Sugars in Honey Using HPAE-PAD: What Is the Best Column?

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Key Words
Honey, CarboPac, adulteration, carbohydrates, consumer protection

Goal
To develop a method to separate and determine fructose, glucose, sucrose, turanose, maltose, trehalose, isomaltose, erlose, raffinose, and melezitose in different honey samples using HPAE-PAD

Introduction
Honey is defined by the Codex Alimentarius as the natural, sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honeycomb to ripen and mature.[1]

A distinction is made between honey and honeydew honey: Blossom honey, also called nectar honey, is the honey that comes from nectar of plants. Examples of blossom honey are acacia honey, lavender honey, and rape honey.[1]

Honeydew honey, on the other hand, is honey that comes mainly from excretions of plant sucking insects (hemiptera) on the living parts of plants or secretions of living parts of plants[1]. Blossom honey and honeydew honey have different legal requirements.[1,2]

The main components of honey are sugars (70–80 %), water (15–20 %), organic acids, enzymes, amino acids, pollen, minerals, and solid particles.[3] The most prominent sugars in honey are fructose and glucose, making up about 80–90 % of the total sugar content. Other sugars are sucrose, maltose, isomaltose, turanose, erlose, trehalose, raffinose, and melezitose. The minimum fructose and glucose content in honey, as a sum-parameter, as well as maximum sucrose content is defined by the Codex Alimentarius (Tables 1 and 2), EU legislation,[2] and national laws. The fructose:glucose ratio has been shown to be characteristic for different unifloral honeys.[4,5]

Moreover, the sugar content in honey varies depending on flower, region of production, and feeding practice of the bees. For this reason, the quantification of a broad range of sugars in honey samples is a useful tool for verifying product declaration and labeling as well as to uncover honey adulteration. Determining the sugar fingerprint of a honey sample may help to differentiate between blossom honey and honeydew honey or may help to determine the purity of unifloral honey, if used together with other analytical tools used for this purpose (e.g. conductivity, water content, isotope ratio measurements, pollen analysis, etc.).

Table 1. Minimum fructose-glucose content in honey. Extracted from Codex Alimentarius.[1]

	Sum Fructose and Glucose
Blossom honey	not less than 60 g per 100 g of honey
Honeydew honey, blends of honeydew honey with blossom honey	not less than 45 g per 100 g of honey

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Table 2. Maximum sucrose content in honey. Extracted from Codex Alimentarius.[1]

	Sucrose
Blossom honey	not more than 5 g per 100 g of honey
Alfalfa (*Medicago sativa*), Citrus spp., False Acacia (*Robinia pseudoacacia*), French honeysuckle (*Hedysarum*), Menzies Banksia (*Banksia menziesii*), Red Gum (*Eucalyptus camaldulensis*), Leatherwood (*Eucryphia*)	not more than 10 g per 100 g of honey
Lavender (*Lavandula spp*), Borage (*Borago officinalis*)	not more than 15 g per 100 g of honey

Equipment

- SP pump, quaternary with degas (P/N 075924)
- DC with dual temperature zone, one injection valve, (P/N 075942)
- EG Eluent Generator (P/N 072045)
- ED Detector, (P/N 072042)
- ED Detector Cell, (P/N 072044)
- ED Detector reference electrode (Ag/AgCl), (P/N 061879)
- Gold ED Electrode (P/N 061749)
- EO Eluent Organizer Trays with four 2 L bottles (P/N 072058)
- AS Autosampler with sample cooling (P/N 063106)
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System 6.8 software

Reagents and Standards

- Deionized water (DI), Type I reagent grade, 18 MΩ·cm resistivity or better, filtered through a 0.2 µm filter immediately before use
- Sodium hydroxide solution 50–52% Fluka (P/N 72064) for applications without eluent generation.

Standards

- D(+)-Glucose Fluka (P/N 49139)
- D(-)-Fructose Sigma (P/N F0127-100G)
- Sucrose Sigma (P/N 84097)
- Isomaltose 98% Sigma (P/N I7253-100G)
- Melezitose hydrate Aldrich (P/N 85,037-3)
- Raffinose pentahydrate Aldrich (P/N 206679-1G)
- Maltose monohydrate Aldrich (P/N M9171)
- Erlose 97% Sigma (P/N E1895)
- Turanose Fluka (P/N 93760)
- Trehalose Fluka (P/N 90208)

Preparation of Reagents and Solutions

Samples

For method development, blossom honey was used. The samples were collected from importers, retailers, and producers active in the Swiss market by the Cantonal Food Enforcements authorities.

Sample Preparation

5 g of honey were dissolved in 50 mL DI water, diluted (suitable concentration), and filtered through a 0.45 µm filter.

Table 3. Example preparations of working standard solutions.

Substance	Stock solution (mg/mL)	Standard 1 (mg/L)	Standard 2 (mg/L)	Standard 3 (mg/L)	Standard 4 (mg/L)
D(+)-Glucose	0.4	7.5	3.7	1.9	0.7
D(-)-Fructose	0.5	10.2	5.1	2.5	1.0
Sucrose	0.6	11.8	5.9	3.0	1.2
Isomaltose 98%	1.8	35.9	17.9	9.0	3.6
Melezitose	0.6	13.0	6.5	3.2	1.3
Raffinose pentahydrate	1.0	19.3	9.6	4.8	1.9
Maltose monohydrate	0.7	13.3	6.6	3.3	1.3
Erlose 97%	1.3	25.8	12.9	6.5	2.6
D(+)-Trehalose	0.5	9.4	4.7	2.4	0.9
D(+)-Turanose 98%	2.0	80.0	40.0	20.0	10.0

Eluent Solution (700 mM NaOH)

Dilute 56 g of the concentrated sodium hydroxide stock solution in thoroughly degassed DI water to a final volume of 1000 mL. The general procedure for eluent preparation is described in Technical Note 71.[6]

Working Standard Solutions

The initial stock solutions for the individual carbohydrates were prepared by dissolving the appropriate amount in DI water. The working standards were obtained after mixing and diluting the initial stock solutions in DI water. The ready-to-use stock solutions and the working standard solutions are stored at 4 °C and are stable for several weeks. For turanose, a separate standard solution is prepared. Example preparations are presented in Table 3.

Conditions

Amperometric Detection

Carbohydrate Waveform

Time (s)	Potential (V)	Integration
0.00	+0.1	
0.20	+0.1	Begin
0.40	+0.1	End
0.41	–2.0	
0.42	–2.0	
0.43	+0.6	
0.44	–0.1	
0.50	–0.1	

CONDITIONS I

Columns	Thermo Scientific™ Dionex CarboPac™ PA10 Guard, 2 × 50 mm (P/N 057181) Thermo Scientific™ Dionex™ CarboPac™ PA10, 2 × 250 mm (P/N 057180)	
Eluent Source	Eluent Generator (KOH)	
Eluent Gradient	Time (min)	KOH (mmol/L)
	0.0	50
	5.0	50
	20.0	95
	27.0	95
	31.0	50
	36.0	50
Flow Rate	0.28 mL/min	
Temperature (Column)	32 °C	
Temperature (Detector)	15 °C	
Injection Volume	10 µL	
Detection	Pulsed Amperometry	
Working Electrode	Au	
Reference Electrode	Ag/AgCl	
Background	12 nC	
Noise	1–10 pC	
System Backpressure	2350 psi	

CONDITIONS II

Columns	Thermo Scientific™ Dionex™ CarboPac™ PA200 Guard, 3 × 50 mm (P/N 062895) Thermo Scientific™ Dionex™ CarboPac™PA200, 3 × 250 mm (P/N 062896)		
Eluents	A: Water (DI) B: 700 mM NaOH		
Eluent Gradient	Time (min)	%A	%B
	0.0	97.1	2.9
	3.0	97.1	2.9
	25.0	64.3	35.7
	30.00	64.3	35.7
	30.01	97.1	2.9
	38	97.1	2.9
Flow Rate	0.40 mL/min		
Temperature (Column)	30 °C		
Temperature (Detector)	15 °C		
Injection Volume	10 µL		
Detection	Pulsed Amperometry		
Working Electrode	Au		
Reference Electrode	Ag/AgCl		
Background	36 nC		
Noise	1–10 pC		
System Backpressure	2670 psi		

CONDITIONS IIA

Columns	Dionex CarboPac PA200 Guard, 3 × 50 mm Dionex CarboPac PA200, 3 × 250 mm		
Eluents	A: Water (DI) B: 700 mM NaOH		
Eluent Gradient	Time (min)	%A	%B
	0.0	97.1	2.9
	9.0	97.1	2.9
	25.0	64.3	35.7
	30.0	64.3	35.7
	30.01	97.1	2.9
	38	97.1	2.9
Flow Rate	0.40 mL/min		
Temperature (Column)	30 °C		
Temperature (Detector)	15 °C		
Injection Volume	10 µL		
Detection	Pulsed Amperometry		
Working Electrode	Au		
Reference Electrode	Ag/AgCl		
Background	38 nC		
Noise	1-10 pC		
System Backpressure	2700 psi		

CONDITIONS III

Columns	Thermo Scientific™ Dionex™ CarboPac™ PA100 Guard, 2 × 50 mm (P/N 057183) Thermo Scientific™ Dionex™ CarboPac™ PA100, 2 × 250 mm (P/N 057182)		
Eluents	A: Water (DI) B: 700 mM NaOH		
Eluent Gradient	Time (min)	%A	%B
	0.0	97.1	2.9
	3.0	97.1	2.9
	25.0	64.3	35.7
	30.0	64.3	35.7
	30.01	97.1	2.9
	38	97.1	2.9
Flow Rate	0.34 mL/min		
Temperature (Column)	25 °C		
Temperature (Detector)	15 °C		
Injection Volume	10 µL		
Detection	Pulsed Amperometry		
Working Electrode	Au		
Reference Electrode	Ag/AgCl		
Background	34 nC		
Noise	1–10 pC		
System Backpressure	2500 psi		

Results

All standards containing turanose showed a characteristic increase of the baseline around six minutes (Figure 1). This effect is due to a pH-dependent, partial on-column degradation of turanose forming fructose and glucose, resulting in the baseline rise at the retention times where they elute. In order to evaluate any impact on the analytical determination, the recovery of turanose was determined by performing standard addition experiments. Recovery was determined to 99 % ± 2% (n=3), proving the analytical concept for turanose.

While turanose decomposes completely to fructose and glucose if stirred in a caustic solution for 2 h, the on-column process only results in a baseline rise. To simulate a potential effect on the determination of fructose and glucose—the main components in honey—a standard solution containing a typical concentration of turanose was injected, with no peaks for fructose and glucose detected. A "forced" integration of the baseline at the times for fructose and glucose resulted in a potential increase of 0.05 % glucose and 0.01 % fructose, compared to the minimum glucose and fructose content to be expected in real honey samples. Hence, the potential impact on the determination of turanose, glucose, and fructose can be considered negligible.

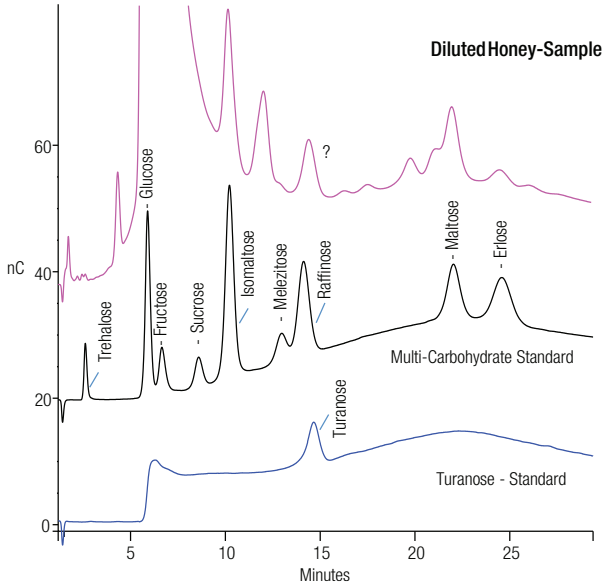

Figure 1. Chromatogram of a diluted honey sample (1 to 1250; pink), sugar standard 2 (black) and turanose standard 2 (blue). Column: Dionex CarboPac PA10; Condition I.

Figure 1 shows the separation of two standard solutions and a diluted honey sample in less than 30 minutes using a Dionex CarboPac PA10 column. The Dionex CarboPac PA10 column is an anion-exchange column optimized to determine the amino, neutral, and acidic monosaccharides found in the carbohydrate moieties of mammalian glycoproteins. It is the column of choice for high sensitivity monosaccharide analyses, and can be used in conjunction with eluent generation. The separation of the diluted honey sample reveals the high analytical complexity of analyzing sugars in honey. Not only are there disparate peak area ratios, but also chromatographic interferences. Comparing the different traces, it becomes obvious that the conditions chosen allow the separation of all carbohydrates except for raffinose and turanose. As turanose is one of the most prominent sugars in honey samples, its quantification is of highest importance to ensure a proper honey characterization. Due to the apparent coelution of raffinose and turanose, the chromatographic conditions applied do not allow a reliable fingerprint analysis and sugar quantification of the target sugars given the high complexity of the matrix analyzed.

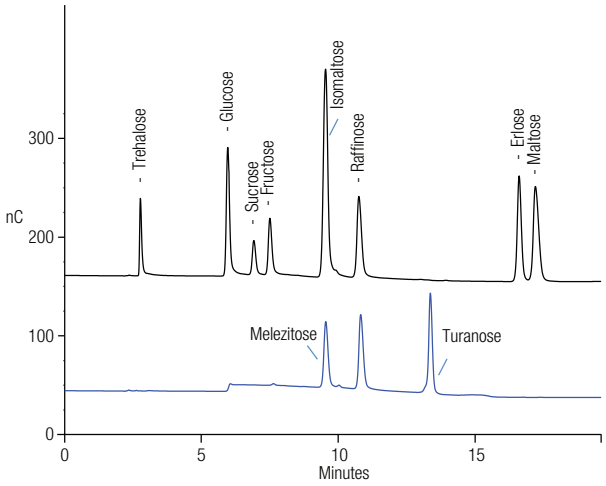

Figure 2. Chromatogram of a turanose-raffinose-melezitose standard solution (blue) and sugar standard 1 solution (black). Column: Dionex CarboPac PA200; Condition II.

The next experiments focused on using the Dionex CarboPac PA200 column. The Dionex CarboPac PA200 column is a solvent-compatible, nonporous, high-efficiency, polymeric anion-exchange column. Its resin consists of 5.5 µm diameter, nonporous beads covered with a layer of functionalized MicroBead latex. This pellicular resin structure permits excellent mass transfer, resulting in high-resolution chromatography and rapid re-equilibration. Though originally designed to provide high resolution separations for oligosaccharide mapping and analysis this column can be used with pure NaOH eluents providing a different chromatographic selectivity than the previously used Dionex CarboPac PA10. Figure 2 shows the separation of all carbohydrates using the chromatographic conditions given as "Condition II". The separation, using an NaOH gradient, is completed in about 20 minutes, providing a good separation of turanose from other sugars present in honey samples. However, this time melezitose coeluted with isomaltose, contradicting the intended purpose of the analysis. In order to address this situation, a modified NaOH gradient was tested (Condition IIa).

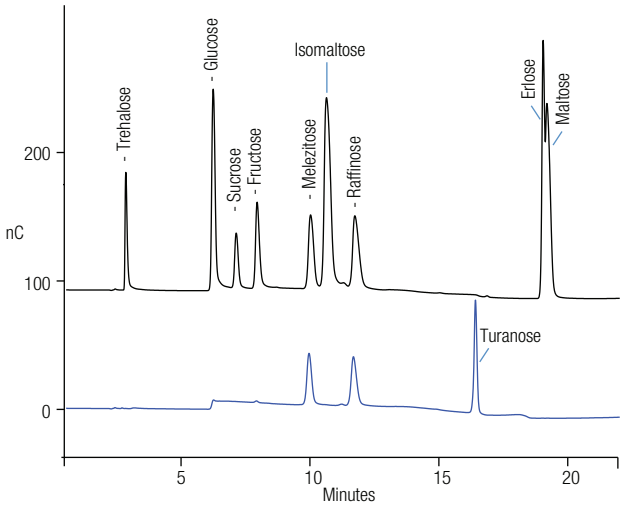

Figure 3. Chromatogram of a turanose-raffinose standard solution (blue) and sugar standard 1 solution (black). Column: Dionex CarboPac PA200; Condition IIa.

The change of the NaOH gradient allowed for the separation of melezitose and isomaltose, and turanose eluted free of interferences. The separation of erlose and maltose, however, was impaired.

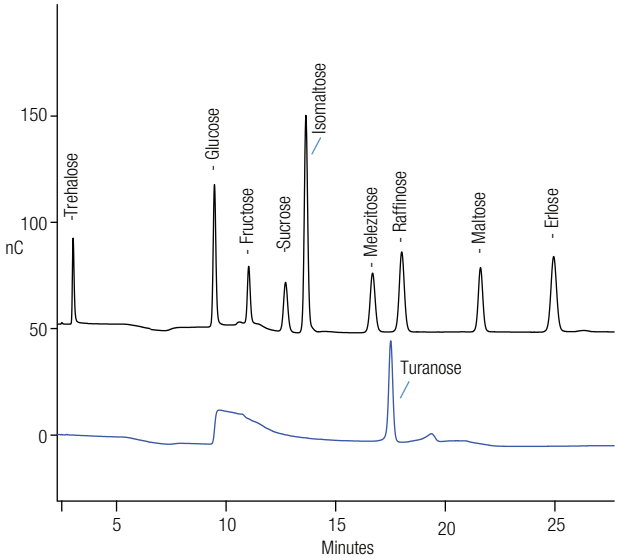

Figure 4. Chromatogram of a turanose standard 2 solution (blue) and sugar standard 2 solution (black). Column: Dionex CarboPac PA100; Condition III.

The Dionex CarboPac PA100 column uses an advanced, high performance, pellicular anion exchange resin consisting of 8.5 µm diameter, nonporous beads to which a layer of functionalized MicroBead latex is attached. Comparing the column parameters, the resin used for the Dionex CarboPac PA100 column shows approximately a 45% higher anion exchange capacity than the Dionex CarboPac PA200, considering similar column formats. Its solvent compatibility made the Dionex CarboPac PA100 an ideal candidate for the analysis of complex honey samples. Applying a similar NaOH gradient (chromatographic conditions see Condition III) resulted in the separation shown in Figure 4. It shows a good separation of all target carbohydrates in standard solutions. The analytical run is completed after 30 minutes. The overall cycle time of the analytical method is close to 40 minutes.

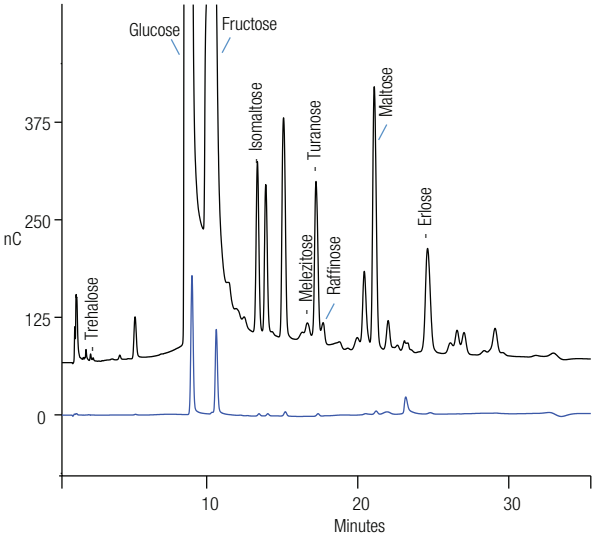

Figure 5. Chromatogram of an acacia honey sample. Black trace: 1:25000, Blue trace: 1:1250. Column: Dionex CarboPac PA100; Condition III.

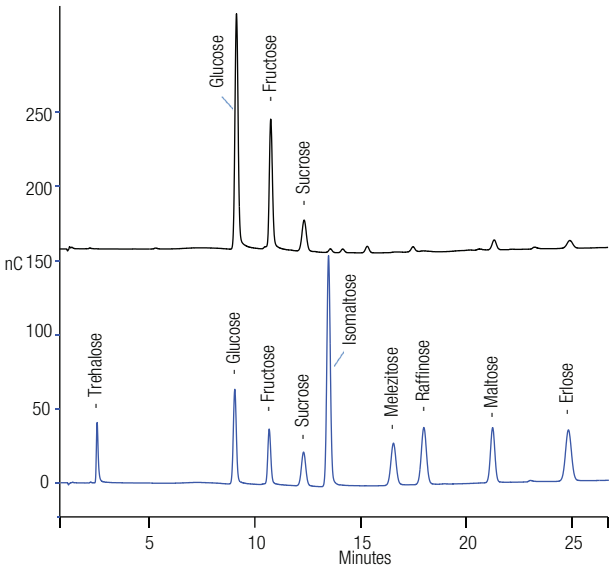

Figure 6. Chromatogram of a Swiss blossom honey sample with atypically high amount of sucrose. Blue trace: Standard 3, black trace: honey 1 at 1:125000 with a final concentration of 144 g/kg sucrose. Column: Dionex CarboPac PA100; Condition III.

In Figure 5, an example of a sugar fingerprint for acacia honey is shown. Despite the complex matrix composition, the separation of all target carbohydrates is achieved with good resolution, allowing the quantitation of even lower concentrations. Due to the high fructose and glucose content, the sample was measured at two different dilutions rates: 1:25000 for the quantification of glucose and fructose, and 1:250 for the remaining sugars.

More than 40 honey samples were collected from importers, retailers, and producers active in the Swiss market by the Cantonal Swiss Enforcement authorities. The origin of the samples was mainly Swiss or European. Monofloral (acacia, chestnut, and lavender), honeydew honey as well as blended blossom honeys were chosen and successfully analyzed using the conditions described above. Figure 6 presents an example of a honey sample with an atypically high concentration of sucrose, which may be explained by additional analytical steps.

Conclusions

The development of an HPAE-PAD method for the determination of sugars in honey samples was achieved using the Dionex CarboPac PA100 column. This column allows the separation of fructose, glucose, sucrose, turanose, maltose, trehalose, isomaltose, erlose, raffinose, and melezitose with minimum sample preparation and an overall cycle time of 38 minutes. The detection method is sensitive enough to allow the determination of lower concentrations of carbohydrates, while also being robust enough to handle higher concentrations of the major components, glucose and fructose.

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