

Determination of *Trans*-Galactooligosaccharides in Foods by AOAC Method 2001.02

INTRODUCTION

Dietary fiber is coarse, indigestible plant matter that, when included in the diet, promotes good gastrointestinal tract health. Typically, dietary fiber is fibrous or gummy material derived from plant cell walls, lignin, polysaccharides, and similar substances that resist hydrolysis by digestive enzymes. About two-thirds to three-fourths of the dietary fiber in a typical diet is classified as insoluble in an aqueous enzyme solution. Soluble dietary fiber is soluble in an aqueous enzyme system, but can be precipitated with four parts of ethanol to one part of the aqueous mixture. Many soluble dietary fibers are food gums, such as pectins, carrageenan, guar, locust bean gum, gum acacia, and xanthan gum. As an example of the chemical composition of these fibers, pectins are polygalacturonic acids with side chains composed of arabinose, xylose, rhamnose, glucose, and galactose.

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 di- to octasaccharides composed of 1–7 galactose units linked to a glucose molecule at the reducing end. Quemener et al.¹ developed a method based on high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) to measure TGOS in food and feed products. Then de Slegte organized a successful AOAC collaborative study of this method and it 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 CarboPac™ PA1 column and detected by pulsed amperometric detection (PAD) using a triple potential waveform (Waveform B, Dionex Technical Note 21³). A quadruple potential waveform (Waveform A, Technical Note 21) provides greater long-term reproducibility of peak area response compared to Waveform B.⁴ During our participation in the Method 2001.02 collaborative study, we analyzed samples with both waveforms. This application note shows that Waveform A produced results equivalent to those obtained with Waveform B. Using Waveform A instead of B for Method 2001.02 will yield equivalent results and greater long-term peak area reproducibility. We previously demonstrated that Waveform A can be used for the determination of polydextrose in foods by AOAC Method 2000.11.^{5,6}

EQUIPMENT

Chromatography Equipment

Dionex BioLC[®] chromatography system consisting of:

GP50 Gradient Pump with vacuum degas option

ED50 Electrochemical Detector

E01 Eluent Organizer

AS50 Autosampler with Thermal Compartment

Dionex Chromeleon 6[®] Chromatography

Workstation

Other Equipment

Vortex mixer (Vortex-Genie[®] 2, VWR Products)

Water bath (Model 1202, VWR Products)

Analytical centrifuge capable of 6000 × *g*
(Spinchron R, Beckman)

REAGENTS AND STANDARDS

Deionized water, 18.0 MΩ-cm resistivity or better (used
for all eluent and standard preparations)

Sodium hydroxide, 50% (w/w) (Fisher SS254)

Sodium acetate, anhydrous (Fluka 71180)

Acetonitrile (Burdick & Jackson 015-4 HPLC-grade)

Hydrochloric acid, 36.5–38.0 % (EM Science HX0603P)

Potassium dihydrogen phosphate (KH₂PO₄, Sigma P 0662)

Dipotassium hydrogen phosphate, anhydrous (K₂HPO₄, Fisher
P288-500)

D(+)-Galactose (Sigma G 0750)

Lactose monohydrate (Merck 7660)

β-Galactosidase, 2000 U/mL (Amano LAF-50 Lactase F,
or equivalent)

OTHER CONSUMABLES

Autosampler vials (0.5 mL, Dionex P/N 055428)

Centrifuge tubes (50 mL, VWR 21008-146)

Syringe filters (0.45-μm, nylon, 13 mm diameter,
Gelman Sciences)

CONDITIONS

Column: CarboPac PA1 Analytical, (4 × 250 mm,
P/N 35391)

CarboPac PA1 Guard, (4 × 50 mm,
P/N 43096)

Expected Operating

Pressure: 9.7 MPa (1400 psi)

Temperature: 25 °C

Degas: 30 sec every 10 min

Injection Volume: 25 μL full loop

Detection: Pulsed amperometry, gold working
electrode, Ag/AgCl reference electrode

ED50 Waveforms for Carbohydrate Analysis

Waveform A:			Waveform B:		
Time (sec)	Potential (V)	Integration	Time (sec)	Potential (V)	Integration
0.00	+0.1		0.00	0.05	
0.20	+0.1	Begin	0.20	0.05	Begin
0.40	+0.1	End	0.40	0.05	End
0.41	-2.0		0.41	0.75	
0.42	-2.0		0.60	0.75	
0.43	+0.6		0.61	-0.15	
0.44	-0.1		1.00	-0.15	
0.50	-0.1				

Note: Waveform A should only be used with a new gold working electrode or a gold working electrode only used previously with this waveform.³

Collection Rate: 2 Hz

Expected
Background: 10–25 nC (Waveform A)
1–15 nC (Waveform B)

Needle Height: 5 mm

Flush Volume: 100 μL

Flow Rate: 1.0 mL/min

Gradient: Eluent A — 12.5 mM sodium hydroxide
Eluent B — 125 mM sodium hydroxide
Eluent C — 125 mM sodium hydroxide/
500 mM sodium acetate

Time (min)	%A	%B	%C
0.0	95	5	0
20.1	95	5	0
35.0	0	100	0
36.0	0	100	0
36.1	0	0	100
46.0	0	0	100
46.1	95	5	0
61.0	95	5	0

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 deionized water. Mix to dissolve and bring to volume with deionized water. Filter through a 0.22- μ m 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 deionized 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 deionized water. Use this acid to adjust the pH of the sample extract.

Buffered Enzyme Mix

Suspend lactase F in phosphate buffer to obtain a final activity of 2000 U/mL. For example, suspend 2 g of lactase F (50,000 U/g) in 50 mL of phosphate buffer. Store the enzyme suspension at 4 °C and use within 8 h. Stir well before using.

ELUENT PREPARATION

12.5 mM Sodium Hydroxide

It is essential to use high-quality water of high resistivity (18.0 M Ω -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 solution. 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 12.5 mM NaOH, use a 10-mL graduated plastic pipette to deliver 1.30 mL (or 2.00 g) of 50% (w/w) sodium hydroxide into a 2-L volumetric flask containing 1.9 L of degassed deionized water. Bring to volume with degassed deionized water. Cap and invert the volumetric flask eight times to mix. (*Note: Do not mix excessively, as this action will increase the carbonate ion in the solution by trapping carbon dioxide from the air.*) Continue degassing for 30 min. After preparation, keep the eluent blanketed under helium at 34–55 kPa (5–8 psi) at all times. If maintained under helium, this eluent can be used for approximately one week.

125 mM Sodium Hydroxide

To prepare 2 L of 125 mM NaOH, use a 10-mL graduated plastic pipette to deliver 13.9 mL (or 21.41 g) of 50% (w/w) sodium hydroxide into a 2-L volumetric flask contain-

ing 1.9 L of degassed deionized water. Bring to volume with degassed deionized water. Cap and invert the volumetric flask eight times to mix. Continue degassing for 30 min. After preparation, keep the eluent blanketed under helium at 34–55 kPa (5–8 psi) at all times. If maintained under helium, this eluent can be used for approximately one week.

125 mM Sodium Hydroxide/500 mM Sodium Acetate

Measure approximately 1.8 L of water into a 2-L volumetric flask. Add a stir bar and begin stirring. Weigh out 82.0 g of anhydrous crystalline sodium acetate. Steadily add the sodium acetate to the briskly stirring water. After the salt dissolves, remove the stir bar with a magnetic retriever. Degas the solution by sonicating under vacuum for 10 min. Use a 10-mL graduated plastic pipet to add 13.9 mL (or 21.41 g) of 50% (w/w) sodium hydroxide to the sodium acetate solution. Bring to volume with degassed deionized water, cap, and invert eight times to mix. Vacuum filter this solution through a 0.2- μ m nylon filter. Filtering may be slow 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.

STANDARD PREPARATION

The AOAC study organizers provided the D(+) – galactose and lactose monohydrate standards. Dry the sugar standards to a constant weight at 103 °C. Prepare the galactose stock standard (800 mg/L) by weighing 0.080 g of anhydrous galactose into a 100-mL volumetric flask. Add deionized water to dissolve, bring to volume with deionized water, and mix. Prepare the lactose stock standard (1425 mg/L as lactose) by weighing 0.150 g of lactose monohydrate into a 100-mL volumetric flask. Add deionized water to dissolve, bring to volume with deionized water and mix.

Prepare the four working standards by diluting the stock standards with deionized water. Dilute 500 μ L each of the galactose and lactose stock standards to 100 mL in a volumetric flask to yield Standard 1 containing 4 μ g/mL galactose and 7.125 μ g/mL lactose. Similarly, dilute 1000 μ L each of the galactose and lactose stock standards to 100 mL in a volumetric flask to yield Standard 2 (8 μ g/mL galactose and 14.25 μ g/mL lactose), 1500 μ L each of the galactose and lactose stock standards to yield Standard 3 (12 μ g/mL galactose and 21.375 μ g/mL lactose), and 2000 μ L each of the galactose and lactose stock standards to yield Standard 4 (16 μ g/mL galactose and 28.5 μ g/mL lactose).

SAMPLE PREPARATION

This section briefly summarizes of the sample preparation procedure outlined in AOAC Method 2001.02. For details, consult the method.² The study organizers provided 14 samples with added TGOS (seven blind duplicates) for analysis. The laboratory samples were (with added concentration of TGOS): yogurt drink (6%), lemonade syrup (15%), custard (5%), orange juice (4%), pet candy (2%), biscuits (8%), and infant formula (4%).
Note: Record all weights measured in the following sections to the nearest milligram.

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 2.5 g of the yogurt drink, 1.5 g of the lemonade syrup, 2 g of the custard, 2.5 g of the orange juice, 1 g of the pet candy, 2 g of the biscuits, and 1 g of the infant formula. 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 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 extract treated**. 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 6000 \times g for 10 min and filter the aqueous supernatant through a 0.45- μ m 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 extract treated**. 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 6000 \times g for 10 min and filter the aqueous supernatant through a 0.45 μ m syringe filter.

Dilute the filtered supernatants with acetonitrile to achieve concentrations within the calibration range for each analyte. The dilution factor (**DF**) will depend 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 2001.02 provides guidelines for choosing appropriate dilution factors. Analyze these solutions by HPAE-PAD within 72 h. Analyze each sample in triplicate.

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 μ g/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 (L_b , g/100 g sample), begin with the lactose concentration determined from Assay 1 (C_{Lb} , μ g/mL). Multiply this value by the dilution factor (**DF**). Convert this value to μ g 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** ($\sim 50 \text{ g}/1.0 \text{ g}$).

Finally, convert from $\mu\text{g}/\text{g}$ to $\text{g}/100 \text{ g}$ by multiplying by 0.0001.

In equation form:

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

For example:

$$L_b = (C_{Lb}) \times (DF) \times (\sim 26 \text{ g}/\sim 20 \text{ g}) \times (\sim 50 \text{ g}/\sim 1.0 \text{ g}) \times (0.0001)$$

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

Calculate galactose released from TGOS (G_g , $\text{g}/100 \text{ g}$ sample) as:

$$G_g = G_t - G_b - G_l$$

Calculate TGOS content ($\text{g}/100\text{g}$ sample) as:

$$\text{TGOS} = k \times G_g$$

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

The procedure described in this application note and in AOAC Method 2001.02 uses a hot phosphate buffer to extract TGOS and lactose from foods. Treatment with β -galactosidase hydrolyzes TGOS to its constituent galactose and glucose. (Any lactose initially present is also hydrolyzed to galactose and glucose. The sample is treated in two steps to account for the initial lactose). The sample is then analyzed by HPAE-PAD. Figure 1 shows the HPAE-PAD separation of Standard 3 (12 $\mu\text{g}/\text{mL}$ galactose and 24.1 $\mu\text{g}/\text{mL}$ lactose). To determine the concentration of TGOS in samples, a four-point galactose/lactose mixed standard calibration curve was established ranging from 4–28 mg/L . Peak areas were plotted against concentration and fit with a linear regression. The correlation coefficient for galactose was 0.9999 and the correlation coefficient for lactose was 0.9997.

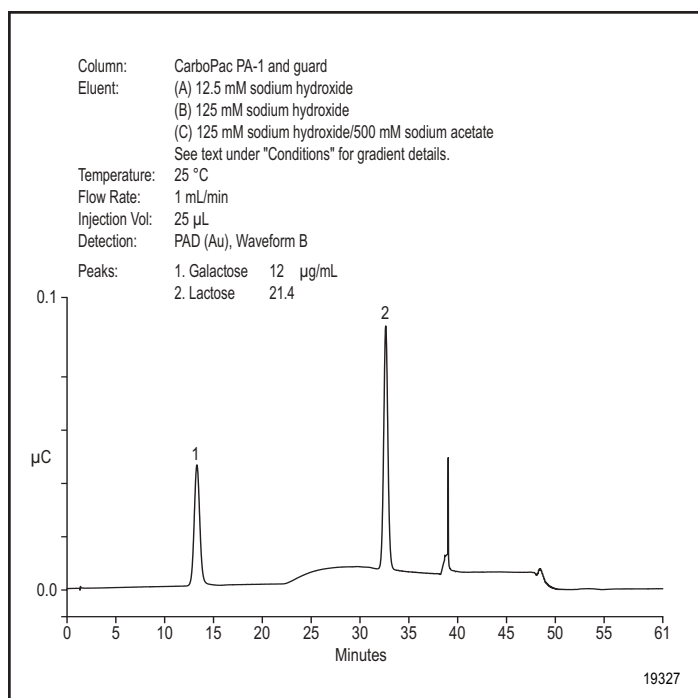


Figure 1. HPAE-PAD separation of 12.0 $\mu\text{g}/\text{mL}$ galactose and 21.4 $\mu\text{g}/\text{mL}$ lactose using Waveform B.

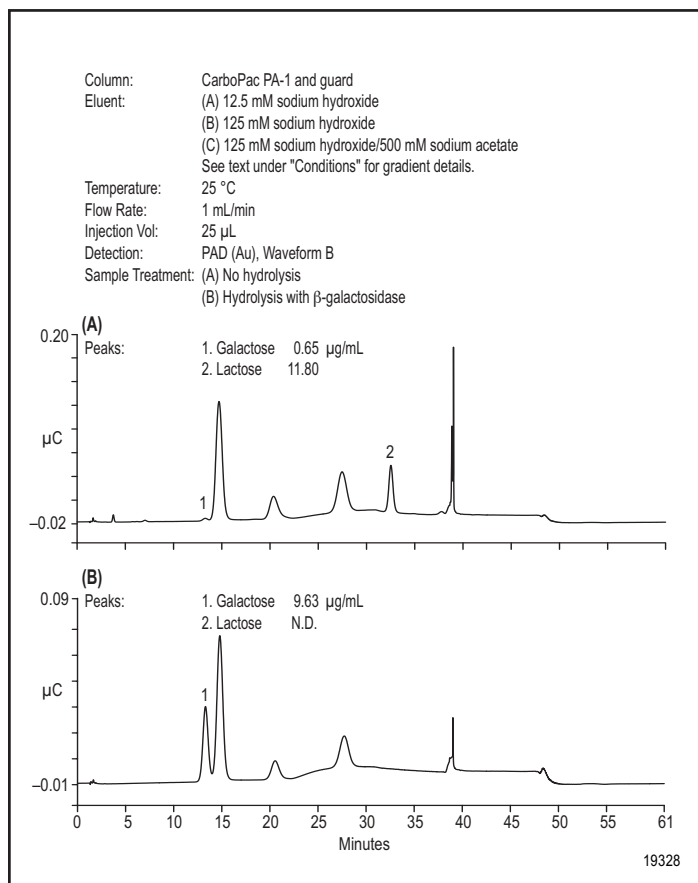


Figure 2. HPAE-PAD analysis of lemonade syrup + 15% TGOS using Waveform B, before (A) and after hydrolysis (B) with β -galactosidase.

We participated in the collaborative study of what is now AOAC Method 2001.02. This method specified the use of a triple potential waveform (Waveform B, Dionex Technical Note 21) for the PAD of transgalactose. We analyzed the seven blind duplicate samples (identities later revealed and shown in Table 1, column 1), and reported the % TGOS values in the second column of Table 1.² These values were consistent with the values determined by the other seven labs participating in the study. Figure 2 shows the HPAE-PAD determination of one of the lemonade syrup samples.

After the originators of this method finished its development, a new quadruple potential PAD waveform was described that yields significantly improved long-term peak area reproducibility for carbohydrate detection.⁴ Immediately after we analyzed the collaborative study samples with Waveform B, we analyzed them with Waveform A. The results using Waveform A are presented in the third column of Table 1. A comparison of Table 1, columns 2 and 3, reveals that Waveform A yields results equivalent to those determined with Waveform B. Because there is greater long-term peak area reproducibility with Waveform A, we recommend using Waveform A when following AOAC Method 2001.02.

PRECAUTIONS AND RECOMENDATIONS

For best results, the Ag/AgCl reference electrode should be replaced every three to six months. Samples containing initially high levels of lactose—for example, infant formula—should be prepared and analyzed in triplicate. AOAC Method 2001.02 recommends using a column temperature of 20–30 °C, ± 5 °C, preferably 20 °C. The method states that column temperature and other chromatographic conditions can be varied to optimize the separation. Our results were obtained at 25 °C.

Table 1. Comparison of Percent TGOS in Food Samples Determined Using AOAC Method 2001.02 with Either Waveform A or Waveform B

Sample	TGOS (%) Determined with Waveform B	TGOS (%) Determined with Waveform A
Yogurt Drink #1	5.35	5.14
Yogurt Drink #7	5.39	5.17
Lemonade Syrup #2	13.10	13.02
Lemonade Syrup #11	13.41	12.88
Custard #3	3.79	3.32
Custard #5	4.19	4.06
Orange Juice #4	3.48	3.35
Orange Juice #13	3.55	3.25
Pet Candy #6	1.92	1.25
Pet Candy #14	2.17	1.66
Biscuits #8	7.68	7.25
Biscuits #12	8.38	8.04
Infant Formula #9	4.12	3.38
Infant Formula #10	4.76	3.96

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LIST OF SUPPLIERS

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Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178 USA, Tel: 1-800-325-3010, www.sigma-aldrich.com.

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