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

Carbohydrates in honey

- **SweetSep™ AEX200 anion-exchange column**
- **High-resolution separation of 15 sugars within 25 min**
- **Sensitive detection using SenCell™**
- **Wild honey, commercial honey, and glucose syrup**

Summary

Honey is a complex natural substance with a promising potential for various health benefits [1] and consists of about 80% carbohydrates. However, it is also a food product that is often affected by frauds and adulterations. In European Union, the composition and definition of honey is regulated by the EU Honey Directive 2001/110/EC [2]. The directive specifies the types of honey products which can be sold under given names and rules on labelling, presentation and information on origin. A study in 2021-2022 reveals that about 46% of honey imported to the European Union does not comply with the EU Honey Directive and these honey samples were tampered with food additives such as sugar syrups [3]. Various analytical methods are used for authenticating original honey. Most of the analytical methods provide indications of pollen distribution, physicochemical parameters, and profile analysis of phenolic, flavonoid, carbohydrate, amino acids, aroma, and individual marker components [4, 5].

In this application note a method is presented for the analysis of carbohydrates in honey. The quantification and identification of carbohydrates are important for determining its floral origin and quality [6]. 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.

Introduction

Honey is a complex natural product made by honeybees from floral nectars. In general, the composition of honey includes about 80% of carbohydrates and a mixture of different substances such as enzymes, proteins, vitamins, minerals, organic acids, pigments, and waxes [7]. In recent years, several analytical methods have been proposed to differentiate honey types and to verify their quality [4, 5]. The carbohydrates in honey consist of mainly fructose and glucose, as well as a mixture of at least 11 disaccharides, 11 trisaccharides, and several oligosaccharides [6]. For identification of the floral origin and evaluating honey quality, the carbohydrate composition is quantified. For example, a high level of sucrose indicates that honey may be tampered with by adding sweeteners. Meanwhile, a high amount of melezitose, a typical indicator of summer honey originating from the forest flora, can lead to honeydew flow disease and causes honey to crystallize in the honeycomb (cement honey) [8].

In this application note a method is presented for the separation and quantification of sugars in honey samples using High-Performance Anion Exchange Chromatography in combination with Pulsed Amperometric Detection (HPAEC-PAD). The method is based on the separation of the honey carbohydrates using a new high-resolution high-capacity anion-exchange column, SweetSep™ AEX200 with a monodisperse 5 µm resin. To demonstrate the performance and versatility of the method a variety of honey samples (wild honey, commercially available honey, and honey-flavored glucose syrup) were analyzed.

Figure 1. Left: SenCell with Au working electrode and Pd/H_2 (HyREF) reference electrode. Right: DECADE Elite electrochemical detector.

Method

The HPAEC-PAD analysis of carbohydrates in honey is performed using a quaternary HPLC system equipped with ET210 eluent tray for nitrogen blanketing, and DECADE Elite electrochemical detector with SenCell electrochemical flow cell (Figure 1) with the LC conditions specified in Table 1. 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. 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 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 using the sparging function of the ET 210 eluent tray. During analysis, the eluent tray is used to pressurize the headspace of the mobile phase with inert Nitrogen 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. Especially, fructose is susceptible to peak tailing due to borate ions. 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 the HPLC solvent glass bottles. To eliminate the presence of borate contaminants in the mobile phase, a Borate trap column was installed in the solvent line between the pump and the autosampler. Additionally, all glass bottles were replaced by pressure resistant PPCO bottles.

Table 1

LC-EC conditions

HPLC system	Quaternary HPLC system
Detector	DECADE Elite electrochemical detector
Columns	SweetSep™ AEX200, 4 × 200 mm column, 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 + 100 mM NaOAc Eluents prepared & blanketed with Nitrogen 5.0
Flow rate	0.7 mL/min
Back pressure	about 210 bar
Injection	10 µL
Temperature	20 °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	2 µA/V

Table 2

Step-gradient program

Time (min)	*Mobile phase*	*Description*
0 - 25	68 mM NaOH	Isocratic elution and detection
25 - 30	100 mM NaOH + 100 mM NaOAc	Column clean-up and regeneration
30 - 45	68 mM NaOH	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. 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. A strong anion-exchange column SweetSep™ AEX200 was chosen for the separation of carbohydrates in honey. This column is based on a monodisperse 5 µm resin coated with quaternary amine functionalized nanoparticles. The high uniformity and monodispersity of the resin allow for fast and high-resolution separation of carbohydrates. The use of a pre-column filter is advised when using samples which might contain particulate matter.

The analysis of carbohydrates is based on isocratic elution using 68 mM NaOH for 25 minutes. Subsequently, a column clean-up step (100 mM NaOH + 100 mM NaOAc) is executed for 5 minutes, followed by 15 minutes of re-equilibration to starting conditions, resulting in a total analysis time of 45 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 20°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 of honey sugars, the Antec SenCell is used. This flow cell [9] has a confined wall-jet design and consists of a Au working electrode (WE), HyREF (Pd-Hydrogen) reference electrode (RE), and 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: Individual sugars (trehalose, glucose, fructose, isomaltose, sucrose, kojibiose, gentiobiose, turanose, palatinose, melezitose, raffinose, 1-kestose, maltose, erlose, and nigerose) were purchased from Sigma-Aldrich or Biosynth. The 10 mM stock standards of individual sugar were prepared in 95/5 (v/v %) water/acetonitrile. A small amount of acetonitrile was added to prevent fast degradation and minimize bacterial or fungal growth. The stock standards were stored in the freezer at –20°C and is stable for more than a month. Working standard mixes in the concentration range of 10 nM— 50 µM were prepared by serial dilution of the stock standards with DI water. The working standard mixes were stored at 4°C prior to use.

Samples: Two honey products were obtained from a Swiss beekeeper. One of the honey samples was harvested during the spring season of 2022, while the other was during the summer season of the same year. Another commercial honey product and honey-flavored glucose syrup were purchased from a supermarket in The Netherlands. The honey-flavored

glucose syrup was taken as an example of a product which does not comply to the definition & composition of honey as defined in the EU Honey Directive 2001/110/EC.

The samples were prepared by weighing 100 mg of the product and dissolving the product in 100 mL DI water (1 g/L concentration). Subsequently, the samples were filtered over a 0.22 µm PES (Polyethersulfone) syringe filter into the vials for injection. Some of the carbohydrates in the samples are in a very high concentration. Therefore, to fit the linear range of the calibration curves, samples with a concentration of 0.1 g/L and 0.01 g/L were also prepared using serial dilution with DI water.

Results

Separation

A chromatogram of 10 µL injection of 10 µM sugar standard mix is shown in Figure 2. The sugar standard mix consists of 15 sugars: 2 monosaccharides (glucose and fructose), 9 disaccharides (trehalose, isomaltose, sucrose, kojibiose, gentiobiose, turanose, palatinose, nigerose, and maltose), and 4 trisaccharides (melezitose, raffinose, 1-kestose, and erlose). All sugars were eluted within 25 minutes, which is similar to the method reported in references [11—13]. Under these conditions, most of the sugars are baseline-separated (resolution > 1.5), except for palatinose and melezitose (resolution 1.1 and 1.2, respectively).

The peak efficiencies found for the sugars range from 40,000 to 80,000 theoretical plates/meter for all sugars in the standard mix. The introduction of a borate trap between the pump and the autosampler successfully suppresses the tailing of fructose (tailing factor of 1.2). The rest of the sugars do not show any significant tailing (tailing factor between 1.0—1.2).

Linearity

Calibration was performed with 14 sugar standards. Linearity was investigated in the concentration range of 0.01 µM to 50 µM. The calibration curves of all sugars are shown in Figure 3. A linear fitting was done to assess the linearity for all sugars, and the fitted lines are extrapolated to the origin. The linearity

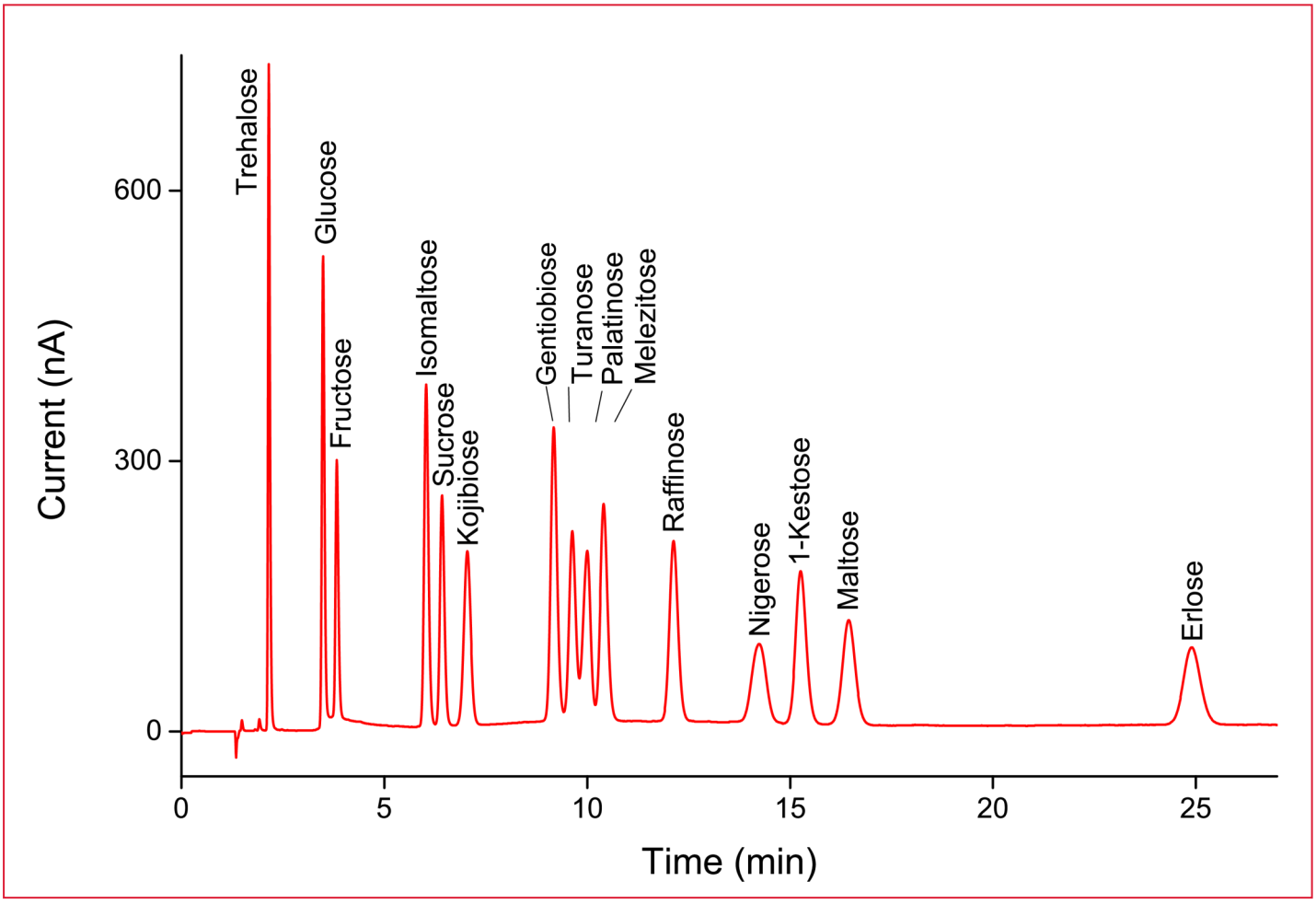

Figure 2. Chromatogram of a 10 µL injection of a 10 µM sugar standard mix in DI water.

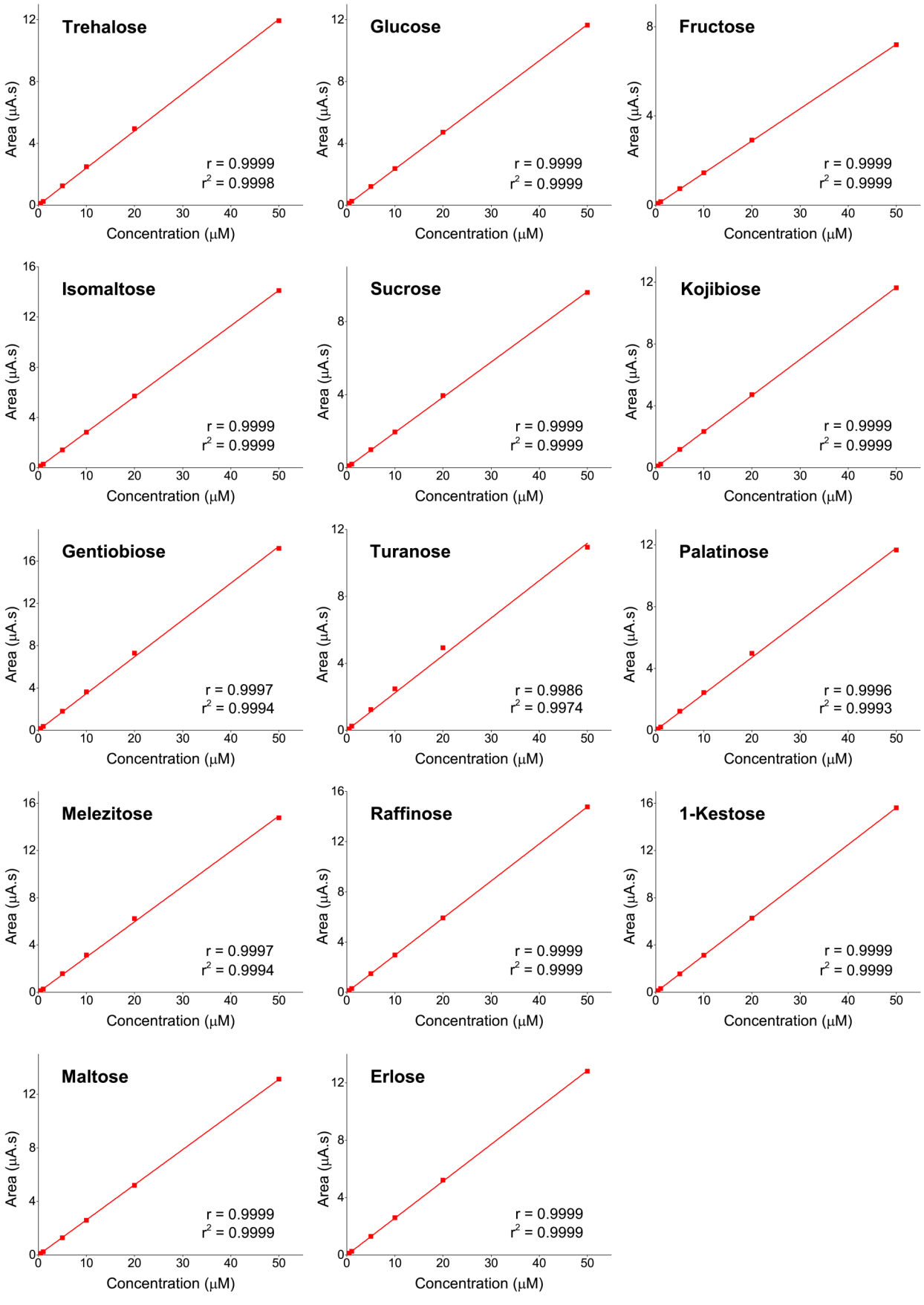

Figure 3. Calibration curves of 14 sugars in the concentration range of 0.1 - 50 µM. The fitted lines are extrapolated to the origin.

is excellent in this concentration range with both correlation coefficients (r) and coefficient of determination (r^2) greater than 0.999 for almost all sugars except turanose (r = 0.9986, r^2 = 0.9974).

The calibration curves in Figure 3 are used for the actual quantification of samples.

Repeatability

The repeatability of the method was evaluated by 10 repetitive injections of the 10 µM standard mix in DI water. The relative standard deviations (RSDs) of the retention time peak height and peak area are listed in Table 3. Excellent repeatability has been found as shown by the low RSD values. RSDs for the retention time are mostly < 0.3% for all sugars except trehalose (RSD = 0.42%). The RSDs for peak height and area for all sugars were < 0.5% and < 0.6%, respectively. These data demonstrate that with this method reproducible analysis of all sugars in the standard mix can be achieved.

Table 3

Repeatability of 10 µL injections of a 10 µM sugar standard mix in DI water (n=10)

Compound	*RSDs (%)*		
	Ret. Time	Peak Height	Peak Area
Trehalose	0.42	0.34	0.33
Glucose	0.29	0.27	0.16
Fructose	0.27	0.39	0.38
Isomaltose	0.20	0.20	0.14
Sucrose	0.20	0.31	0.35
Kojibiose	0.19	0.33	0.25
Gentiobiose	0.18	0.17	0.21
Turanose	0.18	0.21	0.21
Palatinose	0.18	0.21	0.44
Melezitose	0.21	0.27	0.22
Raffinose	0.22	0.42	0.31
1-Kestose	0.24	0.36	0.32
Maltose	0.16	0.50	0.60
Erlose	0.23	0.37	0.46

LOD and LOQ

The Limit of Detection (LOD) and Limit of Quantification (LOQ) for all sugars were determined using a method that was described in the ICH guidelines [14]. The LODs were calculated as the analyte response corresponding to 3× the ASTM noise (average peak-to-peak baseline noise of 10 segments of 0.5 min). The noise was calculated based on a 5-minute section of baseline at t = 18 minutes up to t = 23 minutes. The LOQs were calculated in a similar way to LODs, with a 10× S/N ratio. The response of an injection obtained with a 0.5 µM standard mix was used to calculate the LODs and LOQs for all sugars. The ASTM noise for this injection was 0.18 nA. The LODs and LOQs are shown in Table 4 in µg/L (ppb) and nanomolar concentrations.

Table 4

Calculated Limit of Detection (LOD) and Limit of Quantification (LOQ)

Compound	*LOD*		*LOQ*
	nmol/L	µg/L (ppb)	µg/L (ppb)
Trehalose	7.6	2.6	8.7
Glucose	10.7	1.9	6.4
Fructose	18.6	3.4	11.2
Isomaltose	14.6	5.0	16.6
Sucrose	21.3	7.3	24.2
Kojibiose	29.2	10.0	33.3
Gentiobiose	17.2	5.9	19.6
Turanose	30.3	10.4	34.5
Palatinose	33.2	11.4	37.8
Melezitose	24.0	12.1	40.3
Raffinose	27.5	13.9	46.2
1-Kestose	33.7	17.0	56.7
Maltose	48.9	16.7	55.8
Erlose	67.1	33.9	112.9

The high sensitivity of the method is evident in Table 4. The detection limits for all sugars are below 70 nM. The calculated LOQs range from as low as 6.4 µg/L (for glucose) up to 112.9 µg/L (for erlose). To sum up, the presented method has excellent sensitivity for the detection and quantification of carbohydrates in honey samples.

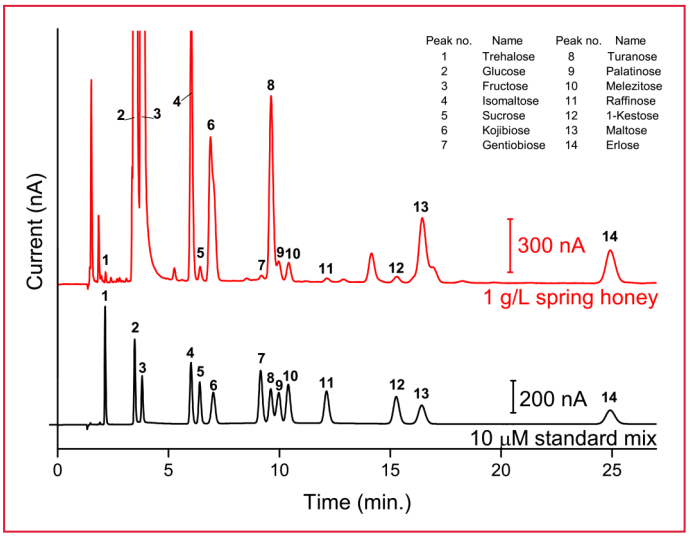

Figure 4. Overlay of chromatograms of: 10 µL injection of 1g/L spring honey sample (red) and a 10 µL injection of 10 µM standard mix (except nigerose) in DI water (black).

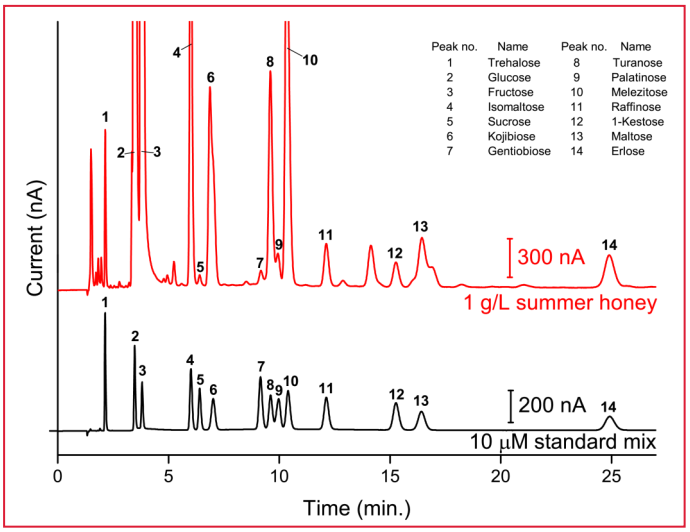

Figure 5. Overlay of chromatograms of: 10 µL injection of 1g/L summer honey sample (red) and a 10 µL injection of 10 µM standard mix (except nigerose) in DI water (black).

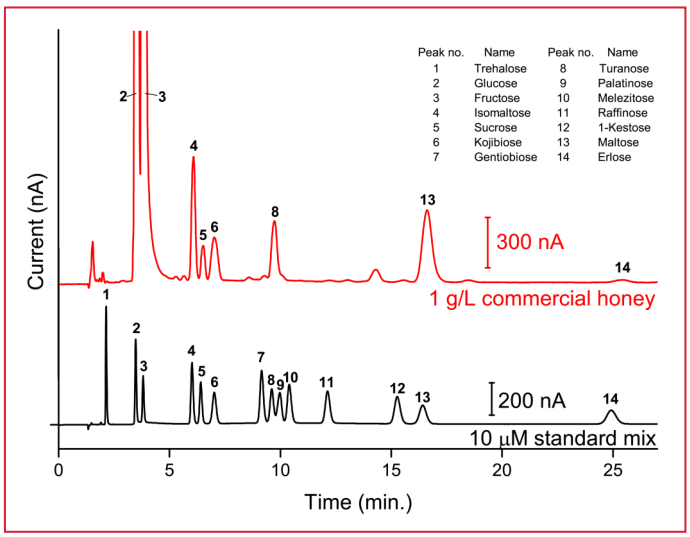

Figure 6. Overlay of chromatograms of: 10 µL injection of 1g/L commercial honey sample (red) and a 10 µL injection of 10 µM standard mix (except nigerose) in DI water (black).

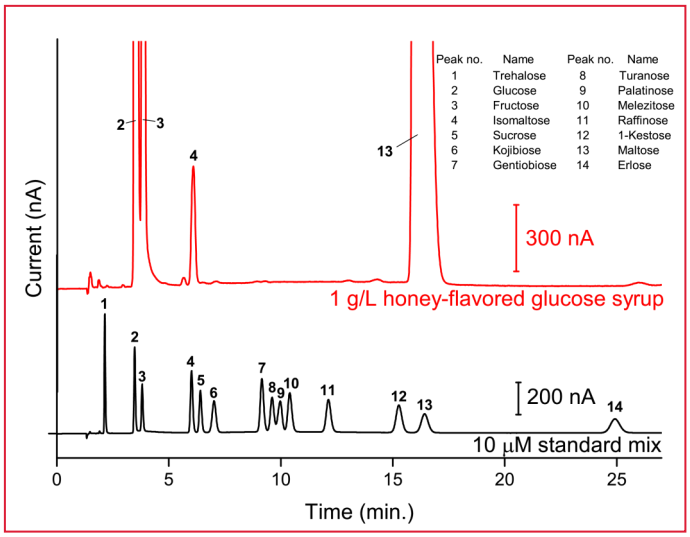

Figure 7. Overlay of chromatograms of: 10 µL injection of 1g/L honey-flavoured glucose syrup sample (red) and a 10 µL injection of 10 µM standard mix (except nigerose) in DI water (black).

Sample analysis

A total of four samples were analyzed, two of which are natural honey sourced from a Swiss beekeeper during the spring and summer seasons of 2022. One commercial honey product and honey-flavored glucose syrup were purchased from a supermarket in The Netherlands. The honey-flavored glucose syrup was to serve as an example of fraudulent honey. The chromatograms of the samples are shown in Figure 4—7. The nutritional labels on the products bought from the supermarket do not show detailed information about the sugar content of these products. The sugar contents in the samples were determined using a calibration curve based on the standards in the concentration range of 0.01 - 50 µM. The amount of sugars in the samples (Table 5) was determined mostly from the undiluted samples (1 g/L concentration). However, during the quantification it was found that the concentration of some sugars based on the undiluted samples exceeded the linear working concentration. Therefore, the amount of some of the sugars was taken from diluted samples, for example, the glucose and fructose contents were calculated based on the 0.01 g/L samples.

Glucose and fructose are the most dominant sugars present in all honey samples. In all honey samples, the amounts of almost all sugars are comparable, except for melezitose. Honey with high melezitose content is well-known to crystallize rapidly within the honeycomb, causing an economic loss to the beekeeper [8]. In spring honey and commercial honey, melezitose contents are low (0-0.2 g/100 g honey), while in

Table 5

Sugar contents (g / 100 g product)

Compound	*Spring honey*	*Summer honey*	*Commercial honey*	*Glucose syrup*
Trehalose	-	0.4	-	-
Glucose	32.4*	27.4*	30.2*	24.3*
Fructose	35.5*	31.9*	42.9*	7.2*
Isomaltose	1.5	2.3[b]	1.0	0.7
Sucrose	0.1	0.1	0.4	-†
Kojibiose	2.0[#]	2.5[#]	0.7	-
Gentiobiose	-	0.1	-	-
Turanose	2.0[#]	2.0[#]	0.9	-
Palatinose	0.2	0.2	-	-
Melezitose	0.2	4.7[#]	-	-
Raffinose	0.1	0.6	-	-
1-Kestose	0.1	0.4	-	-
Maltose	1.2	0.8	1.7	31.1*
Erlose	1.1	1.0	0.1	-

*)calculated from 0.01 g/L samples, [#])calculated from 0.1 g/L samples.
†) No quantifiable amount found in the sample.

Table 6

Unadulterated honey criteria (per 100 g product) & sample tests

Parameter	*Criteria*	*Spring honey*	*Summer honey*	*Commercial honey*	*Glucose syrup*
Sucrose	< 5 g	0.1 g	0.1 g	0.4 g	0.0 g
Maltose	< 4 g	1.2 g	0.8 g	1.7 g	31.1 g
Fructose + Glucose	> 45 g	67.9 g	59.3 g	73.1 g	31.5 g
Fructose/ Glucose	0.9—1.4	1.1	1.2	1.4	0.3

melezitose contents are low (0-0.2 g/100 g honey), while in summer honey, the melezitose contents are almost 40× as high as in spring honey (4.7 g/100 g honey).

To demonstrate the robustness of the method to detect honey fraud, glucose syrup samples were measured. The criteria of the unadulterated honey sample based on the EU Honey Directive and several literatures are listed in Table 6 [2, 15-17]. Based on the specified criteria, the spring and summer honey obtained from a Swiss beekeeper are unadulterated. Commercial honey purchased from a supermarket is also within the criteria of unadulterated honey. On the other hand, glucose syrup contains an amount of maltose about 8× above the criteria. In addition, glucose syrup also has less fructose and glucose contents compared to the criteria, as well as a smaller fructose/glucose ratio. Based on these criteria, the honey-flavored glucose syrup does indeed not comply to the definition & composition of honey as defined in the EU Honey Directive 2001/110/EC.

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Conclusion

The new SweetSep™ AEX200 anion exchange column in combination with SenCell™ provides a sensitive and selective HPAEC-PAD analysis for the quantification of carbohydrates in honey. High-resolution separation is shown for the 15 carbohydrates that commonly occur in honey, within 25 minutes run time. Excellent linearity was obtained for 14 carbohydrates between 0.01 µM to 50 µM, showcasing the method's suitability for the quantification of carbohydrates. The analysis of various samples demonstrated the method's ability to identify adulterated and/or fraudulent honey products based on their sugar contents (Glucose, Fructose, Sucrose and Maltose).

Figure 8. Recommended instrument configuration for this application: the ALEXYS Carbohydrate Analyzer. The system consists of a P6.1L quaternary LPG pump capable of running gradient program, an AS6.1L autosampler, a CT2.1 column thermostat, an ET210 Eluent tray for nitrogen blanketing, and a DECADE Elite electrochemical detector. The ALEXYS Carbohydrate Analyzer can be fully controlled by different Chromatography Data System (CDS) software, namely DataApex™ Clarity™ CDS (version 8.3 and up) or Thermo Scientific™ Chromeleon™ CDS (version 7.2 SR5 and up).

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
260.0030	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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