# Nanoliter Volume Kinetic Assays

Yi-Ming Liu and Jonathan V. Sweedler*

*Department of Chemistry*
*University of Illinois at Urbana–Champaign*
*600 South Mathews Avenue, Urbana, Illinois 61801*

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Determining kinetic parameters from ultrasmall-volume, complex samples such as exploring small-volume chemical reactions and single-cell dynamics is difficult because current methodology does not lend itself to the nanoliter volume range.[1] The major techniques used today are spectroscopic methods[2,3] and miniaturized electrochemical sensors.[4–6] Although sensors of high selectivity exist, specific sensors are not generally available for many compounds of biochemical importance. In addition, sensors usually do not provide the ability to monitor multiple compounds simultaneously.[7] The ideal analytical method should monitor multiple chemical species in real time with chemical specificity in small-volume, complex matrices. Obviously, such a method does not yet exist.

As an approach to gaining "snapshots" of the chemical constituents of a small sample, various microseparations provide detailed compositional information but little temporal information. We present a unique format for a kinetic assay that provides species as well as dynamic (time-based) information from nanoliter volume samples. To demonstrate the performance of this system, amino acids fluorescently labeled with two common reagents, fluorescein isothiocyanate (FITC) and naphthlene-2,3-dicarboxaldehyde (NDA),[8] are separated and detected. Continuous monitoring of multiple fluorescent product formation from a 200 nL sample is demonstrated, allowing rate constants to be obtained for each unique fluorescent product.

Capillary electrophoresis (CE) is an important separation technique for the analysis of a wide variety of complex mixtures, especially as a complementary method to high-performance liquid chromatography (HPLC).[9,10] Although conventional CE allows snapshots of the chemical composition of nanoliter volume samples, it cannot sample material continuously. A method that permits continuous separation and temporal resolution of samples is of great interest in a number of areas and is performed here using a thin rectangular channel.[11] Electrophoresis using rectangular cross sections has been investigated previously because of improved heat dissipation and the optical path length advantage of this geometry.[12–14] Smith et al.[15,16] have developed gel-filled 25–100 $\mu$m thick rectangular channels for automated DNA sequencing, and Ewing et al.[17,18] described continuous electrophoretic separations of dansylated amino acids in narrow channels coupled to small-bore capillaries.

The major goal of the present work is the ability to obtain kinetic information from nanoliter volume samples. The channel width dimension functions as a time axis by moving the outlet end of a sampling capillary across the entrance to the rectangular separation channel. As shown in Figure 1, the simple-to-assemble channel is made from standard microscope slides and an arc lamp excitation source.[11] The movement of the sampling capillary along the rectangular channel entrance is precisely and reproducibly controlled, yielding a high-fidelity time axis. The separated analyte bands are excited and detected using spatially resolved fluorescence detection.[19]

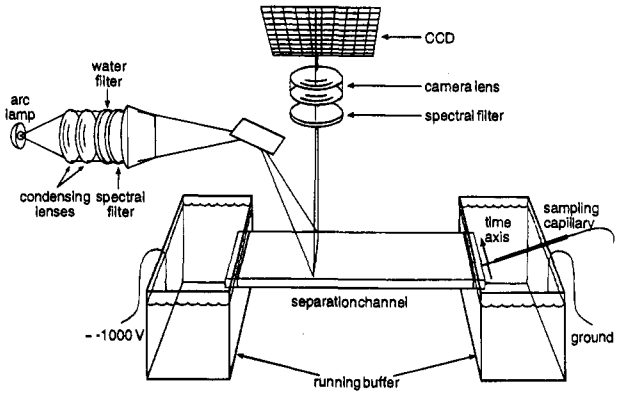


**Figure 1.** Schematic diagram of the two-dimensional system showing the sample introduction capillary, rectangular separation channel, and fluorescence detection system.

The analytical performance of this system is demonstrated by separating the thiocarbonyl derivatives of several amino acids. Figure 2 shows the separation of an amino acid-FITC derivative solution[20] containing arginine, histidine, leucine, and asparagine ($1 \times 10^{-4}$ M each) applied using a gravity injection (30 cm height displacement, 5 s duration) when the sample introduction capillary is stationary. As can be seen, the separation is complete in less than 3 min, with a separation efficiency of ~10 000 and a detection limit (S/N = 3) of 1 $\mu$M for Arg-FITC.

Lateral dispersion, which corresponds to band broadening perpendicular to the electroosmotic flow (EOF), is relatively

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(11) Channels are prepared by gluing two microscopic slides together with 40 $\mu$m glass microspheres (Duke Scientific, Palo Alto, CA), dispersed in the UV-cured adhesive (Norland Products Inc., New Brunswick, NJ) as a spacer between the two channel plates.[17] One edge of the upper channel plate is beveled so that the outlet end of the sampling capillary (50 $\mu$m i.d./365 $\mu$m o.d., Polymicro, Phoenix, AZ) can be held close to the channel entrance. The movement of the sampling capillary along the 2 cm wide entrance is precisely and reproducibly controlled by a two-axis motion controller (860MC2, Newport Research, Irvine, CA). A Hg (Xe) arc lamp (Oriel, Stratford, CT) is used as the excitation source. Although the use of an incoherent arc lamp results in a poorer limit of detection than laser excitation, it allows great flexibility in the choice of excitation wavelengths. After being collimated with a two-element fused silica condenser, the light is filtered with the appropriate spectral interference filter and focused by a silica cylindrical lens to a 2 mm wide image across the rectangular separation channel. The fluorescence emission from the channel is spectrally filtered and focused onto a CCD detector using two camera lenses (105 mm, *f*/1.8 and 50 mm, *f*/1.2 Nikon lenses, Japan) in a relay formation chosen to image a 2 cm wide channel across the 1 cm wide CCD detector. The CCD is a TK512 backside-illuminated CCD controlled with a slow scan camera system (Photometrics Ltd., Tucson, AZ).
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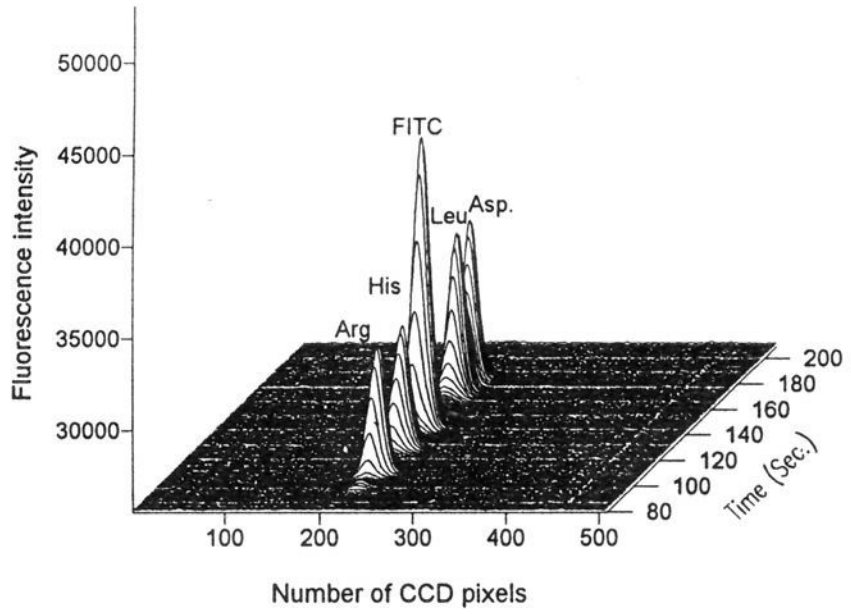


**Figure 2.** Two-dimensional electropherogram when the sample introduction capillary is held stationary in the center of the rectangular separation channel for the injection of a mixture of amino acid-FITC derivatives. The rectangular channel is 7.5 cm long with a 2 cm by 40 $\mu$m cross section. Running buffer: 25 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 10.15. Separation voltage: $-1150$ V. Channel current: 600 $\mu$A. Fluorescence detection: $\lambda_{ex} = 490$ nm; $\lambda_{cm} = 520$ nm.

small. Using the Einstein equation ($\sigma = \sqrt{2D\,t}$), a migration time of 110 s, and an estimated diffusion coefficient of $3 \times 10^{-6}$ cm$^2$/s, the expected full width at half-maximum (FWHM, $2.3\sigma$) is 590 $\mu$m. As we observe a FWHM for Arg of ~620 $\mu$m, this suggests that diffusion is the dominate source of lateral spreading in the channel. Thus, the 2 cm channel achieves 20 independent time points for each pass of the separation capillary for a resolution of one ($4\sigma$) in the time axis. As the sample introduction capillary can be scanned across the rectangular channel entrance in ~1 s in the present system, this corresponds to a minimum time resolution for each time point of ~50 ms. The number of time points for the dynamic separation can be increased by minimizing lateral spreading either by increasing the separation speed (using a higher field strength and a thinner channel) or by using gels.[15,16] A simpler approach to obtain more than 20 time channels is to have the sample introduction capillary move back and forth across the inlet of the rectangular channel (as long as analyte bands do not overtake each other) thus increasing the number of time channels as needed. In the present design, this allows continuous monitoring with ~1 s resolution.

To demonstrate the feasibility of using this system to continuously monitor chemical changes within nanoliter volume samples, the sampling capillary is filled with 200 nL of reaction solution containing Arg, Trp, and NDA/CN$^-$ [21] and injected continuously over 3.5 min into the separation channel. Figure 3A shows the two-dimensional electropherogram, with the increase in the CBI derivatives of Arg and Trp as a function of time. To examine these two derivatization reactions more closely, the peak heights vs elution time for both fluorescent bands are extracted and plotted in Figure 3B. These data demonstrate the kinetic differences in product formation of arginine and tryptophan with NDA. As each species migrates through the rectangular channel at a different rate due to different electrophoretic mobilities, the Arg-CBI and Trp-CBI derivatives have different effective time axes (at a given elution time, the Arg-CBI product reaching the detector corresponds to a different

(21) The sampling capillary contains 200 nL of a mixture of 2 $\mu$L of arginine ($5 \times 10^{-5}$ M) and tryptophan ($1 \times 10^{-4}$ M), 2 $\mu$L of 0.02 M KCN, 4 $\mu$L of 0.1 M borate buffer solution (pH 9.5), and 2 $\mu$L of NDA (0.1 mg/mL) in MeOH. Injection of material from the sampling capillary into the separation channel is performed using hydrodynamic flow by raising the inlet end of the sampling capillary 30 cm over the outlet end/channel entrance.

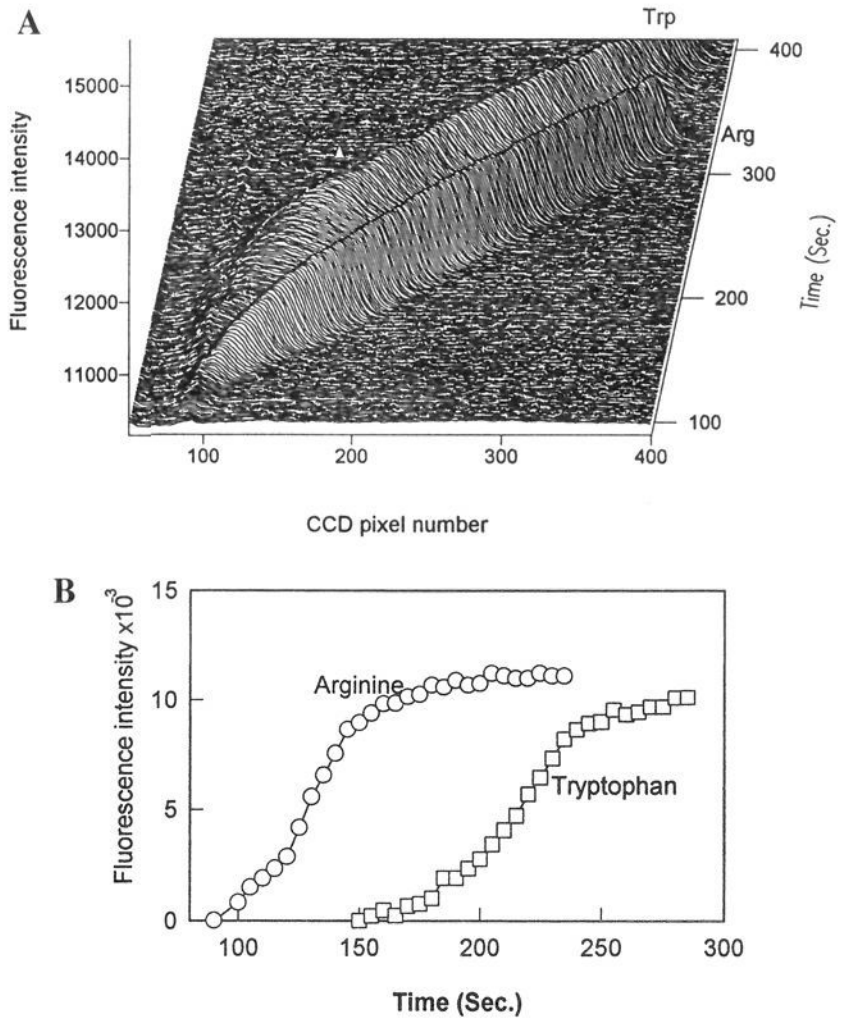


**Figure 3.** (A) Time-resolved, two-dimensional electropherogram when the sample introduction capillary is scanned across the rectangular inlet over 3.5 min for an injection of ~200 nL of the Arg/Trp NDA reaction solution. Running buffer: 25 mM CAPS, pH 10.20. Separation voltage: $-1000$ V. Channel current: 610 $\mu$A. Fluorescence detection: $\lambda_{ex} = 436$ nm; $\lambda_{em} = 490$ nm. (B) Maximum peak height of the Arg-CBI and the Trp-CBI derivatives at each time in part A.

total reaction time than the Trp-CBI product). The CBI product formation follows first-order kinetics, and we can compute a rate constant from the data in Figure 3B assuming that the product peak height is proportional to concentration. For the first-order formation of Arg-CBI, $k = 0.042$ s$^{-1}$, similar to previously reported formation constants of CBI derivatives under similar reaction conditions.[22]

Although rate constants can be determined for individual reactions using a conventional fluorimeter, determining individual rate constants in a more complex situation where multiple competing reactions are occurring or when examining reaction intermediates becomes problematic without separating the individual components using CE or LC. The ability of a conventional separation system to make repeated injections on the 0.1−10 s time scale is difficult. However, the system described here is ideal for following product formation in small-volume complex mixtures.

A time-resolved dynamic separation technique is demonstrated which yields more than 10 000 theoretical plates and a time resolution in the range of 100 ms to several seconds depending on the rate of motion of the introduction capillary. Additional applications of dynamic channel electrophoresis are continuous monitoring of chemical composition in extremely small volume samples such as single cells and coupling the outlet of a capillary LC to this microelectrophoretic technique to achieve unique multidimensional separations.

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