresis buffer was 10 m*M* pH 7 sodium phosphate buffer. The electrophoresis potential was 250 V/cm. Samples were injected by raising the sample vial 10 cm above the grounded reservoir for 10 s.

## RESULTS AND DISCUSSION

Several difficulties associated with using pulsed lasers in CZE have been discussed in earlier papers [2,3]. Specific problems mentioned are poor spatial quality, fluorescence saturation, photodegradation of the sample, capillary damage, and poor intensity stability. The diode-driven Nd:YLF laser used in this study avoids all but one of these problems. The Gaussian-shaped beam profile ($TEM_{00}$) easily focusses to a spot size smaller than a 50 μm I.D. capillary. The fluorescent molecules, 4-MBNA and 2,5-Dns-isoleucine, did not show any measurable saturation or photolysis on the chromatographic time scale. This fact was obtained by observing that the fluorescence intensity is proportional to average power as the repetition rate is varied.

Because of the low Joules per pulse, capillary damage has never been observed. Intensity stability has been a significant problem with the prototype Nd:YLF laser. Pulse-to-pulse power variation is high at 18% relative standard deviation. However, when 1000 pulses are averaged, the value is reduced to 1.96%.

Fluorescence decays for the buffer (background signal) and 4-MBNA within the capillary are shown in Fig. 2. The data shown are the average of 2000 waveforms collected at 2 kHz. The blank has a full width at half maximum (FWHM) of 8 ns. This seemingly large value is caused by convolution of the laser pulse, photomultiplier impulse response, oscilloscope rise-time and the fluorescence decays of the capillary and buffer impurities. Due to the width of the blank, fluorophores with lifetimes greater than 16 ns are best suited for quantitation by temporal resolution. Note the lack of radio-frequency noise in the decays, which is an attribute of the low voltages used to drive all laser components.

4-MBNA is a fluorophore commonly used to assay aminopeptidase enzymes. It has an absorbance that is well-matched to the laser ($\lambda_{max} = 338$ nm) but with a marginal molar absorptivity (1060 l cm$^{-1}$ mol$^{-1}$ at 349 nm). The compound is ideal for time-resolved, fluorescence-based aminopeptidase assays primary for its long lifetime (26 ns), and because it is commercially available both as a pure compound and conjugated with several amino acids. The CZE detection limit ($S/N = 3$) obtained for 4-MBNA was $10^{-9}$ $M$, with 1 s of averaging at 2 kHz.

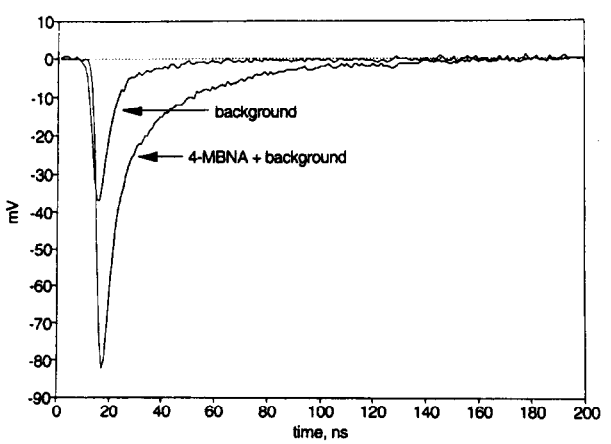


Fig. 2. Fluorescence decay waveforms for $10^{-7}$ $M$ 4-MBNA and the background, collected from within capillary.

2,5-Dns-Cl, an isomer of the more common reagent dansyl chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride), can be used to assay for amino acids. Conjugates formed with this reagent also have long lifetimes, *e.g.* 24 ns for isoleucine. The absorbance maximum for the isoleucine conjugate ($\lambda_{max} = 359$) is also well matched to the Nd:YLF output. This conjugate also has a low molar absorptivity (3100 l cm$^{-1}$ mol$^{-1}$ at 349 nm). The CZE detection limit obtained for 2,5-Dns-Cl was comparable to that for 4-MBNA. The low purity of the preparation precludes giving a more definitive value.

The poorer detection limits reported above, as compared to the picomolar values mentioned earlier, is attributed almost entirely to the smaller molar absorptivities. Fluorescein, fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate have absorptivities of 90 000 [22], 76 000 [22] and 85 000 [4] l cm$^{-1}$ mol$^{-1}$, respectively. Unfortunately, these compounds cannot be successfully employed in measurements that involve temporal solution because of short lifetimes at a pH of *ca.* 7, *i.e.* 4.65 ns [23], 3.5 ns [24], and 2.5 ns [24], respectively.

The major advantage of time-resolved detection is the ability to separate short-lived from long-lived components. Biological samples have considerable short-lived fluorescence caused by the native emission from aromatic moieties. The examples used in this feasibility study are urine and *E. coli* supernatant. Fig. 3 shows two electropherograms of a dilute urine sample. Both were simultaneously generated from the same injection using different temporal delays from the excitation maximum, and different integration windows. In the lower electropherogram, generated using a 60-ns time delay, all of the peaks are considerably smaller in amplitude, indicating most of the native fluorescence from the urine has decayed after 60 ns. In Fig. 4 the urine sample has been spiked with $5 \cdot 10^{-7}$ $M$ 2,5-Dns-isoleucine. In the upper electropherogram it can be seen that the 2,5-Dns-isoleucine is poorly resolved from one of the components of the urine sample. In the lower electrophero-

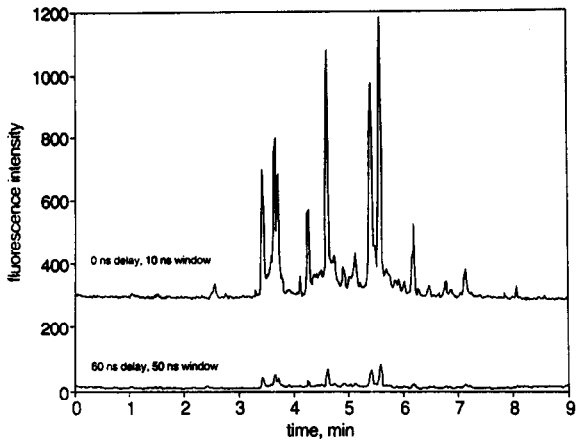


Fig. 3. Electropherograms for human urine sample diluted 1:200. Data were collected simultaneously using different integration delays and windows. Data have been offset for clarity.

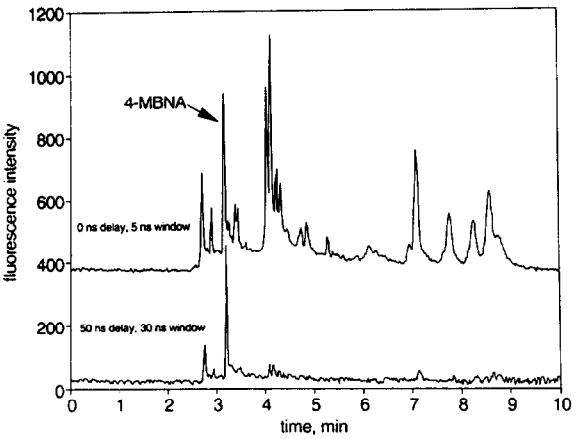


Fig. 5. Electropherograms for *E. coli* supernatant diluted 1:50. Sample was spiked with $3 \cdot 10^{-7}$ *M* 4-MBNA. Data were collected simultaneously using different integration delays and windows. Data have been offset for clarity

gram, collected with a 60-ns time delay, the 2,5-Dns-isoleucine peak has been attenuated less than the native fluorescence peaks. The signal from the interfering peak has been reduced to the extent that it is no longer visible. Similarly, in Fig. 5, a sample of *E. coli* supernatant has been spiked with $3 \cdot 10^{-7}$ *M* 4-MBNA. In the upper electropherogram, collected with no time-delay and a 5-ns integration window, many sample components exhibit native fluorescence, while in the lower electropherogram, the native fluorescence of the sample has been greatly reduced and the 4-MBNA peak is the most prominent feature.

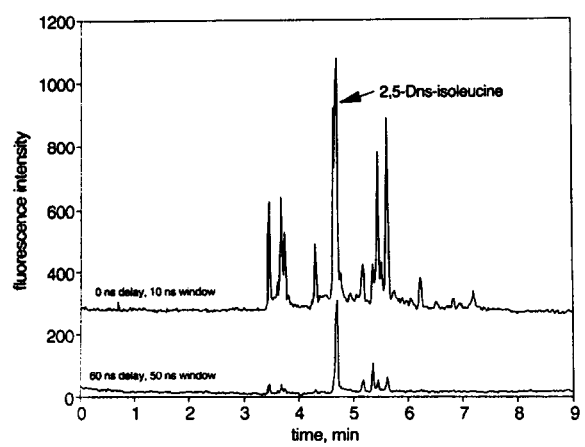


Fig. 4. Electropherograms for human urine sample diluted 1:200 spiked with $5 \cdot 10^{-7}$ *M* 2,5-Dns-isoleucine. Data were collected simultaneously using different integration delays and windows. Data have been offset for clarity.

Future work with this instrument will involve enzyme assays in biological samples, similar to a CZE-based, absorptiometric approach already reported [25]. Our group has a long-standing interest in the identification of bacterial pathogens using aminopeptidase profiling [26,27]. The profile is obtained by measuring enzymatic activity for a suite of amino acids by the hydrolysis of their non-fluorescent, 4-MBNA substrates to produce free 4-MBNA. Converting the published macroscopic analyses into one utilizing CZE should permit identification of fewer than 1000 cells.

The present instrument suffers in two areas. The 18% relative standard deviation associated with the pulse energy appears to be characteristic of the yttrium lithium fluoride (YLF) host crystal. A second generation prototype utilizing a yttrium vanadate host crystal has been built by Continuum. The result was a 1.5% relative standard deviation in the pulse fluctions. Any commercialized version of the diode-driven laser will employ the yttrium vanadate host. This change alone will produce lower detection limits since less noise will need to be averaged. The second change involves switching to OS/2 version 2.0 which permits 32-bit addresses in the C-language. With the attendant ability to com-

pile large data arrays, the entire ensemble of decays transferred to the computer can be stored in memory. Once in memory, post-processing can be used to fine-tune window positions and widths. Additionally, selected data could be processed by non-linear curve fitting routines to deconvolve overlapped peaks. Our ultimate goal is to use the SCSI port of the Analytek digitizer to transfer each decay in real-time to the computer. This will permit much more sophisticated data processing options such as photon counting [19] and computing the fluorescence lifetime of each peak as it elutes.

Future work will also focus on chemically reducing the lower limit of detection (LLD). To some extent this involves conflicting demands upon the fluorescent molecule, *i.e.* long lifetimes are ordinarily produced by transitions with low molar absorptivities. In an attempt to circumvent the problem we are currently searching for classes of molecules that have large molar absorptivities associated with the second, or higher, excited singlet states. This change in strategy should produce a much greater sensitivity combined with a lifetime sufficiently long for temporal rejection of the background interferences. Since the laser wavelength is fixed, an exhaustive examination of many compounds is anticipated.

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