APPLICATION NOTE 73986

Determination of trans-galactooligosaccharides in foods using HPAE-PAD in dual eluent generation cartridge mode

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Goal

To demonstrate that AOAC Method 2001.02 for Trans-Galactooligosaccharides (TGOS) determination in foods can be executed with a Thermo Scientific™ Dionex™ CarboPac™ PA1-1mm column using KOH/KMSA eluent produced electrolytically using Dual Eluent Generation Cartridge (Dual EGC) mode

Introduction

Trans-galactooligosaccharides (TGOS) are soluble galactans that can be classified as dietary fiber because they pass through the small intestine intact but are fermented in the colon by the intestinal flora. TGOS are dietary prebiotics that can improve health by modulating the intestinal microbiota and the immune system. These galactans are di- to octasaccharides composed of 1–7 galactose units linked to a glucose molecule at the reducing end. A high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) method was developed to measure TGOS in food and feed products.[1] The method was approved as AOAC Method 2001.02.[2]

In AOAC Method 2001.02, a buffered extract of the sample containing TGOS is treated with an enzyme, β-galactosidase, that hydrolyzes TGOS to galactose and glucose. Galactose and other sugars are separated on a Dionex CarboPac PA1-4mm column and detected by pulsed amperometric detection (PAD) using a triple potential Waveform (Waveform B, Thermo Scientific Technical Note 21[3]). A quadruple potential waveform (Waveform A, Technical Note 21) provides greater long-

term reproducibility of peak area response compared to Waveform B. Thermo Scientific Application Note 155 showed that Waveform A produced results equivalent to those obtained with Waveform B. Using Waveform A instead of B for Method 2001.02 yields equivalent results and greater long-term peak area reproducibility.[4]

In this application note, AOAC Method 2001.02 was evaluated with a Dionex CarboPac PA1 column (250 mm × 1 mm) using HPAE-PAD in dual eluent generation cartridge (Dual EGC) mode. The 1 mm column requires a flow rate about 16 times less than the 4 mm column, significantly reducing eluent consumption. Dual EGC mode operation avoids the manual preparation of the sodium hydroxide/sodium acetate eluents. This mode uses a methanesulfonic acid (MSA) eluent generation cartridge (EGC) and a potassium hydroxide (KOH) EGC in series to generate an extremely reproducible and accurate KOH/KMSA eluent gradient needed for separating complex carbohydrates. Key performance parameters were evaluated including separation, linearity, and precision. Three samples were analyzed for their TGOS content.

Experimental

Equipment

- Thermo Scientific™ Dionex™ ICS-6000 HPIC system including:
  - Thermo Scientific™ Dionex™ ICS-6000 DP Pump module or SP Pump module
  - Thermo Scientific™ Dionex™ ICS-6000 EG Eluent Generator module
  - Thermo Scientific™ Dionex™ ICS-6000 DC Detector/Chromatography module with ED Electrochemical Detector
  - Thermo Scientific™ Dionex™ AS-AP Autosampler with sample tray cooling (P/N 074926)
  - 4-port valve rebuild kit (P/N 074699), which includes a 0.4 µL injection loop
  - Thermo Scientific™ Dionex™ Dual EG Eluent degasser (P/N 22181-60951)
- Thermo Scientific™ Dionex™ ICS-6000 ED Electrochemical Detector Cell (P/N 072044)
- Gold on PTFE Disposable working electrode including four 2 mil gaskets (P/N 066480)

Note: The 2 mil gaskets are not used in this application.

- Working electrode gasket, 1 mil (P/N 072161)
- Reference electrode, pH, Ag/AgCl (P/N 061879)
- Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) software, version 7.2.9

Assemble the cell following the Dionex ICS-6000 operator's manual [5] and Dionex ED User's Compendium for Electrochemical Detection.[6]

Consumables

- Thermo Scientific™ Nalgene™ Syringe Filter, PES membrane, 0.2 µm (P/N 725-2520)
- Thermo Scientific™ Nalgene™ Rapid-Flow 0.2 µm filter units, 1000 mL, nylon membrane, 90 mm diameter (P/N 164-0020)
- Microcentrifuge tube, 50 mL (Fisher Scientific P/N 06-443-18)
- Nitrogen, ultrahigh purity grade from Airgas
- Thermo Scientific™ Dionex™ EGC 400 KOH Potassium Hydroxide Eluent Generator Cartridge (P/N 302766)
- Thermo Scientific™ Dionex™ EGC 400 MSA Methanesulfonic Acid Eluent Generator Cartridge (P/N 302767)

Reagents and standards

- Deionized (DI) water, Type I reagent grade, 18 MΩ·cm resistivity or better
- D (+) Galactose (Sigma-Aldrich P/N G0625)
- D-Lactose monohydrate (Sigma-Aldrich P/N 61339)
- Glucose (Sigma-Aldrich P/N G8270)
- Fructose (Sigma-Aldrich, P/N F2543)
- Sucrose (Sigma-Aldrich P/N S7903)
- β-Galactosidase (4000 U/mL) (Megazyme, P/N E-BGLAN)
- Acetonitrile, HPLC grade (Fisher Scientific P/N A998-1)
- Sodium hydroxide 50% (w/w) (Fisher Scientific P/N SS254-500)
- Hydrochloric acid, 37% (Fisher Scientific P/N A142-212)

Samples

Three samples were purchased for this study. Sample #1 is a Bimuno GOS powder purchased from www.bimuno.com. Sample #2 are Bimuno Prebiotic Food Supplement Sachets. Sample #3 is a prebiotic GOS dietary supplement caplet from GNC.

Chromatographic conditions

Parameter	Value
Columns	Dionex CarboPac PA1 Guard, 1 × 50 mm (P/N 303273) Dionex CarboPac PA1 Separation, 1 × 250 mm (P/N 303272)
Eluent	Gradient (Table 1)
Flow rate	0.063 mL/min
Column temperature	25 °C
Injection volume	0.4 µL
Autosampler temperature	5 °C
Reference electrode	Ag/AgCl
Working electrode	Gold disposable electrode, with a 1 mil (25.4 µm) gasket
Detection	Pulsed Amperometric Detector (Electrochemical Detection)
Detection compartment temperature	25 °C
Detection waveform	Gold, Carbohydrates, 4-Potential (Table 2)
System backpressure	~4300 psi (100 psi = 0.6894 MPa)
Run time	60 min

Table 1. Eluent gradient

Time (min)	KMSA (mM)	KOH (mM)
0	1	18
10	1	18
18	1	100
18.1	100	100
30	100	100
30.1	1	18
60	1	18

Table 2. Carbohydrates, 4-potential waveform

Time (s)	Potential (V) vs. Ag/AgCl	Integration
0	0.1	Off
0.2	0.1	On
0.4	0.1	Off
0.41	-2.0	Off
0.42	-2.0	Off
0.43	0.6	Off
0.44	-0.1	Off
0.5	-0.1	Off

System installation and precautions

Install the system according to Figure 1. An operational vacuum degasser pump (part of the analytical pump) is important to the success of this and other electrochemical detection methods. This pump can be accidentally turned off during a system restart or instrument configuration. If the vacuum degasser pump is not running, poor baseline performance and loss of column capacity can be observed. Always ensure that the vacuum degas pump is on before running a sequence. To do that, Press F8 or click the "gear" command button on the main Chromeleon instrument panel and locate the pump and then the degasser status. Choose "Expert" mode from the three modes available: "Normal, Advanced, Expert." Make sure the degasser is "On" and "DegasserVaccum" is "OK". If it is "OFF" and "NOT OK", turn on the degasser by selecting "On" from the drop-down menu and wait until "DegasserVacuum" turns to "OK". If the value reads NOT OK, check for leaks at all the connections necessary for vacuum. Install PEEK backpressure tubing (Item # 22181-20031) as needed to achieve an instrument pressure value above 3,000 psi. To ensure the best system performance, only turn on the EGC power when the system pressure exceeds 3,000 psi. Fill a 2 L eluent bottle with degassed DI water. Connect the eluent bottle to the pump and keep the eluent blanket under an inert gas (helium or nitrogen) at 5-8 psi, to minimize absorption of atmospheric carbon dioxide. Turn on the pump and pump DI water through the Dionex EGC 400 MSA cartridge for 15 min at a flow rate of 1 mL/min. Then condition the Dionex EGC 400 MSA cartridge for 30 min using 100 mM MSA at a flow rate of 0.1 mL/min. Connect the Dionex EGC 400 KOH and pump DI water through the Dionex EGC 400 KOH cartridge for 15 min at a flow rate of 1 mL/min. Then condition the Dionex EGC 400 KOH cartridge for 30 min using 100 mM KOH at a flow rate of 0.1 mL/min. After conditioning the EGCs, install the Dionex CarboPac PA1-1mm column set, set KMSA and KOH to the desired concentrations for the application, and keep the flow on at 0.063 mL/min for 60 min.

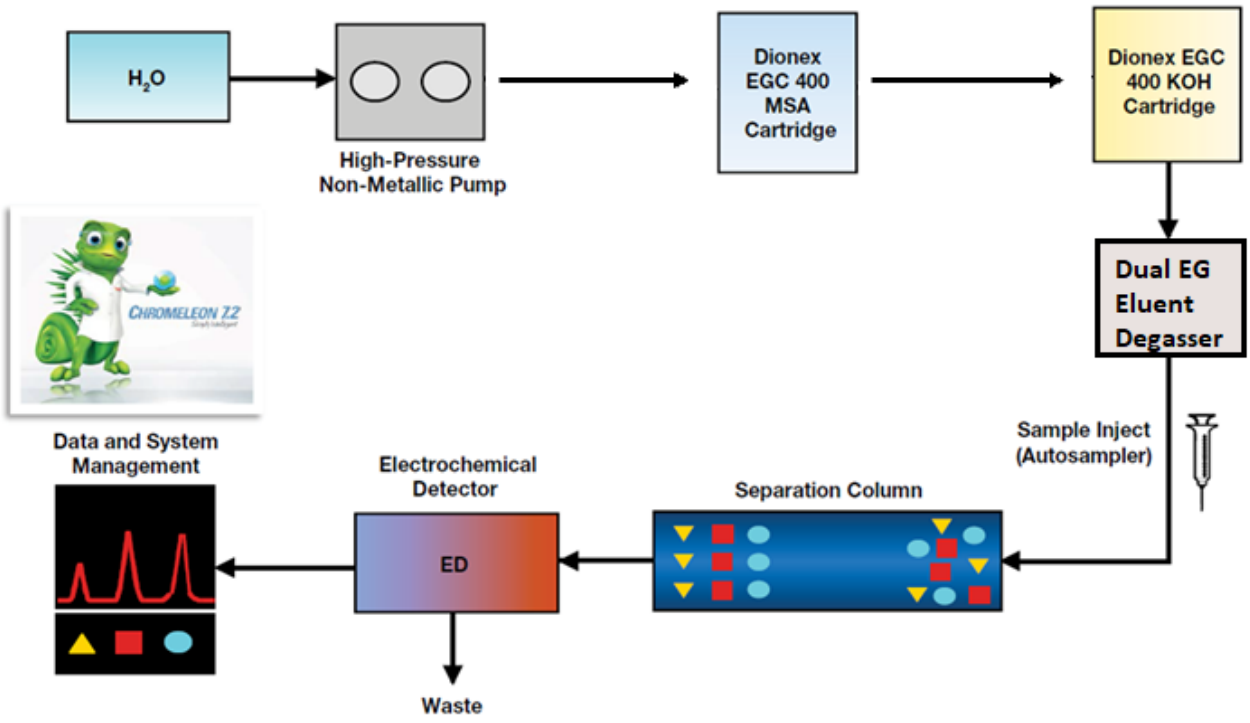

Figure 1. Dionex ICS-6000 HPIC system in Dual EGC mode

Preparation of solutions and reagents

Phosphate buffer, 0.2 M, pH 6.0

Add 22.0 g of potassium dihydrogen phosphate and 4.58 g of dipotassium hydrogen phosphate to a 1 L volumetric flask containing about 500 mL of DI water. Mix to dissolve and bring to volume with DI water. Filter through a 0.22 µm filter before use. Store at 4 °C and use within 8 h.

Sodium hydroxide solution, 1 M

Dilute 5.4 mL (or 8.32 g) of 50% (w/w) sodium hydroxide to 100 mL with degassed DI water. Use this solution to adjust the pH of the sample extract.

Hydrochloric acid, 1 M

Dilute 0.83 mL of HCl to 100 mL with DI water. Use this acid to adjust the pH of the sample extract.

Buffered enzyme mix

Dilute β-galactosidase with phosphate buffer to obtain a final activity of 2,000 U/mL. Store the enzyme suspension at 4 °C and use within 8 h. Stir well before using.

Standards

Stock standard solutions

Prepare the galactose stock standard (800 mg/L) by weighing 0.080 g of anhydrous galactose into a 100 mL volumetric flask. Add DI water to dissolve, bring to volume with DI water, and mix.

Prepare the lactose stock standard (1,425 mg/L as lactose) by weighing 0.150 g of lactose monohydrate into a 100 mL volumetric flask. Add DI water to dissolve, bring to volume with DI water, and mix.

Working standard solutions

Prepare seven working standards by diluting the stock standards with DI water (Table 3). For example, dilute 500 µL each of the galactose and lactose stock standards to 100 mL in a volumetric flask to yield Standard 2 containing 4 µg/mL galactose and 7.125 µg/mL lactose. Four working standards (Std 2–Std 5) were required for AOAC Method 2001.02. To cover wide galactose and lactose concentration ranges in tested samples, the calibration of galactose and lactose was investigated in a wider ranger (Std1–Std 7). Each calibration reference solution was measured in triplicate.

Table 3. Calibration standards

Standard	Stock concentration (mg/L)	L1 (mg/L)	L2 (mg/L)	L3 (mg/L)	L4 (mg/L)	L5 (mg/L)	L6 (mg/L)	L7 (mg/L)
Galactose	800	0.8	4	8	12	16	40	80
Lactose	1425	1.425	7.125	14.25	21.375	28.5	71.25	142.5

Sample preparation

This section briefly summarizes the sample preparation procedure outlined in AOAC Method 2001.02. For details, consult the method.[2]

Extraction

Homogenize liquid samples and pulverize solid samples before weighing a portion for analysis. If the approximate amounts of TGOS and lactose in the sample are known, choose a sample weight corresponding to about 0.1–0.3 g of total TGOS and lactose, but do not exceed 10 g. This study used 1–1.5 g of samples. Weigh a 50 mL centrifuge tube (with screw cap) and record the weight. Tare the balance, add the sample, cap, and record the weight of the sample. Add 40 mL of hot (80 °C) phosphate buffer. Cap the container, mix, and place in the 80 °C water bath for 30 min. Cool to room temperature in an ice bath. Adjust the pH to 5.7–6.3 with 1 M NaOH or 1 M HCl. Dilute the extract to 50 mL with phosphate buffer. Weigh the capped vial with the solution; subtract the net weight of the sample and the weight of the empty tube to determine the weight of sample extract.

Enzymatic hydrolysis

For each food sample, prepare two separate extracts. Use Assay 1 (below) on the first extract to determine the initial concentrations of lactose and galactose. β-galactosidase is added to "matrix match" this extract, but the enzyme is deactivated to halt hydrolysis of TGOS and lactose. Use Assay 2 on the second extract to determine the final concentration of galactose after treating with active β-galactosidase to hydrolyze TGOS and lactose to galactose.

Assay 1

Weigh a 50 mL centrifuge tube (with screw cap) and record the weight. Add 1.0 mL buffered β-galactosidase suspension plus 1.0 mL phosphate buffer. Deactivate the enzyme by placing the tube into the water bath at 100 °C for 10 min. Cool to room temperature. Tare the balance, add 20 g of the sample extract, cap, and record the weight of the treated extract. Gently vortex and place into a water bath at 60 °C. Monitor the temperature. After the samples reach 60 °C, incubate for 30 min with gentle agitation. Cool to room temperature in an ice bath. Add 4 mL of 20% acetonitrile, replace the cap, and mix. Weigh the capped vial with the solution. Subtract the weight of the empty tube to determine the weight of hydrolysate. Centrifuge at 6,000 × g for 10 min and filter the aqueous supernatant through a 0.2 µm syringe filter.

Assay 2

Weigh a 50 mL centrifuge tube (with screw cap) and record the weight. Tare the balance, add 20 g of the sample extract, cap, and record the weight of the treated extract. Add 1.0 mL buffered β-galactosidase suspension, cap, and vortex gently. Place into a water bath at 60 °C. Monitor the temperature. After the samples reach 60 °C, incubate for 30 min with gentle agitation. Cool to room temperature in an ice bath. Add 5 mL of 20% acetonitrile, replace the cap, and mix. Weigh the capped vial with the solution; subtract the weight of the empty tube to determine the weight of hydrolysate. Centrifuge at 6,000 × g for 10 min and filter the aqueous supernatant through a 0.2 µm syringe filter.

Dilute the filtered supernatants with 3% acetonitrile to achieve concentrations within the calibration range for each analyte. The dilution factor (DF) depends upon the expected range of galactose and lactose concentrations. It may be necessary to prepare three different dilutions for initial free galactose, initial free lactose, and final total galactose. AOAC Method 2001.02 provides guidelines for choosing appropriate dilution factors. Analyze these solutions by HPAE-PAD within 72 h.

Calculations

Calculate the concentration of the stock standard by dividing the weight of the sugar used (mg) by 0.1 L. The concentration in mg/L is equivalent to the concentration in µg/mL. Calculate the concentrations of the intermediate standards from the known dilutions of the stock standard. To calculate the initial free lactose in a food sample (Lb, g/100 g sample), begin with the lactose concentration determined from Assay 1 (CLb, µg/mL). Multiply this value by the dilution factor (DF). Convert this value to µg lactose/g sample extract by correcting for dilution during the hydrolysis procedure: multiply by the weight of hydrolysate/weight of extract treated. Convert this value to µg lactose/g sample by correcting for dilution during the extraction procedure: multiply by the weight of sample extract/weight of sample (~50 g/1.0 g). Finally, convert from µg/g to g/100 g by multiplying by 0.0001. In equation form:

$$Lb = (CLb) \times (DF) \times (\text{weight of hydrolysate/weight of extract treated}) \times (\text{weight of sample extract/weight of sample}) \times (0.0001)$$

For example:

$$Lb = (CLb) \times (DF) \times (\sim 26\ g/\sim 20\ g) \times (\sim 50\ g/\sim 1.0\ g) \times (0.0001)$$

Similarly, calculate the initial free galactose in a food sample (Gb) by using the galactose concentration determined in Assay 1. Also, calculate the final total galactose in the hydrolyzed solution (Gt) by using the galactose concentration determined from Assay 2.

Calculate galactose released from TGOS (Gg, g/100 g sample) as:

$$Gg = Gt - Gb - Gl$$

Where:

Gl = galactose released from lactose = Lb/1.9

Calculate TGOS content (g/100 g sample) as:

$$TGOS = k \times Gg$$

Where:

k = (180 + 162n)/(180n) and n is the average number of galactose moieties in the TGOS molecules

For example, if $n = 2$, $k = 1.4$

Results and discussion

Separation

The Dionex CarboPac PA1 column is a general-purpose column for HPAE-PAD methods that separate mono-, di-, and some oligosaccharides.[7] AOAC Method 2001.02 uses a Dionex CarboPac PA1-4mm column with manually prepared sodium hydroxide/sodium acetate eluents. Table 4 shows the AOAC method eluent conditions. The starting 18 mM NaOH (0–20 min) is to separate galactose and other monosaccharides. The gradient from 18 to 125 mM NaOH (20.1–35 min) is to separate lactose and other disaccharides. Finally, 125 mM NaOH, 500 mM NaoAc (36.1–46 min) is used for washing oligosaccharides from the column, and then there is a return to starting conditions (46.1–61 min) to re-equilibrate the column prior to the next injection.

Table 4. Eluent gradient (AOAC method)

Time (min)	NaOH (mM)	NaOAc (mM)
0	18.125	0
20.1	18.125	0
35	125	0
36	125	0
36.1	125	500
46	125	500
46.1	18.125	0
61	18.125	0

Dual EGC mode replaces the manual preparation of the sodium hydroxide/sodium acetate eluents. This mode uses a methanesulfonic acid (MSA) eluent generation cartridge (EGC) and a potassium hydroxide (KOH) EGC in series to generate an extremely reproducible and accurate KOH/KMSA eluent gradient needed for separating complex carbohydrates. A Dionex CarboPac PA1 column in a 1 mm diameter format was recently launched for use with the Dual EGC mode. KMSA is about three times stronger than sodium acetate. To match the strength of 500 mM sodium acetate, 160 mM KMSA is needed at 46 min. Dual EGC mode limits the total concentration of KOH+ KMSA to <200 mM. Therefore, we first tried the conditions in Table 4 and replaced 500 mM NaoAc with 75 mM KMSA. However, we observed that the 20 min column re-equilibrium was not long enough. After adding extra re-equilibrium time, the galactose and sucrose separation was closer to that observed with the AOAC method.

To keep the total run time around 60 min, we decided to develop new Dual EGC mode eluent conditions to achieve a similar separation as the AOAC method. We developed the following conditions: 18 mM KOH + 1 mM KMSA isocratic (0–10 min) to separate galactose and other monosaccharides, 18 mM KOH + 1 mM KMSA to 100 mM KOH 1 mM KMSA (10–18 min) to separate lactose and other disaccharides, 100 mM KOH and 100 mM KMSA (18.1–30 min) to wash the column followed by column re-equilibration (18 mM KOH + 1 mM KMSA). The column washing conditions are not as strong as the AOAC method (125 mM NaOH, 500 mM NaOAc). Therefore, we extended the column wash time 2 min. The column re-equilibrium is 10 min longer than the AOAC method.

Figure 2 shows a separation of sugars of interest (galactose, lactose), and other common sugar in foods (glucose, fructose, and sucrose). Galactose and lactose were well resolved from other sugars. To confirm the 12 min column washing using 100 mM KOH, 100 mM KMSA is enough for this application, retention time stability was monitored. The retention times of galactose and lactose were stable for seven days of continuous injections of samples and standards. If over longer analysis times retention times shorten, column cleaning with stronger manually prepared eluents may be necessary.

Calibration

The calibration standard mixture was prepared with individual carbohydrate concentrations that could be diluted into the concentration range typical for TGOS samples.

Calibration curves with seven concentration levels were constructed for galactose and lactose. The calibration curve showed deviation from linearity in the selected calibration range. Therefore, the peak area versus concentration data were fit using a quadratic regression function. Figure 3 shows the calibration curves. The coefficient of determination (r^2) was greater than 0.999 for each component.

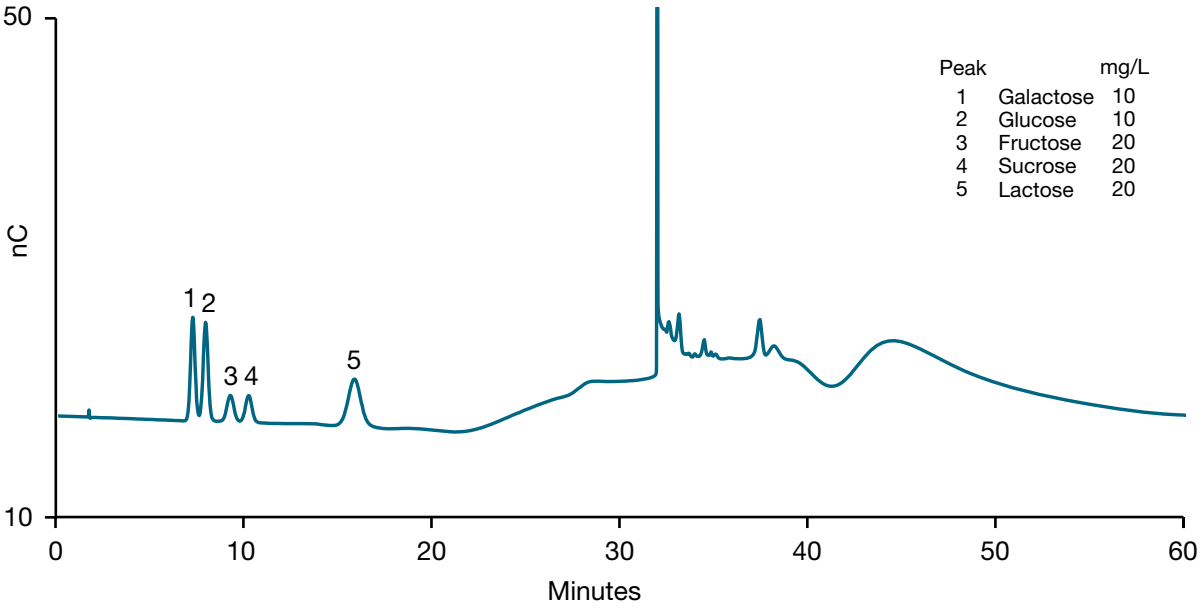

Figure 2. Separation of a five-sugar standard using a Dionex CarboPac PA1-1mm column

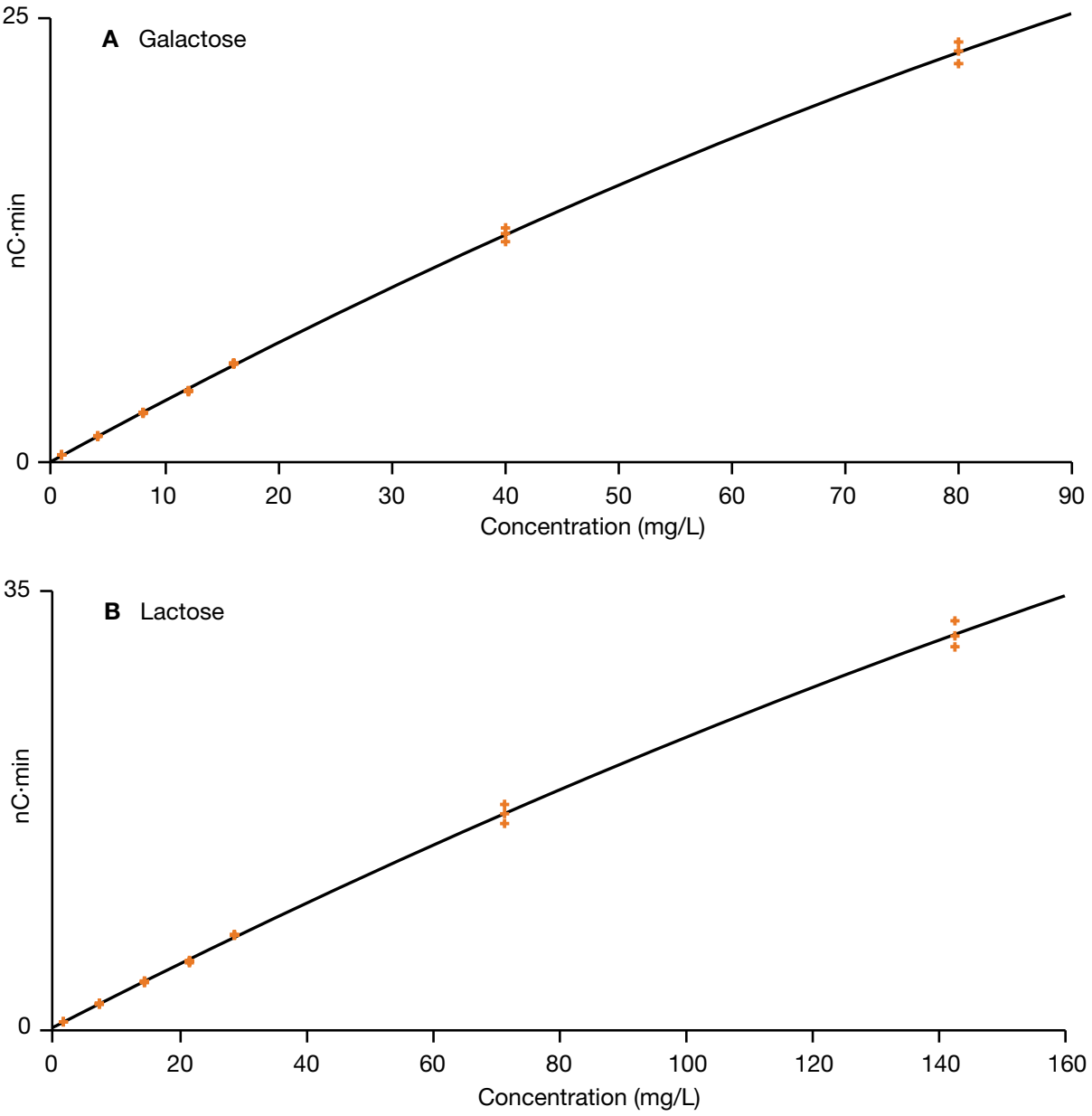

Figure 3. Two carbohydrate calibration curves

Sample analysis

Three GOS samples were obtained. Sample Assay 1 (A1) was used for the determination of galactose and lactose before β-galactosidase hydrolysis. Sample Assay 2 (A2) was used for the determination of galactose after β-galactosidase hydrolysis. Table 5 summarizes the results of the determination of TGOS in the chosen samples. The TGOS ranges from 49 to 80 g/100 g, and the results are very close to the products' nutrition labels.

Figure 4 shows the carbohydrate profile before hydrolysis with β-galactosidase. Figure 5 shows the carbohydrate profile of sample 2 after hydrolysis with β-galactosidase. Some galactooligosaccharides (GOS) between 10 and 30 min were removed after hydrolysis with β-galactosidase, and glucose and galactose increased after hydrolysis with β-galactosidase. This suggests that sample 2 contains a large amount of GOS.

Table 5. TGOS in samples

	Initial free lactose in sample (g/100 g) (Lb)	Initial galactose from free lactose in sample (g/100 g) (Gl)	Initial free galactose in sample (g/100 g) (Gb)	Final total galactose in sample (g/100 g) (Gt)	Galactose release from TGOS (g/100 g) (Gg)	TGOS (g/100 g)
Sample 1	10.4	5.48	0.514	61.7	55.7	78.0
Sample 2	21.0	11.2	4.23	50.6	35.2	49.2
Sample 3	7.44	3.92	0.312	45.8	41.6	58.2

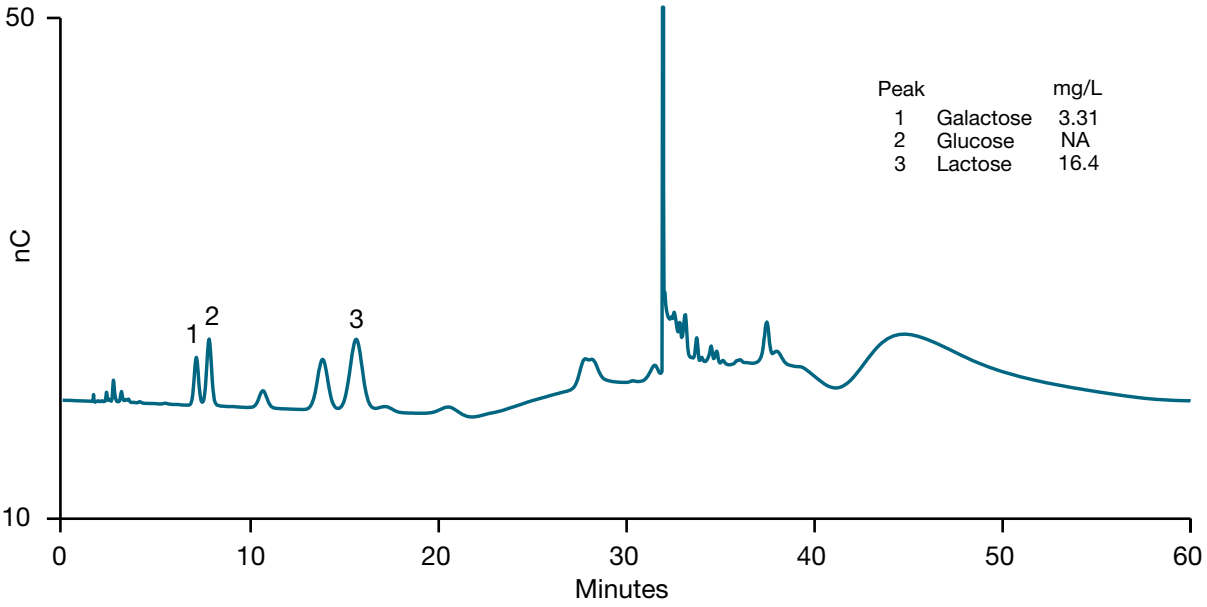

Figure 4. Sample #2 before enzyme hydrolysis (dilute extract 200-fold)

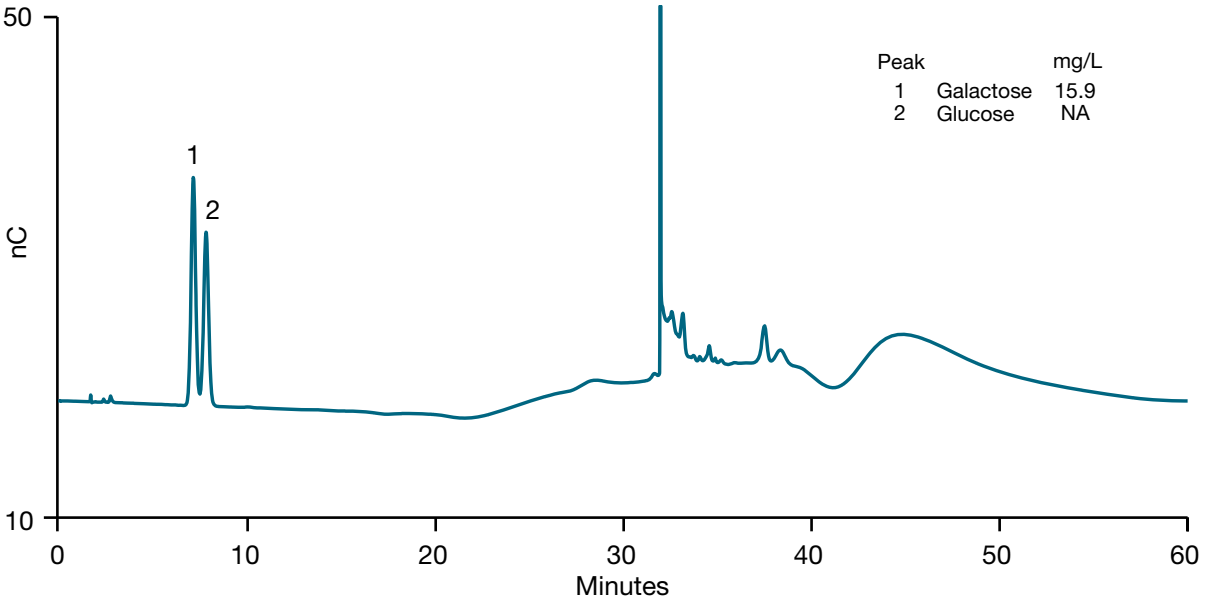

Figure 5. Sample #2 after enzyme hydrolysis (dilute extract 500-fold)

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Precision

The precision of the method was determined by triplicate injection of the level 4 calibration standard. The calculated peak area precision varied from 0.66 to 1.23%, with retention time precision < 0.21% for all target carbohydrates. The high precision of this method is consistent with results typically obtained with a system using eluent generation.

Conclusion

This application note demonstrated that AOAC Method 2001.02 for TGOS analysis could be successfully executed with a Dionex CarboPac PA1-1mm column using HPAE-PAD in Dual EGC Mode. The separation, linearity, and reproducibility were excellent. Comparison with a traditional HPAE-PAD separation of TGOS using sodium hydroxide/sodium acetate eluents showed that the Dual EGC method delivers a similar resolution of carbohydrates, simplifies operation (no eluent preparation), and improves retention time precision.

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