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# Review

# Improved detection and derivatization in capillary electrophoresis

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ABSTRACT

Capillary electrophoresis is well known for its low mass detectabilities, but suffers from poor concentration detection limits. This review will discuss improvements in concentration detectability with an emphasis on derivatization methods. Sample concentration techniques and improved detector designs will also be discussed. Pre- and post-capillary derivatization methods for biofluid analytes such as amino acids, peptides, proteins, oligonucleotides, and oligosaccharides will be examined in detail.

CONTENTS



## 1. INTRODUCTION

Capillary electrophoresis (CE) is rapidly becoming an accepted routine analytical technique, characterized by short run times and high efficiencies. The method is applicable to small molecules, and most importantly, biopolymers such as peptides, proteins, and oligonucleotides. Because the separation mechanism (charge migration, isoelectric focusing, sieving) is orthogonal to chromatographic separation mechanisms, the techniques may be considered complementary in establishing purity, or identifying structural components or impurities in a sample. The major disadvantage to CE is its low concentration sensitivity. Because the entire system volume is only a few $\mu$l, detector flow cells require narrow, short path lengths that ultimate-

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ly limit absorbance or fluorescence sensitivities. The frequently reported mass detection limits in the pmol/fmol range for sample injections of low nanoliter volumes actually translate to only mg/l (ppm) or high $\mu$g/l (ppb) concentration detection limits, easily obtainable in high-performance liquid chromatography (HPLC). Thus, there is a great desire to improve sensitivities for analyte detection in CE. An example of the difference between reported mass and concentration detection limits is the work of Nickerson and Jorgenson [1]. By using laser-induced fluorescence (LIF) for improved detection of naphthalene dicarboxaldehyde (NDA) amino acid derivatives in CZE, they report detection of $2.5 \cdot 10^{-18}$ mol of arginine. These mass detection limits are impressive by any yardstick. However, because of only nl injection volumes, the concentration detection limits are on the order of $10^{-8}$ *M*. Many other reports of detection limits that offer low mass detection limits often have concentration limits of only $10^{-6}$ *M*. Thus, the resolving power and efficiency of CE, coupled with low concentration sensitivites of small sample volumes, is the goal of many analytical laboratories. Progress in the area of CE derivatizations using LIF detection has lowered mass detection limits to the zmol ($10^{-21}$ mol) range, with concentration detection limits on the order of $10^{-12}$ *M*. For instance, Cheng and Dovichi [2] have used LIF detection for fluorescein isothiocyanate (FITC) derivatized amino acids for detectabilities of fewer than 6000 molecules, or $10^{-21}$ mol. These mass detection limits translate into $10^{-12}$ *M* concentration detection limits.

While several recent review articles have addressed the separation mechanisms and methods of detection [3–9] available in CE, less focus has been placed on the discussion of fundamental attempts to lower concentration detectabilities. This review will address the issue of improved detection of trace amounts of analytes in CE through derivatization for improved fluorescence (FL) determination of samples in a biological matrix. However, it should also be noted that there are alternative methods for improving concentration sensitivities. These include alternative detector designs for improved sensitivity, and sample concentration techniques, such as sample stacking or $C_{18}$ or isotachophoretic preconcentration. Detectability of an analyte will ultimately hinge on careful choice of preconcentration conditions, detector design, and derivatization reagent.

## 2. SAMPLE CONCENTRATION TECHNIQUES

The major disadvantage of capillary electrophoresis is the limited amount of sample that can be loaded onto the capillary. By concentrating a large amount of a dilute sample at the beginning of the capillary, prior to separation, loadability is increased, and detectabilities can be lowered several orders of magnitude.

The most popular method of preconcentration in CE is sample stacking. Sample stacking has recently been reviewed [10], and several researchers have been investigating the fundamentals and applications [11,12]. Sample stacking depends on a matrix difference (pH, ionic strength) between the sample buffer and capillary buffer, so that the electric field across the sample zone is more than in the capillary region. In sample stacking, a large volume of sample in a low concentration buffer is introduced for preconcentration at the head of the capillary. The capillary is filled with a buffer of the same composition, but a higher concentration. When the sample ions reach the capillary buffer and the lower electric field, they stack into a concentrated zone. Sample stacking has increased detectabilities 1–3 orders of magnitude.

Another method of preconcentration is to apply isotachophoresis (ITP) prior to the free zone CE separation of analytes [13–15]. ITP is an electrophoretic technique which allows $\mu$l volumes of sample to be loaded onto the capillary, in contrast to the low nl injection volumes typically associated with CE. The technique relies on inserting the sample between two buffers (leading and trailing electrolytes) of higher and lower mobility, respectively, than the analyte. The technique is inherently a concentration technique, where the analytes concentrate into pure zones migrating with the same speed. The technique is less popular than stacking methods because of the need for several

choices of leading and trailing electrolytes, and the ability to separate only cationic or anionic species during a separation. Nevertheless, impressive increases in detectability have been demonstrated. Stegehuis *et al.* [13] used ITP coupled with capillary zone electrophoresis (CZE) for the determination of *o*-phthaldehyde (OPA) and FITC amino acid derivatives. ITP was performed prior to CZE separation in a capillary with a wider diameter than the CZE capillary. This allowed a 5000-fold increase in the loadability of the sample onto the CZE capillary, although the decrease in capillary diameter required a 1:100 split of the running buffer. Fig. 1 shows the remarkable effect of ITP preconcentration on the separation of FITC-derivatized amino acids.

Samples can also be concentrated at the head of the capillary using techniques typically associated with chromatography, notably concentration of hydrophobic analytes using a bonded $C_{18}$ stationary phase, or specific isolation and concentration of an analyte using an affinity ligand [16]. Guzman *et al.* [16] employed an antibody covalently bound to a solid support to concentrate and determine urinary components such as uric acid, amphetamine, and methamphetamine. The antibodies were bound to controlled-pore aminopropyl glass beads and packed into a 100 $\mu$m I.D. capillary. The antibody sites were saturated with sample, then an elution buffer was applied to separate the retained analytes. Although recovery yield varied from 20–65%, because of irreproducibility of the preparation of the bound antibody concentrator, the concentrator is reusable, and up to 350 ng of analyte can be loaded onto the capillary.

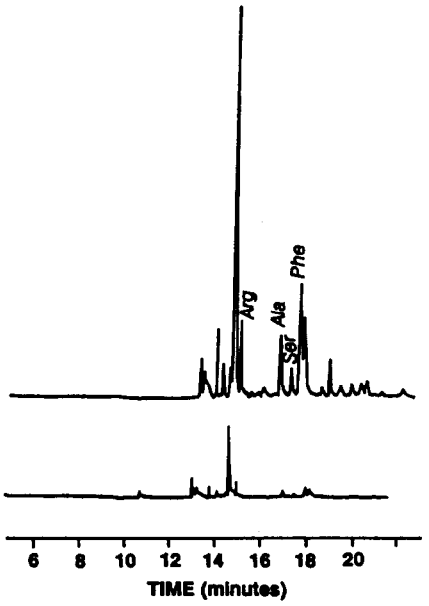



Fig. 1. Electropherograms of an FITC derivatization mixture of some amino acids. LIF detection 488/514 nm. Lower trace: single CE; electrokinetic injection (5 kV, 5 s) of 10 mmol FITC amino acid derivatives; 25 kV applied voltage. Upper trace: coupled ITP–CE; 25-$\mu$l ITP injection; followed by CE electrokinetic injection (5 kV, 5 s). Applied voltage, 10 kV ITP; 25 kV CE. From ref. 13.

## 3. FLUORESCENCE DETECTORS FOR CE

While several alternate modes of detection to UV absorbance have been applied, including electrochemical, mass spectrometric, and indirect detection techniques [4,17,18], the most promising area for lowering detectability of analytes is by LIF. However, there are not many biological samples of interest applicable to analysis by CE that naturally possess a high FL intensity. Also, analysis of natively fluorescent compounds is limited by their differing excitation maxima, and their compatability with available laser excitation wavelengths. Therefore, FL derivatization with a chromopore that fluoresces with an excitation maximum near the wavelength of the laser is an important technique for improved detection in CE. The most popular lasers for excitation in LIF are helium–cadmium lasers, emitting at 325 nm, and argon ion lasers, emitting at 488 nm. An excellent review of the components and optimal parameters for LIF detectors is given by Wu and Dovichi [19].

Sweedler *et al.* [20] developed an improved FL detector for CZE using a charge-coupled device (CCD). Normally with LIF, the laser is focused perpendicularly onto a narrow region of the capillary. With this detector, the output of the laser beam was focussed axially onto the end of the capillary illuminating a 2-cm channel, and the FL emission was collected. The detector operated in two modes, snapshot and time delay integration. Because CCDs have a slow readout

rate, several seconds are required to read a large array and transfer its information into digitalized form. This data transfer is normally performed in the snapshot mode, where, after exposure to the fluorescent signal, the shutter is closed and the array read. Because of the delay between exposures, it is possible to miss an analyte in a narrow observation zone. However, because the laser is focussed axially over 2 cm, it is usually possible to obtain 10–30 exposures per analyte. This process also entails a large amount of data processing from the exposures. Operating in the time delay integration mode eliminated the need for the shutter by synchronizing the transfer of data from the array to the digital processer with the migration of the analyte through the capillary. This technique allowed for less data processing than the snapshot mode, allowed the fluorescence to be integrated over the entire time in the observation zone, and resulted in a 2–5 times decrease in noise. Limits of detection with this detector for FITC-labelled amino acids for a 13-nl injection were in the range of 20–80 zmol.

Hernandez *et al.* [21] used a fluorescence microscope as a detector for CE. The authors report detection of 0.5 fmol of riboflavin, and improved detectabilities using larger diameter capillaries in tandem with either a high-powered UV lamp and photomultiplier tube (PMT), or an argon-ion laser, PMT and photon counter. However, the larger diameter capillaries allow detection of only 0.05 nl volumes, so that there is no real gain in concentration detectabilities.

Cheng *et al.* [22] used a sheath flow cuvette design to improve FL sensitivites. In a sheath flow cuvette, the sample stream is injected into the center of a flowing sheath stream, so that the sample flows as a narrow stream through the center of the capillary. The sheath stream was adjusted to a similar refractive index as the sample stream to minimize light scattering, and flat flow chambers for low FL background were used. Wu and Dovichi [19] used this detector design for a 10-fold improvement in detectability. Analysis of FITC-labeled amino acids yielded detectabilities ranging from arginine $1.3 \cdot 10^{-12}$ $M$ (1.7 zmol) to cysteine $5.6 \cdot 10^{-12}$ $M$ (6 zmol) with signal linearity extending over five orders of magnitude.

Yu and Dovichi [23] have used an argon-ion laser in association with thermoptical absorbance detection for attomole ($10^{-8}$ $M$) detectability for dabsyl-labeled amino acids. Other applications of lasers in novel detection modes have been used to improve selectivity of detection, but not necessarily improved detectability. These include using a He–Cd laser for laser-based refractive index detection [24] and for FL photodiode array detection [25], and an argon-ion laser for FL circular dichroism detection [26].

## 4. DERIVATIZATION IN CE

Chemical derivatization of an analyte improves the sensitivity and detectability of an analyte by introduction of an FL chromophoric group, and increases the selectivity of an analyte if it can be shown that the derivatizing reagent reacts in the biological matrix solely with the analyte of interest. Derivatizations can be performed pre-, post- and on-column with respect to the electrophoretic separation.

Post-capillary techniques allow improved detection of analytes after separation in their native state, while pre-capillary derivatizations change both detection and electrophoretic properties. Post-capillary derivatizations require a rapid reaction time with a reagent that does not share the detection properties of the derivative, so that reagent blank interference is minimal. Detection limits with post-capillary methods are not usually as low as with pre-capillary methods, so that their major application is when derivatives are unstable, or when there are multiple derivatization sites that may yield a mixture of products. Since the reaction is being performed on the analyte electrophoretically resolved from its matrix, it is not necessary for the reaction to go to completion, or for only a single derivative to be formed; however, the percent derivatizations should be reproducible and should not change with concentration. These reactions are often easy to automate for on-line detection of the derivatives. Disadvantages of the technique are possible reagent instability, more complex hardware requirements, and lower sensitivities than pre-capillary derivatization reagents.

For pre-capillary derivatizations, rapid reac-

tion conditions are not necessary. However, percent derivatization of the analyte should be high, 100% if possible, since derivatization may be matrix dependent, and should yield only a single derivative. Pre-column derivatization reagents also often share the detection properties of the derivative, so that it is necessary to remove excess reagent before separation, or to resolve the excess reagent from the derivative. Both pre- and post-capillary methodologies have been developed for derivatization in CE, although the majority of the work has been in pre-capillary derivatizations.

### 4.1. Derivatization of amino acids, peptides and proteins

Both fluorescamine and OPA are reagents with no inherent FL properties, but offer enhanced detectability of primary amino acids with fluorescent detection. They are both common reagents for the pre- and post-capillary derivatization of amino acids and small peptides. Fluorescamine has the disadvantage of being unstable in aqueous eluents, while OPA has better FL yields and is stable in an aqueous environment. Other common derivatization reagents include FITC, 9-fluorenylmethyl chloroformate (FMOC-Cl), 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride), 4-(dimethylamino) azobenzene-4′-sulfonyl chloride (dabsyl chloride), and other acyl chlorides. Because excess reagent undergoes fluorescence and must be resolved from the analytes, these reagents can only be used for pre-capillary derivatizations.

Albin *et al.* [27] have compared FITC, fluorescamine, FMOC-Cl, and OPA for pre- and post-capillary derivatization in CE. For the pre-capillary derivatization and micellar electrokinetic chromatography (MEKC) separation of six amino acids, FMOC-Cl offered the lowest limit of detection, 10 ng/ml, or 0.5 fmol. FMOC-Cl has a rapid reaction time with primary and secondary amino acids, but also yields a FL hydrolysis product that can interfere with detection, unless it is resolved from the analytes or removed by extraction. OPA also has low detection limits (50 ng/ml) for primary amino acids, but the derivatives can be unstable.

While FITC shows good detectability for primary and secondary amino acids, it requires a long reaction time, so that it is impractical for routine use [27]. Excess reagent also interferes with detection. Fluorescamine has a rapid reaction time (milliseconds) with primary amino acids, and excess reagent is hydrolyzed to a non-FL product. Detectabilities are as low as 360 ng/ml. Fluorescamine has also been used for the determination of polyamines in brain, stomach and lung tissue using an internal standard [28]. Guzman *et al.* [29] have also used fluorescamine for the derivatization of proline and hydroxyproline. Although the products were non-FL, they were able to be determined with improved sensitivity (800 fold) at 214 nm, and resolution was also improved. Response was linear from 19–304 nmol/100 $\mu$l.

Nickerson and Jorgenson [30] compared FITC, NDA and OPA for pre-column labeling of amino acids, using phenylalanine as a model. A He–Cd laser operated at 326 nm for OPA and 442 nm for NDA and FITC was used for excitation. Detection limits were 20 n*M* for OPA, 1.6 n*M* for NDA, and 0.22 n*M* for FITC. The better detection limits for FITC and NDA arise from the greater intensity of the 442 nm laser line excitation source. NDA has the further advantage that the excess reagent does not fluoresce, while the FITC reagent must be extracted from the sample or resolved from the analyte.

Ueda *et al.* [31] used cyclodextrin-modified MEKC for the chiral resolution of NDA-labeled amino acids. The excitation maxima of these derivatives coincides with the output of a He–Cd laser. The cyclodextrin-modified running buffer allowed separation of the enantiomers based on differences in partitioning into the cavity of the chiral cyclodextrin to form diastereomeric complexes, while the ability for LIF detection allowed determination of amino acids as low as 0.9 amol/2.5 nl injected.

In CZE, Waldron *et al.* [32] demonstrated improved detectabilities of amino acids with fluorescein thiohydantoin (FTH) and dimethylaminoazobenzene thiohydantoin (DABTH) derivatives. Separation of DABTH derivatives was difficult because of elimination of the primary amine and the carboxylic acid. An

acidic pH was required to protonate the secondary amine, and acetonitrile was added to reduce electroosmotic flow and increase the separation time. The FTH amino acids were anions under basic conditions, and were able to be separated with higher efficiency in CZE than the DABTH derivatives. Although these reagents were applied only to standard solutions of amino acids, the authors point out the application that they would have application as substituted phenyl isothiocyanate derivatives, replacing the phenyl group with a superior chromophore for improved detection of amino acids in Edman degradation sequencing analysis for CZE.

Higashijima *et al.* [33] developed a new derivatization reagent compatible with a semi-conductor laser. Semi-conductor lasers have the advantages of being inexpensive and rugged, but lack chromophores at suitable wavelengths without frequency doubling. The authors developed a thiazine chromophore fluorescent in the deep-red region, compatible with a semi-conductor laser, and with a succinimidyl-activated ester to couple with an amino acid. Detection limits were reported to be only 10 pmol, but the work reported was preliminary, and shows the possible utility of semi-conductor lasers for excitation in CE.

A variety of FL derivatization techniques exist for improving sensitivity and selectivity of peptide fragments. Because peptides contain several functional groups, derivatization generally yields a mixture of products, so that derivatizations are usually carried out post-column, after the separation of the native peptides. Pre-capillary derivatizations must be limited to small peptides or peptides with only a single functional group. In addition, it is desirable for the derivative to fluoresce at wavelengths high enough so that the native fluorescence of Trp or Tyr does not interfere. Many of the derivatization reagents used for amino acids are not useful for peptide derivatizations. OPA does not react significantly with the N-terminal amino group of the peptide, except for dipeptides and other small peptides, and dansyl chloride does not react except at high concentrations of peptides. Fluorescamine has been shown to derivatize the amino group of peptides for sensitive detection in the low pmol range for peptides of less than 20 amino acids in length [34].

Swaile and Sepaniak [35] investigated the FL detection of proteins using native FL, pre-column labeling, and on-column labeling with arylaminonaphthelene-sulfonates. Detection of the protein's (conalbumin) native fluorescence was performed using a frequency doubled argon ion laser (514 nm excitation), for excitation at 257 nm of the aromatic amino acids. The detection limit was 14 n*M*. Precolumn labeling of the protein with FITC lowered the detectability to 0.1 n*M*, but produced a mixture of derivatives. The authors also used fluorescent hydrophobic probes such as 1-anilinenaphthalene-sulfonate (ANS) and 2-*p*-toluidinonaphthalene-6-sulfonate (TNS). These probes undergo non-covalent, hydrophobic interactions with the proteins and change their FL properties. FL properties of the complex are extremely solvent dependent. Quantum efficiencies in aqueous solvents are as low as 0.01, but can increase to 0.6 in viscous or non-polar solvents. Because of solvent requirements for the separation, detection limits with these on-column labeling agents was only 360 n*M* for TNS and 615 m*M* for ANS.

3-(4-Carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA) [36] was used for the improved detection of primary amines, amino acids, and peptides. CBQCA-derivatized amino acids were detectable at masses as low as 10–70 amol. Detection limits for peptides containing a single amino functionality were also in the low amol range, with a dynamic range over 4 orders of magnitude. The reagent has the advantage of being transparent in the reagent blank, and induces an additional charge onto the derivative. Although the reagent was also reactive toward proteins, the formation of multiple peaks negated the reagent's utility for protein derivatization.

The previously described reagents for peptide derivatizations are non-specific and react with free amino groups on all peptides. The reagents yield improved sensitivities for all peptides in a peptide map. For more specificity, reagents can be chosen which react with particular amino acids to selectively detect particular peptides.

Cobb and Novotny [37] used benzoin and 4-methoxy-1,2-phenylenediamine for the derivatization of arginine and tyrosine containing peptides, respectively. FL detection employing a He–Cd laser (325 nm) is well suited to detect the derivatives from both reagents. A tryptic digest of chicken egg white lysozyme was reacted separately with each reagent. Cobb and Novotny [38] further characterized the benzoin derivatization of arginine containing peptides obtained from a tryptic digest of human serum albumin. Attomole mass detection limits were reported. Fig. 2 shows the UV trace for the tryptic digest, and the LIF trace for the benzoin derivatized tryptic digest. There is a $10^3$ difference in the mass injected.

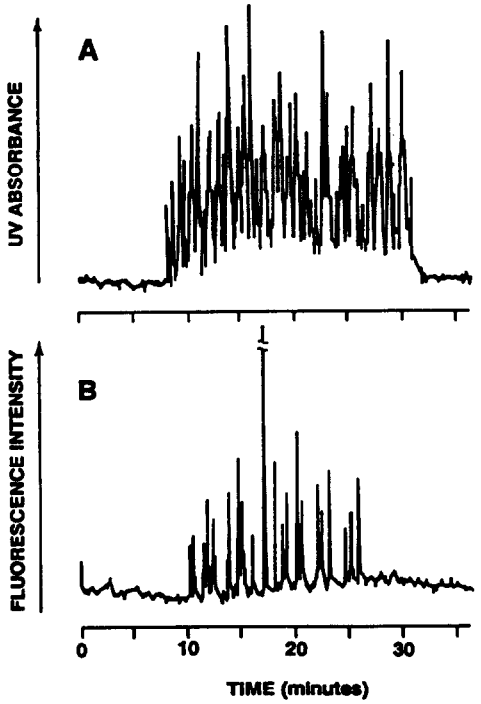



Fig. 2. Tryptic digest of reduced and alkylated human serum albumin (HSA). (A) UV detection 215 nm; 100 fmol of HSA digest; 0.05 *M* 2-(cyclohexylamino)ethanesulfonic acid (CHES) buffer pH 9.7; capillary 55 cm (40 cm to detector) × 50 μm, coated with linear polyacrylamide; 25 kV applied voltage; 12 μA. (B) LIF detection of benzoin-derivatized HSA digest. 360 amol of digest injected; off-scale peak due to derivatization reagent; 0.05 *M* 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 9.1; 0.06 *M* SDS; 10% acetonitrile; capillary 70 cm × 50 μm (50 cm to detector), uncoated capillary; applied voltage 25 kV, 20 μA. From ref. 38 (© American Chemical Society).

Other derivatization reagents useful for FL derivatization of amines, amino acids, peptides, and proteins include dansyl-chloride for both achiral [39,40] and chiral [41] analysis, dabsyl chloride [28,42], N2-(5-fluoro-2,4-dinitrophenyl)-L-alanine amide (Marfey's reagent) [43], phenylisothiocyanate [44], 4-chloro-7-nitrobenzofurazan (NBD-chloride) [45] and NDA [1,46].

### 4.2. Post-capillary reaction detection

Post-capillary derivatization is most often applied to analytes whose derivatives may be unstable, or to analytes that possess multiple derivatization sites that may yield a mixture of products, such as free amino groups on peptides or proteins. The methods rely on either hydrostatic or hydrodynamic addition of the reagent, or to use differential electroosmotic flow to introduce the reagent. Albin *et al.* [27] used a 75-μm reaction capillary separated from a 50-μm separation capillary at a liquid junction containing the buffered reagent, resulting in a flow imbalance where the electroosmotic flow is greater in the larger I.D. capillary. This flow imbalance is made up by introduction of reagent. This system was applied to OPA and fluorescamine derivatization. After optimization of reagent concentrations and temperatures, limits of detection were reported, 60 ng/ml (3.3 fmol) for OPA, and 440 ng/ml (19.2 fmol) for fluorescamine. Fluorescamine was applied to the post-capillary derivatization of a tryptic digest of β-lactoglobulin, Resulting in an 8-fold increase in sensitivity over absorbance detection. The UV and FL traces for the fluorescamine derivatized tryptic digest are shown in Fig. 3. There is a 40-fold decrease in the concentration monitored by the FL trace.

Pentenoy *et al.* [47] designed an OPA post-column reactor fashioned by inserting two 75-μm capillaries into either side and perpendicular to a drilled 75 μm I.D. capillary. Reagent was delivered by hydrostatic pressure, by raising the reagent reservoirs above the buffer reservoirs for a determined amount of time. This was done by filling the separation capillary with running buffer, submersing the ends of the reagent capil-

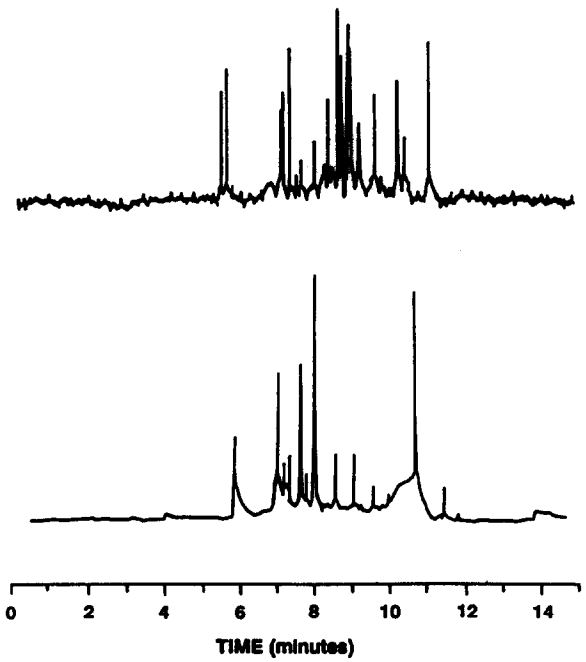


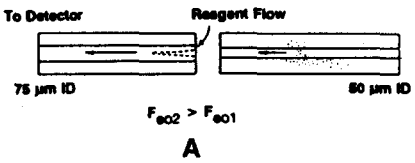

Fig. 3. Free solution electrophoresis of a tryptic digest of β-lactoglobulin. 20 m*M* sodium tetraborate buffer pH 9.5. Field strength 278 V/cm. 30°C. Upper trace: FL detection after post-capillary derivatization with fluorescamine. Xenon lamp 390/450 nm; 0.5 nmol/ml sample concentration; 7 s electrokinetic injection at 5 kV. Lower trace: absorbance detection 200 nm. Deuterium lamp; 20 nmol/ml sample concentration; 1 s vacuum injection. From ref. 27 (© American Chemical Society).

laries in the elevated OPA reagent reservoirs, and applying high voltage for 25 min (Fig. 4). The detection limit for histidine was $9 \cdot 10^{-7}$ *M*. This method is for applications where derivatives are unstable when formed pre-column, or when multiple products can form, since the detection limits are $10^3$ higher than pre-column methods.

Rose and Jorgenson [48] describe a post-capillary reactor for OPA derivatization. The coaxial capillary reactor consists of a separation capillary with a smaller outer diameter, inserted into a reaction capillary of a larger inner diameter. The reagent capillary is perpendicular to these capillaries in a stainless-steel tee. Reagent is delivered by raising the reagent reservoir above the buffer reservoirs. The reactor was optimized for enhanced signal and zone broadening, and characterized using glycine and a mixture of proteins. The FL signal was linear over 3.5 orders of magnitude, with a detection limit of $7.5 \cdot 10^{-8}$ *M* glycine (83 amol) and 22.1 amol for whale skeletal muscle myoglobin. Derivatization resulted in a 100-fold improvement in signal to noise ratio *vs.* UV detection. Fig. 5 shows the UV and FL electropherograms for a mixture of whale skelatal muscle myoglobin, carbonic anhydrase, β-lactoglobulin B, and β-lactoglobulin A. Nickerson and Jorgenson [49] used a similar reactor design for OPA and NDA derivatization, with reagent pumped into the reaction capillary using helium pressure. LIF detection with a He–Cd excitation source was performed on the reaction capillary. The detection limit for horse heart myoglobin derivatized with OPA was $1.2 \cdot 10^{-8}$ *M*, and linear over 3 orders of magnitude.

Rose [50] described a post-column reactor for OPA derivatization in CZE. The electrophoresis capillary was terminated in a static solution of OPA, acting as both a cathodic reservoir and a free solution reactor. Zones from the separation capillary mix and react with the OPA reagent to produce the FL derivative. Only the utility of the reactor was described, emphasizing the importance of convective forces for mixing analyte and reagent. No detection parameters or other analytical figures of merit were reported with this work. Fig. 4 shows the post-column reaction

Fig. 4. Post-capillary reaction schemes. (A) A secondary buffer containing a FL reagent is mixed with the running buffer by virtue of the different electroosmotic flow in the different diameter capillaries; from ref. 27 (© American Chemical Society). (B) Experimental set-up of the CZE–LIF reaction detector: 1 = on-column connector; 2 = buffer reservoirs; 3 = derivatization reagent reservoirs; 4 = LIF detector housing; from ref. 47 (© American Chemical Society). (C) Cross-sectional view of post-column reactor; from ref. 48. (D) Cross-sectional schematic of reactor [(a) top and (b) side view]: RR = reagent reservoir; EC = electrophoresis capillary; SC = glass support capillary; CF = compression fitting; SW = silica window; FO = optical fiber; PA = PMT adapter; CL = collection lens; UF = UV cutoff filter; from ref. 50. (E) Schematic diagram of post-column detection system in CE: 1 = positive terminal; 2 = 4-way connector for earth terminal and mixing of column media and buffer; 3 = 3-way connector for mixing with FL reagent; 4 and 5 = PTFE tubes 0.5 mm I.D. of length 5 and 70 cm, respectively. Column medium, alkaline buffer solution and FL reagent supplied by pumps 1, 2, and 3, respectively; from ref. 51.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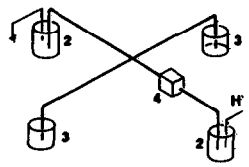
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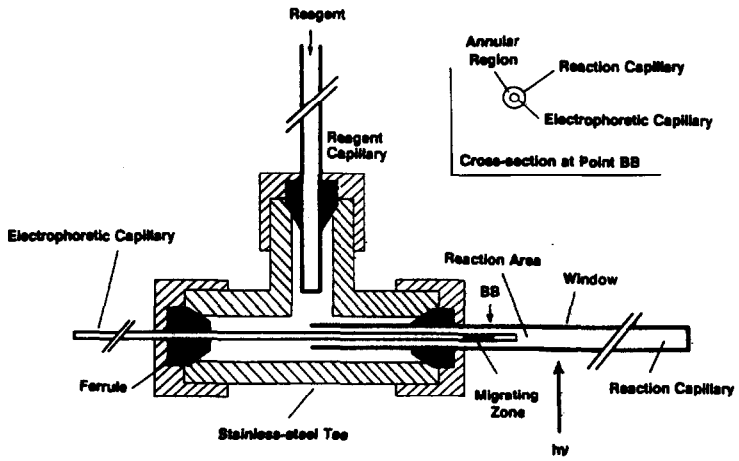
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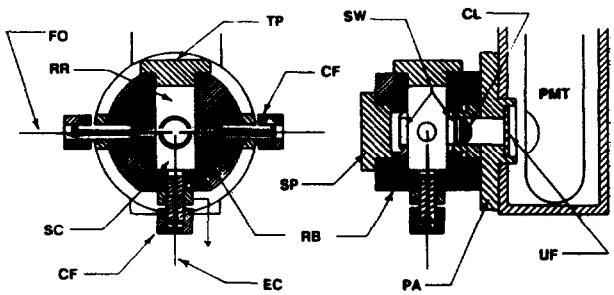
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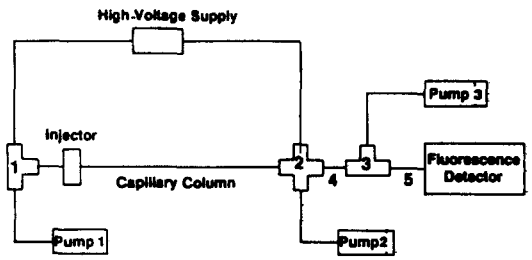
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Fluorescence Detector
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Pump2

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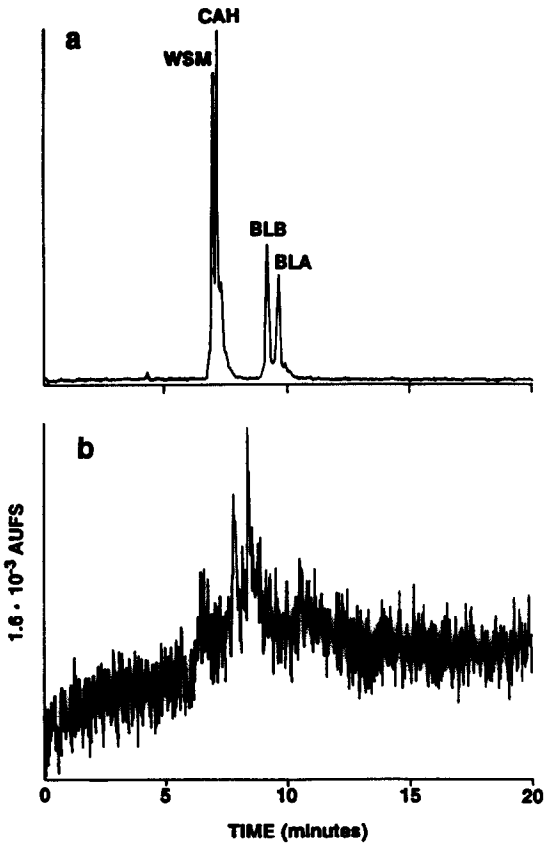


Fig. 5. Comparison of post-capillary FL and UV detection of 0.01% (w/v) whale skeletal muscle myoglobin (WSM), 0.01% carbonic anhydrase (CAH), 0.005% β-lactoglobulin B (BLB), and 0.005% β-lactoglobulin A (BLA). (a) Post-capillary FL detection; (b) UV (229 nm) detection; operating and OPA reagent buffer 50 m*M* borate–50 m*M* KCl, pH 9.5. Sample introduction, 2 s at 30 kV; operating voltage at 30 kV; $\Delta h$ = 16 cm. From ref. 48.

schemes for each of the reactors described above.

Tsuda *et al.* [51] utilized three pumps and two mixing chambers for post-column detection with fluorescamine derivatization (Fig. 4). After the separation, pump 2 delivers alkaline buffer and pump 3 delivers the fluorescamine reagent. Pressure buildup at the outlet resulted in flow reversal, so that pump 1 was operated at a few μl/min to maintain forward flow. The method was applied to acetylpolyamines in urine.

### 4.3. DNA derivatization

Improved detection of oligonucleotides in nucleic acid sequence analysis is necessary for the low mass concentrations associated with microsequencing analysis. Chen *et al.* [52] used tetramethylrhodamine isothiocyanate (TRITC) for coupling to the dideoxynucleotide. The derivatized oligonucleotide was excited by a green helium–neon laser (543.5 nm) using a sheath flow detector design. Mass detection limits of 500 ymol (ymol = $10^{-24}$ mol), or $1.28 \cdot 10^{-10}$ *M*, were reported in the free solution mode.

Chen *et al.* [53] also used a single channel labeling technique, using a fluorescent dye in capillary gel electrophoresis (CGE) for Southern blotting. Drossman *et al.* [54] used fluorescein to label DNA fragments generated in enzymatic DNA sequencing reactions for separation via CGE. Detection limits in unfilled capillaries were 0.1 amol or 60 000 molecules. Swerdlow *et al.* [55] also used fluorescein to label DNA fragments for LIF detection in a sheath-flow cuvette, and reported mass detection limits as low $10^{-20}$ mol.

Swerdlow *et al.* [56] characterized three DNA sequencing methods, utilizing 4 channel, dual channel, and single channel detection. Four spectral channel sequencing is accomplished using four different fluorescent dyes, to be used with each dideoxynucleotide reaction. Two lines from an argon–ion laser (514.5 nm and 488 nm) are used to excite fluorescence, and emission at four wavelengths is achieved using interference filters (540, 560, 580 and 610 nm). Detection limits are on the order of 200 zmol for all four labeled primers.

The two spectral channel sequencing technique was achieved using succinylfluorescein dyes to label the four dideoxynucleotides. A single wavelength (488 nm) is used to excite fluorescence, and emission is monitored at 510 and 540 nm. The ratio of the FL intensities is monitored to distinguish the terminating dideoxynucleotide. The detection limit is 20 zmol for a labeled dideoxynucleotide triphosphate, and 5 zmol for a 100-mer oligonucleotide.

For single channel monitoring, a fluorescent dye was coupled to the nucleotide, and excited with a green helium–neon laser. Detection of 1200 molecules was achieved. Fig. 6 shows the 4-channel, dual-channel and single-channel

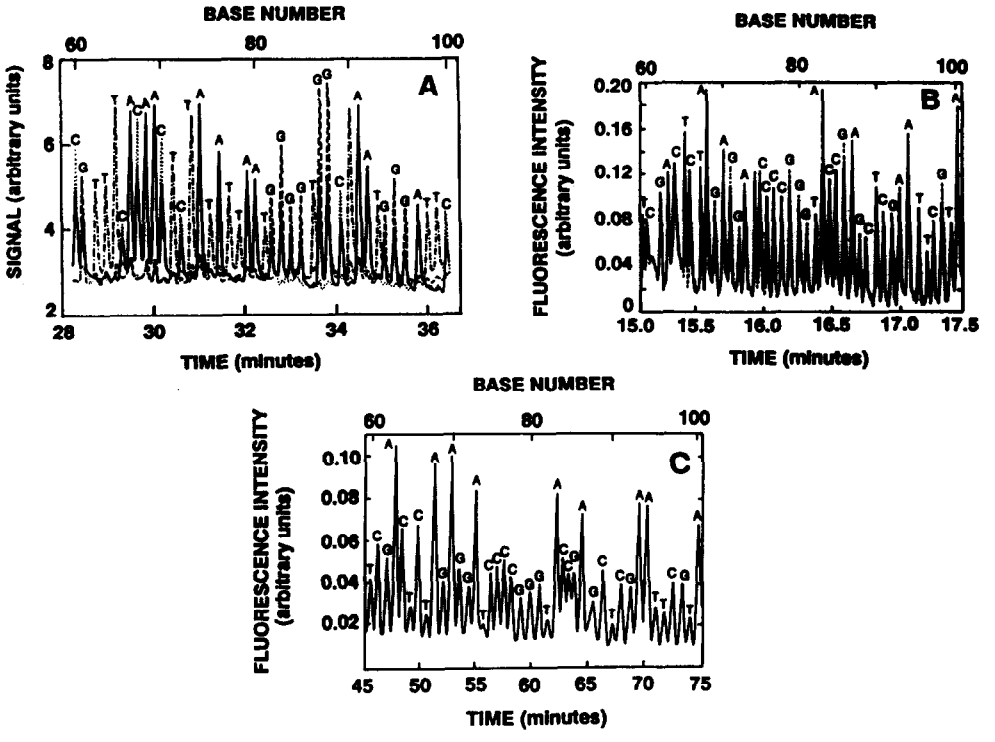


Fig. 6. CGE separation for the pooled reaction products for the DNA sequence of a M13mp18 histidine *t*RNA clone. (A) Four channel sequencing; capillary 41 cm × 50 μm; 27 cm to detector; Electrophoretic injection 30 s at 150 V/cm; traces for C, A, G, and T correspond to emission centered at 540, 560, 580, and 610 nm, respectively. (B) Two spectral channel sequencing; solid trace emission at wavelengths longer than 525 nm; dashed trace emission at wavelengths less than 525 nm. (C) One spectral channel sequencing. From ref. 56 (© American Chemical Society).

traces for the pooled reaction products of a DNA sequence analysis of an M13mp18 histidine *t*RNA clone. The data has not been treated to account for overlap in the spectral channels.

Kasper *et al.* [57] used FL detection (not LIF) for the determination of ethidium bromide stained DNA. Excitation at 232 and emission at 458 nm was used. The greatest sensitivity was obtained at low concentration of reagent, but linearity was lost with high concentration of analyte, necessitating higher concentration of reagent. Detectability was actually 5 times worse than using UV detection, but offered the advantage of elimination of absorbing interferents at 260 nm.

### 4.4. *Oligosaccharide derivatization*

Derivatization of carbohydrate molecules is necessary for spectrophotometric detection because of the lack of chromophoric groups. Derivatizations are most often performed on reducing carbohydrates. Non-reducing oligosaccharides are analyzed after acid hydrolysis to the component monosaccharides. Reagents for oligosaccharide derivatizations in HPLC have recently been reviewed by Honda [58]. Derivatization for CE of oligosaccharides and carbohydrates is somewhat complicated by the fact that the original analytes are uncharged at most pH values. This has been addressed by either inducing a charge onto the analyte with the derivative, or forming charged borate complexes with the derivative.

Liu *et al.* [59] derivatized aldose oligosaccharides into primary amines, followed by derivatization of the reducing end of an amino sugar with 3-(4-carboxybenzoyl)-2-quinoline carboxaldehyde (CBQCA) or 3-benzoyl-2-napthaldehyde (BNA). The reagents show no background fluorescence, and introduction of these FL chromo-

phores also introduce a charged moiety onto the carbohydrate molecule to form highly fluorescent isoindole derivatives. Detection was via LIF using an argon–ion laser operated at 457 nm. The reagents were applied to the derivatization of hydrolyzed maltooligosaccharides and enzymatically degraded samples separated on polyacrylamide gel-filled capillaries. Liu *et al.* [60] further characterized the CBQCA reagent for derivatization of monosaccharides, acid-hydrolyzed polysaccharides, and glycoprotein digested carbohydrates. In this report, the carbohydrate derivatives (maximum 442 nm) were detected with a helium–cadmium laser. This reagent was characterized to yield mass detection limits of 240 amol and concentration detection limits of $8.0 \cdot 10^{-7}$ *M*. Signal was linear over four orders of magnitude. A representative electropherogram for the separation of six CBQCA amino sugars is shown in Fig. 7. Unfavorable mass-to-charge ratios made separation of large oligosaccharides impractical in the free solution capillary format. Liu *et al.* [61] also applied the CBQCA reagent to the derivatization of larger oligosaccharides (degree of polymerization up to 67) for separation via CGE. Low attomole mass detection limits were reported.

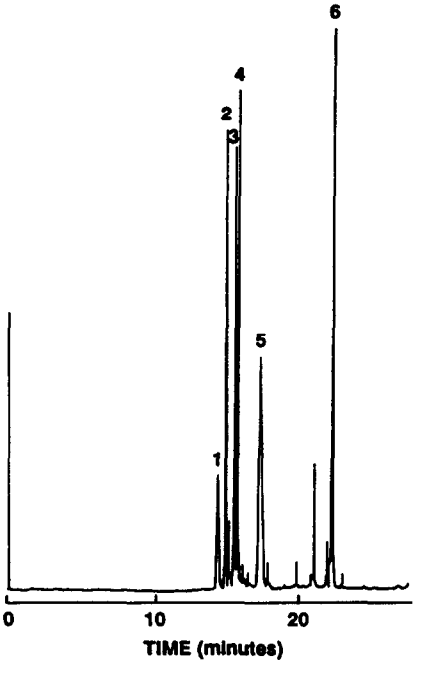


Fig. 7. Electrophoretic separation of six model amino sugars derivatized with CBQCA. 1 = 1-amino-1-deoxyglucose; 2 = 1-amino-2-deoxygalactose; 3 = 2-amino-2-deoxyglucose; 4 = 2-amino-2-deoxygalactose; 5 = 6-amino-6-deoxyglucose; 6 = D-galactosaminic acid. From ref. 60 (© American Chemical Society).

Other reagents for the derivatization of carbohydrates for detection by UV absorbance include 3-methyl-1-phenyl-2-pyrozolin-5-one [62] and 2-amino pyridine [63,64] for separation of borate complexes, and 2,4-dinitrophenylhydrazine [65] for the separation of coupled amino acid–aldehydic sugars.

### *4.5. Polymeric reagents for CE*

One of the disadvantages of all previous pre-column derivatization reagents is dilution of the derivative concentration by the reagent. Post-capillary techniques may minimize the dilution problem, but the detection limits are higher than pre-capillary methods. This is due to the compromises that must be made between the optimum separation conditions and reaction conditions which limit complete formation of the derivative (solvent, time, temperature, pH, etc). We are investigating using a solid-phase reagent containing a fluorescent tag (FMOC) immobilized on a solid polystyrene support. This reagent has been used in HPLC for derivatization of amines [66], amino acids [67], and aminoalcohols [68] in biological fluids. The reagent is stable in an aqueous environment, so that it may be used for on-line derivatizations. By using SDS below its critical micelle concentration (CMC), proteins in biological samples are solubilized, and direct injection and derivatization of analytes in human serum is possible [69] (Fig. 8).

This reagent can be packed into a capillary, so that derivatizations can be performed on-line in free solution CE and MEKC. There are a number of advantages in using solid-phase reactors (SPRs) for CE, beyond eliminating dilution of the analyte with the reagent. The amount of immobilized reagent is in great excess to the analyte concentration, but only the amount that reacts with the analyte is released. The remainder is bound to the solid support, so that the reactor can be used for multiple derivatizations. Because the reagents cannot interact with other

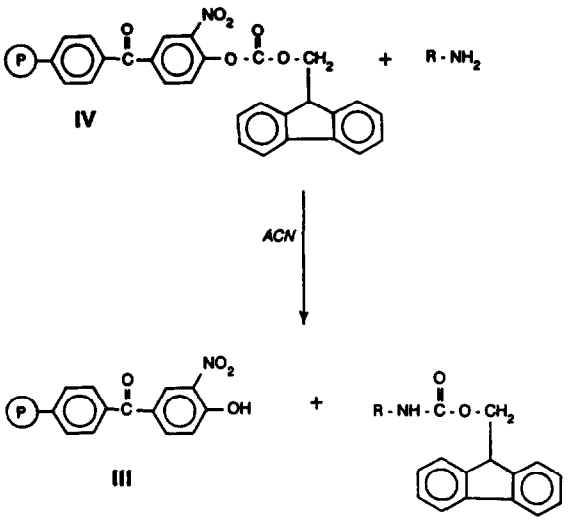


Fig. 8. Reaction of amine with polymeric FMOC reagent. ACN = Acetonitrile.

immobilized reagents, several polymeric reagents may be packed into the same reactor for quantitation using multiple derivatives [66]. Also, when the reagent is exhausted, the tagged reagent can be regenerated in a short, one hour reaction.

By using the CE instrument in the MEKC mode, samples in biological fluid (urine, serum) may be injected directly onto the instrument. This is a significant advantage of MEKC over other modes of capillary separations; however, if derivatization with solution phase reagents is required to lower detectability, analyte extraction and sample clean-up must still be performed prior to injection. SPRs will allow direct injection of analytes in biological fluids onto the capillary. The hydrophobic nature of the SPR will act to preconcentrate the analyte, or it can be used in tandem with an affinity concentrator [16]. By using SPRs, analytes in biological samples may be injected directly onto the capillary for derivatization, followed by separation and FL detection. SPRs contribute to a significant decrease in analysis time, sample clean-up, detectability, and expense for analyses in CE.

## 5. CONCLUSIONS

Capillary electrophoresis has emerged as a premier technique for the rapid, high-resolution separation of analytes of biological interest. It is especially valuable for biotechnology related samples, such as DNA sequencing products, and biotechnology synthesized peptides and proteins. By careful choice of detector design, derivatization reagent, laser excitation wavelength, and preconcentration steps, low concentration detection limits are possible. The method is complementary to HPLC in the information it provides, so that with low concentration detectability, CE becomes an attractive method for the routine analysis and characterization of samples with biological interest.

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