Electrophoresis 2000, *21*, 3305–3310

Ying Zhang
Frank A. Gomez

Department of Chemistry and Biochemistry,
California State University,
Los Angeles, CA, USA

On-column derivatization and analysis of amino acids, peptides, and alkylamines by anhydrides using capillary electrophoresis

This work demonstrates the use of an in-capillary procedure for derivatization of amino acids, peptides, and alkylamines by anhydrides using capillary electrophoresis (CE). Migrating in an uncoated fused-silica capillary, plugs of substrate and anhydride are injected separately and electrophoresed. Differential transport velocities permit the separate zones to penetrate each other under an applied field, thereby facilitating reaction. In initial experiments the extent of reaction between tryptophan and acetic anhydride was examined and product amounts quantitated by CE. In separate experiments a series of amino acids and peptides were injected into the capillary and reacted with phthalic anhydride on-column to yield the phthalic derivatized species. Finally, on-column derivatization of alkylamines with phthalic anhydride was investigated and electrophoretic mobility related to molecular weight of the derivatized amines. These procedures illustrate the use of the capillary as a microreactor in the facile synthesis of derivatized molecules and ease of quantitation of reaction products under conditions of electrophoresis.

Keywords: Capillary electrophoresis / In-capillary microreactions / Amino acids / Phthalic anhydride / Acetic anhydride
EL 4117

1 Introduction

Over the past ten years capillary electrophoresis (CE) has become an important analytical technique, particularly in the analysis and separation of biological species [1, 2]. This versatility coupled with a mechanism of analyte resolution that is complimentary to more traditional chromatographic techniques has led to widespread application of this technology in many laboratories [3]. CE differentiates charged species on the basis of mobility under the influence of an applied electric field. The value of the electrophoretic mobility (μ) of a species is directly related to its net charge and inversely related to its hydrodynamic drag. CE requires only small quantities of material, is applicable to water-soluble, nonvolatile, high molecular weight species, is readily automated, and can be manipulated by the alteration of electrolyte properties such as pH, ionic strength, and electrolyte composition, or by the incorporation of electrolyte additives.

Recent studies have documented the use of the capillary as a microreactor and have focused on the analysis of enzyme-mediated reactions, enzyme assays and immunoassays [4–23]. These assays have been applied to a wide variety of biochemical and analytical problems. For example, Jin *et al.* [4] used an on-column approach to monitor the oxidation of glucose by glucose oxidase in submicroliter samples. Kwak *et al.* [5] related electrophoresis conditions of in-capillary enzyme-catalyzed microreactions to product distribution profiles. Finally, Harmon *et al.* [6] used an in-capillary technique to monitor the oxidation of ethanol to acetaldehyde by alcohol dehydrogenase. These studies and others have concentrated on using electrophoretically mediated microanalysis (EMMA) [4–23]. In EMMA, differential electrophoretic mobility is utilized to merge distinct zones of analyte and analytical reagent(s) under the influence of an electric field [7, 8]. The reaction is then allowed to proceed within the region of reagent overlap either in the presence or absence of an applied potential, and the resultant product is transported to the detector under the influence of the applied electric field [9].

The natural progression of in-capillary reaction techniques to include nonenzymatic reactions has only recently been examined [24]. One reason for this is that many of these types of reactions must be conducted under inert atmospheres and/or under dry solvent conditions, thereby making analysis by CE problematic since the technique is predominantly a nonaqueous one. Secondly, reaction rates *vis-á-vis* kinetics of nonenzymatic reactions are not always on the timescale of CE separations; hence, monitoring of the reaction by CE is difficult.

Correspondence: Dr. Frank A. Gomez, Department of Chemistry and Biochemistry, California State University, Los Angeles, 5151 State University Drive, Los Angeles, CA 90032-8202, USA
E-mail: fgomez2@calstatela.edu
Fax: +323-343-6490

Abbreviation: ***N*-Ac-Trp**, *N*-acetyltryptophan

CE and CEC

© WILEY-VCH Verlag GmbH, 69451 Weinheim, 2000
0173-0835/00/1515-3305 $17.50+.50/0

Finally, systems to be studied are limited by their physicochemical behavior under electrophoresis and must first be evaluated prior to the study by CE. Although there are inherent difficulties in studying nonenzymatic reactions, these obstacles should not lessen the resolve to study them from a microreactor approach.

Herein, we outline the use of the capillary as a microreactor in the on-column synthesis and analysis of derivatized amino acids, peptides, and alkylamines by acetic and phthalic anhydride (Fig. 1). Plugs of substrate and anhydride are injected onto the capillary and electrophoresed. Spatially distinct zones of the chemical reagents with differential transport velocities are made to interpenetrate under the influence of an applied electric field. Reaction products are then detected and quantitated. This study provides the basis for the use of the capillary as a microreactor in the analysis of nonenzymatic reactions. The importance of studying nonenzymatic systems on-column by CE is severalfold. First, small-scale synthesis and analysis of molecular targets by CE is economically sound. Second, analysis of small quantities of material decreases the toxic effects inherent in large-scale synthesis of materials. Third, there is potential for coupling the in-capillary approach to drug design and development, in particular combinatorial approaches to the synthesis of potential drug candidates.

2 Materials and methods

2.1 Chemicals and reagents

All chemicals were of analytical grade. Acetic anhydride, phthalic anhydride, *N*-acetyl-tryptophan (*N*-Ac-Trp), glycine, serine, threonine, methionine, leucine, tryptophan, histidine, D-Ala-D-Ala, D-Ala-D-Ala-D-Ala, Gly-Gly-His, propylamine, butylamine, isoamylamine, and hexylamine were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Stock solutions of acetic anhydride and phthalic anhydride (0.01 mg/mL each) were prepared by dissolving the solid in acetonitrile. Stock solutions of amino acids, peptides, and alkylamines (0.01 mg/mL each) were prepared by dissolving the solids

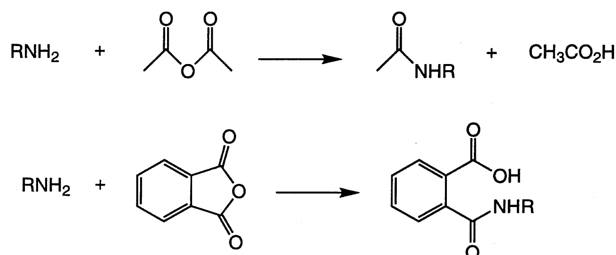

Figure 1. Reaction of acetic acid and phthalic anhydrides with amines.

in acetonitrile:water 9:1 v/v. Electrophoresis was carried out using a 20 mM phosphate buffer, adjusted to pH 10 by addition of 0.1 M NaOH.

2.2 Apparatus

The capillary electrophoresis system used in this study was a Beckman Model P/ACE 5510 (Fullerton, CA, USA). The capillary tubing (Polymicro Technologies, Phoenix, AZ, USA) was of uncoated fused silica with an internal diameter of 50 μm, a length from inlet to detector of 40.5 cm, and a length from detector to outlet of 6.5 cm. Data were collected and analyzed with Beckman System Gold software. The conditions used in CE were as follows: voltage, 24 kV; current, 50 μA; detection, UV detection at 200 nm; temperature, $25 \pm 0.1^{o}C$.

2.3 Procedures

For the quantitation of amino acid derivatization reactions, 1.2 nL of sample solute (a 1 s time of pressure injection equates 1.2 nL of volume of liquid) containing amino acid in acetonitrile:water was introduced by pressure injection onto the capillary equilibrated with buffer. A solution (1.2 nL) of anhydride in acetonitrile at increasing concentration was subsequently introduced by pressure injection and electrophoresis was run at 24 kV to complete elution of all species. Experiments involving increasing plug lengths of anhydride (1.0 mM) were similar to the above except that various volumes of anhydride were injected at constant concentration. For derivatization reactions not involving quantitation, experiments were similar to the above.

3 Results and discussion

3.1 Amino acid derivatization and quantitation

In the first experiment we examined the in-capillary derivatization of a single amino acid by acetic anhydride and subsequent quantitation of reaction products. In this experiment plugs of Trp and acetic anhydride were introduced into the capillary from reagent vials by pressure injection and electrophoresed in buffer (Fig. 2). Differences in transient velocities cause the faster migrating plug (acetic anhydride) to penetrate the zone of the slower migrating plug of sample (Trp) under an applied field. Trp was dissolved in a 9:1 acetonitrile:water mixture because it is only slightly soluble in acetonitrile. Acetic anhydride was dissolved in acetonitrile because it is readily hydrolyzed in aqueous solution and would hinder the amount of *N*-Ac-Trp that could form in the capillary.

Figure 3A shows the electropherograms obtained for the conversion of Trp to *N*-Ac-Trp using acetic anhydride. Trp elutes near the migration time of neutral species. Acetic anhydride only weakly absorbs at 200 nm and is not ob-

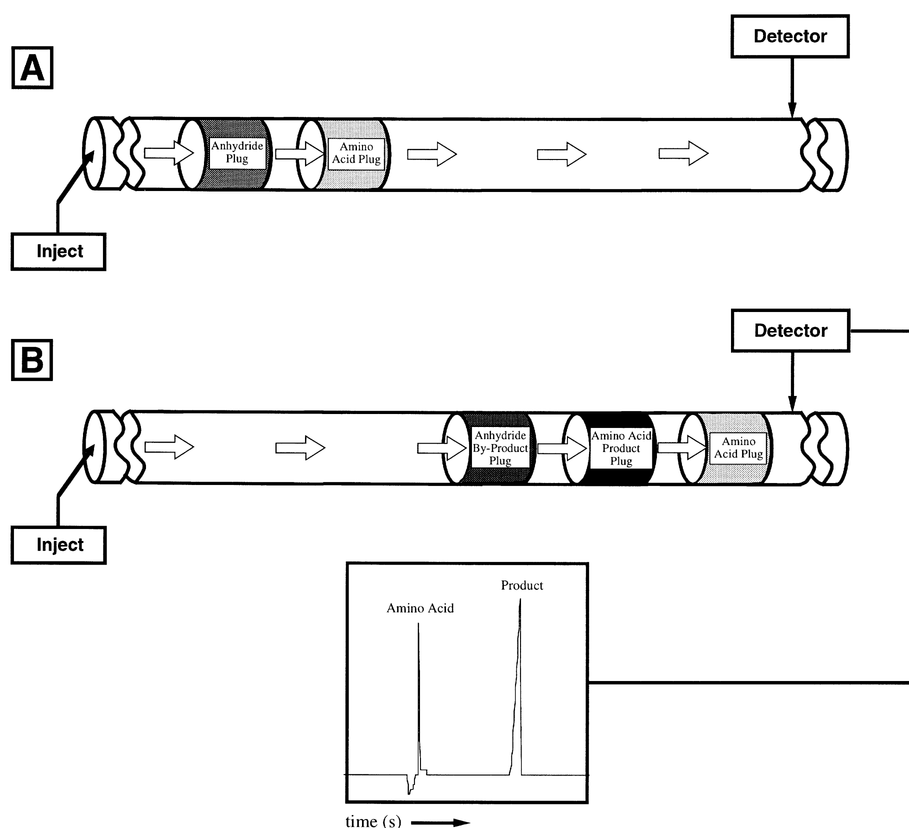

Figure 2. Schematic of an in-capillary microreaction (A) before and (B) after reaction.

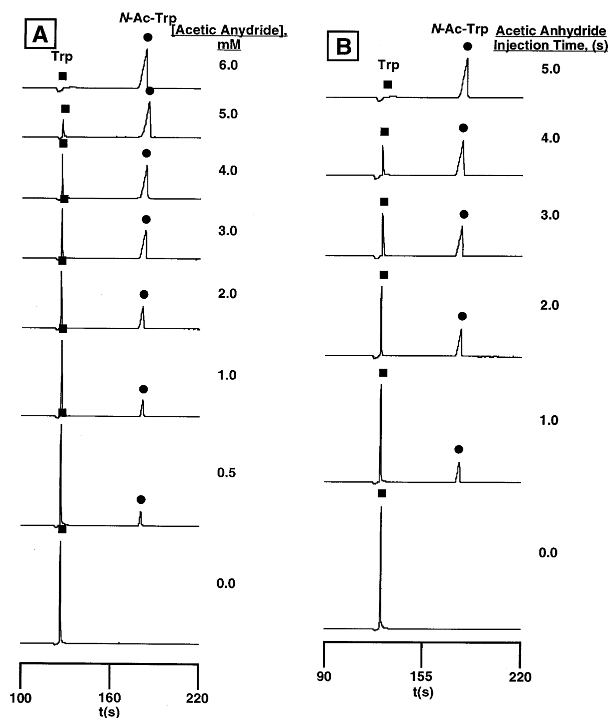

Figure 3. Conversion of Trp to *N*-Ac-Trp on (A) increasing the concentration of acetic anhydride in the capillary column and (B) increasing the injection time of acetic anhydride in the capillary column.

served in the electropherogram. In addition, the by-product of the reaction, acetic acid, is not observed in the series of electropherograms since it too has no appreciable absorbance at 200 nm. *N*-Ac-Trp has a larger electrophoretic mobility than both Trp and acetic anhydride and is observed last in the electropherogram. The concentration of acetic anhydride was sequentially increased during the course of the experiment. The conversion of Trp to *N*-Ac-Trp is complete at the highest concentration of acetic anhydride. Upon electrophoresis Trp penetrates the anhydride zone and is no longer in the original injected sample zone. Because of the lower conductivity of the sample plug and the reagent plug, stacking occurs. Proof of the stacking process can be seen in the front end of the Trp plug and back end of the *N*-Ac-Trp plug. Where the two interact is the tailing on the Trp plug and fronting of the *N*-Ac-Trp plug. Figure 4A shows the response of the electropherograms to changes in the concentration of acetic anhydride in the plug. Calibration curves for Trp were constructed with several concentrations of Trp. Increasing consumption of Trp and generation of *N*-Ac-Trp correlated with increasing concentration of acetic anhydride in the plug.

A number of general interpretations can be drawn from these results. First, on-column derivatization of an amino acid easily proceeds under conditions of electrophoresis

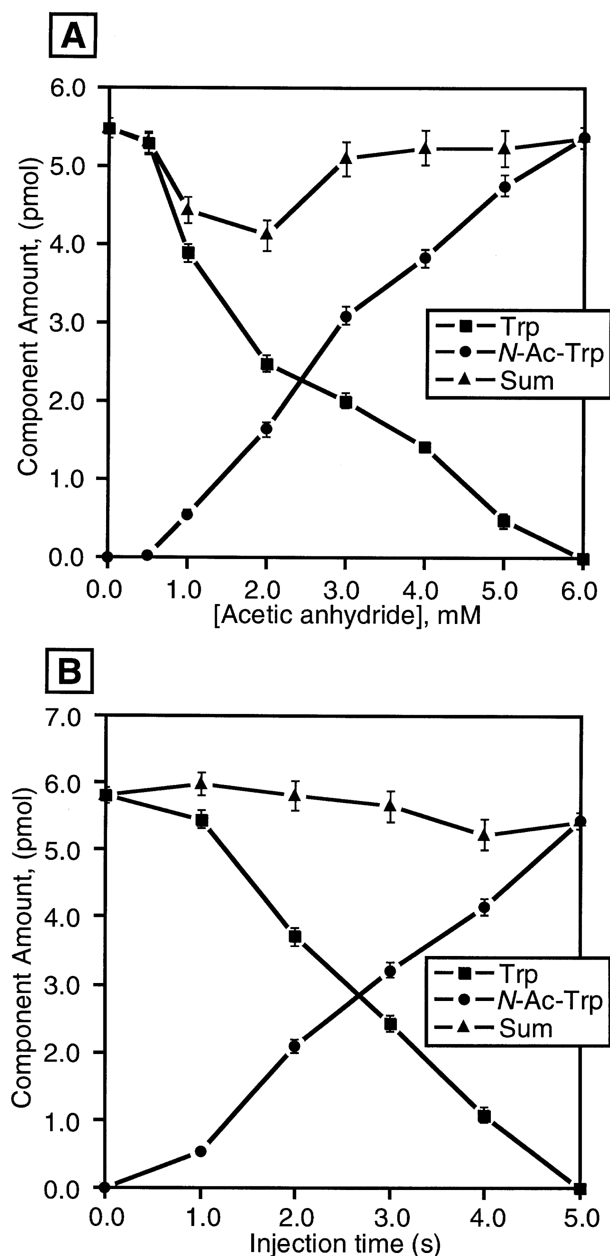

Figure 4. Response of the electropherograms to (A) changes in the concentration of acetic anhydride in the capillary column and (B) changes in the injection time of acetic anhydride in the capillary column.

using differential electrophoretic mobilities to cause penetration of one zone of sample into another. Second, products of in-capillary reactions are easily quantitated as shown by the near horizontal line in Fig. 4A representing the summation of Trp and *N*-Ac-Trp in picomoles. Third, there is only minimal mixing of sample plug zones upon injection and little sample plug broadening during electrophoresis. Fourth, reactions requiring nonaqueous conditions can be used if reagents are dissolved, and initially electrophoresed, in organic media.

In the second type of experiment, we examined the conversion of Trp to *N*-Ac-Trp when a plug of Trp is introduced into the region occupied by an increasing plug length (injection time) of acetic anhydride in the plug at a constant concentration. Figure 3B shows a representative series of electropherograms obtained for the conversion of Trp to *N*-Ac-Trp by an increasing plug length of acetic anhydride. A 1 s injection time equates to 1.2 nL of liquid. Figure 4B shows the response of the electropherograms to changes in the injection time of acetic anhydride in the plug and, hence, to the contact time between acetic anhydride and Trp. Increasing consumption of Trp and generation of *N*-Ac-Trp correlated with increasing injection time of acetic anhydride in the capillary at constant voltage. These observations were a direct result of a stacking phenomenon in the sample plug zones. We believe that the limit of detection (LOD) for on-column microreactions using UV/Vis detection is approximately 0.1 pmol.

3.2 Derivatization of amino acids with phthalic anhydride

In a second series of experiments we reacted seven aromatic (His, Trp) and nonaromatic amino acids (Gly, Leu, Met, Ser, Thr) with phthalic anhydride using the in-capil-

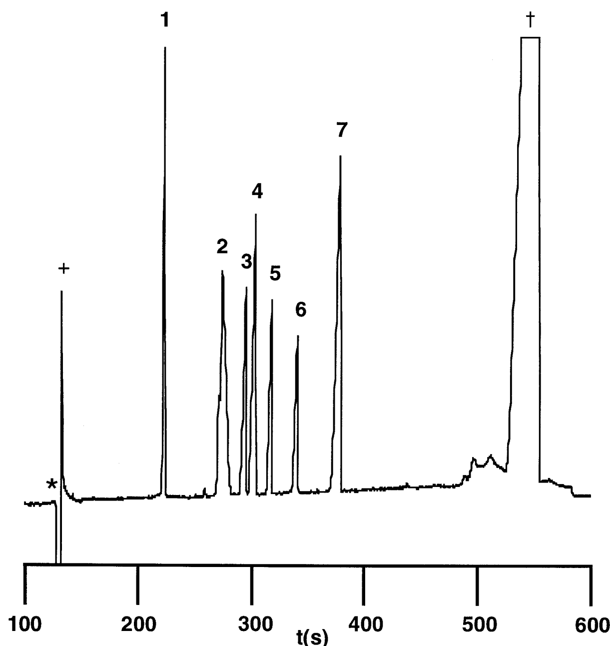

Figure 5. Conversion of seven amino acids after reaction with phthalic anhydride in the capillary column, Peak 1, *N*-R-His; 2, *N*-R-Trp; 3, *N*-R-Leu; 4, *N*-R-Met; 5, *N*-R-Thr; 6, *N*-R-Ser; 7, *N*-R-Gly; R = *o*-(-O_2C)C_6H_4C(O); (*) electroosmotic flow; (+) unreacted amino acids; (+) phthalic acid.

lary technique. In this experiment, separate plugs of sample containing the seven amino acies (total concentration of amino acids in 10 mM) and phthalic anhydride (10 mM) were injected and electrophoresed. The amino acids are negatively charged at pH 10 and migrate through the capillary at a rate slower than phthalic anhydride. In organic media phthalic anhydride readily reacts with primary amine moieties of amino acids.

Figure 5 is a representative electropherogram showing the derivatization of the amino acids. Peak resolution of all seven derivatized amino acids in achieved. In the present experiment the amino acids are shifted to longer migration times upon derivatization due to changes in their charge-to-mass ratio. The relative standard deviations (RSDs) for peaks 1–7 vary from 2.4–3.8%. The number of plate counts varies from $1.3–6.3 \times 10^5$ for the seven derivatized amino acids. Small amounts of unreacted amino acids are observed in the electropherogram near the electroosmotic flow (EOF). The hydrolysis product, phthalic acid, is observed at approximately 9 min.

In a separate experiment three small peptides, D-Ala-D-Ala, D-Ala-D-Ala-D-Ala, and Gly-Gly-His were reacted on-column with phthalic anhydride to form the respective phthalic derivatized peptides. Figure 6 contains representative electropherograms showing the derivatization of the three peptides before and after reaction with phthalic anhydride. The reaction products *N*-D-Ala-D-Ala, *N*-D-Ala-DAla-D-Ala, and *N*-Gly-Gly-His are all negatively charged and flow toward the cathodic end at different electrophoretic mobilites. The peak shapes for all three peptides are good and the efficiencies high. The earliest eluting peak (*i.e.*, closest to EOF) was that for *N*-Ac-Gly-Gly-His, which also happens to have the highest molecular weight of the three derivatized peptides.

3.3 Derivatization of alkylamines with phthalic anhydride

In a final series of experiments, we reacted a series of alkylamines (propylamine, butylamine, isoamylamine, and hexylamine) on-column with phthalic anhydride. Under similar conditions as stated in the previous experiments, plugs of alkylamines and phthalic anhydride were injected and electrophoresed. Figure 7A is a representative electropherogram showing the derivatization of four alkylamines by phthalic anhydride. The alkylamines range in length from three to six carbon atoms and are structurally similar. The earliest eluting peak was that for *N*-Ac-hexylamine, which also has the greatest molecular mass of the four derivatized alkylamines. Figure 7B shows the relationship between electrophoretic mobility (μ) and molecular mass. As can be seen, a good correlation between μ and molecular mass is observed.

4 Concluding remarks

This report demonstrates the ease of conducting in-capillary microreactions using CE and the practicality in quantitating the reaction products. We have shown this by ex-

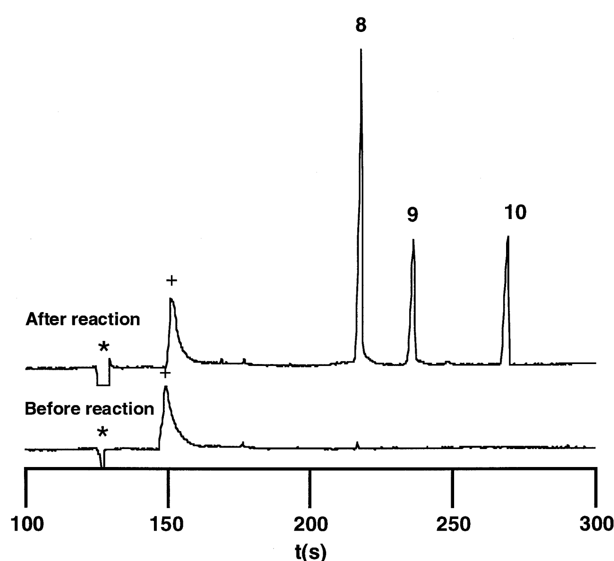

Figure 6. Derivatization of three peptides by phthalic anhydride. Peak 8, *N*-R-Gly-Gly-His; 9, *N*-R-D-Ala-D-Ala-D-Ala; 10, *N*-R-D-Ala-D-Ala; R = $o(\text{-}O_2C)C_6H_4C(O)$; (*) electroosmotic flow; (+) unreacted peptides.

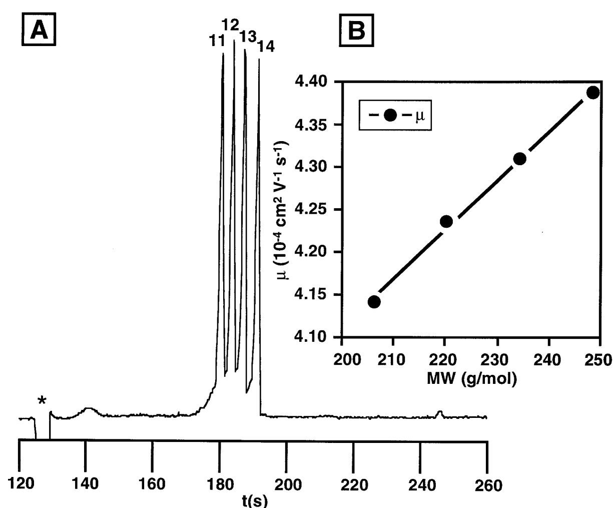

Figure 7. (A) Derivatization of four alkylamines with phthalic anhydride; peak 11, *N*-R-hexylamine; 12, *N*-R-isoamylamine; 13, *N*-R-butylamine; 14, *N*-R-propylamine; R = $o(\text{-}O_2C)C_6H_4C(O)$; (*) electroosmotic flow. (B) Electrophoretic mobility *versus* molecular mass for four derivatized alkylamines.

amining the on-column synthesis and analysis of derivatized amino acids, peptides, and alkylamines by acetic and phthalic anhydride. On-column synthesis of molecular targets is both economically sound and environmentally appealing, given the small quantities of materials used in the technique. There is great potential in coupling on-column techniques to other analytical techniques, including mass spectrometry and polymerase chain reaction. This study points the way to further applications of the capillary as a microreactor in the study of small-scale reactions.

The authors gratefully acknowledge financial support for this research by grants from the National Science Foundation CAREER (Grant CHE-9703142) and CRUI Programs (Grant DBI-9710796).

Received March 6, 2000

5 References

[1] Nashabeh, W., El Rassi, Z., *J. Chromatogr.* 1992, *596*, 251–264.

[2] Zhao, D. S., Gomez, F. A., *Chromatographia* 1997, *44*, 514–520.

[3] Tomlinson, A. D., Benson, L. M., Guzman, N. A., Naylor, S., *J. Chromatogr. A* 1996, *744*, 3–15.

[4] Jin, Z., Chen, R., Colon, L. A., *Anal. Chem.* 1997, *69*, 1326–1331.

[5] Kwak, E.-S., Esquivel, S., Gomez, F. A., *Anal. Chim. Acta* 1999, *397*, 183–190.

[6] Harmon, B. J., Leesong, I., Regnier, F. E., *Anal. Chem.* 1994, *66*, 3797–3805.

[7] Wu, D., Regnier, F. E., *Anal. Chem.* 1993, *65*, 2029–2035.

[8] Harmon, B. J., Patterson, D. H., Regnier, F. E., *Anal. Chem.* 1993, *65*, 2655–2662.

[9] Patterson, D. H., Harmon, B. J., Regnier, F. E., *J. Chromatogr. A* 1996, *732, 119–132.*

[10] Chu, Y.-H., Avila, L. Z., Gao, J., Whitesides, G. M., *Acc. Chem. Res.* 1995, *28*, 461–468.

[11] Bao, J., Regnier, F. E., *J. Chromatogr. A* 1992, *608*, 217–224.

[12] Avila, L. Z., Whitesides, G. M., *J. Org. Chem.* 1993, *58*, 5508–5512.

[13] Xue, Q., Yeung, E. S., *Anal. Chem.* 1994, *66*, 1175–1178.

[14] Chang, H.-T., Yeung, E. S., *Anal. Chem.* 1993, *65*, 2947–2951.

[15] Mechref, Y., El Rassi, Z., *Electrophoresis* 1995, *16*, 2164–2171.

[16] Emmer, A., Roeraade, J., *J. Chromatogr. A.* 1994, *662*, 375–381.

[17] Perron, M. J., Page, M., *J. Chromatogr. A.* 1994, *662*, 383–388.

[18] Licklider, L., Kuhr, W. G., Lacey, M. P., Keough, T., Purdon, M. P., Takigiku, R., *Anal. Chem.* 1995, *67*, 4170–4177.

[19] Maeda, M., Tsuji, A., Ohshima, N., Hukuoka, M., *J. Biolumin. Chemilumin.* 1993, *8*, 241–246.

[20] Patterson, D. H., Harmon, B. J., Regnier, F. E., *J. Chromatogr. A* 1994, *662*, 389–395.

[21] Cole, L. J., Kennedy, R. T., *Electrophoresis* 1995, *16*, 549–556.

[22] Lada, M. W., Schaller, G., Kennedy, R. T., *Anal. Chim. Acta* 1995, *307*, 217–226.

[23] Amankwa, L. A., Kuhr, W. G., *Anal. Chem.* 1992, *64*, 1610–1613.

[24] Taga, A., Sugimura, M., Honda, S., *J. Chromatogr. A* 1999, *802*, 243–248.