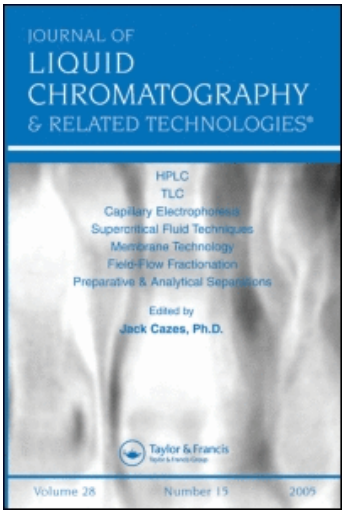

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Laser-Based Fluorimetric Detection Schemes for the Analysis of Proteins by Capillary Zone Electrophoresis

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LASER-BASED FLUORIMETRIC DETECTION SCHEMES FOR THE ANALYSIS OF PROTEINS BY CAPILLARY ZONE ELECTROPHORESIS

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ABSTRACT

Presented herein are three laser-based fluorimetric detection schemes for the analysis of proteins by capillary zone electrophoresis (CZE). Experimental parameters are discussed for high peak efficiency along with detection considerations. Detection of proteins is performed using the native fluorescence of the protein, precolumn labeling and on-column labeling with arylaminonaphthalene-sulfonates. Minimum detectable concentrations are presented as well as a discussion of the merits of each method.

INTRODUCTION

Capillary zone electrophoresis (CZE) is an efficient method for separating charged species that is based on differences in electrophoretic mobility. Because of the plug-

like flow profile of CZE, molecular (axial) diffusion generally limits peak efficiency, provided solute-capillary wall interactions and thermal gradients are eliminated(1-3). Since proteins are generally charged and possess very low diffusion coefficients, they are amenable to efficient separation by CZE. Numerous reports in the literature illustrate high efficiency and resolving power in the separation of proteins, however, very little information about detection has been presented(4-10).

The diminutive capillaries employed in CZE, and the concomitant small peak volumes, greatly complicate detection. Detection is generally performed on-column using optical detectors that employ relatively short time constants(11, 12). Short pathlength, low throughput, and the potential for large amounts of stray light are consequences of on-column detection, which can have deleterious effects on detectability. To provide adequate detectability, a variety of detection methods (for example conductivity(13), electrochemistry(14-16), fluorimetry(8, 17-22) and mass spectrometry(23)) have been developed for CZE. However, not all of these methods have been applied to the detection of proteins. Herein we discuss and demonstrate the use of several laser-based fluorimetric detection schemes for the detection of proteins in CZE.

A near zero background and a direct proportionality between excitation power and signal intensity render fluorimetry a very sensitive detection method. Although

fluorescence detection with conventional excitation sources has been applied to CZE(17), it has become more common to use a laser as an excitation source(8, 19-22). Lasers exhibit high excitation powers and are well-collimated, so their outputs are easily focused into the small flow channel of the capillary. Using this method, detection of less than 1 femtomole of strong fluorophores is easily achieved(19, 20, 24, 25). The limited number of easily used lasers and available fluorophores are the major obstacles to the general application of this method to CZE.

Laser-based fluorescence detection of nonfluorescent or difficult to excite species (such as proteins) has been preformed using pre-(12, 20, 25), on-(22, 26), or post-column(18, 19) derivatization, or "indirect" fluorimetry(8). The use of fluorescent labels (e.g. fluoroscein isothiocyanate, FITC), not only facilitates direct fluorimetric detection but the spectroscopic characteristics of the label can be matched to a particular laser wavelength so maximum detectability can be achieved. For example amino acids that were pre-column labeled with FITC have been detected in quantities as low as 10^{-21} moles injected(20). However, the application of pre-column labeling to proteins has been less successful (see later discussion), due to a loss in the integrity of the protein peak after labeling. On- and post- column derivatization schemes have been developed to address this problem(18, 19, 22). However, post-column schemes generally complicate the

experimental apparatus and are restricted to labels that exhibit no or little native fluorescence.

Indirect fluorescence detection can also be used for the detection of nonfluorescing compounds(8). This method involves "doping" the electrophoretic buffer with a fluorophore and detecting the reduction in fluorescence after displacement of the dopant. The displacement mechanism most typically used is ion repulsion. The potential for large charges on proteins suggest that each protein could displace numerous fluorophores, resulting in excellent detectability(8). However, the existence of other ions in the buffer will reduce displacement efficiency. Moreover, in electrophoresis it is generally observed that resolution is optimum when the pH of the electrophoretic buffer is near the isoelectric point (pI) of the protein, which minimizes the net charge on the protein(4, 6). These considerations can result in undesirable compromises in buffer composition that effect both detectability and separation.

The remainder of this paper will examine the applicability of various fluorimetric detection schemes to the analysis of proteins by CZE. We will describe the use of the native fluorescence of proteins for detection, the difficulties of pre-column labeling, and a novel method of on-column labeling with "hydrophobic protein probes". Conditions for accomplishing the latter method will be investigated and analytical figures of merit will be presented.

EXPERIMENTAL

Conalbumin (CON), bovine serum albumin (BSA), beta lactoglobin A and B (BLG A and BLG B), fluoroscein isothiocyanate (FITC), 1-anilinonaphthalene-8-sulfonate (ANS), 2-p-toluidinonaphthalene-6-sulfonate (TNS) and 2-[Cyclohexylamino]-ethanesulfonic acid (CHES) were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals employed were reagent grade and obtained from Baxter Healthcare (Stone Mountain, GA).

Separations and detection studies were performed using 25, 50, and 75 μm capillaries obtained from Polymicro Technologies (Phoenix AZ), which were washed with 1M KOH, 0.1 M KOH, and distilled water prior to initial use. Between injections the capillary was washed for 5 minutes with 0.1 M KOH, water, and buffer. Electrophoretic buffers were made with HPLC grade water, contained 20 mM CHES, and adjusted to pH 10.2 with KOH. High voltage was applied across the capillary using a Hipotronics (Brewster, NY) model 840A power supply.

Laser-based fluorescence detection of native proteins was performed using a Coherent (Palo Alto, CA) Inova 100 argon ion laser (514 nm, 7 W) that was frequency doubled to produce 257 nm radiation using an InRad (Northvale, NJ) model 527-003 harmonic generator. The excitation power was approximately 9 mW. Detection of FITC labeled proteins was accomplished using a Uniphase Cyonics model 2001SL (San

Jose, CA) argon ion laser (488 nm, 10 mW) for excitation. Excitation of ANS and TNS was performed using a Liconix 4230NB (Santa Clara, CA) helium-cadmium laser (325 nm, 15 mW). Fluorescence was collected normal to the excitation beam and isolated with a Instruments SA H-10 (Metuchen, NJ) monochromator. Emission wavelengths were as follows: native fluorescence - 325 nm, FITC labeling - 520 nm, ANS labeling - 500 nm, and TNS labeling - 450 nm. Detection was performed with a Hamamatsu (Bridgewater, NJ) 1P28 photomultiplier tube and photocurrents were processed with a Pacific Precision Photometer Model 126 (Concord, CA). Nonseparation fluorescence measurements were performed using a SpectroVision (Chelmsford, MA) fluorimeter.

Minimum detectable concentrations (MDC) for native fluorescence and FITC labeling were determined using static tests that have been described elsewhere and were based on a S/N = 2(12). Injections were performed hydrostatically, by raising the inlet of the capillary 15 cm for 10 to 30 seconds. The injection volume was determined by continuous injection of sample as described previously(27). Minimum injectable amounts and linear dynamic ranges were determined using calibration curves. Minimum detectable concentrations for ANS and TNS labeling were determined by calculating the concentration at band center for the lowest concentration on the calibration plot (S/N < 6) and extrapolating to S/N = 2.

Conalbumin was labeled with FITC by stirring 2 mg of protein, in 0.02 M sodium borate solution, with a the desired

amount of label for 24 hours at 4° C. Subsequently, a small amount of sample was removed and the remainder dialyzed against phosphate buffered saline to remove excess fluoroscein. The dialyzed sample was used to determine the MDC for a given dervatization ratio. Electrophoretic studies were performed using undialyzed samples.

RESULTS AND CONCLUSIONS

Considerations for High Peak Efficiency. Under ideal conditions efficiency in CZE is determined by axial diffusion(3). Therefore, due to their low diffusion coefficients, protein separations can result in very high plate numbers. However, experimental parameters such as pH, buffer conductivity, applied voltage, and capillary diameter must be optimized to achieve high efficiency. For example, it is possible to reduce protein wall interactions, which greatly reduce the efficiency of the band, by employing an electrophoretic buffer with a pH that is above the pI of the protein(4, 5, 28). Although this procedure reduces the selectivity of CZE, it will be employed in this work to assure high efficiency. Table 1 illustrates the effects of several other experimental parameters on the efficiency of conalbumin at a pH of 10.2.

A major advantage of CZE over traditional electrophoretic techniques is the ability to use high electric field strengths without experiencing detrimental thermal

Table 1.

Effects of Experimental Parameters on Peak Efficiency for Conalbumin.

Capillary Diameter	Mobile Phase Concentration of KCl					
	15 mM		30 mM		45 mM	
	N (m^{-1}) N/time ($m^{-1}min^{-1}$)	Power (Wm^{-1})	N (m^{-1}) N/time ($m^{-1}min^{-1}$)	Power (Wm^{-1})	N (m^{-1}) N/time ($m^{-1}min^{-1}$)	Power (Wm^{-1})
25 μm [a]	52,000 4,600	0.12	474,000 37,600	0.24	230,000 17,500	0.40
25 μm [b]			156,000 18,300	0.71		
75 μm [c]	52,000 2,600	0.30	10,000 400	0.53		

a) applied field 235 V/cm
b) applied field 350 V/cm
c) applied field 150 V/cm

Note: Electrophoretic buffer contained 20 mM CHES. Detection was preformed by native fluorescence.

effects(3). However, it is possible to overcome the capillary's ability to dissipate heat (e.g. by using high ionic strength electrophoretic buffers) and produce a radial temperature gradient within the capillary. Under these conditions the plug-like flow profile of electroosmotic flow is distorted and band broadening due to slow mass transfer across the flow profile occurs(2). However, it should be noted, if the conductivity of

the buffer is significantly lower than the conductivity of the sample, band "fronting" will be observed(29). This is responsible for the dramatic increase in efficiency in Table 1 when the KCl concentration was increased from 15 mM to 30 mM (25 μm capillary). Further increasing the KCl concentration (to 45 mM) increases the heating effect to the point of degrading efficiency. Increasing the applied voltage also increases the power that the capillary must dissipate. This results in a decrease in plate number, which is illustrated by the 25 μm i.d./30 mM KCl data at the two different field strengths. Since the power that can be dissipated by the capillary is determined by the surface to volume ratio of the capillary, the use of smaller diameter capillaries will allow the application of higher electric fields to minimize analysis time and axial diffusion without incurring thermal-related band broadening. This is illustrated by the higher efficiency of the 25 μm i.d./45 mM KCl data as compared to 75 μm i.d./30 mM KCl case, even though the smaller capillary has a greater thermal load. Moreover, the smaller diameter capillary results in lower electrophoretic currents, which reduces the power that must be dissipated by the capillary (compare the 30 mM KCl data for the two diameters).

Analysis time is another important consideration. Grushka et al. have calculated that band broadening due to temperature gradients is most detrimental at high flow rates in large diameter capillaries(2). Therefore, to achieve high

efficiency and short analysis times, small capillaries must be employed. This is illustrated by the larger number of theoretical plates per minute when the small 25 μm capillary is employed. This is a result of the decrease in thermal band broadening and a large increase in electroosmotic flow at the higher field strength. However, for these advantages to be realized detection schemes compatible with these capillaries must be developed.

Laser-based fluorescence detection can be utilized in very narrow-bore capillaries. With the high exciting power of laser sources it is possible to overcome the short pathlengths that result from on-column detection. Additionally, the collimation of the laser output permits tight focusing of the beam through the capillary. If a more divergent source were employed, it would be difficult to focus all of the excitation power into capillary and scatter from the capillary wall (hence optical background level) would be increased.

Native Fluorescence Detection. In order to enhance analytical selectivity, it is desirable to base detection on some intrinsic characteristic of the protein. This is achieved when using commercial CZE instruments by detecting the UV absorbance (λ between 200 and 230 nm) of the peptide bonds in the protein. Although somewhat complicated by the lack of "convenient" (i.e., reliable, low cost, and easy to operate) UV laser sources, laser-based detection can be accomplished by exciting the aromatic amino acid residues of the protein. The amino acids tyrosine, tryptophan and phenylalanine have

excitation maxima between 260 and 280 nm and fluoresce (albeit fairly weakly) between 280 and 350 nm, with some band shifts due to their incorporation into the structure of the protein(30). Therefore, the emission spectrum of the protein is the combination of the fluorescence of the three fluorophores and sensitivity is determined by the number of aromatic amino acids in the protein.

Since there is not a convenient continuous-wave laser that operates between 260 and 280 nm, we employed the frequency doubled 514 nm output of an argon ion laser for excitation. Although not optimum, the wavelength (257 nm) produced by this system can be use to excite the aromatic amino acids. The minimum detectable concentration (MDC) for conalbumin (conalbumin has 61 aromatic amino acids(31)) using this method with different diameter capillaries is presented in Table 2, along with the MDC for pre-and on-column labeling (see later discussion). The increase in MDC with decreasing capillary diameter is expected due to the decrease in pathlength. However, the loss in detectability would be somewhat compensated by the higher efficiency (i.e. higher concentration at band center for a given injected concentration) of the smaller capillary and the possibility of more rapid analysis (see 25 and 75 μm i.d. data in Table 1).

Unfortunately, the utility of native fluorescence detection detection method is reduced by the expense and complexity of the required instrumentation, which required a 4 hour warm up time and exhibited large changes in power during

Table 2

Minimum Detectable Concentration of Conalbumin

Detection Method	Capillary Diameter (μm)	Minimum Detectable Concentration (nM)
Native Fluorescence	50	14
	25	25
FITC Labeling	50	0.1
TNS Labeling	25	360
ANS Labeling	25	615

Note: MDC for FITC labeling was determined by static measurement after dialysis and does not account for loss of peak integrity during separation.

the course of a day. However, this method could become viable for daily operation if convenient low power UV lasers are developed in the future.

Pre-column Covalent Labeling. As stated previously, the detection of non-fluorescent species is often performed by the labeling of the analyte with a fluorescent tag prior to injection. This has been done in CZE separations of small molecules, using FITC(20), which reacts with amine functionalities and can be excited with the 488 nm line of an argon ion laser. Although this method permits the use of a

convenient laser, it requires time consuming sample preparation. More significantly, the labeling of proteins with FITC destroys the integrity of the CZE band (by producing a complex mixture of derivatives) and renders this method unusable for quantitative analysis of proteins.

The peak profiles of conalbumin detected by the native fluorescence and pre-column labeling are shown in Figure 1. For native fluorescence a sharp conalbumin peak and a small impurity peak are observed. However, in the case of pre-column labeling, multiple peaks are observed. Furthermore, increasing the derivatization ratio ([FITC]/[conalbumin]) exacerbates this problem and reduces the observed peak height. This same problem has been observed by other researchers(19) and is probably the result of heterogeneous labeling of the protein. Conalbumin has a total of 102 side chain amine groups that can react with FITC(31). Conalbumin molecules labeled with different numbers of FITC molecules will, hence, possess different mass to charge ratios and different electrophoretic mobilities. This range of electrophoretic mobilities results in a very broad band.

On-Column Labeling with with Hydrophobic Probes. In an attempt to alleviate the aforementioned problems, we investigated the use of fluorescent hydrophobic probes to label proteins. These compounds undergo changes in their fluorescent characteristics as a result of noncovalent interactions with proteins. 1-anilinonaphthalene-8-sulfonate (ANS) and 2-p-toluidinonaphthalene-6-sulfonate (TNS) are two

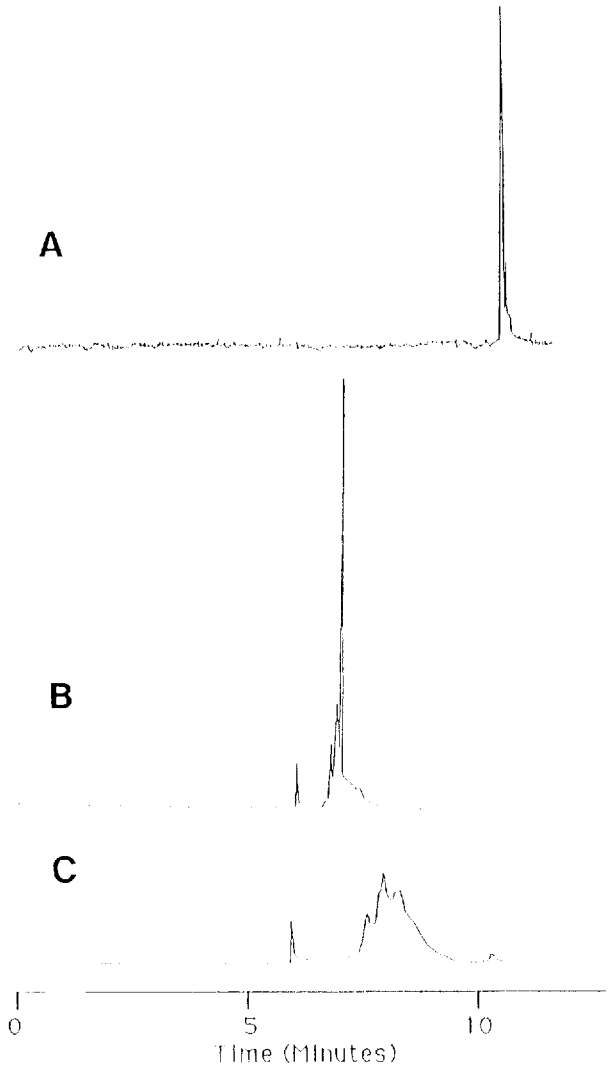

FIGURE 1. Peak profiles for conalbumin using native fluorescence detection and precolumn labeling with FITC. A) Native fluorescence detection, conditions: 25 μm i.d. x 80 cm capillary , 20mM CHES/ 30 mM KCl buffer, and 1 mg/ mL conalbumin. B) FITC labeled detection, conditions: 25 μm i.d. x 60 cm capillary, 5:1 FITC/conabumin derivatization ratio, same buffer as A, and 0.5 mg/mL conalbumin. C) FITC labeled detection,conditions: 10:1 FITC/conabumin derivatization ratio, same capillary, buffer and conalbumin concentration as B. Note: B and C are on same scale.

such compounds(32, 33) that have been used to investigate the secondary structure of proteins(33). These compounds intercalate into the hydrophobic regions of proteins with binding constants that range between 10^4 and 10^6(32). Upon intercalation the fluorescence quantum efficiency of the probe increases dramatically. Therefore, it should be possible to detect proteins by simply incorporating these compounds into the electrophoretic buffer.

The mechanism for this effect is based on an unusually large increase in dipole moment upon excitation, which causes the fluorescent characteristics of ANS and TNS to be dependent on environment. In polar solvents (e.g. water) the solvent cage around the molecule rapidly rearranges following excitation to form a lower energy Franck Condon state that facilitates non-radiative decay via intersystem crossing to a energetically similar triplet state(32-36). The resulting fluorescence quantum efficiency is generally less than 0.01. In viscous or non-polar solutions the solvent cage rearrangement occurs at times longer than the fluorescent lifetime of the probe, thereby reducing intersystem crossing. Under these conditions, quantum efficiencies as high as 0.6 are observed(37). When intercalated into proteins the the probe experiences no solvent cage rearrangement and fluorescence is observed(35).

Since the protein probe interaction is an equilibrium process, the response factor of this detection scheme will be

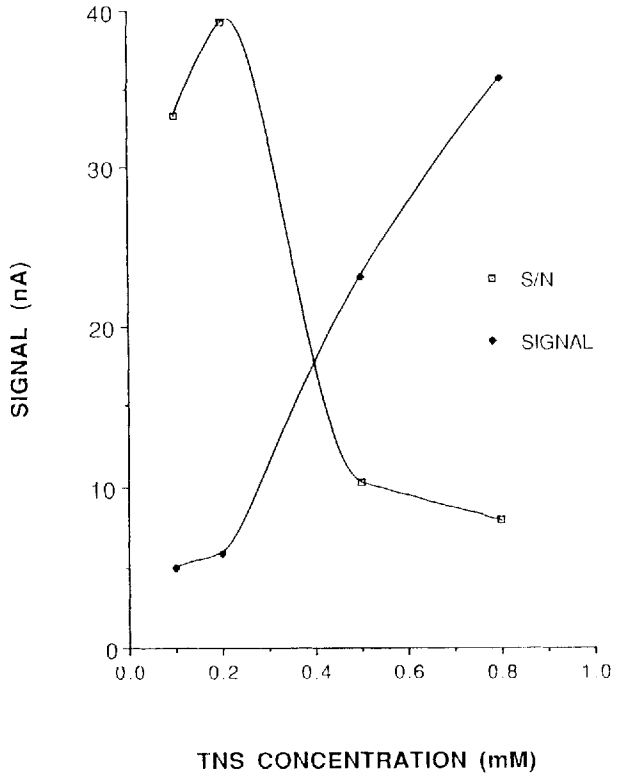

FIGURE 2. Effects of TNS concentration on signal and signal to noise ratio for conalbumin. Conditions: 25 µm i.d. x 60 cm capillary, 0.5 mg/mL conalbumin, 20mM CHES/ 30 mM KCl buffer and 235 V/cm.

determined by both the fluorescent characteristics of the probe and solution parameters that influence the equilibrium. The effects of TNS concentration in the electrophoretic buffer are illustrated in Figure 2. As the concentration of TNS increases the signal (as measured in peak height) increases due to the intercalation of more of the probe into the protein.

The signal should continue to increase with concentration until the binding sites on the protein are completely filled. However, the expected increase in detectability with probe concentration is not achieved due to a large increase in the fluorescent background and baseline noise when the TNS concentration exceeds approximately 0.2 mM. Above that concentration there is about a 25 fold increase in the baseline noise which substantially reduces the signal to noise ratio for conalbumin. This could be the result of intra-TNS interactions, that influence the aforementioned relaxation process and enhance fluorescence.

The effects of pH on the fluorescent signal of TNS in a conalbumin solution are illustrated in Figure 3. Although this study was performed using conventional fluorimetry, it does show that the pH of the solution does affect the equilibria. It should be noted that in this study, no fluorescent signal was observed, at any pH, if conalbumin was not present in the solution. The increased signal is probably a result of an increase in positive charge on conalbumin at neutral pH, which would increase the interaction of the anionic probe with the protein. However, as discussed earlier, the use of neutral pH buffers can reduce efficiency in CZE. Therefore the detection limits shown in Table 2, determined at pH 10.2, are not at optimum conditions for fluorescence. However, this parameter could be optimized if coated capillaries were employed to reduce solute-wall interactions (see ref 38 this same journal).

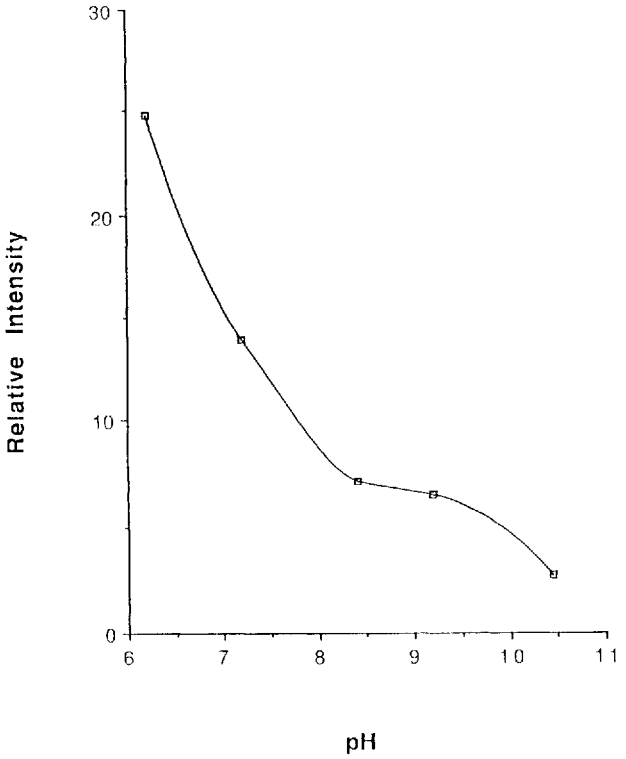

FIGURE 3. Effects of buffer pH on fluorescence signal of TNS in conalbumin. Conditions: 1 mg/mL conalbumin, 0.2 mM TNS, 20 mM CHES /30 mM KCL buffer, $\lambda_{excitation}$ = 325nm, and $\lambda_{emission}$ = 450.

The minimum detectable concentrations for ANS and TNS were presented in Table 1. These were determined using the probe concentration that produces the highest signal to noise ratio for conalbumin at a pH 10.2. Although these minimum detectable concentrations are higher than the other fluorescence detection schemes, they represent a significant

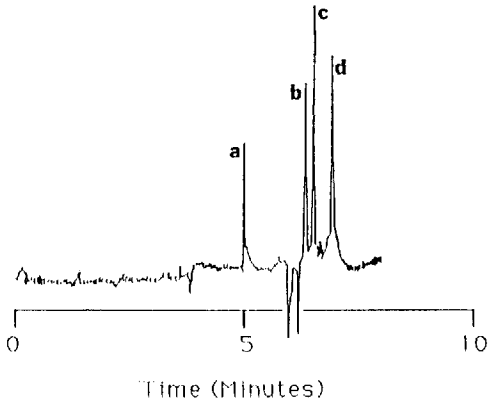

FIGURE 4. Separation of conalbumin (a), beta lactoglobin B (b), beta lactoglobin A (c) and bovine serum albumin (d). Conditions: 25 μm i.d. x 60 cm capillary,1 mg/mL CON, 0.3 mg/ mL BLG B, 0.3 mg/mL,BLG A, 0.25 mg/mL BSA, 20mM CHES/ 30 mM KCl buffer and 235 V/cm.

improvement over conventional spectrophotometric detection, as observed in our laboratory. Furthermore, the peak profile for conalbumin and several other proteins are not distorted by the labeling process (see Figure 4). This is probably the result of the dynamic nature of this labeling mechanism, created by the moderate binding constant with these proteins. Work in our laboratory with other on-column labeling mechanisms demonstrated that large equilibrium constants can result in band broadening due to slow exchange between different species present in the band(26).

To show the applicability of this method to the determination of other proteins, the minimum injectable

amount (MIA) for the two probes for conalbumin, bovine serum albumin and beta lactoglobin A are given in Table 3. Of particular interest, is the difference in detectability of the two different probes. The MIA for TNS are not only lower than ANS but also more uniform. The increased detectability with TNS is probably the result a larger change in dipole moment upon excitation, which results in a larger change in quantum yield upon binding(33). The greater uniformity in MIA for TNS could possibly be explained by TNS having a generally higher binding constant for these proteins than ANS. This would force the labeling equilibrium toward completion for each protein and result in similar detectability.

Future plans include optimizing parameters for the on-column labeling of proteins with TNS and other

Table 3

Minimum Injectable Amounts of Protein for ANS and TNS

Protein	ANS (femtomoles)	TNS (femtomoles)
Conalbumin	15	1
Bovine Serum Albumin	0.6	0.2
Beta Lactoglobin A	not detected	0.4

Note: The linear dynamic range for the proteins shown is 1.5 orders of magnitude for ANS and 2 orders of magnitude for TNS.

arlyaminonaphthalene-sulfonate derivatives. By employing neutral pH buffers it should be possible to improve the detectability of this method. Furthermore, it should be possible to reduce the fluorescent background by the use of polarized fluorescence detection to discriminate against fluorescence from unbound label molecules. In addition, we are investigating the utility of this type of labeling mechanism for other analytes.

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