Table I. Comparison of Isocratic Elution Times (in minutes) for BPD Isomers from β- and γ-CyD Columns with Water Mixtures of Methanol or Acetonitrile as Mobile Phases

| % X/% water | X = methanol | | | | X = acetonitrile | | | |
|---|---|---|---|---|---|---|---|---|
| | β-CyD | | γ-CyD | | β-CyD | | γ-CyD | |
| | 7,8 | 9,10 | 7,8 | 9,10 | 7,8 | 9,10 | 7,8 | 9,10 |
| 60/40 | 5.3 | 4.8 | 6.0 | 5.2 | 3.5 | 3.5 | 3.6 | 3.6 |
| 50/50 | 7.2 | 6.1 | 10.6 | 7.8 | 3.7 | 3.7 | 4.0 | 3.9 |
| 40/60 | 12.5 | 9.1 | 22.5 | 12.3 | 4.2 | 4.1 | 4.9 | 4.5 |
| 30/70 | 17.7 | 11.4 | 57.5 | 24.6 | 5.5 | 5.3 | 7.4 | 6.2 |
| 20/80 | 58.5 | 26.0 | >60 | >60 | 11.8 | 9.0 | 19.2 | 12.3 |
| 10/90 | >60 | 59.2 | >60 | >60 | 56.2 | 27.2 | >60 | 44.4 |

also have application when using the cyclodextrins as vehicles for drug delivery.

**HPLC Separation with β- or γ-CyD as Stationary Phase.** Our spectroscopic results clearly indicate that the separation of 7,8-BPD from 9,10-BPD may be accomplished by HPLC methodology using β- or γ-CyD either as a stationary or a mobile phase. Such a feasibility study was carried out and the results of the isocratic runs are shown in Table I.

It is apparent from Table I that a base-line separation of 7,8-BPD from its 9,10-isomer can easily be achieved by using the mobile phase of MeOH/water with a water content between 40% and 70% or a MeCN/water mixture with a water content between 70% and 85% for a bonded γ-CyD column. A water content between 50% and 80% for the methanol mixture or 80% and 90% for the MeCN mixture is optimal for the bonded β-CyD column. It is also noteworthy that under a given percentage of water content, the elution time with the MeCN mixture is shorter than that of the methanol mixture, consistent with the greater hydrophobicity of the former. As expected from our binding studies, the 7,8-isomer is being retained longer than its 9,10-counterpart using the same column, and the gamma column is more effective than the beta column in trapping these benzo[a]pyrene metabolites.

## ACKNOWLEDGMENT

We thank D. Vines for carrying out some of the experiments.

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RECEIVED for review July 18, 1988. Accepted September 6, 1988. This research was supported by USPHS Grant CA-42682 and a subproject of NIH-MBRS Grant S06RR0892.

# On-Line Connector for Microcolumns: Application to the On-Column *o*-Phthaldialdehyde Derivatization of Amino Acids Separated by Capillary Zone Electrophoresis

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**The design of a simple, on-column connector for microcolumn separations is described. The connector, in the form of a cross or tee, is fabricated from the fused silica capillary tubing, itself, and has sufficiently low dead volume to be compatible with the tubing dimensions normally associated with microcolumn separation techniques. As a demonstration of one possible application, a mixture of amino acids is separated by capillary zone electrophoresis (CZE), the amino acids are derivatized on-column with *o*-phthaldialdehyde (OPA) introduced from a cross connector, and the highly fluorescent adducts are detected downstream by laser-induced fluorescence. Zone broadening by the connector is determined to be approximately 10% for CZE separations performed in 75 μm i.d. capillary tubes. Detector response is found to be linear over more than 3 orders of magnitude with minimum limits of detection in the subfemtomole range.**

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It is difficult to transfer all of the versatility associated with conventional separation techniques to modern, analytical scale, separations because the latter rely heavily upon the use of microcolumns. Great care must be taken to minimize sources of extracolumn band broadening in order that the advantages of microcolumn techniques may be fully realized (*1*). This situation becomes especially apparent in the case of microcolumn techniques involving the use of capillary tubes (internal diameter less than 100 μm). Injectors, detectors, and connecting tubing must be designed in such a manner that the increase in system variance caused by each is far less than the fundamental column variance. As a result, the use of connecting devices (tubing, fittings, etc.) for capillary tubes has been avoided whenever possible. Sample introduction is often accomplished in a split fashion with a small sample plug being delivered directly onto the head of the capillary column, while solute detection is commonly made in an on-column configuration.

The ability to make extremely low volume connections with capillary tubes would facilitate the extension of various sep-

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aration and detection schemes currently employed in conventional separation techniques to microcolumn separations. We report here the design, construction, and use of a simple, low volume, on-column capillary connector that is fashioned from the fused silica tubing, itself. As a demonstration of the utility of the connector, we describe one possible application, namely, the on-column derivatization of molecules separated by capillary zone electrophoresis (CZE) using the fluorogenic reaction of *o*-phthaldialdehyde (OPA) and 2-mercaptoethanol (MERC) with amino acids.

Capillary zone electrophoresis is rapidly becoming a valuable analytical tool for the separation of mixtures of biomolecules and exemplifies one of the modern separation techniques that is entirely dependent upon the use of capillary-dimension separation channels (*2–4*). Several of the common chromatography detection schemes have been successfully coupled with capillary zone electrophoresis including: ultraviolet absorbance (*5*), fluorescence (*4*, *6*, *7*), electrochemistry (*8*), conductivity (*9*, *10*), and mass spectrometry (*11*, *12*). Of these detectors, fluorescence affords typically the greatest sensitivity for those applications in which it can be used.

Fluorescence detection, in conjunction with high-performance liquid chromatography (HPLC) or ion chromatography (IC), has gained widespread use for the analysis of amino acids. In such applications, the nonfluorescent amino acids are derivatized prior to detection by one of several standard fluorogenic reactions (*13–15*). Several reports to date have described the analysis of prederivatized amino acids by capillary zone electrophoresis (*4*, *7*, *16*), in which case the conditions of the separation are dictated by the physical properties (pI, mobility, interaction with buffer additives, etc.) of the labeled molecules. Postcolumn or on-column derivatization of molecules separated by CZE is difficult to accomplish because of the small internal diameter of the capillary tubing (usually 75 μm or less). Since typical peak volumes are on the order of tens of nanoliters, connections made before the detector generally cause a significant loss in resolution by zone broadening in the connecting volume. Recently, Nickerson, Rose, and Jorgenson (*17*) described a method for the postcolumn CZE derivatization of primary amines that utilized the fluorogenic *o*-phthaldialdehyde reaction. In their work, the end of the capillary column was sheathed by a section of larger inside diameter fused silica capillary and the fluorogenic reagent stream was introduced into the sheath and mixed with the separation buffer by way of a tee connection. Detection of the fluorescent derivatives (1-alkylthio-2-alkylisoindoles) was then made in the larger capillary tube in a postcolumn manner. Arguments will be presented that on-column CZE derivatization of primary amines with OPA, made possible by an on-line connector with negligible dead volume, represents an appealing alternative.

## EXPERIMENTAL SECTION

**Construction of the On-Column Connector.** The on-column capillary connector was constructed by drilling a hole (approximately 60 μm in diameter) through 75 μm i.d. polyimide-clad fused silica capillary tubing (Scientific Glass Engineering or Polymicro Technologies, Inc.) with a 40-W $CO_2$ laser (Laakmann Electro-Optics, Inc.), which was equipped with a computer-controlled x-y translation stage. With the laser beam focused into the center of the capillary tube and both the laser spot size and pulse rate optimized, a hole was formed at a right angle to the internal channel of the capillary tube (Figure 1a). The hole produced by the laser was slightly tapered with the entrance diameter being somewhat larger than the exit diameter. The polyimide coating was removed from the surface of the capillary surrounding the hole and a stainless steel wire (38–50 μm in diameter, California Fine Wire) was inserted through the hole to act as a guide for two additional 75 μm i.d. fused silica capillaries that butt against the main capillary (Figure 1b,c). The three capillaries were then temporarily sealed together by allowing warm poly(ethylene glycol)

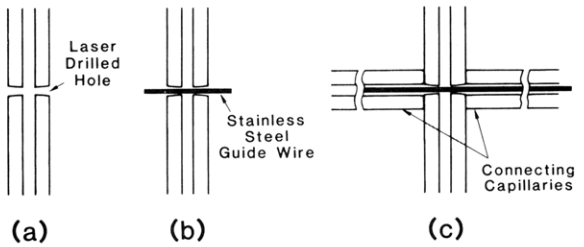


**Figure 1.** Construction of the on-column capillary connector. In part c the guide wire extends beyond one connecting capillary to facilitate removal.

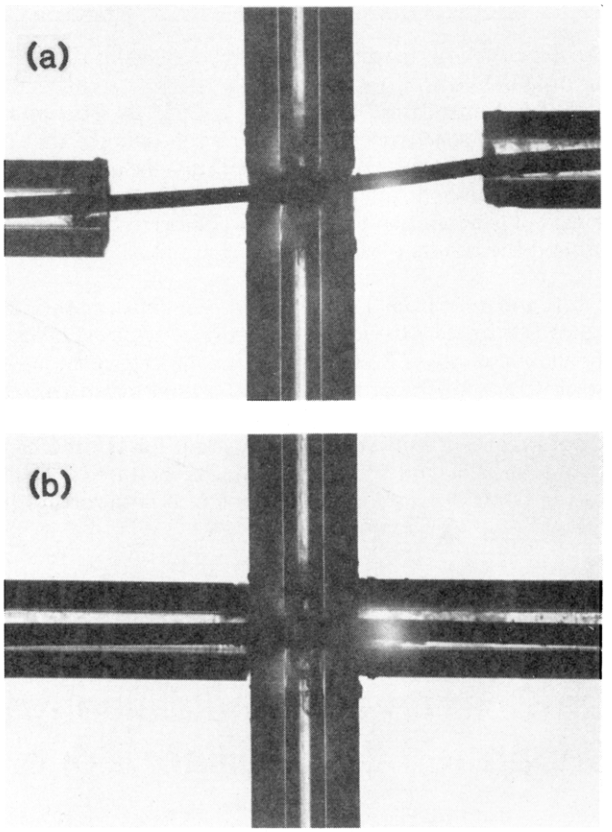


**Figure 2.** Photograph of an on-column capillary connector taken (a) before and (b) after the connecting capillaries are positioned along the guide wire for permanent attachment. The capillary tubing dimensions are 75 μm i.d. and 375 μm o.d.; the guide wire is 38 μm.

to flow into the gaps between the capillary surfaces and solidify. A photograph of an on-column capillary connector taken before application of the poly(ethylene glycol) is shown in Figure 2. A permanent seal was made by applying a small amount of epoxy (Miller-Stephenson 907) to the connection and allowing it to cure at room temperature for 24 h. The poly(ethylene glycol) served to prevent the epoxy from flowing into the capillaries while curing. After the epoxy was fully cured, short lengths of protective Teflon tubing were slid up to the connection along all four sections of fused silica and epoxied into place. The connector was then gently warmed to liquify the poly(ethylene glycol) and the stainless steel guide wire was removed by hand. The capillary system was thoroughly rinsed with methanol to remove any remaining poly(ethylene glycol) from the inner surfaces of the capillary tubing.

**Apparatus.** The experimental setup of the capillary zone electrophoresis/laser induced fluorescence (CZE/LIF) detection system was similar to that described previously (*4*, *7*) and is illustrated in Figure 3. A 1-cm section of the protective polyimide coating was removed from the surface of the fused silica capillary at a distance of 6–8 cm from the connector and this section of the capillary was fixed in space between two Swagelok fittings. Each end of the main capillary tubing was dipped in a 4-mL glass

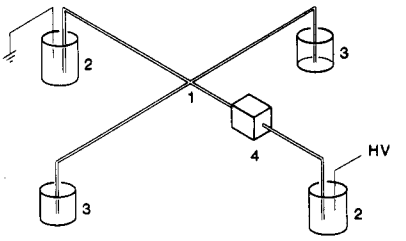


**Figure 3.** Experimental setup of the CZE/LIF detector system: 1, on-column connector; 2, buffer reservoirs; 3, derivatization reagent reservoirs; 4, LIF detector housing.

vial containing approximately 3 mL of electrolyte–buffer solution. Connection to high voltage was provided by a strip of Pt foil submersed in each of the buffer reservoirs. The injection end of the capillary tubing was connected to ground while the detection end was held at a high negative potential typically −25 kV. The current through the system was monitored as a potential drop across a 1-kΩ resistor in the ground side of the circuit. The capillary system and buffer reservoirs were enclosed in a Plexiglas box to prevent operator exposure to high voltage. Electroosmotic flow rates were measured in a manner similar to that described by Huang et al. (*18*) in which the capillary column was filled with supporting buffer diluted by 5%, the buffer reservoirs were filled with supporting buffer, and the current was monitored as one tubing volume was displaced by supporting buffer under the influence of the applied potential. Sample volumes for hydrostatic injections were estimated by peak area comparison with electrokinetic injections performed under conditions of known electroosmotic flow. Fluorescence detection was accomplished by using the 325-nm line of a He–Cd laser (Liconix Model 4240NB). The UV excitation light (7 mW) was directed perpendicular to the bare section of capillary by a 100-μm fused silica optical fiber (Polymicro Technologies, Inc.). The fluorescent emission was collected in a coplanar, right-angle geometry by a 600-μm optical fiber. The fluorescent light was directed to a photomultiplier tube (Centronic Q4249B) equipped with a fast monochromator (ISA Model H10) and a 450-nm (or 520 nm for dansyl amino acid analysis) interference filter (10 nm fwhm Corion). The photomultiplier tube (PMT) photocurrent was converted to voltage, amplified (100 ms risetime), and plotted on a stripchart recorder. Electropherograms could also be recorded digitally at 12 Hz and stored in a computer (IBM PC-XT).

**Reagents.** Dansyl-L-proline, *o*-phthaldialdehyde, and 2-mercaptoethanol were purchased from Sigma. Water used to prepare solutions was freshly deionized and distilled with a water purifier (Model LD-2A coupled with a Mega-Pure Automatic Distiller, Corning Glassworks). The supporting electrolyte for all experiments was 0.2 M borate buffer (pH 7.8) prepared from reagent grade sodium borate decahydrate and boric acid (J. T. Baker). Stock solutions of OPA were prepared by dissolving 50 mg of OPA in 5 mL of methanol. Stock solutions of MERC were prepared by mixing 20 μL of 2-mercaptoethanol with 4.0 mL of methanol. The OPA reagent was prepared fresh each day by adding 1.2 mL of each of the above reagent solutions to 2.5 mL of the supporting buffer and adjusting the pH to 9.5 by the dropwise addition of 1.0 M NaOH.

## RESULTS AND DISCUSSION

Band broadening caused by the on-column capillary connector was evaluated by measuring peak variances 6.5 cm before and 6.5 cm after the connector. This was accomplished by installing a second LIF detector before the capillary connector. Both detectors were fabricated from identical optical and electronic components. The reagent reservoirs (see Figure 3) were filled with running buffer and positioned 6.5 cm above the buffer reservoirs. Approximately 50 nL of the probe molecule, DNS-L-proline, was injected at a concentration of $10^{-5}$ M by dipping the ground end of the capillary tube into the sample solution and raising the sample vial 8.5 cm above the reagent reservoirs for 10 s. The separation was performed at an applied voltage of −20 kV and DNS-L-proline was observed to migrate at a rate of 0.8 mm/s under these conditions.

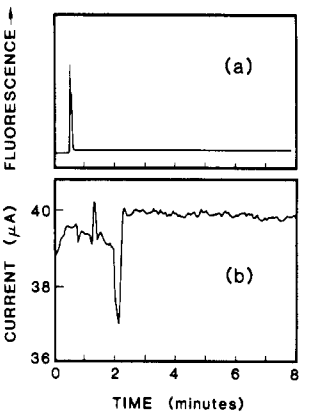


**Figure 4.** CZE electropherograms: (a) fluorescence response observed as the reagent-rich zone traverses the LIF detection region and (b) current profile observed as the same zone passes from the connector to the exit of the capillary tube. Voltage is −25 kV and the distance from the connector to the detector is 8 cm. Connector to column exit distance is 34 cm and the total capillary length is 74 cm.

Electropherograms were recorded digitally at 12 Hz and peak variances were calculated by using the method of statistical moments. The variance measured in these experiments represents the sum of the capillary connector variance and the column variance along 13 cm of the separation channel centered about the connector. An increase in peak width (fwhm) of approximately 20% was observed with the on-column connector in line. Comparison with a single capillary tube (no connector in line) under similar conditions indicated that approximately half of this broadening was caused by connector dead volume. We therefore estimate that the on-column capillary connector introduced roughly a 10% increase in peak width under these conditions.

Hydrostatic pressure was used to introduce the reagent stream into the separation channel by raising the reagent reservoirs (see Figure 3) 14 cm above the buffer reservoirs. This was accomplished at the beginning of each set of experiments by filling the entire capillary system with running buffer (by syringe), submersing the ends of the connecting capillaries in the elevated reagent reservoirs, and applying high voltage (typically −25 kV) for approximately 25 min. Both the background signal associated with the LIF detector and the current through the system exhibited a change as the reagent stream was entrained in the separation stream. Both of these signals were used as a means of determining the rate at which reagent was being introduced into the main capillary. The length of time required to reach a steady state of reagent introduction through the on-column connector is dependent upon the length of the capillary tubing leading to the main capillary, the height of the reagent reservoirs (hydrostatic pressure), and the magnitude of the electroosmotic flow in the main capillary. As the reagent stream became entrained in the effluent, the LIF background and the current through the system were observed to increase rapidly and then settle to a steady state. The LIF signal leveled off at a value that was typically 5 times the background associated with the running buffer. Under our conditions, the reagent flow rate (3 nL/s) was determined to be approximately one-third of the electroosmotic flow rate.

During the time in which the voltage is switched off to allow sample introduction, the reagent stream remains free to flow into the separation channel unguided by electroosmosis. As a result, a reagent-rich zone is formed near the connector, and a corresponding disturbance is observed in both the current and LIF profiles shortly after the running voltage is reapplied (see Figure 4). The elution time of the fluorescent signal corresponds to the amount of time required for the zone to travel from the connector to the detection volume, while the

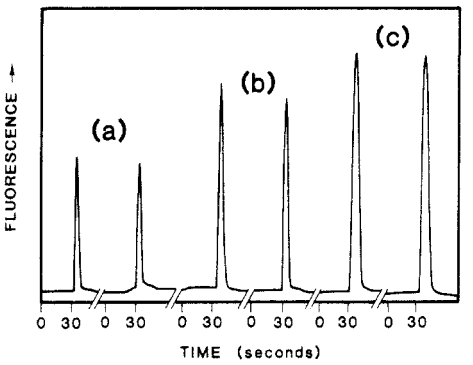


**Figure 5.** Fluorescence response observed as the reagent-rich zone reaches the LIF detection region for different voltage-off periods of (a) 10, (b) 20, and (c) 40 s. Other conditions are the same as in Figure 4.

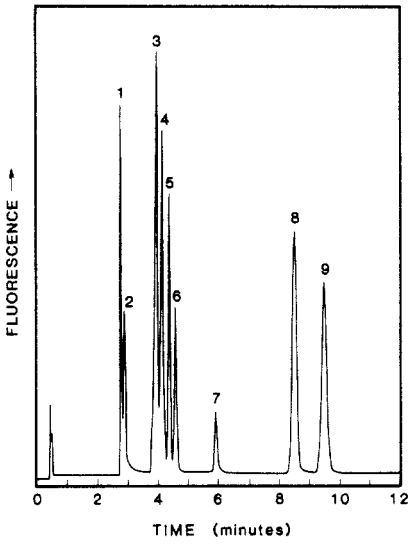


**Figure 6.** CZE separation of a nine-component amino acid mixture with on-column OPA derivatization and subsequent detection by LIF. Sample components are as follows: 1, L-arginine; 2, L-lysine; 3, L-isoleucine; 4, L-histidine; 5, L-serine; 6, L-asparagine; 7, L-cysteine; 8, L-glutamic acid; 9, L-aspartic acid. Separation conditions are as follows: 75 μm i.d. capillary, 40 cm to connector, 48 cm to LIF detector, 74 cm total length, −25 kV. Approximately 15 nL of the sample was injected by raising the sample vial 1 cm above the reagent reservoirs for 15 s.

period of time over which the disturbance in the current profile occurs corresponds to the time required for the zone to pass from the connector to the exit of the capillary tube. The magnitude of the signal observed by LIF is dependent upon the length of time that the voltage is switched off, as is illustrated in Figure 5. Because the reagent zone elutes in the first 40 s of the separation, we do not believe that this disturbance poses a serious problem, although this effect does become more apparent as the sample quantities are reduced. It should be possible to eliminate this problem by stopping the reagent flow with valves that close when the voltage is switched off.

Figure 6 shows a representative electropherogram illustrating the separation of a nine-component L-amino acid mixture with on-column OPA derivatization using the on-column capillary connector. Each of the amino acids was injected at a concentration of $10^{-4}$ M except for cysteine, which was present at $10^{-3}$ M. The OPA adduct of cysteine has previously been reported to yield a relatively low fluorescence signal (*13*, *19*). The residence time of each amino acid in the reaction volume is dependent upon the electrophoretic mobility of the molecule, the electroosmotic flow rate through the capillary tube, and the distance from the connector to the

**Table I. Response Index, $n$, and Correlation Coefficient, $r$, for a Four-Component Amino Acid Mixture[a]**

| amino acid | $n$ | $r$ |
|---|---|---|
| L-Arg | 0.96 | 0.998 |
| L-His | 1.02 | 0.997 |
| L-Glu | 1.04 | 0.998 |
| L-Asp | 1.06 | 0.998 |

[a] 75 μm i.d. capillary, 39 cm to on-column connector, 46.6 cm to LIF detector, 65.5 cm total column length, reagent reservoirs 14 cm above buffer reservoirs, potential = −25 kV, current = 40 μA.

detection volume. Under the conditions of our experiment, the residence time ranged from 28 s for L-arginine, which is sufficient time for adduct formation (*20*), to 98 s for L-aspartic acid. The peak shapes and resolution appear to be quite good, which is a further indication that the on-column capillary connector and derivatization process introduce little zone broadening. Both L-arginine and L-lysine are positively charged under the conditions of this separation (pI = 10.7 and 9.4, respectively) and therefore elute quite early. Even so, they are observed to be well resolved from the LIF disturbance, as illustrated in Figure 6. Run-to-run variation of peak heights is less than 10% and is most likely caused by variations in injection volume rather than by the derivatization process.

Detector linearity and minimum limits of detection for the method were examined by using a four-component L-amino acid mixture. Standard solutions of the amino acid mixture were prepared in running buffer and were serially diluted to cover a concentration range of nearly 4 orders of magnitude. The data were treated in a manner similar to that described previously (*7*, *21*) in which the function

$$Y = C[\text{analyte}]^n \quad (1)$$

was used to describe the detector response. In eq 1, $Y$ represents the PMT photocurrent, $C$ is a constant, [analyte] is the amino acid concentration in the injected sample solution, and $n$ is the response index of the detector. Note that the logarithmic form of eq 1 is linear with slope = $n$, where $n$ = 1 would represent a perfectly linear detector. The results of a linear least-squares fit to the data are presented in Table I. Although $n$ deviates slightly from unity, we believe that the detection system yields a linear response for well over 3 orders of magnitude as evidenced by the fact that the coefficient of correlation is quite close to unity. The lower limit of $9 \times 10^{-7}$ M (roughly 14 fmol injected) obtained for L-histidine corresponds to a signal to noise ratio of approximately 2 (peak-to-peak noise). With the 100-μm excitation fiber, we estimate our detection volume to be approximately 0.9 nL, which corresponds to about 800 amol of L-histidine (neglecting sample dilution, which occurs during separation).

The intersection of three capillary flow streams and diffusion in the aqueous buffer provide the only means of mixing reagent and analyte solutions together in this detection system. A comparison was made between on-column derivatization and precolumn derivatization of the four-component amino acid mixture in order to determine the relative extent of reaction for the on-column derivatization process. Precolumn derivatization was accomplished in the following manner; the reagent in the elevated reservoirs was replaced with running buffer, the standard four-component amino acid mixture was prepared with each component present at a concentration of $10^{-4}$ M in a 10-fold excess of reagents (OPA and MERC), the mixture was vigorously shaken, and an aliquot was withdrawn by gravity injection onto the CZE system.

Parts a and b of Figure 7 show the electropherograms corresponding to the CZE separation of the four-component amino acid mixture with precolumn and on-column OPA

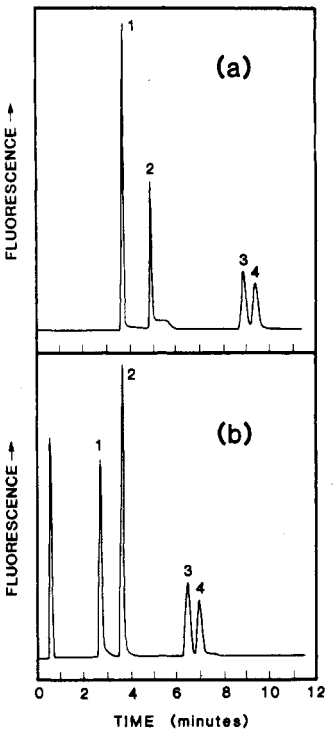


**Figure 7.** CZE electropherograms comparing (a) precolumn and (b) on-column OPA derivatization of a four-component amino acid mixture. Both samples were prepared from the same stock solution and were present in the injected sample solution at a concentration of $10^{-4}$ M. Separation conditions are the same as in Figure 6. The ordinate in part b has been scale expanded to twice that of part a.

derivatization, respectively. A series of timed injections revealed that the fluorescence of the L-histidine derivative decreased rapidly with time (caused by decomposition of the fluorescent adduct). This accounts for the difference in relative intensities of the first two peaks in the electropherograms presented in Figure 7. The origin of the response observed just after the elution of L-histidine in the top trace is unknown. However, it is reproducible and was observed to decrease in intensity along with the L-histidine signal. The intensity of the L-arginine peak remained constant for over 35 min after the mixing of reagents and was therefore selected for use in the comparison of the two detection schemes. By comparison of the L-arginine peak in the two electropherograms in Figure 7, we estimate that there is roughly a factor of 3 decrease in sensitivity, for on-column derivatization versus precolumn derivatization, for analytes that form stable adducts. The on-column derivatization method, however, appears to afford greater sensitivity than precolumn derivatization for analytes that react to produce unstable adducts. Additionally, on-column derivatization eliminates the need to time carefully the reaction period prior to sample introduction in order to make quantitative determinations.

## CONCLUSION

A simple, low volume microcolumn connector and its application to the on-column OPA derivatization of amino acids separated by capillary zone electrophoresis have been described. The detection system has been characterized and was observed to yield a linear response that extends over 3 orders of magnitude in solute concentration. Possible improvements to the current derivatization system would include an alternate method of reagent introduction (i.e. a low flow rate pumping system) as well as a means of stopping the reagent flow during sample introduction. There are a large number of other uses for this on-column connector; additional applications need not be restricted to capillary zone electrophoresis but include other separation techniques that utilize capillary tubing.

## ACKNOWLEDGMENT

We thank Lyn Bowman, Electrical Engineering Department, Stanford University, for his assistance in forming holes in the fused silica capillary tubing with a $CO_2$ laser.

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RECEIVED for review June 3, 1988. Accepted August 30, 1988. Support for this work by Beckman Instruments, Inc., is gratefully acknowledged.