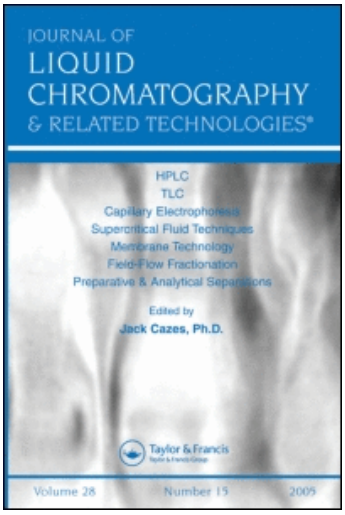

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EVALUATION OF DEBRANCHED RICE STARCH SAMPLE PREPARATION METHODS FOR ANION-EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTOR

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EVALUATION OF DEBRANCHED RICE STARCH SAMPLE PREPARATION METHODS FOR ANION-EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRIC DETECTOR

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ABSTRACT

Rice starch was debranched with the debranching enzyme pullulanase. Anion-exchange chromatography with pulsed amperometric detection (AEC-PAD) was used to study effect of time on the degree of enzyme catalyzed debranching. The stability of starch sample in different starch solvents during extended storage was evaluated. Lyphylized, non-debranched starch samples were prepared in 0.0375 M HCl , 0.0375 M NaOH and water, followed by heating at 121°C for 30 min. Samples were stored for 21 days. HCl hydrolyzed the starch. Starch samples prepared in water were also unstable with progressively increasing peak areas up to 21 days. Starch samples prepared in NaOH are most stable on storage. Lower concentration (0.009, 0.0046, 0.0023, and 0.0012 M) of HCl hydrolyzed freshly prepared non-debranched starch auto-

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claved at 121°C for 30 min in respective concentration of HCl to different degrees dependent on the concentration of HCl used.

Starch samples prepared in water from freshly cooked or lypholized non-waxy and waxy starch samples were also unstable with progressively increasing peak areas up to 7 days of storage. To determine the effect of time on degree of enzyme catalyzed debranching, debranched starch samples were removed at various time intervals and analyzed immediately after sampling using water as the solvent.

INTRODUCTION

Starch is composed of two different α-glucan polymers: amylose and amylopectin. Amylose is essentially linear (1-4)-α-D-glucan chains; whereas, amylopectin is a branched component composed of short (1-4)-α-D-glucan chains linked to each other by α-(1-6) linkages. These two components, with widely different properties, are assembled and placed in the same granule. Amylopectin, one of the largest molecules in nature, is the principal component in the majority of starches. The amount of amylopectin and its branched-chain length distribution are important in influencing the functional and nutritional properties of the starch(1). Starch retrogradation occurs during cooling of a cooked paste and has been described in a review by Hoover(2). When cooled, the starch chains in a gelatinized paste associate leading to the formation of a more ordered structure. These molecular interactions are termed collectively "retrogradation" and have important textural and dietary implications. Branched- chain length distribution plays one of the most important roles in retrogradation of starches.

Alteration of the branched-chain structure of amylopectin has resulted in development of commercial products. Debranched starches have been used to make starch resistant to digestion(3,4,5). Recently, Guraya et al.(6,7) have reported a process for making slowly digestible starches from cooling enzymatically catalyzed debranched starches.

Amylopectin chain length distribution has been determined by using debranching enzymes (e.g., pullulanase and isoamylase) which specifically hydrolyze α-(1-6)-D-glycosidic interchain linkages,(8) followed by size exclusion chromatography (SEC) or anion-exchange chromatography with pulsed amperometric detection (AEC-PAD). SEC gives an overall view and does not quantify each individual chain length type. AEC-PAD has been found to separate and detect each individual chain length type. The main problem with PAD is different response for chains of different chain length(9,10). Therefore, peak area in a AEC-PAD chromatogram is not directly related to the quantity of a particular chain length type. This problem can be solved by using a quantification standard (9), post column enzyme reactor,(11) or a relative response factor(12).

The stability of debranched rice samples prepared for AEC-PAD has not been evaluated for changes during extended storage. Koch et al.(12) studied the stability of debranched amylopectin from wheat, pea, and potato. The samples were prepared in 100 mM NaOH and examined using AEC-PAD over a period of three days. They found no significant differences in the peak areas or the number of peaks during storage. Koizumi et al.(13) stored samples of short chain amylose (degree of polymerization, ≈ 17) in high pH eluents (150 mM NaOH solution) and deionized water for three days in a refrigerator. Chromatograms were similar for both storage solvents. However, in addition, they found small peaks of epimerization products in the chromatogram of the alkaline sample solution using AEC-PAD.

In this investigation, AEC-PAD was used to determine individual glucose polymers and the effect of reaction time on the degree of debranching by pullulanase. The effects of storage time on the stability of the starch samples in various solvents were also examined. Determining which solvent results in stable peaks over extended periods of storage was essential, since repeated injections needed to be made during the course of the study.

EXPERIMENTAL

Preparation of Freshly Cooked and Lypholized Non-Debranched Waxy and Non-waxy Rice Starch

Waxy and non-waxy rice starch were purchased from A & B Ingredients, Fairfield, NJ. A two liter batch of either 10% (w/v) waxy or non-waxy rice starch (db) solution was prepared by placing 200 g (db) of starch in a tared 2 liter stainless steel container. Distilled water (450 mL) was stirred into the starch to create a slurry. The rice starch slurry was slowly added to a 3 liter stainless steel beaker containing 1250 mL of boiling distilled water which was then placed in a boiling water bath. Distilled water (100 mL) was used to rinse the rice starch slurry remaining in the container. The starch solution was allowed to cook for 1 hr. The samples were cooled to ambient temperatures and freeze dried. When only freshly prepared samples were needed for analysis, the cooked slurry was not freeze dried and samples were used as needed.

Preparation of Debranched Waxy and Non-waxy Rice Starch

After the waxy and non-waxy rice starches were cooked and cooled as described above, each starch solution was separated into two 1-liter batches. The solutions were cooled to about 60°C in an ice water bath and the pH of each batch was adjusted to 4.8-4.9 with 1 N HCl. The containers were then placed into a

water bath at 57.5°C (optimum temperature for debranching enzyme) and allowed to reach equilibrium while being stirred continuously with a GlassCol (Terre Haute, IN) electric stirrer with paddle attachment set at 133 rpm. Ten percent (8 mL / L of 10% starch solution) by weight pullulanase (200 PUN/mL, 1.25 gm/mL, Novo Nordisk, Franklinton, NC), per dry weight of starch was added to each of the containers. The starch solutions were continuously stirred for 48 hrs. To determine effects of time on degree of debranching, aliquots of debranched starch containing 10 gm/100 mL of starch solution were removed for analysis at 0, 1, 2, 4, 8, 12, 24, and 48 hr time intervals and autoclaved at 121°C for 30 min (to stop further enzymatic activity). The 0 h sample served as the control for comparing debranched and non-debranched starch samples.

Anion-Exchange Pulse Amperometric Detection Chromatography

Chromatography was performed on a Dionex (Sunnyvale, CA) Series 4000i system equipped with a gradient pump system, an eluant degassing module, and a pulsed amperometric detector (PAD). Detection was by triple-pulsed amperometry with a gold working electrode and silver-silver chloride reference electrode(14). A 50 μL sample (0.2 mg starch /mL) filtered through 0.45 μm Millex-HV Durapore membrane (Millipore Corp, Bedford MA) was injected into 50 μL sample loop. A Dionex CarboPac PA1 pellicular anion exchange resin (250 x 4 mm) column and a CarboPac PA1 guard column (50 x 4 mm) were used for separation of debranched starch polymers. All eluants were prepared using Milli-Q water (resistivity: 8-12 megohm-cm) and degassed with helium prior to use to prevent absorption of carbon dioxide producing carbonate. Carbonate will act as a displacing ion and shorten retention times.

Eluant 1 was 0.15 M NaOH and Eluant 2 was a mixture of 0.15 M NaOH and 0.5 M NaOAC. The flow rate was set at 1.0 mL/min. The mobile phase was 70 % eluant 1 and 30 % eluant 2 at time 0 min; from 7.5 to 7.7 min the mobile phase was brought to 50 % eluant 1 and 50 % eluant 2; sample was injected at 7.7 min and eluant was brought to 100% eluant 2 from 7.7 to 16.5 min. Preliminary studies showed that these conditions were necessary to achieve good separation of peaks. It was found that switching eluant 1: eluant 2 ratio from 70:30 to 50:50 at 7.5 min before bringing it to 100% eluant 2 at 7.7 min resulted in better separation of glucose and maltose peaks. The eluant stayed at 100% eluant 2 for additional 5 min for cleanup before returning to 70:30 eluant 1:eluant 2 for 7.5 min. This was done to equilibrate the column prior to injection. The cell stays on throughout the gradient including the initial equilibration step but the data collection only starts at 7.7 min. Data was collected using the Peak Net Chromatography Workstation by Dionex, Release 4.30.

The following working pulse potentials and durations were used for detection of saccharides: E_1 = 0.05V(420 ms); E_2 = 0.75V(80 ms); E_3 = -0.20V (360 ms). E_1 was for oxidation of CHOH groups on the saccharides, E_2 was to remove the reaction product and cleans the electrode surface, E_3 was to reduce gold oxide back to gold. The sampling period was set to 200 ms, and the response time of the detector was set for 1s. Samples were low in concentration therefore the response was measured at 300 nA.

Approximately 2 milligrams of each standard from DP-1 to DP-7 glucose units (Sigma Chemical Co. St. Louis, MO), were dissolved in 10 mL of Milli-Q deionized water (resistivity: 8-12 Megohm-cm). An amount of 10 - 50 µL of this solution was diluted with 10 mL in volumetric flasks to give final concentrations of approximately 200-1000 ppb for each glucose polymer. Standard samples were injected to make a standard curve and injections were made at the start and end of each sample analysis. This was done to make sure that consistent operating conditions were maintained. No standards were available for sugars beyond DP-7 glucose units.

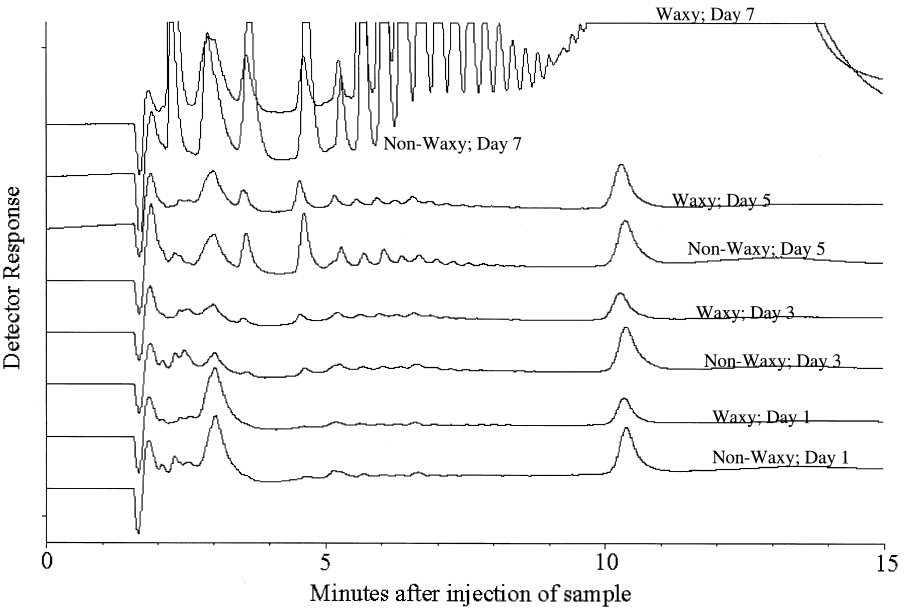

Figure 1. Effect of time of storage, on the detector response of non-debranched freeze dried non-waxy and waxy starch autoclaved at 121°C 30 minutes in water and injected over a period of 7 days.

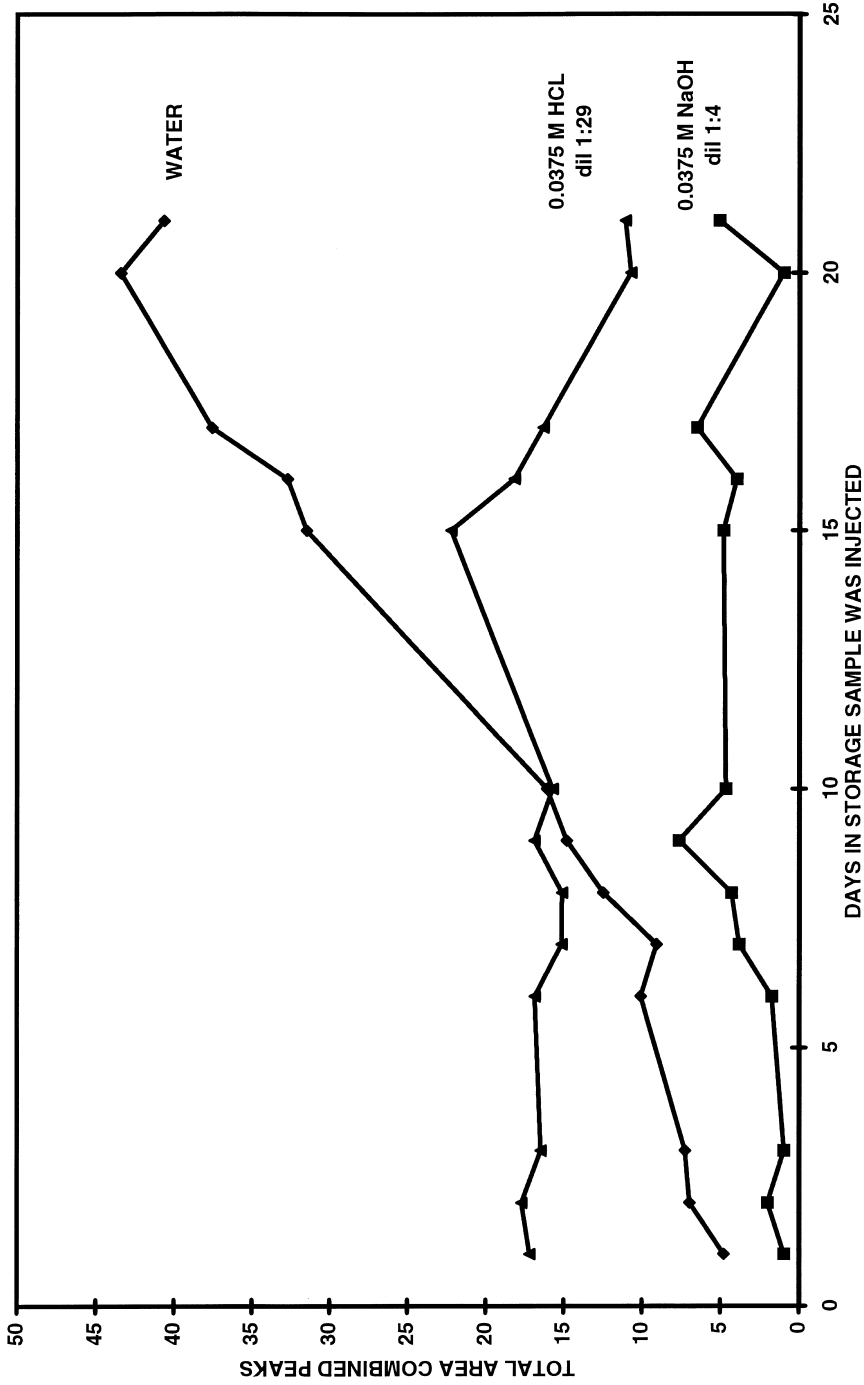

Figure 2. Effect of time of storage, on the detector response of freeze dried non-debranched non-waxy starch samples prepared in water, 0.0375M HCl and 0.0375M NaOH and autoclaved at 121°C for 30 minutes injected over a period of 21 days.

RESULTS AND DISCUSSION

Several problems were encountered in the development of a method to measure degree of debranching. Initially, we hypothesized that freeze-dried, cooked starch could be rehydrated and solubilized in water at 121°C for 30 minutes. Approximately 2 mg each of freeze-dried, cooked waxy and non-waxy starches were dissolved in 5 mL of water and autoclaved at 121°C for 30 minutes. Samples were injected into the Dionex system over a period of 7 consecutive days to determine the stability of the prepared samples. It was determined that rehydrated samples prepared in this manner did not give consistent results with time (Fig. 1). The chromatogram shows increasing concentrations of starch with increased sample storage time when the same concentration of starch was injected each of the 7 days. All the peaks were off-scale on the seventh day. We suggest that the structure of starch in water changes with time, thereby exposing more oxidative sites which results in bigger peaks in the chromatogram.

Since we could not use water alone to achieve freeze-dried starch sample stability on storage, we hypothesized that freeze-dried waxy and non-waxy samples, when prepared in 0.0375 M HCl or 0.0375 M NaOH and autoclaved at 121°C for 30 minutes, might ionize and would result in improved stability. Prior to injection, the samples prepared in HCl were diluted 1 to 29 and the NaOH

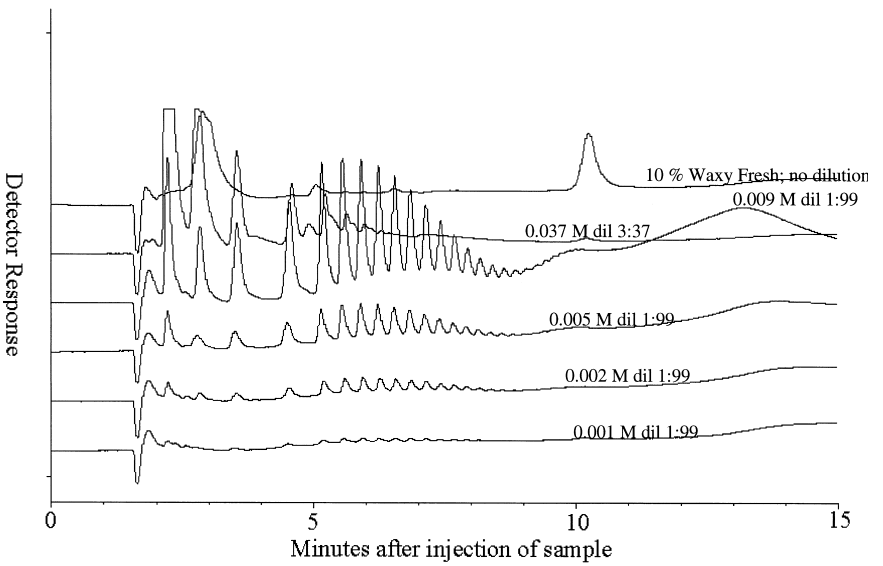

Figure 3. Effect of concentration of HCl, non-debranched freshly prepared waxy starch autoclaved at 121°C for 30 minutes in appropriate concentration of HCl, on the detector response.

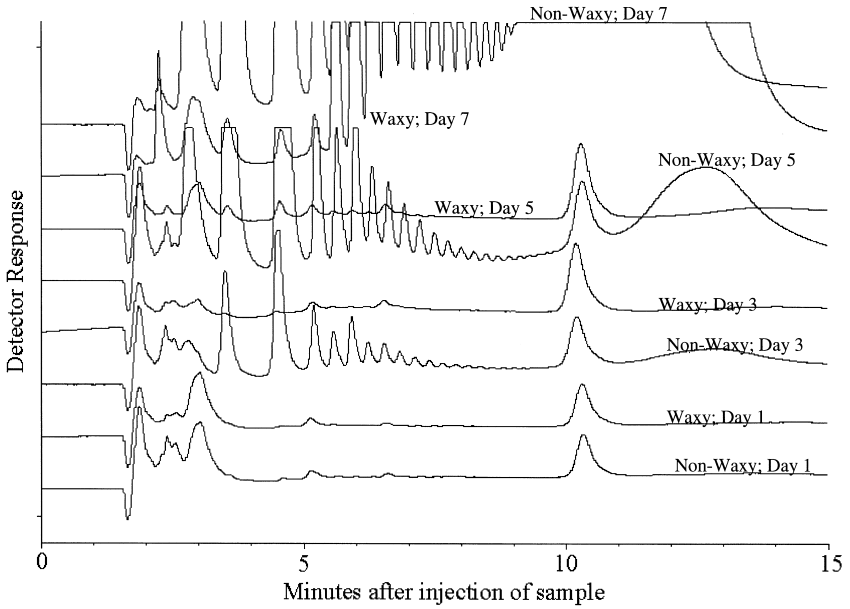

Figure 4. Effect of time of storage, on the detector response of non-debranched freshly prepared non-waxy and waxy starch autoclaved at 121°C for 30 minutes and injected over a period of 7 days.

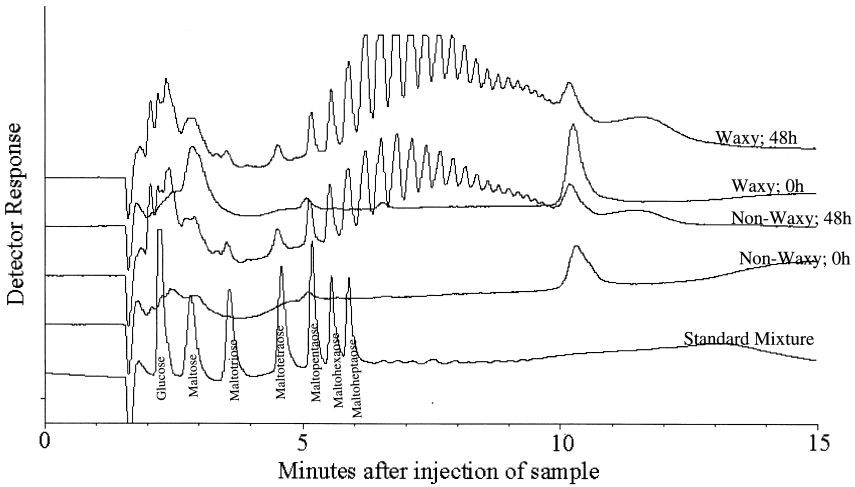

Figure 5. Sample chromatogram comparing freshly cooked non-debranched non-waxy and waxy starch with respective starch debranched for 48h.

samples were diluted 1 to 4 with water. Injections were made onto the HPLC over a twenty-one day period. Results (Fig. 2) indicate that the total area for the samples prepared in NaOH was stable as compared to the samples prepared in HCl and water. The total peak area of control samples prepared in water were only stable for 10 days after which the peak areas increased.

It was then decided to prepare freshly cooked starch samples by adding decreasing HCl concentration to find the concentration at which there was minimal hydrolysis of starch. Two mg (db) of 10 % freshly cooked non-waxy and waxy starch were mixed with 10 mL of various concentrations of HCL (0.009, 0.0046, 0.0023, 0.0012, and 0.0375 M HCl) and autoclaved at 121°C for 30 minutes. Samples were cooled and diluted 1:99 for waxy and 3:37 for regular starch with milli-Q water, then injected into the HPLC. Figure 3 shows that as the concentration of HCl increased, starch hydrolysis increased. At 0.0375 M HCl concentration, all the high molecular weight peaks disappeared, showing that complete hydrolysis had taken place.

Preparation of starch samples in NaOH gave the best results as far as stability of the starch is concerned. This is probably due to ionization of the hydoxyl groups on starch, which stabilizes the structure of starch in solution. The increase in concentration of debranched starch prepared in water over time could be due to unstable structure of starch in the presence of water. This instability could lead to continuous relaxation of the starch molecule, which might allow the oxidation or dehydrogenation of a greater number of hydroxyl groups, which would then lead to greater response concentration.

A decision was made to prepare freshly cooked non-waxy rice and waxy rice samples in water and conduct a storage stability study, since we thought that the instability of starch in water could be due to the freeze-drying and rehydration steps. Peak areas increased with time for both non-waxy and waxy (Fig. 4) starches. Therefore, samples cannot be prepared in advance and injected to obtain reliable results. Therefore, for our debranching study, we injected freshly debranched samples immediately after preparation in water to make reliable comparisons for studying debranching.

To determine effects of time on degree of debranching, 2 mg (db) samples withdrawn at 0, 1, 2, 4, 8, 12, 24, and 48 hr intervals during debranching with pullulanase were mixed with 10 mL of water and autoclaved at 121°C for 30 min. Samples were immediately injected into the HPLC. Figure 5 shows a sample chromatogram of a standards (DP 1-7) and, 0 h and 48 h debranched waxy and non-waxy starch samples injected immediately after being withdrawn. Each peak in the chromatogram showed an increasing degree of polymerization of maltooligosaccharide with glucose as the first peak. The AEC-PAD method was successful in determining the effect of time of debranching on the debranching of starch. The total areas under the respective peaks of glucose polymers using AEC-PAD increased with time (Fig. 6). The waxy starch was not completely

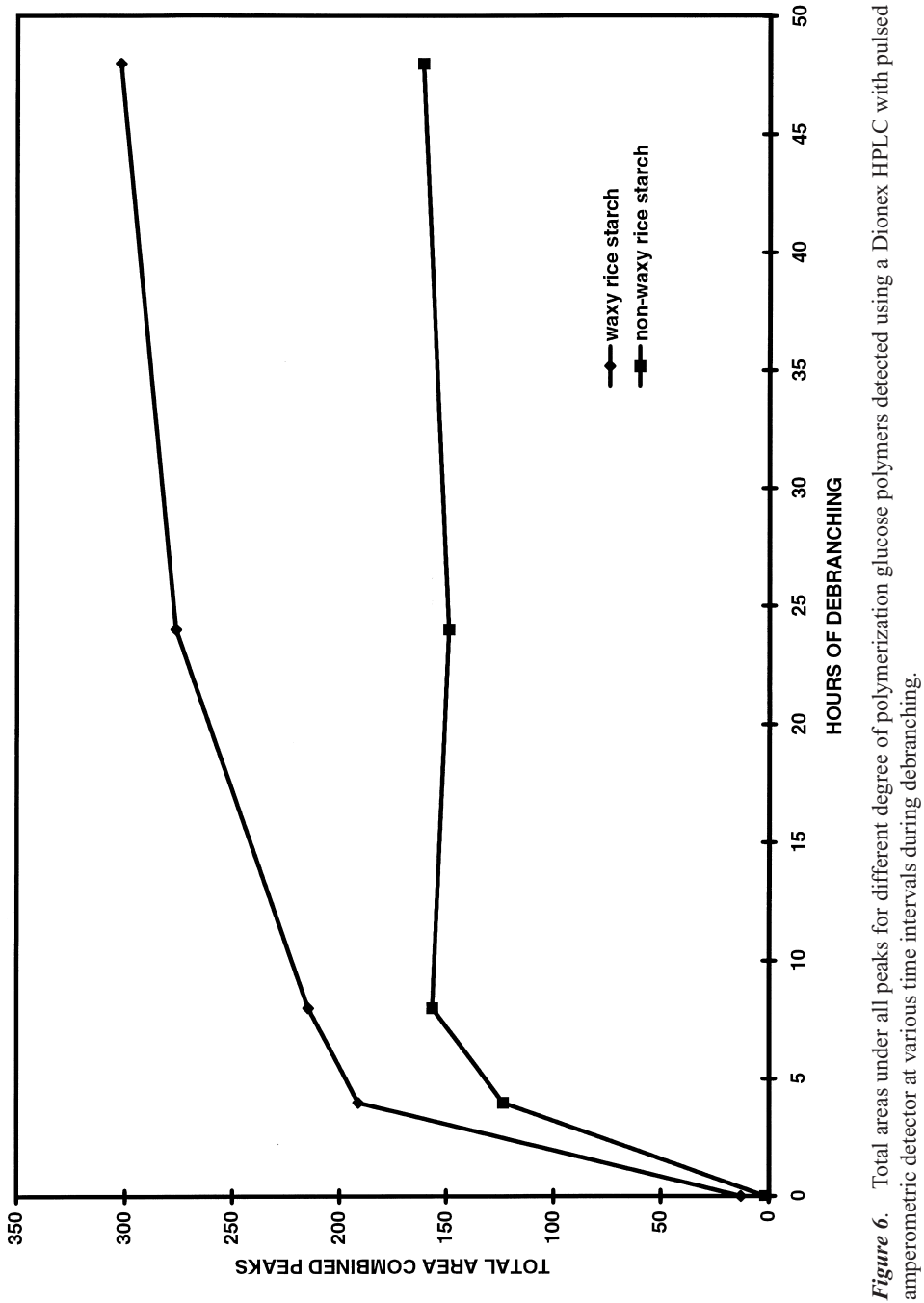

Figure 6. Total areas under all peaks for different degree of polymerization glucose polymers detected using a Dionex HPLC with pulsed amperometric detector at various time intervals during debranching.

debranched, even after 48 h of debranching because of onset of retrogradation. Similar results were reported by Guraya et al. (2000 a,b) using other methods of determining degree of debranching. No increase in total area of non-waxy starch occurred after 8 h of debranching. This would be expected because waxy starch is highly branched as compared to non-waxy starch.

CONCLUSIONS

Debranched starch samples have to be analyzed immediately after preparation in water or prepared in NaOH in order to get reliable results. Storage of starch samples affects its stability and, therefore, no comparisons can be made between same samples prepared in water and injected on different days.

ACKNOWLEDGMENTS

The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

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