PROFILING OF SACCHARIDES IN HONEY BY HILIC-MS

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INTRODUCTION

Honey is a popular natural food that is consumed by people and used as an ingredient in many processed foods. The main constituents of honey are fructose and glucose. Other minor carbohydrates in honey include di and trisaccharides. There are more than two dozens of di and trisaccharides have been identified in honey [1]. Besides carbohydrates, other minor honey constituents are organic acids, proteins, amino acids, minerals, polyphenols, vitamins and aroma compounds.

Honey has a relatively high value, and is prone to be adultered by less expensive syrup or sweetener substituents. The botanical and the geographical origin of honey could also be fraudulently claimed to gain economic benefit. Codex has clear definition of blossom honey and honeydew honey, and set the limits for its quality [2]. However, those criteria and limits are not complete enough to detect potential adulteration. Many labs look into the minor carbohydrate profiles in honey, along with other methods, to differentiate authentic honey from fraudulent or adulterated honey products [3].

High Performance Liquid Chromatography (HPLC) techniques, such as Hydrophilic Interaction Liquid Chromatography (HILIC) and High Performance Anion Exchange Chromatography (HPAEC), are commonly used for the sugar analysis. Recently a new HILIC method using Waters XBridge™ BEH Amide column and Waters QDa™ Mass detector has been developed for the sugar analysis [4]. It has a better chromatographic resolution for challenging sugars, and a better detection selectivity and sensitivity. Here we extend this HILIC-MS method to the profiling and quantification of saccharides in honey.

ACQUITY Arc System with PDA, XBridge BEH Amide XP column, and QDa Mass Detector

METHODS

Standard Preparation:
A 10 mg/mL stock of the standard saccharides was prepared in 1:1 acetonitrile-water. A reference internal standard (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 individual standard solutions at levels 1, 2, 5, 10, 20, 50, 100, and 200 μ g/mL (ppm) with the reference IS at about 25 μ g/mL.

Sample Preparation:
Samples of various honey products and syrups were purchased from local grocery stores. 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 micron PVDF membrane filter. Aliquots of these filtered solutions were diluted with acetonitrile-water (1/1 v/v) solvent at different dilution ratios (25 and 2500 were used) for the minor and the major saccharides analysis. Aliquots of the reference IS stock solution were added to each final sample solution. The reference IS concentration in the standard and the sample solutions were kept the same at about 25 μ g/mL.

LC conditions

LC system:	ACQUITY Arc™ System
Runtime:	25.0 min
Column:	XBridge BEH Amide XP (2.5 µm, 3.0 × 10 mm)
Injection vol.:	1 µL
Mobile phase:	A) 90:6:4 v/v/v Acetonitrile-Water-Methanol B) 48:50:2 v/v/v Acetonitrile-Water-Methanol Both mobile phases contain 0.05 v/v% Diethylamine and 500 ppb guanidine hydrochloride
Flow rate:	0.8 mL/min

Table 1. *Gradient elution conditions used for quantification of sugars in samples.*

Time (min)	Flow Rate (ml/min)	%A	%B	Curve
Initial	0.800	100	0	Initial
4.00	0.800	100	0	6
13.00	0.800	60	40	6
20.00	0.800	60	40	6
20.10	0.800	100	0	6

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 90:6:4 Acetonitrile-Water-Methanol mixture with 0.05 v/v% Diethylamine and 5 mg/L guanidine hydrochloride. Once new column has been such conditioned, no further conditioning was conducted.

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*
Curve Fit:	Linear
Smoothing:	Mean, Level 19
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

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1) Method optimization

The original analysis conditions used in the previous publication[4] were optimized to improve separation resolution and robustness of the analysis. One of the mobile phase solvents, isopropanol, was replaced with methanol to reduce the system pressure. The mobile phase composition was also adjusted. The QDa detector parameters have been investigated to ensure optimal detection sensitivity. Figure 1 shows the effect of the cone voltage on the analyte ion [M+Cl]⁻ abundance and fragmentation. Cone voltage of 5 Volt was the optimal value.

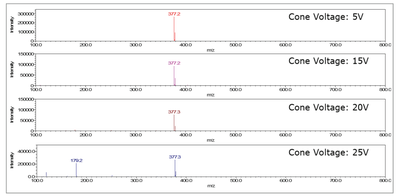

Figure 1. *Overlay of extracted mass spectra of the turanose peak under different QDa cone voltages. Other experimental conditions were the same. The effects of cone voltage on the turanose chloride adduct ion [M+Cl⁻] abundance and fragmentation is shown.*

2) Saccharide profile in honey

Profiles of saccharides in honey were obtained (Figure 2). The mono, di and trisaccharides chromatograms were obtained from the single ion recording (SIR) detection at their chloride adduct ions [M+Cl]⁻. The peak ID were tentatively assigned by comparing the retention times (RT) of standards with the saccharide peaks' RT. If two or more standards' RTs match to a saccharide peak, all the standards are assigned to this peak.

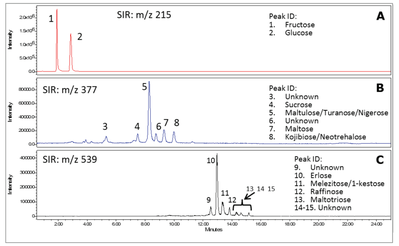

Figure 2. *Chromatograms of mono, di and trisaccharides in honey A. (A) SIR channel at m/z 215 for monosaccharide chloride adduct ions [M+Cl]⁻. Sample concentration: 0.5 mg/mL. (B) SIR channel at m/z 377 for disaccharide ions [M+Cl]⁻. Sample concentration: 0.5 mg/mL. (C) SIR channel at m/z 539 for trisaccharide ions [M+Cl]⁻. Sample concentration 5 mg/mL. A steep gradient composition change to 100% mobile phase B from 13 to 15 min, and stay at 100% B for 5 min was used in obtaining the trisaccharide chromatogram.*

RESULTS AND DISCUSSION

3) Peak identification

In Fig. 2, two monosaccharides, six disaccharides, and seven trisaccharides with descent peak intensities were detected. The obtaining of these 15 well resolved peaks demonstrates the excellent separation selectivity and efficiency of the XBridge BEH Amide XP column. The elution order of saccharides is significantly different than the elution order in HPAEC. This HILIC method provides an alternative way in sugar analysis.

In order to identify these peaks, 25 standards have been screened and their RTs have been compared to the honey peaks for peak identification. These standards have been reported may exist in honey. They include 5 monosaccharides, 14 disaccharides, and 6 trisaccharides. The names and their k' (capacity factor) of these standards are listed in Table 2.

All disaccharides and trisaccharides on the list have fructose and/or glucose as basic unit. Some of them have structures extremely similar to each other, and very close RTs. Some honey peaks have multiple standards matched in RT, and when this happened, multiple IDs were assigned to these peaks.

Although the standards list is quite extensive, there are still some disaccharide peaks and trisaccharide peaks could not be identified. This could because that the excellent separation efficiency offered by the XBridge BEH Amide column, and the highly selective and sensitive detection by the QDa detector made it possible to see these unknown peaks that previously could not be seen.

The characteristics of saccharide profiles in honey could be used as an effect way to differentiate honeys from different origins or from adulteration.

Table.2 *List of capacity factors (k') of standards under the gradient elution conditions in Fig. 2. The analytes in bold are identified as constituents in the honey A.*

K' values of saccharides under the gradient elution conditions in Fig. 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	**Maltotriose**	24.55
Sucrose	12.54	**Maltose**	16.86	Gentiobiose	20.43	Isomaltotriose	28.70
Isomaltulose	13.67						

4) Comparison of saccharide profiles in honey and syrup samples

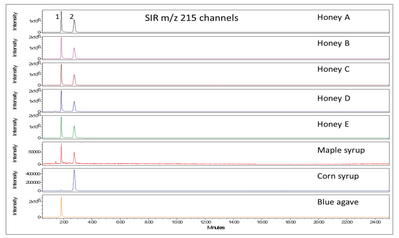

Figure 3. *Comparison of monosaccharide profiles in honey and syrup samples. Chromatograms are from SIR channels at m/z 215 for monosaccharide chloride adduct [M+Cl]⁻. Peak 1: fructose, Peak 2: glucose. Sample concentrations are at 0.5 mg/mL. Honey sample info is in Table 3.*

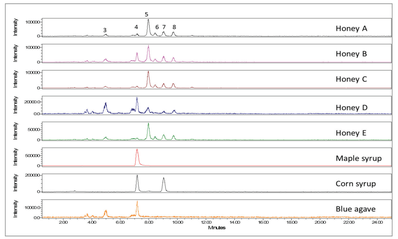

Figure 4. *Comparison of disaccharide profiles in honey and syrup samples. Chromatograms are from SIR channels at m/z 377 for disaccharide chloride adduct [M+Cl]⁻. Peak assignment are the same as in Figure 1. Sample concentrations are at 0.5 mg/mL. Honey sample info is in Table 3.*

These honey samples were from different sources and of different origins (Table 3). Their monosaccharide profiles and disaccharide profiles were shown and compared with syrups Figure 3 and 4.

These honey samples have significant different profiles than the syrup samples' profiles.

Table 3. *Honey samples description.*

Sample	Description
Honey A	Raw organic, USA
Honey B	Clover, USA
Honey C	wild flower, USA
Honey D	Turkey
Honey E	Canadian

4) Calibration plots for fructose, glucose, and sucrose

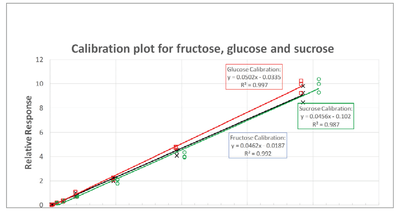

Figure 4. *Relationship between the relative responses (analyte peak over internal standard peak) and the analyte concentration for fructose (black), glucose (red) and sucrose (green). Fitting mode: linear with 1/x weighing. Calibration ranges: fructose 1 - 190 ppm, glucose: 1 - 190 ppm, sucrose: 5 - 200 ppm. The fitted calibration equations and R^2 are shown in the plots.*

5) Quantification of sugars in honey and syrup samples

The fructose, glucose, and sucrose in honey and the syrup samples were quantified under the conditions listed in the method section. The fructose, glucose and sucrose contents were shown in Table 4.

Table 4. *Quantification of sugars in honey and syrup samples. The content results are average of three measurements.*

Sample	Content (g/100g)			
	Fructose	Glucose	Fruc. + Gluc.	Sucrose
Honey A	28.7	29.9	58.6	0.3
Honey B	30.7	23.5	54.2	0.9
Honey C	33.3	26.7	60.0	0.3
Honey D	36.6	25.4	62.1	0.5
Honey E	32.5	28.7	61.2	0.2
Maple syrup	0.5	0.5	1.0	54.2
Corn syrup	0.1	11.2	11.4	5.5
Blue agave	52.2	1.0	53.2	0.2

CONCLUSION

- **Well resolved disaccharide peaks in honey samples were obtained on XBridge BEH Amide XP column. The characteristics of disaccharide profiles of honey from different origins are useful in differentiating honey from different floral or geographic origins. They are also useful in testing adulteration.**
- **Unknown saccharide peaks were uncovered in honey samples. These peaks do not belong to the known saccharides that have been identified in honey before. 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 Alimemtarius guide.**
- **The elution order of saccharides under this HILIC condition is significantly different from that in HPAEC. This HILIC-MS method could be an useful alternative method for the honey analysis.**

References

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