Practical and Quantitative Aspects in the Analysis of FITC and DTAF Amino Acid Derivatives by Capillary Electrophoresis and LIF Detection

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Key Words

Capillary electrophoresis
Laser induced fluorescence (LIF) detection
Amino acid analysis
FITC and DTAF derivatives

Summary

Practical aspects related to the preparation of the fluorescein isothiocyanate (FITC) and dichlorotriazinylaminofluorescein (DTAF) derivatives of amino acids for purposes of quantitative analysis are examined and factors affecting quantification are discussed. It is shown that the labelling reaction for both reagents can be speeded up by operating at 40 °C. The difficulties with derivatizing amino acids at detectable concentrations are highlighted. In spite of the high sensitivity of CE-LIF, detection sensitivity in real applications is limited by factors external to the analytical process, such as the label chemistry.

Introduction

The determination of amino acid composition and sequencing of small quantities of proteins is a very important area of research in chemistry and biochemistry. Most of the practical applications involving amino acid analysis are performed using LC methods [1–3]. At the same time, electro-driven separation has, as a prime advantage the fact that very small sample volumes are required. This makes it very attractive for the analysis of biomolecules such as proteins, peptides and amino acids, where sometimes only small quantities of sample are available. The electrokinetic methods also offer significant advantages over liquid chromatography in terms of speed of analysis [1].

One of the on-going problems faced by researchers in this field is the relatively poor detectability of UV detection, resulting in poor concentration limits of detection (CLOD), notwithstanding the apparently low mass detection limits. This restricts the use of UV detection in ultratrace level analysis. More sensitive detection methods are therefore much to be desired.

Laser induced fluorescence (LIF) detection, first introduced in CE by Zare and co-workers [4] and used later by Sepaniak and co-workers [5] has the potential to enhance sensitivity, since more monochromatic light can be focused into the small inner diameter (50–70 µm) fused silica capillaries. This results in the excitation energy being more effectively applied to the very small sample volume. More recently, LIF detection applied to CE, with emphasis on detector technology, has received considerable attention demonstrating that vast improvement in instrumental detection sensitivities can be achieved [6–12].

Since most biological compounds such as proteins, peptides and amino acids do not possess native fluorescence properties at any one of the commonly available laser lines, chemical derivatization with a fluorescent label is required. However, this approach is not too simple as problems with reaction kinetics and label chemistry can adversely affect sensitivity resulting in a much higher concentration limit of detection than would normally have been achieved or expected.

In most reported work, test mixtures are derivatized at relatively high concentrations, typically 10^{-3}–10^{-4} M, and subsequently diluted to demonstrate detection limits in the 10^{-11}–10^{-12} M range. Furthermore, difficulties associated with ultratrace level derivatization have not been clearly spelt out in the current literature. Obviously, CE-LIF can be considered as a practical ultrasensitive analytical method only if it is possible to perform derivatization at a concentration level that is comparable to the detectable concentrations.

We present a critical review of the literature so as to properly assess and evaluate the historical approach to these derivatization methods with the aim of further improvement. The most commonly used fluorescent label, FITC, has been evaluated with regard to its merits for quantitative analysis when used in conjunction with LIF-CE and compared to another, albeit less-frequently used reagent, DTAF.

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FITC and DTAF as Derivatizing Reagents

FITC

Fluorescein isothiocyanate (FITC), has been widely used as a fluorescent labelling agent [13]. It is a commonly used derivatization reagent for antibody labelling [14], immunofluorescence procedures [15] and it is also frequently applied in the labelling of proteins [16] and amino acids [17]. It was first introduced as a fluorescence reagent in 1958 by Riggs et al. [14] and is available in two isomeric forms [13] (Figure 1). Isomer 1 is the form most often used as a fluorescence labelling reagent. In fact, there is only one report in the literature on the use of isomer 2 as labelling reagent [18]. Throughout this text, FITC refers to isomer 1 unless otherwise stated.

The use of FITC as a more sensitive alternative to phenyl isothiocyanate in the Edman degradation [19] was suggested by Maeda and Kawauchi [20]. They determined the N-terminal amino acids of proteins and peptides by first forming the thiocarbamylated (FTC) protein (Figure 2), followed by acidic cleavage to the fluorescein thiohydantoin amino acid (FTH). Muramoto et al. successfully demonstrated the application of FITC in the microsequencing of proteins and peptides [21]. The binding of fluorescein isothiocyanate to proteins and amino acids was described by Maeda et al. [15] who also suggested that the overall reaction mechanism for FITC with proteins is similar to that for Edman's phenyl isothiocyanate.

The thiocarbamylation reaction is sensitive to pH and temperature [17]. The best condition for this reaction was stated to be reacting in 0.2 M carbonate buffer at pH 9.0. Various reaction times have been described in the literature for FITC, depending on the product to be labelled. Mahoney et al. [22] reported that for the labelling of concanavalin A at room temperature, at least 30 hours are required before labelling is complete. In the first paper on FITC as an alternative reagent to PITC, Maeda and Kawauchi [20] mentioned a reaction time, with proteins, of 2 hours at 25 °C in the dark. In a subsequent report the authors described a method using FITC containing traces of pyridine [17] which was allowed to react with a neutral amino acid, alanine, at 23 °C for a period of 4 hours. The authors also reported that the acidic or basic amino acids reacted relatively slowly in comparison to the others. In a recent review [23], the reaction time for this derivative was described as being between 4–24 hours at room temperature.

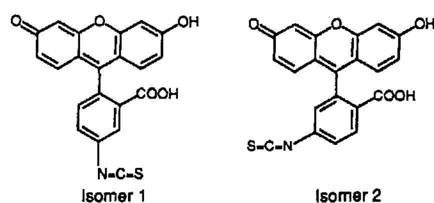


Figure 1
Isomeric forms of fluorescein isothiocyanate (FITC).

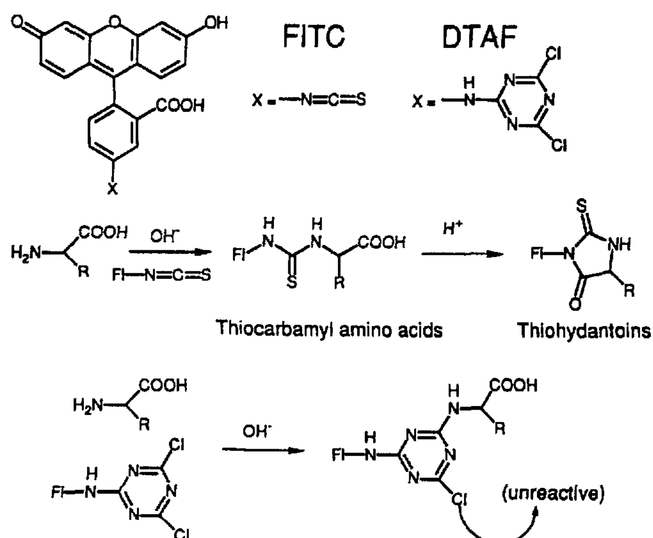


Figure 2
Derivatization of amino acids with FITC and DTAF.

DTAF

A fluorescein analogue, DTAF, introduced in 1976 as a fluorescing reagent by Blakeslee et al. [14, 15], is claimed to be superior to FITC in cost, purity and stability. DTAF has absorption and emission properties nearly identical to fluorescein isothiocyanate (FITC). Major absorption peaks occur at 492 nm and a single emission peak at 513 nm [14]. These absorption and emission data have been confirmed in other reports [22, 24]. DTAF is very stable when dry but it hydrolyses in alkaline solutions (pH 9.0). The half life has been estimated to be 4.1 hours at pH 9.0 and room temperature [22]. Therefore, stock solutions are to be kept at 0 °C.

DTAF was first used as a labelling reagent in chromatographic analysis by Siegler et al. [24, 25], who evaluated its application as a derivatization reagent for primary and secondary amines. Blakeslee et al. [14] claimed fast and smooth reaction at room temperature for DTAF with IgG, 80 % reacted in 1 hour at pH 9.0 and room temperature. Reaction rates at pH 8.0 have also been studied, 70 % labelling was observed for IgG. Unfortunately, lower pH values facilitate the formation of degradation by-products (Figure 3). The reaction

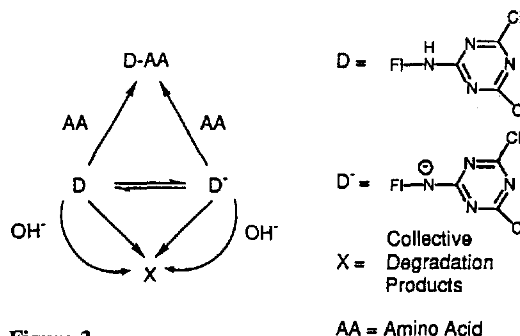


Figure 3
Degradation pathways of DTAF.

with amines slows down above pH 10.0 due to deprotonation of the bridging amine (Figure 3) and is most effective when both DTAF and amine are not ionized [24].

Reports in the literature are conflicting as to the time required for maximum labelling with DTAF. Mahoney et al. reported that at room temperature and at pH 8.0 at least 6 hours are required for the labelling of concanavalin A with DTAF [22]. This is in contrast to the 1 hour described by Blakeslee and Baines for the labelling of rabbit immunoglobulin [14]. A labelling time of 1 hour was also described by authors who evaluated DTAF as a derivatizing agent for amino acids with primary and secondary functions [26, 27]. When considering those data with the aim of choosing derivatization conditions, one must also take into account other factors which will affect reaction kinetics such as steric hindrance and reagent concentration.

LIF Detection with FITC/DTAF in Capillary Electrophoretic Methods

Most FITC applications in CE concern the thiocarbamyl derivatives (FTC). The first use of this reagent in electrokinetic methods was reported by Cheng and Dovichi in 1988 [8] when subattomole ($< 10^{-18}$ M) analysis of some amino acids was demonstrated. Concentration detection limits range from $9 \cdot 10^{-11}$ M for LYS to $5 \cdot 10^{-12}$ M for ALA. The high sensitivities achievable with laser induced fluorescence [6, 8, 12, 23, 28] and lamp fluorescence [23, 29] were demonstrated. Generally, limited sets of FTC amino acids were used as analytical probes in instrumental development rather than as the subject of analysis in their own right and little attention as been devoted to the quantitative aspects. Dovichi and co-workers used FTC amino acids as model compounds in the development of the sheath-flow cuvette [6, 9, 10]. The sheath-flow cuvette is a device used to minimize the scatter of the excitation laser beam from the walls of the capillary. These derivatives were also used in the development of CCD (charge-coupled devices) based on multichannel fluorescence detectors [7]. Exceptional detection limits were achieved with these sophisticated home-built detection systems. In most cases, standard solutions were used, with the amino acids being derivatized at fairly high concentrations (most often with the amino acid in excess of the labelling reagent, i.e. with FITC as the limiting factor rather than the amino acid which is the analyte of interest) followed by dilutions to arrive at a particular desired concentration.

On the other hand, a review of the literature has shown that DTAF has rarely been used in electrokinetic methods. It has been shown to be a potential derivatization reagent with some advantages over FITC [26, 27].

Experimental

Chemical Reagents

For all experiments L-amino acids (Sigma Chemical Co., St. Louis, MO, USA) were used. Fluorescein isothiocyanate isomer 1 (FITC) was obtained from Sigma Chemical Co., St. Louis, MO, USA and dichlorotriazinylaminofluorescein (DTAF) was obtained from Fluka Biochemika, Buchs, Switzerland. Absolute ethanol was obtained from Merck, Darmstadt, Germany, dichloromethane from AnalytiCals Carlo Erba and the acetone used was HPLC/Spectro grade (Alltech Assoc. Inc., Deerfield, IL, USA).

Derivatization Procedure

The FIT reagent solution was 5 mM FITC in HPLC grade acetone. The DTAF reagent solution was 5 mM DTAF in a 9-to-1 (by volume) mixture of absolute ethanol and dichloromethane. Stock amino acid solutions were 0.2 mM each in 0.2 M carbonate buffer (pH 9.0) for FITC derivatization or 0.1 M borate buffer (pH 8.0 or 9.0) for DTAF derivatization. For comparative studies, the total amount of buffer and organic solvent (from the reagent stock solutions) were held constant, irrespective of the actual volume ratios used. A typical procedure (Table 1) consisted of mixing e.g. 5 µL amino acid stock solution with 20 µL of carbonate buffer, 24 µL of reagent solvent and 1 µL of reagent stock solution. With a total volume of 50 µL, the final concentrations were 10^{-5} M for each amino acid and 10^{-4} M for the reagent. All cited derivatization concentrations refer to the final concentration in the 50 µL derivatization mixture.

Table I. General scheme used to prepare amino acid derivatives.

Vial#	1	2	3	4	5	6
Amino acid stock (µL)	5.0	4.0	3.0	2.0	1.0	0.0
Reagent stock (µL)	1.0	1.0	1.0	1.0	1.0	1.0
Buffer (µL)	20.0	21.0	22.0	23.0	24.0	25.0
Organic solvent (µL)	24.0	24.0	24.0	24.0	24.0	24.0
Total volume (µL)	50.0	50.0	50.0	50.0	50.0	50.0
Final conc of each A.A. (µM)	2.0	1.6	1.4	1.2	0.8	(blank)
Final conc of reagent (mM)	0.1	0.1	0.1	0.1	0.1	0.1

Analytical Conditions

All separation buffers were based on phosphate/borate and were prepared with deionized water (Milli – Q, Millipore Corp., Bedford, MA, USA). The pH of the buffers was controlled by the phosphate/borate ratio.

Electrokinetic separations were performed on a Beckman P/ACE System 2100 equipped with a Beckman Laser Module 488 which consists of a 3 mW, 488 air cooled argon ion laser (Beckman Instruments Inc., Palo Alto, CA, USA). Fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were 50 µm ID, 375 µm OD, 57 cm in length, with the detection window at 50 cm.

A four-step injection procedure was applied in all experiments. In the first step, the capillary was rinsed with running buffer for 2 minutes. This was followed by pneumatic sample introduction (2 seconds). In the third step, the capillary was placed in a vial containing separation buffer and a 0.1 second pressure injection performed. The function of this step is to rinse the outer side of the capillary and to avoid carry-over from the sample to the separation buffer vial. Just dipping should be sufficient, however the P/ACE control software does not allow 0-time injections. No detrimental effects were observed from the 0.1 second injection of separation buffer. Finally the capillary was placed in the separation buffer vial and the separation voltage (20 kV) applied. The column temperature was maintained at 25 °C at all times.

Results and Discussion

Optimization of the Derivatization Conditions

Sample derivatization. Evaluation of the derivatization procedure was performed at room temperature (21 °C) and at an elevated temperature of 40 °C. From the stock solutions, microliter aliquots were mixed in a 1.5 mL disposable vial as shown in Table I. Six vials were prepared, five of them contained five amino acids at equal concentration. The sixth vial was a blank, containing all the reagents but no amino acid. The concentration of the derivatization reagent was ten times higher than the total highest concentration of amino acids. After the appropriate aliquots were added, the vials were immediately stoppered and then shaken to ensure thorough mixing of the contents. The vials were then placed in an oven or kept at room temperature for the prescribed time depending on the reagent. For derivatization at lower or higher concentrations, the appropriate volumes were taken from the appropriate stocks to give the desired concentrations of amino acids and reagents. After derivatization, samples were diluted with deionized water (Milli-Q). A 10 µL Hamilton syringe was used to take the appropriate quantities of derivative for the respective dilutions. The diluted solutions were kept at 4 °C prior to analysis. After the reaction was initiated, sampling was done at 0.5, 1, 2, 4, 6, and 8 hour intervals. For DTAF, evaluation was performed at pH 8.0 and pH 9.0. Plots were made of response versus time of reaction for both FITC and DTAF.

FITC. The derivatization conditions were adapted from the method developed by Kawauchi et al. [17]. These authors reported complete reaction between FITC and alanine within less than 2 hours at room temperature. At a concentration of $2 \cdot 10^{-5}$ M, we found the reaction to be much slower. The amino acid concentration for the Kawauchi experiments was not mentioned but from other data in their experimental descriptions it can be inferred that it was possibly 2 orders of magnitude larger than ours. The FITC-reagent produces a few typical large background peaks but also some minor but very disturbing peaks. In a standard mixture, containing five amino acids (Figure 4), GLN was not further considered because of coelution with a reagent peak. Figure 5A shows the formation of the derivatives in the standard mixture as a function of time at room temperature (21 °C). After 8 hours there is no indication that the reaction with the amino acids is near completion.

Traces of pyridine (0.001 %) have been claimed [22] and used to accelerate the reaction [8, 18]. In our experience, with 10^{-4} M of FITC containing 0.001 % pyridine, no effect could be observed in the labelling of ALA at room temperature. After 24 hours the reaction was still not complete. On the other hand, raising the reaction temperature to 40 °C was found to be effective in reducing the reaction time. Figure 5B demonstrates that at least 4 hours are required for FITC (with no traces of pyridine) to react completely with an amino acid.

DTAF. DTAF poses more solubility problems than FITC. Although preparation of stock solutions in water has been claimed [14, 15] we could only obtain slurries in this medium. Even in acetone, DTAF occurs as a suspension [31]. Homogenous derivatization solutions were obtained when the DTAF stock was prepared in 9 : 1 (v/v) ethanol/dichloromethane. The volume fraction of dichloromethane was selected so that a clear solution was obtained after mixing with an equal volume of aqueous buffer. Figure 6 shows the CE separation of a standard mixture containing five amino acids. Like FITC, DTAF gives a few large background peaks with some minor peaks which are just as disturbing as those in FITC.

As with FITC, a comparable reduction in reaction time with increased temperature was observed for DTAF. A further improvement is obtained by increasing the reaction pH from 8.0 to 9.0 [30]. The labelling of primary and secondary functions at pH 8.0 and pH 9.0 at 40 °C are shown in Figure 7. These data show that maximum labelling is achieved at pH 9.0 in 2 hours but this is achieved in a somewhat longer time at the lower pH.

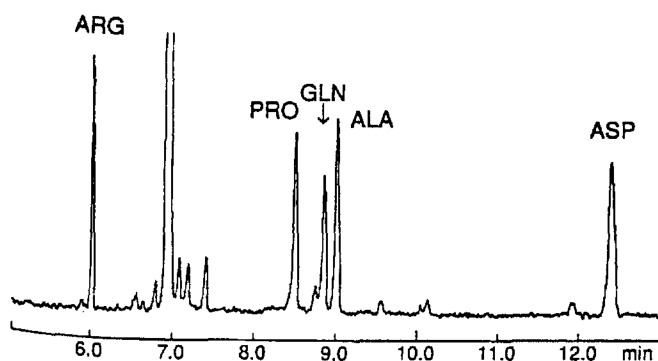


Figure 4

CE separation of a standard mixture containing 5 amino acids derivatized with FITC. Derivatization: each amino acid at $2 \cdot 10^{-5}$ M with 10 fold excess of reagent.

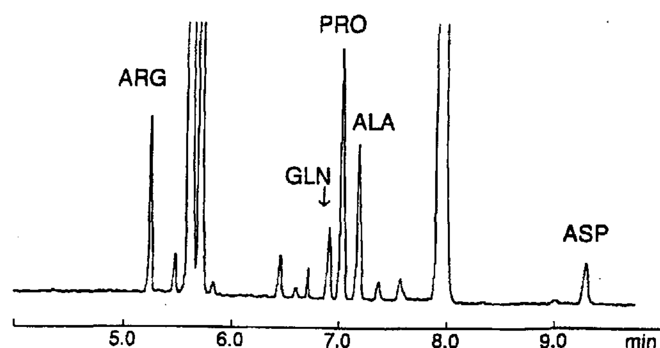
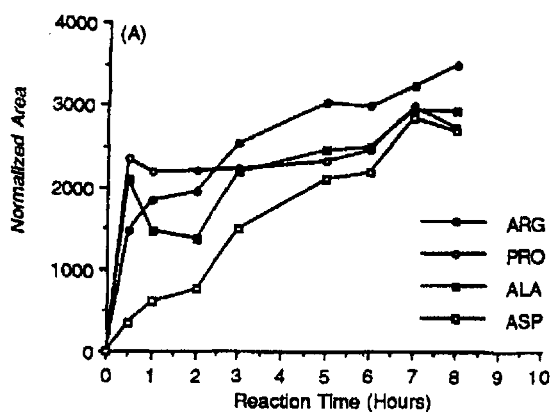


Figure 6

CE separation of a standard mixture containing 5 amino acids derivatized with DTAF. Derivatization: each amino acid at $2 \cdot 10^{-5}$ M with 10 fold excess of reagent.



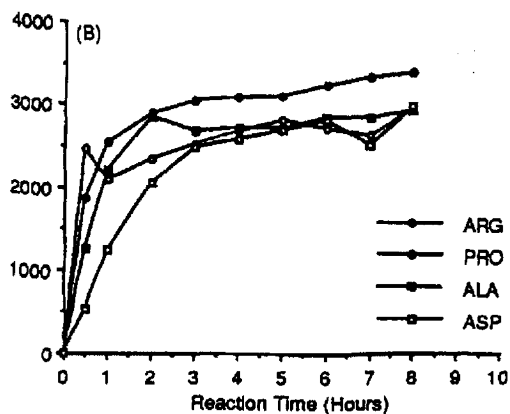
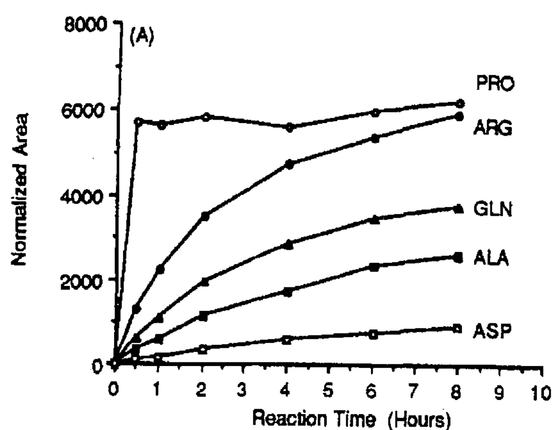


Figure 5

Derivatization of amino acids ($2 \cdot 10^{-5}$ M each) with tenfold excess of FITC. (A) at room temperature, (B) at 40 °C.



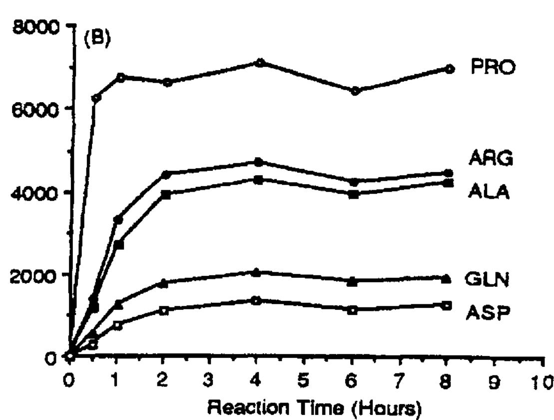


Figure 7

Derivatization of amino acids ($2 \cdot 10^{-5}$ M each) with tenfold excess of DTAF at (A) pH 8.0 and (B) pH 9.0 (reaction temperature: 40 °C).

Final comment. Compared to FITC, DTAF reacts faster with the amino acids. Both derivatization reactions can be speeded up by operating at higher temperatures.

Instrumental Sensitivity (Dilutions after Derivatization)

To evaluate instrumental sensitivity, a mixture of 5 amino acids was derivatized with both reagents at a relatively high concentration (0.4 mM) using the optimized conditions as described in the previous section. After derivatization, several dilutions were made in deionized water to give a dynamic range of 10,000. Care was taken to avoid serial dilutions. All dilutions were done within the shortest possible time. Immediately after the dilutions were made, the solutions were placed in a refrigerator at 0 °C until analysis. Each diluted concentration was analyzed in triplicate. The various concentrations were analyzed randomly. Linearity and qualitative concentration limits of detection (CLOD) were determined. Under the described conditions the reagent peaks do not interfere with quantitation. Figure 8 shows that a linear relation (in a log-log scale) was obtained in the range $2 \cdot 10^{-10}$–$2 \cdot 10^{-6}$ M for both FITC- and DTAF-derivatized amino acid.

From a practical point of view, this lower value can be considered as close to the concentration detection limit (Figure 9, $2 \cdot 10^{-10}$ M each). It must be observed that these experiments were performed with short injection times, 2 seconds. By increasing sample loading (e.g. with sample stacking) it is very likely that a further reduction in the instrumental detection limit could be demonstrated. However, for reasons which are explained below, this was not attempted.

The observed linearity is indicative of the stability of the diluted solutions for both reagents, since they were kept at 0 °C from the time of preparation until they were ready to be analysed. The analysis at each concentration was performed in triplicate, resulting in a considerable time lag between the analysis of the first concentration to be analyzed and the last concentration. From the slopes obtained from the calibration data shown in Figure 8 (Table II) it can be inferred that the FITC and DTAF have a similar labelling efficiency when derivatization is performed at fairly high concentration. The concentration limit of detection mentioned in Table II was obtained from the regression analysis:

$$\text{LOD} = \frac{3.S_{y/x}}{\text{slope}} \qquad (1)$$

Derivatization Sensitivity (Dilution before Derivatization)

Derivatization was performed at different concentrations with both reagents, starting from a high of $4 \cdot 10^{-5}$ M to a low of $4 \cdot 10^{-9}$ M, covering a dynamic range of 10,000. Reagent concentration was kept at 10 times excess of the total highest concentration. Preparation of the derivatives was based on the scheme described in Table I. Each concentration was analysed in triplicate and responses plotted against concentration. The same correlation was not found when the derivatization step was performed at low analyte concentrations. A lower limit of 10^{-7} M can be estimated from Figure 10.

Even above that concentration, response and concentration are less well correlated than in the case with derivatization at high concentration followed by dilution. This is indicative of the problems that are associated with derivatizing at low analyte concentration levels. Therefore, derivatization is clearly the sensitivity determining step. That is why, as previously noted, ostensible sensitivity enhancing procedures such as on-column focusing, are not effective as a means of increasing sensitivity.

In addition to the problem with derivatization chemistry, the determination of small amounts is hindered in another way. In a complex mixture, the reagent concentration must be in excess of the total amount of reacting species, which means that the excess in terms of an individual species is extremely large. In this

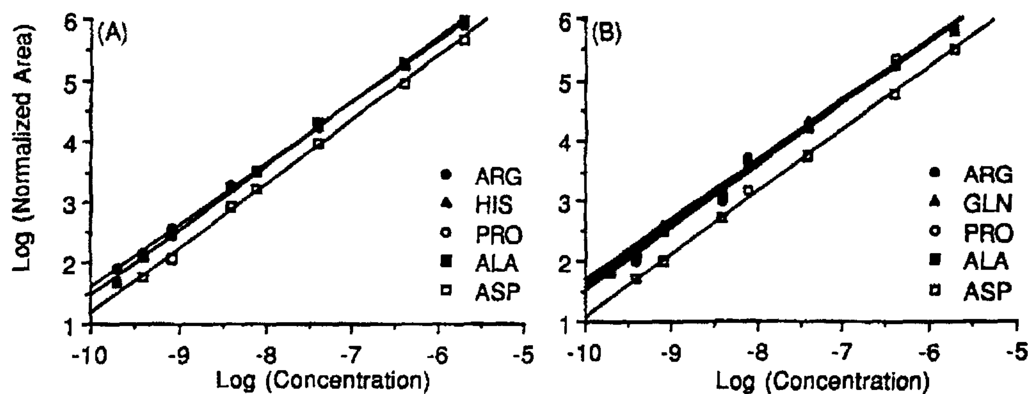


Figure 8
Response curves for (A) FITC- and (B) DTAF-derivatized amino acids. Derivatization at high concentration ($4 \cdot 10^{-4}$ M each) followed by dilution.

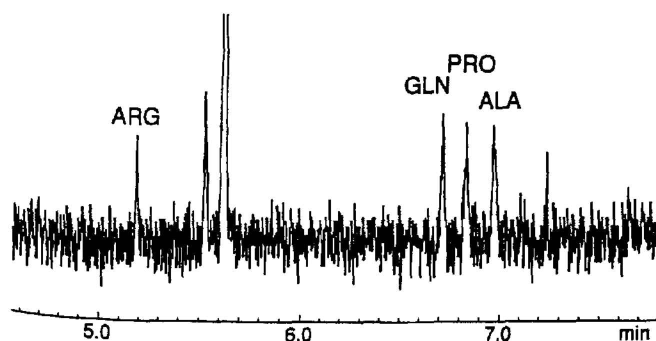


Figure 9
DTAF amino acids, diluted to $2 \cdot 10^{-10}$ M each.

Table II. Area-concentration relationships for some FITC- and DTAF-derivatized amino acids: slopes and limits of detection.

	FITC		DTAF	
	Slope	LOD	Slope	LOD
ARG	$4.8. 10^{11}$	$1.1. 10^{-10}$	$5.3. 10^{11}$	$2.7. 10^{-10}$
GLN	–	–	$5.4. 10^{11}$	$6.2. 10^{-11}$
PRO	$4.1. 10^{11}$	$3.9. 10^{-10}$	$5.2. 10^{11}$	$2.7. 10^{-10}$
ALA	$4.1. 10^{11}$	$1.4. 10^{-10}$	$4.0. 10^{11}$	$3.0. 10^{-10}$
ASP	$1.9. 10^{11}$	$3.1. 10^{-10}$	$1.6. 10^{11}$	$3.0. 10^{-10}$
HIS	$3.9. 10^{11}$	$1.0. 10^{-10}$	–	–

ARG = arginine, GLN = glutamine, PRO = proline, ALA = alanine, ASP = aspartic acid, HIS = histidine.

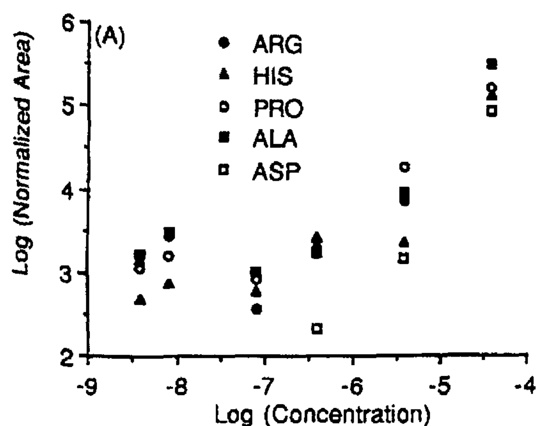


Table III. Migration time data obtained from triplicate analysis of FITC- and DTAF-derivatized amino acids.

Compound	FITC Mean (min)	FITC % rsd (n = 3)	DTAF Mean (min)	DTAF % rsd (n = 3)
ARG	6.53	0.64	5.31	0.08
GLN	–	–	6.92	0.07
HIS	9.19	0.73	–	–
PRO	9.58	0.87	7.04	0.15
ALA	10.21	0.95	7.19	0.09
ASP	14.83	1.49	9.13	0.07

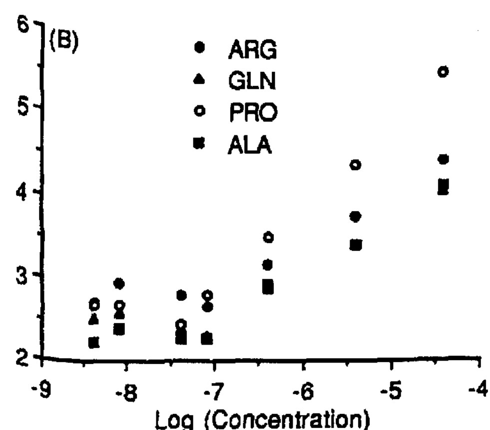


Figure 10
Response curves for FITC (A) and DTAF (B) derivatized amino acids. Derivatization at indicated concentration.

situation the magnitude of fluorescent side-products, whose exact nature is difficult to elucidate, becomes comparable to, or even larger than the peaks of interest. Using methods that are at present to hand, complete separation of all those peaks, which is necessary for reliable quantitation, is extremely difficult if not impossible.

Variability of Migration Times

In the quantitative experiments each analysis was carried out in triplicate, resulting in very good run-to-run variability in the migration times for both FITC and DTAF (Table III). On the other hand, FITC shows less day-to-day variability than DTAF (Table IV). Since the capillary in the Beckman P/ACE 2100 is liquid cooled and thermostated, this variability is not related to variations in temperature but may be attributed to the presence of the excess reagent in the sample. It is possible that the excess reagent causes some interaction with the capillary surface and that this leads to variability in migration times. A two minutes rinse with buffer prior to injection did not seem to have any effect on this variability.

Table IV. Run-to-run and day-to-day variability in the migration times in the analysis of FITC and DTAF-derivatized amino acids.

	FITC				DTAF			
	Run-to-Run		Day-to-Day		Run-to-Run		Day-to-Day	
	Mean (min)	% rsd (n = 28)	Mean (min)	% rsd (n = 4)[a]	Mean (min)	% rsd (n = 28)	Mean (min)	% rsd (n = 4)[a]
ARG	6.06	0.82	5.96	5.13	5.33	2.34	5.67	9.38
GLN	–	–	–	–	6.94	3.09	7.58	12.87
HIS	8.08	1.71	8.15	6.18	–	–	–	–
PRO	8.60	1.44	8.50	6.31	7.07	2.99	7.73	13.02
ALA	9.09	2.30	8.96	6.88	7.20	3.23	7.92	13.27
ASP	12.75	3.20	12.23	10.76	9.20	3.56	10.50	17.61

[a]Based on observations taken on 4 days over a period of 2 months.

Conclusion

Notwithstanding the high potential sensitivity of detection of CE-LIF, the difficulty in derivatizing amino acids at low concentrations with FITC and DTAF leads to the conclusion that from a practical and quantitative point of view, sensitivity with LIF detection is dictated mainly by the reagent chemistry. Other ways must be found to improve on the derivatization with these reagents not only in terms of reaction kinetics but also to produce cleaner derivatives so as to fully utilize the high sensitivity of laser induced fluorescence detectors. More time and resources could usefully be invested in the synthesis of new fluorescent labels which are tailor-made for CE-LIF work and are amenable to the commercially available LIF detection systems. Advances recently made in instrumental technology have not been paralleled by a similar evolution in required chemical technology. To fully exploit the potential of extremely low detectability, further development in derivatization chemistry will be needed. Considerations on suitability as labels should include the compatibility of the labelling chemistry i.e. fast reaction kinetics, size of the fluorescent moiety and little or no contribution to the fluorescent background signal.

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