Determination of Plant-Derived Neutral Oligo- and Polysaccharides

Jeffrey Rohrer
Thermo Fisher Scientific, Sunnyvale, CA, USA

Application Note 67

Introduction

Since it was first reported that high-performance anion-exchange chromatography (HPAE) could be combined with pulsed amperometric detection (PAD) to separate and detect carbohydrates with high sensitivity and without derivatization,[1] HPAE-PAD has been applied to the determination of many types of carbohydrates in a large variety of samples.[2] Carbohydrates were separated with the Thermo Scientific™ Dionex™ CarboPac™ PA1 Column, a strong anion-exchange column specifically designed to separate mono- and oligosaccharides. In 1989, Koizumi et al. reported that HPAE-PAD could be used to separate linear glucose polymers with degrees of polymerization (DP) as high as 50.3 This method was a significant improvement over size-exclusion chromatography, which could not resolve polymers greater than DP12, and reversed-phase chromatography, which could not determine polymers greater than DP30 (due to the poor solubility of the large carbohydrates in reversedphase eluents). More recently, Hanashiro et al. reported an HPAE-PAD separation of glucose polymers greater than DP80.[4]

The high-resolving power of HPAE and its ability to determine higher DPs than other techniques, allowed Koizumi et al. to use HPAE-PAD to "fingerprint" the distribution of α1,4-linked glucose polymers from six natural sources.[5] This fingerprinting capability, combined with the sensitivity of PAD, allowed the determination of beet sugar adulteration of orange juice[6] and the determination of the geographical origin of honey from its oligosaccharide profile.[7]

Since 1989, numerous publications have featured HPAE-PAD analysis of oligo- and polysaccharides, including amylopectins,[3–5] arabinans,[8] arabinoxylans,[8] fructooligosaccharides,[9] fructans,[10] galactans,[11] glucoarabinoxylans,[12] mannans,[13] xylans,[14] and xyloglucans.[15] These carbohydrate polymers were isolated from a variety of sources. In one study, the authors determined the fructooligosaccharide content of over 80 fruits, vegetables, and grains.[9]

This application note describes how to develop an HPAE-PAD method for separating plant-derived neutral oligo- and polysaccharides. Amylopectin and fructan separations demonstrate column, eluent, and gradient choices for developing rugged HPAE-PAD methods for neutral oligo- and polysaccharides.

Equipment

- Thermo Scientific Dionex BioLC chromatography system consisting of:
  - GP50 Gradient Pump with vacuum degas option
  - ED50 Electrochemical Detector
  - E01 Eluent Organizer
  - AS50 Autosampler with Thermal Compartment
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System, 6 Workstation

Reagents and Standards

- Deionized water, 17.8 MΩ-cm resistivity or better (used for all eluent and standard preparations)
- Sodium hydroxide, 50% (w/w)
- Sodium acetate, anhydrous
- Inulin, chicory root
- Inulin, dahlia tuber
- Maltrin® MO40

Other Consumables

- Syringe filters (0.45 µM PVDF)
- Nylon filter disks (0.2 µM, 47 mm)
- Autosampler vials

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CONDITIONS	
Columns:	Dionex CarboPac PA1 or Dionex CarboPac PA100 Analytical (4 × 250 mm)
	Dionex CarboPac PA1 or Dionex CarboPac PA100 Guard (4 × 50 mm)
Expected Operating	
Pressure:	17.2 MPa (2500 psi)
Degas:	30 s every 10 min
Injection Volume:	10 µL
Injection Loop:	25 µL
Eluents:	A: 100 mM or 150 mM NaOH
	B: 100 mM or 150 mM NaOH/500 mM or 1 M sodium acetate
Flow Rate:	1.0 mL/min
Detection:	Pulsed amperometry, gold working electrode, Ag/AgCl reference

Waveform[16]

Time (sec)	Potential (V)	Integration
0.00	+0.1	
0.20	+0.1	Begin
0.40	+0.1	End
0.41	–2.0	
0.42	–2.0	
0.43	+0.6	
0.44	–0.1	
0.50	–0.1	

Note: This waveform should only be used with a new gold working electrode or gold working electrode that has only been previously used with this waveform.

Collection Rate:	2 Hz
Expected	20–37 nC
Background:	
Temperature:	30 °C
Needle height:	2 mm
Cut Segment	
Volume:	4 µL
Flush Volume:	100 µL
Example* Gradients:	
	Eluent A: 100 mM sodium hydroxide
	Eluent B: 100 mM sodium hydroxide/ 1.0M sodium acetate
Flow Rate:	1.0 mL/min

M040 Separation

Time (min)	%A	%B	Curve
0.0	95	5	6
60.0	55	45	6
61.0	95	5	6

Autosampler Cycle Time: 75 min

Inulin Separation

Time (min)	%A	%B	Curve
0.0	85	15	6
60.0	45	55	6
61.0	85	15	6

Autosampler Cycle Time: 75 min

* Other gradients in this application note are shown in the figures or described in the text.

Preparation of Solutions and Reagents

Sample Preparation

Maltrin M040

Weigh out 25 mg of M040 and add water to prepare a 5.0 mg/mL solution. All work presented in this application note was performed with 5.2 mg/mL M040 solutions.

Inulins

Weigh out 25 mg of inulin and add 100 mM sodium hydroxide, prepared in the same manner as the eluent, to prepare a 5.0 mg/mL solution. All work presented in this application note was performed with 5.0 mg/mL chicory root inulin and 5.5 mg/mL dahlia tuber inulin solutions.

General Advice on Preparing Samples Containing Oligosaccharides for HPAE-PAD

Samples can be dissolved in water or 100 mM sodium hydroxide (reconstitute just prior to analysis). Higher concentrations of sodium hydroxide may be used, but the effect on the chromatography for a given sample, sample concentration, and injection volume must be evaluated. Cloudy samples should be filtered using a 0.45 µM syringe filter. If samples that are cloudy, even after filtration, are injected, they will damage the guard column and clog the injector, injection valve, injection loop, or tubing leading to the guard column. For additional advice on sample considerations for Dionex CarboPac columns, see the Dionex CarboPac column manual (Document #031824).

Eluent Preparation

100 and 150 mM Sodium Hydroxide

It is essential to use high-quality water of high resistivity (17.8 MΩ-cm or better) that contains as little dissolved carbon dioxide as possible. Biological contamination should be absent. Sodium hydroxide eluent should be prepared with 50% (w/w) sodium hydroxide. Sodium hydroxide pellets are covered with a thin layer of sodium carbonate and should not be used under any circumstances. To prepare 2 L of 100 mM NaOH, use a 10 mL graduated plastic pipet to deliver 10.4 mL 50% (w/w) sodium hydroxide into 1 L of water and then add water to reach 2 L. Stir this solution only 1–2 min. Excessive mixing will increase the carbonate ion in the solution by trapping carbon dioxide from the air. After preparation, keep the eluent blanketed under helium at 34–55 kPa (5–8 psi) at all times. If maintained under helium, the eluent is viable for approximately one week. To prepare 2 L of 150 mM NaOH, dilute 15.6 mL 50% (w/w) sodium hydroxide using the same procedure described for the 100 mM eluent.

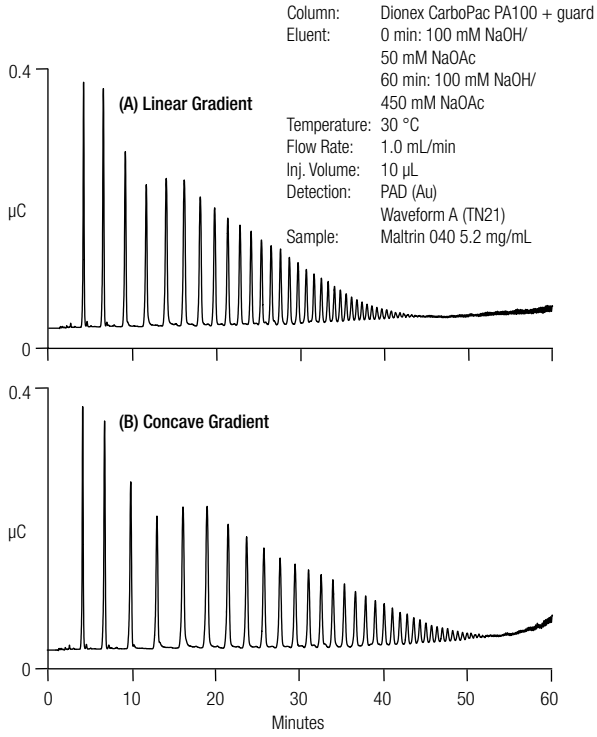

Figure 1. Comparison of a linear and curved gradient for a polysaccharide separation.

100 or 150 mM Sodium Hydroxide with 0.5 or 1.0 M Sodium Acetate

Measure approximately 800 mL of water into a 1 L graduated cylinder. Add a stir bar and begin stirring. Weigh out 41.0 g (0.5 M) or 82.0 g (1 M) of anhydrous crystalline sodium acetate. Steadily add the sodium acetate while briskly stirring the water. After the salt dissolves, remove the stir bar with a magnetic retriever and use a 10 mL graduated plastic pipet to add 5.2 mL (100 mM sodium hydroxide) or 7.8 mL (150 mM sodium hydroxide) 50% (w/w) sodium hydroxide to the sodium acetate solution. Add water to the solution to reach a final volume of 1 L, replace the stir bar, and stir briefly to mix. Vacuum filter this solution through a 0.2 µM nylon filter. This step may proceed slowly if the filter clogs with insoluble impurities from the sodium acetate. After preparation, keep the eluent blanketed under helium at 34–55 kPa (5–8 psi) at all times. If maintained under helium, this eluent is viable for approximately one week.

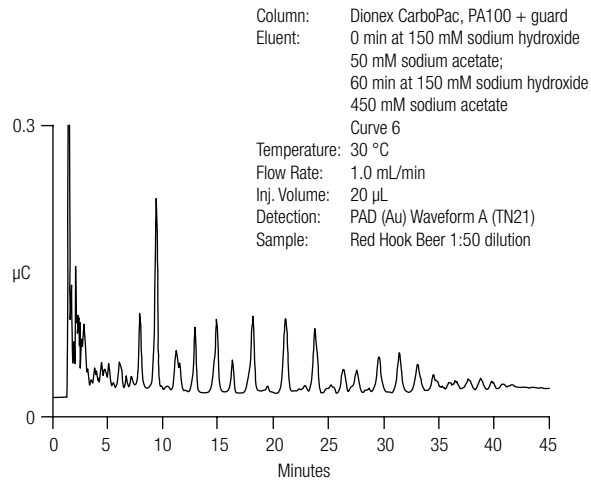

Figure 2. HPAE-PAD analysis of Red Hook amber beer.

Result and Discussion

Gradient Choice

Uncharged oligo- and polysaccharides with little or no branching are generally separated on either a Dionex CarboPac PA1 or Dionex CarboPac PA100 column set using a sodium acetate gradient in an eluent containing either 100 or 150 mM sodium hydroxide. The sodium hydroxide is present to ionize the saccharide hydroxyl groups to oxyanions so that the saccharide binds to the Dionex CarboPac column, a strong anion exchanger. The acetate ion is a stronger eluent than the hydroxide ion and is used to elute the bound saccharide. Figure 1, Panel A shows the separation of Maltrin M040 on a Dionex CarboPac PA100 column using a gradient of 50 to 450 mM sodium acetate in 100 mM sodium hydroxide. Maltrin M040 is a maltodextrin sample prepared from cornstarch. Maltodextrins are nonsweet nutritive polymers of glucose. Maltodextrins can also be classified as amylopectins, glucose polymers in which the glucoses are joined by α1,4 linkages with occasional branches of α1,6 linked glucose. Changing the linear gradient (curve 5) in Figure 1, Panel A to a slightly concave gradient (curve 6), yields the separation in Figure 1, Panel B. Although no additional peaks are identified, the spacing between the later-eluting peaks is improved. This gradient may improve the reproducibility of integration and will make it easier to collect individual peaks for purification or additional analyses. We have found that a slightly concave gradient improves separations of carbohydrate polymers in which the later-eluting peaks are less resolved as the polymer gets larger. Figure 2 shows this method applied to the analysis of beer, which is known to contain amylopectins.

Column Choice

Figure 3 compares the Maltrin M040 separation on a Dionex CarboPac PA100 column set (Panel A) with the same separation on a Dionex CarboPac PA1 column set (Panel B). This figure highlights the resin differences between the Dionex CarboPac PA1 and Dionex Carbo PA100 columns that lead to improved oligo- and polysaccharide separations on the Dionex CarboPac PA100. The Dionex CarboPac PA100 has a smaller latex particle size and increased latex cross-linking compared to the Dionex CarboPac PA1 column.

These differences yield more efficient chromatography and 10% less column capacity (90 meq vs 100 meq). The improved efficiency is shown numerically by comparing the efficiency of peaks from both separations (Table 1). This improved efficiency leads to the identification (same detection parameters used for both chromatograms) of three more peaks on the Dionex CarboPac PA100 column. The shorter retention times in Panel A compared to Panel B demonstrate the lower capacity of the Dionex CarboPac PA100. This reduced capacity causes larger carbohydrate polymers to elute with less sodium acetate. A change in the sodium acetate gradient of 50 to 450 mM to a gradient of 80 to 480 mM, to account for the higher capacity of the Dionex CarboPac PA1 column, did not identify a greater number of peaks (chromatogram not shown).

100 mM Sodium Hydroxide or 150 mM Sodium Hydroxide

In the many HPAE-PAD publications of oligo- and polysaccharide separations, the authors have used either 100 mM sodium hydroxide or 150 mM sodium hydroxide. We compared the Maltrin M040 separation with 100 mM sodium hydroxide (Figure 4, Panel A) with the same separation using 150 mM sodium hydroxide (Figure 4, Panel B). Although the concentration of eluent anions is greater at all points in the separation with the 150 mM sodium hydroxide eluent, only the first peak (not shown) elutes earlier in the 150 mM sodium hydroxide separation. We believe longer retention with the 150 mM sodium hydroxide eluent is due to the greater ionization of sample hydroxyl groups that leads to tighter binding to the Dionex CarboPac column.

This result has been previously observed for separations of branched mammalian oligosaccharides.[17] The 150 mM sodium hydroxide eluent did not identify any more peaks in the Maltrin M040 sample compared to the 100 mM eluent. Using 150 mM rather than 100 mM, sodium hydroxide may be beneficial for the separation of some samples due to improved solubility of some larger carbohydrate polymers.

Table 1. Comparison of peak efficiencies.

Peak #	Dionex CarboPac PA100	Dionex CarboPac PA1
4	17087	9378
8	39445	24417
12	66978	45667

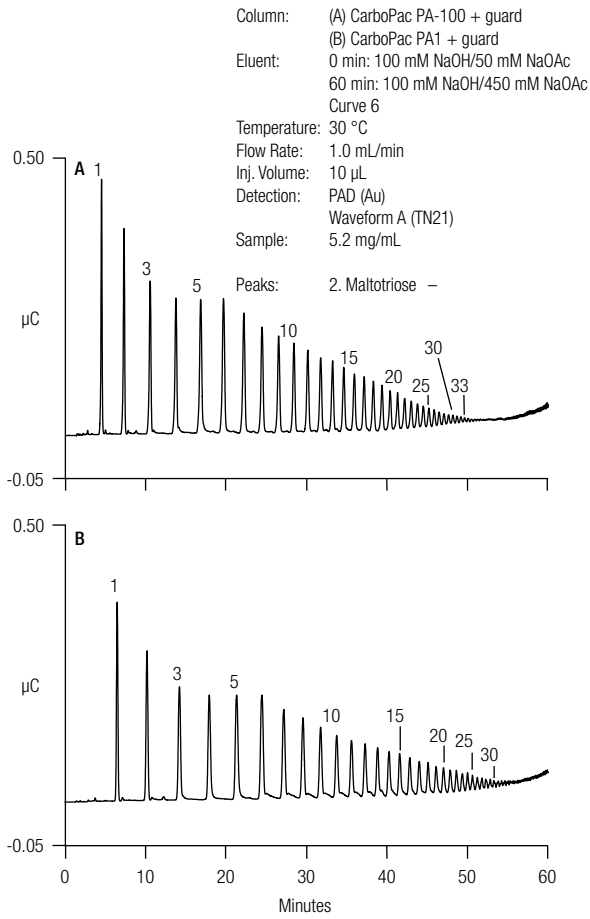

Figure 3. Comparison of the Dionex CarboPac PA1 and Dionex CarboPac PA100 columns for a polysaccharide separation.

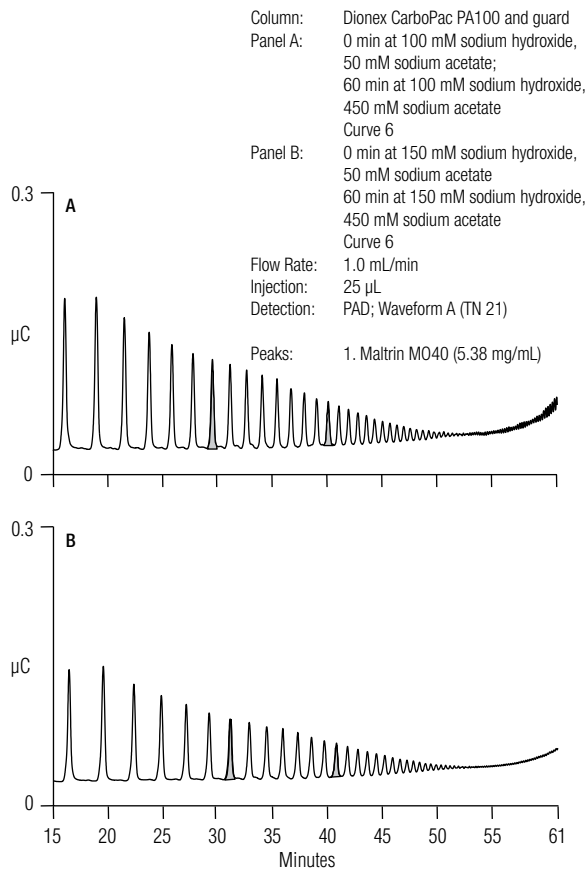

Figure 4. Maltodextrin separation with 100 mM and 150 mM NaOH.

Inulin Analysis

We used what we learned developing amylopectin separation to develop inulin separations. Inulins are fructans that are linear β2-1 linked polymers of fructose as large as DP60 with a terminal glucose. Figure 5 shows a comparison of inulin from chicory root (Panel A) and dahlia tuber (Panel B). The inulins are separated on a Dionex CarboPac PA100 column using a 100 mM sodium hydroxide eluent and a curved gradient (Curve 6) of 150 to 550 mM sodium acetate. Figure 5 demonstrates the "fingerprinting" ability of the HPAE-PAD technique, as the inulins isolated from different sources have different polymer distributions. HPAE-PAD can also be used to compare different isolations or lots of an oligo- or polysaccharide isolated from the same source. Figure 6 shows a comparison of two lots of chicory root inulin purchased over ten years apart. Comparison of the peak areas (Table 2) reveals that the older lot has a greater percentage of larger polymers.

Developing Rugged Assays of Oligo- and Polysaccharides

To develop rugged HPAE-PAD assays of oligo- and polysaccharides, the analyst should optimize the column reequilibration, temperature, nature of the sample, amount of sample injected, choice of electrochemical waveform, and autosampler injection mode.

For good retention time reproducibility, the column must be equilibrated to the starting gradient conditions prior to each injection, and the reequilibration period should be tightly controlled. In all separations shown in this application note, the column set was reequilibrated at initial conditions for 15 min (15 mL), with the first minute used to return from the final gradient condition to the starting condition, prior to the next injection. We controlled the reequilibration time by setting the Thermo Scientific Dionex AS50 Autosampler cycle time to 15 min (flow rate was 1 mL/min) after the separation's final gradient time point.Controlling the column temperature by placing the column set in the Thermo Scientific Dionex AS50 Autosampler Thermal Compartment (TC) also increases retention time reproducibility.

Table 2. Comparison of two lots of chicory inulin.

Peak Number	Peak Height Ratio (old lot/new lot)
3	0.76
6	0.89
10	0.97
12	0.93
14	0.92
18	0.96
23	0.99
27	1.00
31	1.08
35	1.13

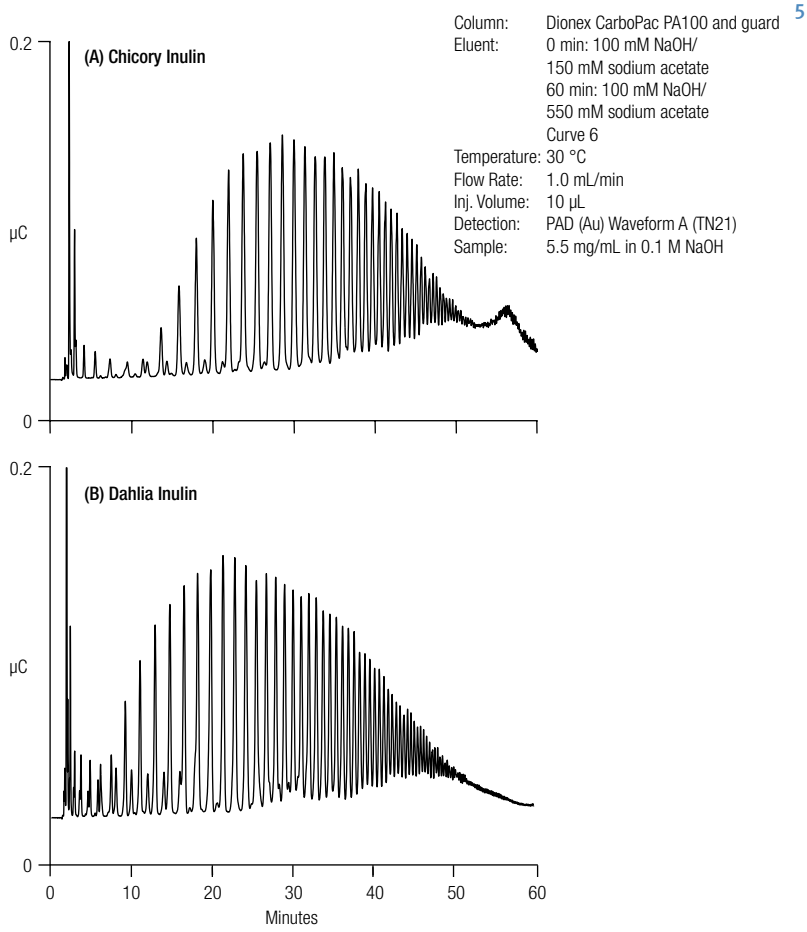

Figure 5. HPAE-PAD comparison of chicory and dahlia inulins.

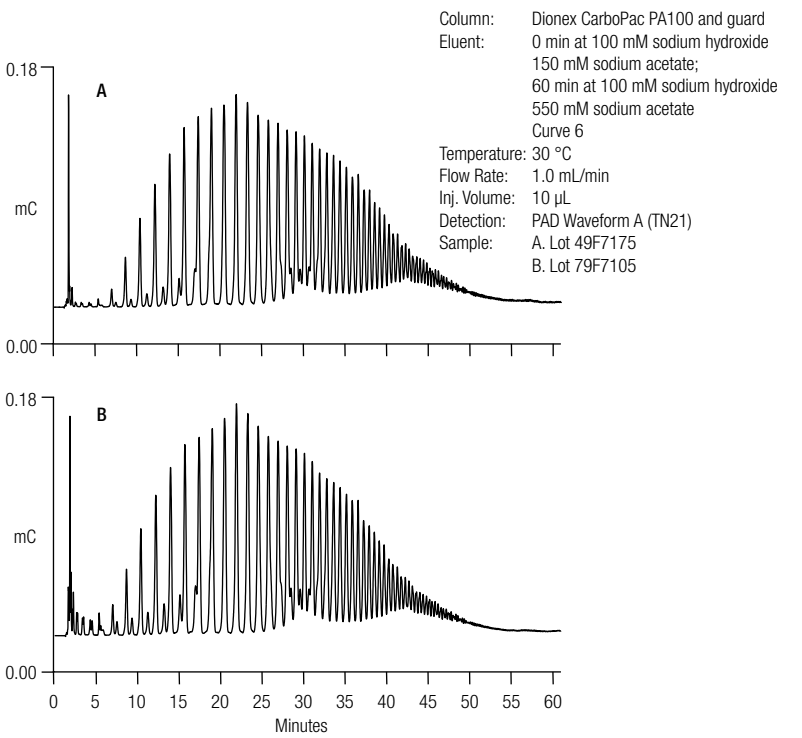

Figure 6. Comparison of two lots of chicory inulin.

The nature of the sample(s) must also be considered. Many oligo- and polysaccharide samples are prepared by the acid or enzymatic hydrolysis of a larger polymer. For example, amylose and amylopectins are prepared by hydrolyzing starch. Samples prepared in this manner can still contain small amounts of very high-molecular-weight material that will not elute from the column during a method designed to elute all the major peaks. After multiple injections, the loss of column capacity due to these large polymers will cause the retention times of later-eluting peaks to shorten. To demonstrate this retention time loss, we made 32 consecutive separations of Maltrin M040 using the method in Figure 1A. Figure 7 compares the first injection to the last injection. The retention times are the same for the first 18 peaks, but are shorter for all the later-eluting peaks in the 32nd injection. Figure 8 shows that this effect is a result of tightly bound sample that is not eluted by the separation method. The lower chromatogram is a blank injection (10 µL water) made prior to the 32 Maltrin M040 injections, and the upper chromatogram is a blank injection made immediately after the 32 Maltrin M040 injections. The separation method used for this comparison was the same as that used for the 32 Maltrin M040 injections, except that the column was washed for 10 min with 100 mM sodium hydroxide 1 M sodium acetate after the final gradient point. This comparison shows that there were sample components that did not elute from the column during the 32 injections. Although we could eliminate this problem by washing the column after each injection, extra analysis time would be required. The amount of time between column cleanings can be determined based on which peaks one is interested in measuring (i.e., if one wished to quantify any or all of the first 18 peaks of this separation, a column cleaning would only be needed every 32 injections). If, in this separation, only the first 20 peaks are of interest, the injection amount could be reduced, which would further increase the times between column cleanings. To determine if the Dionex CarboPac column needs to be cleaned, inject a known sample, such as the chicory root inulin shown in Figure 5, Panel A. A standard of chicory root inulin aliquoted into a number of small vials and stored in a freezer could be used as a system suitability standard to judge retention, response, and overall performance of the chromatography system.

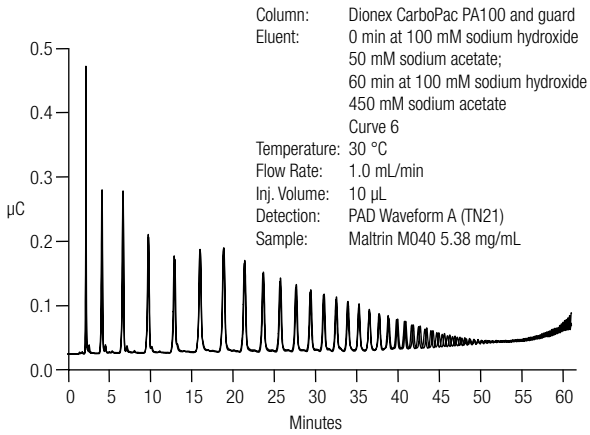

Figure 7. Comparison of maltodextrin separations 1 and 32.

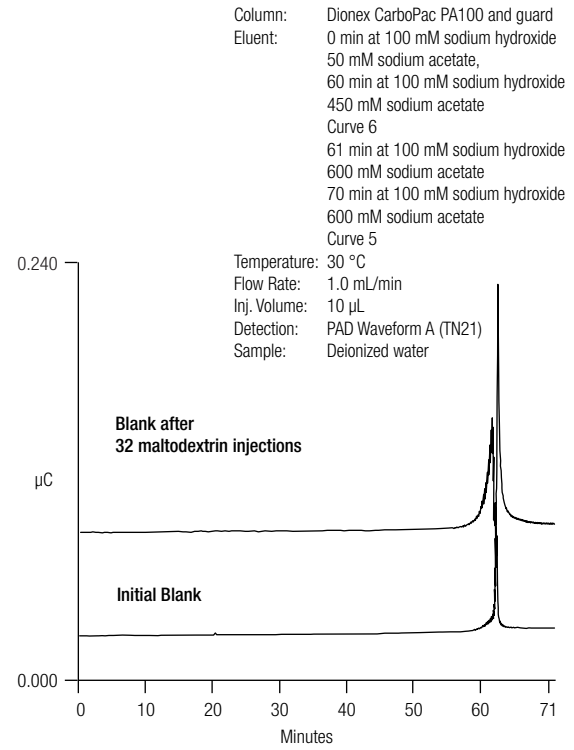

Figure 8. Blank injections (with wash) before and after 32 maltodextrin separations.

Another possible cause of reduced retention times, especially for later-eluting peaks, is an injection of too much sample. Figure 9 shows the effect of injecting increasing amounts of Maltrin M040 on the Dionex CarboPac PA100 column set. Note the flattened peak shapes and reduced retention times of the later-eluting peaks. Sometimes, sample is intentionally overloaded to collect larger amounts of a given peak or peaks for a separate analysis. Alternatively, the Dionex CarboPac PA100 is available in a 9 × 250 mm format or custom packed in a 22 × 250 mm column body. When collecting fractions from a 4 × 250 mm Dionex CarboPac column, the Thermo Scientific Dionex CMD Carbohydrate Membrane Desalter can be used to convert the sodium hydroxide and sodium acetate eluent into dilute acetic acid that can be removed by vacuum drying.[18] The Dionex CMD device can remove up to 350 mM Na+ at 1.0 mL/min. Fractions collected from larger Dionex CarboPac columns can be neutralized with acetic acid and dialyzed against water using 1000-dalton mw-cutoff dialysis tubing.

To obtain the best reproducibility of electrochemical response, use Waveform A16 (the same as used here), control the temperature of the electrochemical cell, use a properly functioning autosampler, and choose an autosampler injection mode that balances the reproducibility desired with the amount of sample you wish to expend (the best reproducibility requires using more sample). Waveform A provides better long-term reproducibility than the previously recommended waveforms[16] with sodium acetate-containing eluents.[19] To control temperature, the electrochemical cell can be installed inside the Dionex AS50 TC, along with the column set.

The precision and accuracy of Dionex AS50 autosampler injections, and therefore peak area reproducibility, depends on the mode of injection. The most accurate and precise injections are made in full-loop injection mode using a calibrated sample loop. In this mode, the Dionex AS50 autosampler draws four times the loop volume from the sample vial and delivers it to the injection valve. The middle portion of the sample is positioned in the loop and injected. For loop sizes less than 17 µL, 2.5 times the loop volume plus 25 µL is delivered to the valve. Peak area

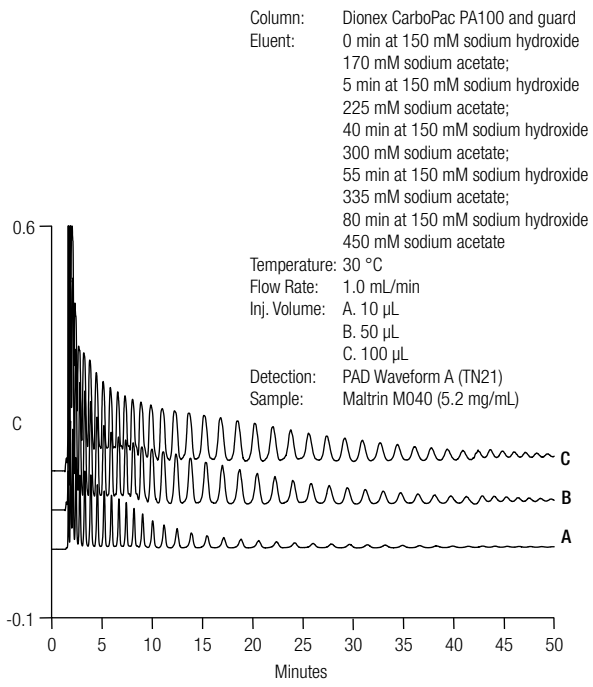

Figure 9. Effect of sample overload on oligosaccharide chromatography.

precision for full-loop injections of 25 µL is typically 0.3% RSD. To conserve sample, use either the partial-loop or the partial-loop, limited-sample (Partial LS) injection mode.

In the partial-loop mode, the Dionex AS50 autosampler draws the volume to be injected from the sample vial, plus two times the cut segment volume. (The cut segment volume is a portion of the sample discarded from each end of the aspirated sample to improve accuracy.) The middle portion of the sample is positioned in the loop and injected. For best precision and accuracy when using one of the partial-loop injection modes, install a sample loop that is at least two times the injected volume. Peak area precision for partial-loop injections of 10 µL is typically 1% RSD. A 10 µL partial-loop injection with a cut volume of 10 µL uses 30 µL of sample. The Dionex AS50 autosampler in Partial LS mode wastes no sample and usually provides precision of 1–2% RSD. See the Dionex AS50 autosampler reference manual for a complete discussion of the different injection modes.

Table 3. Electrochemical response of amylose oligosaccharides.

DP	# HCOH	Relative Response[a]	Relative Response[b]	Relative Response per HCOH[a]	Relative Response per HCOH[b]	Response per µmole[c]	Response per µg[c]
2	8	1.00	–	–	1.00	17.8	51.9
3	11	1.39	–	–	1.01	22.3	44.2
4	14	1.72	–	–	0.98	29.0	43.6
5	17	2.06	–	–	0.97	29.8	35.9
6	20	2.33	0.74	1.08	0.93	34.5	34.8
7	23	2.59	0.82	1.03	0.90	35.7	31.0
8	26	–	0.89	0.99	–	–	–
9	29	–	1.00	1.00	–	–	–
10	32	–	1.10	1.00	–	–	–
11	35	–	1.20	1.00	–	–	–
12*	38	–	1.31	1.00	–	38.8	20.6
13*	41	–	1.38	0.99	–	40.0	19.6
14*	44	–	1.46	0.97	–	50.9	21.5
15	47	–	1.55	0.96	–	–	–
16*	50	–	1.59	0.92	–	46.0	18.8
17*	53	–	1.65	0.90	–	52.2	18.3
18–19	–	–	–	–	–	44.6	14.8
19–21	–	–	–	–	–	44.3	13.6
20–22	–	–	–	–	–	54.4	15.9

Electrical Response of Oligo- and Polysaccharides

Although not investigated in this application note, a few publications have studied the relative electrochemical responses of oligo- and polysaccharides.[3,5,20,21] These publications investigated the change in electrochemical response as amylose and amylopectin DP increased. Koizumi et al.[3,5] showed that response increased with DP. This change could be normalized to an increase per hydroxyl group (Table 3). Consistent with those observations, Ammeraal, et al.[20] showed that as DP increased, there was a linear increase in response per micromole injected, and the response per microgram injected decreased. A more recent publication confirmed these observations.[21]

Other Considerations

For analysts who wish to consume less eluent and produce less waste per injection, the Dionex CarboPac columns are available in 2 × 250 mm formats. The use of sodium nitrate in place of sodium acetate has been reported for amylopectin separations.[22] The authors reported that nitrate, a stronger eluent that acetate, affected better separations between linear and branched, and was able to distinguish a higher DP for amylopectin (DP58 for acetate and DP66 for nitrate). Dionex has not evaluated the long-term effects of using sodium nitrate eluents for HPAE-PAD.

Precautions and Recommendations

For best results, the Ag/AgCl reference electrode should be replaced every three to six months.

Appendix

Because it is not entirely accurate to compare two gradient separations by using theoretical plates (efficiencies), in this appendix we use the peak capacity method reported by Giddings to compare the maltodextrin separations on the Dionex CarboPac PA1 and Dionex CarboPac PA100 columns.[23]

To calculate the peak capacity of a gradient separation, the following formula is used:

$$\text{Peak Capacity} = 1.5 \times \text{Run Time} \times (1/\text{Average Peak Width})$$

The 1.5 is the resolution factor for baseline resolution. Using this formula, we calculated a value of 191 for the Dionex CarboPac PA1 column and 209 for the Dionex CarboPac PA100 column. This 10% increase matches that seen for the number of peaks identified using the same integration parameters in the software (30 peaks for Dionex CarboPac PA1 column and 33 for the Dionex CarboPac PA100 column).

References

1. Rocklin, R. D.; Pohl, C. A. *J. Liq. Chromatogr.* **1983**, *6*, 1577-1590.
2. Lee, Y. C. *J. Chromatogr. A* **1996**, *720*, 137-149.
3. Koizumi, K.; Kubota, Y.; Tanimoto, T.; Okada, Y. *J. of Chromatogr.* 1989, 464, 365-373.
4. Hanashiro, I.; Abe, J.; Hizukuri, S. *Carbo. Res.* **1996**, *283,* 151-159.
5. Koizumi, K.; Fukuda, M.; and Hizukuri, *S. J. of Chromatogr.* **1991**, *585*, 233-238.
6. Thermo Fisher Scientific. Application Note 82; Sunnyvale, CA.
7. Swallow, K.W.; Low, N.H. *J. Agric. Food Chem.* **1990**, *38,* 828-832.
8. van der Hoeven, R. A. M.; Niessen, W. M. A.; Schols, H. A.; Bruggink, C.; Voragen, A. G. J.; van der Greef, J. *J. Chromatogr.* **1992**, *627*, 63-73.
9. Campbell, J. M.; Bauer, L.L.; Fahey, G.C., Jr.; Hogarth, A. J. C. L.; Wolf, B.W.; Hunter, D. E. *J. Agric. Food Chem.* **1997**, *45*, 3076-3082.
10. Chatterton, N. J.; Harrison, P. A. *New Phytol.* **1997**, *136*, 3-10.
11. Schols, H. A.; Lucas-Lokhorst, G.; Voragen, A. G. J.; Niessen, W. M. A. *Carbohydrates in the Netherlands* **1993**, *9*, 11-13.
12. Verbruggen, M. A.; Spronk, B. A.; Schols, H. A.; Beldman, G.; Voragen, A. G. J.; Thomas, J. R.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbo. Res.* **1998**, *306*, 265-274.
13. Torto, N.; Buttler, T.; Gorton, L.; Marko-Varga, G.; Stalbrand, H.; Tjerneld, F. *Anal. Chim. Acta* **1995**, *313*, 15-24.
14. Mou, S.; Sun, Q.; Lu, D. *J. Chromatogr.* **1991**, *546*, 289-295.
15. McDougall, G. J.; Fry, S. C. *Carbo. Res.* **1991**, *219*, 123-132.
16. Thermo Fisher Scientific. Technical Note 21; Sunnyvale, CA.
17. Cooper, G. A.; Rohrer, J. S. *Anal. Biochem.* **1995**, *226*, 182-184.
18. Thayer, J.; Rohrer, J. S.; Avdalovic, N.; Gearing, R. P. *Anal. Biochem.* **1998**, *256*, 207-216.
19. Rocklin, R. D.; Clarke, A. P.; Weitzhandler, M. *Anal. Chem.* **1998**, *70*, 1496-1501.
20. Ammeraal, R. N.; Delgado, G. A.; Tenbarge, F. L.; Friedman, R. B. *Carbo. Res.* **1991**, *215*, 179-192.
21. Koch, K.; Andersson, R.; Aman, P. *J. of Chromatogr. A* **1998**, *800*, 199-206.
22. Wong, K. S.; Jane, J. *J. Liq. Chromatogr.* **1995**, *18*, 63-80.
23. Giddings, J. C. *United Separation Science*; John Wiley and Sons: New York, 1991, p 105.

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