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Migration time correction for the analysis of derivatized amino acids and oligosaccharides by micellar capillary electrochromatography

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Abstract

Migration-time reproducibility is essential in the use of capillary electrophoresis to identify components in mixtures. Two methods based on the migration time of either one or two reference markers are proposed for improving migration time reproducibility. These methods were evaluated to determine the migration time reproducibility for phenylthiohydantoin-amino acids, fluorescein thiohydantoin-amino acids, and tetramethylrhodamine labeled oligosaccharides. In the best case, the relative standard deviation of the migration time was reduced from >3% without correction to <0.04% with the two-marker correction.

Keywords: Migration time correction; Derivatization, electrophoresis; Amino acids; Oligosaccharides

1. Introduction

Capillary electrophoresis is a powerful analytical tool that produces rapid and extremely high-resolution separation of complex mixtures [1,2]. In many applications, components in a sample are identified by comparing their migration time with the migration time of a set of standards. Drifts in migration time can make this comparison difficult, particularly for components with small differences in migration time. While the identity of components can be confirmed by co-migration with an authentic standard, this procedure requires at least two electrophoresis runs. It is preferable to ensure sufficient migration time reproducibility between runs so that a separation performed on a sample can be directly compared with the separation of standards.

Several groups have proposed methods to improve migration time reproducibility. Lee and Yeung [3] used an adjusted migration index to improve the precision of migration time. This method requires accurate knowledge of the internal diameter of the capillary, which is not easy to obtain with current technology of capillary production. Bocek and co-workers proposed the use of migration times of two standards and an unknown to determine the electrophoretic mobility of the unknown [4]. Jumppanen and Riekkola developed a similar method using up to four markers of known electrophoretic mobilities [5]. The authors [4,5] demonstrated significant improvement in migration time reproducibility with relative

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standard deviation as low as 0.01% after correction. However, for these methods the electrophoretic mobilities of the markers must be known. This requires determination of the electrophoretic mobilities of the markers in a specific separation system. Other methods such as relative migration time and relative mobility have also been studied [6,7]. Palmer and Vandeginste found that the relative mobility of the analyte to the reference standard gave better reproducibility than the relative migration time and actual mobility methods [8].

This group has considered three analyses that are particularly sensitive to variations in migration time. First, we have reported the use of capillary electrophoresis to identify the phenylthiohydantoin (PTH)-amino acid produced by the classic Edman degradation reaction [9–11]. There are 22 components commonly observed in this reaction: 19 amino acids (cysteine is not stable in the reaction) plus three common impurities (diphenylthiourea, dimethylphenylthiourea, and diphenylurea). Most of these components are neutral and their separation requires the use of micellar electrokinetic chromatography. Several components produce closely spaced peaks whose identification requires very precise migration times.

Second, we have performed similar studies based on a fluorescent Edman reagent that produces fluorescein thiohydantoin (FTH)-amino acids [12,13]. The identification of these components is more challenging than for the PTH-amino acids. The bulky fluorescein group dominates the mobility of each component. The best separation conditions require careful adjustment of the buffer pH to near the pK_a of fluorescein; the amino acid side-chains modulate the pK_a of the derivative, facilitating separation based on differences in ionization at the pH of the separation. The migration times are sensitive to slight changes in buffer pH and ionic strength.

Third, we have studied a set of metabolic products generated by biosynthesis and biodegradation of fluorescently labeled oligosaccharides [14–17]. Some of the biosynthetic products are very similar in structure, and their separation is based on complexation with boric and phenylboronic acids and partitioning into SDS micelles. The separation of the components requires a relatively long time and produces a set of closely spaced peaks. The identification of these products is sensitive to slight changes in migration time.

In this paper, we report two methods to correct for variations in migration time. The first method corrects for variation in electroosmotic mobility while the second method corrects for variations in both electroosmotic and electrophoretic mobility.

2. Theory

In capillary electrophoresis, the migration time of an analyte x is given by

$$t_x = \frac{LL_{\text{eff}}}{V[\mu_{\text{eof}} + \mu_{\text{ep},x}]} \tag{1}$$

where L_{eff} is the effective length of the capillary from inlet to the detection window, L is the total length of the capillary, V is the applied potential, μ_{eof} is the electroosmotic mobility, and μ_{eof} is the electrophoretic mobility of the analyte.

2.1. Method 1

A single marker (m) is used to eliminate the effect of variations in electroosmotic flow from run to run. To perform this one-marker correction, we first obtain a standard electropherogram containing the marker and the analyte of interest. The overall electrophoretic mobility of the marker is given by

$$\mu_{\text{overall},m} = \mu_{\text{eof}} + \mu_{\text{ep},m} = \frac{LL_{\text{eff}}}{Vt_m} \tag{2}$$

where $\mu_{\text{ep},m}$ is the electrophoretic mobility of the marker.

If only electroosmotic flow varies between runs, then the overall mobility of the marker under nonstandard conditions is given by

$$\hat{\mu}_{\text{overall},m} = \mu_{\text{eof}} + \Delta\mu_{\text{eof}} + \mu_{\text{ep},m} = \frac{LL_{\text{eff}}}{V\hat{t}_m} \tag{3}$$

where $\hat{\mu}$ is the observed mobility under the nonstandard conditions, $\hat{t}$ is the observed migration time, and $\Delta\mu_{\text{eof}}$ is the change in electroosmotic mobility.

The change in electroosmotic mobility is given by

$$\hat{\mu}_{\text{overall},m} - \mu_{\text{overall},m} = \Delta\mu_{\text{eof}} = \frac{LL_{\text{eff}}}{V\hat{t}_m} - \frac{LL_{\text{eff}}}{Vt_m} \quad (4)$$

The corrected mobility of component x is given by

$$\hat{\mu}_{\text{overall},x} - \Delta\mu_{\text{eof}} = \frac{LL_{\text{eff}}}{V\hat{t}_x} - \left(\frac{LL_{\text{eff}}}{V\hat{t}_m} - \frac{LL_{\text{eff}}}{Vt_m}\right) = \frac{LL_{\text{eff}}}{Vt_{\text{corrected},x}} \quad (5)$$

The corrected migration time is given by

$$t_{\text{corrected},x} = \left[\frac{1}{\hat{t}_x} - \left(\frac{1}{\hat{t}_m} - \frac{1}{t_m}\right)\right]^{-1} \quad (6)$$

2.2. Method 2

In the one-marker correction method, only the change in electroosmotic flow from run to run was considered. A two-marker method considers variations in both electroosmotic and electrophoretic mobilities. Electrophoretic mobility might change due to a change in the mobility of the micelle in micellar electrokinetic chromatography, a change in viscosity that accompanies a temperature change, etc.

Under standard conditions, the overall mobility is given by Eq. (3). Under nonstandard conditions, the overall mobility is

$$\hat{\mu}_{\text{overall}} = \mu_{\text{eof}} + \Delta\mu_{\text{eof}} + \gamma\mu_{\text{ep}} \quad (7)$$

where γ is the fractional change in electrophoretic mobility; by definition $\gamma = 1$ in the standard electropherogram. We assume that the electrophoretic mobility changes proportionally for all components, so that γ is the same for all components in any run.

The difference between the overall mobility of an analyte (x) and the overall mobility of marker 1 (m_1) in standard conditions is independent of the electroosmotic mobility

$$\mu_x - \mu_{m_1} = \mu_{\text{ep},x} - \mu_{\text{ep},m_1} \quad (8)$$

Similarly, the difference in overall mobility between the components under nonstandard conditions is given by

$$\hat{\mu}_x - \hat{\mu}_{m_1} = \gamma[\mu_{\text{ep},x} - \mu_{\text{ep},m_1}] \quad (9)$$

The term γ is evaluated from the ratio of the differences in mobility

$$\gamma = \frac{\hat{\mu}_{m_1} - \hat{\mu}_x}{\mu_{m_1} - \mu_x} = \frac{\frac{1}{\hat{t}_{m_1}} - \frac{1}{\hat{t}_x}}{\frac{1}{t_{m_1}} - \frac{1}{t_x}} \quad (10)$$

The term γ is constant for all components in the sample. In practice, it is calculated by comparing the migration times of the two markers that were added to the separation mixture

$$\gamma = \frac{\frac{1}{\hat{t}_{m_1}} - \frac{1}{\hat{t}_{m_2}}}{\frac{1}{t_{m_1}} - \frac{1}{t_{m_2}}} \quad (11)$$

Ideally, these two markers differ widely in their migration times in order to improve the precision of the calculation.

The corrected migration time of an analyte is then determined by rearranging Eq. (10)

$$t_{\text{covered}} = \left[\frac{1}{t_{m_1}} - \frac{1}{\gamma}\left(\frac{1}{\hat{t}_{m_1}} - \frac{1}{\hat{t}_x}\right)\right]^{-1} \quad (12)$$

3. Experimental

3.1. PTH-amino acids

The determination of PTH-amino acids was reported elsewhere [9–11]. Briefly, the fused-silica capillary was 40 cm in total length ($L_{\text{eff}} = 35$ cm), 50-μm I.D. and 185-μm O.D. This capillary was preconditioned by gravity flow of the running buffer for 24 h. The separation was performed at 10 kV running voltage with 15-s hydrodynamic injection. The running buffer (pH = 6.8) was composed of 10.7 m*M* sodium phosphate, 1.8 m*M* sodium borate, and 25 m*M* sodium dodecylsulfate (SDS).

The mixture of 19 PTH-amino acids, diphenylthiourea (DPTU), dimethylphenylthiourea (DMPTU), and diphenylurea (DPU) was purchased from Applied Biosystems (Foster City, CA). A working solution (2.5×10^{-5} *M*) of each PTH-amino acid was prepared by diluting the stock solution (1×10^{-4} *M* in acetonitrile) in running buffer. Fused-

silica capillary was purchased from Polymicro Technologies Inc.

3.2. FTH-amino acids

The FTH amino acid standards were prepared in our laboratory [12,13]. The coupling reaction was carried out by adding 20 μl of aqueous solution of 0.01 *M* individual amino acids (Sigma, Oakville Canada) and 20 μl of 1×10^{-3} *M* fluorescein isothiocyanate (Molecular Probes, Eugene, OR) solution in HPLC-grade acetone (Aldrich, Milwaukee, WI, USA) into 120 μl of a 0.2 *M* carbonate buffer at pH 9.1. The solution was mixed and then stored at room temperature in the dark for six to eight hours to complete the reaction. No wash or extraction steps were performed. The thiazolinone intermediate was converted to FTH amino acid by adding 120 μl of neat trifluoroacetic acid (Caledon, Edmonton, Canada) to each reaction vial and reacting for 12 h in the dark. The mixture was then evaporated on a Speed Vac (Fisher, Edmonton Canada) to obtain a dry FTH-amino acid, which was dissolved in 500 μl of HPLC grade acetonitrile (Aldrich) to give approximately 4×10^{-5} *M* solution and stored at 4°C until needed. The concentration of the stock solution was calculated assuming the reaction yields were 100%.

To prepare the working solution of mixed FTH-amino acids, a 5 μl aliquot of each stock solution was added to a vial, and the mixture was made up to 500 μl with acetonitrile to give a 4×10^{-7} *M* solution. The mixture was diluted 100-fold again with running buffer to obtain a solution containing 18 FTH-amino acids each at approximately 4×10^{-9} *M*. Cysteine was not included in this solution because it was not stable in the reaction. Lysine was not included because of side reactions involving the ε-amine. Because no extraction was used, some by-products were present in this solution; they have not been identified.

The instrument for FTH-amino acid analysis was described elsewhere [17]. The separation buffer (pH 6.86) was composed of 15 m*M* sodium dihydrogen phosphate, 3.75 m*M* sodium tetraborate, 7.7 m*M* SDS, and 2.0 m*M* magnesium acetate. The working solution was electrokinetically injected at 540 V for 2 s and separated in a 60-cm long, 30-μm I.D., and 150-μm o.d. capillary at 18 kV.

3.3. Tetramethylrhodamine-labeled oligosaccharides

A tetramethylrhodamine (TMR) labeled disaccharide, βGal(1→4)βGlcNAc-O–TMR (LacNAc), was used as a substrate. The biodegradation of LacNAc-O–TMR by galactosidase gives *N*-acetyl-β-D-glucosaminide-O–TMR (GlcNAc), and further degradation by hexosaminidase gives $HO(CH_2)_8CONH$-CH_2CH_2NHCO–TMR (Linker arm: -O–TMR). Biosynthesis by fucosyltransferases can result in the formation of αFuc(1→2)βGal(1→4)βGlcNAc-O–TMR (H-Type II), βGal(1→4)[αFuc(1→3)]-βGlcNAc-O–TMR (Lewis X), and αFuc(1→2)-βGal(1→4)[αFuc(1→3)]βGlcNAc-O–TMR (Lewis Y). Synthesis of the substrate and products has been described previously [14].

The instrument used for the determination of the fluorescent substrate and enzyme products has been described [16]. Briefly, separation was carried out in a 50–60 cm long, 10-μm I.D., and 150-μm O.D. capillary at an electric field of 400 V/cm. The electrophoresis buffer contained 10 m*M* each of phosphate, tetraborate, phenylboronic acid, and SDS, at pH 9.3. The sheath fluid was identical to the running buffer.

4. Results and discussion

4.1. PTH-amino acids

Ten replicate runs of standard solutions containing the 19 PTH-amino acids, DMPTU, DPTU, and DPU, all at a concentration of 2.5×10^{-5} *M*, were obtained on the same day. The first electropherogram was taken as the standard, and subsequent electropherograms were normalized to the standard using the one-marker method. Fig. 1 shows the relative standard deviation (RSD) values for the 22 analytes. Generally, the RSD values were reduced from about 1% without correction to less than 0.5% with 1-marker correction based on PTH-Y or DPTU.

The relative positions of the marker and analyte in the electropherogram affected the reproducibility of the corrected migration time. When PTH-Y was used as the marker (dashed curve), the early-migrating analytes had a RSD in migration time $\leq$0.5%, but

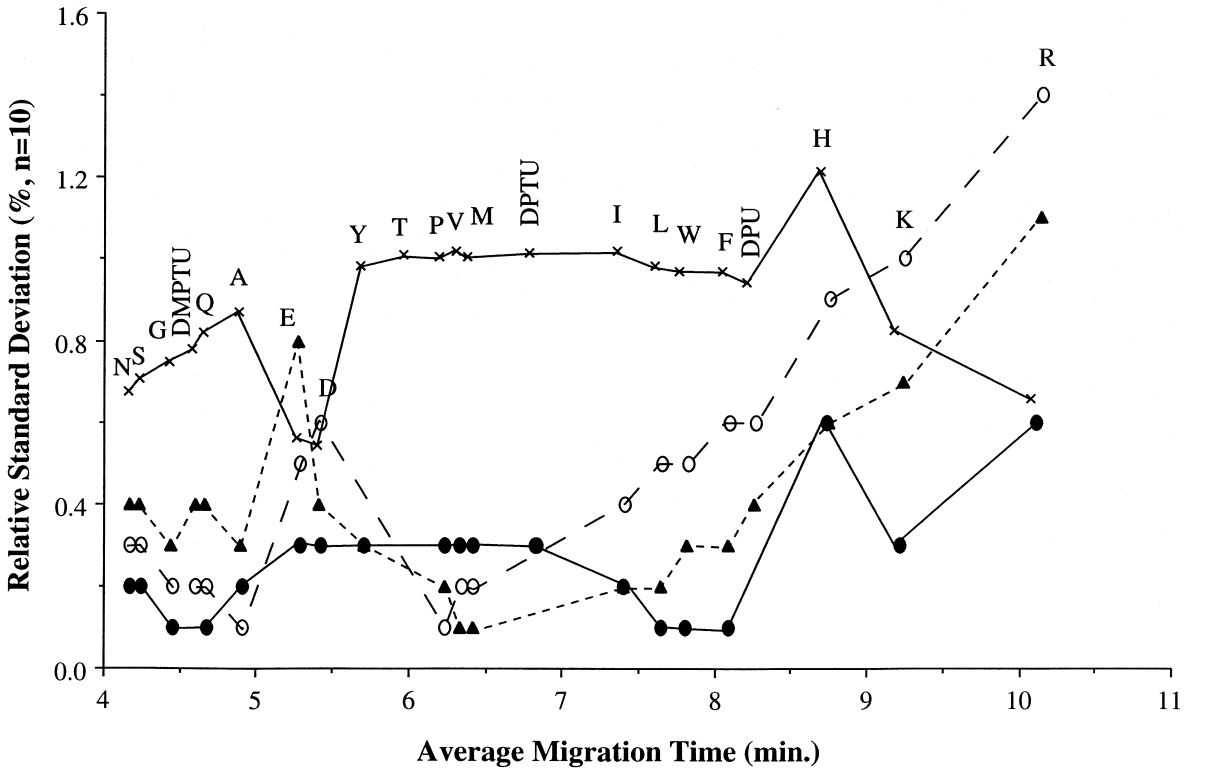

Fig. 1. Relative standard deviations (RSD) of migration times for 19 PTH-amino acids, DMPTU, DPTU, and DPU before correction (×) and after one-marker correction based on PTH-Y (○) or DPTU (△) and two-marker correction based on DMPTU and DPU (●). Letters represent PTH-amino acids with single letter abbreviation. Separation conditions described in the text.

the RSD of migration time from the last six peaks increased monotonically from 0.5% to 1.3%. DPTU migrated in the middle of the electropherogram. When it was used as a marker (dotted curve) the relative precision of migration time for all analytes, except PTH-E and the last three peaks, was better than 0.4%.

The two-marker method corrects for drift in both electroosmotic and electrophoretic mobilities. DMPTU and DPU were used to correct the migration times in the same ten runs as above (solid curve). These components are common impurities generated in the Edman degradation reaction and make ideal markers for correction of migration time. The two-marker method significantly improved the reproducibility of migration times for all the analytes. After two-marker correction, the RSD of the migration times for all the analytes were $\leq$0.4% except for the PTH-H and PTH-R, which were about 0.6%.

Fig. 2 presents four successive electropherograms obtained from the mixture. Fig. 2A presents the raw data. Identification of any single component is difficult due to the variation in migration time. The single-marker migration time correction, Fig. 2B, improves the migration time precision for the early migrating components, but actually degrades the migration time precision of the last few components. The two-component migration time correction, Fig. 2C, does an outstanding job of correcting for migration time variations across the electropherograms, which simplifies identification of each component.

4.2. Between-day precision

To demonstrate the longer-term reproducibility of the migration times of PTH-amino acids, we calculated the RSD values of migration times from ten electropherograms that were obtained over a two

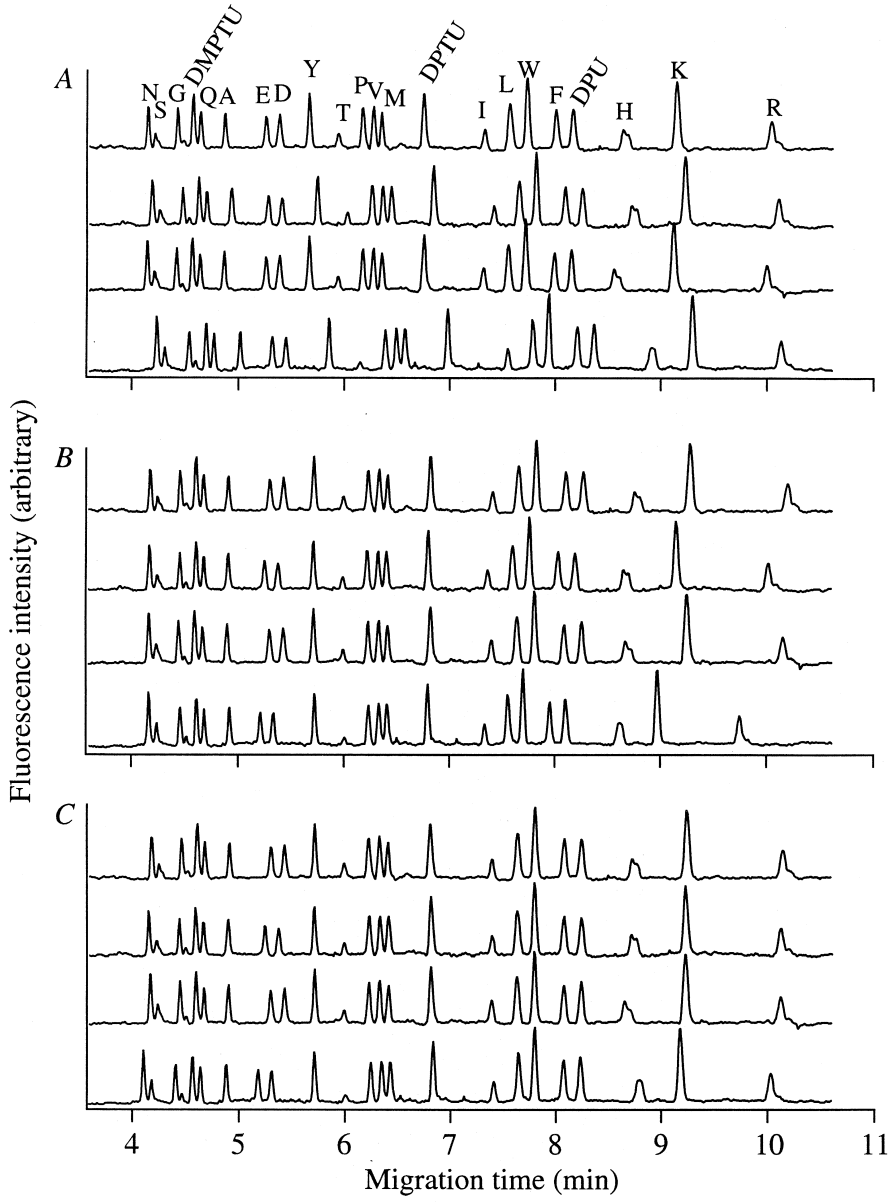

Fig. 2. Comparison of electropherograms for raw data (A), one-marker corrected data (B), and two-marker corrected data (C) for separation of PTH-amino acids. PTH-Y was used as the marker for the one-component correction and DMPTU and DPU were used for the two-marker correction.

month period. The RSD values from the raw data and after one- or two-marker corrections are shown in Fig. 3. The raw migration times without correction gave RSD from 3 to 10%. After the one-marker (DPTU) correction, the RSD values were generally reduced to about 1%. A V-shaped distribution of RSD values vs. average migration time was observed. As the migration time of the analytes approached the marker, DPTU, the RSD values became smaller. When DMPTU and DPU were used in the two-marker method, the RSD values from these ten runs on different days were reduced to $<0.5\%$ for all

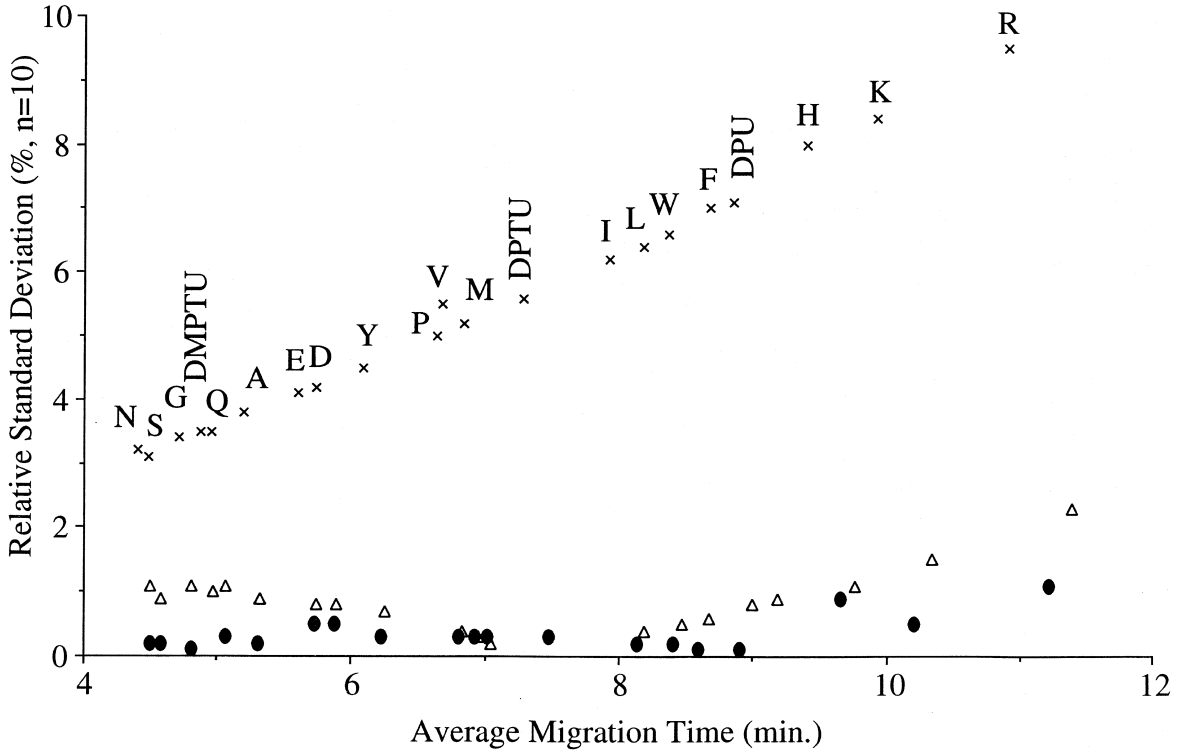

Fig. 3. Relative standard deviations (RSD) of migration times of 19 PTH-amino acids, DMPTU, DPTU, and DPU from ten runs obtained over two months before correction (×), after one-marker (DPTU) correction (△), and two-marker (DMPTU and DPU) correction (●). Separation conditions are the same as for Fig. 1 except the running voltage was 9 kV.

the analytes except for PTH-H and PTH-R which gave RSD values of 0.8% and 1%, respectively.

4.3. FTH-amino acids

We also applied the two correction methods for the analysis of FTH-amino acids. These compounds were much more difficult to analyze than the PTH-amino acids. Our separation condition was based on the careful adjustment of the pH of the buffer near the pK_a for fluorescein; slight differences in ionization affected the separation. As a result, the migration times were quite sensitive to the buffer composition.

Four electropherograms were obtained from four sequential runs. The RSD values obtained from the raw data, after the one-marker correction, and after the two-marker correction are shown in Fig. 4. Before correction, the original migration time data gave RSD values from 2 to 4%. When peak B1 at 19.09 min was used as the marker for the one-marker correction, the RSD of migration times were reduced to less than 1% for all except the last five peaks. The RSD of migration time for each peak improved as its migration time approached the marker B1 at 19.09 min.

Further improvement in the reproducibility of migration times for FTH-amino acids was obtained using the two-marker correction method. When the two peaks FTH-N and FTH-D at 15.05 and 28.67 min were used as the markers, RSD values were generally reduced to less than 0.4% for all the FTH-amino acids, which was at least 8 times better than the uncorrected data.

4.4. Oligosaccharide analysis

As our last demonstration of the migration time correction methods, five runs of a standard solution containing five tetramethylrhodamine-labeled oligo-

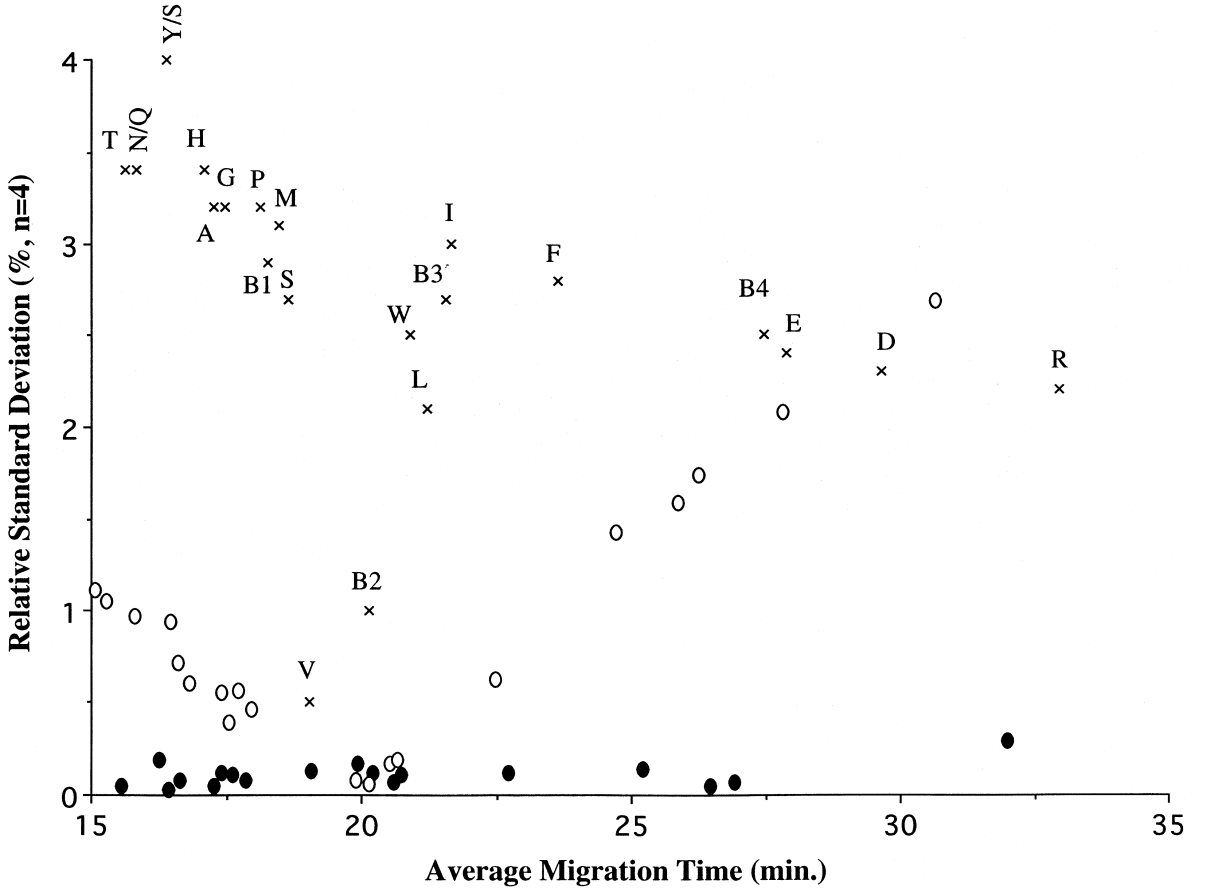

Fig. 4. Relative standard deviations (RSD) of migration times for 19 FTH-amino acids and unknown by-products without correction (×), with one-marker correction using the peak B1 at 19.09 min (○), and two-marker correction using the two peaks (N, D) at 15.05 and 28.67 min (●). B1, B2, B3, and B4 are unidentified by-products. Other letters represent FTH-amino acids with single letter abbreviation. Separation conditions described in the text.

saccharides were performed within one day. Approximately 3% RSD in peak migration time was observed from the raw data, Fig. 5. The large variation of migration time is probably due to a change of temperature between runs; the experiments were performed in a poorly thermostated room on a warm summer's day.

The variation in migration time is dramatically reduced using the correction methods. The RSD values for peaks 1, 2, 4, and 5 were reduced to less than 0.5% after one-marker correction using peak 3 as the marker. These are further reduced to less than 0.03% after two-marker correction with peaks 3 and 5 as the markers.

5. Conclusion

The use of markers to improve migration time precision is not without problems. In particular, it may be difficult to choose markers that do not co-migrate with components in the standard mixture. Also, it is unlikely that the use of markers will be useful for large changes in buffer conditions that

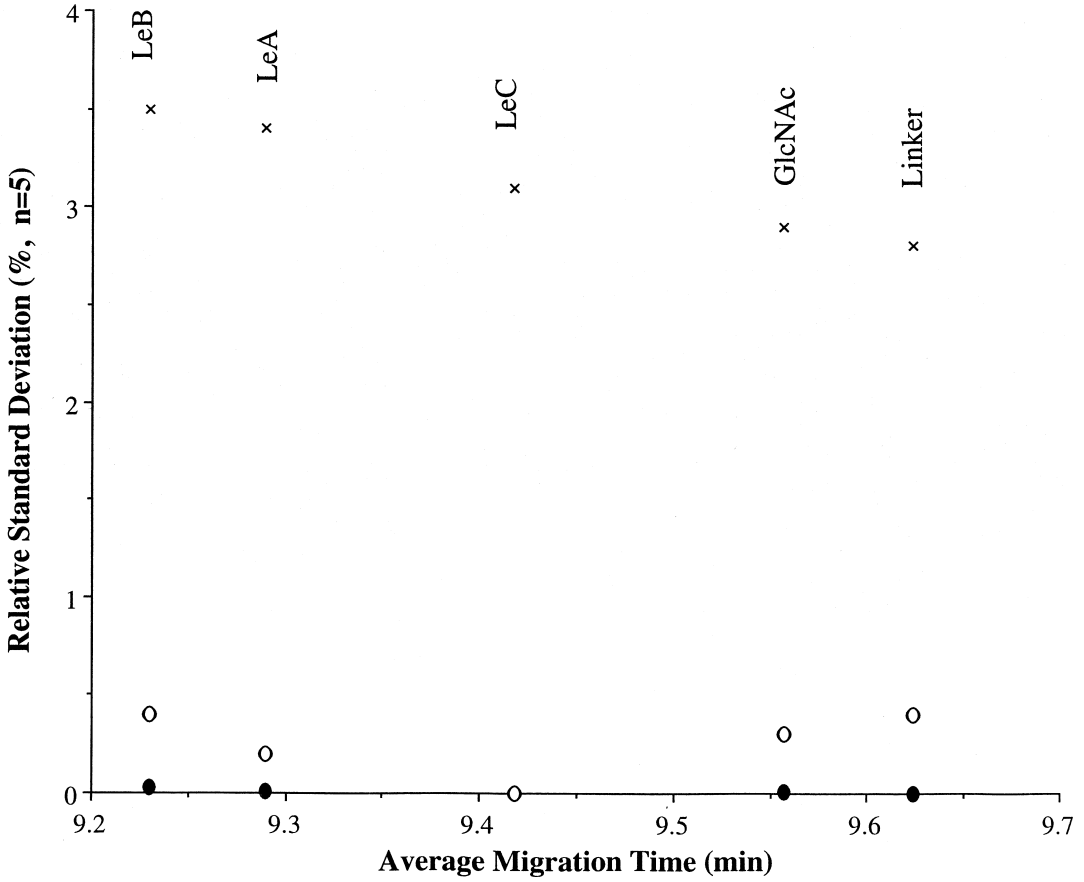

Fig. 5. Relative standard deviations of migration times of five oligosaccharides separated using CE/LIF before (×) and after one-marker correction using peak 3 (○) and two-marker (●) corrections using peaks 3 and 5.

result in band-reversal. However, the technique dramatically reduces variation in migration time when applied to the separation of PTH-amino acids, FTH-amino acids, and oligosaccharides.

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