



The finest HPAEC-PAD  
Applications for  
Carbohydrate Analysis

**Food and Beverage**

Mono- and disaccharides  
Sugars in meat & fish  
Carbohydrates in food  
according to AOAC  
Carbohydrates in instant  
coffee  
Carbohydrates in honey

**Prebiotics Food Additives**

Fructans in infant formula  
TGOS in food products  
Profiling of FOS

**Lactose Free Products**

Lactose in dairy & meat  
Lactose in lactose-free  
products

**Artificial Sweeteners**

Sugar alcohols

## Characterization of Inulin-type fructan mixtures

- **SweetSep™ AEX200 column**
- **High-resolution HPAEC-PAD separation**
- **Profiling of Inulin and fructo-oligosaccharide**
- **Quantification of short-chain FOS**
- **Dietary fiber, prebiotic and food additive**

Fructans are polymers consisting of fructose found widely distributed in nature as plant storage carbohydrates. Inulin-type fructans (ITF) are present in many plant species including wheat, onion, bananas, garlic and chicory. Estimates suggest a person consuming an average European diet ingests 3-11 g inulin-type fructans daily [1]. Fructans are a form of dietary fiber as they are not digested in the upper gastrointestinal tract. They can act as an energy source for the gut microbiota and have the potential to promote the growth of specific beneficial bacteria. Furthermore, ITF is increasingly being used as a food additive, for example as a low-caloric sweetener or a texture-improving ingredient of low-fat foods [2].

Fructans exist as heterogeneous blends of polymers, with degrees of polymerization ranging from 2 to 60 subunits. The properties of inulin-type fructan as a functional food ingredient depend mainly on the average degree of polymerization (DP). The average DP can vary, depending on source, harvest time, and processing conditions [3]. In this application note a method is presented for the analysis of the chain-length distribution of Inulin-type fructans. The use of High-performance anion-exchange chromatography (HPAEC), coupled with pulsed amperometric detection (PAD), enables the complete separation and sensitive detection of individual oligosaccharides. To demonstrate the specificity and sensitivity of this method, the oligo- and polysaccharide distribution of several inulin samples was characterized by HPAEC-PAD using a new anion-exchange column SweetSep™ AEX200.



## Introduction

Fructans are polymers consisting of fructose building blocks and serve as an energy-storage carbohydrate in many plants. Inulin-type fructans (ITF) are present in over 36,000 plant species and are widely used in the food industry because of their nutritional and functional properties. Estimates suggest a person consuming an average European diet ingests 3-11 g of inulin-type fructans daily [1]. Inulin-type fructans are linear polymers where the fructose residues are bound to one another by  $\beta$ -2,1 linkage with an optional terminal glucose unit. Hence, all inulin-type fructans can be described with the generic chemical structure  $GF_n$  (with G as optional glucose, F as fructose, and n indicating the number of fructose moieties).

Inulin is typically extracted as native inulin from a food source such as chicory root. Native inulin is a heterogenous blend of oligo- and polysaccharides with a degree of polymerization (DP) ranging from 2 to 60. Some of the fructans have a glucose unit at the reducing end ( $GF_n$  type fructans), while others do not include a glucose residue at all ( $F_n$  type fructans). The functional properties of inulin mainly depend on the degree of polymerization. To achieve its desired properties, native inulin can be processed into more purified food ingredients, such as long-chain inulin (DP 10-60) or the shorter fructooligosaccharides (FOS, DP 2-7). Short-chain FOS (scFOS, DP 2-4) can be obtained by the enzymatic elongation of sucrose with fructose moieties [2].

The  $\beta$ -2,1 linkages prevent inulin from being digested like a typical carbohydrate. The fructans are not broken down by human digestive enzymes and are therefore available as a nutrient for beneficial bacteria. The shorter fructans are fermented first by the colonic microflora. The longer chain inulin is fermented twice slower than FOS, and therefore stimulates the metabolic activity of the improved flora in more distal parts of the colon [4]. As the fructans resist enzymatic digestion and reach the gut intact, they are also considered dietary fiber from a food-labeling perspective.

Inulin-type fructans are also increasingly being used as food additives. Several commercial grades of inulin are available that have a neutral, clean flavor and are used to improve the mouthfeel, stability, and acceptability of healthier food products. For example, short-chain inulin is soluble and sweet with minimal caloric value and can be used to partially replace sucrose in low-sugar products. Long-chain inulin is less soluble and more viscous and can be used to simulate the presence of fat in low-fat products [5].

High-performance anion-exchange chromatography (HPAEC) coupled with pulsed amperometric (PAD) detection has already been demonstrated as a powerful tool to profile the chain-length distribution of FOS and inulin [6]. Evaluation of the chain length distribution of Inulin-type fructans is important as it affects the nutritional and functional properties. This application note describes a method to profile Inulin-type fructans using HPAEC-PAD. Several grades of Inulin-type fructans with varying chain length distributions were analyzed to demonstrate the performance of the method.

Additionally, quantification of Inulin-type fructans as dietary fiber is also required for food-labeling purposes. However, the lack of commercial standards hampers the direct quantification of fructans with a DP over 5. A method based on enzymatic hydrolysis followed by analysis HPAEC-PAD suitable for the quantification of all lengths of fructans is described in application note 220\_022: Determination of Fructans in infant formula and adult nutritionals. The quantification of scFOS is possible with the same method for profiling as commercial standards are available for these short oligosaccharides.



Figure 1. ALEXYS™ Carbohydrate Analyzer consisting of the ET210 eluent tray (for N<sub>2</sub> blanketing), a P6.1L quaternary LPG pump, AS6.1L autosampler, CT2.1 column thermostat, and the DECADE Elite electrochemical detector.



**Table 1**

Conditions	
HPLC system	ALEXYS™ Carbohydrate Analyzer
Detector	DECADE™ Elite electrochemical detector
Columns	SweetSep™ AEX200, 4 × 200 mm column, 5 μm SweetSep™ BIT, 4 × 50 mm borate ion trap All columns: Antec Scientific
Filter	Pre-column filter PEEK, 0.5 μm
Mobile phase (MP)	A: 100 mM NaOH B: 100 mM NaOH, 500 mM NaOAc Eluents prepared & blanketed with Nitrogen 5.0
Flow rate	0.8 mL/min
System backpressure	About 250 bar at the start of run, about 275 bar at the end of the NaOAc gradient (t=60 min)
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 1
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 0.5 μA
ADF	0.05 Hz
Range	10 μA/V

**Table 2**

Gradient program for Inulin ( $DP_{max} \geq 9$ )		
Time (min)	Mobile phase	Description
0	100 mM NaOH	Elution & detection
12	100 mM NaOH, 180 mM NaOAc	
60	100 mM NaOH, 450 mM NaOAc	
60 - 75	100 mM NaOH	Equilibration, starting conditions

**Table 3**

Gradient program for FOS ( $DP_{max} \leq 8$ )		
Time (min)	Mobile phase	Description
0	100 mM NaOH	Elution & detection
12	100 mM NaOH, 180 mM NaOAc	
12 - 22	100 mM NaOH, 450 mM NaOAc	Column clean-up and regeneration
22 - 37	100 mM NaOH	Equilibration, starting conditions

## Method

The method for profiling of Inulin-type fructans was evaluated using ALEXYS™ Carbohydrate Analyzer (Figure 1). The system consists of an ET210 eluent tray for Nitrogen blanketing, a P6.1L quaternary LPG pump, an AS6.1L autosampler, a CT2.1 column thermostat, and the DECADE Elite electrochemical detector. Take into account that the selection of a specific quaternary HPLC systems may influence the separation performance, and may require some small customization of the conditions to achieve the results outlined in this application note. The SenCell with Au working electrode and HyREF reference electrode was selected for sensitive detection of the carbohydrates.

## Separation

Under alkaline conditions (pH > 12) carbohydrates can be separated using 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. Only polymeric anion-exchange columns are suitable for carbohydrate separation at such alkaline conditions. The retention time of carbohydrates is inversely correlated with the pKa value and increases significantly with molecular weight. A high-resolution anion-exchange column with a small particle size (5 μm), SweetSep™ AEX200, was chosen for the separation of the fructans. The morphology and exchange capacity of this new AEX200 stationary phase was specifically optimized to enable the analysis of a wide variety of carbohydrates samples ranging from monosaccharides present in food, plants and glycoproteins up to polysaccharides such as FOS. The use of a pre-column filter is advised when using samples which might contain particulate matter.

The separation was performed at 25°C. For the analysis of inulin with a degree of polymerization up to 65, the gradient shown in Table 2 was used. All FOS oligo- and polysaccharides are eluted within 60 minutes using up to 450 mM sodium acetate gradient in 100 mM NaOH. Subsequently, the column is equilibrated for 15 minutes at the starting conditions, resulting in a total run time of 75 minutes.

For the analysis of shorter oligos with a max DP of 8 a shorter gradient program was used (table 3). The gradient is exactly the same for the first 12 minutes. After elution of the oligosaccharides with a DP ≤ 8, a strong column clean-up and regeneration step is executed at t =12 min to elute strongly retaining components and to remove carbonate ions (CO<sub>3</sub><sup>2-</sup>)



## Profiling of Inulin-type fructans

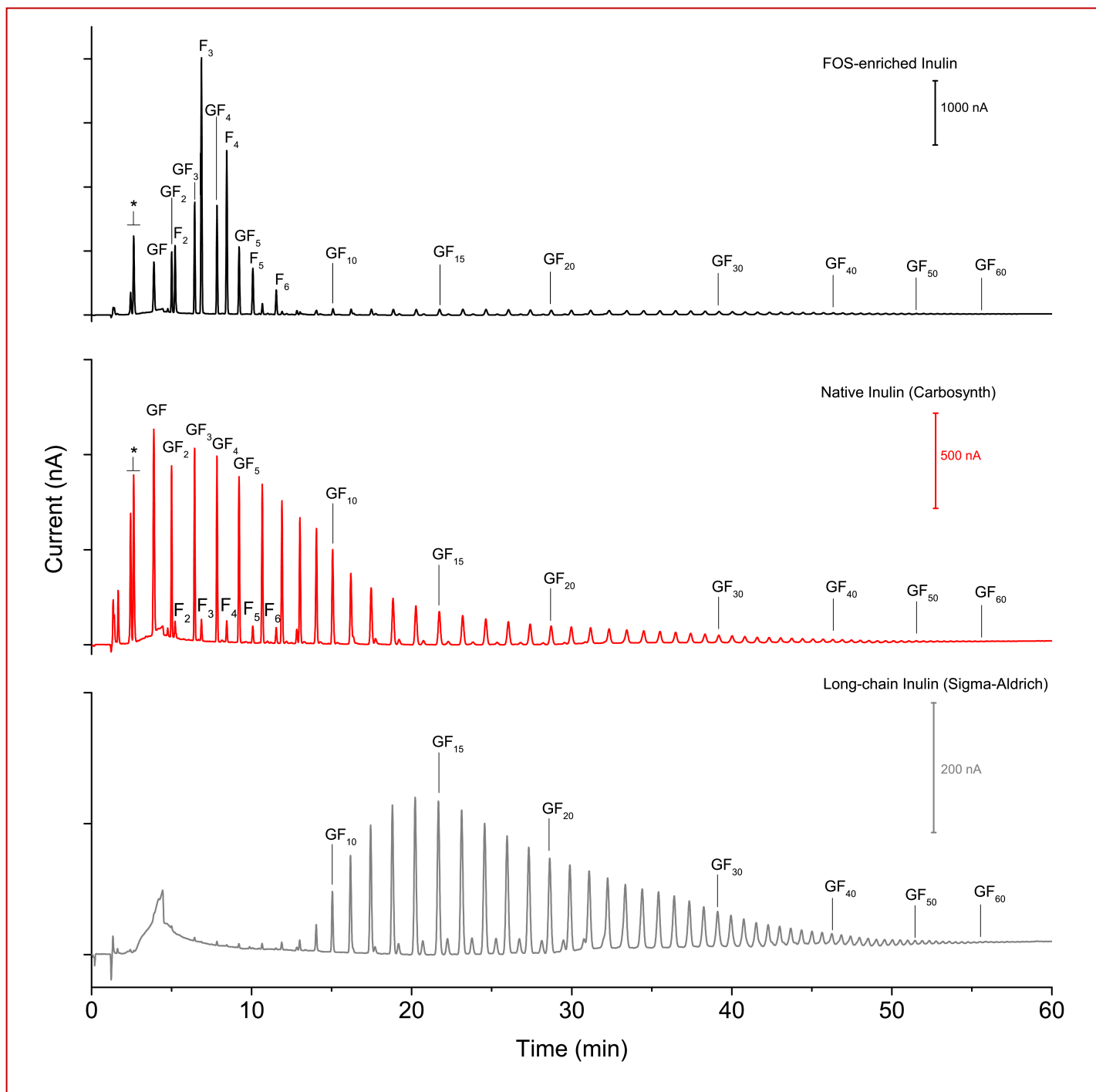


Figure 2. Overlay of 10  $\mu\text{L}$  injections of 200 ppm of inulin-type fructan samples. The Inulin-FOS mixture (Jarrow Formulas) is shown in black and was confirmed a mix of FOS and long-chain inulin. The Inulin from chicory from Carbosynth is shown in red and was characterized as native inulin. The Inulin from chicory from Sigma-Aldrich is shown in grey and was characterized as purified long-chain inulin. Part of the fructans are labeled with their respective generic chemical structure  $\text{GF}_n$  (with G as glucose, F as fructose, and n indicating DP). The monosaccharides (glucose and fructose) are indicated with an asterisk.

build up on the column. After the clean-up step, the column is equilibrated for 15 minutes at the starting conditions, resulting in a total run time of 37 minutes.

### Detection

For the pulsed amperometric detection of oligosaccharides 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/  $\text{H}_2$ ) reference electrode (RE) and stainless steel auxiliary electrode (AE). A 4-step potential waveform was applied as described in Table 1. The detection temperature was set to 35°C. The cell current was typical about



0.5  $\mu\text{A}$  with these PAD settings under the specified conditions. This particular 4-step waveform has the following benefits: (1) a consistent long-term peak area response and (2) minimal electrode wear [7], resulting in less flow cell maintenance and system down time.

## Sample preparation

Three products containing inulin-type fructans were obtained for this study. Inulin powder from chicory was purchased from 2 suppliers: Carbosynth (Compton, United Kingdom) and Sigma-Aldrich (Saint Louis, USA). One commercial consumer product was obtained from local stores: Jarrow Formulas Inulin-FOS food supplement containing Orafiti® Synergy1.

One gram of powder was dissolved in 50 mL of DI water, filtered over a 0.22  $\mu\text{m}$  Polyethersulfone (PES) syringe filter (25 mm  $\varnothing$  FFL/MLS), and diluted to its final concentration (200 ppm) with DI water. Samples were kept at 4°C and injected within 24 hours.

## Results

### Characterization of Inulin

The 3 samples containing inulin were characterized according to the method described in Tables 1 and 2. An overlay of the chromatograms is shown in Figure 2. Note that this chain-length distribution can be primarily interpreted qualitatively; the response factor decreases linearly with the chain length [8], hence the qualitative distribution is biased towards the lower mass fructans and does therefore not represent the exact quantitative distribution.

The chromatogram of a 10  $\mu\text{L}$  injection of 200 ppm Inulin from Carbosynth is shown as the red trace in Figure 2. The inulin consists predominantly of  $\text{GF}_n$  type fructans ranging from DP 3 ( $\text{GF}_2$ ) to approximately DP 61 ( $\text{GF}_{60}$ ). Additionally, this sample contains a substantial amount of free sugars (glucose, fructose, and sucrose). Based on the chain-length distribution of this mixture and the presence of free sugars this was characterized as native inulin, which is the crude hot-water extract of inulin. This sample demonstrates the excellent separation of the presented method. The  $\text{GF}_n$  and  $\text{F}_n$  type fructans are baseline separated until  $\text{GF}_7$  and  $\text{F}_7$ . However, the  $\text{GF}_n$  type series and the  $\text{F}_n$  type series show slightly different retention behavior, and therefore, they inevitably overlap, resulting in the coelution of components starting with  $\text{GF}_8$  and  $\text{F}_8$ .

The chromatogram of a 10  $\mu\text{L}$  injection of 200 ppm inulin from Sigma-Aldrich is shown as the grey trace in Figure 2. The chain

length of this mixture varies from DP 4 ( $\text{GF}_3$ ) to approximately DP66 ( $\text{GF}_{65}$ ) and is free of any mono- and disaccharides. This mix of inulin has been processed to remove all oligomers resulting in high molecular weight inulin and is also known as long-chain inulin.

The two inulin powders from both manufacturers were labeled as “Inulin from chicory” without any additional specification. Further characterization revealed that inulin powders consisted of different grades of Inulin. The terms Inulin, FOS, and terms of proprietary mixes of the two are often used inconsistently throughout industry and academia [9]. In the most generic sense, inulin is a term that covers all fructans of the inulin-type, however, it can also be used to specifically describe the hot-water extract from an Inulin-containing material.

The Inulin-FOS food supplement, shown as the black trace in Figure 2, was confirmed to be inulin enriched with FOS. This blend of inulin-type fructans consists of two distinct chain-length distributions. The first distribution consists of  $\text{F}_n$  and  $\text{GF}_n$  type oligomers up to a DP of 7 ( $\text{GF}_6$ ). The second distribution consists of solely  $\text{GF}_n$  type fructans ranging from DP 9 ( $\text{GF}_8$ ) to DP 66 ( $\text{GF}_{65}$ ).

### Characterization and quantification of free sugars and short chain FOS

The short chain fructooligosaccharide (scFOS) samples with DP < 8 were analyzed according to method described in Tables 1 and 3. The gradient used for these scFOS samples has exactly the same slope as the gradient used for inulin samples, hence the retention times of the oligomers match the retention times from the low-mass components of the inulin samples exactly. The quantification of most inulin-type fructans is limited by the lack of commercially available sugar standards. However, for scFOS and mono- and disaccharides all standards are available and therefore mixtures containing only scFOS can be quantified using the presented method.

A chromatogram of a 10  $\mu\text{L}$  injection of a 10 ppm sugar standard containing glucose (G), fructose (F), sucrose (GF), kestose ( $\text{GF}_2$ ), nystose ( $\text{GF}_3$ ), and fructosyl nystose ( $\text{GF}_4$ ) is shown in Figure 3. The monosaccharides (glucose and fructose) are baseline separated ( $R_{\text{G-F}} = 1.6$ ), and can be reliably quantified as shown by the repeatability (Table 4) and linearity data (Figure 4). All oligosaccharides are baseline separated ( $R > 1.5$ ) and show no significant tailing with symmetry factors around 1.0–1.2.





## Profiling of Inulin-type fructans

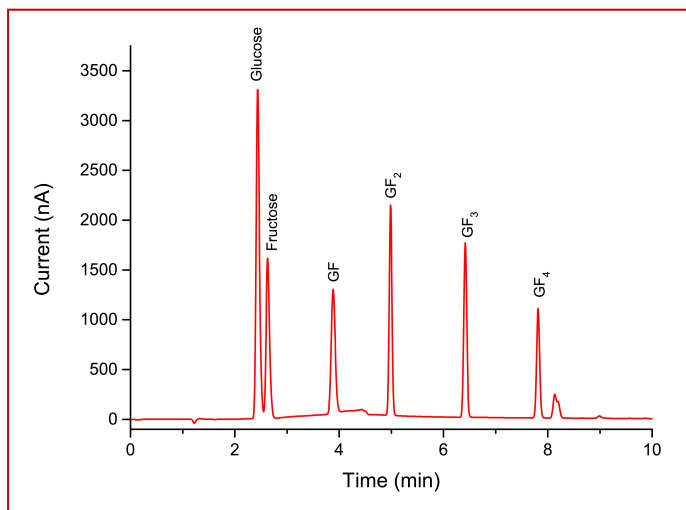


Figure 3. Chromatogram of 10 µL injections of 10 ppm standard solution containing glucose (G), fructose (F), sucrose (GF), kestose (GF<sub>2</sub>), nystose (GF<sub>3</sub>), and fructosyl nystose (GF<sub>4</sub>).

### Linearity and LOQ

The linearity was investigated with standards dissolved in DI water in the concentration range of 0.25 - 25 mg/L. The obtained calibration curves are shown in Figure 4. The linearity is excellent in this concentration range with correlation coefficients (*r*) for peak area better than 0.9991 for all 6 sugars except for kestose (GF<sub>2</sub>, *r* = 0.9987).

The LOQ was calculated as the analyte response corresponding to 10x the ASTM noise determined on a 5-minute section of the

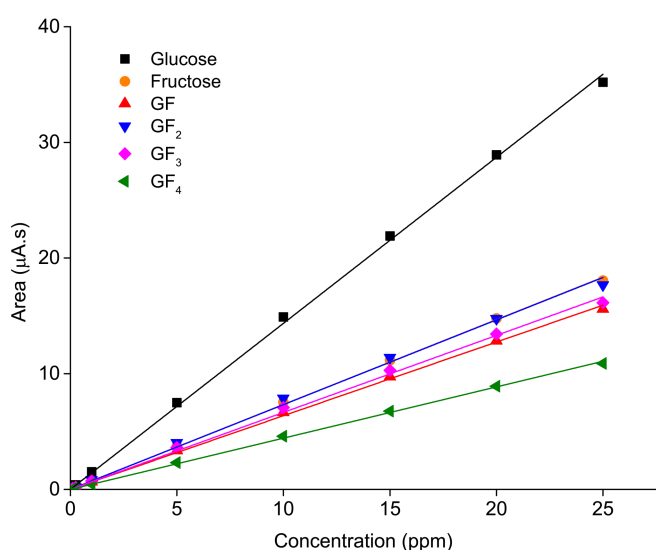


Figure 4. Calibration curve of the glucose (G), fructose (F), sucrose (GF), kestose (GF<sub>2</sub>), nystose (GF<sub>3</sub>), and fructosyl nystose (GF<sub>4</sub>) in the concentration range of 0.25 - 25 mg/L.

baseline (average peak-to-peak baseline noise of 10 segments of 0.5 min). The average ASTM noise was 0.27 nA and the calculated concentration LOQs were 8, 17, and 24 µg/L for glucose, fructose, and sucrose, respectively. The concentration LOQs for the scFOS were 12, 15, and 25 µg/L for GF<sub>2</sub>, GF<sub>3</sub>, and GF<sub>4</sub>, respectively. During the sample preparation 1 g is dissolved in 50 mL of water, hence the highest LOQ of 25 µg/L for GF<sub>4</sub> corresponds to 0.125 mg/100 g product, which is a factor 800 below the required LOQ for labelling purposes (0.1g/100g).

### Repeatability

The relative standard deviation (RSD) of the retention time and peak area were determined for 5 repetitive injections with a 1 mg/L and 0.25 mg/L sugar standard in water. Retention times were stable, with RSD values < 0.20% for all oligos and free sugars independent of concentration. The RSD values for the peak area are shown in Table 5. The RSDs for peak area are ≤ 1.65% for the 0.25 mg/L standard and typically < 0.65% for the 1 mg/L standard. The data demonstrate that with this method reproducible analysis of all the components, including the free sugars, can be achieved.

Table 4

Repeatability of 10 µL injections of a 0.25 and 1 mg/L sugar standard mix in DI water (n=5)

Compound	RSD's (%)		RSD's (%)	
	1 mg/L		0.25mg/L	
	<i>t<sub>R</sub></i>	Area	<i>t<sub>R</sub></i>	Area
G	0.18	0.39	0.07	0.74
F	0.17	0.40	0.06	0.45
GF	0.12	0.64	0.08	1.65
GF <sub>2</sub>	0.09	0.57	0.04	0.43
GF <sub>3</sub>	0.08	0.29	0.04	0.39
GF <sub>4</sub>	0.07	0.24	0.03	1.37

### Quantification of free sugars

The free sugars (glucose, fructose, and sucrose) content for all three samples was quantified using the presented method. The obtained values and the specified maximum free sugar content for the fructan powders are summarized in Table 5. The mono- and disaccharide content in the inulin samples are all in accordance with the specifications.



**Table 5**

Free sugars content (n = 4)

Fructan type	Glucose [g/100g]	Fructose [g/100g]	Sucrose [g/100g]	Total [g/100g]	Specified [g/100g]
Inulin-FOS	0.54	3.79	3.20	7.53	≤ 8
Native inulin	1.05	2.71	4.46	8.22	≤ 10
Long-chain Inulin	-	-	-	-	≤ 0.1

## References

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## Conclusion

The ALEXYS™ Carbohydrate Analyzer in combination with the SweetSep™ AEX200 offers a dedicated and optimized analysis solution for the characterization of mixtures of inulin-type fructans, quantification of short-chain FOS, and determination of free sugar content. The chain-length distribution of complex Inulin mixtures can be determined within 75 minutes, quantification of short-chain FOS and free sugars can be performed in just 37 minutes. The results obtained from various samples, ranging from short-chain FOS to long-chain inulin, show that this method is well suitable for the characterization of heterogeneous blends of inulin-type fructans.



# Profiling of Inulin-type fructans

## Ordering information

<b>Detector only</b>	
176.0035B	DECADE Elite SCC electrochemical detector
116.4321	SenCell 2 mm Au HyREF
<b>Recommended ALEXYS analyzer</b>	
180.0057W	ALEXYS Carbohydrates Analyzer - gradient (quaternary LPG)
116.4321	SenCell 2 mm Au HyREF
186.ATC00	CT2.1 Column Thermostat
<b>Column</b>	
260.0010	SweetSep™ AEX200, 4 x 200 mm column, 5 µm
260.0030	SweetSep™ BIT, 4 x 50 mm borate ion trap
260.0100#	Pre-column filter PEEK, 0.5 µm
<b>Software*</b>	
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.

\*) The ALEXYS Carbohydrates Analyzer can also be controlled under Thermo Fisher Scientific Chromeleon™ CDS. For the DECADE Elite electrochemical detector only also control drivers are available in Waters Empower™, Agilent OpenLab CDS and Agilent OpenLab CDS Chemstation Edition. Please contact Antec for more details.

*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 and may be adjusted accordingly. Specifications mentioned in this application note are subject to change without further notice.

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