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Improvement of laser-induced fluorescence detection of amino acids in capillary zone electrophoresis

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Abstract

Laser-induced fluorescence detection in capillary zone electrophoresis of fluorescein isothiocyanate (FITC I)-derivatized amino acids is a very sensitive detection technique. Unfortunately, the excess of FITC I and additional compounds interferes with the separation and detection of the amino acids. Therefore, the interferences were minimized by using optimal derivatization conditions. A liquid ion-exchange resin LA-2 was used to extract FITC I from the sample solution after derivatization. A better long-term stability was one of the results we obtained from optimizing the extraction step. The separations of the FITC-amino acids were performed in borate buffer solution of pH 9.5. Not all amino acids can be separated completely. The detection limits of two selected compounds, proline and arginine, were 0.3 and 0.5 n*M*, respectively.

1. Introduction

Laser-induced fluorescence (LIF) detection is the most sensitive detection technique [1–4] in capillary zone electrophoresis (CZE). Amino acids and primary amines can react with fluorescein isothiocyanate (FITC) to form highly fluorescent compounds [5–8]. The efficiency of the derivatization reaction depends on many factors; there are many interfering reactions, partly attributed to the impurities in the reagents. Therefore, it is difficult to identify the FITC-amino acid signals unambiguously and to distinguish them from the interfering peaks [5]. Previous results [9] showed that the stability of the derivatives decreases with time, leading to bad reproducibility and high detection limits. The aim of this work was to minimize the interfering effects of the amino acid determination after derivatization in different ways.

First, decreasing interferences implies that the derivatization conditions should be optimized, such as variation of buffer concentration, buffer components, buffer additives, FITC concentration and timing of the preparation.

Second, the reaction of the impurities with the FITC can be stopped by deactivating the derivatives or by minimizing their concentration to a very low level after complete reaction with the amino acids. Furthermore, it should be of interest to improve the reproducibility of the method, too. The basic idea is to decrease the FITC excess by reaction with an water-insoluble ion-exchange resin modified with amine groups simi-

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lar to the derivatization of the amino acids, but differing in the FITC derivative being fixed in the organic phase. The result should be a simple liquid–liquid separation of the aqueous and organic phases.

2. Experimental

2.1. Equipment

A commercial Beckman P/ACE System 2050 with a laser-induced fluorescence detector was used for all experiments. The excitation was performed by an air-cooled argon ion laser (3 mW) at a wavelength of 488 nm. The emission intensities were measured at a wavelength of 520 ± 10 nm filtered by a band pass filter. Unless otherwise specified, the separations took place in a 57 cm (50 cm effective length) $\times$ 75 μm I.D. capillary and a voltage of 20 kV with the cathode on the detector side. The capillary was thermostated at 25°C. Sample injection was accomplished by voltage (5 kV, 5 s) or mainly by pressure (0.5 p.s.i.; 1 p.s.i. = 6894.76 Pa) for a time of 10 s. The instrument was controlled and data were collected with software Gold. The detector signals were amplified by a factor of 10 by the software.

2.2. Materials

The L-amino acids alanine (Ala), arginine (Arg), asparagine (Asn), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), phenylalanine (Phe), proline (Pro) and tyrosine (Tyr) were purchased from Merck (Darmstadt, Germany), the FITC isomer I (FITC I) and the ion-exchange resin Amberlite LA-2 from Fluka (Buchs, Switzerland). The 1 m*M* stock solutions of the amino acids were stored at +4°C. The FITC I stock solution was prepared by dissolution of 19.5 mg FITC I in 100 ml acetone. After derivatization overnight 9 ml of the solution were mixed (by shaking) with a known volume of resin LA-2.

3. Results and discussion

3.1. Optimization of the derivatization reaction with FITC I

To show the influence of the kind and concentration of the buffer solution on the efficiency of the derivatization reaction L-Arg was selected as a model substance because of its short migration time. The comparison of phosphate and carbonate buffer solutions is shown in Fig. 1. The peak intensity and the peak shape strongly depend on the concentration of the buffer which varied from 4.4 to 133 m*M*. The best results (small and high peaks) were obtained at lower buffer concentrations. Similar results were also observed for disodium hydrogenphosphate solution, which was preferred for the higher sensitivity (expressed as peak area) at a concentration of 0.022 *M* and the shorter derivatization time. Working without any buffer components gives very low derivatization efficiencies and totally disturbed peak shapes. The carbonate and phosphate buffer conditions for maximum sensitivity are shown in Table 1.

Experiments on the time dependence of the derivatization have shown that pyridine recommended as activator [5] for the derivatization had no significant influence on the degree of formation of FITC-amino acid derivatives (Fig. 2). In addition, the pyridine impurities produced additional FITC derivatives which overlapped with the amino acid peaks of interest. This means that derivatization without pyridine is possible without a great decrease of efficiency.

Decreasing FITC concentrations ranging from 55.5 to 0.55 μM also led to decreasing absolute intensities of the components FITC-Arg and FITC but in every case in the same intensity ratios of FITC and FITC-amino acid (Fig. 3). It was not possible to get a high constant intensity of the analyte by decreasing the FITC concentration. Therefore, the higher concentration (55.5 μM) of FITC was used for the following investigations. A additional increase in FITC concentration to improve sensitivity is possible but interfering peaks have to be eliminated more effectively then.

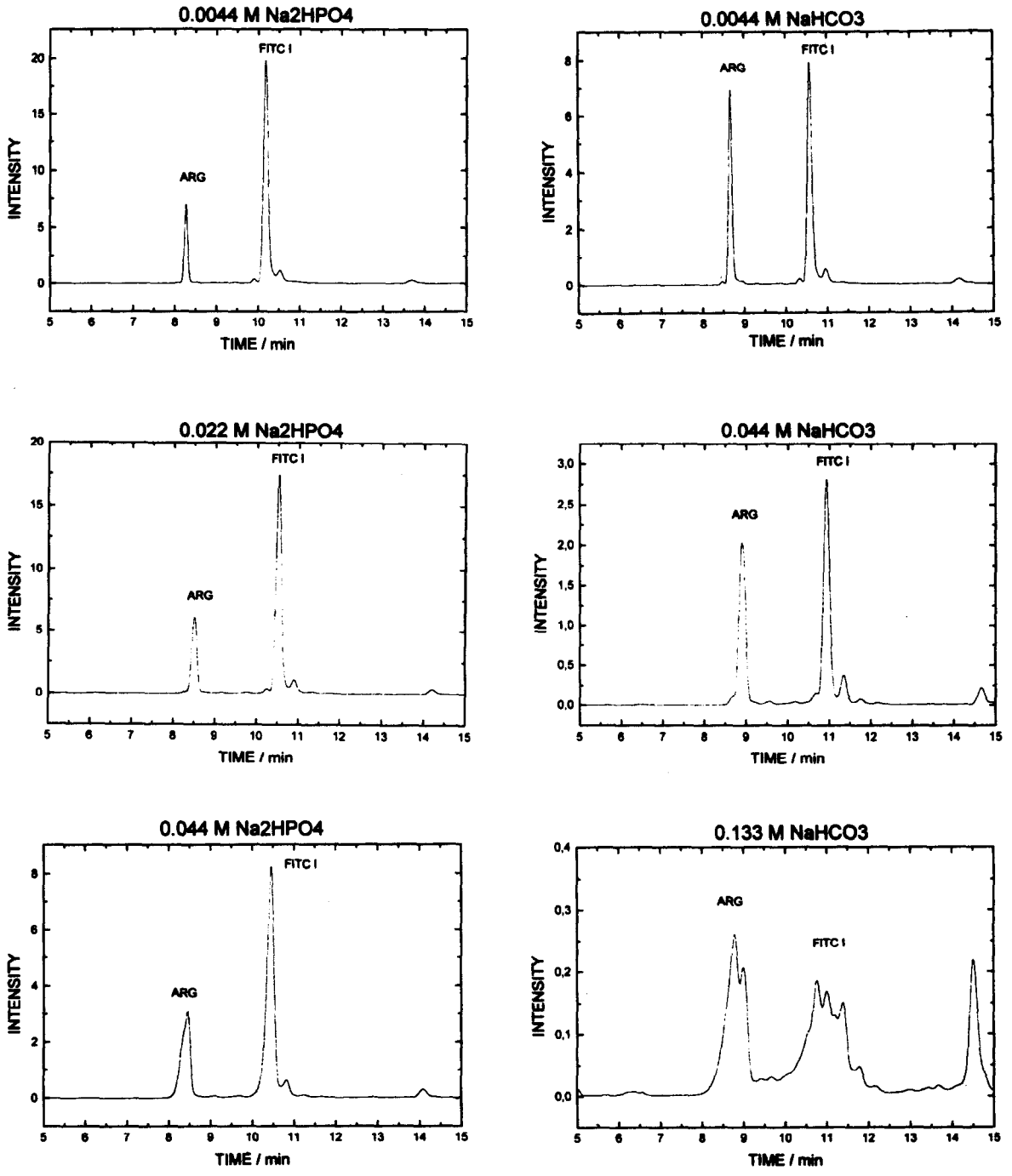

Fig. 1. Electropherograms of FITC-Arg and FITC using different buffer solutions. Derivatization conditions: FITC (11 μM), Arg (10 μM); separation conditions: borate buffer pH 9.

Table 1
Conditions for effective derivatization

Buffer	Concentration (mM)	pH	Reaction time (h)
Hydrogencarbonate	4.4	9	14
Hydrogenphosphate	22	9	12

Derivatization stock solutions: sodium hydrogencarbonate (0.2 M) and disodium hydrogenphosphate (0.2 M), both analytical-reagent grade.

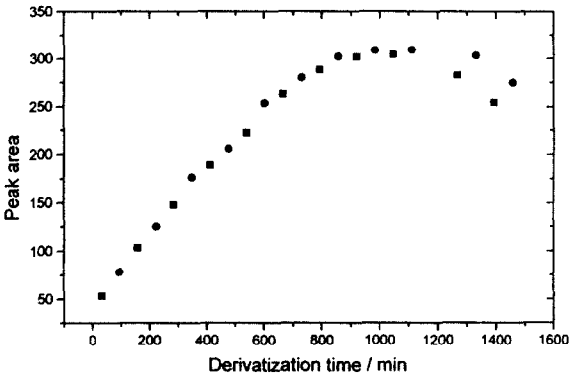

Fig. 2. Plot of peak area of FITC-Arg as a function of derivatization time with (●) and without (■) pyridine. Derivatization conditions: carbonate buffer (0.022 M) pH 9, FITC (11 μM), without or 10 μl pyridine/4.5 ml derivatization solution, Arg (10 μM).

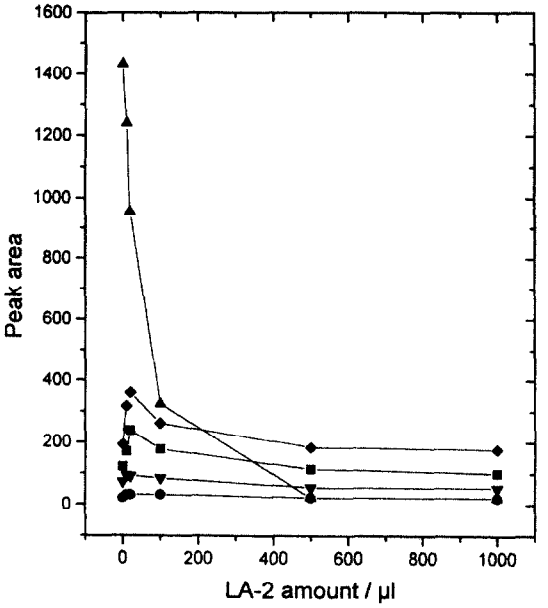

Fig. 4. Plot of peak areas of FITC-Arg (■), FITC-Pro (◆), FITC (▲) and two unknown compounds (●, ▼) as a function of added Amberlite LA-2 amount. LA-2 amount (μl)/6 ml derivatization solution, Pro (10 μM), other conditions as in Fig. 2.

3.2. Elimination of interfering components

To eliminate the interfering components corresponding to the higher FITC concentration for a more effective derivatization an ion-exchange

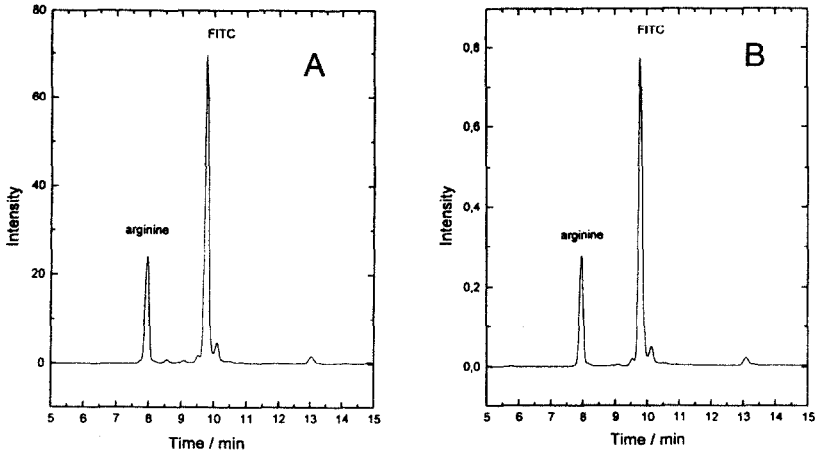

Fig. 3. Electropherograms of FITC-Arg and FITC using different FITC concentrations. Derivatization conditions: 0.022 M phosphate buffer pH 9.4, arginine (10 μM), (A) 55.5 μM FITC, (B) 0.55 μM FITC.

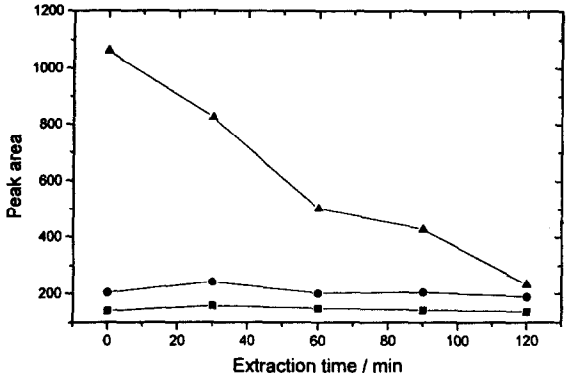

Fig. 5. Dependence of peak area of FITC-Pro (●) and FITC-Arg (■) on extraction time. Extraction condition: 10 μl LA-2/9 ml derivatization solution, other conditions as in Fig. 3. ▲ = FITC.

resin with secondary amine functionalities to react with FITC was tested. This resin was selected for three reasons: (1) the water-insoluble resin reacts with the FITC excess to decrease their concentration in the aqueous solution by liquid–liquid extraction, (2) it may stop the reaction with the amino acids at a specific time and (3) it may prevent further side reactions with impurities. Depending on the amount of the resin LA-2 the FITC intensity (expressed as peak area) is drastically reduced (Fig. 4). Unfortunately, some of the interfering peaks, the two unknowns, do not follow in the same matter. But the peak areas of FITC-Arg and FITC-Pro are unchanged so that a reaction between the derivatives and the resin did not take place. The interfering FITC resin adduct is easily separated by the distribution in the organic phase and/or a simultaneous formation of an orange precipitate in the organic phase. The aqueous phase separated from the resin phase can be used without further pretreatment for analysis. The plot of peak area *versus* extraction time in Fig. 5 with 10 μl LA-2/9 ml aqueous solution shows the reaction of LA-2 and FITC without changes in Arg and Pro intensities. One of the advantages of the FITC excess deactivation by LA-2 is also the improvement of the long-term stability of the amino acid derivatives demonstrated on Arg and Pro in Fig. 6. After a derivatization time of 12 h the liquid phases of the three samples were separated and analyzed over a period of 20 h. Contrary to the continuous increase of the peak intensities of the FITC-amino acids without LA-2 to reach a maximum the peak intensities decreased slightly in the case of LA-2 extracts (100 and 500 μl/9 ml) to reach stable signals over the time. Summarizing the results, the following conditions were selected for the most effective derivatization procedure: 55 μ*M* FITC, 22 m*M* Na_2HPO_4, reaction time 12 h, room temperature, 0.2 ml Amberlite LA-2, 30 min shaking.

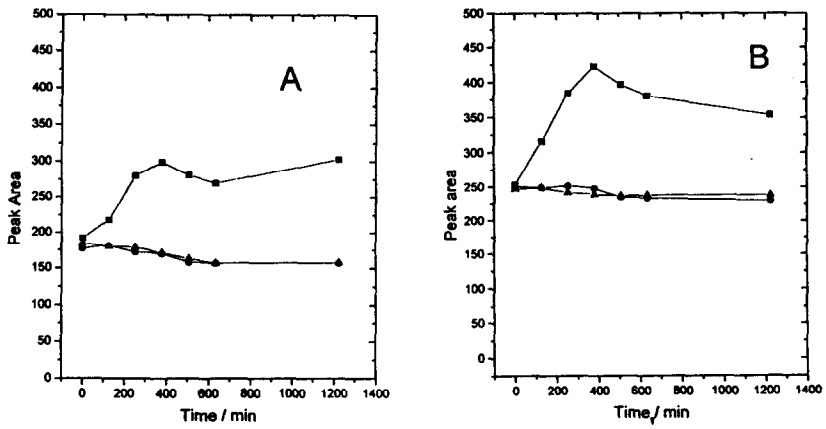

Fig. 6. Dependence of peak area of FITC-Arg and FITC-Pro on time after extraction of FITC with different amounts of LA-2 (long-term stability). LA-2 amounts/6 ml derivatization solution, time was measured after phase separation, other conditions as in Fig. 3. ■ = Without LA-2; ● = 100 μl LA-2; ▲ = 500 μl LA-2.

3.3. Optimization of the electrophoretic separation

To optimize the electrophoretic separation two main parameters were considered, *e.g.* the pH and the concentration of the buffer solution. Our previous investigations shown that the best results for FITC-amino acid separations with respect to short analysis time and high separation efficiency were obtained with a borate buffer. An increase in pH from 8.6 to 9.9 led to longer migration times for all amino acids investigated without drastic changes in sequence (Fig. 7). The elution order of FITC-amino acids depends only on the degree of the electroosmotic flow showing a maximum between pH 8 and 9. For the best combination resolution and migration time a pH value of 9.5 was chosen. By dilution of the original borate solution the migration time can be decreased even further if a complete analysis of all amino acids is not required. The migration times for all amino acids investigated may be found in Table 2. The pressure injection technique (10 s) has produced the highest sensitivity without distortion of peak shape for the FITC-amino acid detection.

The calibration for FITC-Arg and FIT-Pro has given detection limits ($S/N = 3$, 5 s peak width) of 0.5 and 0.3 nM, respectively, with correlation coefficients of 0.999 in both cases. The relative standard deviations are lower then 10% for a concentration of 10 nM. The dynamic range is more than three orders of magnitude. The proposed method will be applied to the determination of amino acids in needles of trees and other environmental samples in addition and comparison to the traditional HPLC determination. Further investigations on this subject will be aim at a more effective separation.

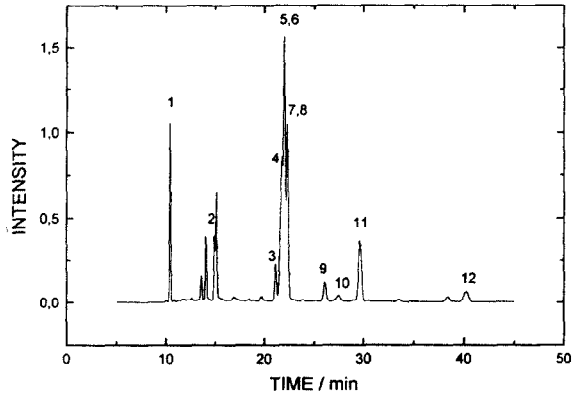

Fig. 7. Electropherogram of selected FITC-amino acids. Separation buffer: 0.4% boric acid, 0.3% sodium tetraborate pH 9.5; derivatization conditions: 0.022 M phosphate pH 9.4; extraction: 0.2 ml LA-2/6 ml sample solution. Peaks: 1 = Arg; 2 = $(Cys)_2$; 3 = Ile; 4 = His; 5 = Pro; 6 = Tyr; 7 = Phe; 8 = Gln; 9 = Ala; 10 = $(Cys)_2$; 11 = Gly; 12 = Glu.

Table 2
Migration times of the FITC-amino acids

Derivatives	Migration time (min)[a]	
	A	B
Ala	9.15, 13.25	26.79
Arg	7.36	10.4
Asn	11.8	15.05
$(Cys)_2$	13.45	14.28, 27.73
Gln	13.21	14.72, 22.87
Glu	19.84	41.13
Gly	14.09	31.03
His	12.21	22.12
Ile	12.07	14.51, 21.93
Phe	12.19	22.95
Pro	12.6	22.00
Tyr	12.24	22.49

Borate buffer: A = 0.2% boric acid, 0.15% sodium tetraborate, pH 9.5; B = 0.4% boric acid, 0.3% sodium tetraborate, pH 9.5

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