



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 as fraud marker in honey

- **ALEXYS™ Carbohydrate Analyzer**
- **SweetSep™ AEX200 anion-exchange column**
- **High-resolution separation of oligo– and polysaccharides**
- **Honey profiling and detection of adulteration in honey**

### Summary

In a 2013 EU report concerning food safety honey is listed as one of the top 10 food products at risk of food fraud [1]. Despite the tight regulation of honey in the European Union (EU Honey Directive 2001/110/EC) [2], instances of fraudulent honey still remain. The main reason for the undetected honey fraud is due to the large compositional variations of natural honey, which complicates the distinction from non-authentic honey [3]. Furthermore, the existing quality parameters of authentic honey are limited, not to mention the limited analytical techniques as a verification tool for honey authenticity [2, 4]. To address this gap, a 2023 report describes additional quality parameters and analytical techniques for verification of honey authenticity, one of which concerns the significance of oligo– and polysaccharides in honey [5]. These long carbohydrate chains exhibit specific profiles for different types of honey, making them potential markers in detecting honey fraud.

The analysis of oligo– and polysaccharides in honey is demonstrated in this application note. Given the small quantity of these long carbohydrate chains in honey, a high-resolution separation and a sensitive detection technique is essential. Therefore, the method is based on separation and detection by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) using the SweetSep™ AEX200 high-resolution anion-exchange column. To demonstrate the applicability of the method, a number of samples were investigated.



## Introduction

The market demand for honey as a natural sweetener is substantial. In 2022, European Union countries imported about 190.000 tons of honey, despite producing 286.000 tons [6]. The average import price for honey in the EU in 2022 is €2.65 per kilogram [6]. In contrast, cheaper sweeteners such as sugar syrups are available for about €0.4 — €0.6 per kilogram [6]. This significant price difference between authentic honey and the cheaper sweetener serves as a primary motivation for honey fraud through addition or blending.

Regulatory frameworks, for example the EU Honey Directive 2001/110/EC [2] or the USDA CID A-A-20380 [7], specify compositional parameters of authentic honey. These parameters include the level of mono- and disaccharides in the honey, along with other parameters such as moisture content, diastase activity, and amino acid content. Despite these strict regulations, instances of fraudulent honey remain undetected and therefore require additional authenticity testing parameters. A recent report highlights the significance of oligo- and polysaccharides as additional parameters for determining honey authenticity [5].

This application note describes a method for the profiling of oligo- and polysaccharides in honey sample using High-Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD). HPAEC-PAD stands out as the most sensitive technique for the

measurement of adulterants in honey samples [8]. The method is based on gradient separation of longer carbohydrate chains using the new SweetSep™ AEX200 high-resolution anion-exchange column. The AEX200 stationary phase consists of highly monodisperse 5 µm ethylvinylbenzene-divinylbenzene copolymer particles coated with quaternary amine functionalized nanoparticles [9]. As a proof of principle, several honey samples were analyzed. In addition, the obtained carbohydrate profiles of an authentic honey sample were compared to those of intentionally adulterated honey sample to assess the method's applicability in fraud detection.

## Method

The HPAEC-PAD analysis was conducted using the ALEXYS Carbohydrate Analyzer, consisting of a P6.1L quaternary LPG pump, an AS6.1 autosampler, a CT2.1 column thermostat, an ET210 eluent tray for nitrogen blanketing, and equipped with a DECADE Elite electrochemical detector (Figure 1). The SenCell with Au working electrode and HyREF reference electrode was selected for sensitive detection of the carbohydrates. The HPAEC-PAD system was operated using the settings listed in Table 1. Take into account that the selection of a specific quaternary HPLC system 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 were made to guarantee method reproducibility and system stability, particularly when working with ion exchange chromatography using a mobile phase at a high pH.

### Carbonate ions

CO<sub>2</sub> present in the air can get easily dissolved in the mobile phase and form carbonate ions (CO<sub>3</sub><sup>2-</sup>). These carbonate ions 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 manually using a commercially available carbonate-free 50% w/w NaOH solution. The diluent was DI water (resistivity >18 MΩ.cm), which was sparged with Nitrogen 5.0 (≥ 99.999% pure). During analysis, the ET210 eluent tray is used to pressurize the headspace of the mobile phase with inert Nitrogen gas (0.2—0.4 bar N<sub>2</sub> overpressure).



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

HPAEC-PAD conditions	
System	ALEXYS™ Carbohydrate Analyzer - gradient (quaternary LPG)
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	0.7 mL/min
Pressure	about 180–200 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	Description
0	92	8	Gradient elution and detection
30	10	90	
30–35	10	90	Column clean-up and regeneration
35–50	92	8	Equilibration to starting conditions

## 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. The retention time of carbohydrates is inversely correlated with the pKa value and increases significantly with molecular weight. Only polymeric anion-exchange columns are suitable for carbohydrate separation in this alkaline condition. Therefore, a strong anion-exchange column SweetSep™ AEX200 was chosen for the separation of the oligo- and polysaccharides. The high uniformity and monodispersity of the AEX200 resin allow for fast and high-resolution separation of carbohydrates [9]. The use of a pre-column filter is advised when using samples that might contain particulate matter.

The analysis of oligo- and polysaccharides is based on gradient

elution outlined in Table 2. The initial condition was set to 100 mM NaOH + 40 mM NaOAc. A linear gradient to 100 mM NaOH + 450 mM NaOAc was applied for 30 minutes, during which the elution of oligo- and polysaccharides took place. Subsequently, a column clean-up step (100 mM NaOH + 450 mM NaOAc) was executed for 5 minutes, followed by 15 minutes of re-equilibration to starting conditions, resulting in a total analysis time of 50 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

The Antec SenCell was used for the pulsed amperometric detection of carbohydrates. This flow cell [10] 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 [11]. The oven temperature was set at 35°C. Under the specified conditions the cell current was typically about 0.3 μA.

## Preparation of standard and samples

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

**Samples:** Five honey samples (H1–H5) and one rice syrup sample (RS) were obtained from third parties. The honey samples H1–H4 were tested for oligo- and polysaccharide content by a third party using various analytical methods such as NMR (Nuclear Magnetic Resonance) spectroscopy, IRMS (Isotope Ratio Mass Spectrometry), LC-HRMS (Liquid Chromatography—High Resolution Mass Spectrometry), and EA-CRDS (Elemental Analysis—Cavity Ring-Down Spectroscopy). Sample H5 is an authentic avocado honey originating from Mexico. The details of all samples are provided



in Table 4.

The samples were prepared by dissolving 1.25 g of sample in 50 mL DI water (25 g/L concentration). The rice syrup sample is further diluted 10× with DI water to a final concentration of 2.5 g/L. Subsequently, the samples were filtered over a 0.22 μm PES (Polyethersulfone) syringe filter to remove any particulate matter prior to analysis. To demonstrate the method's applicability in fraud detection based on honey profiles, sample H5 (avocado honey) was intentionally adulterated with the rice syrup sample. The adulterated sample was prepared by mixing the solution of sample H5 (25 g/L) with the solution of RS (2.5 g/L) in a ratio of 9:1. The adulterated sample contains a final concentration of 22.5 g/L of avocado honey and 0.25 g/L of rice syrup (approximately 1.1% adulteration level).

Table 4

List of samples	
Sample	Description / Labels
H1	Negative for oligo- and polysaccharides
H2	Positive for oligosaccharides and negative for polysaccharides
H3	Negative for oligosaccharides and positive for polysaccharides
H4	Positive for oligo- and polysaccharides
H5	Authentic avocado honey (geographical origin: Mexico)
RS	Rice syrup for adulteration of honey sample

## Results

### Separation of oligo- and polysaccharides

A chromatogram of a 10 μL injection of 0.5 g/L maltodextrin standard is presented in Figure 2. The peak assignments were established based on the injection of glucose (DP<sub>1</sub>), maltose (DP<sub>2</sub>), and maltotriose (DP<sub>3</sub>) standards (data not shown here). A high-resolution separation of each degree of polymerization (DP) up to DP<sub>40</sub> was achieved within 25 minutes.

### Sample analysis

Several honey samples were analyzed to obtain their oligo- and polysaccharide profiles. These carbohydrates are present in relatively low concentrations in honey. Therefore, several papers describe the sample clean-up workflow to concentrate these carbohydrates for better profiling [12, 13]. Note that in this application note, no sample clean-up was performed due to the use of PAD as a sensitive detection technique. The chromatograms of 10 μL injection of the honey samples H1—

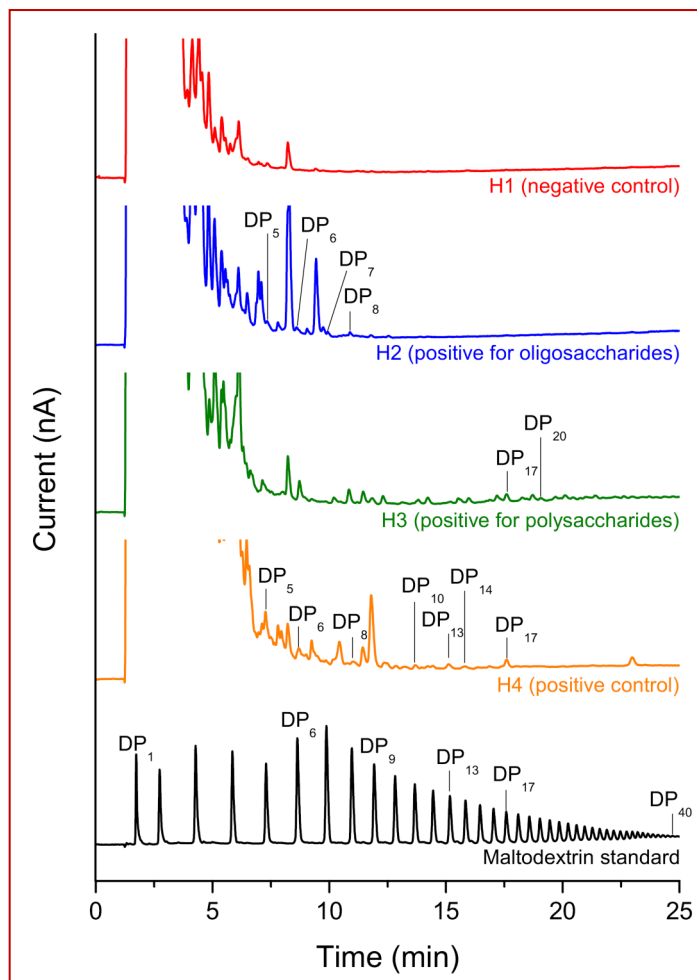


Figure 2. Chromatograms of 10 μL injection of sample H1 (negative control, red line), H2 (positive for oligosaccharides, blue line), H3 (positive for polysaccharides, green line), H4 (positive control, orange line), and maltodextrin standard as the reference (black line).

H4 are depicted in Figure 2. Peak assignments on the chromatograms were made with reference to the maltodextrin standard. The oligo- and polysaccharide profiles in H1—H4 samples were in accordance to the description from the third party. In sample H1, labelled as the negative control, no peaks corresponding to polysaccharides or oligosaccharides attributable to maltodextrin were observed. Malto-oligosaccharide peaks corresponding to DP<sub>5</sub> up to DP<sub>8</sub> were identified on sample H2. Meanwhile, several peaks with retention times > 12 minutes indicated the presence of polysaccharides in sample H3. However, only DP<sub>17</sub> and DP<sub>20</sub> were identified based on the maltodextrin standard. The sample H4, labelled as a positive control, consists of various DPs from DP<sub>5</sub> up to DP<sub>17</sub>. It is worth noting that the chromatograms of sample H1—H4 exhibit multiple peaks between t = 5 minutes to t = 10 minutes which does not correspond to any of the maltodextrin standard peaks. These unknown peaks may correspond to different types of oligosaccharides or another component in honey matrices.

\*) 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 polymerisation (DP) for starch sugars.



The chromatogram of authentic avocado honey sample H5 (Figure 3, black line) shows various peaks attributed to DP<sub>5</sub> up to about DP<sub>17</sub> of malto-oligosaccharides. The authentic avocado honey sample was intentionally adulterated with RS to assess the method's applicability to detect honey adulterations. Rice syrup is a popular adulterant originated from C3 plants (plants with Calvin photosynthesis cycle) and shares a similar carbon isotope profile with sugars in natural honey. Consequently, honey adulteration using rice syrup is more difficult to detect by another analytical method, such as IRMS or NMR spectroscopy [8].

The chromatogram of RS (Figure 3, blue line) shows the presence of oligo- and polysaccharides, some of which match the oligo- and polysaccharides in the maltodextrin standard, specifically DP<sub>4</sub>, DP<sub>5</sub>, and DP<sub>10</sub>–DP<sub>17</sub>. The oligosaccharide peaks marked with asterisks in the rice syrup chromatogram do not match the DP<sub>6</sub>–DP<sub>8</sub> peaks found in maltodextrin.

The chromatogram of the adulterated honey sample, overlaid in Figure 3, demonstrates the challenges associated with detecting fraudulent honey. The oligo- and polysaccharide profiles of the adulterated honey sample closely resemble those of the authentic honey sample. However, a comparison between the authentic and the adulterated sample shows several signs of adulteration by the addition of rice syrup. Two additional peaks, marked with asterisks, appeared only in the adulterated sample. In addition, several other peaks corresponding to the DP<sub>10</sub>–DP<sub>12</sub> malto-oligosaccharides of the rice syrup are more prominent in the adulterated sample. These results highlight the potential of HPAEC-PAD to detect honey adulteration by syrup addition at levels of as low as 1%, based on the oligo- and polysaccharide contents. These results are in accordance to the findings described in literature [12, 13].

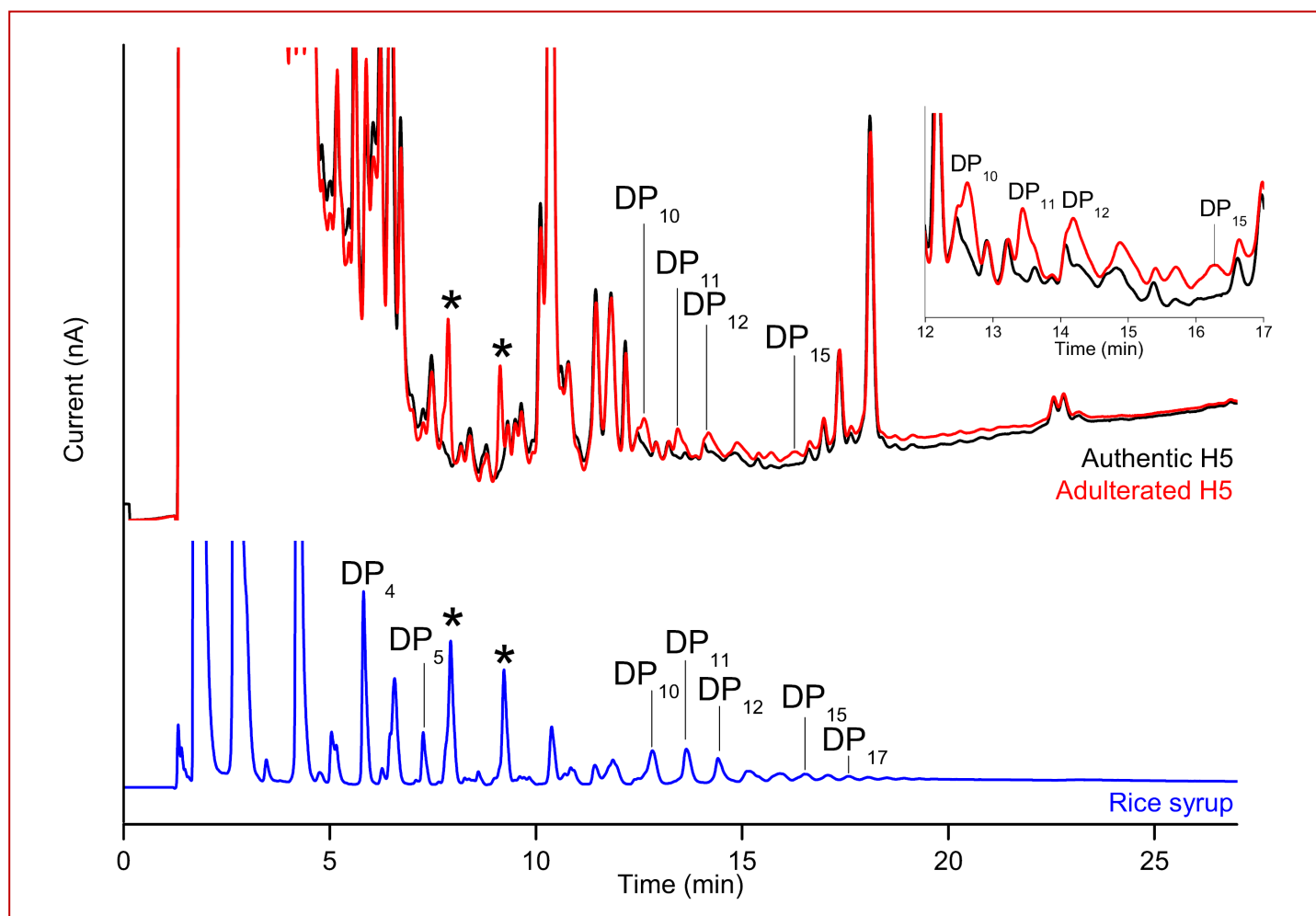


Figure 3. Overlay of chromatograms of a 10  $\mu$ L injection of the 2.5 g/L rice syrup sample (blue trace), 25 g/L authentic avocado honey sample H5 (black trace), and avocado honey sample H5 adulterated with 1.1% rice syrup (red trace). Inset shows a zoom-in on the chromatogram between  $t=12$  min and  $t=17$  min. Peaks marked with (\*) are from the rice syrup.



## References

1. European Commission, Report on the food crisis, fraud in the food chain and the control thereof (2013/2091 (INI)), [https://www.europarl.europa.eu/doceo/document/A-7-2013-0434\\_EN.html](https://www.europarl.europa.eu/doceo/document/A-7-2013-0434_EN.html)
2. European Union Honey Directive, 2001/110/EC, European Union, <https://eur-lex.europa.eu/eli/dir/2001/110/oj>
3. Elflein, L., Authenticity of honey, *eFoodLab International*, 2015, 3.
4. Chin, N.L., Sowndhararajan, K., A review on analytical methods for honey classification, identification, and authentication, *Honey Analysis—New advances and challenges*, 2020, Ch. 5, <https://www.intechopen.com/chapters/71122>
5. Ždiniaková, T., et al., EU coordinated action to deter certain fraudulent practices in the honey sector—analytical testing results of imported honey, *JRC Technical Report*, 2023, <https://publications.jrc.ec.europa.eu/repository/handle/JRC130227>
6. European Commission, Honey Market Presentation, 2023, [https://agriculture.ec.europa.eu/system/files/2023-12/market-presentation-honey\\_autumn2023\\_en.pdf](https://agriculture.ec.europa.eu/system/files/2023-12/market-presentation-honey_autumn2023_en.pdf)
7. The U.S. Department of Agriculture (USDA) Commercial Item Description (CID) A-A-20380, 2019 [https://www.ams.usda.gov/sites/default/files/media/AA20380\\_Honey.pdf](https://www.ams.usda.gov/sites/default/files/media/AA20380_Honey.pdf)
8. Se, K.W., et al., Detection techniques for adulterants in honey: Challenges and recent trends, *J. Food Compos. Anal.*, 2019, 80, 16-32
9. Marvelous, C., et al., Evaluation of a novel stationary phase for the separation of lactose and isomers in lactose-free products using high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD), *J. Chrom. A.*, 2024, 1716, 464661
10. Louw, H., Brouwer, H.J., Reinhoud, N., Electrochemical flowcell, 2014, US Patent 9310330
11. Rocklin, R.D., Clarke, A.P., Weitzhandler, M., Improved long-term reproducibility for pulsed amperometric detection of carbohydrates via a new quadruple-potential waveform, *Anal. Chem.*, 1998, 70, 1496-1501
12. Morales, V., Corzo, N., Sanz, M.L., HPAEC-PAD oligosaccharide analysis to detect adulterations of honey with sugar syrups, *Food Chem.*, 2008, 107, 922-928
13. Megherbi, M., et al., Polysaccharides as a marker for detection of corn sugar syrup addition in honey, *J. Agric. Food Chem.*, 2009, 57, 2105-2111

## Conclusion

ALEXYS Carbohydrate Analyzer with SweetSep™ AEX200 anion exchange column offers selective HPAEC-PAD analysis of oligo– and polysaccharides in honey. High-resolution separation up to DP<sub>40</sub> was achieved within 25 minutes run time, enabling fast profiling of these larger carbohydrates in honey samples. The analysis of an authentic and adulterated avocado honey sample demonstrates the method's ability to detect low-level adulterations of honey by addition of starch-based syrups (rice, wheat, etc.). Therefore, making HPAEC-PAD a potentially valuable tool to detect fraudulent honey products based on the oligo– and polysaccharide contents.



## 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>Columns</b>	
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 <sup>#</sup>	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 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.

SweetSep, DECADE Elite, ALEXYS, SenCell, FlexCell and HyREF are trademarks of Antec Scientific. Clarity™ and DataApex™ are trademarks of DataApex Ltd. Chromeleon™ is a trademark of Thermo Fisher Scientific, Empower™ is a trademark of Waters corporation, OpenLAB™ and Chemstation are trademarks of Agilent Technologies, Inc. All other trademarks are the property of their respective owners.

**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

