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Capillary electrophoretic analysis of carbohydrates derivatized by in-capillary condensation with 1-phenyl-3-methyl-5-pyrazolone

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Abstract

Our previous papers on capillary electrophoresis (CE) have shown that samples can be derivatized in a capillary and the derivatives can be analyzed immediately after derivatization, provided that the derivatization reaction is so rapid as to complete in seconds. The present paper presents extended application of in-capillary derivatization to a much slower reaction such as the condensation of reducing carbohydrates with 1-phenyl-3-methyl-5-pyrazolone (PMP) which requires 30 min at 70°C in pre-column derivatization by manual operation. It was necessary to first drive the introduced plugs of sample and reagent solutions to put them together at the entrance of the heated portion of a capillary, then to allow the superimposed plugs to react for a relevant period. We showed how to determine the introduction times of the sample and the reagent solutions as well as intermediate running buffer, the voltages to be applied for plug driving and product analysis, and the duration of voltage application, all of which are important for effective in-capillary derivatization. An example of the analysis of maltooligosaccharides by this technique is presented. It was shown that maltooligosaccharides were quantitatively derivatized with PMP in 35 min at 57°C, and the derivatives could be analyzed in ca. 15 min by CE immediately after derivatization. Separation was satisfactory in 200 m*M* borate buffer, pH 8.2 containing sodium dodecyl sulfate to a concentration of 200 m*M*. Although the theoretical plate number, and accordingly the resolution, were significantly lower than the corresponding values in pre-capillary derivatization, reasonable reproducibility was ensured for both migration time (RSD 3.5% on average) and peak area (RSD less than 3%) under the optimized conditions. It is notable that sample amount could be lowered to the 10 fmol level, in contrast to the 10 pmol level in pre-capillary derivatization. In addition, since the technique employed here (the modified at-inlet technique of in-capillary derivatization) is easily automated, the established system will be highly beneficial for routine analysis of carbohydrates. Analysis by this technique was also shown to be useful for kinetic study of the derivatization reaction.

Keywords: Derivatization, electrophoresis; In-capillary derivatization; Automation; Carbohydrates; Phenylmethylpyrazolone

1. Introduction

Recent advancement of capillary electrophoresis (CE) has realized high-resolution separation with column efficiency typically of several ten thousands theoretical plates. This method also allows on-column detection with high reproducibility. Owing to these advantages CE has been utilized for analysis of variety of compounds including pharmaceutical, biomedical, environmental, and process substances,

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which are closely related to our everyday life. We have been attracted by another advantage of this method based on single-phase property, and developed various methods for in-capillary chemical derivatization of analytical samples [1–3] and for binding studies between substances by in-capillary physical interaction [4–6].

There are three types of in-capillary derivatization, which are performed at the inlet of [1], in the middle of [2] and throughout [3] a capillary. We have pointed out the advantages and problems of each type, but all the successful examples so far reported involves only a rapid reaction such as the condensation of the amino group with *o*-phthalaldehyde. Since in-capillary derivatization is an important technique for biomedical and pharmaceutical analysis, application should be extended to a wider range of reactions by appropriate devices.

This paper reports examples of application to much slower reaction such as the condensation of reducing carbohydrates with 1-phenyl-3-methyl-5-pyrazolone (PMP). The condensation with PMP is an excellent method developed in our laboratory for pre-column derivatization of reducing carbohydrates for high-performance liquid chromatography (HPLC) [7] and CE [8–12]. It gives quantitative yields of derivatives, and the reaction conditions are so mild that it causes neither desialylation nor desulfation during derivatization. The derivatives can be sensitively detected by conventional methods based on UV absorption and electrolytic oxidation on a glassy carbon electrode. However, the reaction rate is slow requiring as long as 30 min at 70°C for completion, though it proceeds much faster than most of other derivatization reactions for carbohydrates.

We discuss herein appropriate conditions for in-capillary derivatization involving such slow reaction and presents some data using oligosaccharides as model carbohydrates.

2. Experimental

2.1. CE

A CE system from Applied Biosystems was used, composed of a high-voltage supply, a sample introduction device using a small vacuum pump, an air-circulating type capillary oven capable of controlling the temperature with accuracy of ±1°C (upper limit 60°C), a UV monitor, and a data processor. The capillary oven was separated from the injection portion and the detector block by panels, and the capillary length in the oven was freely changeable by fitting both ends of the capillary in the oven to the panels using silicon septa. A roll of 50-μm I.D. fused-silica capillary was supplied by Polymicro Technologies (Phoenix, AZ, USA). In the analysis of the in-capillary derivatized product a 71-cm piece was cut out from the roll and a 2-mm length detection window was created at the 49-cm position from the inlet by burning the polyimide coating. The window was fixed on the detector block, and the 4~49-cm portion from the inlet was disposed in a temperature-controlled oven. In the analysis of the pre-capillary derivatized product a piece of the same length was cut out and a window was made at the 45-cm position from the inlet. The capillaries were flushed with 1 *M* sodium hydroxide and before each run equilibrated with the running buffer. The running buffer was prepared by dissolving boric acid (and sodium dodecyl sulfate, SDS, when necessary) in hot water at around 50°C and after cooling adjusting pH to 8.2 with sodium hydroxide. Thus-prepared solutions were degassed before use.

2.2. Materials

PMP was purchased from Kishida (Osaka, Japan) and recrystallized from methanol. Carbohydrate samples of analytical grade were obtained from Wako (Osaka, Japan). The chemicals for preparing running buffer were of the highest grade commercially available. Water was deionized and glassware-distilled before use.

2.3. In-capillary derivatization of maltooligosaccharides with PMP

For the application to such slow reaction as the condensation of carbohydrates with PMP, a special device is required to shorten the reaction time by elevating reaction temperature as high as possible. Among commercially available apparatus, the above-mentioned CE system from Applied Biosystems was

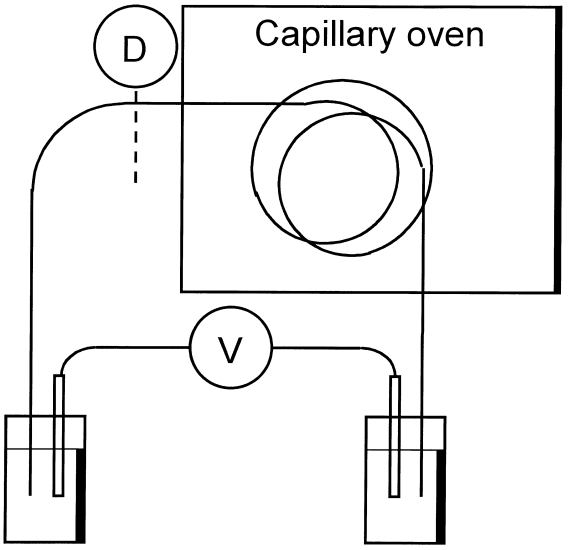

Fig. 1. Diagrammatic expression of the set-up of the equipment for in-capillary derivatization involving a slow reaction. A greater portion of the capillary is placed in a heated oven to enhance reactivity. D = Detector, V = voltage supply.

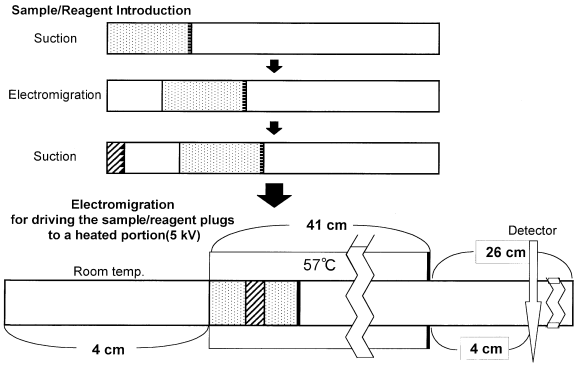

Fig. 2. Illustration of the introduction of a sample and a reagent solutions and driving the plugs to the heated portion of the capillary. Plugs of the reagent solution, the running buffer and the sample solution were introduced in this order, and a voltage was applied to both ends of the capillary to drive the sample and reagent plugs to the entrance of the heated portion of the capillary. ▨ Sample solution, □ running buffer, ▩ reagent solution. These solutions can be introduced by pressurization, suction, or electromigration. In this case the sample and the reagent solutions were introduced by suction and the running buffer was introduced by electromigration by applying a voltage.

the most suited for such a special device. Using this apparatus we could dispose of the 4–45-cm portion of a 71-cm capillary in the oven, thus making the set-up as shown in Fig. 1.

Sample and a reagent solutions and the running buffer were introduced by a program using appropriate indices calculated based on the discussion in Section 3.1, and the product was analyzed with borate buffer or borate buffer containing SDS.

2.4. Pre-column derivatization of maltooligosaccharides with PMP

This was performed primarily according to the procedure in our previous paper [7]. More details are described in Section 3.4 and in the captions to Fig. 4.

3. Results and discussion

3.1. Determination of various indices for in-capillary derivatization

Fig. 2 illustrates the procedure for in-capillary derivatization involving a slow reaction.

When reagent moves faster than sample, the reagent solution, the running buffer, and the sample solution are introduced in this order by suction. If sample moves faster than reagent, the introduction order of the sample and the reagent solutions should be reversed. It is necessary to drive the sample and the reagent plugs to the entrance of the heated portion of a capillary by applying a voltage for relevant time, and thereby to make the plugs overlapped on each other. In order to perform in-capillary derivatization successfully, we should know important indices, such as the voltage to be applied for sample/reagent driving, duration of voltage application, position of the reagent solution at the initial stage, and the length of intermediate buffer plug (if necessary). Among them the voltage to be applied can be fixed to an arbitrary value, as far as the sample and reagent solutions can be introduced within a short period of time not to make total analysis time remarkably longer (periods within 1 min are desirable). Therefore, it was fixed at 5 kV in the present study. The other indices must be determined by reasonable procedures. The followings are the simplified procedures for the determination of such indices.

3.1.1. Duration of voltage application for sample/reagent driving

The proper duration of voltage application for sample/reagent driving can be estimated as follows. The duration of voltage application (t) is equal to the ratio of the distance (d_S or d_R) to be traveled by the sample or the reagent to its velocity, v_S or v_R, respectively, and t can be expressed as the ratio of d_S to v_S or d_R to v_R, respectively.

It should be noticed that d_S is the center to center distance between the sample plug at the initial and the final positions (Fig. 3). Similarly d_R is that between the reagent plug at the initial and the final positions. The terms v_S and v_R can be estimated by using the migration times of the sample and the reagent (t_s and t_R, respectively) obtained by using the same system operated at the same applied voltage, but without heating (at room temperature). Thus, v_S and v_R can be expressed as ι_D/t_S and ι_D/t_R, respectively. where ι_D is the distance between the capillary inlet and the detection window. Therefore, t can be estimated as:

$$t = \frac{t_S d_S}{\iota_D} \text{ or } \frac{t_R d_R}{\iota_D} \quad (1)$$

In the system shown in Fig. 3 an ample plug length was chosen to allow quantitative in-capillary derivatization without incomplete overlapping of plugs. Since the capillary temperature at the pre-oven portion is slightly higher than room temperature, the migration velocities of the sample and the reagent are considered to be slightly higher than those at room temperature by the effect of capillary heating at the next portion (oven). This may cause slight mismatch between the actual and estimated values of t_S as well as t_R, and accordingly t. However, such mismatch was easily eliminated by introducing an amply long reagent plug, and sufficiently reproducible in-capillary derivatization was realized.

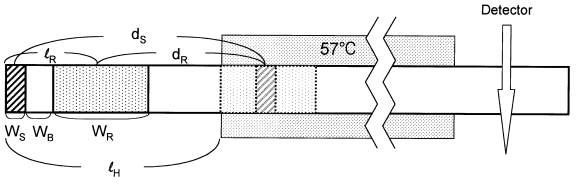

Fig. 3. Details of the inlet and the heated portion of the capillary. The expression of plugs as in Fig. 2.

The relationship between sample plug length (w_S) and the corresponding introduction time (τ) is varied dependent on the pressure and capillary inner diameter, but if these factors are fixed to constant values, plug length is generally the first-order function of introduction time ($w_S = a\tau + b$, where a and b are constants). In the present system, a was $2.0 \cdot 10^{-3}$ m s^{-1} and b was $4.0 \cdot 10^{-4}$ m. The sample plug should be as short as possible in order to obtain high column efficiency, but too short plug is not beneficial with respect to detection sensitivity. In the present system of derivatization of carbohydrates with PMP introduction for 1.5 s, which was equivalent to 3.4 mm of plug length, was appropriate. The reagent plug should be sufficiently long to avoid incomplete overlapping of the plugs at the entrance of the heated portion of the capillary. Introduction for 10 s was appropriate in the present system. This was corresponding to a plug length of 20.4 mm.

3.1.2. The center position of the reagent plug

In the technique of in-capillary derivatization the sample plug is at the inlet of the capillary (Fig. 3). Therefore, d_S is equal to $\iota_H - (w_S - w_R)/2$, where ι_H is the distance between the capillary inlet and the entrance margin of the heating block, and w_S and w_R are the lengths of the sample and the reagent plugs, respectively. On the other hand the distance between the capillary inlet and the center of the reagent plug at the initial stage (ι_R), which expresses the position of the reagent plug, is $\iota_H + w_R/2 - d_R$. Therefore, ι_R can be calculated as follows:

$$\iota_R = \iota_H + \frac{w_R}{2} - \frac{t_S}{t_R} \cdot \left(\iota_H + \frac{w_R - w_S}{2} \right)$$
$$= \frac{(t_R - t_S)}{t_R} \cdot \iota_H + \frac{1}{2} \cdot \frac{t_S}{t_R} \cdot w_S + \frac{1}{2} \cdot \frac{(t_R - t_S)}{t_R} \cdot w_R \quad (2)$$

Thus, the position of the reagent plug (ι_R) can be determined by Eq. (2) using the migration times of the sample as well as the reagent, and the lengths of their plugs.

3.1.3. Length of the intermediate buffer plug

In many cases of in-capillary derivatization an

appropriate length of running buffer plug must be placed between the sample and reagent plugs. The length of the intermediate plug (w_B) can be estimated as follows:

$$w_B = \iota_H - w_S - \frac{t_S}{t_R} \cdot \left(\iota_H + \frac{w_R - w_S}{2} \right)$$
$$= \frac{(t_R - t_S)}{t_R} \cdot w_S + \frac{(t_S - 2t_R)}{t_R} \cdot w_R - \frac{1}{2} \cdot \frac{t_S}{t_R} \quad (3)$$

The intermediate plug can be introduced by either pressure (pressurization or suction) or voltage application. In either mode relevant time for the introduction of the intermediate buffer plug must be estimated using the w_B values obtained above. Therefore, the relationship between plug length (w_B) and introduction time (in the case of introduction by pressurization/suction) or applied voltage/duration (in the case of electromigration) should be established by a prior experiment using the same CE system.

Thus, various indices mentioned above must be obtained in order to perform in-capillary derivatization effectively. The estimation of such indices seems to be rather cumbersome, but once suitable values are established based on the above considerations, samples can be easily be automatically derivatized and analyzed repeatedly.

According to the above inference a solution of PMP, the running buffer, and a sample solution of maltopentaose were introduced in this order from the anodic end. In this in-capillary derivatization 200 m*M* borate buffer, pH 8.2, was used as reaction medium, and the sample and the reagent solutions were prepared in this medium. When the introduction of the sample and the reagent solutions was carried out for 1.5 and 10 s, respectively, which corresponded to the plug lengths of 3.4 and 20.4 mm, respectively, the required plug length of the intermediate running buffer was estimated to be almost zero (the introduction of the running buffer was not necessary). In the standard manual operation of precolumn derivatization [7] the reagent solution is made in methanol, but in this technique of in-capillary derivatization reaction medium was changed to borate buffer, to facilitate condensation and to prevent broadening of the product peak which otherwise would be caused by perturbation of the medium due to mixing of methanol with the running buffer. The pH value in this system was approximately the same as in the manual operation. Reagent concentration was reduced to 25 from 500 m*M* to minimize blank peak. Sample concentration was also decreased to 0.2 m*M*, less than 1% of reagent concentration. The t_S and t_R values were measured for a mixture of maltopentaose and PMP, without heating and standing at the entrance of the heated portion of the capillary. Since maltopentaose could not be detected at 245 nm (the wavelength of the absorption maximum of the PMP derivative) it was monitored at a shorter wavelength of 195 nm, where maltopentaose was barely detected as the borate complexes with enhanced sensitivity [13]. PMP could also be detected at this wavelength. After the introduction of the reagent and the sample solutions a potential of 5 kV was applied for 3 min between the inlet and the outlet of the capillary, in order to drive the introduced plugs by electromigration. The sample, maltopentaose, moved as the borate complexes, whereas the reagent, PMP, was migrated as the enolate ion. The electrophoretic movement of these compounds was toward the anode, but since electroosmotic flow was rapid toward the cathode, these compounds eventually moved to the cathode by the combined effects of electrophoresis and electroosmosis. Thus, the plugs were migrated at different velocities to be successfully superimposed on each other at the entrance of the heated portion of the capillary. The temperature of the oven was maintained at 57°C, just below the upper limit.

After standing the superimposed plugs for various periods of time the product was analyzed by the zone electrophoresis mode by applying a voltage of 10 kV. The running buffer was the same as the reaction medium, i.e., 200 m*M* borate buffer, pH 8.2. The derivatives were separated as borate complexes.

Fig. 4 shows the thus-obtained electropherogram.

3.2. Effect of heating time

In this technique of in-capillary derivatization the plugs of the carbohydrate solution and the reagent solution were driven to the heated portion of a capillary and superimposed on each other by the program outlined above. Since sample/reagent concentrations (0.5 m*M*/25 m*M*), reaction medium (200

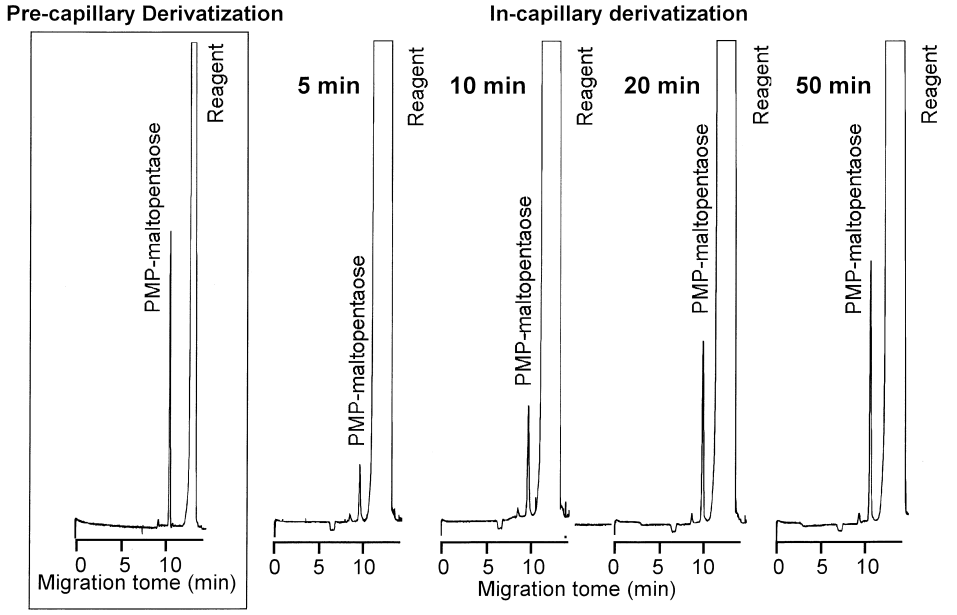

Fig. 4. Time course of the in-capillary derivatization of maltopentaose with PMP, as observed from the peak intensities of the derivative. Capillary, uncoated fused-silica (71 cm×50 μm I.D.); running buffer, 200 m*M* borate buffer, pH 8.2; sample solution, 0.5 m*M* maltopentaose in the running buffer; reagent solution, 25 m*M* PMP in the running buffer. In-capillary derivatization: introduction time, 1.5 s (sample solution) and 10 s (reagent solution); applied voltage for plug driving, 5 kV; duration, 3 min. Product analysis: applied voltage, 10 kV; detection, UV absorption at 245 nm. Pre-capillary derivatization was performed by heating a mixture of 2 m*M* maltopentaose (10 μl) and 200 m*M* borate buffer, pH 8.2, containing PMP (100 m*M*) (30 μl) for 50 min at 70°C. The product was analyzed using 200 m*M* borate buffer, pH 8.2, as running buffer. The total length of the capillary was 71 cm and the length from the capillary inlet to the detector window was 49 cm.

m*M* borate buffer, pH 8.2) and temperature (57°C) were fixed considering individual situations, only standing time (i.e., heating time or reaction time) was allowed to be altered. Fig. 4 compares the result of analysis of the product from maltopentaose obtained by various standing times (various reaction times).

In the technique of in-capillary derivatization the reagent concentration was far higher than sample concentration in order to facilitate the reaction and to increase the yield of the product. In the manual operation of pre-column derivatization the excess amount of the reagent is removed by solvent extraction, but such operation is difficult in the in-capillary derivatization. The huge peak following the product peak in Fig. 4 was of the excess reagent, but it did not interfere with the determination of maltopentaose under the conditions employed. It is clearly shown that the peak intensity increased as the standing time became longer. It reached a plateau after 50 min and the yield was almost quantitative at 50 min. Fig. 4 also gives an electropherogram obtained by pre-capillary derivatization under similar conditions, for reference. The yield of PMP-maltopentaose by pre-capillary derivatization was almost quantitative.

3.3. Analysis of a maltooligosaccharide mixture

Fig. 5 shows the result of the analysis of a maltooligosaccharide mixture (0.2 m*M* each) under similar conditions.

In this case running buffer was changed to 200 m*M* borate buffer, pH 8.2, containing SDS to a concentration of 200 m*M* to facilitate separation. Although t_S was slightly varied among the components, the variation was so small that the use of the value of maltopentaose for other oligosaccharides

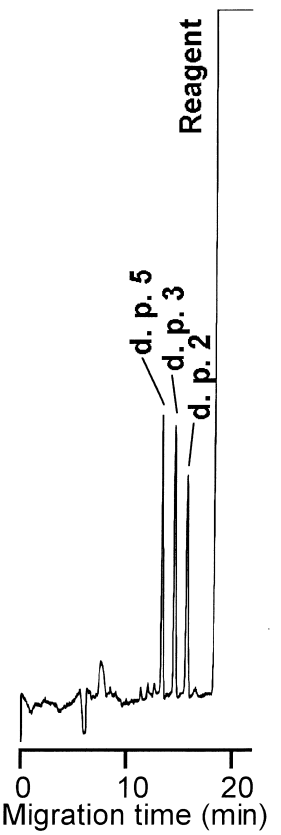

Fig. 5. Analysis of a mixture of maltooligosaccharides as in-capillary derivatized PMP derivatives. Running buffer was 200 m*M* borate buffer, pH 8.2, containing SDS to a concentration of 200 m*M*. The running buffer was introduced for 4 min at 5 kV between the sample and the reagent plugs. All other conditions for in-capillary derivatization and product analysis as in Fig. 4.

did not pose any problem to achieve complete plug overlapping, resulting in reproducible in-capillary derivatization. Thus, the introduction times of the sample and reagent solutions were 1.5 and 10 s, respectively, as in the analysis of maltopentaose (Fig. 4), but the running buffer was introduced between the plugs of the sample and the reagent by applying a voltage of 5 kV for 4 min based on the calculation by Eq. (3). All of the di-, tri- and pentamers of glucose were well separated from each other without interference from the excess reagent. Interestingly the maximum yields were obtained for all members of maltooligosaccharides in 35 min. The reduced analysis time as compared to that in the absence of SDS (Fig. 4) was probably due to the enhanced effect of sample/reagent mixing due to the presence of a high concentration of SDS, a powerful detergent.

3.4. Comparison with pre-capillary derivatization

As a reference experiment to Fig. 5 we analyzed the pre-capillary derivatized product from maltooligosaccharides. In this experiment the total length of the capillary was maintained the same as in Fig. 5, but the length from the capillary inlet to the detector window was reduced to 45 cm in order to match the analytical conditions. It was indicated that the theoretical plate numbers of the di-, tri- and pentaoligosaccharides derivatized by in-capillary derivatization ($1.3\cdot10^4$, $1.5\cdot10^4$, and $1.4\cdot10^4$, respectively) were significantly lower than the values obtained by pre-capillary derivatization ($5.8\cdot10^4$, $5.1\cdot10^4$, and $5.1\cdot10^4$, respectively), obviously due to peak broadening during the standing process for reaction. Resolution of peaks also became lower (3.9→2.2 for di-/trisaccharides, 4.4→2.6 for tri-/pentasaccharides) as a result of the decrease in column efficiency. The relative standard deviation of migration time (including the derivatization process), however, gave no significant change (3.0→3.5% on average for five repeated analyses). The limit of detection in terms of the concentration of the final analytical solution was at the same level of 10^{-6} *M*, but the amount of oligosaccharide actually required was different between the derivatization methods (in-capillary derivatization, 10 fmol level; pre-capillary derivatization, 10 pmol level). It is quite important that in-capillary derivatization allows remarkable sample reduction by three orders of magnitude.

3.5. Calibration curves and reproducibility

The calibration graphs of peak area vs. oligosaccharide concentration for all these oligosaccharides were linear at least in a range of 10–500 μ*M* with high coefficients of correlation, 0.998–0.999. The relative standard deviation (RSD) values of the peak area estimation was less than 3% ($n=7$) for all these oligosaccharides at the 50 μ*M* level. High reliability was thus demonstrated.

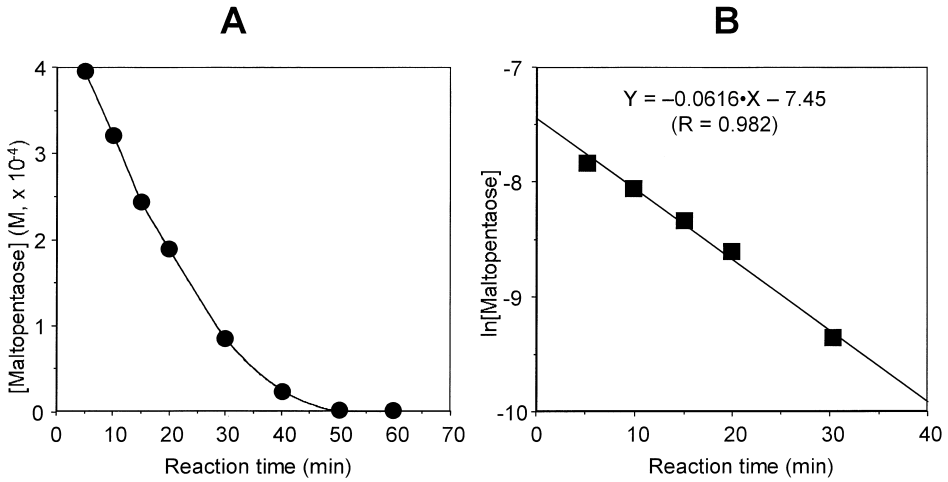

Fig. 6. (A) Plot of maltopentaose concentration in the reaction mixture vs. reaction time. Maltopentaose concentration was calculated from the yield of PMP-maltopentoase, determined by using the calibration graph. (B) Plot of ln [maltopentaose concentration] vs. reaction time for kinetic study of the reaction of this oligosaccharide with PMP. The data were obtained from Fig. 4.

3.6. Kinetic study of the derivatization

We have already pointed out that in-capillary derivatization is useful for the determination of rate constant of derivatization reaction, especially rapid reaction [2,3]. Plot of the concentration of the remaining maltopentaose calculated from the yield of the PMP derivative estimated from the data in Fig. 4 against reaction time gave a descending curve as shown in Fig. 6A, and the plot of ln [maltopentaose concentration] vs. reaction time gave a straight line as shown in Fig. 6B.

The slope of this straight line ($6.16 \cdot 10^{-2}$ min^{-1}, i.e., $1.03 \cdot 10^{-3}$ s^{-1}) means the pseudo first-order rate constant under the conditions employed. A rather higher value of $1.30 \cdot 10^{-3}$ s^{-1} was obtained for a reaction rate constant when SDS was added to running buffer. Thus, the described technique was shown to be useful also for the determination of rate constant of such slow reaction of carbohydrates with PMP.

4. Conclusion

It has been demonstrated that in-capillary derivatization technique is applicable to not only rapid reactions but also a slow reaction such as the condensation of a reducing carbohydrate with PMP, which requires long reaction time at high temperature in pre-capillary derivatization by manual operation. Among the three techniques of in-capillary derivatization, the ones in the middle of [2] and throughout [3] the capillary are not applicable to such slow reaction, because they do not allow long reaction time. The at-inlet technique [1] employed here, though slightly modified to drive the plugs to heated portion of a capillary, has an advantage that reaction time can be freely changed depending on situation. Although the same reaction medium was used for derivatization reaction and electrophoretic separation of the derivatives in the present case, use of different media will also be possible, as already demonstrated in the amino acid analysis as *o*-phthalaldehyde derivatives [1]. The observed column efficiency (theoretical plate number $1.3 \sim 1.5 \cdot 10^4$) was lower than in pre-capillary derivatization, but the extent of decrease was within one fifth. Reproducibility of migration time (RSD 3.5% on average) was somewhat lower than in pre-capillary derivatization (3.0% in average). However, the decrease was within allowable range for practical analysis. The reproducibility of peak area (RSD less than 3%) also ensured routine analysis. Notwithstanding such inferiority in separation capability, in-capillary derivatization can be done with only one thousandth of

the sample amount required in pre-capillary derivatization. It should also be emphasized that this technique is suitable for automated analysis. Once the appropriate values for the introduction times of the sample as well as the reagent solutions, the standing time, the voltages to be applied for plug driving and product analysis, and the duration of voltage application are determined and put into program, the introduced samples can be automatically analyzed as their derivatives. Extension to wider application is on going.

Acknowledgements

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