Analysis of Neuropeptides Using Capillary Zone Electrophoresis With Multichannel Fluorescence Detection

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ABSTRACT

Capillary zone electrophoresis is fast becoming one of the most sensitive separation schemes for sampling complex microenvironments. A unique detection scheme is developed in which a charge-coupled device (CCD) detects laser induced fluorescence from an axially illuminated electrophoresis capillary. The fluorescence from an analyte band is measured over a several centimeter section of the capillary, greatly increasing the observation time of the fluorescently tagged band. The sensitivity of the system is in the 1-8 x 10^{-20} mol range for derivatized amino acids and peptides. Subattomole quantities of bag cell neuropeptides collected from the giant marine mollusk *Aplysia californica* can be measured.

1. INTRODUCTION

1.1 Background

The ability of the cellular biochemist to unravel the complex nature of an individual cell depends on the quality of the available analytical techniques. The assay of single neurons provides information which is only available through discrete cellular sampling schemes. Furthermore, the resolution of many important questions demands analytical schemes that sample extremely small subcompartments of individual neurons.

This article describes new analytical methodology: capillary zone electrophoresis followed by multichannel laser-induced fluorescence (LIF), and its applications to studying the distribution of neuropeptides found within single cells. The sensitivity of the approach allows measurements in the low zeptomole (10^{-21} mol) range, improving the sensitivity over conventional cell sampling techniques by almost one million fold.

The giant marine snail *Aplysia californica* is an ideal system to study the roles of peptides as neurotransmitters, as well as their roles in the regulation of behavior. With approximately 20,000 neurons, one million synapses, and neurons as large as 500 μm, *Aplysia* are extremely amenable to discrete cellular stud-

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ies. The neuropeptides found within a neuron are not evenly distributed throughout the cytoplasm, but are found in discrete, small packets called vesicles. It is estimated that single dense-cored vesicles found in the bag cells have diameters of between 100 and 300 nm, and contain between 5,000 and 50,000 neuropeptide molecules.

Previous research has shown that four neuroactive peptides are found within the bag cell neurons; more significantly, they appear to be differentially packaged within individual vesicles,[1-3] and not evenly distributed throughout the cell.[4] Thus, the development of an analytical methodology to study the distribution and distribution mechanisms of neuropeptides within a single cell has been the major goal of this research.

Unfortunately, the demands upon an analytical scheme to separate and detect the amounts of neuropeptide found within a small subcompartment, and ultimately, a single vesicle are severe. Even for a material present at high concentration (0.01 M) within a typical vesicle, only 10^{-20} mol or ≈10,000 molecules are present.

1.2 Capillary Zone Electrophoresis

Capillary zone electrophoresis (CZE) is an extremely efficient, ultra-small-volume separation method suitable for the analysis of charged species in aqueous media. Injection volumes are from nanoliters to picoliters; thus, small volume samples such as the contents of individual cells are easily accommodated.

CZE is conceptually a very simple technique; samples are separated in an open fused-silica capillary tube with an inside diameter of between 2 and 200 μm and a length between 10 and 100 cm. The separation is based on the electrophoretic mobility of the analyte species; because electroosmotic flow is typically much greater than electrophoretic migration rates, all species, regardless of charge migrate down the column past an on-column detector. Extremely high separation efficiencies approaching 1,000,000 theoretical plates are often achieved. Figure 1 shows a block diagram of the CZE system; several recent review articles describe in much greater detail the operation and capabilities of CZE.[5-7]

1.3 Detection in CZE

Because the capillaries used with CZE have inside diameters less than 100 μm, detecting a few nanoliters of a low concentration sample is difficult. To date, there have been a number of detection schemes employed in CZE, including electrochemical, radioisotope, and optical detection methods. Currently the most sensitive is laser induced fluorescence (LIF), with detection limits for most systems in the low attomole

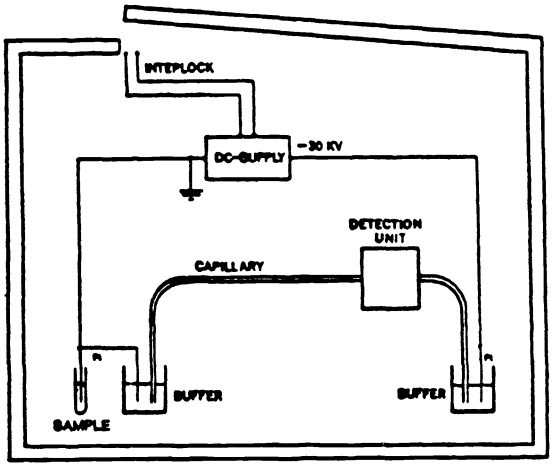

Figure 1. Capillary electrophoresis system showing the buffer filled capillary; 20,000 V is applied across the capillary during an electrophoretic separation.

range.[8-11] In these systems, the laser illumination is perpendicular to the capillary, the capillary is used as the flow cell, and the fluorescence emission is detected with a photomultiplier tube. Dovichi and co-workers[12-13] have developed a more sensitive method that also uses perpendicular illumination but uses a sheath flow cuvette as the sample cell. This greatly reduces the scattered light and the luminescence from the capillary walls; with this method, detection limits are in the low zeptomole range for fluorescently tagged amino acids.

For the work described here, we use the most sensitive low-light level detector available-- the cooled, slow-scan, scientific charge-coupled-device (CCD). Several recent review articles describe the operating and performance characteristics of CCDs in detail,[14-16] with additional articles describing the use of CCDs for analytical fluorescence detection.[17,18] CCDs can have a lower dark count rate and much higher quantum efficiencies than PMTs, but suffer from a finite, but exceedingly small read noise. The biggest obstacle to overcome with CCDs is their slow readout rate. While the individual elements in the CCD can be read at 50-200 kHz with cooled, scientific slow-scan systems, the frame rate is still several seconds for the entire array. An analyte band passes a given point on the capillary in milliseconds so that a several second long (shutter closed) deadtime during readout is problematic.

Because of the slow readout rate, the CCD cannot simply replace a PMT in the CZE/LIF system. In the one published report of the use of a CCD with CZE, after each 0.2-s exposure of the CCD to the fluorescence emission, approximately 5-s were required to readout the CCD and transfer the information to the host computer.[19] Due to the low duty cycle of the detector (most of the time the shutter is closed), the sensitivity of this system is not high

enough for the proposed neuropeptide experiments.

We circumvent the array readout problem by using a unique axial illumination system where the laser is focused through the end of the capillary and the fluorescence from the analyte is collected over a several centimeter capillary section. The emission is imaged onto the entrance slit of the spectrograph and detected with the CCD. Thus, the flow cell is on-column, and the fluorescence emission from a single analyte band is collected for between 2 and 45 seconds, depending on the electrophoretic mobility of the analyte. Because of the long observation time, the sensitivity of the system is high--in the low zeptomole range for derivatized amino acids and peptides. In order to demonstrate the ability to separate and detect the quantities of peptides found within a small section of a cell, a series of experiments are presented showing the detection and semiquantitation of less than 10^{-18} moles of neuropeptides from extracts of the bag cells of *Aplysia*.

2. CZE/LIF/CCD SYSTEM

This section describes the LIF/CCD detection system, the CZE separation system, and the derivatization and *Aplysia* work. For greater detail on the axial illumination, optical and detection system, the interested reader is referred to Reference 20.

2.1 Optics and Separation System

The goal of the axial illumination system is to focus the laser into the buffer-filled capillary as efficiently as possible. The capillary does not fulfill the requirement of a light-pipe in that the index of refraction is higher for the walls than for the aqueous center. Thus light tends to travel through the walls of the capillary rather than through the channel. We use this to advantage by focusing the light carefully into the capillary, and after the 2 cm observation zone, bend the capillary sharply. The bend causes any remaining light in the center channel to radiate into the walls and out of the aqueous channel. In this way, photodestruction of the fluorophores only occurs in the region where the fluorescence emission is collected.

The overall system is shown in Figure 2. The end of the capillary is mounted in a quartz cuvette held at ground potential; this end is the output of the capillary electrophoresis channel. In these experiments, a 63 μm I.D. capillary is used, and the focusing lens is chosen for a <50 μm 1/e spot size. The imaging optics are designed to image a 2.5 cm section of the 63 μm channel onto the 6 mm by 0.25 mm entrance slit of an ISA CP200 spectrograph. The mirror and lenses are chosen to

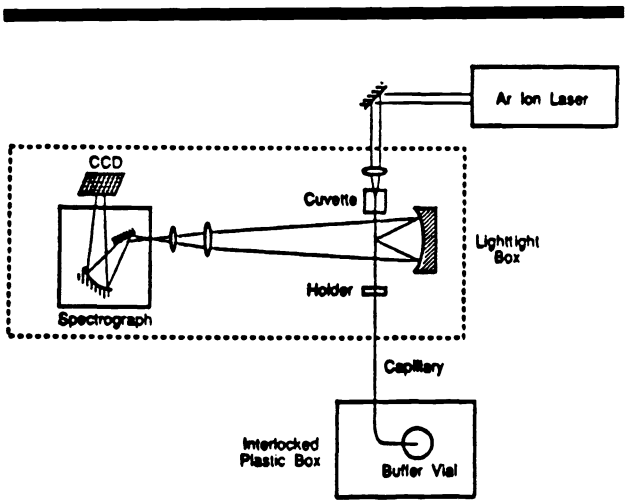

Figure 2. Diagram of the Ar Ion laser, axially illuminated separation capillary, optics and the CCD detector.

optimize light collection; because the requirements of the optics are different for the two dimensions, cylindrical optics are used. The Photometrics PM512 CCD is located at the focal plane of the spectrograph. Thus, 516 516-point fluorescence spectra are obtained from each exposure, with each spectrum corresponding to the image of a different point on the capillary.

The capillary electrophoresis system is similar to that described previously.[21] A 4-cm section of the protective polyimide coating from the 63-μm i.d., 363-μm o.d. capillary (Polymicro, Phoenix, AZ) is burned off with gentle heating in a flame. The inlet end of the capillary, held at positive high voltage, is placed in a 4-ml vial containing several ml of electrolyte. The electrical circuit is completed by placing strips of Pt foil submersed in both buffer reservoirs. All separations employ a voltage of 20 kV potential across the capillary. The capillary inlet is contained in an interlocked plexiglass box, and the outlet in a light-tight optical enclosure. This prevents possible exposure to the high voltage during operation.

All samples are introduced onto the capillary by gravity injection. The inlet end of the capillary is removed from the buffer reservoir and placed in an elevated sample microvial. For the experiments reported here, the height displacement is 5 cm, and the injection times range from 10 to 30 s, which introduces between 4 and 13 nl of sample.

2.2 Reagents and Sample Preparation

The water used to prepare all solutions is freshly deionized and distilled. The supporting electrolyte for all the experiments is 50 mM borate at pH 9.0, prepared from reagent grade sodium borate decahydrate and boric acid (Mallinckrodt). Stock solutions of fluorescein isothiocyanate (FITC) isomer 1 (Aldrich) fluorescein (Sigma) and sulforhodamine 101 (Exciton) are prepared at approximately 10^{-5} M and used without further purification. All low concentration solutions are made

fresh daily by serial dilutions from the stock solutions. Amino acids are purchased from Sigma and used without further purifications. Synthetic neuropeptide standards are from Peninsula Laboratories and have been purified by reversed phase HPLC.

The amino acids are individually derivatized with FITC isomer 1 using the procedure described by Kawauchi[22] which we have modified.[20] The derivatization of the amino acids and peptides are carried out at high concentrations and diluted immediately prior to use, while the derivatization of the cell extract is performed at considerably lower concentration.

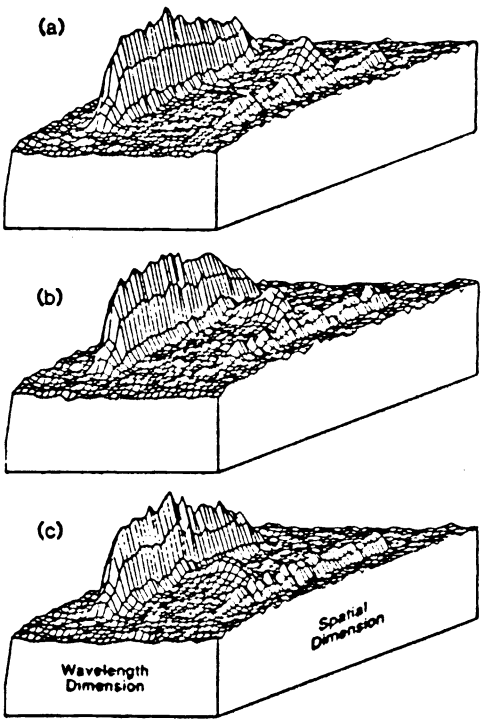

Figure 3. Snapshots of the CCD output for a single 3 amol injection of FITC: (a) after 13 minutes, (b) 4-s later, and (c) 6-s after (b).

3. TIME-DELAYED INTEGRATION

The ability of the optical system to image an analyte band undergoing electrophoresis is demonstrated in Figures 3a to 3c. These focal plane outputs represent 0.5 s exposures of the CCD to the capillary. In Figure 3a, the 3 amol injection of FITC has just entered the observation region, and Figure 3c is an exposure taken 10 s later of the same band during the same electrophoretic run. The Rayleigh and Raman scattering bands are clearly visible, along with the FITC fluorescence. Notice that even with the very low laser power (approximately 30 μW) the FITC has undergone considerable photodegradation in the 10 s between these images. Even though the analyte bands migrate during the time the shutter is closed during CCD readout, the band is still in the observation zone after many exposures.

Problems still exist with this method of data acquisition: the low duty cycle of the detector is far from ideal, the use of the CCD to take a series of exposures

creates the need to extract the information from multiple exposures and these multiple exposures increase the effective read noise of the CCD. An improved method of operating the system, with reduced data generation, lower read noise, and most importantly, a 100% duty cycle employs the time-delayed integration readout method.

The time delayed-integration (TDI) method involves synchronizing the CCD readout rate with the analyte band migration rate. Figure 4 shows a simplified block diagram of the CZE capillary and the CCD system demonstrating the

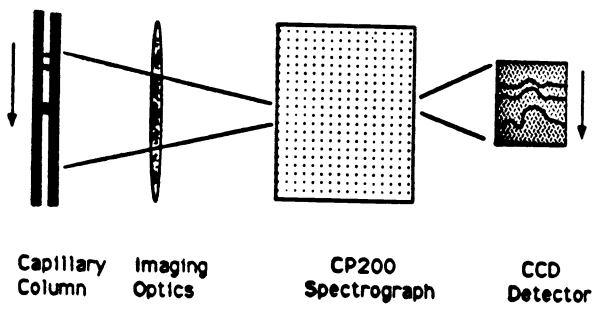

Figure 4. In the TDI mode, the CCD shift rate is synchronized to the analyte band migration rate.

TDI method. As the band migrates down the capillary, its fluorescence image moves across the CCD. When the CCD shift rate is matched to the band migration rate, the fluorescent image always falls on the moving accumulating band of photogenerated charge. Because the high voltage start time is known, the geometry of the system is fixed, and the electrophoretic mobility of a sample is essentially constant during a run, it is an easy matter to calculate the optimum shift rate for any given time.

A TDI electropherogram is shown in Figure 5.[20] In this run, 3 amol of fluorescein and sulforhodamine are injected, and every 16th spectrum plotted. The Rayleigh

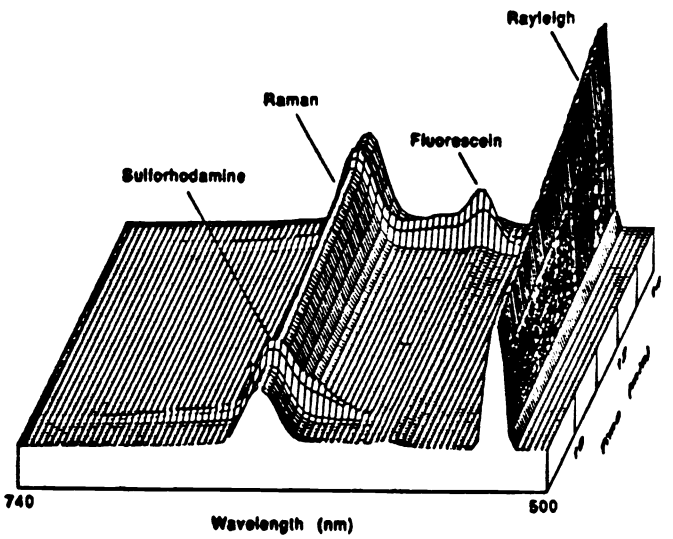

Figure 5. TDI Electropherogram of 3 amol of sulforhodamine and fluorescein, with the major Rayleigh and Raman bands of water clearly visible.

and Raman bands are clearly visible. Also obvious is the increase in the Rayleigh and Raman bands as a function of time. The integration time for each succeeding

spectrum is longer than the proceeding spectrum to make up for the slower moving analyte bands. For all subsequent electropherograms, two wavelength cutoff points are used, with the intensity values between the cutoff values averaged.

4. RESULTS AND DISCUSSION

The major goal of this project is the separation and analysis of amino acids and peptides from extremely small samples. In order to demonstrate this capability, approximately 300 zmol (≈3 x 10^{-19} mol) of four FITC labeled amino acids have been injected onto the column. Figure 6 shows the resulting electropherogram.[20] The detection sensitivity for these amino acid ranges from 2-8 x 10^{-20} mol.

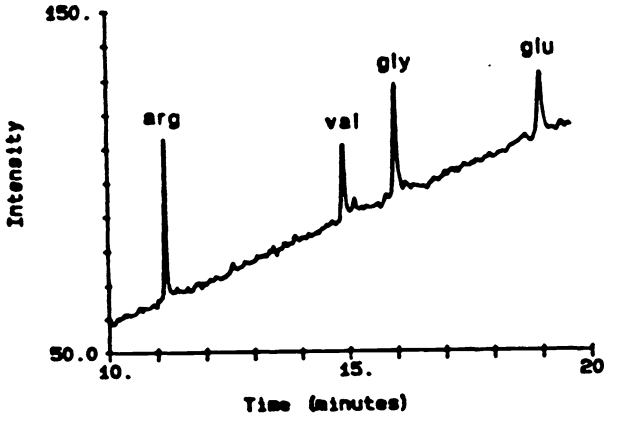

Figure 6. Electropherogram of four FITC-amino acids, with less than 0.3 amol of each injected onto the column.

The sloping baseline is an artifact of the changing shift rate; the integration time is continually increasing to allow for the slower moving, later analyte bands. Figure 7 shows an electrophoretic run of 25 zmol of FITC, corresponding to approximately 14,000 molecules, with a limit of detection of several thousand molecules.[20] This result is particularly exciting because these quantities are roughly the values expected within a single synaptic vesicle.

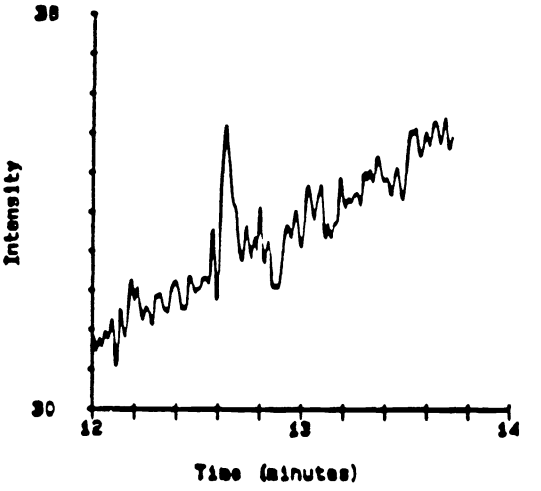

Figure 7. Electropherogram of 25 zmol FITC; this corresponds to approximately 10,000 molecules, or roughly the amount expected in a single synaptic vesicle.

In order to demonstrate the ability of this system to analyze peptides from a small section of a single cell, a single bag cell cluster containing approximately 400 cells is removed from an Aplysia, homogenized and diluted to 2

ml; the extract, after purification, is derivatized, and the resulting material diluted to ≈1000 ml. Approximately 10 nl of this solution is injected and a three minute section of the resulting electropherogram is shown in Figure 8. This injection corresponds to the amount of material contained in approximately two millionths of a single cell. The object of these manipulations is to demonstrate the sensitivity of the system independent of the sample handling and derivatization problems of trace analysis. In Figure 8b and 8c, the sample has been spiked with synthetic γ and ELH; thus, two of the major peaks appear to be important bag cell neuropeptides. The additional peaks in 8c are impurities in the HPLC purified synthetic peptides.

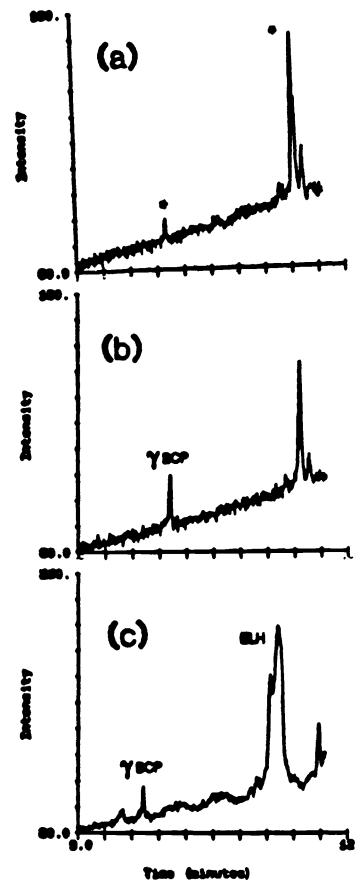

Figure 8. Electropherogram of approximately two millionths of a single bag cell neuron: (a) extract, (b) spiked with γ, and (c) spiked with both γ and ELH.

In conclusion, the combination of a CCD used in the TDI mode with an axially illuminated capillary electrophoresis system provides sensitivity for derivatized amino acids and peptides in the low zeptomole (10^{-21} mol) range, opening up new realms for small volume analysis. The sensitivity and assaying capabilities allow studies of the differential distribution and distribution mechanisms of neuropeptides within an individual cell. We expect the sensitivity and utility of the system to be improved as improvements in the optical detection and axial illumination system are made; however, great attention is needed to develop the new methods of sample handling and derivatization needed to work with these ultra-small samples.

5. ACKNOWLEDGEMENTS

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6. REFERENCES

1. Fisher, J.M.; Sossin, W.S.; Newcomb, R.; Scheller, R.H. *Cell* **1988**, *54*, 813.

2. Newcomb, R.W.; Scheller, R.H. *Brain Research* **1990**, *51*, 229.

3. Sossin, W.S.; Fisher, J.M.; Scheller, R.H. *Neuron* **1989**, *2*, 1407.

4. Sossin, W.S.; Sweet-Cordero, A.; Scheller, R.H. *Neurobiology* **1990**, in press.

5. Gorden, M.J.; Huang, X.; Pentoney, S.L., Jr.; Zare, R.N. *Science* **1988**, *242*, 224.

6. Ewing, A.G.; Wallingford, R.A.; Oleferowicz, T.M.; *Anal. Chem.* **1989**, *61*, 292A.

7. Jorgenson, J.W.; Lukacs, K.D. *Science* **1983**, *222*, 266.

8. Roach, M.; Gozel, P.L.; Zare, R.N. *J. Chromatogr.* **1988**, *426*, 129.

9. Drossman, H.; Luckey, J.A.; Kastichka, A.J.; D'Cunhan, J.; Smith, L.M. *Anal. Chem.* **1990**, *62*, 900-903.

10. Nickerson, B.; Jorgenson, J.W. *HRC&CC. J. High Resolut. Chromatogr., Chromatogr. Commun.* **1988**, *11*, 533-34.

11. Kuhr, W.G.; Yeung, E.S. *Anal. Chem.* **1988**, *60*, 2642-2646.

12.Cheng. Y.-F; Dovichi, N.J. *Science* **1988**, *242*, 562-4.

13. Wu, S.; Dovichi, N.J. *J. Chromatogr.* **1989**, *480*, 141-55.

14. Sweedler, J.V.; Bilhorn, R.B.; Epperson, P.M.; Sims, G.R.; Denton, M.B.; *Anal. Chem.* **1988**, *60*, 282A-91A.

15. Epperson, P.M.; Sweedler, J.V.; Bilhorn, R.B.; Sims, G.R.; Denton, M.B. *Anal. Chem.* **1988**, *60*, 327A-35A.

16. Blouke, M.M.; Janesick, J.R.; Elliott, T.; Hall, J.E.; Cowens, M.W.; May, P.J. *Opt. Eng.* **1987**, *26*, 864.

17. Epperson, P.M.; Denton, M.B. *Anal. Chem.* **1989**, *61*, 1513-19.

18. Epperson, P.M.; Jalkian, R.D.; Denton, M.B. *Anal. Chem.* **1989**, *61*, 282-5.

19. Cheng, Y.-F.; Piccard, R.D.; VoDinh, T. *Applied Spectrosc.* **1990**, *44*, 755-65.

20. Sweedler, J.V.; Shear, J.B.; Fishman, H.A.; Zare, R.N.; Scheller, R.H. *Anal. Chem.*, in press, 1991.

21. Gassman, E.; Kuo, J.E.; Zare, R.N. *Science* **1985**, *230*, 813-4.

22. Kawauchi H.; Tuzimuri, K.; Maeda. H.; Ishida, N. *J. Biochem.* **1969**, *66*, 783-9.