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Micellar electrokinetic capillary chromatographic separation and fluorescent detection of amino acids derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole

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Abstract

Another method has been developed for the separation of amino acids (1 min derivatization plus 22 min separation) by micellar electrokinetic capillary chromatography (MECC) with laser-induced fluorescence detection. Interestingly enough, such work has never been performed on essential amino acids derivatized by 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). Fifteen L-amino acid standards were labelled with NBD-F at 60°C for 1 min, and separated in a buffer system containing 20 m*M* borate, 25 m*M* sodium cholate, 10 m*M* Brij 35 and 2.5% methanol. Methanol was employed to expand the MECC migration time window; whereas Brij 35 was used to improve the fluorescence intensity of amino acid derivatives. This method also indicates that bile salt is effective for MECC separation of ionic analytes. Surprising though, improvements in resolution, sensitivity and speed for amino acids analysis are obtained in this work, which are not initially apparent in just employing another derivatizing reagent. Under optimal conditions, 15 amino acids were separated in a short 22 min analysis time, the shortest ever reported, and detection limits of nanomolar concentration and attomole mass were obtained. Furthermore, RSDs of migration time and peak height are better than 1% and 1.8%, respectively, again the smallest ever reported in the literature.

Keywords: Derivatization, electrophoresis; Amino acids; Fluoronitrobenzoxadiazole

1. Introduction

Capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection is a powerful method for separation of biomolecules. This method possesses the advantageous characteristics of high separation efficiency, high detection sensitivity and short analysis time, so it has become a popular method for rapid and high-resolution separation and determination of amino acids. Selection of fluorogenic and fluorescent reagents is very important for CE–LIF separation of amino acids. An excellent reagent for labeling amino acids should have the advantages of rapid reaction speed, high derivatization efficiency and less side products. For instance, fluorescein isothiocyanate (FITC) provides very high detection sensitivity for amino acids when excited by a commonly used argon ion laser at 488 nm. However, its derivatization reaction with amino acids is slow and is accompanied with many fluorescent side

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products [1]. Tetramethylrhodamine isothiocyanate (TRITC) is another fluorescent reagent that produces high detection sensitivity and can be excited by an inexpensive He–Ne laser at 543.5 nm. Nevertheless, the derivatization reaction is extremely slow [2]. On the other hand, fluorogenic reagents: *o*-phthalaldehyde (OPA) [3], naphthalene-2,3-dicarboxyaldehyde (NDA) [4], and fluorescamine (FS) [5] react with amino acids very quickly, but these reagents only react with primary amino acids, but not secondary amino acids, such as proline and hydroxyproline, to yield fluorophores. Furthermore, the fluorescent derivatives produced by these three reagents need to be excited by a He–Cd laser at UV wavelengths. This laser is more expensive and less popular than the argon ion laser. Another fluorescent reagent used for CE–LIF of amino acids is 3-(4-carboxybenzoyl)-2-quinolinecarboxyaldehyde (CBQCA). However, its derivatization reaction with amino acids takes 1 h, and similarly, the derivatives are only excited by a He–Cd laser at 442 nm [6]. Table 1 compares the use of different fluorogenic and fluorescent reagents in the analysis of amino acids.

Compared to other fluorescent reagents, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), which was introduced by Watanabe and Imai for labeling analytes [7,8], has several important advantages. First, it can react with almost all common amino acids, including both primary and secondary amino acids. Second, its reaction with amino acids is very fast, and there are only two fluorescent side products. In addition, NBD-F has high derivatization efficiency for amino acids, resulting in high detection sensitivity for amino acids when excited by an argon ion laser at 488 nm. NBD-F has been identified to be a useful and sensitive fluorescent agent for high-performance liquid chromatographic separation of amino acids [7,8], and CE–LIF separations of D- and L-phenylalanine [9], D- and L-aspartic acid [10] and dipeptides [11]. Although CE–LIF using NBD-F derivatization has been employed for enantiometric separations of phenylalanine or aspartic acid, this

Table 1
Comparison of the performance of CE separations of amino acids using NBD-F and other labelling reagents

Reagent[a]	Reaction time	Stability of derivatives[b]	Reagent fluorescence	Amino acids	Field strength (V/cm)	Buffer pH	Analysis time (min)	Detection limit
FITC	4 h	Stable (within 24 h)	High	R, K, Y, L, M, T, A, S, G, E, D, W	303	10	26	9–150 zmol
TRITC	20 h	Unstable	High	20 amino acids	326	9.0	12	1–2 zmol
OPA	Few s	Unstable	None	R, K, H, V, L, M, T, A, S, G, E, Q, W	267	9.5	38	3.0–100 amol
NDA	25 min	Stable	None	R, Y, H, F, L, V, M, T, A, S, G, E, D, I	214	9.0	27	0.7–2.3 amol
FS	Few ms	Stable	None	G, V, M, F, L, R	278	9.5	13	15.8 fmol (for glycine)
CBQCA	1 h	Stable (>24 h)[c]	None	R, Y, H, F, L, V, M, T, A, S, G, E, D, W, I, Q, N	240	7	30	10–70 amol
NBD-F	1 min	Stable (2 days)	High	R, K, Y, H, F, L, V, M, P, T, A, S, G, E, D	319	9.0	22	6.89–35.1 amol
FMOC	1 min	Stable[d]	High	R, K, Y, H, F, L, V, M, P, T, A, S, G, E, D, I, C	286	9.2	17	0.2 amol (for alanine)

[a] FITC: Fluorescein isothiocyanate [1], TRITC: tetramethylrhodamine isothiocyanate [2], OPA: *o*-phthalaldehyde [3], NDA: naphthalene-2,3-dicarboxyaldehyde [4], FS: fluorescamine [5], CBQCA: 3-(4-carboxybenzoyl)-2-quinolinecarboxyaldehyde [6], NBD-F: 4-fluoro-7-nitro-2,1,3-benzoxadiazole (this work), FMOC: 9-fluorenylmethyl chloroformate [12].

[b] Derivatization achieved at room temperature (RT), except at 60°C for NBD-F; all except FITC produce few reaction side products; only NBD-F and FMOC can derivatize both primary and secondary amino acids.

[c] Two weeks if derivatives are in dry states.

[d] Thirteen days at 4°C (and at RT) in daylight (or in darkness) [23].

method has not been fully exploited for the determination of standard amino acids found in proteins. Another fluorescent reagent, 9-fluorenylmethyl chloroformate (FMOC) which has similar merits as NBD-F (see Table 1), has the drawback of being excited only by an expensive pulsed KrF laser at 248 nm [12].

Capillary zone electrophoresis (CZE) has been used for separation of amino acids. However, this CE mode cannot provide enough resolution, especially for total amino acid analysis, since neutral amino acids migrate at similar velocities in CZE without separation [4,13]. To improve resolution, another CE mode, micellar electrokinetic capillary chromatography (MECC), has also been applied for amino acid separations. The most popular surfactant used for this purpose is sodium dodecyl sulfate (SDS) [3,6]. Nevertheless, SDS involves long analysis time, and results in slow analysis speed and irreproducible results. Another surfactant: sodium cholate (SC), which is a bile salt, produces micelles with hydrophobic surfaces, so it was generally considered for separation of hydrophobic analytes, especially for separation of highly hydrophobic analytes such as polychlorinated biphenyls (PCBs) [14] or polycyclic aromatic hydrocarbons (PAHs) [15,16]. However, SC has also been used for MECC separation of ionic vitamins and vitamin cofactors [17]. Moreover, bile salt was often used for chiral separation of amino acids [18] and enantiometric compounds [19–22]. In addition, bile salt can tolerate high concentration of organic solvents without drastic loss of efficiency and dramatic increase in analysis time. So we believe it should provide both good resolution and high speed for MECC separation of amino acids derivatized by NBD-F.

In MECC, organic solvents were often used to expand the migration time window and improve selectivity. Methanol was a commonly used organic additive in both SDS-MECC [3,14] and SC-MECC [14] systems for separations of hydrophobic molecules. However, in the SC system, the use of methanol is much favored because this system can tolerate much more methanol than the SDS system, which results from the unique bile salt micelle structure. For instance, it has been reported that the critical micelle concentration (CMC) of SDS begins to rise if methanol content is over 10%, while that of SC stays nearly constant up to 30% of methanol content [14]. Therefore, methanol has been added to the separation buffer in this work to achieve better selectivity. Furthermore, it was reported that Brij 35 could produce a three-fold signal enhancement in the detection of NBD-F derivatized dipeptides [11]. In order to achieve the same goal, we adopted the use of Brij 35 in our work.

In this work, SC was employed in MECC–LIF for separation of 15 essential amino acids, which, surprisingly though, has never been performed. Moreover, methanol was used to enhance resolution and Brij 35 was employed to improve detection sensitivity. The results obtained demonstrate that this is an excellent MECC system for amino acid separation.

2. Experimental

2.1. Instrumentation

All CE experiments were performed on the Beckman P/ACE 5000 system (Fullerton, CA, USA) equipped with a LIF detector using the argon ion laser line at 488 nm. An uncoated fused-silica capillary tube of 47 cm×375 μm O.D.×75 μm I.D. was used for all CE separations. A short section (ca. 3 mm) of polyimide coating was removed to form a detection window at a location 7 cm from the cathodic end of the capillary. Before use, the capillary was washed consecutively with 0.1 *M* NaOH, doubly distilled water and the separation buffer. Sample introduction was performed by electrokinetic injection at 15 kV for 2 s. The applied voltage for CE separation was typically 15 kV. The injection volumes, which vary with the migration times of the amino acid, range from 2.7 to 8.6 nl. All CE experiments were conducted at room temperature (25°C) using a sodium borate buffer at pH 9.0, which has been commonly used (see Table 1).

2.2. Precolumn derivatization of standard amino acids

Precolumn derivatization of standard amino acid solutions was conducted in microvials. A 5-μl volume of solution of amino acids at 100 μ*M* each (50 μ*M* for proline) was mixed with 5 μl of 50 m*M*

NBD-F solution. Typically, derivatization reaction was conducted at 60°C for 1 min [8]. Then the mixture was diluted with the running buffer solution to quench the labeling reaction.

2.3. Reagents

Unless otherwise stated, all chemicals were of analytical-reagent grade. L-Amino acids kit, NBD-F [CAS 29270-56-2], SC and Brij 35 were purchased from Sigma (St. Louis, MO, USA). 5.0 m*M* stock solutions of amino acids were prepared in borate buffer and diluted to the desired concentration prior to use. All buffer solutions were prepared by doubly distilled water and passed through a 0.22-μm cellulose acetate filter before use. NBD-F stock solution was prepared in ethanol. To simplify sample preparation for initial method development, the sample mixture only contains 11 NBD-F tagged amino acids listed in Table 2, except Arg, Lys, Glu and Asp, since these four NBD-F labelled derivatives were easily resolved from other 11 derivatives. Final experiments using optimal conditions were performed using a 15-amino acid mixture. Separation of Ile, Trp, Cys, Gln and Asn from the other 15 amino acids has been unsuccessful, and therefore they were not included.

3. Results and discussion

Borate was usually employed as the running buffer for CZE separations of amino acids. This buffer gives a high and reproducible electroosmotic flow, and provides high speed and good reproducibility for the separation of amino acids. However, since the electroosmotic flow in borate is too fast, the resolution is not enough for total amino acid analysis. To improve resolution, we used SC as the pseudostationary phase for MECC separation of amino acids. Fig. 1 compares the electropherograms obtained for separation of 11 amino acids in borate and borate–SC. From Fig. 1A, we can see that only seven peaks were obtained for 11 amino acids mixture in borate buffer. These labelled amino acids have similar mobilities and co-eluted in borate buffer. Separation has not been improved at 10 m*M* SC (Fig. 1B). Since the CMC of SC is 13 m*M* [15], that means SC at lower concentration than CMC does not improve resolution. Complete resolution of 11 amino acids is almost achieved when SC concentration is 25 m*M*, except that a peak overlaps with the reagent peak a (Fig. 1C). However, the resolution becomes worse when SC concentration is 50 m*M* (Fig. 1D). It is noted that the resolution is improved at the expense of analysis time as migration is finished within 9 min in C, as compared to 7

Table 2
Migration times (t_m), number of theoretical plates (N), RSD of t_m (RSD_t), RSD of peak height (RSD_H), and detection limits (LODs) for amino acids ($n=6$, $S/N=3$)[a]

Peak No.	Analyte	t_m (min)	N	RSD_t (%)	RSD_H (%)	LOD (n*M*)	LOD (amol)
1	Arg (R)	6.85	189 000	0.56	1.01	4.09	35.1
2	Lys (K)	7.85	158 000	0.72	1.41	4.31	32.3
3	Tyr (Y)	8.71	175 000	0.63	0.88	3.75	25.4
4	His (H)	10.01	346 000	0.47	1.24	3.91	23.0
5	Phe (F)	10.29	208 000	0.55	1.82	1.22	6.98
6	Leu (L)	10.62	338 000	0.68	0.99	2.20	12.2
7	Val (V)	10.76	396 000	0.47	1.69	2.43	13.3
8	Met (M)	10.87	354 000	0.50	1.35	1.31	7.10
9	Pro (P)	11.04	281 000	0.42	1.70	1.50	8.00
10	Thr (T)	11.46	291 000	0.53	1.80	1.70	8.74
11	Ala (A)	11.56	298 000	0.60	1.75	1.96	9.99
12	Ser (S)	12.13	364 000	0.71	1.29	2.09	10.1
13	Gly (G)	12.39	341 000	0.64	1.18	1.45	6.89
14	Glu (E)	18.85	254 000	0.96	1.02	7.63	23.8
15	Asp (D)	21.47	211 000	0.82	1.33	7.31	20.1

[a] Separation conditions as in Fig. 4.

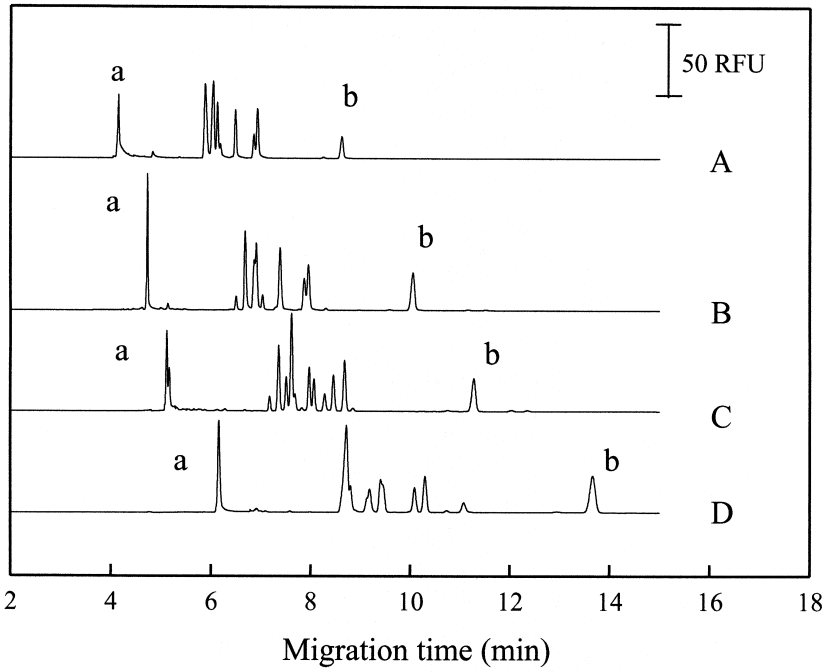

Fig. 1. Electropherograms obtained for separations of 11 NBD-F labelled amino acids in 20 m*M* borate buffer (pH 9.0) at different SC concentrations. (A) No SC, (B) with 10 m*M* SC, (C) with 25 m*M* SC, (D) with 50 m*M* SC. Amino acid concentrations: 5.0 μ*M*, except 2.5 μ*M* for Pro; separation voltage: 15 kV; injection: 15 kV for 2 s. The first and last peaks (a and b) in each electropherograms are NBD-F reagent peaks.

min in A. Reagent peaks a and b are probably due to reaction side products: NBD-OH and NBD-NH_2 [8].

Our experiments also demonstrated that although SC is commonly used to improve separation of highly hydrophobic molecules, it is also effective for separation of ionic amino acids. The resolution enhancement indicates that SC has different retention for NBD-F labelled amino acids, which is resulted from different interaction between SC and NBD-F labelled amino acids. We also attempted to use SDS as the surfactant for MECC separation of NBD-F labelled amino acids, but our results (not shown) demonstrated that this strategy was not effective to improve resolution.

Although it is well known that fluorescent intensity strongly depends on the environment around the fluorophore, we can see that the addition of SC micelles in the borate buffer does not enhance the detection sensitivity of NBD-F labelled amino acids (see Fig. 1). It has been reported that the fluorescence intensity of NBD-F labelled dipeptides increased when Brij 35, or polyoxyethylene(23)lauryl ether, a non-ionic surfactant, was added in the separation buffer [11]. In this work, we also found that the addition of Brij 35 in borate–SC buffer resulted in an enhancement of detection sensitivity for NBD-F labelled amino acids. Fig. 2 compares the electropherograms obtained in the borate–SC buffer with or without 10 m*M* Brij 35. The intensity increased to about five times for the reagent peaks a and b when Brij 35 was used as an additive. However, only three-fold enhancements were observed for most of the NBD-F labelled amino acids. Higher concentration of Brij 35 than 10 m*M* in the MECC buffer did not further improve the sensitivity (results not shown). But too high a concentration of Brij 35 has caused unstably high current in the separation capillary and influenced the separation reproducibility.

Fig. 3 compares the effect of different concentrations of methanol on the MECC separation of NBD-F labelled amino acids. From the elec-

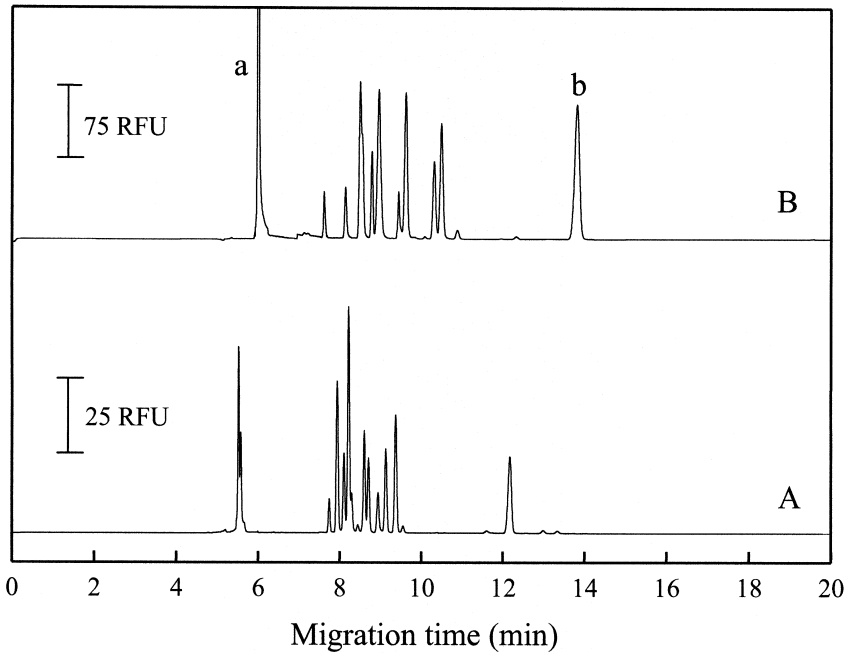

Fig. 2. Electropherograms obtained for separations of 11 NBD-F labelled amino acids in 20 m*M* borate–25 m*M* SC buffer (A) with no Brij 35, and (B) with 10 m*M* Brij 35. Amino acid concentrations: 5.0 μ*M*, except 2.5 μ*M* for Pro. Other conditions as in Fig. 1.

tropherograms we can see that the use of methanol expands the migration time window of SC system, resulting in a better resolution of amino acids (Fig. 3B). However, higher methanol content results in longer separation time and lower separation efficiency. For instance, peak tailing is very obvious when methanol content is high (see Fig. 3D). Furthermore, when the methanol content was 10%, the migration time became 40 min for the separation of all 15 amino acids (results not shown). Therefore, a methanol content of 2.5% was found to be the best choice, considering both optimal resolution and short analysis time (see Fig. 3B).

Fig. 4 shows the MECC separation of 15 NBD-F labelled amino acids under optimal conditions. The separation time is within 22 min. If only the first 13 amino acids had been analyzed, the total separation time could have been achieved within 13 min. This value is the smallest analysis time ever reported, with the consideration of the separation field strength, running buffer pH and the number of amino acids analyzed (Table 1). Based on this optimized method, separations of the 15-amino acid mixture at different concentrations were performed six times to establish limits of detection (LODs) and relative standard deviations (RSDs). Migration times, theoretical plate numbers (N), RSDs of migration time and peak height, and LODs are tabulated in Table 2. The N values for amino acids range from 158 000 to 396 000, indicating that the SC-MECC system can provide high separation efficiency for NBD-F labelled amino acids. The detection limits, calculated based on $S/N=3$, range from 1.22 to 7.63 n*M* or from 6.89 to 35.1 amol for 15 amino acids. A 1000-fold reduction in the LOD was achieved, as compared to the results obtained in a previous report based on HPLC separations [7]. Our findings (i.e., 7.31 n*M* aspartic acid and 1.22 n*M* phenylalanine) also compare favorably with a detection limit of 140 ppm or 0.85 m*M* in D- and L-phenylalanine [9], and 100 n*M* in D- and L-aspartic acid [10]. This improvement is attributed to the use of laser excitation and the addition of Brij 35 in the running buffer. The RSDs of migration time are less than 1% and that of peak height are better than 1.8%. For comparison, it is surprising to find that RSDs of other amino acid

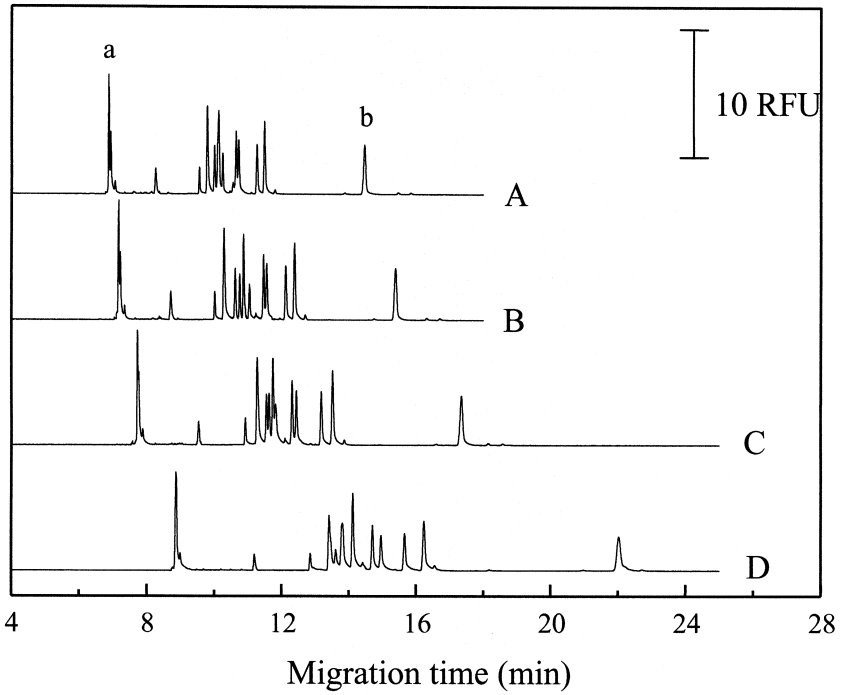

Fig. 3. Electropherograms obtained for separations of 11 NBD-F labelled amino acids in 20 m*M* borate–25 m*M* bile salt–10 m*M* Brij 35 buffer (pH 9.0) (A) with 1.0% methanol, (B) with 2.5% methanol, (C) with 5.0% methanol, (D) with 10% methanol. Amino acid concentrations: 0.5 μ*M*, except 0.25 μ*M* for Pro. Other conditions as in Fig. 1.

studies are seldom reported, except that a peak area RSD of 3% was reported for Phe [9], and a peak height RSD of up to 4.8% and migration time RSD of up to 1.71% were reported for Ser, Ala, Glu and Asn [3]. This shows that the RSD values for amino acid separation in this work are the smallest ever reported in the literature.

4. Conclusions

We have shown that SC-MECC–LIF with NBD-F derivatization is a simple, efficient method for separation of amino acids. It should be mentioned that this method is rapid in that the experiment, including sample derivatization and MECC separation, can be accomplished within 25 min. The only faster amino acid separation reported was achieved in 17 min (see Table 2). However, a special and expensive KrF pulsed laser should be used to excited the FMOC derivatives at 248 nm [12]. It is noted that the migration order of amino acids depends on the fluorogenic or fluorescent reagent being used. Incidentally, NBD-F derivatives are highly compatible with SC so that good separation can be achieved within a reasonably short period of time, with additional resolution enhancement accomplished by the addition of methanol. Therefore, the selection of a suitable labeling reagent is not only dictated by the rate and efficiency of derivatization, the quantum yield and stability of fluorescent derivatives, and the number of interfering fluorescent side products, it is also influenced by the resolution and reproducibility achieved in the separation of derivatized analytes. NBD-F is a good example of providing good resolution within reasonably short analysis time for analysis of standard amino acids. A limitation of this method is that Ile, Trp, Cys, Gln and Asn cannot be resolved from the other 15 amino acids. Future work is to apply this method to analyze L-amino acids in biological samples, which should not be affected by the temperature of 60°C used for derivatization.

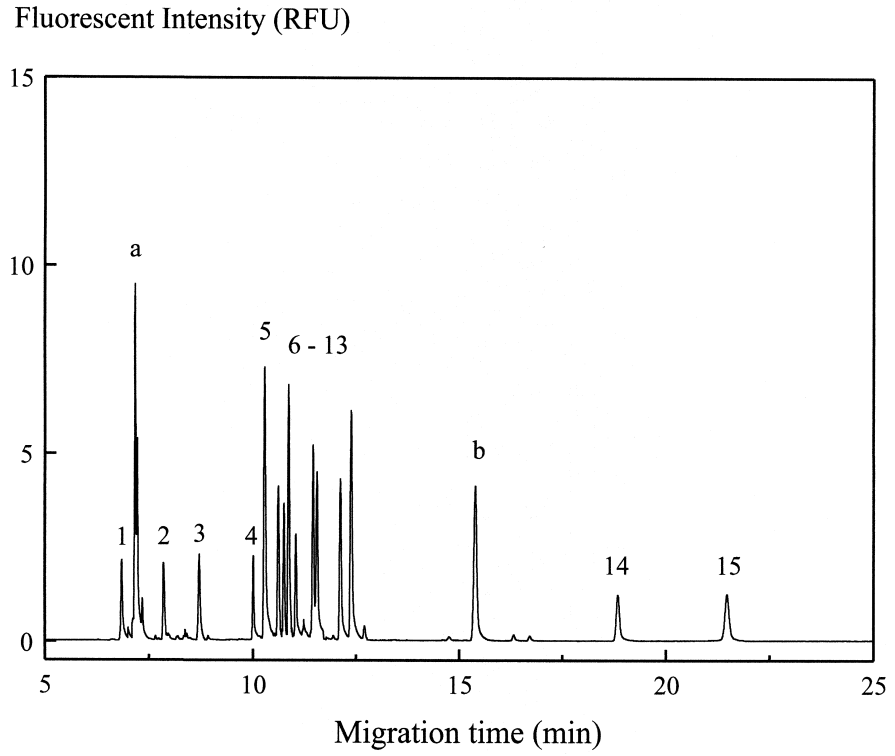

Fig. 4. Electropherogram obtained for separations of 15 NBD-F labelled amino acids in 20 m*M* borate–25 m*M* bile salt–10 m*M* Brij 35 buffer (pH 9.0) with 2.5% methanol. Amino acid concentrations: 0.5 μ*M*, except 0.25 μ*M* for Pro. Other conditions as in Fig. 1. Peak identification for amino acids is shown in Table 2.

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