Application Note
Carbohydrate Analysis

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

Oligo– and polysaccharides in honey & agave syrup

- **ALEXYS Carbohydrate Analyzer**
- **SweetSep™ AEX200 anion-exchange column**
- **High-resolution separation of oligo– and polysaccharides**
- **Separation of fructo– and maltooligosaccharides**
- **Honey and agave syrup samples**

Summary

Natural sweeteners such as honey and agave syrups are vulnerable to adulterations with cheaper sweeteners, compromising the nutritional value and health benefits from these natural sweeteners. Oligo– and polysaccharides are one of the crucial parameters to detect adulteration in these food products [1]. Due to the small quantities of these longer carbohydrate chains, they are often separated and detected using a sensitive detection method such as HPAEC-PAD. HPAEC-PAD enables oligo– and polysaccharides profiling and allows for accurate assessment of the authenticity of honey and agave syrups [2, 3].

This application note is complementary to the previously published application note “220_028— Oligo– and polysaccharides as fraud marker in honey”. The previous application note shows the value of oligo– and polysaccharide profile of a honey sample to detect low level adulteration. In this application note, a HPAEC-PAD method is presented to improve the existing oligo– and polysaccharides profiling method by separation of two different types of oligosaccharides (fructo– and maltooligosaccharides), leading to identification of the adulterant present in the sample. Honey and agave syrup samples were analyzed using the presented method, highlighting the method’s ability to obtain a detailed carbohydrate profiling and to detect adulteration.

Introduction

Consumption of foods with a high glycemic index (GI) is often associated with several health conditions such as diabetes, obesity, and cardiovascular diseases. Consequently, there is a growing trend on adopting a low GI diet, which recommends replacing table sugar with sweeteners with a smaller GI, such as agave syrup. Agave syrup is a natural sweetener produced from agave plants with 3—5× lower GI index compared to table sugar [4]. However, it is also a frequent target of food fraud due to its high production cost and lengthy production process, which involves harvesting mature agave plants that are 5 to 7 years old [4].

Similarly, honey is another popular natural sweetener that is vulnerable to adulteration despite regulatory frameworks in the EU and USA [5, 6]. Instances of fraud often involve the addition of cheaper sweeteners, such as corn syrup or rice syrup, to honey. These fraudulent practices go undetected due to the diverse compositions of authentic honey, which vary based on geographical origin and floral sources [7]. One parameter with potential for detecting honey adulteration is the oligo- and polysaccharide profile [1], which exhibits specific characteristics in each type of honey. Significant changes in the carbohydrate profile can indicate the presence of adulterants. Therefore, the carbohydrates profile is a good screening tool for honey adulteration.

Figure 1: ALEXYS Carbohydrate Analyzer.

Building upon previous application note 220_028, which focused on oligo- and polysaccharides in honey, this application note delves deeper into profiling by separating two types of oligosaccharides (fructo– and maltooligosaccharides) present in samples. Separation of these oligosaccharides in honey samples is crucial, as various syrups used as adulterants contain distinct types of oligosaccharides. For instance, corn syrup is primarily composed of maltooligosaccharides [2, 3]. By determining the type of oligosaccharides, it is possible not only to detect adulteration but also to identify the specific adulterants present.

In this application note, high-performance anion exchange chromatography (HPAEC) was selected as the preferred separation technique, with a strong anion-exchange SweetSep™ AEX200 column employed for this purpose. To demonstrate the method's applicability, two honey samples and agave samples were analyzed, providing insights into the value of the approach in detecting and identifying adulterants in these natural sweeteners.

Method

The HPAEC-PAD analysis of oligo– and polysaccharides was performed using the ALEXYS Carbohydrate Analyzer, consisting of an ET210 eluent tray for nitrogen blanketing, P6.1L quaternary LPG pump, AS6.1 autosampler, CT2.1 column thermostat, and DECADE Elite electrochemical detector (Figure 1). The SenCell electrochemical flow cell with Au working electrode and HyREF reference electrode was selected for sensitive detection of the carbohydrates. The HPAEC-PAD system was operated using the method settings specified in Table 1. Note: in case a third-party quaternary HPLC system is used this may influence the separation performance, and may require some small customization of the conditions to achieve the results outlined in this application note. A few precautions are made to guarantee method reproducibility and system stability. Those precautions are related to working with ion exchange chromatography using a mobile phase at a high pH.

Carbonate ions

Carbonate ions (CO_3^{2-}), which can be formed from CO_2 originating from the air can get easily dissolved in the mobile phase at high pH and can interfere with carbohydrate retention on anion exchangers due to their strong binding properties as a divalent ion. This will lead to shorter retention times, decreased column selectivity, loss in resolution, and poor reproducibility. To minimize the introduction of carbonate ions in the mobile phase the eluents were carefully prepared

Table 1

HPAEC-PAD conditions

HPLC system	ALEXYS™ Carbohydrate Analyzer - gradient (quaternary low pressure gradient)
Detector	DECADE™ Elite electrochemical detector
Columns	SweetSep™ AEX200, 4 × 200 mm column, 5 µm SweetSep™ AEX200, 4 × 50 mm precolumn, 5 µm SweetSep™ BIT, 4 x 50 mm borate ion trap All columns: Antec Scientific
Mobile phase (MP)	A: 100 mM NaOH B: 100 mM NaOH + 500 mM NaOAc Eluents prepared & blanketed with Nitrogen 5.0
Flow rate	0.7 mL/min
Back pressure	about 220—245 bar
Injection	10 µL
Temperature	30 °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 0.3 µA
ADF	0.5 Hz
Range	5 µA/V

Table 2

Gradient program

Time (min)	*%A*	*%B*	*Mobile Phase*	*Description*
0	95	5	100 mM NaOH + 25 mM NaOAc	Gradient elution and detection
20	70	30	100 mM NaOH + 150 mM NaOAc	
45	10	90	100 mM NaOH + 450 mM NaOAc	
45 - 50	10	90	100 mM NaOH + 450 mM NaOAc	Column clean-up and regeneration
50 - 60	95	5	100 mM NaOH + 25 mM NaOAc	Equilibration to starting conditions

manually using a commercially available carbonate-free 50% w/w NaOH solution. The diluent was DI water (resistivity >18 MΩ.cm, TOC < 5 ppm), which was sparged with Nitrogen 5.0 using the sparging function of the ET210 eluent tray. During analysis, the eluent tray is used to pressurize the headspace of the mobile phase with inert Nitrogen 5.0 gas (0.2—0.4 bar N_2 overpressure).

Borate ions

Borate ions (BO_3^{-3}) can pair with the vicinal hydroxyls present in some carbohydrates. This may lead to peak tailing and loss of peak symmetry of the affected carbohydrates even when borate is present at low ppb concentrations in the mobile phase. Possible sources of borate contaminants entering the mobile phase are via (1) the DI water system, borate is one of the first ions released when the filters lose their capacity or (2) it can leach from borosilicate glass bottles at high pH. To eliminate the presence of borate contaminants in the mobile phase, PPCO eluent bottles are used and a Borate ion trap column was installed in the solvent line between the pump and the autosampler.

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 in this alkaline condition. The retention time of carbohydrates is inversely correlated with the pKa value and increases significantly with molecular weight. The SweetSep™ AEX200 column (4 × 200 mm) and precolumn (4 × 50 mm) were chosen for the separation of oligo– and polysaccharides, ultimately enabling the separation of two types of oligosaccharides (fructo– and maltooligosaccharides). This column is based on a monodisperse 5 µm resin coated with quaternary amine functionalized nanoparticles [8]. The resin's high uniformity and monodispersity allow for rapid, high -resolution separations of carbohydrates [8].

The separation of the oligo– and polysaccharides is based on the gradient profile depicted in Table 2. The initial condition was set to 100 mM NaOH + 25 mM NaOAc. A linear gradient to 100 mM NaOH + 150 mM NaOAc was applied for 20 minutes, followed by another linear gradient to 100 mM NaOH + 450 mM NaOAc for 25 minutes. All components of interest eluted within the total gradient run time of 45 minutes. Subsequently, a column clean-up step (100 mM NaOH + 450 mM NaOAc) was executed for 5 minutes, followed by 10 minutes of re-equilibration to starting conditions, resulted in a total analysis time of 60 minutes. During the clean-up and regeneration step, all late eluting interferences and carbonate ion build-up will be removed from the column, ensuring reproducible analysis. The separation temperature was set at 30°C. Note, that it usually takes a few runs to equilibrate the HPAEC-PAD system and get stable retention times.

Detection

For the pulsed amperometric detection, the Antec SenCell is used. This flow cell [9] has a confined wall-jet design and consists of a Au working electrode (WE), a HyREF (Pd-

Hydrogen) reference electrode (RE), and a stainless-steel auxiliary electrode (AE). For detection, a 4-step potential waveform was applied. The choice of the 4-step potential waveform resulted in excellent reproducibility and minimal electrode wear [10]. The oven temperature was set at 35°C. Under the specified conditions the cell current was typically about 0.3 µA.

Preparation of standards and samples

Standards: 50 g/L stock standards of maltodextrin (DE* 4—7) and inulin were prepared in 95/5 (v/v%) water/acetonitrile. Acetonitrile was added to suppress bacterial and fungal growth and to prevent fast degradation. The stock standards under these conditions are stable for more than a month in the fridge at 4°C. The working standards of maltodextrin and inulin were prepared by serial dilution of the stock standards with DI water, with a final concentration of 0.5 g/L and 0.2 g/L, respectively. The working standard solution was filtered over a 0.22 µm PES (polyethersulfone) syringe filter into the vials for injection.

Samples: A total of four samples were kindly provided by a testing laboratory located in Germany. Two samples (H1 and H2) are authentic honeydew honey containing natural oligosaccharides from different geographical origin. These honey samples were confirmed to be authentic by NMR spectroscopy, and were also previously tested using HPLC-ELSD. Two other samples (A1 and A2) are agave syrups. The agave syrup sample A1 was authentic and contains inulin. The sample A2 is agave syrup sample spiked with 2.5% corn syrup.

The samples were made by dissolving 1.0 g of sample in 4.2 mL DI water. The samples were filtered over a 0.22 µm PES (Polyethersulfone) syringe filter. Afterward, the samples were diluted 10× using serial dilution with DI water and transferred into the vials for injection.

Table 4

List of samples

Sample	*Description / Labels*
H1	Authentic honeydew honey with natural oligosaccharides (origin: Brazil)
H2	Authentic honeydew honey with natural oligosaccharides (origin: Greece)
A1	Authentic natural inulin-containing agave syrup
A2	Agave syrup spiked with 2.5% corn syrup

*) DE = Dextrose Equivalent is a measure of the amount of reducing sugars present in a sugar product, expressed as a percentage on a dry basis relative to dextrose. The dextrose equivalent gives an indication of the average degree of polymerization (DP) for starch sugars.

Results

Separation of oligo– and polysaccharides

Maltodextrin comprises of maltooligosaccharides, while oligosaccharides from inulin are mixture of linear fructooligosaccharides (labelled as F_n, with n indicating the number of fructose moieties) with an optional terminal glucose unit (shown as DP_n). The overlay of maltodextrin and inulin chromatograms is presented in Figure 2. The peak assignments were based on the injection of glucose (DP_1), maltose (DP_2), and maltotriose (DP_3) standard (data is not shown). Figure 2 shows that both oligosaccharides (DP_2 up to DP_{10}) from inulin and maltodextrin eluted at distinct retention times, demonstrating the method's capability to separate fructo– and maltooligosaccharides within 25 minutes. Due to a different retention behaviour of the polysaccharides (DP > 10) from maltodextrin and inulin, some of the polysaccharides peaks elute at the same retention time. Nevertheless, a high-resolution separation in each sample was achieved.

Sample analysis

Profiling the oligo– and polysaccharides in honey samples has been shown to be an effective method to detect honey adulteration. However, this method is only feasible if the profile of the authentic honey is available. The oligo– and polysaccharide profile of the two authentic honey samples

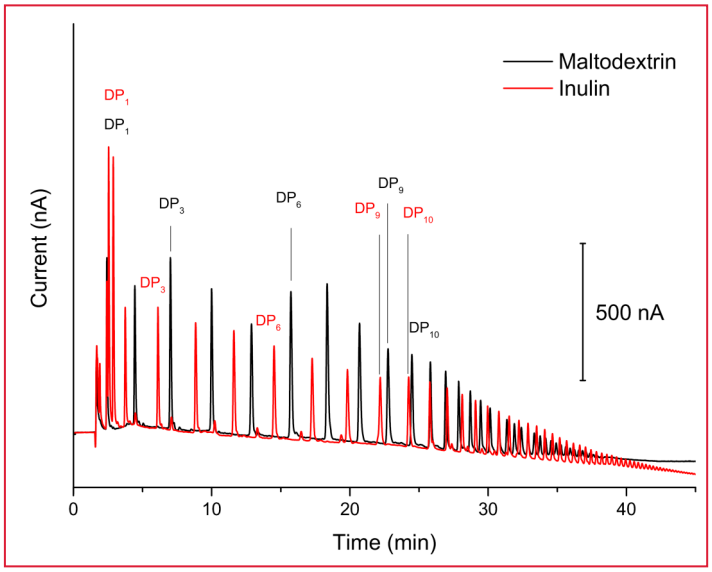

Figure 2. Overlay chromatogram of 10 µL injection of 500 ppm maltodextrin DE 4—7 standard (black) and 200 ppm of inulin standard (red).

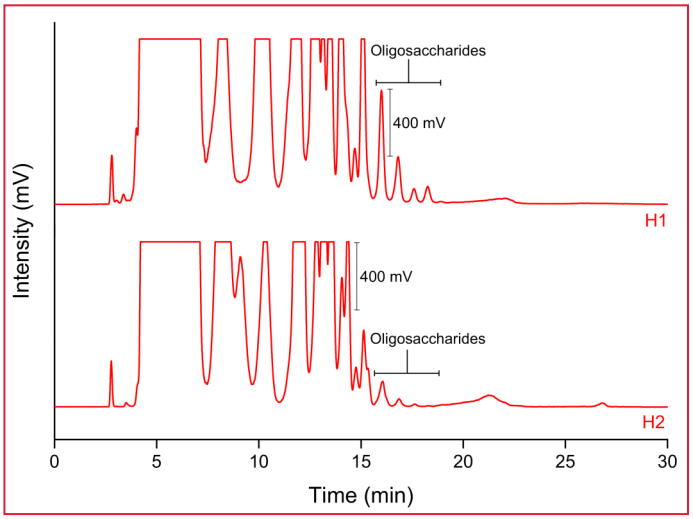

Figure 3. ELSD chromatograms of sample H1 (top) and sample H2 (bottom).

were initially obtained using a HPLC-ELSD method, as illustrated by the chromatograms in Figure 3. Both ELSD chromatograms of the honey samples show the presence of oligosaccharides in the samples. The amount of oligosaccharides in the sample H1 is larger compared to sample H2. However, these chromatograms do not provide further information about the type of oligosaccharides, nor the degree of polymerization of these oligosaccharides. Furthermore, the chromatograms do not indicate the presence of polysaccharides in both samples.

The HPAEC-PAD chromatograms of samples H1 and H2 are presented in Figure 4 and Figure 5. While both chromatograms show the same complexity as shown in the ELSD chromatograms, the HPAEC-PAD chromatograms offer a better resolved oligosaccharide profile for both samples up to a larger DP. Sample H1 consists of numerous oligosaccharides, some of which can be attributed to maltooligosaccharides type from DP_2 to DP_7, and confirmed using ESI-MS (data is not shown here). Furthermore, the sample H1 shows several peaks that may correspond to the polysaccharides (DP > 10), showcasing the superior sensitivity of PAD over ELSD as detection method. Sample H2 also shows several oligosaccharide peaks which are attributed to maltooligosaccharides from DP_2 to DP_8. Unlike sample H1, sample H2 does not contain polysaccharides. The presence of oligosaccharides in honey sample is often linked to adulteration. The HPAEC-PAD profile of these larger sugars in adulterants usually has an unique pattern because it represents homologous series. However, in the sample H1 and H2, the oligosaccharides composition is rather random. Therefore the

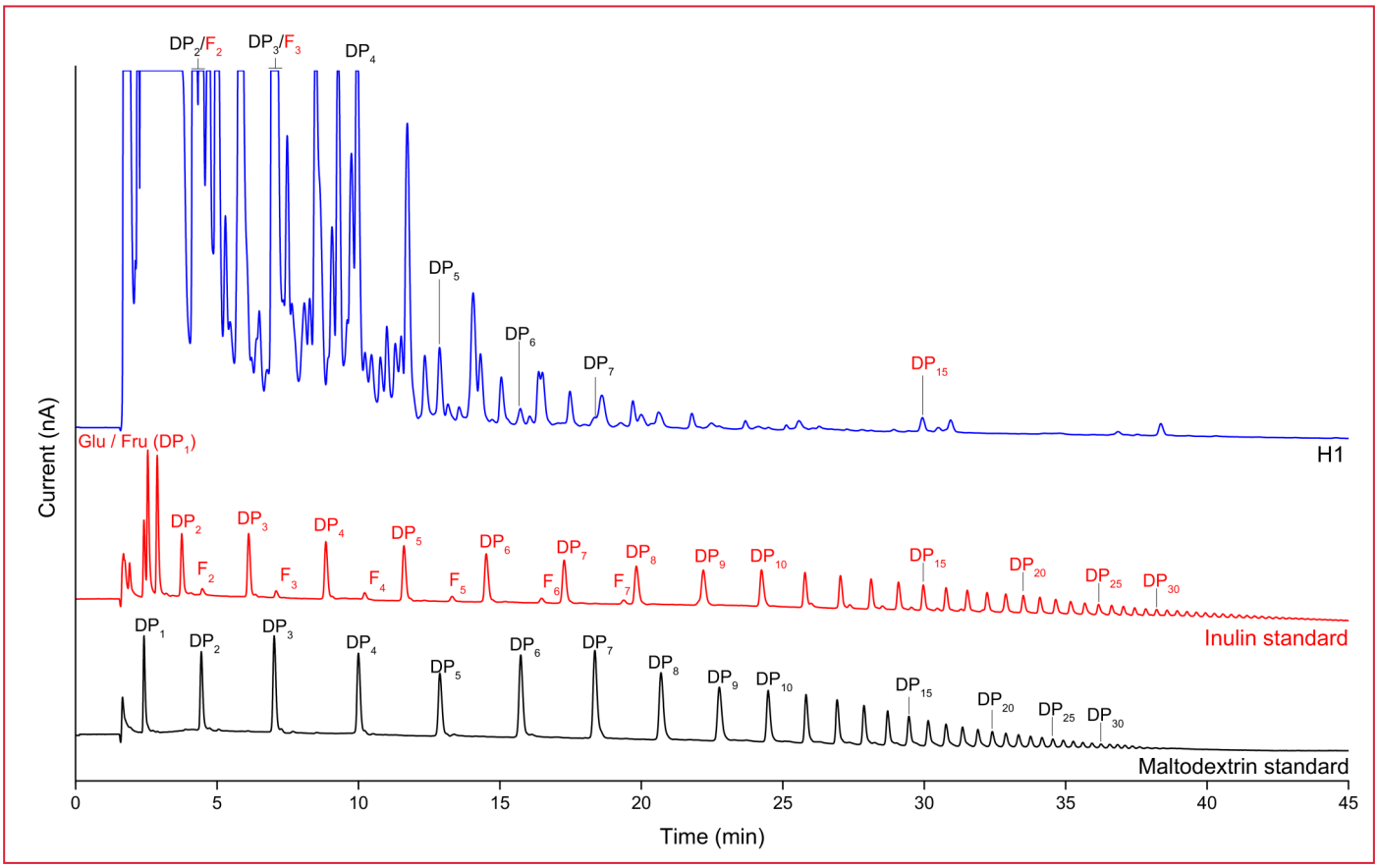

Figure 4. Chromatograms of 10 µL injection of sample H1 (blue line), 200 ppm inulin standard (red line), and 500 ppm maltodextrin standard (black line). Color of peak labels correspond to the oligosaccharides type (red = fructooligosaccharides, black = maltooligosaccharides).

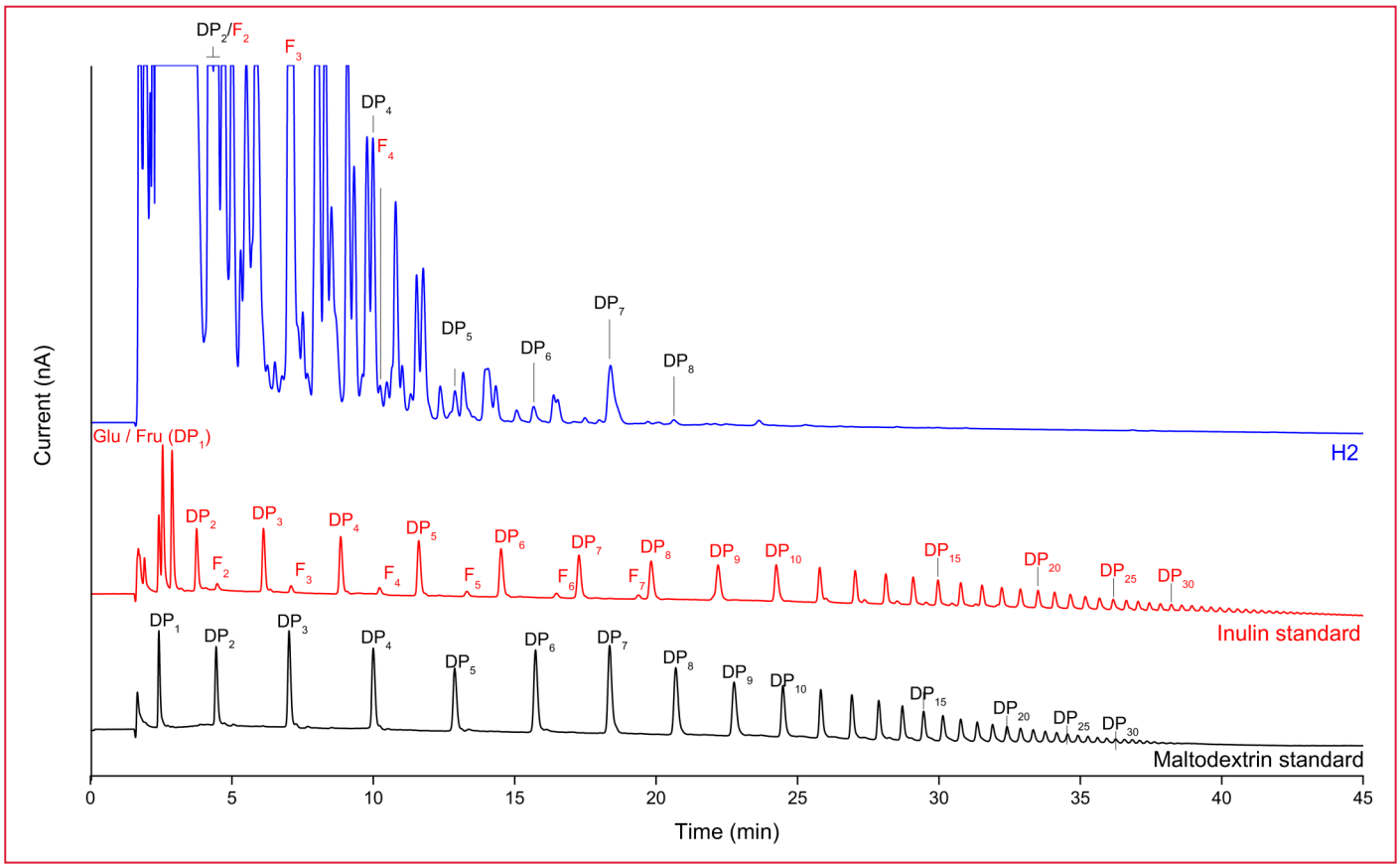

Figure 5. Chromatograms of 10 µL injection of sample H2 (blue line), 200 ppm inulin standard (red line), and 500 ppm maltodextrin standard (black line). Color of peak labels correspond to the oligosaccharides type (red = fructooligosaccharides, black = maltooligosaccharides).

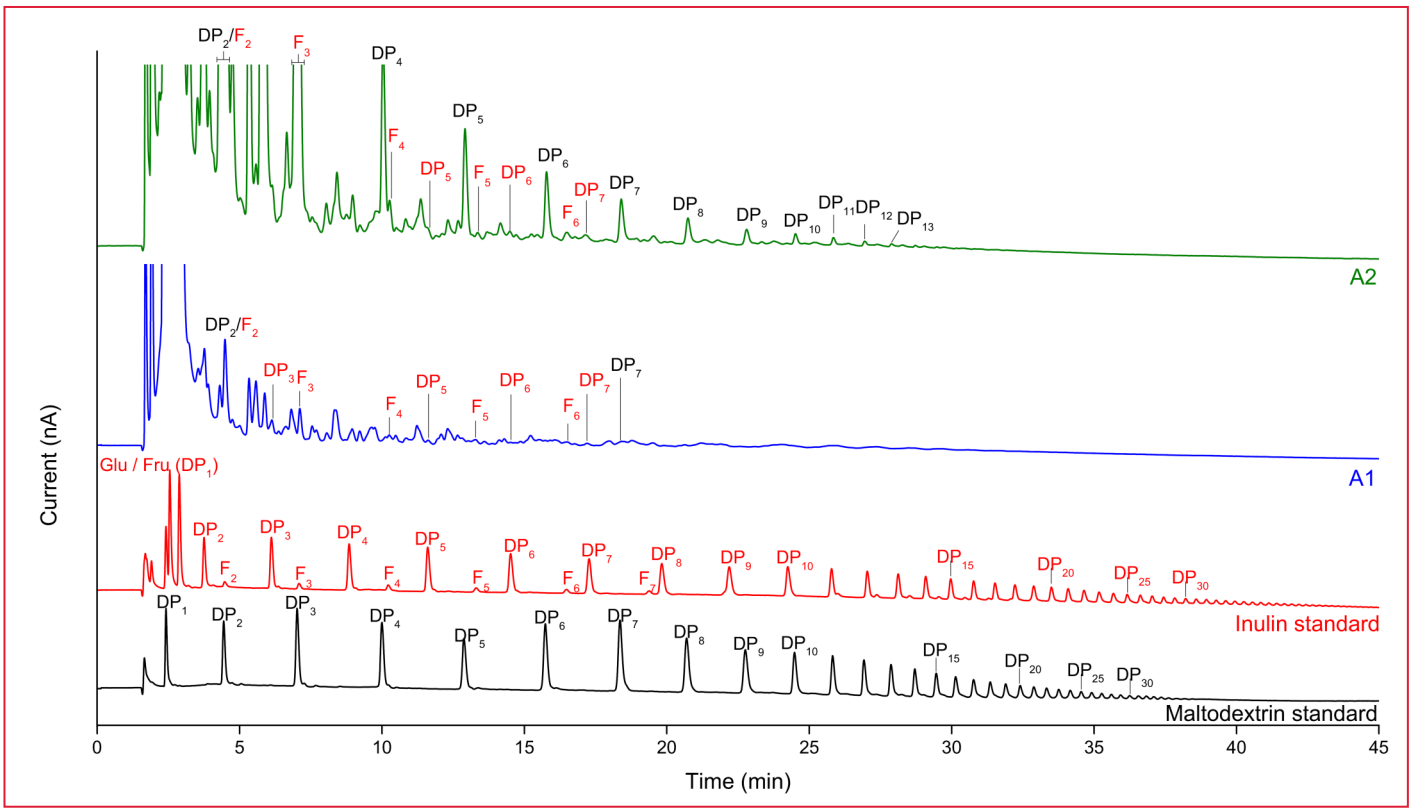

Figure 6. Chromatograms of 10 µL injection of sample A1 (blue line), A2 (adulterated with 2.5% corn syrup, green line), 200 ppm inulin standard (red line), and 500 ppm maltodextrin standard (black line). Colors of peak labels correspond to the oligosaccharides type (red = fructooligosaccharides, black = maltooligosaccharides).

oligosaccharides in these samples do not correlate with addition of adulterants, but instead are of natural origin [11].

The analysis of samples A1 and A2 shows the importance of determining the type of oligosaccharides. As depicted in the chromatograms in Figure 6, the sample A1 (agave syrup with natural inulin) contains fructooligosaccharides from DP_3 up to DP_7 and a small amount of maltooligosaccharides DP_7. The chromatogram of the sample A2 (agave syrup spiked with 2.5% corn syrup) also shows fructooligosaccharides peaks similar to the sample A1. In addition, maltooligosaccharides from DP_4 to DP_{13} were also detected. These maltooligosaccharides are typical for corn-syrup based adulterants [2, 3]. Notably, these maltooligosaccharides were detected in a 10× diluted sample, corresponding to a 0.25% addition of corn syrups. This demonstrates the capability of HPAEC-PAD to detect a very low level of adulteration, as well as a possibility to separate the type of oligosaccharides, leading to identification of adulterants.

References

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Conclusion

ALEXYS Carbohydrate Analyzer with SweetSep™ AEX200 anion exchange column offers selective HPAEC-PAD analysis of oligo– and polysaccharides. The presented method allows high-resolution separation of fructooligosaccharides from maltooligosaccharides, as well as separation of oligo– from polysaccharides, offering a detailed carbohydrates profiling. The analysis of honey samples indicates the superior sensitivity and selectivity of HPAEC-PAD over HPLC-ELSD as detection method. The analysis of an authentic and an adulterated agave syrup highlights the capability of the method in detecting adulteration as well as the potential of identifying adulterants based on the oligosaccharides type present in the samples.

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	
260.0010	SweetSep™ AEX200, 4 x 200 mm column, 5 µm
260.0015	SweetSep™ AEX200, 4 x 50 mm precolumn, 5 µm
260.0030	SweetSep™ BIT, 4 x 50 mm borate ion trap
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.

*) 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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