Application Note

Food & Beverage

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Phenols
Bisphenol A
Catechins
Flavonoids
Phenols
Antioxidants
Resveratrol
Epicatechin
Quercetin
Other polyphenols

Carbohydrates
Monosaccharides
Disaccharides
Lactose
Galactooligosaccharides
Other oligo- and polysaccharides

Vitamins, minerals etc.
A, C, D, E, and K
Iodide
Q10, ubiquinols

Trans-galactooligosaccharides in Food Products

- **ALEXYS Carbohydrate Analyzer**
- **Improvements on AOAC 2001.02**
- **SweetSep™ AEX200 column**
- **Galactose, glucose, lactose & isomers**
- **'Fast and green' analytical method**

Summary

Prebiotics are nondigestible food ingredients that provide numerous health benefits and help maintain a healthy and balanced gut microflora. They beneficially affect the host by selectively stimulating the growth or activity of health promoting bacteria in the colon, such as bifidobacteria [1]. Trans-galactooligosaccharides (TGOS) are prebiotic water-soluble carbohydrates consisting of a chain of 1-7 galactose units with a terminal glucose. TGOS are not broken down by human digestive enzymes and are therefore available as a nutrient for beneficial bacteria. As a prebiotic, TGOS has a wide application in human and animal foods.

One of the most commonly-used method to quantify TGOS is the AOAC 2001.02 method [2]. This AOAC approved method is based on enzymatic hydrolysis of TGOS and subsequent analysis of the reaction products (galactose & glucose) with High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD). In this application note an adapted method was evaluated, using the novel Antec Scientific SweetSep™ AEX200 column with 5 µm particle size. The adapted method resulted in better separation under isocratic conditions and a shorter analysis time. Both the original and adapted methods provide excellent sensitivity, linearity and reproducibility, and are suitable for the routine analysis of TGOS in food and feed using the ALEXYS Carbohydrates Analyzer.

Introduction

Trans-galactooligosaccharides (TGOS) are water-soluble oligosaccharides consisting of a chain of galactose units with a terminal glucose and typically have a degree of polymerization (DP) between 2 and 8. TGOS are not digested in the small intestine but are fermented in the colon by the bacterial flora. By stimulating the growth of the beneficial bacteria, the consumption of TGOS as a prebiotic supplement may result in a broad range of positive health effects.

TGOS are produced by the trans-galactosylation activity of β-galactosidase enzymes, using lactose as both donor and acceptor substrate. During the synthesis, the glycosyl group of one or more D-galactosyl units is transferred onto the D-galactose moiety of lactose. The yield depends on the enzymatic source and reaction condition, and TGOS are produced as a complex mixture of branched and linear oligosaccharides with varying chain length and glycosidic linkages [3]. For example, up to 8 different disaccharides consisting of galactose and glucose with different β-glycoside bonds can be present in a TGOS mix [4]. All galactose-glucose based disaccharides except lactose are not susceptible to decomposition by human digestive enzymes and are therefore considered TGOS [5].

The principle of the AOAC 2001.02 method to determine the total content of TGOS is based on the enzymatic treatment of a sample with a β-galactosidase enzyme and subsequent analysis of the reaction products using High Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD). β-galactosidase catalyzes the hydrolysis of glycosidic bonds in the galactooligosaccharides, resulting in the release of the monosaccharides glucose and galactose. The galactose release is subsequently quantified in the AOAC method and the TGOS content is then calculated by multiplying the amount of released galactose by the so-called 'k-factor' to correct for the terminal glucose units in TGOS. The k-factor is based on the ratio glucose and galactose in the TGOS ingredient and can be calculated using the average degree of polymerization.

So, a boundary condition to analyze TGOS in a sample is that the k-factor or average degree of polymerization must be provided by the manufacturer of the GOS ingredient to be able to accurately quantify the total TGOS contents by the AOAC 2001.02. Alternatively, the total TGOS content can be determined without the use of the k-factor, by taking into account the quantification of the released glucose in the analysis [6].

Figure 1: ALEXYS Carbohydrates analyzer consisting of the ET 210 eluent tray, P6.1L analytical pump, AS6.1L autosampler, CT2.1 column thermostat and DECADE Elite electrochemical detector.

Such approach could be employed in case the k-factor is not known of the GOS ingredient.

In this application note an improved separation method for the analysis of TGOS in food products is presented using the ALEXYS carbohydrates analyzer in combination with a microbore SweetSep AEX200 column, and compared to the performance using the original AOAC 2001.02 method. The adapted method is based on simple isocratic elution and resulted in shorter run times, less mobile phase consumption, better separation of lactose-allolactose and other lactose isomers, and more accurate quantification of TGOS. Furthermore, in the presented method the quantification of the glucose release is also included in the analysis to determine the amount of TGOS in test samples, both with and without the use of the k-factor.

Method

The original and adapted AOAC method for total TGOS analysis was evaluated using the ALEXYS Carbohydrates Analyzer as shown in figure 1. The HPAEC-PAD system consists of an ET210 eluent tray for inert gas blanketing (using N_2), P6.1L quaternary LPG pump, AS6.1L autosampler, and the DECADE Elite electrochemical detector. The SenCell with Au working electrode and HyREF reference electrode was selected for sensitive detection of the sugars of interest.

Table 1

Original AOAC 2001.02 method	
HPLC system	ALEXYS Carbohydrates Analyzer
Detector	DECADE Elite electrochemical detector
Columns	Thermo Scientific™ Dionex™ CarboPac™ PA1 guard column, 50 x 4.0 mm ID Thermo Scientific™ Dionex™ CarboPac™ PA1 analytical column, 250 x 4.0 mm ID
Mobile phase (MP)	A: 12.5 mM NaOH B: 125 mM NaOH C: 125 mM NaOH, 500 mM NaOAc Eluents prepared & blanketed with Helium 5.0
Flow rate	1.0 mL/min
Back pressure	about 110 bar
Injection	10 µL
Temperature	25 °C for separation, 35 °C for detection
Flow cell	SenCell with Au WE, stainless steel AE and HyREF RE, AST 2
Potential waveform (4-step)	E1, E2, E3, E4: +0.1, -2.0, +0.6, -0.1 V ts, t1, t2, t3, t4: 0.2, 0.4, 0.02, 0.01, 0.07 s
I-cell	about 0.2— 0.4 µA
ADF	0.05 Hz
Range	10 µA/V

Table 2

Gradient program		
Time (min)	*Mobile phase*	*Description*
0 - 24	18.75 mM NaOH	Elution & detection
24 – 39	18.75 mM - 125 mm NaOH	
39 - 40	125 mm NaOH	
40 - 50	125 mm NaOH, 500 mM NaOAc	Column clean-up and regeneration
50 - 65	18. 75 mM NaOH	Equilibration, starting conditions

Separation

Under alkaline conditions (pH > 12) carbohydrates can be separated by means of HPAEC. Carbohydrates are weak acids with pKa values ranging between 12 and 14. At high pH they will be either completely or partially ionized depending on their pKa value. Due to the extreme alkaline conditions only polymeric anion-exchange columns are suitable for carbohydrate separation.

AOAC 2001.02 method: The recommended HPAEC column with 10 µm particles size and quaternary ammonium functionalized latex as mentioned in the AOAC method was chosen for method evaluation, using the conditions given in Table 1 and

Table 3

Adapted AOAC 2001.02 method	
HPLC system	ALEXYS Carbohydrates Analyzer
Detector	DECADE Elite electrochemical detector
Columns	SweetSep™ AEX200, 2.1 × 200 mm column, 5 µm SweetSep™ AEX200, 2.1 × 50 mm precolumn, 5 µm Borate ion trap, 2.1 × 50 mm column, 10 µm (Antec Scientific)
Mobile phase (MP)	A: DI Water B: 100 mM NaOH C: 100 mM NaOH + 100 mM NaOAc Eluents prepared & blanketed with Nitrogen 5.0
Flow rate	0.18 mL/min
Back pressure	about 200 bar, column net pressure about 130 bar
Injection	3 µL
Temperature	25 °C for separation, 45 °C for detection
Flow cell	SenCell with Au WE, stainless steel AE and HyREF RE, AST 2
Potential waveform (4-step)	E1, E2, E3, E4: +0.1, -2.0, +0.6, -0.1 V ts, t1, t2, t3, t4: 0.2, 0.4, 0.02, 0.01, 0.07 s
I-cell	about 0.2— 0.4 µA
ADF	0.05 Hz
Range	10 µA/V

Table 4

Step-gradient program		
Time (min)	*Mobile phase*	*Description*
0 - 15	12 mM NaOH	Elution & detection
15 - 20	100 mM NaOH, 100mM NaOAc	Column clean-up and regeneration
20 – 45	12 mM NaOH	Equilibration, starting conditions

Table 2. The temperature for separation was set at 25 °C using the CT 2.1 column thermostat. The separation is based on isocratic elution of galactose followed by a gradient for the elution of lactose. A strong column clean-up and regeneration step is executed at t = 40 min in every run to elute late eluting components. After the 10 min clean-up step the column is equilibrated for 15 minutes at the starting conditions, resulting in a total run time of 65 minutes.

Adapted method: To improve the separation of the mono– and disaccharides the SweetSep™ AEX200 column with 5 µm particle size was chosen. The SweetSep™ AEX200 column is a strong anion-exchange column containing highly monodisperse 5 µm ethylvinylbenzene-divinylbenzene copolymer (80% crosslinked) coated with functionalized nanoparticles with quaternary amine. This type of column enables fast and high resolution separations of sugars with short analysis time. The

conditions are shown in Table 3 and Table 4. The CT 2.1 column thermostat was used to perform the separation at 25°C. Due to its smaller ID (2.1 mm) a microbore column exhibits less sample dilution and therefore requires a smaller injection volume compared to a standard bore column. Therefore, an injection volume of 3 µL was chosen to ensure that all analyte signals fall within the linear range of the detector and to avoid any additional dilution steps, when measuring the exact same samples and calibration standards. All analytes of interest (galactose, glucose, allolactose and lactose) are separated under isocratic elution. A strong column clean-up and regeneration step is executed at t =15 min to elute strongly retaining components and to remove carbonate ions (CO_3^{2-}) build up on the column. After the clean-up step the column is equilibrated for 25 minutes at the starting conditions, resulting in a total run time of 45 minutes.

Detection

For the pulsed amperometric detection of simple sugars the Antec SenCell electrochemical flow cell is used. This flow cell [6] has a confined wall-jet design and consists of a Au working electrode (WE), HyREF (Pd/ H_2) reference electrode (RE) and stainless steel auxiliary electrode (AE). The AOAC method mentions the use of a 3-step waveform. However, a 4-step waveform yields significantly improved long-term peak area reproducibility compared the described 3-step waveform while yielding equivalent results [7, 8], and is therefore the preferred waveform. Hence, the 4-step potential waveform was used for both methods. The temperature for detection was set to 35°C for the original AOAC method and 45°C for the adapted method. The cell current was typical about 0.2—0.4 µA using these PAD settings under the specified conditions.

Preparation of standards and reagents

Standards: 5 g/L stock standards of the individual sugars were prepared in 95/5 (v/v%) water/acetonitrile to increase the storage life. Stock standards under these conditions are approximately stabile for more than a month in the fridge at 4° C. Working standards in the concentration range of 0.5 - 48 mg/L were prepared by dilution of the stock standards with DI water.

Carrez reagents: a Carrez clarification procedure is used for deproteination of the dairy samples. By removing all proteins also the enzymatic activity will be quenched, eliminating any unwanted conversion of the sugars inside the sample during the analysis process. The Carrez I solution was prepared by dissolving 15.0 g potassium hexacyanoferrate(II) trihydrate in 100 mL of DI water in a volumetric flask. The Carrez II solution was prepared by dissolving 30.0 g zinc sulfate heptahydrate in 100 mL of water in a volumetric flask. Both Carrez solutions were filtered over a qualitative filter paper (Whatman™ 590/1) prior to use.

Buffers: A phosphate buffer (200 mM, pH 6) was prepared by dissolving 11.0 g KH_2PO_4 and 2.3 g K_2HPO_4 in 0.5 L of DI water. The buffer was sterilized by filtration over a 0.2 µm Polyethersulfone (PES) syringe filter (25 mm Ø FFL/MLS) and kept at 4°C until use.

Enzyme solution: β-galactosidase (EC 3.2.1.23, Megazyme) was diluted with phosphate buffer to obtain a final activity of 2,000 U/mL. and kept at 4°C until use.

Sample preparation

Two TGOS ingredients were obtained for this study. Vivinal® GOS Powder (>68% GOS content , TGOS A) was kindly provided by FrieslandCampina Ingredients and TGOS powder (>70% GOS content, TGOS B) was purchased from Carbosynth (Compton, United Kingdom). Two test samples were prepared based on the TGOS powders by dissolving 0.5 g of TGOS in 9.5 g of DI water. Additionally, 3 commercial food products, an infant formula containing GOS (0.48 g / 100 mL), a semi-skimmed milk, and a buttermilk were obtained from the local supermarket. To simulate a high TGOS supplemented food product, 9.5 g of dairy products was spiked with 0.5 g TGOS A.

All samples were prepared according to AOAC Method 2001.02 followed by a Carrez clarification for the dairy samples as summarized below. As a comparison, only the TGOS A and TGOS B ingredients were measured using both methods. The dairy samples were measured only with the adapted method to demonstrate the accuracy of the method. An extensive description of sample preparation can be found in the method [2]:

- TGOS A in DI water
- TGOS B in DI water
- Infant formula (sample S1)
- Infant formula spiked with TGOS A (sample S2)
- Semi-skimmed milk spiked with TGOS A (sample S3)
- Buttermilk spiked with TGOS A (sample S4)

Extraction: 40 mL of hot phosphate buffer was added to 2g of sample. The mixture was incubated for 60 min at 80 °C and subsequently cooled down to room temperature in an ice bath. The pH of the extract was verified to be pH 6 and subsequently the extract was diluted to a the total volume of 50 mL with phosphate buffer.

Enzymatic Hydrolysis: For each samples 2 separate extracts were prepared. Assay 1 for the determination of the initial concentrations of the free lactose , glucose, and galactose present in the sample. Assay 2 for the determination of the final concentration of galactose and glucose after hydrolysis of the TGOS present in the sample.

Assay 1 (initial test solution A1): 250 µL of phosphate buffer was added to 250 µL of β-galactosidase suspension. The enzyme was deactivated by incubating the suspension at 100 °C for 10 minutes. The deactivated enzyme solution was cooled down to room temperature and 5 g of extract solution was added. The mixture was incubated at 60 °C for 30 minutes under gentle agitation, and cooled down to room temperature in an ice bath. Subsequently, 1 mL of 20% acetonitrile was added.

Assay 2 (hydrolyzed sample solution A2): 5 g of extract solution was added to 250 µL of enzyme solution. The mixture was incubated at 60°C for 30 minutes under gentle agitation, cooled down to room temperature in an ice bath, and 1.25 mL of 20% acetonitrile was added.

Carrez precipitation: The dairy-based samples were clarified using a carrez precipitation. For both assays 50 µL Carrez I and 50 µL carrez II reagent was added, followed by an addition 3% acetonitrile up to a total volume of 50 mL. The solution was incubated for 30 minutes at room temperature and centrifuged 10 min at 4000 xG to obtain a clear supernatant.

All samples were further diluted 5 times with 3% acetonitrile and filtered over a 0.2 µm Polyethersulfone (PES) syringe filter (25 mm Ø FFL/MLS) prior to injection.

Calculations

Calculation TGOS concentration AOAC Method 2001.02

The initial concentration of free galactose ($Gal_{initial}$, in mg/L) and lactose ($Lac_{initial}$, in mg/L) are determined directly in assay 1. The final concentration galactose (released + free) in the hydrolyzed solution (Gal_{total}, in mg/L) is determined in assay 2. The galactose released from lactose ($Gal_{lactose}$, in mg/L) in assay 2 can be calculated using the following formula:

$$Gal_{lactose} = \frac{180}{342} x\ Lac_{initial} = \frac{Lac_{initial}}{1.9}$$

Where, 180/342 is a factor based on the molar masses of Galactose and Lactose. The total amount of galactose released from TGOS (Gal_{TGOS}, in mg/L) is calculated by subtracting the initial galactose and the galactose released from lactose from the total galactose:

$$Gal_{TGOS} = Gal_{total} - Gal_{initial} - Gal_{lactose}$$

The concentration of TGOS in mg/L is then calculated by multiplying the galactose released from TGOS by the k-factor [2]:

$$TGOS = k\ x\ Gal_{TGOS}$$

The k-factor can be calculated using:

$$k = \frac{180 + 162n}{180n}$$

With n being the average number of galactose moieties in the TGOS molecules (i.e. the average degree of polymerization -1). For example, if n = 2, k is 1.4. An accurate estimation of the n factor, and consequently the k factor is needed to determine the TGOS contents following the AOAC 2001.02 method. The k-factor may vary among GOS manufacturers and even between GOS batches. Ideally, each manufacturers should determine the k factor for each batch and provide it to their customers.

Calculation TGOS concentration without k-factor

Alternatively, the TGOS concentration can be calculated without the k-factor by including the released glucose in the calculations [10]. The initial concentration glucose ($Glu_{initial}$, in mg/L) and glucose in the hydrolyzed solution (Glu_{total} , in mg/L) are determined directly in assay 1 and 2, respectively. The glucose in mg/L released from lactose can be calculated using the following formula:

$$Glu_{lactose} = \frac{180}{342} x\ Lac_{initial} = \frac{Lac_{initial}}{1.9}$$

The released amount of glucose is calculated in a similar way as galactose, with:

$$Glu_{TGOS} = Glu_{total} - Glu_{initial} - Glu_{lactose}$$

Subsequently, the total concentration of TGOS is calculated by adding the glucose released from TGOS to the galactose released from TGOS:

$$TGOS = \frac{162}{180} x\ Gal_{TGOS} + Glu_{TGOS} = 0.9\ x\ Gal_{TGOS} + Glu_{TGOS}$$

Results - Original AOAC Method

In figure 2 a typical chromatogram of the sugar standard mix is shown in black. The standard consist of 12 mg/L galactose, glucose and lactose in DI water. For reference a chromatogram of an injection of allolactose in DI water is shown as a red overlay. The resolution of galactose and glucose is 1.4, allowing quantification of both monosaccharides if required. However, allolactose and lactose are not well-separated, which could lead to the incorrect quantification of lactose if allolactose is present in the sample. Moreover, the increasing NaOH concentration during the gradient (from 18.75 mM at t = 24 min to 125 mM at t = 39 min) resulting in a gradually rising background current (baseline), which could potentially complicate integration of the lactose peak. The peak efficiencies found for the sugars ranged from 15.000 theoretical plates per meter for galactose and glucose to 75.000 theoretical plates per meter for lactose. All peak tailing factors ranged from 1.0 to 1.1.

Linearity

The linearity was investigated with standards dissolved in DI water in the concentration range of 0.5 - 48 mg/L. This concentration range is much larger than described in the AOAC method. The obtained calibration curves are shown in figure 3. Linearity is excellent with correlation coefficients for peak area of 1.0000, 0.9997 and 1.0000 for galactose, glucose and lactose, respectively. The larger concentration range of the calibration curve enabled that all samples (hydrolyzed and non hydrolyzed extracts) could be measured and quantified with the same dilution factor, and no additional sample specific dilution steps were required.

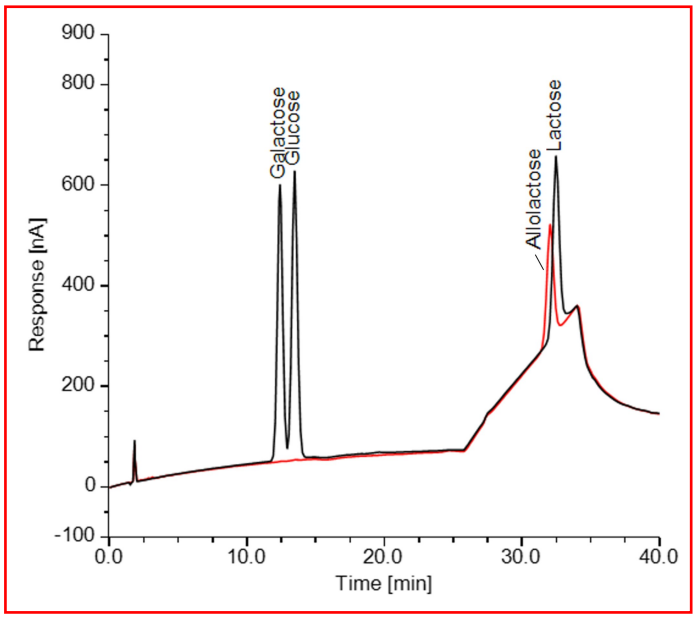

Figure 2: Overlay of a chromatogram of a 10 µL injection of 12 mg/L sugar standard mix (black) in DI water and a chromatogram of a 10 µL injection of 10 mg/L allolactose in DI water (red).

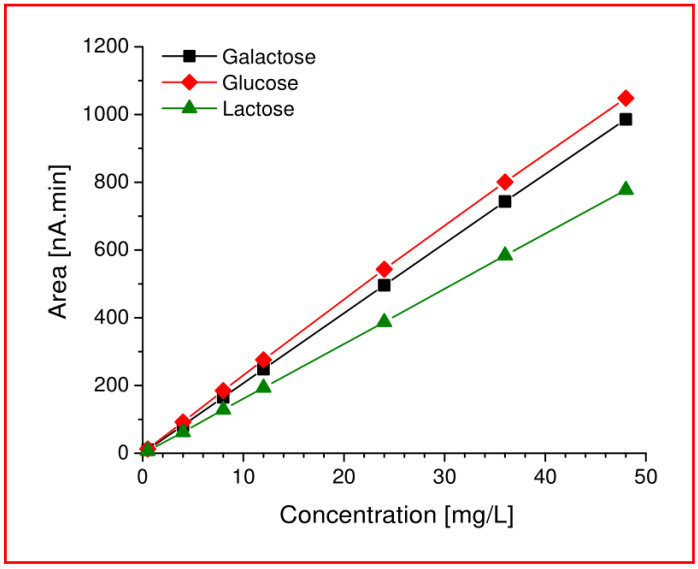

Figure 3: Calibration curves of galactose, glucose and lactose in the concentration range of 0.5 - 48 mg/L (standards in DI water).

Repeatability

The relative standard deviation (RSD) of the retention time and peak area were determined for 10 repetitive injections with a 0.5 and 4 mg/L standard (table 5). The RSD for peak areas for all sugars was < 1.5% for the 0.5 mg/L standard and < 0.6% for the 4 mg/L standard. These data demonstrate that with this method reproducible analysis of all the analytes of interest can be achieved using the ALEXYS Carbohydrates analyzer.

Detection limit

The LOD was calculated as the analyte response corresponding to 3x the ASTM noise determined on a 5-minute section of the baseline (average peak-to-peak baseline noise of 10 segments of 0.5 min). The noise level was 0.19 nA and the calculated LOD's were in the range of 10 - 22 µg/L or 120 - 220 pg on-column (Table 6).

Table 5

Repeatability of 10 µL injections of a 0.5 and 4 mg/L sugar standard mix in DI water (n=10)

	RSD's (%) 4 mg/L		*RSD's (%)* 0.5 mg/L	
Compound	t_R	*Area*	t_R	*Area*
Galactose	0.14	0.27	0.27	0.83
Glucose	0.13	0.32	0.30	0.75
Lactose	0.06	0.56	0.17	1.41

Table 6

Calculated Limit of Detection (LOD)

	Concentration		On-column amount	
Compound	*nM*	*µg/L*	*pmol*	*pg*
Galactose	69	12	0.69	120
Glucose	62	11	0.62	110
Lactose	64	22	0.64	220

Sample analysis

Two TGOS ingredients (TGOS A and B) were prepared and analyzed using the AOAC Method 2001.02. An overlay of the initial test solutions (assay 1) of the TGOS ingredients is shown in figure 4. The TGOS A sample (red curve) contains a small amount of free glucose and almost no galactose. In the TGOS B sample (black curve) a small amount of galactose is present and almost no glucose. Both TGOS samples contain a significant amounts of lactose. In addition, the peak shape of lactose (peak 3) does not look symmetrical and in case of TGOS B a clear shoulder peak is visible, which is likely caused by coelution of allolactose present in the TGOS ingredients.

The results for both samples are summarized in table 7. The TGOS content calculated using the k-factor and based on the released amount of glucose and galactose are both listed in the table. The difference between the calculated TGOS contents with both methods is not more than 2% for all samples. Note that a k-factor of k = 1.4, corresponding with an average degree of polymerization of n = 2, was used for all samples.

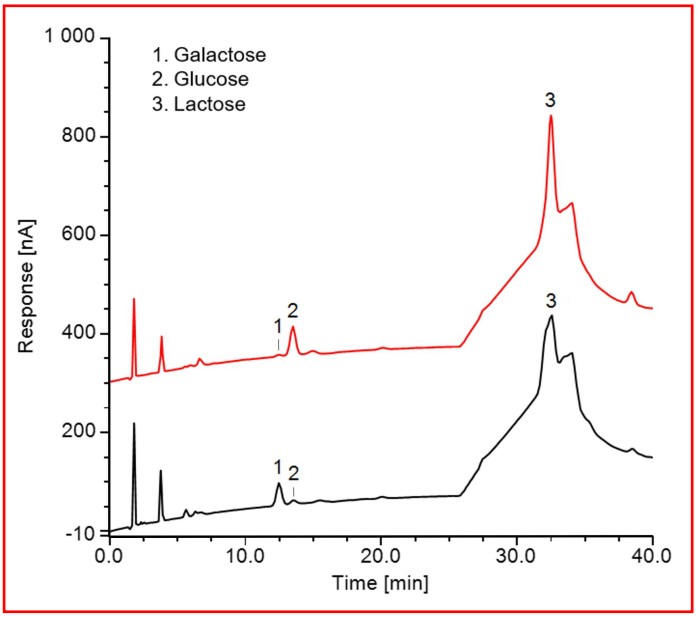

Figure 4: Overlay of the chromatograms obtained with the non hydrolyzed initial test solutions (assay 1) of TGOS A (red, +35% off-set) and TGOS B (black).

The actual degree of polymerisation and k-factor of the products might differ.

Table 7

TGOS content (g/100g sample)

Sample	Gal release from TGOS	Glu from TGOS	TGOS (0.9 x Gal + Glu)	TGOS (k x Gal)
TGOS A	2.66	1.37	3.76	3.72
TGOS B	2.82	1.34	3.87	3.94

Results - Adapted AOAC method

A typical chromatogram of the sugar standard mix obtained with the adapted method is shown in figure 5. The 12 mg/L calibration standard in DI water is shown in black and an 3 µL injection of 5 mg/L standard mix containing calibration standard and 3 lactose isomers (allolactose, lactulose, and epilactose) is shown as an overlay in red. The separation is significantly improved, galactose-glucose are well-separated (R ≥ 2.4), and lactose is baseline separated with its isomers (R ≥ 2.2). Due to the isocratic separation the background current is constant resulting in a flat baseline. All analytes of interest (galactose, glucose, and lactose) elute within 12 minutes. The total analysis time for each sample is reduced to 45 min, which includes a 5 min post-run step gradient for column clean-up and 25 min re-equilibration time. The peak efficiencies found for the sugars are in the range of 40000 - 55000 theoretical plates per meter. All peak tailing factors are in the range of 1.1 to 1.2.

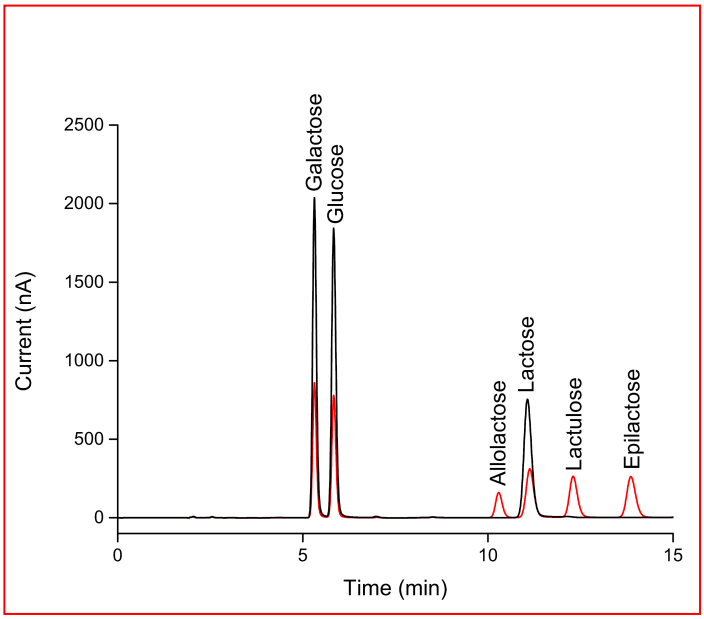

Figure 5: Overlay of a chromatogram of 3 µL injection of 12 mg/L sugar standard (black) and a chromatogram of 3 µL injection of 5 mg/L standard mix with lactose isomers (red).

Linearity

The linearity was investigated with standards dissolved in DI water in the concentration range of 0.5 - 48 mg/L . This improved method resulted in excellent linear response (correlation coefficient > 0.999 for all compounds). Despite that, a quadratic fit, ignoring origin and weighted with 1/ concentration^2, was used to further improve the quantification. The obtained calibration curves are shown in figure 6. The relative standard error of the calibration curves are 0.46%, 0.58%, and 0.52% for galactose, glucose, and lactose, respectively. Additionally, the relative residual error for all compounds in all calibration levels are within ± 1%. Overall, the obtained calibration curves indicate excellent accuracy and large dynamic range. Therefore, all samples (hydrolyzed and non hydrolyzed extracts) could be measured and quantified accurately with the same dilution factor using this calibration curve, and no additional sample specific dilution steps were required.

Repeatability

The relative standard deviation (RSD) of the retention time and peak area for 10 repetitive injections are summarized in table 8 for a 0.5 and 4 mg/L standard in DI water. Retention times were stable, with RSD values for retention time in the range of 0.15 - 0.18% for all analytes. The RSD for peak areas for all sugars are exceptionally good, with values of < 0.3% for the 0.5 mg/L standard and < 0.2% for the 4 mg/L standard. These data demonstrate that reproducible analysis of all the analytes of interest can be achieved with the ALEXYS carbohydrate analyzer using the improved method.

Table 8

Repeatability of 3 µL injections of a 0.5 and 4 and mg/L sugar standard mix in DI water (n=10)

	RSD's (%) 4 mg/L		*RSD's (%) 0.5 mg/L*	
Compound	t_R	*Area*	t_R	*Area*
Galactose	0.16	0.16	0.18	0.18
Glucose	0.15	0.06	0.17	0.17
Lactose	0.18	0.11	0.18	0.26

Detection limit

The LOD was calculated as the analyte response corresponding to 3x the ASTM noise determined on a 5-minute section of the baseline (average peak-to-peak baseline noise of 10 segments of 0.5 min, calculated on the baseline from t = 12 min to t = 17 min). The ASTM noise was 0.13 nA and the calculated concentration LODs were in the range of 2.3 - 6.3 µg/L.

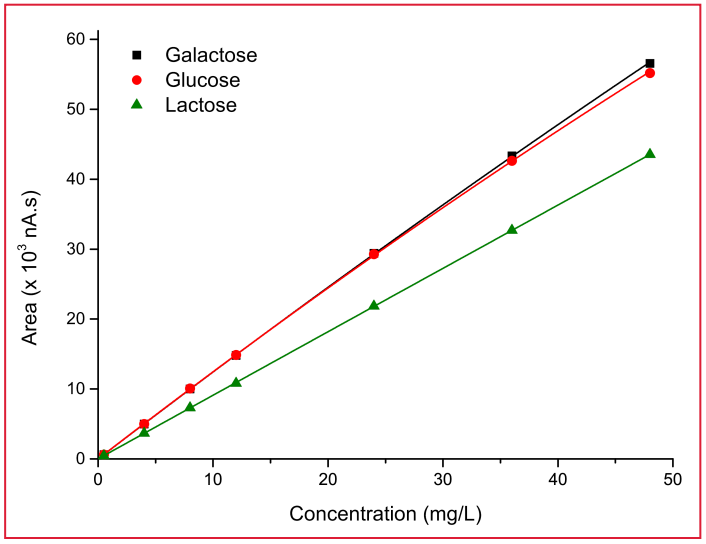

Figure 6: Calibration curve of galactose, glucose and lactose maltose in the concentration range of 0.5 - 48 mg/L

It is evident that the concentration sensitivity increased by a factor of 3 - 5× compared to the official method with the conventional 4.0 mm ID column. The on-column LOD is also significantly improved with this adapted method and ranges from 7.0 to 19.0 pg.

Table 9

Calculated limit of Detection (S/N = 3)

	Concentration		*On-column load*	
Compound	*nM*	*µg/L*	*fmol*	*pg*
Galactose	12.9	2.3	39	7.0
Glucose	13.9	2.5	42	7.5
Lactose	18.5	6.3	56	19.0

Sample analysis

The same TGOS ingredient samples were analyzed using the improved method. An overlay of the initial test solution of the two TGOS ingredients (assay 1) is shown in figure 7. Allolactose was found to be present in both samples, particularly in the TGOS B sample the response of allolactose is significant and as large as the response of lactose, indicating that the isomers may be present in equimolar concentrations.

Also, small amounts of the isomers lactulose and epilactose are identified in both samples. In addition to the identified peaks, numerous putative TGOS component are present in the chromatogram, but they are all well-separated and do not interfere with the quantification of galactose, glucose and lactose, either before or after hydrolysis (see figure 8).

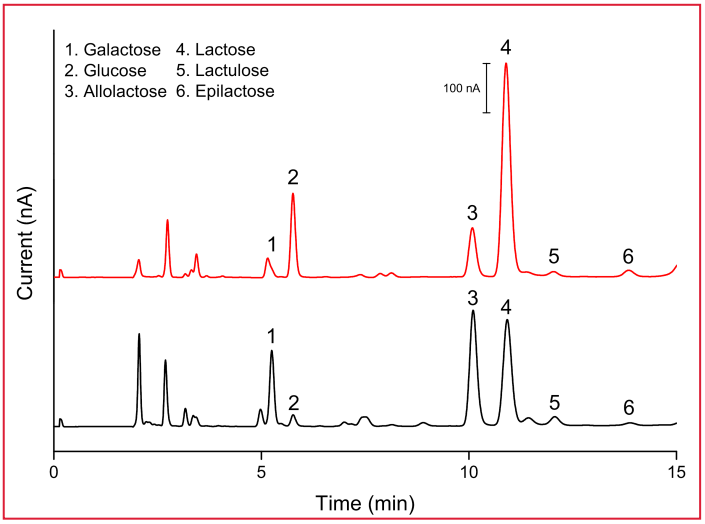

Figure 7: Overlay chromatograms obtained with the non hydrolyzed initial test solutions (assay 1) of TGOS A (red) and TGOS B (black).

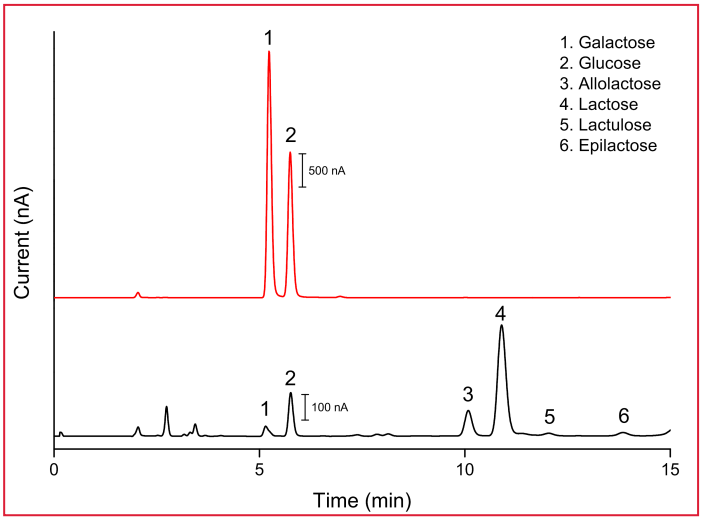

Figure 8: Overlay chromatograms obtained with the TGOS A sample. The initial test solution (assay 1) is shown in black. The hydrolyzed sample (assay 2) is shown in red.

An example chromatogram of milk spiked with TGOS A (sample S3) is shown in figure 9. The untreated sample (assay 1) shows a high level of lactose, of which the large portion is originating from the unspiked milk, and a small portion from TGOS A. The hydrolyzed sample (assay 2) is depicted in red. Lactose and allolactose are not detected in the enzymatically treated sample, indicating that the sample is fully hydrolyzed. The obtained results from all samples are summarized in table 10. The TGOS content calculated via the released glucose and galactose and the TGOS content calculated via the k-factor are both listed in the table. For all samples the calculation based on glucose and galactose and the k-factor correspond very well.

The measured TGOS A content of 3.3 g/100 g and TGOS B content of 3.7 g/100 g are consistent with the typical values

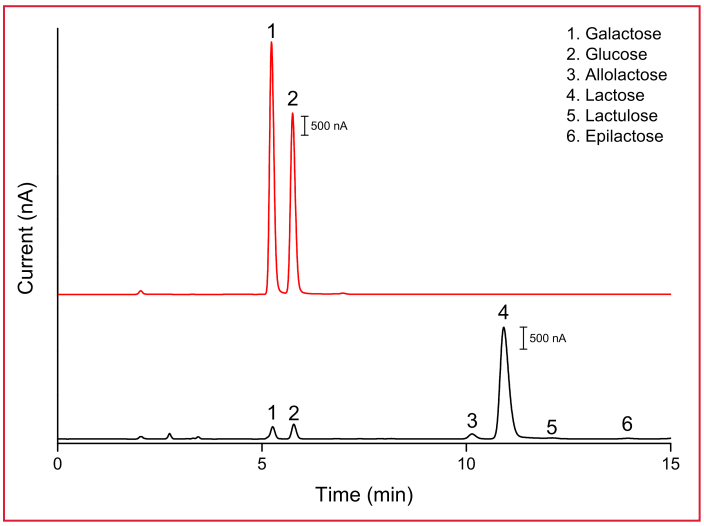

Figure 9: Overlay of chromatograms obtained with the TGOS spiked milk sample (S3). The initial test solution (assay 1 is shown in black). The hydrolyzed sample (assay 2) is shown in red.

stated on the product label (3.5 g/100 g for TGOS A and 3.65 g/100 g for TGOS B).

Table 10

TGOS content (g/100g sample)

Sample	Galactose from TGOS	Glucose from TGOS	TGOS (0.9 x Gal + Glu)	TGOS (k x Gal)
TGOS A	2.29	1.20	3.27	3.21
TGOS B	2.62	1.33	3.68	3.66
Sample S1	0.52	0.24	0.71	0.73
Sample S2	2.62	1.36	3.71	3.67
Sample S3	2.35	1.23	3.34	3.29
Sample S4	2.51	1.34	3.60	3.51

Comparison of methods

It can be concluded that the TGOS content obtained with the original AOAC method and the adapted method with improved separation (isocratic elution on the SweetSep AEX200 2.1 mm ID column are in accordance with the product labeling information. However, the resulting TGOS values determined based on the improved separation method are slightly smaller. This could be related to the shelf life of the TGOS samples. The experimental work based on the original AOAC method was executed in 2021, the evaluation of the adapted method in 2025. Both TGOS A and B samples expired in 2023 and may have undergone chemical degradation.

The SweetSep AEX200 is well suited for fast, high-resolution separation of lactose and isomers under isocratic conditions, as demonstrated in the application note “Analysis of Lactose and Isomers in ‘Lactose-free’ Labelled Products” [9]. Therefore, the application of this column for the determination of TGOS following the AOAC 2001.02 leads to a simpler, faster and significantly better separation. Furthermore, the shorter run time in combination with a lower flow rate, due to the reduced column ID, resulted in a higher sample throughput and about 80% reduction in mobile phase consumption and thus less waste (‘green’ analytical method).

To summarize the improvements on the AOAC (2001.02):

- Simple isocratic separation (within 15 minutes)
- Stabile baseline during separation (no NaOH gradient)
- Shorter run time (45 min) & higher sample throughput
- Less mobile phase consumption (80% reduction)
- Separation of lactose from all relevant isomers (R_s > 2.2)
- Better on-column LOD’s (almost 10—15× better)

The excellent sensitivity and linear range of detection of the DECADE Elite in combination with the SenCell allowed calibration over a larger concentration range of 0.5 - 48 mg/L (correlation coefficients > 0.999 for all sugars (linear fitting), or < 1% relative standard error for all sugars (quadratic fitting)) than specified in the AOAC 2001.02. The larger calibration range may help to simplify and reduce the large number of sample-specific dilution factors advised in the 2001.02, into a fixed set of dilution factors for the majority of food samples.

AOAC 2001.02 limitations

It should be mentioned in general that the AOAC 2001.02 method has some limitations with respect to applicability to certain food samples [11]. The method works well for samples with a high TGOS content and/or if the lactose contents is low. For samples like infant formula the analysis is challenging and not accurate enough, because such samples contain a high concentration of lactose and a low TGOS-to-lactose ratio (TGOS content typically below 10g/100g). As an example, the product label of the sample S1 (infant formula) specifies the TGOS content of 0.48 g/100 g. However, the TGOS content calculated based on the adapted method is 0.73 g/100 g. As another example, sample S2 (infant milk spiked with TGOS A) contains 3.67 g TGOS /100 g, while the TGOS A contains 3.21 g TGOS /100 g. The difference between these two samples are 0.46 g TGOS /100 g, which is closer to the content on the product label. TGOS-to-lactose content in the sample S2 was increased significantly by spiking, and therefore this standard addition method may result in a more accurate estimation of the actual TGOS content in a sample. Otherwise, for such samples containing high levels of lactose the AOAC 2021.01 method based on HPLC-FLD is a more suitable approach [12]. Furthermore, it is anticipated that when quantifying the TGOS contents based on the release of both galactose and glucose, after enzymatic conversion (so without using the k-factor), this will also become challenging in samples with high levels of glucose and a low TGOS contents.

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Conclusion

The ALEXYS Carbohydrate Analyzer with DECADE Elite detector and SenCell provides a fast and user-friendly analysis solution for the quantification of the TGOS content in food products following the AOAC 2001.02. An optimized method is presented using a SweetSep AEX200 high-resolution HPAEC column with 5 µm particle size. The column enables fast isocratic separation of all analytes of interest within 15 minutes, combined with excellent resolution for lactose and relevant isomers (Rs > 2.2). Furthermore, a shorter run time (45 min) in combination with a lower flow rate resulted in a higher sample throughput and a reduction of 80% in mobile phase consumption. As an alternative, the TGOS contents could be quantified by analysing both the galactose as well as the glucose release after hydrolysis. In that case product information from the manufacturer about the k-factor or degree of polymerization is not required.

Ordering information

Detector only	
176.0035B	Decade Elite SCC electrochemical detector
116.4321	Sencell 2 mm Au HyREF
Recommended ALEXYS analyzer	
180.0057W	ALEXYS Carbohydrates Analyzer - gradient (quaternary LPG)
116.4321	SenCell 2 mm Au HyREF
186.ATC00	CT2.1 Column Thermostat
Column	
260.0020	SweetSep™ AEX200, 2.1×200 column, 5 µm
260.0025	SweetSep™ AEX200, 2.1×50 precolumn, 5 µm
260.0031	Borate ion trap, 2.1×200 column, 10 µm
260.0100*	Pre-column filter PEEK, 0.5 µm
Software#	
195.0035	Clarity CDS single instr. incl LC, AS module

*) In case samples might contain particulate matter it is advised to use a pre-column filter.

#) Antec ECD drivers are available for Chromeleon CDS, OpenLAB CDS and Empower CDS. The ALEXYS Carbohydrates Analyzer (full system) can also be controlled under ThermoFisher Scientific Chromeleon™ CDS. Please contact Antec for more details.

Reagents, chemicals and sample prep accessories

Chemicals	
50% (w/w) NaOH, carbonate-free	Fisher Scientific, pn SS254-500
Sodium acetate trihydrate, HPLC grade	Fisher Scientific, pn 10122400
Galactose	Sigma, pn Car-11 (Carbohydrate kit)
Glucose	Sigma, pn Car-11 (Carbohydrate kit)
Lactose	CarboSynth, pn OL04771
Allolactose	CarboSynth, pn OG09259
Lactulose	Sigma, pn 61360-5G
Epilactose	CarboSynth, pn OG04727
β-galactosidase	Megazyme, pn E-BGLAN
Acetonitrile, 99.99% LC-MS grade	Fisher Scientific, pn A955-212
Potassium hexacyanoferrate trihydrate	Fluka, pn 60280
Zinc sulfate heptahydrate	Sigma, pn 31665
Potassium dihydrogen phosphate	Fisher, pn P-4806-60
Potassium hydrogen phosphate	Fisher, pn 11593
TGOS B	BioSynth, pn OG32134
DI water 18.2 MΩ.cm, TOC < 5 ppb	YoungIn Chromass Aquapuri Essence+ 393
Choice™ PES (Polyethersulfone) Syringe Filters, 0.22 µm, 25 mm Ø FFL/MLS	ThermoFisher Scientific, pn CH2225-PES

For research purpose only. The information shown in this communication is solely to demonstrate the applicability of the ALEXYS system and DECADE Elite detector. The actual performance may be affected by factors beyond Antec's control. Specifications mentioned in this application note are subject to change without further notice.

Antec Scientific (USA)
info@AntecScientific.com
www.AntecScientific.com
T 888 572 0012

Antec Scientific (worldwide)
info@AntecScientific.com
www.AntecScientific.com
T +31 (172) 268888

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