

## PROFILING OF CARBOHYDRATES IN HONEY BY HILIC-MS

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### INTRODUCTION

Honey is a popular natural product that is commonly consumed either as a food or as an ingredient in processed foods. The main constituents of honey are fructose and glucose. The minor carbohydrates in honey include di- and tri-saccharides. About two dozen di- and tri-saccharides in honey have been identified<sup>(1)</sup>. Most of these di- and tri-saccharides consist of fructose and/or glucose exclusively as the saccharide units. This makes the separation of these minor carbohydrates very challenging.

Honey is prone to be adulterated by syrup or sweetener substitutes. The botanical and the geographical origins of honey could also be fraudulently claimed to gain economic benefit. Codex has a definition for honey and its quality specification<sup>(2)</sup>. Many researchers have investigated the minor carbohydrate composition to characterize authentic honey of various botanical and geographical origins<sup>(3)</sup>.

Ion-Exchange Chromatography (IC) and Hydrophilic Interaction Liquid Chromatography (HILIC) are common technologies used for the carbohydrate analysis. As to the honey analysis, since its carbohydrate composition is so complicated, the separation efficiency attained by current methods is not enough, a better analysis method with higher separation efficiency is still desired.

Recently a new HILIC-MS method using a Waters XBridge™ BEH Amide column and a Waters ACQUITY QDa™ Mass detector has been developed for sugar analysis<sup>(4)</sup>. It has excellent separation efficiency for carbohydrates and has good column stability (column lifetime). Here this HILIC method was modified for the profiling and quantification of carbohydrates in honey. Excellent separation and sensitive detection of mono-, di- and tri-saccharides in various honey and syrup samples has achieved.

### METHODS

#### Standard Preparation:

Stock solutions of each carbohydrate standard were prepared at 10 mg/mL in acetonitrile-water (1/1 v/v) mixture. A reference Internal Standard (IS) mixture of stable isotope labeled fructose-<sup>13</sup>C<sub>6</sub>, glucose-<sup>13</sup>C<sub>6</sub>, and sucrose-<sup>13</sup>C<sub>6</sub> was prepared at about 0.5 mg/mL. The stock solution and the reference IS mixture were used to prepare standard mix solutions at levels 1, 2, 5, 10, 20, 50, 100, and 200 µg/mL (ppm) with the reference IS at 25 µg/mL.

#### Sample Preparation:

Honey and syrup samples were purchased from local stores. Their label description and country of origin are listed in Table 1. These samples were dispersed in acetonitrile-water mixture (1/1 v/v) to form stock sample solutions at about 50 mg/mL. These solutions were filtered through 0.2 µm PVDF membrane syringe filters. Aliquots of the filtrates were further diluted with acetonitrile-water (1/1 v/v) at various dilution ratios (5 and 500 were used) for the carbohydrate analysis. Aliquots of the reference IS stock solution were added to each final sample solution at 25 µg/mL prior to the analysis.

#### Column care:

New XBridge BEH Amide XP columns were flushed with 50 column volumes of 80/20 v/v acetonitrile-water, followed by 100 column volumes of the conditioning solvent mixture (90:6:4 v/v/v Acetonitrile-Water-Methanol mixture with 0.05 v/v% Diethylamine, or DEA, and 5 mg/L guanidine hydrochloride, or GdnHCl).

#### LC conditions

LC system: ACQUITY Arc™ System with Column Heater (CH30-A)  
Runtime: 25.0 min (include 5 min conditioning time)  
Column: XBridge BEH Amide XP (2.5 µm, 3.0 × 10 mm)  
Column temp.: 90°C  
Injection vol.: 1 µL  
Mobile phase: A) 90:6:4 v/v/v Acetonitrile-Water-Methanol (0.05% DEA and 0.5 mg/L GdnHCl)  
B) 78:20:2 v/v/v Acetonitrile-Water-Methanol (0.05% DEA and 0.5 mg/L GdnHCl)  
C) 90:6:4 v/v/v Acetonitrile-Water-Methanol (0.05% DEA and 0.5 mg/L GdnHCl)  
Flow rate: 0.8 mL/min  
Elution program: Table 2

Table 1. Honey and syrup samples label description and country of origin.

Sample	Description
Honey A	Raw organic, USA
Honey B	Clover, USA
Honey C	wild flower, USA
Honey D	Turkey
Honey E	Canada
Maple syrup	Vermont
Corn syrup	No high fructose, USA
Blue agave	Mexico

Table 2. Gradient elution conditions used for quantification of sugars in samples.

Time (min)	Flow Rate (mL/min)	%A	%B	%C	Curve
Initial	0.800	100	0	0	Initial
4.0	0.800	100	0	0	6
13.0	0.800	0	100	0	6
20.0	0.800	0	100	0	6
20.1	0.800	0	0	100	6
25.0	0.800	0	0	100	6
25.1	0.800	100	0	0	6

#### MS conditions

MS system: ACQUITY QDa™ (Performance)  
Ionization mode: ESI-  
Capillary voltage: 0.8 kV  
Cone Voltage: 5 V  
Probe temp: 600°C  
Acquisition Rate: 5 Hz  
Full Scan: 100-1250 m/z  
SIR [M+Cl]<sup>-</sup>: 215.0 Monosaccharides  
221.0 Fructose-<sup>13</sup>C<sub>6</sub>, glucose-<sup>13</sup>C<sub>6</sub>  
377.1 Disaccharides  
383.1 Sucrose-<sup>13</sup>C<sub>6</sub>  
539.2 Trisaccharides  
701.2 Tetrasaccharides  
863.2 pentasaccharides  
1025.2 hexa-saccharides  
1187.3 hepta-saccharides

#### 1) Method optimization

The analysis conditions used in the previous study<sup>(4)</sup> were modified to include a gradient ramp to 20% water in the mobile phase for a faster elution of oligosaccharides. Figure 1 shows the effect of the cone voltage on the turanose MS spectrum. Other standards showed a similar effect of cone voltage on MS signal.

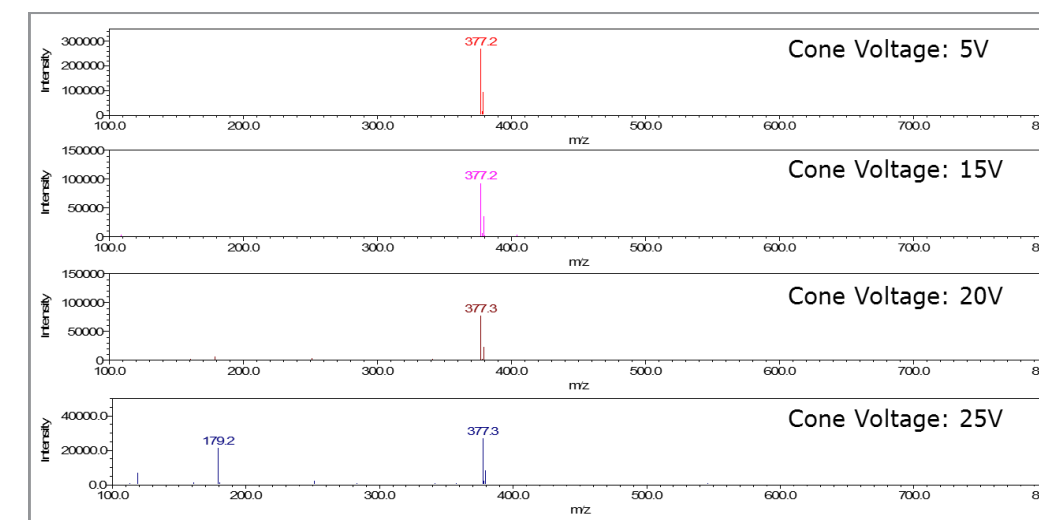


Figure 1. Overlay of extracted mass spectra of the turanose peak under different QDa cone voltages. Other experimental conditions were the same. Under the 5V cone voltage, the most abundant chloride adduct ions [M+Cl]<sup>-</sup> were obtained.

#### 2) Carbohydrates in honey

Figure 2 shows chromatograms of mono-, di- and tri-saccharides in honey from the Single Ion Recording (SIR) detection of carbohydrate chloride adduct ions [M+Cl]<sup>-</sup>. These chromatograms feature well resolved peaks and flat baseline.

The identities of the peaks were tentatively assigned to standards that had the same RT by comparing the honey peaks' RT with those of standards. 25 standards, including 14 disaccharides and 6 trisaccharides, were screened for honey peak identification. The 25 standards and their estimated capacity factor (k') are listed in Table 3.

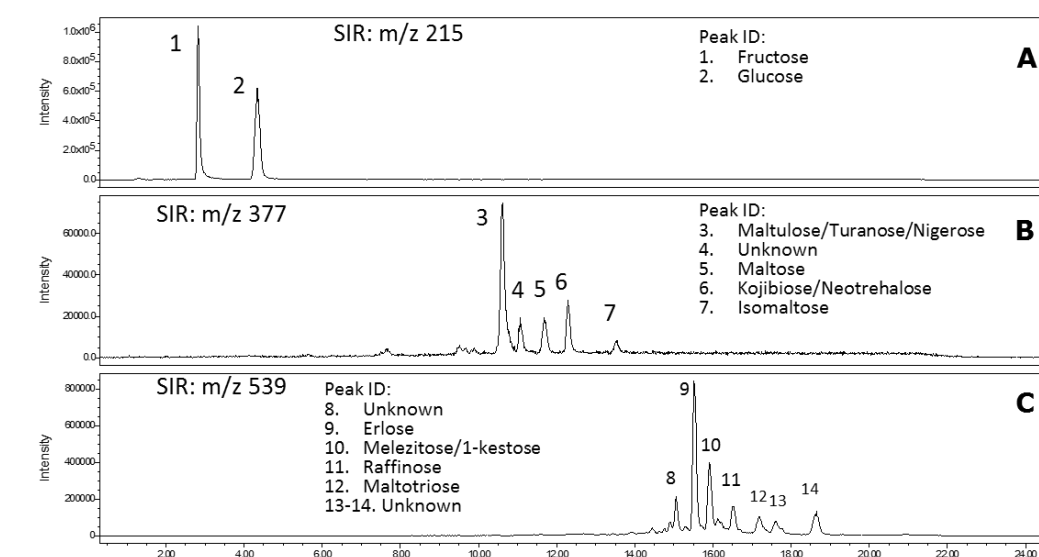


Figure 2. Chromatograms of mono-, di- and trisaccharides in Honey A. (A) SIR channel at m/z 215 for monosaccharide chloride adduct ions [M+Cl]<sup>-</sup>. Sample concentration: 0.1 mg/mL. (B) SIR channel at m/z 377 for disaccharide ions [M+Cl]<sup>-</sup>. Sample concentration: 0.1 mg/mL. (C) SIR channel at m/z 539 for trisaccharide ions [M+Cl]<sup>-</sup>. Sample concentration 10 mg/mL. The peak ID were tentatively assigned to standards that had the same RT.

Table 3. List of capacity factors (k') of standards under the same conditions in Fig. 2. The analytes in bold are identified as possible constituents in Honey A.

k' values of saccharides under the gradient elution conditions in Fig. 2							
Arabinose	1.77	Laminaribiose	13.87	Kojibiose	18.79	Eriose	22.64
Fructose	2.45	Maltulose	14.05	Neotrehalose	19.21	Melezitose	23.37
Mannose	3.43	Turanose	14.09	Trehalose	19.55	1-Kestose	23.39
Galactose	3.62	Nigerose	14.19	Melibiose	19.61	Raffinose	23.92
Glucose	4.09	Cellobiose	16.22	Isomaltose	20.07	Maltotriose	24.55
Sucrose	12.54	Maltose	16.86	Gentobiose	20.43	Isomaltotriose	28.70
Isomaltulose	13.67						

### RESULTS AND DISCUSSION

#### 3) Carbohydrate profiles in honey and syrup samples

The mono-, di-, and tri-saccharide profiles in 5 honey samples and 3 syrup samples were compared in Figures 3, 4, and 5. Between the honey and the syrup groups, honey has significantly different profiles. Within the honey group, Honey D has quite different di- and tri-saccharide profiles than other honey samples. A closer look at the rest honey samples' disaccharide profiles at a higher sample concentration (10 mg/mL) shows discernable differences in Honey B than the rest of North American honey samples A, C, and E (Fig. 6). Since Honey B was labelled as clover honey, while other North American honeys were not labelled as any specific blossom honey (Table 1), one possible hypothesis would be that the different disaccharide profile in Honey B might be related to the single botanical source of clover. This demonstrates that this HILIC-MS method is excellent in providing the fine details of carbohydrate profiles in honey samples.

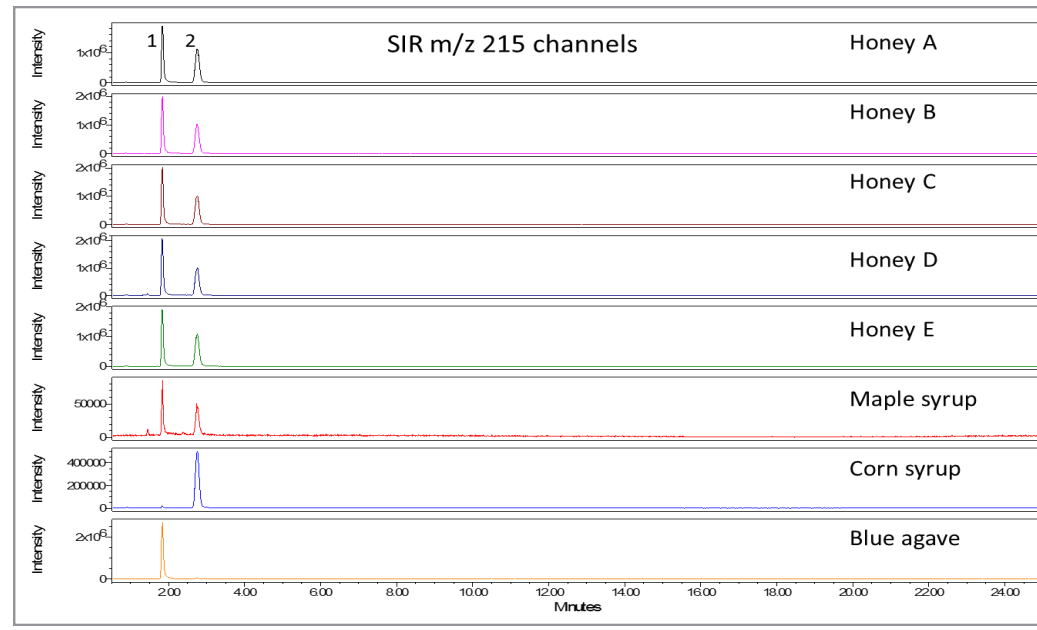


Figure 3. Comparison of monosaccharide profiles in honey and syrup samples. Chromatograms are from SIR channels at m/z 215 for monosaccharide chloride adduct [M+Cl]<sup>-</sup>. Peak 1: fructose, Peak 2: glucose. Sample concentrations are at 0.1 mg/mL. Honey sample info is in Table 3.

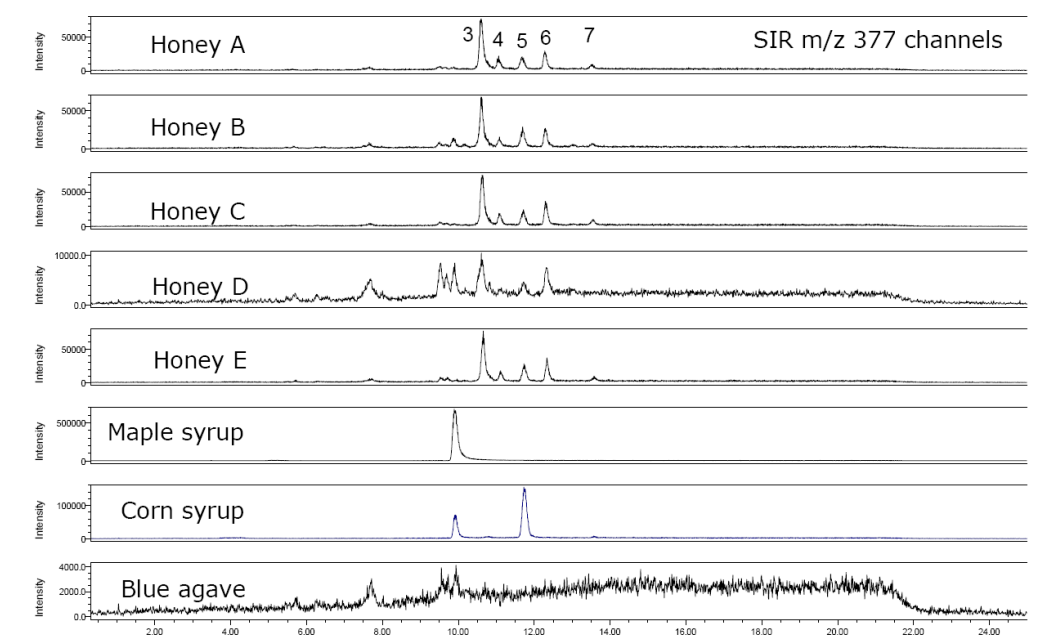


Figure 4. Comparison of disaccharide profiles in honey and syrup samples. Chromatograms are from SIR channels at m/z 377 for disaccharide chloride adduct [M+Cl]<sup>-</sup>. Peak assignment is the same as in Figure 2. Sample concentrations are at 0.1 mg/mL.

#### 4) Quantification of sugars in honey and syrup samples

The fructose, glucose, and sucrose in honey and the syrup samples were quantified (Table 4). Figure 7 shows the calibration curves for fructose, glucose, and sucrose. The limit of quantitation (LOQ) were estimated at 0.02% (0.02 g/100g of honey) using the lowest concentration level in the calibration plots and the sample amount in the sample preparation. The relative standard deviation (RSD) from 4 measurements on two separate days are also shown in Table 4. The accuracy of the fructose and glucose measurements was evaluated by spiking the maple syrup sample with fructose and glucose. Spike recovery of 94% and 91% were obtained for fructose and glucose, respectively (Table 5).

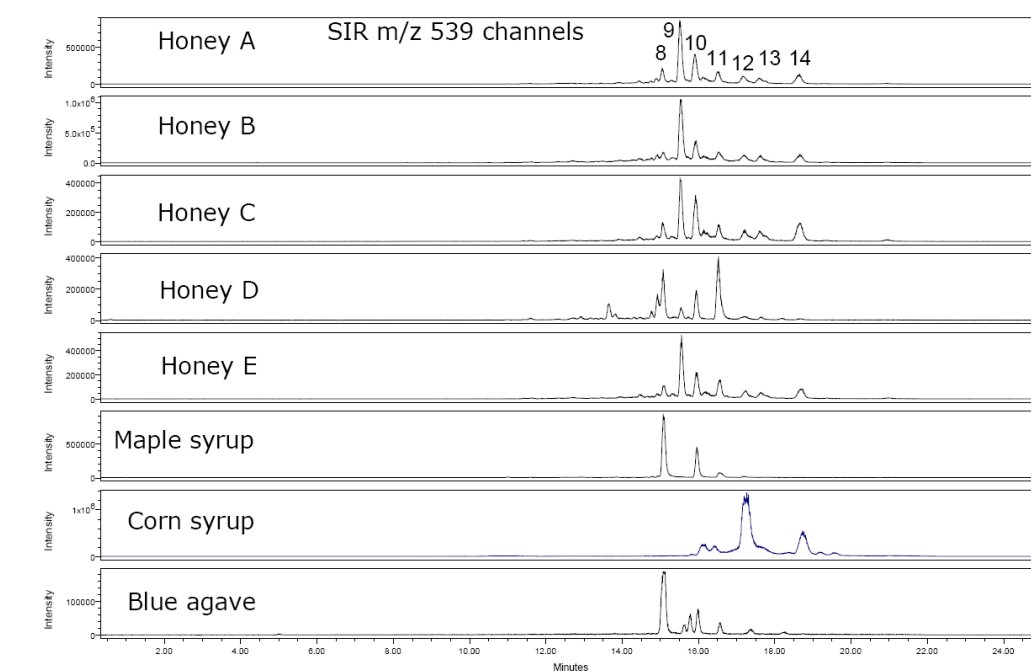


Figure 5. Comparison of trisaccharide profiles in honey and syrup samples. Chromatograms are from SIR channels at m/z 539 for trisaccharide chloride adduct [M+Cl]<sup>-</sup>. Peak assignment is the same as in Figure 2. Sample concentrations are at 10 mg/mL.

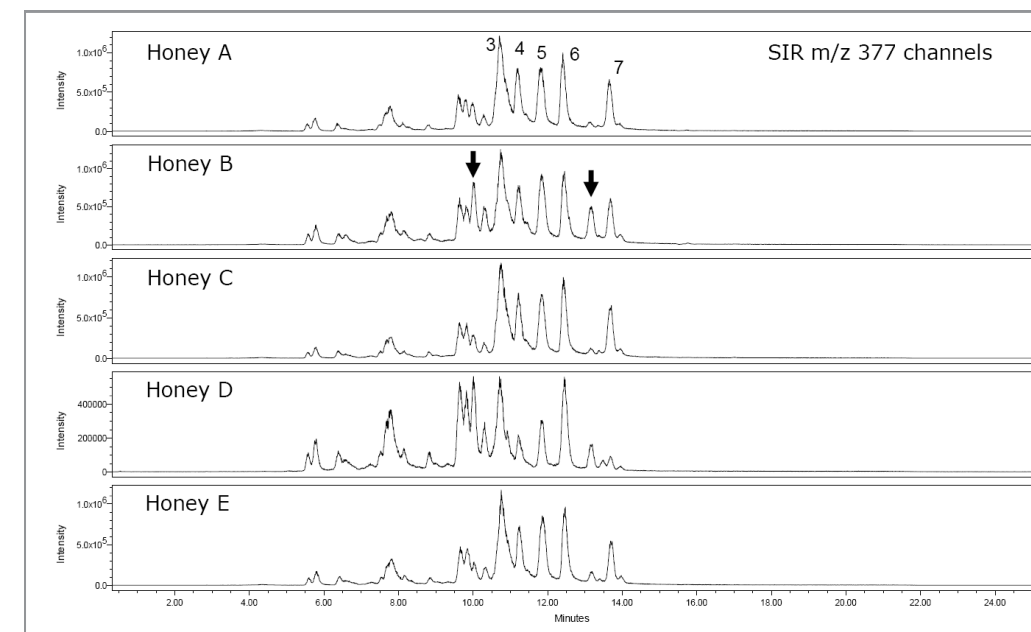


Figure 6. Comparison of disaccharide profiles in honey samples. Chromatograms are from SIR channels at m/z 377 for disaccharide chloride adduct [M+Cl]<sup>-</sup>. Peak assignment is the same as in Figure 2. Sample concentrations are at 10 mg/mL. The arrow indicates the difference between Honey B and other North American honey samples (A, C, and E). Honey D is a product of Turkey.

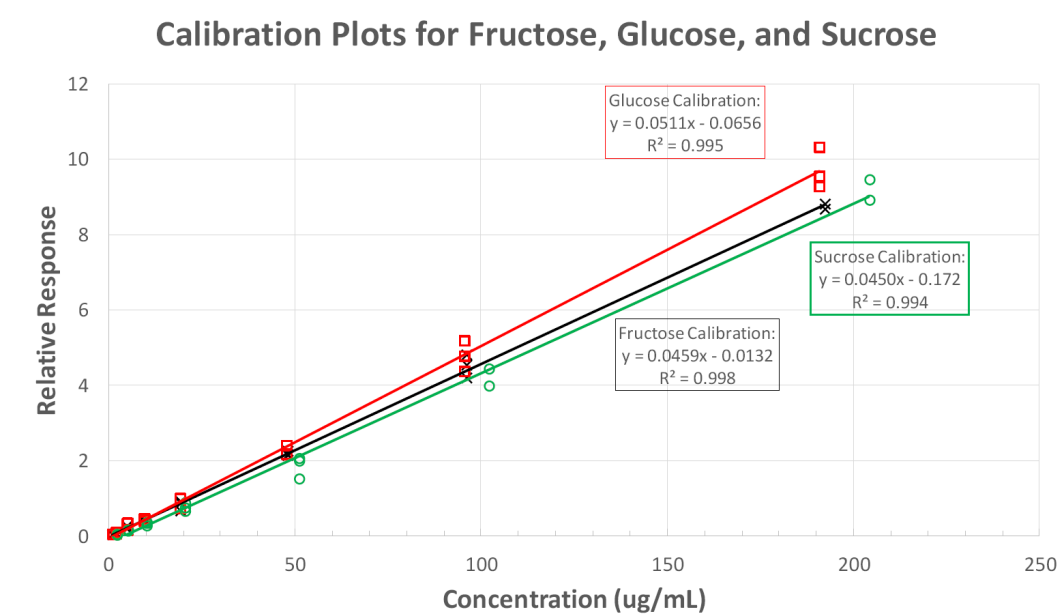


Figure 7. Relationship between the relative responses (analyte peak over reference IS peak) and the analyte concentration for fructose (black), glucose (red) and sucrose (green). Fitting mode: linear with 1/x weighing. Calibration range is 2-200 ppm (µg/mL). The fitted calibration equations and R<sup>2</sup> are shown in the plots.

Table 4. Quantification results on fructose, glucose, and sucrose in honey and syrup samples. The results are average of at least 4 measurements conducted on two separate days. The relative standard deviation of these measurements are also shown.

sample	Fructose		Glucose		Fruc. + Gluc. (g/100g)	Sucrose	
	Avg. (g/100g)	RSD (%)	Avg. (g/100g)	RSD (%)		Avg. (g/100g)	RSD (%)
Honey A	34.2	7%	36.1	4%	70.3	0.2	6%
Honey B	39.5	2%	32.8	4%	72.3	0.7	3%
Honey C	37.8	8%	31.4	4%	69.2	0.2	11%
Honey D	44.9	3%	32.9	6%	77.8	0.3	6%
Honey E	36.9	2%	34.4	9%	71.2	0.1	16%
Maple syrup	0.5	9%	0.6	3%	1.1	53.5	8%
Corn syrup	0.1	9%	12.3	9%	12.4	4.2	3%
Blue agave	71.9	5%	0.8	8%	72.7	0.2	7%

### CONCLUSION

- Well resolved mono-, di-, and tri-saccharide peaks in honey samples were obtained on XBridge BEH Amide XP column.
- Unique characteristics of the di- and tri-saccharide profiles of honey from different botanic and geographic origins were observed. This is useful in differentiating honey from different floral or geographic origins, and in adulteration testing.
- Unknown carbohydrate peaks were uncovered in honey samples. These peaks do not belong to any screened carbohydrate. Further investigation is needed.
- The fructose, glucose, and sucrose content in honey samples were quantified. Their contents can be used to evaluate the quality of the honey using the Codex Alimentarius guide.
- The elution order of saccharides under this HILIC condition is significantly different from that in HPAEC. This HILIC-MS method could be a useful method for the honey analysis.

### References

- Doner LW: The sugars of honey - a review. J Sci Food Agric 28:443-456, 1977.
- Codes Alimentarius Commission. Revised Codex Standard for honey; Codex STAN, 12-1981, Rev. 1 (1987), Rev. 2 (2001).
- J.M. Da Costa Leite et al. Determination of oligosaccharides in Brazilian honeys of different botanical origin, Food Chemistry 70 (2000) 93-98
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ACQUITY Arc System with PDA, XBridge BEH Amide XP column, and QDa Mass Detector

