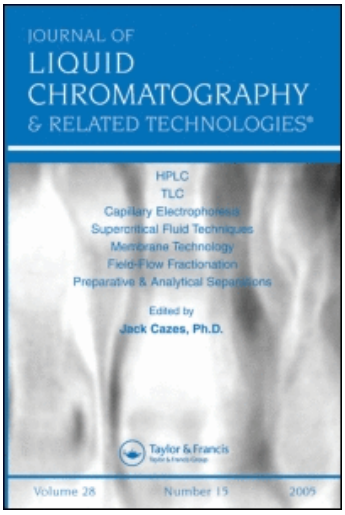

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Effects of Pushing Agents on the Separation and Detection of Debranched Amylopectin by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection

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EFFECTS OF PUSHING AGENTS ON THE SEPARATION AND DETECTION OF DEBRANCHED AMYLOPECTIN BY HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTION

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ABSTRACT

The effects of acetate and nitrate as pushing agents on the high-performance anion-exchange chromatography with pulsed amperometric detector (HPAEC-PAD) for the separation and detection of debranched amylopectin were evaluated. Our experimental data indicated that nitrate could differentiate branched from linear isomers, whereas acetate could not. Therefore, the nitrate system had better separating power than the acetate system provided that the chromatograms under both gradient systems were completed within 100 min. Nitrate was more compatible with the detector than acetate; thus, a higher detector response resulted. Under our experimental conditions, with a signal-to-noise ratio of two, the number of DP (degree of polymerization) at various concentrations that was resolved by the nitrate system (62 at 0.5 mg/mL, 65 at 1.0 mg/mL, and 66 at 1.5 mg/mL) was larger than those resolved by the acetate system (56 at 0.5 mg/mL, 58 at 1.0 mg/mL, and 58 at 1.5 mg/mL). The chromatograms obtained from the nitrate gradient system were more reproducible than those from the acetate gradient system in terms of the consistencies of retention time and the peak area

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of the chromatographic peak. To various concentrations of the component with the same DP, the detector responses were more consistent with nitrate as a pushing agent compared with acetate. Neither pushing agent, however, resulted in a quantitative response for homologous debranched amylopectin.

INTRODUCTION

Starches from different botanical sources have diverse functional properties that make them useful for applications in food, pharmaceutical, paper, plastic, and other industries. The functionality of starch is allied to its chemical structure. Therefore, knowledge of its structure will help in the understanding of the relationship between the physicochemical properties and the functional properties of starch. The chain-length distribution is an important parameter for characterizing the molecular structure of amylopectin. Although size exclusion chromatography with refractive index detector (SEC-RI) is one of the most frequently used techniques, it cannot separate each debranched chain of a specific chain-length. Furthermore, the refractive index detector often lacks sensitivity and selectivity, and it cannot be used with a gradient. In the early 1980's, Hughes and Johnson [1,2] successfully applied a triple-pulsed amperometric detection method for the detection of carbohydrates. Rocklin and Pohl [3] had combined the high-performance anion-exchange chromatography with pulsed amperometric detection for the determination of carbohydrates. Since then, there has been a significant increase in the application of high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) in carbohydrate research [4-9].

The affinities of anions to the strongly basic anion-exchange resin are based on their charges and radia of the hydrated ions and are increased in the order of OH^- < acetate < nitrate < sulfate [10]. Therefore, sulfate is the strongest pushing agent and hydroxide is the weakest pushing agent in the anion-exchange chromatography. By using an isocratic system, Rocklin *et al.* [3] have evaluated acetate, carbonate, nitrate, and sulfate as pushing agents for the determination of carbohydrates by HPAEC-PAD. They preferred acetate

as a pushing agent because acetate and hydroxide have similar affinity for the anion-exchange resin, and all other pushing agents reduced the column loading capacity. Since then, acetate has become the most commonly used pushing agent in the HPAEC-PAD for the separation of carbohydrate. Recently, Lu *et al.* [11] have studied the effect of acetate, nitrate, and sulfate as pushing agents in the isocratic system on the determination of malto-oligosaccharides by HPAEC-PAD. They concluded that nitrate had a greater ability to reduce the capacity factor for better resolution and thus nitrate should be a better pushing agent than acetate. All the studies, so far, on pushing agents were performed under the conditions of an isocratic system. For the study reported here, we evaluated the function of acetate and nitrate as pushing agents in a gradient system for quantitative and qualitative analysis of debranched amylopectin. Our results should provide additional information on exploring the HPAEC-PAD in starch analysis.

EXPERIMENTAL

Materials

Sodium hydroxide solution (5 N), sodium acetate, sodium nitrate, and glacial acetic acid were obtained from Fisher Scientific (Fair Lawn, NJ). Sodium azide was obtained from Eastman Organic Chemicals (Rochester, NY). Glucose (G1), maltose (G2), and corn starch were obtained from Sigma Chemical Co. (St. Louis, MO). Maltotriose hydrate (G3, 95%), maltotetraose (G4), maltohexaose (G6, Tech., 90%), and maltoheptaose hydrate (G7, 90%) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Maltopentaose (G5) and iso-amylase (EC 3.2.1.68, crystal, from Pseudomonas amyloderamosa, 59,000 units/mg protein) were obtained from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan). All chemicals and enzymes were used without further purification. The corn amylopectin was fractionated from corn starch by the method of Schoch [12] and purified three times by

recrystallization. The isolated amylopectin was washed with methanol and oven dried at 50°C for 12 hr. The tapioca amylopectin and wheat amylopectin were provided by Dr. J.-F Chen. The acetate buffer was prepared by mixing 16 mL of 0.1 N acetic acid with 1 mL of 0.1 N sodium acetate solution. The standard solution containing G1 to G7 was composed of glucose (31 μg/mL), maltose (42 μg/mL), maltotriose hydrate (23 μg/mL), maltotetraose (42 μg/mL), maltopentaose (39 μg/mL), maltohexaose (33 μg/mL), and maltoheptaose hydrate (48 μg/mL). Water (18 MΩ cm) used in all of the preparations was obtained from the Milli-Q Reagent Water System (Millipore, Bedford, MA).

HPAEC-PAD

The HPAEC was performed on a Dionex (Sunnyvale, CA) DX30 gradient pump module equipped with a Dionex pulsed amperometric detector. Sample injection was *via* a Dionex microinjector valve with a 200 μL sample loop. Sample solution was filtered through a 0.45 μm Magna nylon membrane (Fisher Scientific, Fair Lawn, NJ) before injection. A CarboPac PA1 anion-exchange column (250 x 4 mm) and a CarboPac PA1 guard column (25 x 3 mm) were used for sample separation. Two HPLC gradient systems were applied for the separation of debranched amylopectin. The acetate gradient system employed two eluents. Eluent 1 was 150 mM sodium hydroxide and eluent 2 was a mixture of 150 mM sodium hydroxide and 500 mM sodium acetate. The gradient was programmed as follows: 0-0.1 min, 75% eluent 1 and 25% eluent 2; 0.1-15 min, linear gradient to 45% eluent 2; 15-45 min, linear gradient to 60% eluent 2; 45-80 min, linear gradient to 70% eluent 2; 80-100 min, linear gradient to 80% eluent 2. The nitrate gradient system employed eluent 1 and eluent 3, which was a mixture of 150 mM sodium hydroxide and 500 mM sodium nitrate. The following gradient was used: 0-5 min, 94% eluent 1 and 6% eluent 3; 5-10 min, linear gradient to 8% eluent 3; 10-30 min, linear gradient to 13% eluent 3; 30-80 min, linear gradient to 20% eluent 3; 80-100 min, linear gradient to 25% eluent 3. The flow rate for both

gradient systems was 1.0 mL/min throughout the program. The column was allowed to equilibrate for 10 min with the initial condition of the gradient system before each run. All eluents were filtered through a 0.45 μm Supor-450 membrane (Fisher Scientific, Fair Lawn, NJ) and degassed by sparging with helium gas before use. The debranched amylopectin was detected by a pulsed amperometry using a gold electrode with the following repeating sequences of potentials and durations: E1 = 0.05 V (480 ms); E2 = 0.6 V (120 ms); E3 = -0.6 V (60 ms). E1 was for sample oxidation, E2 was to clean the electrode surface, and E3 was to reduce gold oxide back to gold. The sampling period was set to 200 ms, and the response time was set to 1 s.

Enzymatic Debranching of Amylopectin

Amylopectin was debranched by using iso-amylase according to the procedures described by Jane *et al.* [13] with some modifications. Briefly, amylopectin (50 mg) was suspended in 9 mL of water and heated in a boiling water bath with constant stirring for 15 min. The suspension was cooled down to 25°C, then 1 mL of acetate buffer (0.1 N) was added and followed with a 5 μL of iso-amylase. The reaction mixture was incubated for 48 h in a shaker water bath (Versa Bath S, model 236, Fisher Scientific, Fair Lawn, NJ) at 40°C with 100 strokes/min. The mixture was then adjusted to pH 6 with 5 N NaOH solution, followed by the addition of sodium azide solution (10%, 20 μL), and heated in a boiling water bath for 15 min to inactivate the enzyme.

RESULTS AND DISCUSSION

Separation of Debranched Amylopectin

Debranched corn amylopectin with concentrations of 0.5 mg/mL, 1.0 mg/mL, and 1.5 mg/mL were subjected to HPAEC-PAD analysis by the acetate and the nitrate gradient systems. The resulting chromatographic

profiles of debranched corn amylopectin from the acetate and the nitrate gradient systems are shown in Figure 1. Both gradient systems were designed so that the separations for the debranched amylopectin were completed within 100 min. The standards G1 to G7 were used to identify the chromatographic peaks with the DP (degree of polymerization) from 1 to 7. The assignment for the chromatographic peaks with DP higher than 7 was based on the generally

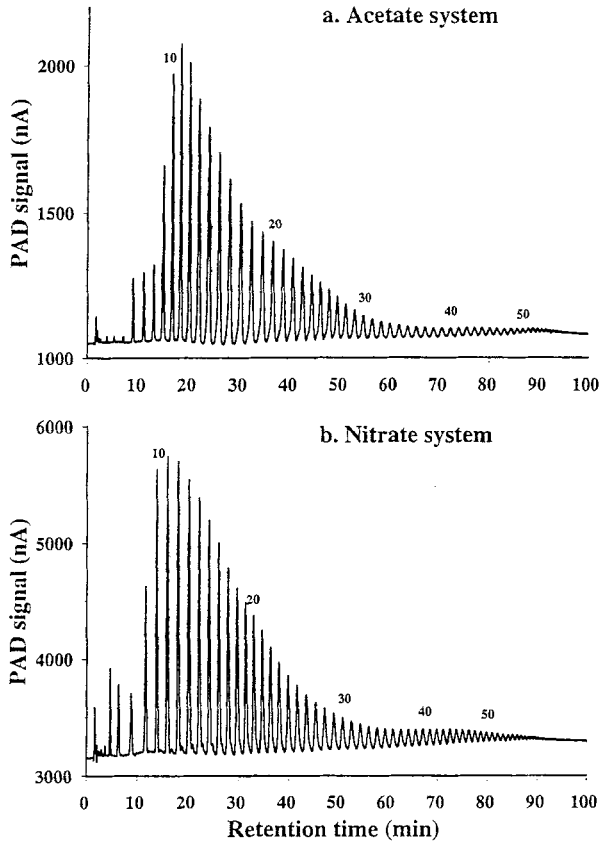

FIGURE 1. HPAEC-PAD chromatographic profiles of the enzymatic debranched corn amylopectin using (a) the acetate gradient system and (b) the nitrate gradient system. Peak numbers indicate the degree of polymerization.

accepted assumptions that (1) the retention time of the homologous series of saccharides increased as the DP increased, and (2) each successive peak represented a saccharide which was 1 DP longer than that of the previous peak. The detector response for the saccharides was much more sensitive under the nitrate system (Figure 1b) than the acetate system (Figure 1a). This observation suggested that the nitrate system was more compatible with the detection system (PAD). The number of DPs resolved by the acetate and nitrate systems is summarized in Table 1. With a signal-to-noise ratio of two or three, the number of DPs resolved by the nitrate system was always greater than that by the acetate system at all concentrations of the samples. The greater number of DPs detected in the nitrate system was attributed to the increase in the detection sensitivity of the system. Also, some minor peaks, which were suggested to be the isomers with maltosyl branches [14], were observed in the chromatograms using the nitrate system (Figure 1b) but not in those using the acetate system. For the acetate system, the isomers could be separated by increasing the resolution; however, the higher the resolution, the greater the disengagement of the two adjacent peaks, which meant a longer

TABLE 1. Degrees of Polymerization[a] Resolved by the Acetate and the Nitrate Gradient Systems

Sample concentrations mg/mL	Degree of polymerization			
	Acetate gradient system		Nitrate gradient system	
	S/N[b] = 3	S/N = 2	S/N = 3	S/N = 2
0.5	53	56	59	62
1.0	56	58	62	65
1.5	56	58	63	66

a. Average of triplicate analysis.
b. Signal-to-noise ratio.

separation period would be required. Thus, the nitrate system was more effective in differentiating the branched isomers, which were often found in the debranched amylopectin samples.

Reliability of the Systems

The reliability of the analytical system is an important issue especially when the characterization of amylopectin was based on the distribution of over a hundred saccharides, and standards for each saccharide are not always available. Therefore, the reliability of the acetate system and the nitrate system was investigated *via* the evaluation of the reproducibility and the accuracy of the acetate and the nitrate systems.

Reproducibility

The reproducibility of the chromatograms was judged by (1) the consistency of retention times (t_R) and (2) the consistency of detector responses determined by peak areas that were measured after repeated injections of the sample. Figure 2a is the plot of $\bar{t}_R$ *vs.* DP for both gradient systems. Each data point represented an average of retention time of the corresponding component resulted from the triplicate injections of the samples with concentration of 0.5 mg/ml, 1.0 mg/ml, and 1.5 mg/ml under the same chromatographic conditions. Figure 2b was the plot of the observed maximum shift (t_{Rmax} - t_{Rmin}) of the corresponding component *vs.* DP. The maximum shift of the chromatographic peaks ranged from 0.05 min to 3.72 min in the acetate system and from 0.02 min to 1.39 min in the nitrate system. The standard deviation plot of the $\bar{t}_R$ *vs.* DP for each system clearly (Figure 3) showed that the overlapping regions between peaks in the acetate system were significant. In contrast, the deviations of t_R were small in the nitrate system. For every sample analysis, the column was equilibrated with the initial chromatographic conditions for 10 min before each corresponding gradient

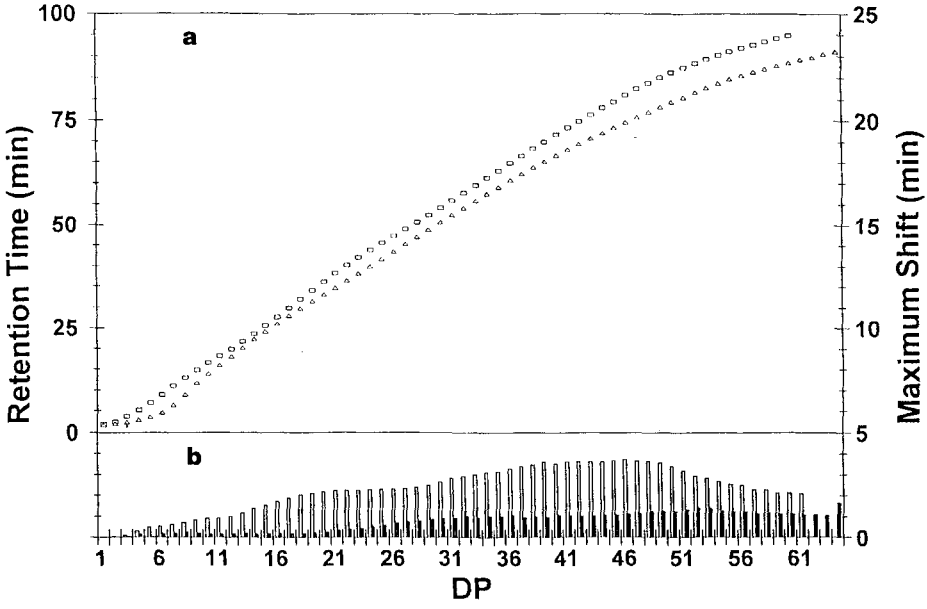

FIGURE 2. (a) Average retention time ($\bar{t}_R$) *vs.* DP of the corresponding component in the chromatograms of debranched corn amylopectin under the acetate gradient system (□) and the nitrate gradient system (△). (b) The observed maximum shift of each component in the acetate system (empty bar) and the nitrate system (shaded bar).

system was applied. The experimental results suggested that the acetate system might require a longer period of equilibration to condition the column. The initial and final concentrations of the acetate in the acetate gradient system were 125 mM and 400 mM, respectively, and those of the nitrate in the nitrate gradient system were 30 mM and 125 mM, respectively. The profound difference in the concentration of the pushing agent between the initial stage and the final stage (275 mM) of the acetate system also suggested that the column might need a longer time for equilibration.

The debranched tapioca and wheat amylopectin samples were also analyzed with the nitrate system. Their chromatographic profiles are shown in

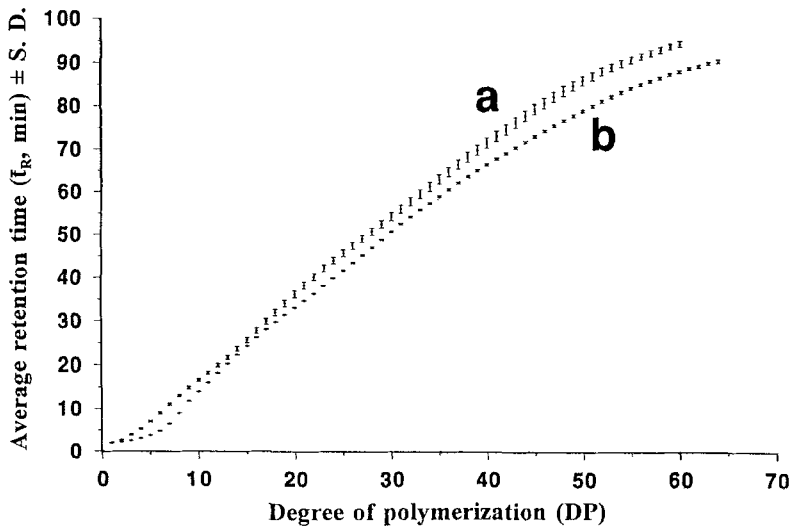

FIGURE 3. The plot of average retention time (from 9 analyses) and its standard deviation *vs.* DP of the corresponding component in the chromatograms of the debranched corn amylopectin with various concentrations under (a) the acetate system and (b) the nitrate system.

Figure 4. Figure 5 shows a plot of $\bar{t}_R$ with deviation *vs.* DP where $\bar{t}_R$ was an average of the retention time of the corresponding saccharide resulted from replicate injections of debranched corn, tapioca, and wheat amylopectin. As shown in Figure 5, the retention times of the saccharides with the same chain-length were very reproducible even though they were from different sources.

The reproducibility of the detector response was evaluated by the standard deviation of the peak areas, which resulted from the triplicate analyses of each sample. The peak area and its standard deviation for each component in the sample versus DP were plotted and are shown in Figure 6. Figure 6a is of the acetate system; Figure 6b is of the nitrate system. For the samples with concentration of 0.5 mg/mL (6ai), the standard deviations of the detector responses in the acetate system were comparable to those in the nitrate system (6bi), however, for the samples with a concentration of 1.0 mg/mL

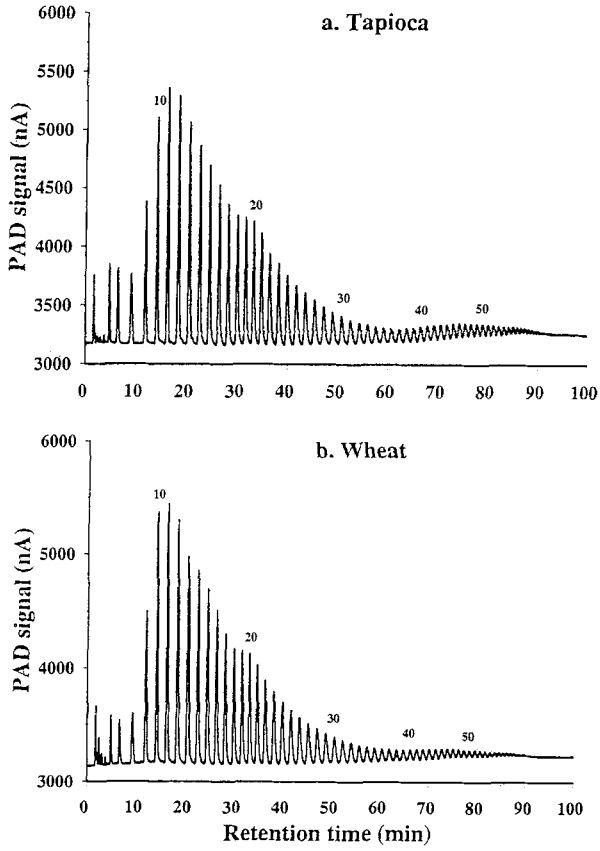

FIGURE 4. HPAEC-PAD chromatographic profiles of the enzymatic debranched (a) tapioca amylopectin and (b) wheat amylopectin under the nitrate system. Peak numbers indicate the degree of polymerization.

(6aii and 6bii) and 1.5 mg/mL (6aiii and 6biii), the deviations of the detector responses were relatively lower in the nitrate than in the acetate system.

Accuracy

To determine the accuracy of a measurement, knowledge of the true value from the sample is required. However, it is very difficult, if not

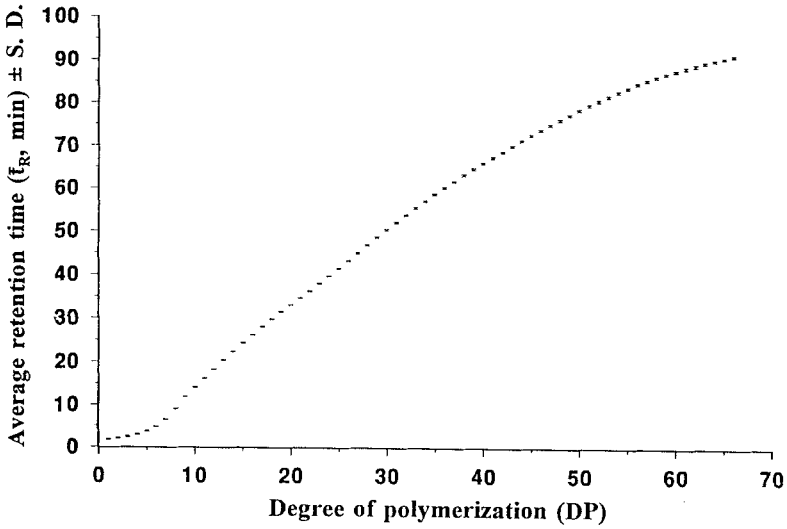

FIGURE 5. The plot of average retention time (from 6 analyses) and its standard deviation *vs.* DP of each corresponding component in the chromatograms of debranched amylopectin samples from corn starch, tapioca starch, and wheat starch.

impossible, to obtain standards for each of the saccharides in our samples. Therefore, the accuracy of the analysis of debranched amylopectin was determined by comparing the signal ratio of the corresponding component in the samples with different concentrations to the concentration ratios (i.e., true value) of the samples. The plots in Figure 7 are the signal ratios of the corresponding component of sample concentration ratios between 1.5 and 1 mg/mL, 1.0 and 0.5 mg/mL, and 1.5 and 0.5 mg/mL. With a concentration ratio of 1.5 (1.5 to 1 mg/mL), both systems exhibited a relatively consistent signal ratio with higher accuracy (7ai and 7bi). On the other hand, with a concentration ratio of 3 (1.5 to 0.5 mg/mL), both systems showed fluctuation in the response ratio with poor accuracy (7aiii and 7biii). These experimental results indicated that the appropriate sample size for the analysis would be 1 mg/mL to 1.5 ml/mL. Overall, the detector response was more consistent with nitrate as the pushing agent (Figure 7b) compared to that with acetate as

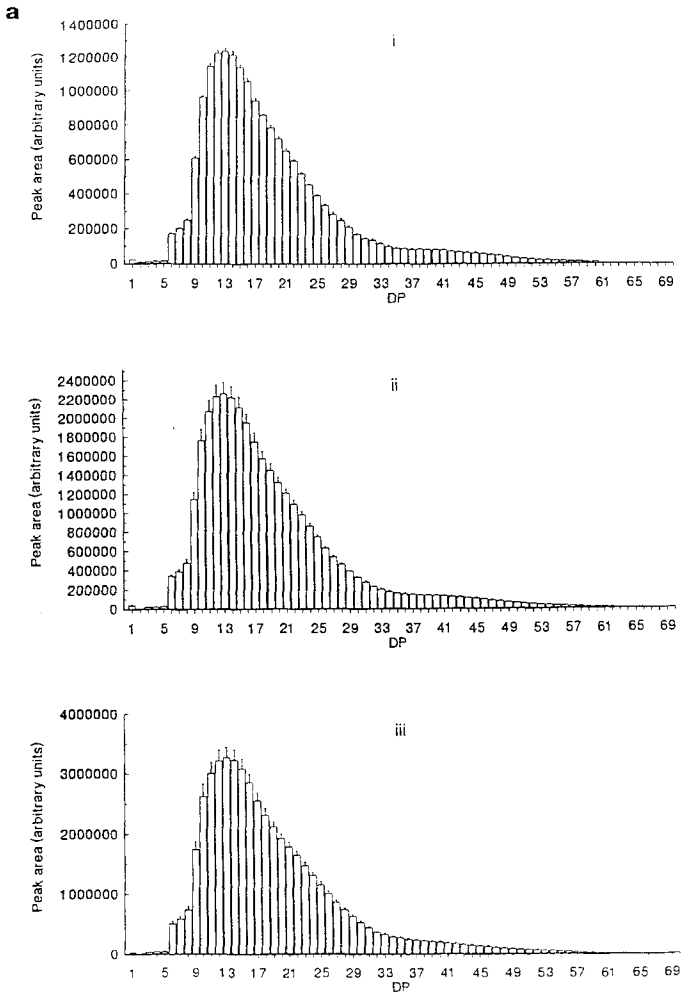

FIGURE 6. Peak areas and their standard deviations *vs.* DP in (a) the acetate system and (b) the nitrate system with sample concentrations of (i) 0.5 mg/mL, (ii) 1.0 mg/mL, and (iii) 1.5 mg/mL.

(continued)

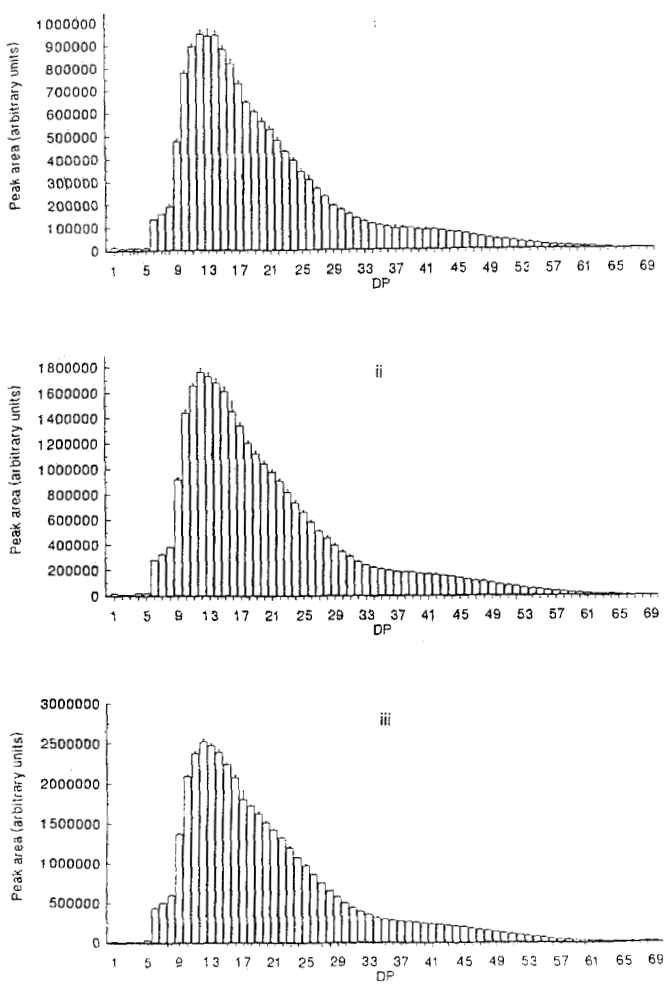

FIGURE 6 (continued).

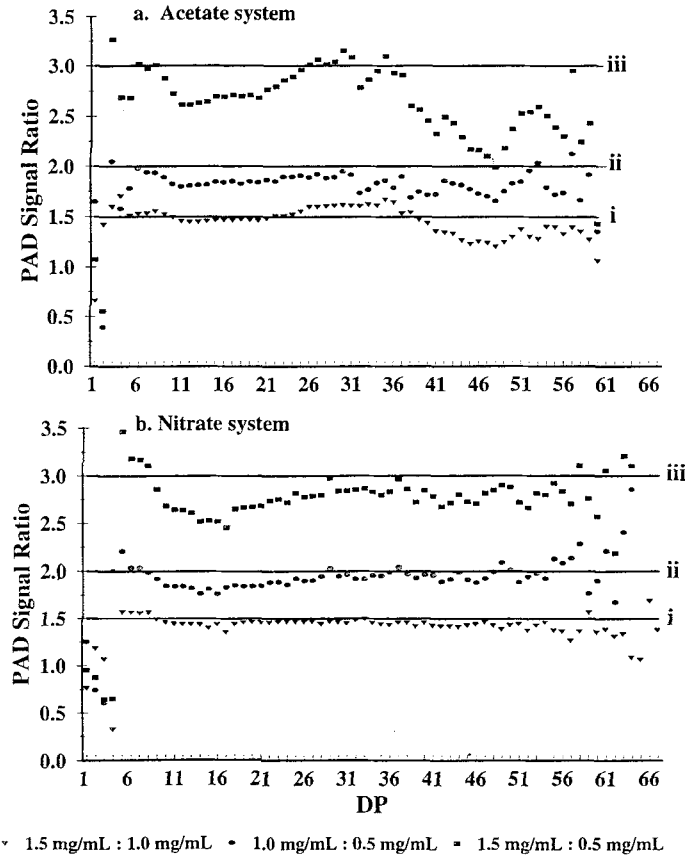

FIGURE 7. The signal ratios *vs.* DP of the corresponding components in the debranched corn amylopectin with sample concentrations of (i) 1.5 and 1 mg/mL, (ii) 1.0 and 0.5 mg/mL, and (iii) 1.5 and 0.5 mg/mL under (a) the acetate system and (b) the nitrate system. The solid lines represent the true values of the corresponding ratios.

the pushing agent (Figure 7a). This observation was supported by the mean signal ratios and the standard deviations (calculated from DP 5 to DP 56) for various concentration ratios of the sample (Table 2). In most occasions, the signal ratios obtained under the nitrate system were closer to the corresponding true values and the deviations were low.

TABLE 2. Statistic Data for the Signal Ratios from DP 5 to DP 56

Stats.[a]	Acetate System			Nitrate System		
True Value	1.5[b]	2[c]	3[d]	1.5[b]	2[c]	3[d]
Mean	1.46	1.83	2.67	1.45	1.93	2.80
Std. Dev.	0.12	0.08	0.29	0.05	0.09	0.17
Maximum	1.67	1.95	3.06	1.57	2.21	3.46
Minimum	1.20	1.65	2.10	1.34	1.76	2.46

a. Calculated from 52 data points.
b. Calculated from sample concentration ratio of 1.5 $mgmL^{-1}$/1.0 $mgmL^{-1}$
c. Calculated from sample concentration ratio of 1.0 $mgmL^{-1}$/0.5 $mgmL^{-1}$
d. Calculated from sample concentration ratio of 1.5 $mgmL^{-1}$/1.5 $mgmL^{-1}$

CONCLUSION

In comparison with the commonly used pushing agent, acetate, nitrate offered greater reproducibility and accuracy, and lower detection limit in the HPAEC-PAD; thus, it is a promising pushing agent for the quantitative and qualitative analysis of the debranched amylopectin. Our study further supported the view of Lu *et al.* [11] that nitrate was a better pushing agent than acetate for the separation of saccharides. However, neither pushing agent resulted in a quantitative response for homologous debranched amylopectin. In addition, the HPAEC-PAD can separate the saccharides but cannot provide the information of total carbohydrate for each saccharide. On the other hand, the size exclusion chromatography with refractive index detector (SEC-RI) can provide information of total carbohydrates; however, it cannot separate the saccharides as the HPAEC-PAD does. Therefore, an alternative detection technique for HPAEC is needed so that the information for both the fine chain-length distribution and the total carbohydrates for each saccharide in the debranched amylopectin sample can be obtained simultaneously.

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