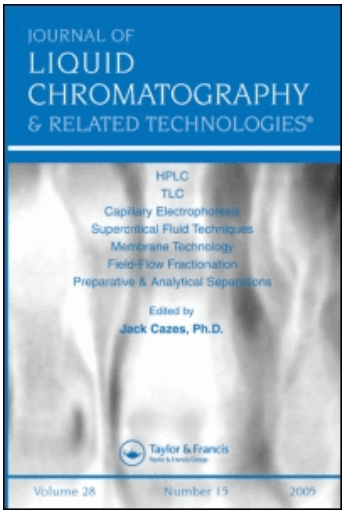

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SIMULTANEOUS MEASUREMENTS OF CAPILLARY ELECTROPHORESIS FLUORESCENCE PEAKS AND THEIR CORRESPONDING SPECTRA

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ABSTRACT

Capillary electrophoresis coupled to laser induced fluorescence detection is one of the most sensitive techniques for chemical analysis and its applicability could be remarkably increased if spectral analysis is incorporated to it. With this view we have modified the conventional system by introducing CCD and PMT measurement simultaneously. The data obtained from PMT was processed and the peak position was determined by second derivative to trigger CCD system. With this

method the electropherogram for peak identification by migration time and the spectrum corresponding to each peak were simultaneously recorded. The obtained spectra for various amino acids attached to Fluorescein Isothiocyanate (FITC) are also reported.

INTRODUCTION

Capillary Electrophoresis (CE) with Laser induced fluorescence detection (LIFD) is known to be one of the most sensitive separation and detection techniques [1,2,3]. Using LIFD as low as 10^{-13} molar concentrations of FTC-amino acids has been reported by us [4]. In addition, the method is suitable for detection of very small quantities of analyte enabling useful application in biology, analytical chemistry and medical sciences where the detection of minute traces of some chemicals plays a key role. Laser induced fluorescence detection is normally performed only with a sensitive photomultiplier tube (PMT) to detect fluorescence labeled species. However, the applicability of this technique will be enhanced if it can be used to record the spectrum of each analyte which passes through the capillary window since the spectral analysis can be considered as the fingerprints of the particular substances.

Among the spectral analysis devices, CCD offers a high photoresponse and high dynamic range. Moreover, essential virtue of CCD system for this type of investigation is that the spectral measurements are carried out with a single shot rather than conventional scanning procedure. But a note of caution should be introduced. Being the CCD a charge storing device, its sensitivity increases with exposure. For extremely short collection times (picoseconds) the PMT is more sensitive.

Cheng et al incorporated for the first time a Charge Coupled Device in the snap shot mode to detect amino acids labeled with Fluorescein Isothiocyanate in a CE-LIFD instrument [5]. The CCD was placed as a camera oriented orthogonally to the laser and pictures of the capillary were taken. The fluorescent images were built as peaks by a computer. In this seminal experiment more than 5 seconds were required to transfer the data to the computer and each exposure lasted 0.2 second enhancing the chances of missing bands. In addition, for a typical run massive amount of data were collected.

Using axial illumination and a 2 cm window on the capillary, Sweedler et al at Zare's laboratory analyzed

fluorescence with a CCD in a time delayed integration mode [6]. By synchronizing the movement of the fluorescent band with the clock of the CCD so that the photogenerated charge moved with the band they enhanced the sensitivity of the detection system. Picomolar concentration and zeptomole amounts of Fluorescein and Sulforhodamine were detected. At the same time they were able to obtain the spectra of both dyes. These achievements were due to an elaborated optics and careful considerations in the synchronization procedure. The optics had to expand the laser beam to illuminate the whole 2 cm window and to focus its image on a column of the photosensitive detector array. The clock of the CCD had to be synchronized with a band which moved in nonuniform fashion, a non trivial task and certainly not suitable for routine analytical work.

Taylor and Yeung axially excited an array of capillaries by means of optical fibers [7]. This excitation method simplified cumbersome alignment procedures. The fluorescence was collected with CCD and bands for each capillary were recorded. Since no synchronization of the moving band and the clock of the CCD was used, 0.1 sec snap shot had to be taken and that

diminished the sensitivity. Later on Ueno and Yeung expanded the capillary array to 100 but had to give up to the axial excitation and resorted to a more complicated excitation optics [8]. Although they sacrificed optical simplicity they successfully detected the bands by means of a CCD camera in an array of 100 capillaries.

Nilsson et al followed a different strategy for CCD application in CZE-LIFD [9]. They expanded the laser beam to illuminate a substantial part of the length of the capillary and collected the whole image of the capillary at a given angle with a CCD camera. This allowed to follow all the fluorescent bands in real time on the screen of a computer. By binning the CCD over 50 pixels they obtained a line profile showing the fluorescence intensity versus position along the capillary. Interestingly they found that peak width changes in unpredictable way due to imperfections of the capillary and this should hinder the time delayed integration mode used by Sweedler et al.

Takahashi et al have used the orthogonal arrangement with the sheath flow cuvette method in capillary array electrophoresis [10, 11]. An arrangement of sheath flow

cuvettes was illuminated with a single laser beam that crossed the cuvettes for excitation. A CCD camera was placed perpendicular to the laser beam for simultaneous detection of the bands in the cuvettes.

In most of the above experiments the CCD was used as an imaging facility (i.e. in camera mode) to record the illuminating spot, tube or band. In principle, this does not provide additional information as compared to the conventional PMT detector. In the present work, we are employing a CCD as a detector attached to a spectrometer which permits to record the spectrum of the moving molecules.

In all those CCD experiments the collinear geometry was never employed. Optical simplicity as well as excellent stray radiation rejection are among the main advantages of this geometry. These advantages cooperate for enhanced sensitivity for CE-LIFD applications[12]. In preliminary experiments we have hydrodynamically injected FITC labeled amino acids and analyzed their spectra by means of a liquid nitrogen cooled CCD in a collinear LIF detector. High sensitivity as well as slight but significant differences in the spectra of FTC-amino acids have been observed [13]. However, the

small magnitude of the differences necessarily required a high resolution.

If the CCD system collects in the snap shot mode all the time then, the store devices of the data acquisition system will be quickly saturated. This trouble gets worse when high resolution CCD is employed. Ideally in the snap shot mode the CCD should collect data just for the time that the analyte goes through the detection window of the capillary. This reasoning suggested that the combination of a photomultiplier and a high resolution CCD would allow to record the peak and to trigger the CCD, providing the electropherogram plus the relevant spectral data. Such PMT-CCD combination should not collect data when there is no band in the window.

In the present article we report the successful combination of a PMT and a CCD for the detection of electrophoretically separated FTC-amino acids and their spectral measurements in a narrow bore capillary.

EXPERIMENTAL CONDITIONS

Instrument: Figure 1 shows a diagram of the experimental set up. The CE system is a collinear instrument already described [3,12]. Briefly, a 3 mW Argon ion laser (Ion

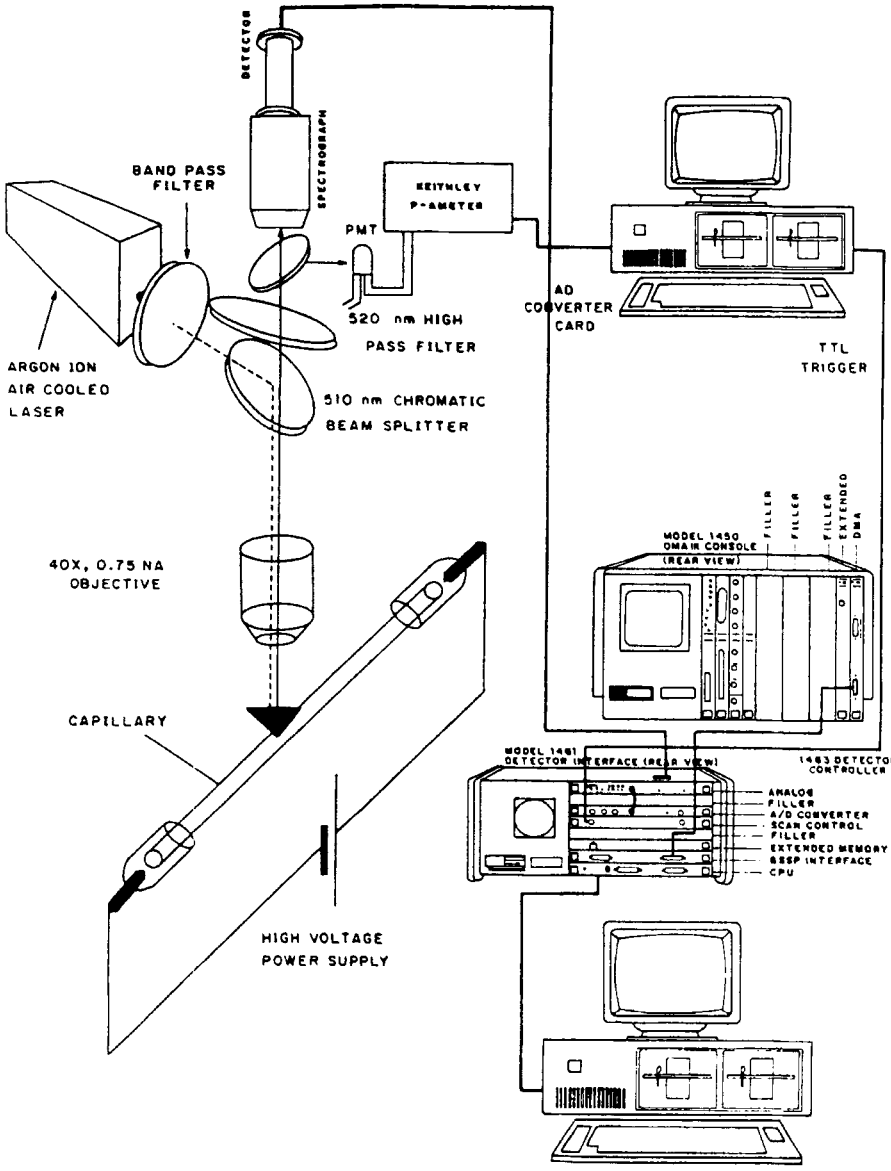

Figure 1.-

Experimental set up to measure CE peaks and corresponding spectra simultaneously.

Laser Technology, Salt Lake City, USA) was tuned to 488 nm and reflected by a dichroic mirror centered at 510 nm (Carl Zeiss, Caracas, Venezuela). The laser was focused by means of a .75 NA objective (Carl Zeiss, Caracas, Venezuela) on the window of the capillary (Polymicro Technologies, Phoenix, AZ, USA). The window was located at 20 cm of the anodic end of a 30 cm long, 20 μm bore fused silica capillary filled with a 20 mM carbonate buffer at pH 9.4. The fluorescence was collected by the same objective and stray radiation was attenuated by a high pass filter centered at 520 nm (Carl Zeiss, Caracas, Venezuela) and a notch filter centered at 488 nm (Andover, Salem, NH, USA). Then the radiation was split by a beam splitter centered at 530 nm (Andover, Salem, NH, USA). One beam was focused on a R1477 multialkali PMT (Hamamatsu, Bridgewater, NJ, USA) driven by a HC-123 miniaturized high voltage power supply (Hamamatsu, Bridgewater, NJ, USA). The other beam was focused on the entrance slit of the monochromator [13].

The spectrometer had a 0.5 m optical path(EG&G, Princeton, NJ, USA, model 1236) and was employed with a cryogenic cooled CCD detector model 1433-C with a 1433-1 controller and a 1461 detector interface system(EG&G,

Princeton, NJ, USA). The temperature of the detector was maintained at -140°C throughout the experiment. In the present investigation, the scan control board in the detector interface permits TTL level (0-5 Volt) for external triggering. A stand-alone 386 MS-DOS pc runs the Princeton Applied Research OMA-2000 software to collect spectral data via the detector interface.

The PMT anode output is monitored with an Electrometer (Keithley, Cleveland, OH, USA, model 614) which allows us to use the analog preamplifier voltage output between 0 and 5 Volts. The Electrometer signal is sampled with a 5 Volt AD voltage converter card (resolution was 2.4 mV) AI08G type from ICS (Industrial Computer Source, San Diego, CA, USA) with its corresponding PASCAL software specially prepared for this purpose. It was most convenient to use a second stand-alone 386 microprocessor type MS-DOS PC to process the digitized PMT data. A special program was written to measure the CE recording data and trigger the CCD detector via the scan control board input on the CCD detector interface.

Real-time synchronization of the experiment is performed by using simple build-in PASCAL function

calls. The sampling rate is limited due to computing power to calculate the derivatives. We could sample up to 700 AD conversions and process (including first and second derivatives) per second with the present computer configuration. Averaging a high number of AD conversions is an indispensable tool to reduce the noise. The number of stored data points in memory is limited in our computer configuration to some 4000 time/intensity data points. So averaging about 120 AD conversions permitted us to sample 6-10 data points per second for the required maximum 15 minutes of measuring the electropherogram. The effective sampling rate of 6-10 Hz during 15 minutes turned out to be enough for this purpose.

Recently, Timperman et.al.[14] have demonstrated the feasibility of wavelength resolved fluorescence detection by using a CCD system which was triggered at a certain predetermined time interval independent of whether the analyte was passing through the window or not. This required excessive (and also unnecessary) use of memory of the computer and hence it is not convenient for routine measurements. Moreover duration time used for data collection is limited by triggering

interval. These short comings are overcome by different approach. During the measurements, in the time between each stored data point in memory the program calculates and estimates the first and second derivative which permits us to find the best moment to trigger the CCD detector system to measure a complete luminescence spectrum i.e. somewhere at or just before the CE peak maximum. Triggering on second derivative gave the best result, i.e. near the highest CE peak intensity. The first derivative indicates the beginning of the peak; obviously all the small peaks originated from noise are handled by the software. The triggering due to noise is avoided by setting a certain offset level (see figure 2). The triggering event on the second derivative is typically some 2-3 seconds before the peak maximum. With a typical 3 seconds CCD shutter opening time, it can be covered more or less the whole peak during the CCD exposure. During 1.5 seconds after closure of the CCD shutter (the time needed to read the CCD pixels and transferring the data to the detector interface memory) the CE measurement program does not deliver triggering pulses. This domain is considered as a blind period for which the other spectral measurement cannot be carried out.

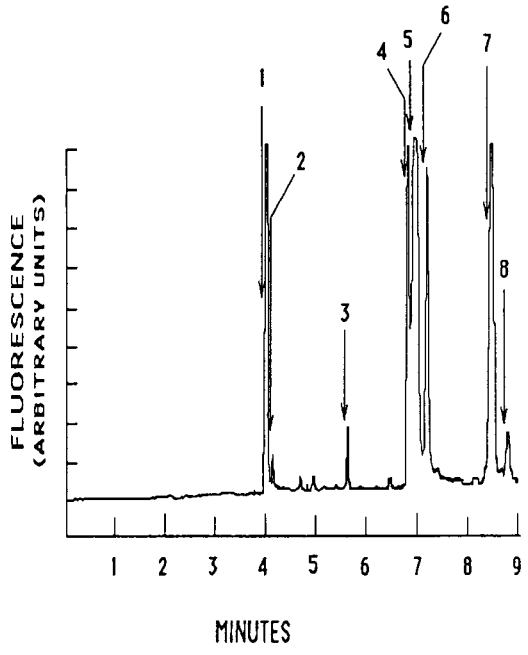

Figure 2.-

Electropherogram of a mixture of 8 amino acids. The arrows indicate the time when the CCD camera was triggered. Notice the absence of arrows on small peaks. 1= FTC-Arginine, 2= FTC-Asparagine, 3= FTC-Gutamine, 4= FTC-Proline, 5= FTC-Threonine, 6= FTC-Glycine, 7) FTC-Tryptophane and 8) FTC-Methionine.

Reagents: Sodium Carbonate, Sodium Bicarbonate, Sodium hydroxide, Arginine, Glutamine, Methionine, Asparagine, Proline, Threonine, Glycine, Tryptophan, Fluorescein Isothiocyanate (Isomer 1) HPLC grade Acetone were obtained from Sigma Chemical Co (Sigma Chemical, ST. Louis, MO, USA).

Derivatization procedure: A 20 mM Carbonate buffer at pH 9.4 was filtered through .22 μm filters (Millipore, Bedford, MA, USA). The amino acids were dissolved in carbonate buffer to obtain a 1 mg/ml solution. A $4x10^{-4}$ M solution of FITC in acetone was prepared. Then 1 ml of the amino acid solution was combined with 10 μl of FITC solution and allowed to react for 4 hours in the dark. Since FITC was the limiting reactant, the final concentration of FTC-Amino acid was $4x\ 10^{-6}$ M. This solution was diluted in steps of 10 to measure the limit of detection of the system.

Capillary Electrophoresis: Each amino acid was run individually and also combined with the other amino acids in a single solution. The electrophoretic run consisted in injecting a plug of the test solution by the hydrodynamic method. The anodic end of the capillary was immersed in the sample reservoir and a pulse of -19 psi was applied for 0.3 sec at the cathodic end. The anodic end was withdrawn from the sample reservoir and immersed in a buffer reservoir. Both the cathode and the anode were made of Platinum-Iridium wire. 20 KV from a high voltage power supply (Bertan, Hicksville, NY, USA, model 30R) were applied between the two ends of the

capillary. After each run the capillary was rinsed with 0.1 N sodium hydroxide solution for 2 minutes followed by water for 2 minutes and 20 mM carbonate buffer for 3 minutes.

RESULTS AND DISCUSSION

Figure 2 shows the conventional CE peaks of the mixture of eight amino acids recorded with the PMT detector. The figure also shows the exact time when the trigger was activated. Differences in peak amplitude are due to the reaction time of individual amino acids and different photoluminescence efficiency. Fig 3 and Fig 4 show the spectra corresponding to each peak in CE measurement. Curve number 8 of figure 4 looks like a straight line because of its low intensity as compared with the other curves. We have, therefore, plotted the curves number 1 and 8 with different scales as shown in figure 5. Each peak is identified with the migration time as mentioned in the caption of figures 3, 4 and 5. Intensity of the peak in CE corresponds to the area under the curve of each spectrum and does not show any relation with the peak intensity of the spectrum. It is worth mentioning that with the present experimental set

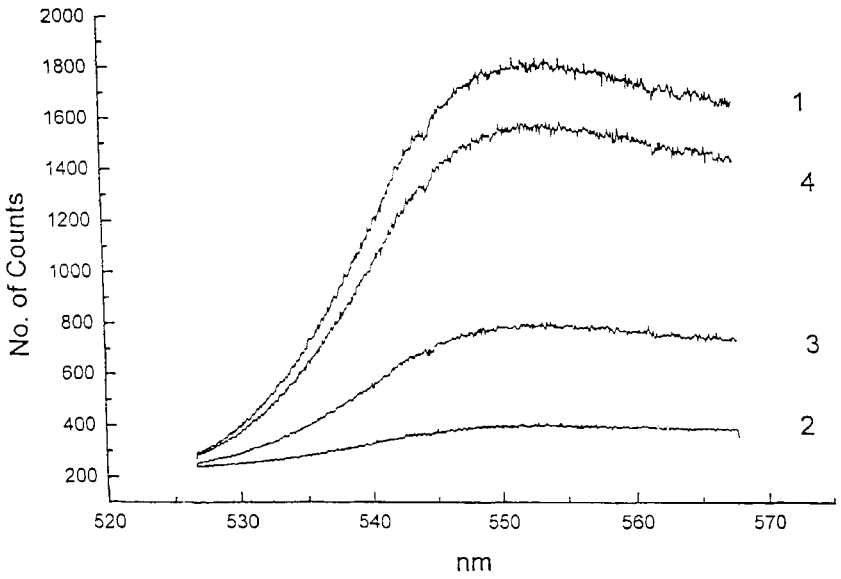

Figure 3.-

Spectra recorded for 1= FTC-Arginine, 2= FTC-Asparagine, 3= FTC-Glutamine and 4= FTC-Proline.

up we could measure photoluminescence spectra down to 10^{-09} molar concentration with a noticeable peak intensity with only 3 seconds exposure. This nanomolar sensitivity is three orders of magnitude lower than the picomolar sensitivity easily reached with the collinear geometry together with a PMT detector. Several factors explain this sensitivity reduction. For instance, compared to the conventional collinear instrument, in the present instrument the optical path to the CCD is

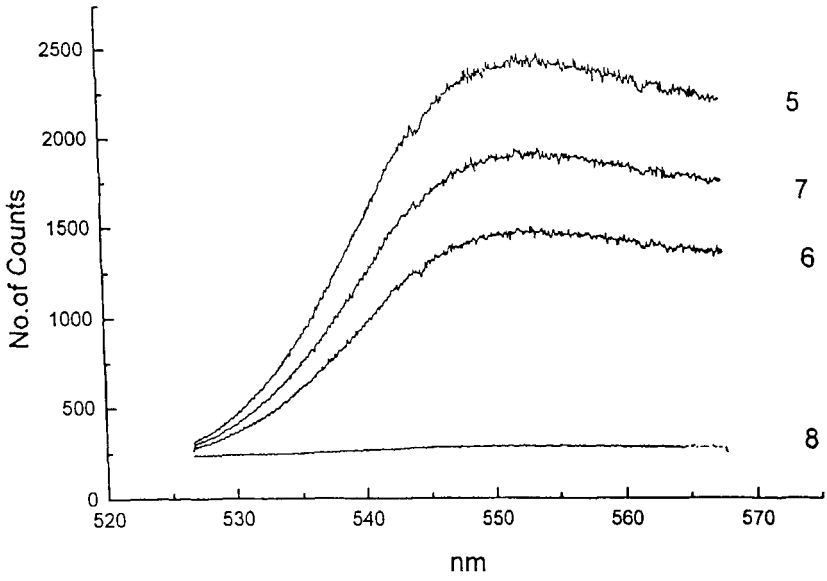

Figure 4.-

Spectra recorded in the same experiment as in figure 3. 5= FTC-Threonine, 6= FTC-Glycine, 7= FTC-Tryptophane and 8= FTC-Methionine. Notice that the spectrum of FTC-Methionine looks flat due to the high scale.

more than 50 cm longer, the fluorescence is split for two photodetectors and the slit of the monochromator limits the amount of fluorescence reaching the CCD. Nevertheless, it is possible to push down the detection limit in the present design by selecting the proper optical component such as a beam splitter with 90 % transmission intensity, using cylindrical mirrors for fluorescence collection (15) and so on. In any event

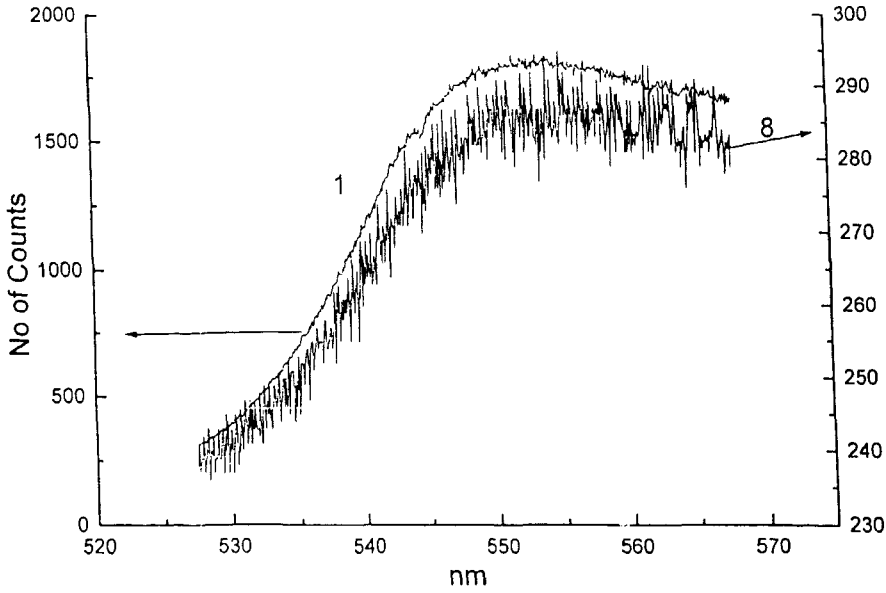

Figure 5.-
Spectra of FTC-Arginine (1) and FTC-Methionine (8). Notice the different scales for the FTC-Arginine spectrum (left scale) and for the FTC-Methionine spectrum (right scale)

this sensitivity is two orders of magnitude higher than the required sensitivity for DNA sequencing applications. In addition, the present design simplifies the discrimination of four different fluorochromes. Other designs have used a complicated combination of filters and until four PMTs to distinguish the four fluorochromes (16). The present design uses a single CCD for the analysis of the four fluorochromes.

In previous experiments we observed slight but consistent spectral differences between the FTC-amino acid spectra. This differences included disparate relative intensity at the peak position and non proportional shape on the high energy side of the FTC-amino acid spectra (13). Similar differences were observed in the present experiments.

In short, the present experimental set up along with the tailored software permit to record simultaneously CE peak and its corresponding photoluminescence spectrum with ease. It is clear that this experimental set up is easy to automate and speed up the measurement capabilities. Reasonable amount of spectral data and their theoretical interpretation will be necessary to explore full possibilities of capillary electrophoresis. Such study will certainly have several applications in ultra sensitive spectroscopy based clinical diagnostic systems. Moreover, in recent investigation on diagnosis of certain diseases, it has been found that spectral information is very useful, particularly in early diagnosis of Cancer [17].

The current approach of analysis is based on the retention time of a particular species. Spectral

analysis will be an additional support for classification and analysis. If properly applied , this methodology can also be useful to improve DNA sequencing techniques based on capillary-array electrophoresis.

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