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Derivatization at capillary inlet in high-performance capillary electrophoresis
Its reliability in quantification

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Abstract

The possibility of using derivatization at the capillary inlet for quantitative analysis by capillary electrophoresis was explored using amino acids as model compounds. It was shown that by using an appropriate introduction reagent and sample introduction program, i.e. reagent, then sample and once again reagent (so-called sandwich mode), selected amino acids were derivatized in high yields with *o*-phthalaldehyde as in pre-capillary derivatization.

Analyses of a number of amino acids by this at-inlet derivatization technique, followed by separation in a low pH phosphate buffer containing CHAPS, demonstrated the efficacy of this technique in automated amino acid analysis.

Keywords: Derivatization, electrophoresis; Amino acids

1. Introduction

In liquid chromatography (LC) and capillary electrophoresis (CE) separated components of a sample are most frequently monitored by absorption in the ultraviolet or visible region. Monitoring by fluorescence also is sometimes useful. However, such direct detection methods have a limitation in that the components must have either chromophores or fluorophores. Chemical transformation to UV–Vis-absorptive or fluorescent derivatives plays an important role in cases where such conditions are not satisfied.

Generally, there are two modes of chemical derivatization, i.e., those in pre-separation and post-separation fashions. Post-separation derivatization has the advantages that the derivatization reaction proceeds spontaneously simply by adding a stream of a reagent solution to the eluate or the electrophoretic solution containing the separated components and that the reaction does not necessarily need to give quantitative yields of derivatives. In LC, the derivatization reaction occurs in a tube long enough to give sufficient concentrations of derivatives for detection. In CE, however, the distances between separated zones of the components and the detector window are too short to allow for the reaction to proceed quantitatively. Although a few skillful techniques were reported by Rose and Jorgenson [1], Pentoney et al. [2] and Albin et al. [3], post-separation derivatization in CE is far from a practical technique at this point. For these reasons, derivatization in CE is usually performed in a pre-separation (pre-capillary) fashion.

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Pre-capillary derivatization has an advantage in that one can convert the components of a sample to derivatives having the desired chemical properties, by choosing an appropriate derivatization reagent. Therefore, even non-ionic components may be transformed to either anionic [4] or cationic [5] derivatives, and both of them may be direct objects of electrophoresis. In contrast, the minimum volume that can be handled by manual operation is generally several microliters, whereas the volume introduced to a capillary is several nanoliters in usual procedures for high-performance capillary electrophoresis (HPCE), hence, approximately one thousandth of the solution of the derivatives is analyzed and the rest remains unused or discarded. Although a few devices for automated pre-column derivatization for high-performance liquid chromatography (HPLC) are commercially available, there seem to have been no devices for automated pre-capillary derivatization in HPCE.

Under these circumstances, one should draw attention to a hidden characteristic property of HPCE that analysis can be performed in free solutions. This means that the capillary as a separation tool can be used also as a small reaction vessel. A reaction can occur at any region in the capillary. If it occurs at the inlet of the tube, it leads to a new type of derivatization. Fischman et al. [6] pointed out the importance of this technique and demonstrated its usefulness in the derivatization of a few amino acids with carboxytetramethylrhodamine succinimidyl ester and separation of the derivatives by CE. Tjaden et al. [7] also presented some derivatization data obtained by this technique. On the other hand, Gilman and Ewing [8] used a variation of this technique for the analysis of the amino group-containing small molecules in the fluid of a single animal cell. In this experiment, a single mammalian cell was introduced to a capillary inlet, where a plug of a reagent solution containing naphthalenedicarboxyaldehyde, sodium cyanide, 4-(2-hydroxy-ethyl)-piperazineethanesulfinic acid (HEPES), and digitonin in the same buffer as the electrophoretic carrier had been created by prior injection of this solution. The cell fluid was lysed and the components were derivatized to fluorescent products, which were subsequently separated by CE. Detection of the He–Cd laser (422 nm)-induced fluorescence enabled simultaneous analysis of a few catecholamines and amino acids at the fmol level. Although these previous works suggested the advantage of this technique, none of them discussed in detail quantification in such narrow tubes in relation to the problem of sample/reagent mixing.

It will be well recognized that this "at-inlet" derivatization technique is also important for automated analysis of a group of compounds having a number of homologues but neither chromophores nor fluorophores, such as proteins, carbohydrates and related substances. We describe herein the results of quantification by various sample/reagent introduction modes and under various conditions, taking derivatization of selected amino acids with *o*-phthalaldehyde as a model system.

2. Experimental

2.1. Reagents

The samples of amino acids, *o*-phthalaldehyde (OPA), cinnamyl alcohol (C_{Al}) and cinnamic acid (C_{Ac}) were of analytical grade and were commercially available. Acetonitrile was of HPLC grade. Water was deionized and distilled before use in a glass vessel. All carrier and reagent solutions were made in these solvents and degassed before use.

The sample solutions were prepared by mixing equal volumes of 100 m*M* borate buffer, pH 10.0, and an aqueous amino acid solution containing four or sixteen amino acids in various concentrations, together with C_{Ac} to a concentration of 1.5 m*M*. The reagent solutions were made by mixing equal volumes of a 100 m*M* OPA solution in either water or acetonitrile and 100 m*M* borate buffer, pH 10.0. Both the sample and reagent solutions were prepared fresh before use.

2.2. HPCE

HPCE was performed with an ABI-270A capillary electrophoresis system from Perkin Elmer (Norwalk, CT, USA), equipped with a thermostated oven for controlling capillary temperature and a multi-wavelength UV detector. A capillary of fused silica (50 μm I.D., 72 cm) was obtained from Polymicro

Technologies (Phoenix, AZ, USA) and the detection window was made at the 50 cm position from the anodic end by removing a 1-mm portion of polyimide coating by burning. The polyimide coating of the 0–5 cm portion from the anodic end of the capillary was also removed for the estimation of plug length. The sample and reagent solutions were introduced automatically by suction for specified periods. Introduction and standing times were freely programmable. The temperature of the oven was maintained at 30°C throughout analysis.

2.3. Estimation of plug length

A dye solution, prepared by mixing equal volumes of an aqueous 0.05% (w/v) solution of bromophenol blue and 1 *M* sodium hydroxide, was introduced to the inlet end of the capillary (that had been made transparent by removing the polyimide coating) for specified periods, and the lengths of the blue zones were measured using a scale against yellow background.

2.4. Pre-capillary derivatization (as reference)

Selected amino acids were derivatized with OPA by a slight modification of the method of Roth [9]. A sample solution (2 μl) of the four selected amino acids – phenylalanine (Phe), methionine (Met), alanine (Ala) and glutamic acid (Glu) – was mixed with the aqueous reagent (OPA) solution (12 μl), and the mixture was allowed to stand for 20 min at room temperature. The reaction solution was introduced to the capillary and analyzed immediately by HPCE.

2.5. At-inlet derivatization

A sample solution of selected amino acids and the reagent (OPA) solution were introduced successively to the capillary for various periods of time, in either tandem or sandwich mode (diagrams in Fig. 1), and the resultant succession of plugs of the sample and reagent solutions were allowed to stand for specified times.

A potential of 30 kV was applied between both ends of the capillary to transport the resultant OPA derivatives of the amino acids to the detector window. Detection was carried out at 230 nm.

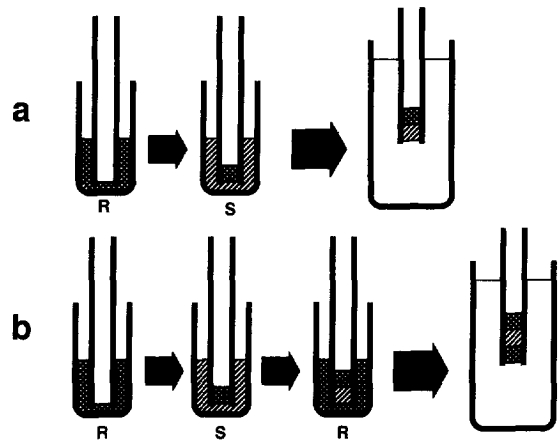

Fig. 1. Diagram of the introduction of sample and reagent solutions to the inlet of a capillary by tandem (a) and sandwich (b) modes. ▨ sample solutions (S), ■ reagent solution (R), □ electrode solution.

3. Results and discussion

3.1. Observation of the state of mixing

The capillaries for HPCE are narrow, their inner diameter being usually in the order of 10–50 μm, and the hydrodynamics in such narrow tubes is considered to be somewhat different from that in ordinary columns used for LC. The state of mixing in flow injection analysis was studied by Scholten et al. [10], but little has been known about the mixing of overlayed plugs in such narrow tubes.

Fig. 2 shows the result of a model experiment to demonstrate what happens when plugs of various lengths are overlayed in a 50-μm I.D. tube. Two solutions of C_{A1}, one 5×10^{-3}% (w/v) and the other 1×10^{-2}% (w/v) in electrophoretic buffer, were the models of a sample (S) and a reagent (R) solution, respectively. The use of a common solution was to observe the state of mixing of these solutions simultaneously and at a single wavelength.

It is theoretically possible to choose different solutes as models for the sample and the reagent. However, since the mixed zone should be monitored at the detector window in the same state as that at the

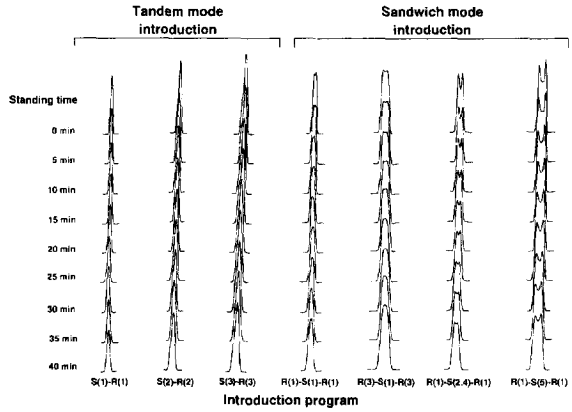

Fig. 2. Comparison of peaks observed when 5×10^{-3}% (w/v) and 1×10^{-2}% (w/v) c_{A1} solutions in 50 m*M* borate buffer (pH 10.0) (assumed as sample solution, S, and a reagent solution, R, respectively) were introduced to a capillary (50 μm I.D., 72 cm) using various programs, and a potential of 30 kV was applied between both ends after standing the overlayed plugs for various times. Electrophoretic carrier, 50 m*M* borate buffer (pH 10.0). The distance between the inlet and the detector window was 50 cm. Detection was performed by measuring the absorption at 230 nm. The numbers in parentheses for these programs are the periods (in seconds) of introduction.

inlet, without being disturbed during transportation to the detector window, the sample and the reagent should have exactly the same mobility; otherwise they will be separated into two zones during transportation, which would make it impossible to observe the state of mixing. The most suitable pair that meets this requirement is that of non-ionic compounds. In addition, each of the pair should have different absorption maxima and should be monitored separately by dual-wavelength measurement without interference from each other. Unfortunately, it is quite difficult for the selected pair to meet this second requirement as well. In addition, an expensive dual-wavelength monitoring system for HPCE is required for such an experiment. The single-compound model employed in this work is the second best method of obtaining sufficient information on mixing.

Thus, the two solutions of C_{A1} were overlayed at the anodic end of the capillary in either tandem (S–R) or sandwich (R–S–R) mode for varying periods of time, by suction, using the automated sampling system installed in the HPCE apparatus. After the overlayed plugs were allowed to stand for various times, a potential was applied between the tube ends. The plugs in the capillary were gradually mixed on standing, then the mixed zone was driven to the detector window by electroosmotic flow, where it was monitored by UV absorption at 230 nm. Since the media of sample and reagent were common and also the same as the electrophoretic carrier, peak broadening arising from disturbance due to the mixing of media is minimized. It is observed that introduction by the S(1)–R(1) program in the tandem mode, where the number in parentheses is the introduction period in seconds, gave a symmetric peak, irrespective of standing time, although standing for longer times gave wider peaks. The formation of the symmetric peaks, in spite of the difference of C_{A1} concentrations, is presumably due to molecular diffusion that occurred while they were standing at the inlet. Elongation of the introduction period caused distortion of peaks. Thus, the S(2)–R(2) program gave rather fronting peaks at short standing times (0 and 5 min), but the peak became symmetric after standing for more than 10 min, obviously due to molecular diffusion. In the S(3)–R(3) program, a shoulder was observed at 0 min, but the peak became fronting, then symmetric upon longer standing. On the other hand, the sandwich-mode introduction by the R(1)–S(1)–R(1) program caused peak splitting under conditions where the time of standing was short (0 and 5 min). The peak became flat between 10 and 20 min and sharp thereafter. In this program, the peak splitting disappeared after 10 min, whereas in the R(1)–S(2.4)–R(1) and R(1)–S(5)–R(1) programs peak splitting remained even after 40 min. When the introduction period of the reagent solution was longer in the sandwich mode, as in the R(3)–S(1)–R(3) and R(5)–S(1)–R(5) programs, peaks were symmetrical and not splitting even in shorter standing. For example, the R(3)–S(1)–R(3) program gave a broad but symmetric peak after standing for 20 min. Introduction of the reagent solution for longer times will be favorable for derivatization causing higher yield of derivatives because of broader reagent zones. However, introduction of the reagent solution for excessively long times caused peak broadening, hence was not practical for the present work.

In all these observations the effect of zone dispersion during transportation to the detector window

should be taken into account, but the change of peak shape during transportation is generally small, compared to that caused by standing at the inlet. Fig. 2 shows only a part of the results obtained by this series of experiments, the rest not being displayed. The introduction periods for sample and reagent solutions, mentioned above, were obtained with the HPCE apparatus at hand and such values will vary depending on the apparatus. Therefore, the introduction period should be replaced by plug length, which is a much more universal index. It was confirmed that there was a linear relationship ($y=2.1x+0.35$, $r=1.000$) between the introduction period (x, in seconds) and the plug length (y, in mm), when estimated using a certain dye (bromophenol blue). From this linear relationship, the various programs mentioned above can be expressed in terms of plug length.

3.2. Selection of a model system for at-inlet derivatization

Pre-column (for LC) and pre-capillary (for CE) derivatizations require quantitative yields of derivatives to achieve sensitive detection as well as reliable quantification. When underivatized components of a sample interfere with the analysis of derivatives, this requirement has a critical meaning that the method in question is invalid. In the analysis of biological materials, the derivatization reaction must proceed under mild conditions so that it does not decompose the materials. It is also important that the excess reagent and the reagent blank do not interfere with the analysis of the derivatives. All these requirements should be fulfilled also for at-inlet derivatization. In addition, the derivatization reaction should be rapid, because a slow reaction causes zone broadening.

For these reasons we adopted the derivatization of amino acids with OPA (e.g., first-order rate constant for phenylalanine, 1.06×10^{-2} s^{-1} [11]) as a model system and discuss advantages and problems of at-inlet derivatization.

3.3. Sample/reagent introduction scheme

The results in Section 3.1 help us image the state of mixing of sample and reagent overlayed at the inlet of a capillary. In the present section, the derivatization of the four selected amino acids with OPA at the inlet of a 50-μm I.D. capillary is presented, which was observed from the peaks of the derivatives monitored by UV absorption at 230 nm. The separation of the OPA derivatives was performed by zone electrophoresis mode at pH 10.0, using a 50 m*M* borate buffer as carrier, which is the same medium as that of the derivatization reaction. Under these conditions, the OPA derivatives of the amino acids appeared in the order of Phe, Met, Ala, Glu. The peak of reagent (OPA) appeared first, well isolated from the peaks of the OPA–amino acids. For the purpose of quantitation, C_{Ac} was added as an internal standard, and it appeared between OPA–Ala and OPA–Glu. All these compounds were baseline separated in ca. 8 min. Since the reaction medium for the derivatization was the same as that of the electrophoretic carrier, the zones of the separated derivatives were not disturbed by differences in medium composition. The resolution (R_s) of the first two derivatives (OPA–Phe and OPA Met) was less than 1.3, whereas those for any other neighboring compounds exceeded 2.

Table 1 gives some of the data on the relative response of the OPA derivatives to C_{Ac} for various introduction programs. The standing time was kept constant at 20 min.

As anticipated from the experiments in Section 3.1, the sandwich mode of reagent–sample–reagent introduction generally gave higher efficiency for derivatization than did the tandem mode. In particular, the R(3)–S(1)–R(3) program gave the highest relative response for all OPA derivatives of the

Table 1
Comparison of the yields of the OPA derivatives from the selected amino acids obtained by various sample/reagent introduction programs, as observed from relative peak responses

Introduction program	Relative peak response to C_{Ac}			
	Phe	Met	Ala	Glu
R(1)–S(1)	0.70	0.88	1.02	1.33
R(3)–S(1)	0.70	0.85	0.91	1.32
R(1)–S(1)–R(1)	1.04	1.13	1.03	1.86
R(3)–S(1)–R(3)	1.10	1.17	1.06	1.86
R(5)–S(1)–R(5)	0.95	1.10	0.96	1.82
R(3)–S(2.4)–R(3)	0.93	0.98	0.83	1.58
R(5)–S(2.4)–R(5)	0.91	0.97	0.85	1.61

amino acids. With longer sample/reagent introduction periods in the tandem mode, the peaks of OPA derivatives were broadened to a significant extent (data not shown).

3.4. Effect of standing time

Fig. 3a shows the dependence of the relative peak response of each OPA derivative on standing time at the inlet.

It is observed that the relative response of every OPA-derivative reached a plateau after standing for 20 min. This change was similar to the time course of the pre-capillary derivatization using the sample and reagent concentrations identical with those in at-inlet derivatization (Fig. 3b).

3.5. Comparison of the electropherogram with that in pre-capillary derivatization

Fig. 4a shows the electropherogram obtained after the at-inlet derivatization by the R(3)–S(1)–R(3)

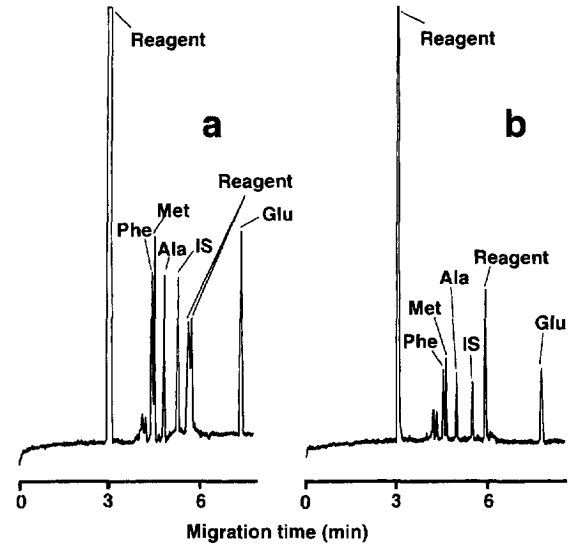

Fig. 4. Analysis of OPA derivatives of selected amino acids formed by at-inlet (a) and pre-capillary (b) derivatizations. Standing time in (a), 20 min. The other conditions are the same as in Fig. 3. Reagent=OPA, IS=internal standard (C_{Ac}).

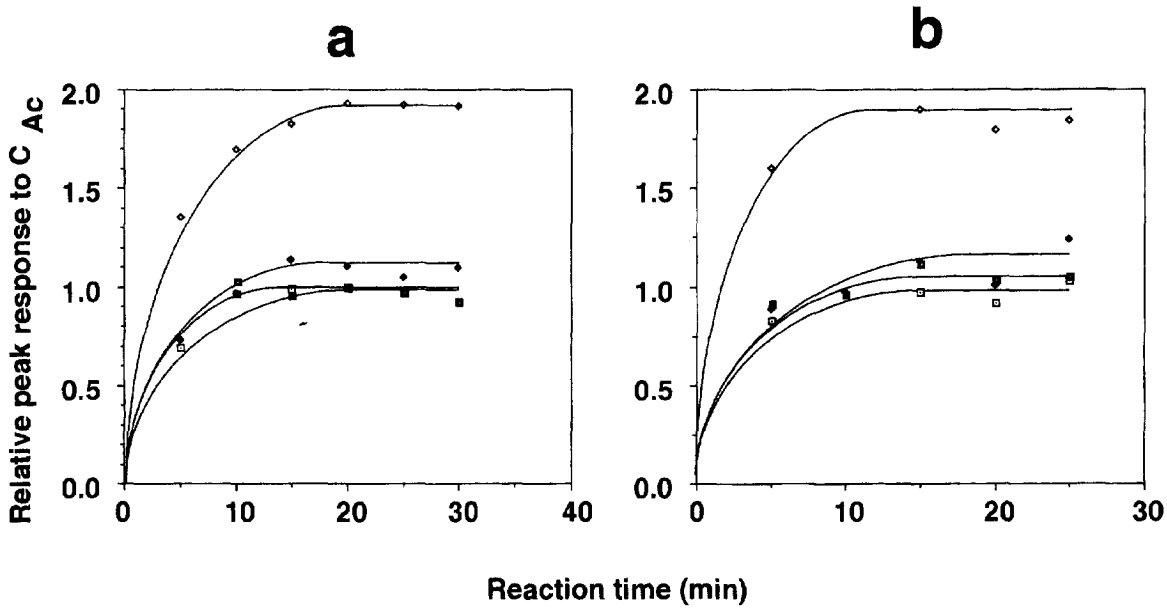

Fig. 3. Change of the yields of OPA derivatives of selected amino acids. (a) At-inlet derivatization, (b) Pre-capillary derivatization. Sample/reagent introduction program in (a), R(3)–S(1)–R(3). The concentrations of the amino acids and OPA were identical in both derivatizations. The conditions for the analysis of the derivatives are the same as in Fig. 2. ⊡ phenylalanine (Phe), ◆ methionine (Met), □ alanine (Ala), ◇ glutamic acid (Glu).

program. Fig. 4b shows the corresponding electropherogram obtained by the pre-capillary derivatization using the same sample and reagent concentrations. In both cases the reaction solutions were analyzed directly without clean-up.

There are plural peaks of the reagent (ca. 3 min and 6 min). The faster moving peak arose obviously from the excess reagent, because it appeared from the reagent preparations from all companies examined, but the slower-moving peak was specific for the preparation we used (from Nakarai tesque, Karasuma-Kyoto). It is considerd to be due to a by-product in manufacturing. It is also notable that the peaks at ca. 6 min are twin-headed in the electropherogram obtained by the at-inlet derivatization. The splitting of the peak top was probably due to incomplete mixing, because such splitting was not observed for pre-capillary derivatization. Notwithstanding this splitting of the reagent peak, all OPA derivatives of the amino acids gave single peaks, and the average values of relative response were in good agreement with those obtained in pre-capillary derivatization (Table 2). The faster-moving peak from the reagent was also twin-headed, though the splitting was not visible, because the peak top was flattened due to low attenuation.

3.6. Calibration and reproducibility

All the calibration curves of relative peak response vs. amino acid concentration obtained by at-inlet derivatization were linear ($y=1.88x+0.0402$, $r=0.992$ for Phe; $y=2.02x+0.0303$, $r=0.993$ for Met; $y=2.01x+0.0157$, $r=0.994$ for Ala; $y=3.47x-0.0169$, $r=0.995$ for Glu, where x and y are the amino acid concentration (in mM) and the relative response to IS, i.e., 0.5 mM Gly, respectively) for at least the 0.1–1.0 mM range, with all four amino acids. This concentration range corresponds to an introduced amount of 1–10 pmol. Gly was adopted as an internal standard instead of cinnamic acid in this case, because it is much more similar to the analytes (other amino acids); it is also derivatized with OPA.

The relative standard deviation of relative peak response at a 1.0 mM concentration of amino acids was less than 3% (Phe, 2.6%; Met, 2.3%; Ala, 2.7%; Glu, 2.2%). At lower concentrations, slightly higher values were obtained.

3.7. Attempt for automated amino acid analysis

Separation of amino acids by HPCE was investigated by a few groups: [12] as dansyl derivatives, [13] as phenylthiocyanate derivatives, [14] as phenylthiohydantoin derivatives. The analysis as phenylthiohydantoin derivatives by the micellar electrokinetic chromatography mode [14] gave good separation of 23 amino acids, but the derivatization to phenylhydantoin is so drastic that it cannot be performed by the at-inlet derivatization technique. Conversion to dansyl, as well as phenylthiocyanate, derivatives is not as rapid as conversion to OPA derivatives. Since the amino group is substituted by OPA in the present derivatization, all derivatives behave as anions in alkaline media, due to the dissociation of the carboxy group. For this reason, zone electrophoresis in an alkaline medium, such as borate buffer, pH 10.0, is the first choice. However, separation of isomeric amino acids (for example, leucine–isoleucine) is difficult, because their charge and molecular mass are exactly the same. The

Table 2
Comparison of the yields of the OPA derivatives of the selected amino acids between at-inlet and pre-capillary derivatizations, as observed from relative peak responses

Method for derivatization	Average relative response to C_{Ac} ($n=10$)			
	Phe	Met	Ala	Glu
At-inlet[a]	1.00	1.05	1.04	1.85
Pre-capillary[b]	1.00	1.09	1.07	1.88
Relative yield $\left(\frac{\text{At-inlet}}{\text{pre-capillary}}\times 100\right)$	100	96.3	97.2	98.4

[a] Sample/reagent introduction, R(3)–S(1)–R(3) sandwich mode; standing time, 20 min.
[b] The concentrations of the amino acids and OPA were the same as in the at-inlet derivatization. Reaction time, 20 min.

derivatives of amino acids of similar size (for example, valine–threonine, alanine–serine) were only poorly resolved under these conditions. Our search for an appropriate additive to improve separation was only partially successful, but lowering the pH to 6.8 and the addition of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) to a concentration of 3.2 m*M* gave rather good separation. Fig. 5 shows the electropherogram of amino acids as their OPA derivatives obtained automatically by setting the introduction program to 3 s (R)–1 s (S)–3 s (R), standing time at 20 min and applied voltage at 30 kV, using the electrophoretic carrier mentioned above. Although the separation condition was slightly changed in this manner, the reaction condition was not altered in this case.

The basic separation mode was obviously zone electrophoresis, but additional modes such as hydrophobic interaction and ion-pair formation with the additive should be taken into consideration. Even under these conditions, the separation of leucine and isoleucine was not complete. The difference of the reaction medium for derivatization from the electrophoretic carrier may be one of the reasons for incomplete separation, though it is considered to be minor. Further investigation into additional separation modes will conquer this problem. Anyway, although analysis time was long and a number of peaks due to the reagent blank were present, a greater proportion of the amino acid components could be separated and identified by co-migration with authentic specimens.

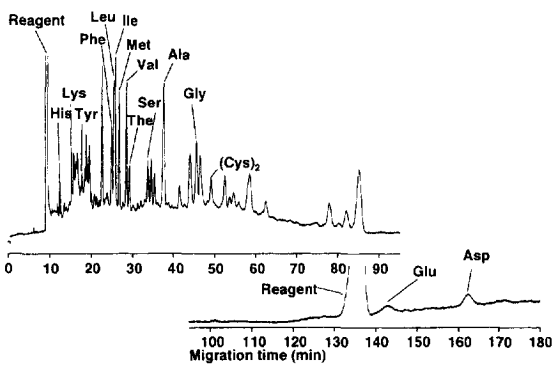

Fig. 5. Analysis of amino acids derivatized with OPA by the at-inlet technique using the R(3)–S(1)–R(3) program. Standing time, 20 min. Electrophoretic carrier, 50 m*M* phosphate buffer (pH 6.8) containing CHAPS at a concentration of 3.2 m*M*. The other analytical conditions are the same as in Fig. 4a. His=histidine, Lys=lysine, Tyr=tyrosine; Leu=leucine; Ile=isoleucine, Val=valine, Thr=threonine, Ser=serine, Gly=glycine, $(Cys)_2$=cystine, Asp=aspartic acid. The other abbreviations are the same as in Figs. 3 and 4.

4. Conclusion

At-inlet derivatization is a special technique advantageous for HPCE, but there is a limitation that the derivatization should be rapid enough to give quantitative yields of derivatives in allowable time, without zone broadening to such an extent that it hampers the separation of the components to be analyzed from accompanying substances. However, the example presented here demonstrates its usefulness, provided such conditions are satisfied. Development of a much more suitable chemistry of derivatization in the future will add greatly to the value of this technique.

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