Waters™

Note d'application

Profiling of Carbohydrates in Honey by HILIC-MS

Jinchuan Yang, Paul D. Rainville

Waters Corporation

Abstract

Here HILIC method was modified for the profiling and quantification of carbohydrates in honey.

Carbohydrate composition in honey can be assessed by HILIC-MS method using a gradient elution with a solvent mixture of acetonitrile, water, and methanol on an XBridge BEH Amide XP Column. Individual chromatograms for the mono-, di-, and trisaccharide groups can be obtained. These chromatograms have excellent separation efficiency for the structurally, closely-related di- and trisaccharides in honey. Differences in the di- and trisaccharide chromatograms were found in honey samples from different countries and from different botanical origins.

Benefits

- Excellent separation efficiency for carbohydrates
- Profiles of di- and trisaccharides in separate chromatograms with flat baseline
- Unique sugar separation selectivity

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 trisaccharides. About two dozen di- and trisaccharides have been identified in honey.[1] Most of these di- and trisaccharides consist of fructose and/or glucose exclusively as the saccharide units. This makes the separation of these minor carbohydrates very challenging. Besides carbohydrates, other minor honey constituents include organic acids, proteins, amino acids, minerals, polyphenols, vitamins, and aroma compounds.

Honey is a relatively high value product and is prone to be adulterated by syrup or sweetener substituents. 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.[2] Many researchers and labs have investigated the minor carbohydrate composition of honey to characterize authentic honey of various botanical and geographical origins.[3] Ion-Exchange Chromatography (IC) and Hydrophilic Interaction Liquid Chromatography (HILIC) are common technologies used for carbohydrate analysis. IC provides excellent separation efficiency for

carbohydrates, but special equipment and technical expertise is often needed to generate good results. HILIC also provides excellent separation efficiency for carbohydrates and its analysis time is usually shorter. However, HILIC column stability is often a concern. As to the honey analysis, since its carbohydrate composition is so complicated, the separation efficiency attained by the current methods is not enough. A better analysis method with higher separation efficiency is still desired.

Recently a new HILIC-MS (mass spectrometry) method using a Waters XBridge BEH Amide XP Column and a Waters ACQUITY QDa Mass Detector has been developed for sugar analysis.[4] It has excellent separation efficiency for carbohydrates and 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 was achieved for mono-, di-, and trisaccharides in various honey and syrup samples.

Experimental

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 an 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) for the carbohydrate analysis. Aliquots of the reference internal standard (IS) stock solution were added to each final sample solution prior to the analysis.

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 1. Honey and syrup sample label descriptions and country of origin.

Standard preparation

Stock solutions of each carbohydrate standard were prepared at 10 mg/mL in an acetonitrile:water (1/1 v/v) mixture. A reference IS mixture of stable isotope labeled fructose-$^{13}C_6$, glucose-$^{13}C_6$, and sucrose-$^{13}C_6$ 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 of 1, 2, 5, 10, 20, 50, 100, and 200 µg/mL (ppm) with the reference IS at 25 µg/mL.

Method conditions

Column conditioning

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 acetonitrile:water:methanol mixture with 0.05 v/v% diethylamine, or DEA, and 5 mg/L guanidine hydrochloride, or GdnHCl).

LC conditions

System:	ACQUITY Arc with Column Heater CH30-A
Runtime:	25.0 min (include 5-min conditioning time)

Column:	XBridge BEH Amide XP, 2.5 µm, 3.0 × 100 mm (p/n: 186006095)
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 5.0 mg/L GdnHCl)
Flow rate:	0.8 mL/min
Elution program:	Table 2

Gradient

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

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

MS conditions

System:	ACQUITY QDa (Performance)
Ionization mode:	ESICapillary
voltage:	0.8 kV
Cone voltage:	5 V
Probe temp.:	600 °C
Acquisition rate:	5 Hz
Full scan:	*m/z* 100–1250
SIR $[M+Cl]^-$:	215.0 Monosaccharides

221.0 Fructose-$^{13}C_6$, glucose-$^{13}C_6$

377.1 Disaccharides

383.1 Sucrose-$^{13}C_6$

539.2 Trisaccharides

701.2 Tetrasaccharides

863.2 Pentasaccharides

1025.2 Hexasaccharides

1187.3 Heptasaccharides

Results and Discussion

Method Development

The analysis conditions used in the previous study4 were modified to include a gradient ramp to 20% water in the mobile phase fora faster elution of oligosaccharides. The XBridge BEH Amide XP Column is very stable under this gradient elution condition. There was no significant column pressure increase during this work (over 400 injections). It was found necessary to condition new BEH amide columns as recommended in the experimental section. The QDa detector parameters (probe temperature, capillary voltage, and cone voltage) have been investigated. 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.

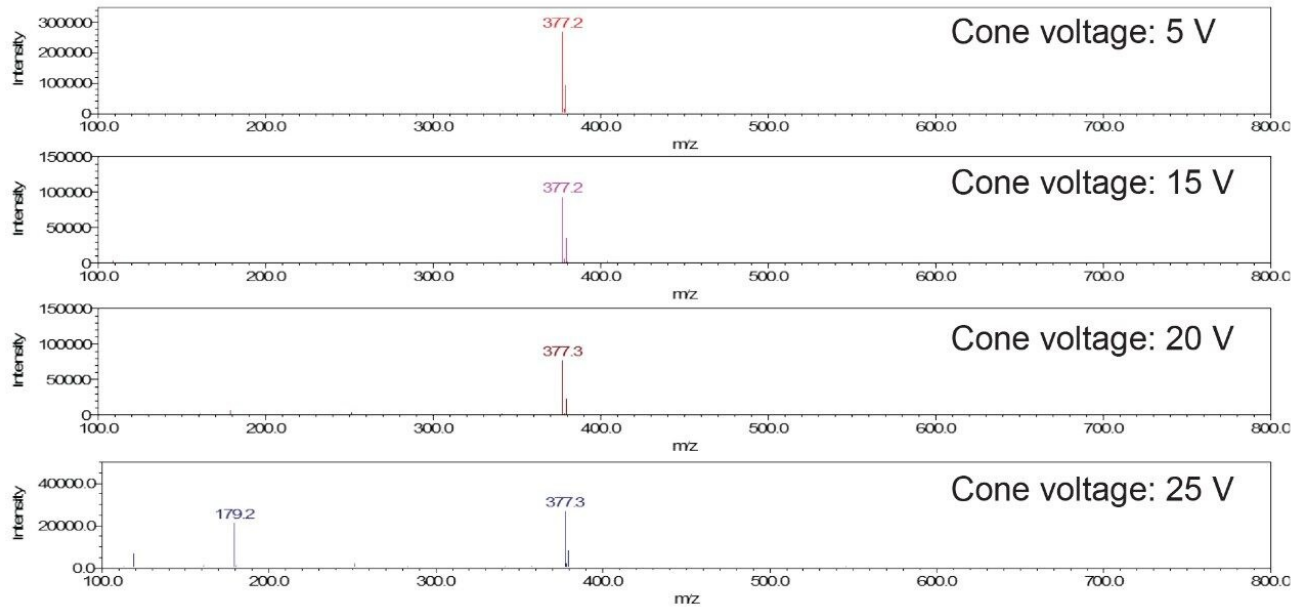

Figure 1. Overlay of extracted mass spectra for the turanose under different QDa cone voltages. Other experimental conditions were the same. The most abundant chloride adduct ions $[M+Cl]^-$ were obtained under the 5 V cone voltage.

Carbohydrates in Honey

Figure 2 shows chromatograms of mono-, di-, and trisaccharides in honey from the single ion recording (SIR) detection of carbohydrate chloride adduct ions $[M+Cl]^-$. These chromatograms feature well-resolved peaks and flat baseline. Fourteen wellresolved main peaks were obtained from the honey sample. This level of separation efficiency is hard to match with other methods. The mono-, di-, and trisaccharide groups eluted in different and wide retention time (RT) regions, so there is no chance for one carbohydrate group to interfere with other groups. The flat baselines in these chromatograms are the results of the highly selective MS detection. Compared to the optical and the electrochemical detectors, the MS detection (SIR) in this method selectively detected the target compounds and ignored non-isobaric compounds. This makes the chromatogram baseline flat and has little interference from the matrix.

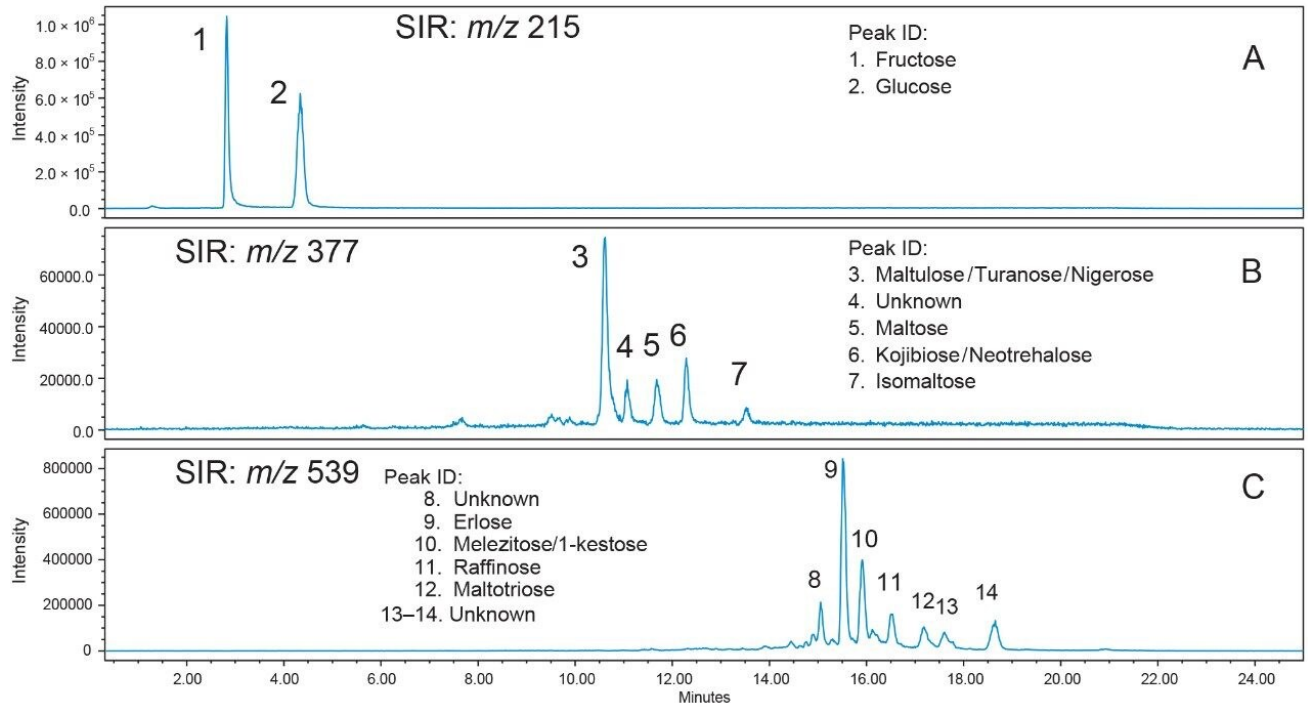

Figure 2. Chromatograms of mono-, di-, and trisaccharides in Honey A. (A) SIR channel at m/z 215 for monosaccharide chloride adduct ions $[M+Cl]^-$. Sample concentration: 0.1 mg/mL. (B) SIR channel at m/z 377 for disaccharide ions $[M+Cl]^-$. Sample concentration: 0.1 mg/mL. (C) SIR channel at m/z 539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

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. Twenty-five standards, including 14 disaccharides and six trisaccharides, were screened for honey peak identification. The 25 standards and their estimated capacity factor (k') are listed in Table 3. These di- and trisaccharides have been reported to be present in honey.[1] Although the list of the standards was quite comprehensive, there were still a few honey peaks that did not have any standard matched in RT. These peaks, specifically 4, 8, 13, and 14, were labeled as unknown peaks in Figure 2. On the other hand, some honey peaks had more than one standard matched in RT, such as 3, 6, and 10 in Figure 2. These peaks were labeled with all the matched standards. Interestingly, there were some di- and trisaccharides in Table 3 that have been reported to be present in honey but did not appear to be present in the honey samples in this study

K' values of saccharides under the gradient elution conditions in Figure 2							
Arabinose	1.77	Laminaribiose	13.87	**Kojibiose**	18.79	**Erlose**	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	**Maltoriose**	24.55
Sucrose	12.54	**Maltose**	16.86	Gentiobiose	20.43	Isomaltotriose	28.70
Isomaltulose	13.67						

Table 3. List of carbohydrate standards and their capacity factors (k') under the gradient elution conditions shown in Fig. 2. The standards in bold font were found possibly present in the studied honey samples.

Carbohydrate Profiles in Honey and Syrup Samples

The mono-, di-, and trisaccharide profiles in five honey samples and three syrup samples were compared in Figures 3, 4, and 5. Between the honey and the syrup groups, honey has significantly different profiles than those from the syrup group. Within the honey group, it is obvious that Honey D has quite different di- and trisaccharide profiles than those from the other honey samples. Honey D is an imported product from Turkey, while other honey samples are from North America. The geographic origin of Honey D might contribute to the big difference in the di- and trisaccharide profiles. A closer look at the rest of the 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) in Figure 6. The differences are indicated in the chromatogram of Honey B by arrows in Figure 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 its single botanical source (clover). These findings need to be further investigated with authentic samples from various botanical and geographical origins for potential practical use. However, this is evidence that this HILIC-MS method is excellent in providing the fine details of carbohydrate profiles in honey samples.

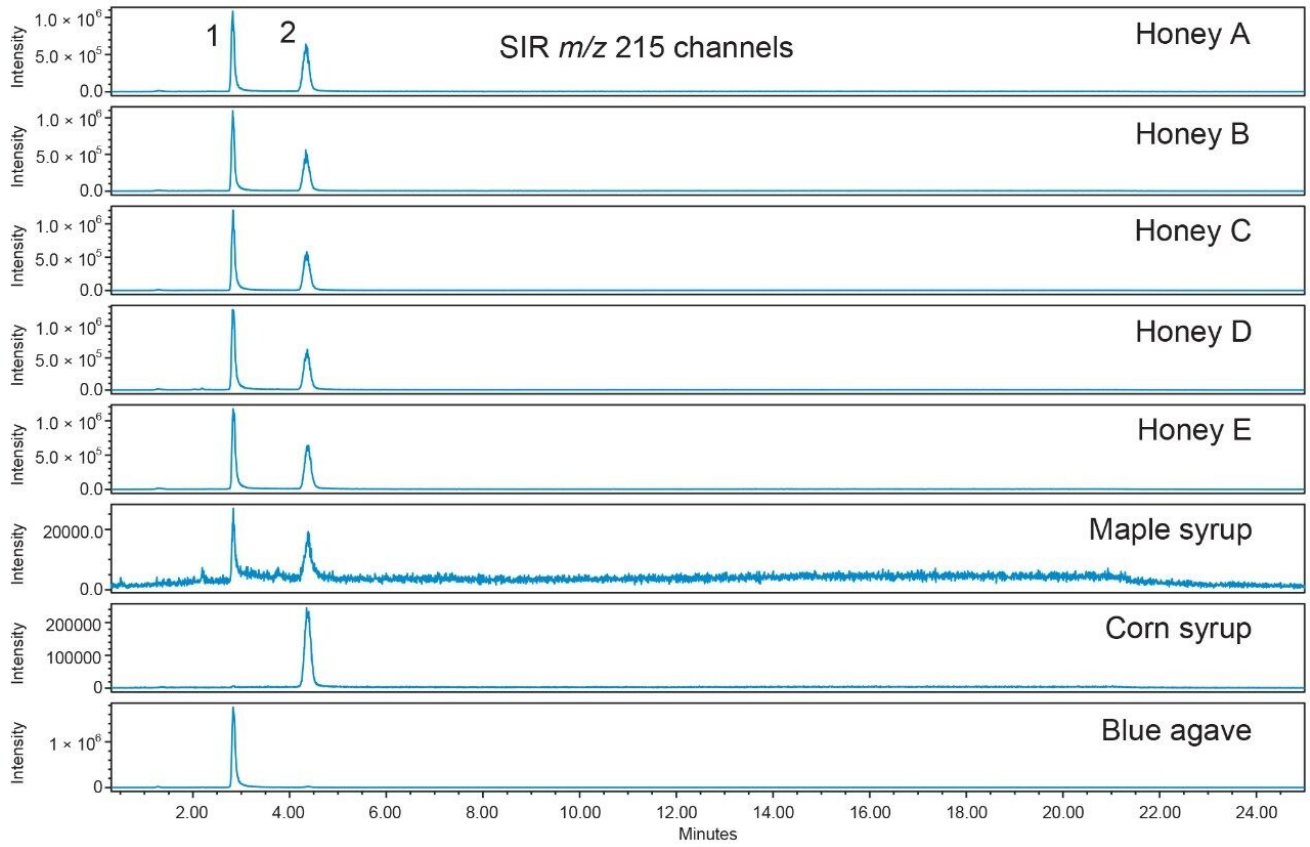

Figure 3. Comparison of monosaccharide profiles in honey and syrup samples. Chromatograms are from SIR channels at m/z 215 for monosaccharide chloride adduct [M+Cl]-. Peak 1: fructose, Peak 2: glucose. Sample concentrations are at 0.1 mg/mL. Honey sample information is in Table 3.

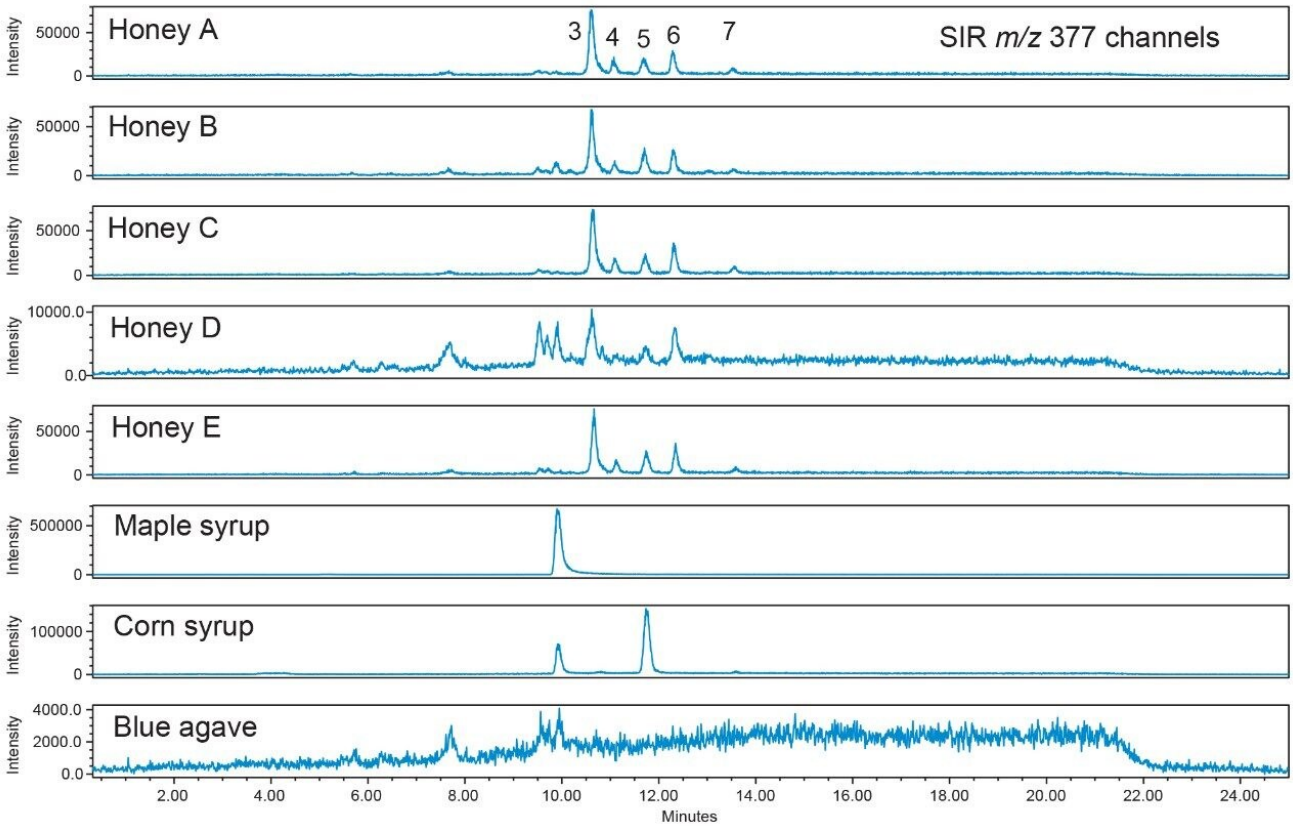

Figure 4. Comparison of disaccharide profiles in honey and syrup samples. Chromatograms are from SIR channels at m/z 377 for disaccharide chloride adduct $[M+Cl]^-$. Peak assignments are the same as in Figure 2. Sample concentrations are at 0.1 mg/mL.

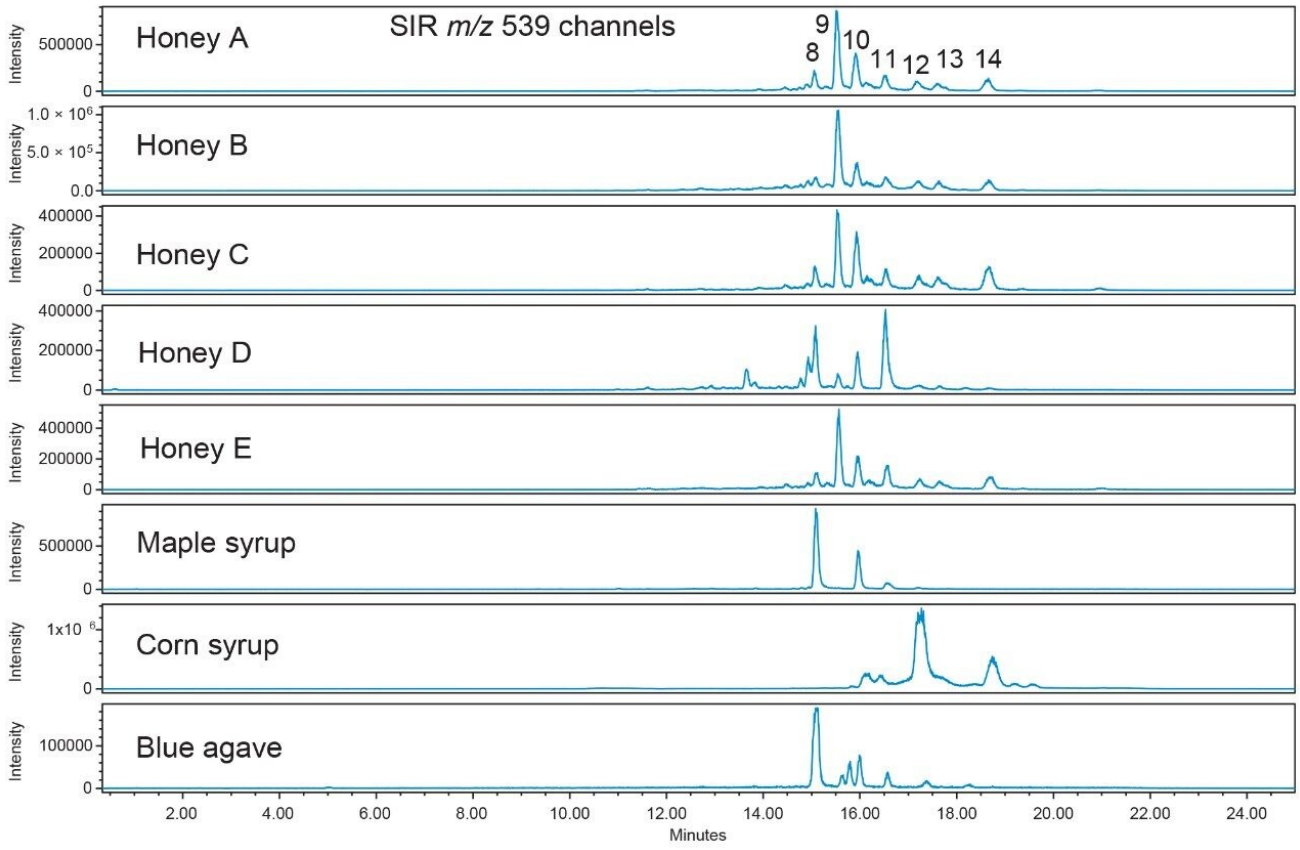

Figure 5. Comparison of trisaccharide profiles in honey and syrup samples. Chromatograms are from SIR channels at m/z 539 for trisaccharide chloride adduct $[M+Cl]^-$. Peak assignments are the same as in Figure 2. Sample concentrations are at 10 mg/mL.

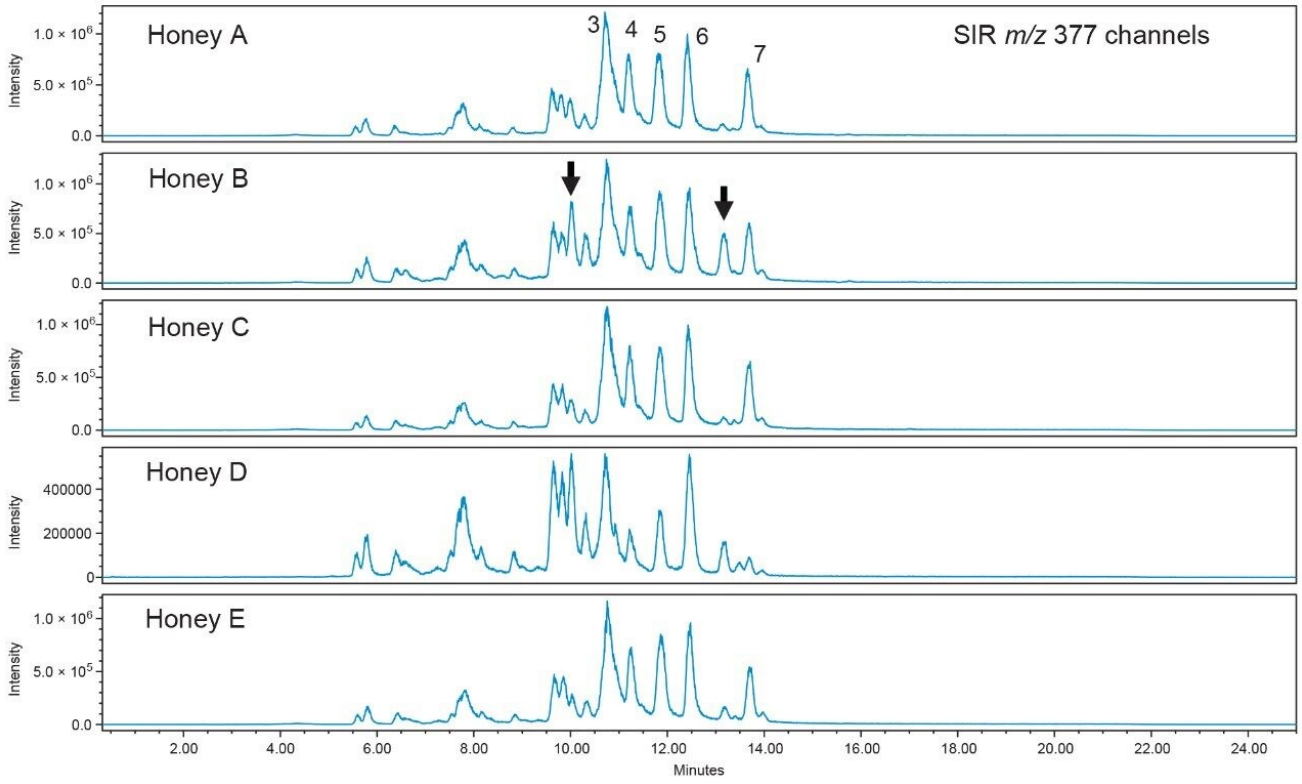

Figure 6. Comparison of disaccharide profiles in honey samples. Chromatograms are from SIR channels at m/z 377 for disaccharide chloride adduct $[M+Cl]^-$. Peak assignments are the same as in Figure 2. Sample concentrations are at 10 mg/mL. The arrow indicates the difference between Honey B and other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

Quantification of Sugars in Honey and Syrup Samples

The fructose, glucose, and sucrose in the honey and the syrup samples were quantified and the results are shown in Table 4. The sum of the fructose and glucose content and the sucrose content are part of the honey quality factors in the Codex standard for honey.2 Figure 7 shows the calibration curves for fructose, glucose, and sucrose. The limit of quantitation (LOQ) for these sugars was estimated at 0.02% (0.02 g/100 g of honey), using the lowest concentration level in the calibration plots and the sample amount in the sample preparation. The intermediate precision, or the relative standard deviation (RSD) from four measurements in two days, is 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 recoveries of 94% and 91% were obtained for fructose and

glucose, respectively (Table 5).

Sample	Fructose		Glucose		Fruc. and Gluc.	Sucrose	
	Avg. (g/100 g)	RSD (%)	Avg. (g/100 g)	RSD (%)	(g/100 g)	Avg. (g/100 g)	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%

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

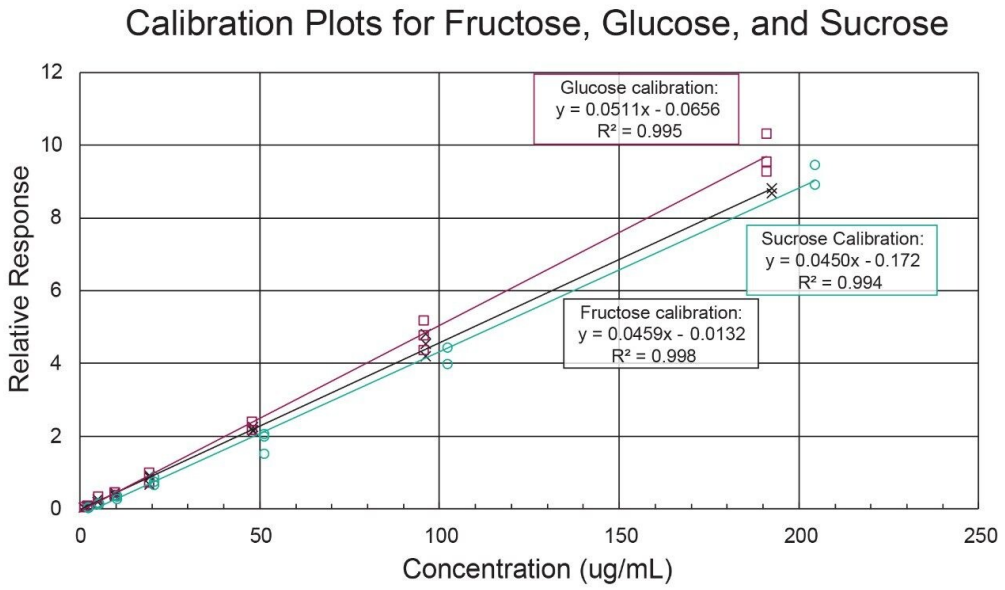

Figure 7. Relationship between the relative responses (analyte peak over reference IS peak) and the analyte concentration for fructose (black), glucose (red), and sucrose (green). Fitting mode: linear with 1/x weighing. Calibration range is 2–200 ppm (ug/mL). The fitted calibration equations and R^2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

	Fructose (g/100 g)	Glucose (g/100 g)
Maple syrup	0.514	0.584
Spiked maple syrup	0.968	1.021
Difference	0.453	0.436
Spiking level	0.481	0.477
Recovery (%)	94.2	91.5

Table 5. Spiking experiment result using a maple syrup sample.

Conclusion

Carbohydrate composition in honey can be assessed by this HILIC-MS method using a gradient elution with a solvent mixture of acetonitrile, water, and methanol on an XBridge BEH Amide XP Column. Individual chromatograms for the mono-, di-, and trisaccharide groups can be obtained. These chromatograms have excellent separation efficiency for the structurally, closely-related di- and trisaccharides in honey. Differences in the di- and trisaccharide chromatograms were found in honey samples from different countries and from different botanical origins. The total run time for each injection was 25 minutes, which is faster than a typical IC analysis. The intermediate precision of less than 9% in RSD was obtained in the quantitation of fructose and glucose, and less than 16% for the low level sucrose in honey. The recovery for the fructose and the glucose was above 90%. The LOQ of fructose, glucose, and sucrose are about 0.02 g/100 g in honey. This method is suitable for quantitation of fructose, glucose, and sucrose and for the profiling of carbohydrate in honey for various purposes, such as quality assessment, botanical and geographical origin study, or adulteration testing.

References

1. Doner, L.W. The Sugars of Honey - A review. *J. Sci. Food Agric*. 1977, 28,443–456.
2. Codes Alimentarius Commission. Revised Codex Standard for Honey; Codex STAN, 12-1981, Rev. 1 (1987), Rev. 2 (2001).
3. Da Costa Leite, J. M.; et al. Determination of Oligosaccharides in Brazilian Honeys of Different Botanical Origin. *Food Chemistry* 2000, 70, 93–98.
4. Yang, J.; Rainville, P.; Liu, K.; Pointer, B. Quantification of Mono and Disaccharides in Foods, Application Note, Waters Corporation, 720006575EN, 2019.

Featured Products

ACQUITY Arc System <https://www.waters.com/134844390>

ACQUITY QDa Mass Detector <https://www.waters.com/134761404>

Empower 3 Chromatography Data Software <https://www.waters.com/10190669>

720006700, October 2019