

Nota applicativa

## Analysis of Carbohydrates by UltraPerformance Liquid Chromatography and Mass Spectrometry

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## Abstract

In this application, UPLC-MS is used for the rapid and direct analysis of simple and complex carbohydrates in a variety of sample matrices. The stability of the ACQUITY UPLC BEH Amide Column allows the use of high pH mobile phases that improve chromatographic resolution and enhance MS signal intensity without the need for derivatization, metal complexing agents, or post-column addition.

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## Introduction

Carbohydrates are the most abundant class of organic compounds in nature, and they play essential roles in many biological processes. These include providing storage and transport of energy for cells in most living organisms, as well as providing structure for plants and animals.

The term “carbohydrate” can refer to monosaccharides, disaccharides, oligosaccharides (3 to 10 sugar units), or polysaccharides (up to thousands of sugar units). The number of possible isomers for each monosaccharide (e.g., fructose, glucose, galactose) increases with the number of carbons. For more complex carbohydrates (i.e., oligosaccharides and polysaccharides), it is even more difficult to distinguish between isomers with simple analytical techniques.

Carbohydrates are typically analyzed with amino or polyamine columns in HILIC mode with refractive index (RI) detection since these analytes have weak UV chromophores. However, these methods suffer from salt interferences, anomer mutarotation, and incompatibility with gradient separations. In addition, reducing sugars (glucose, maltose, and lactose) are lost at elevated temperatures on amino and polyamine columns due to Schiff base or enamine formation, thus reducing column lifetime. Techniques that use ion-exchange chromatography are not compatible with MS detection due to the use of high salt buffers.

Mass spectrometry is a desirable technique for detecting carbohydrates since it is approximately 10-fold more sensitive than evaporative light scattering (ELS) and 100-fold more sensitive than RI detection. Outside the areas of proteomics and biopharmaceuticals, there are only a few reports using MS detection for analysis of carbohydrates. Two reports used flow injection analysis (FIA) into the MS instrument<sup>1,2</sup>, and another used rubidium in the mobile phase as a complexing agent for NMR and ESI-MS<sup>3</sup>.

ICP-MS and ESI-MS have also been used to study glucose, sucrose, and fructose fragmentation<sup>4</sup>. The lack of

chromatographic separation with FIA prohibits separation of isomers and accurate quantitation due to matrix interferences. The use of metal complexing agents in the mobile phase or post-column addition of an ionizing agent prior to MS detection can also complicate analysis and foul the MS source. HILIC coupled with ESI-MS has been successfully used for analysis of amino acids, peptides, glycoconjugates, and organic acids in foods without derivatization using an amide stationary phase<sup>5</sup>. These separations were performed using larger 5 µm particle columns and run times were in excess of 100 minutes.

In this application, UPLC-MS is used for the rapid and direct analysis of simple and complex carbohydrates in a variety of sample matrices. The stability of the ACQUITY UPLC® BEH Amide column allows the use of high pH mobile phases that improve chromatographic resolution and enhance MS signal intensity without the need for derivatization, metal complexing agents, or post-column addition.

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## Experimental

### LC conditions

Instrument:	Waters ACQUITY UPLC System with ACQUITY. SQD
Data system:	MassLynx version 4.1
Columns:	ACQUITY UPLC BEH Amide 2.1 x 50 mm, 1.7 µm 2.1 x 100 mm, 1.7 µm or 2.1 x 150 mm, 1.7 µm
Weak Needle Wash:	75/25 ACN/H <sub>2</sub> O (500 µL)
Strong Needle Wash:	20/80 ACN/H <sub>2</sub> O (800 µL)
Seal Wash:	50/50 ACN/H <sub>2</sub> O

Injection Mode: Partial loop with needle overfill (PLNO)

Mobile phases, flow rate, gradient conditions, column temperature, and injection volume are all listed in figure captions.

## MS conditions

Ionization mode:	ES
Capillary:	2.8 kV
Cone:	25 V
Source Temp.	120 °C
Desolvation Temp.:	350 °C
Desolvation Gas:	500 L/hr
Cone Gas:	50 L/hr
Dwell Time:	40 or 80 ms
SIR <i>m/z</i> :	179.2 (fructose, glucose) 341.3 (sucrose, maltose, lactose) 503.4, 665.5, 827.6, 989.7, 1151.8 (maltooligosaccharides n=1 to 5) 121.1 (erythritol), 803.8 (stevioside), 950.1 (rebaudioside C),

## Sample preparation

The food sugar standard (fructose, glucose, sucrose, maltose, and lactose) was prepared at 10 µg/mL in 50/50 ACN/H<sub>2</sub>O. Other standards were prepared in 50/50 ACN/H<sub>2</sub>O at the specified concentrations. Beer samples were prepared by diluting 1:1 with 50/50 ACN/H<sub>2</sub>O. Natural sweetener powder was dissolved in 50/50 ACN/H<sub>2</sub>O. Maple syrup samples were prepared at a concentration of 0.5 to 1 mg/mL in 50/50 ACN/H<sub>2</sub>O. All samples were filtered using a 0.45 µm PVDF syringe filter.

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## Results and Discussion

In solution, reducing sugars can undergo mutarotation, in which the cyclic saccharide is in equilibrium with its  $\alpha$  and  $\beta$  anomeric forms. While these anomers can be separated for some sugars under certain conditions, the presence of two peaks for one carbohydrate complicates identification and quantitation. In Figure 1, the anomers of the reducing sugars in the standard mixture are clearly visible under acidic and unmodified conditions, and baseline separation of the five food sugars is not achieved.

When a basic mobile phase modifier is used, the anomers are collapsed and the ionization efficiency of the carbohydrates is increased. These conditions result in simpler chromatography, thus leading to more accurate carbohydrate identification and quantitation. Also note that retention for all sugars increases with increasing pH.

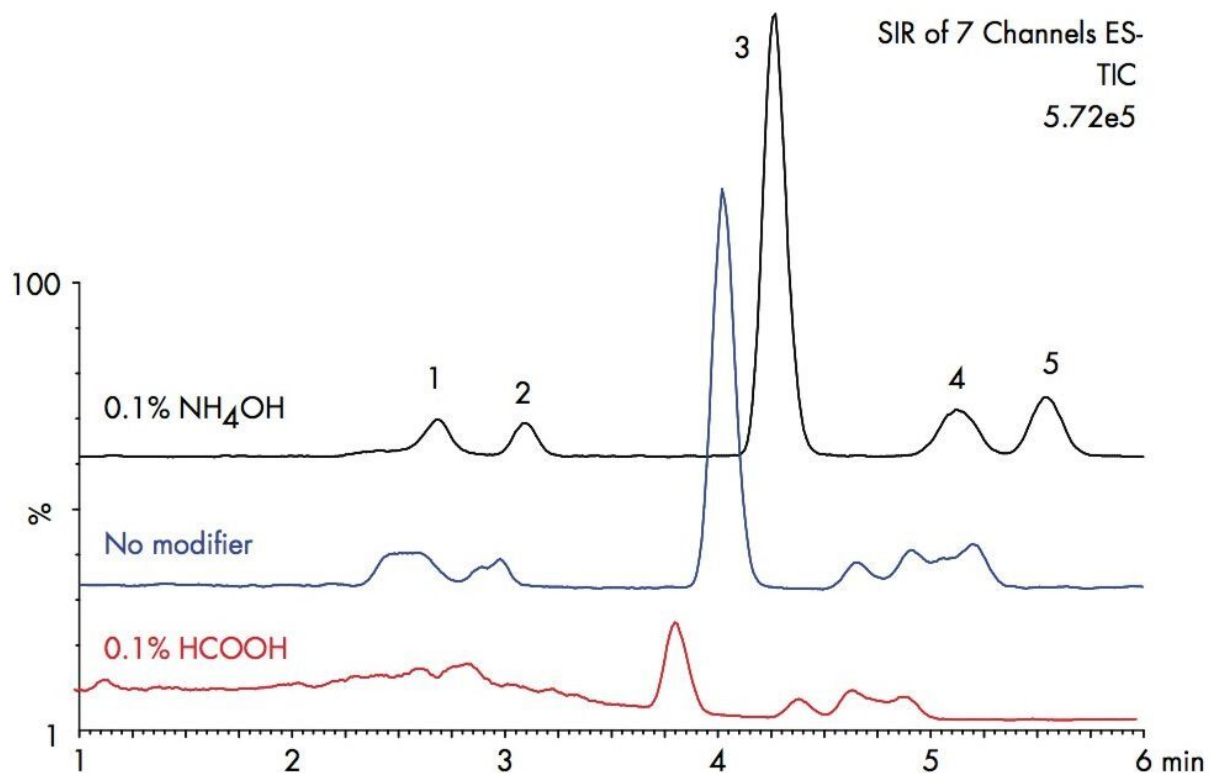


Figure 1. UPLC-MS analysis of carbohydrates in 75/25 ACN/H<sub>2</sub>O containing formic acid, no modifier, and ammonium hydroxide. The flow rate is 0.13 mL/min, the column temperature is 35 °C, and the injection volume is 0.7 µL. The concentration of each analyte is 10 µg/mL in 50/50 ACN/H<sub>2</sub>O. The column dimensions are 2.1 x 50 mm, 1.7 µm. Peaks: (1) fructose, (2) glucose, (3) sucrose, (4) maltose, and (5) lactose.

Similar separation can be achieved for the same carbohydrate mixture using acetone as the organic solvent instead of ACN (Figure 2). In addition, resolution is improved with increasing column length, as is expected. This is important given the recent shortage of ACN experienced by the entire analytical community, and thus gives an alternative set of conditions for analyzing carbohydrates by UPLC-MS. Also, unlike amino-based columns, increased temperature can be used to further facilitate anomer collapse and resolution. Under the elevated temperature and pH conditions shown in Figure 2, the lifetime of the ACQUITY UPLC BEH Amide Column is more than 2,000 injections (~ 8 days of continuous testing) with minimal loss in carbohydrate retention (data not shown).

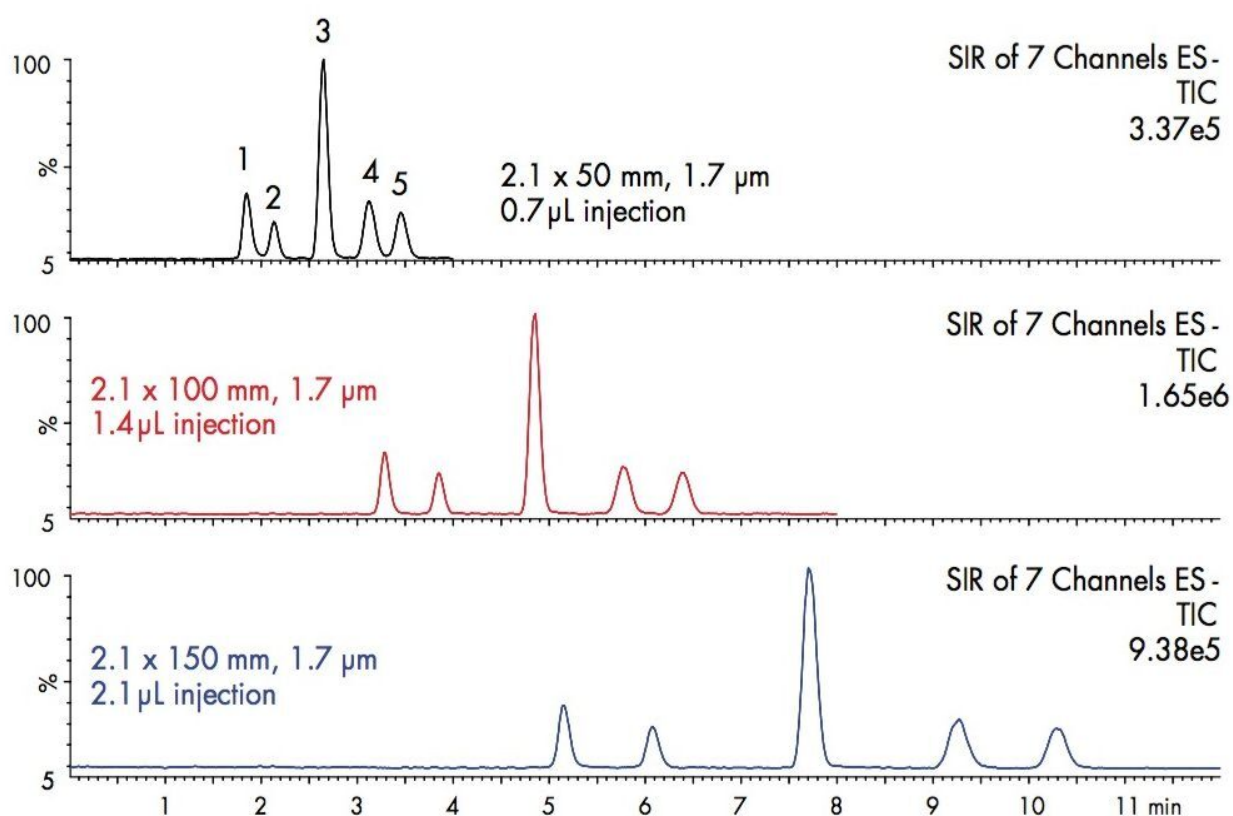


Figure 2. UPLC-MS analysis of carbohydrates using acetone as the organic solvent. The isocratic mobile phase was 77/23 acetone/H<sub>2</sub>O with 0.05% NH<sub>4</sub>OH. The flow rate is 0.13 mL/min and the column temperature is 85 °C. The column dimensions and injection volumes are shown on the figure. The sample and peak elution order is identical to Figure 1.

Figure 3 shows the separation of a more complex mixture of carbohydrates, which include maltooligosaccharides. This particular separation is performed under gradient conditions, which are required for more complex carbohydrate mixtures. Under these conditions, no significant rise in the baseline is observed throughout the gradient, indicating that the BEH Amide column does not exhibit MS bleed, which is a common problem with amino and polyamine columns.

One of the major application areas for this method is the analysis of carbohydrates in commercial food and beverage products. This applies to the analysis of incoming raw materials, in-process samples, and quality control of finished product. For example, beer is made, in part, with malted barley, which contains many simple and complex carbohydrates. During fermentation, yeast consumes the sugars and converts them to alcohol and carbon dioxide, thus leaving the finished product with little or no maltose or other simple sugars. Figure 4 shows the analysis of a commercial beer sample compared to a food sugar standard.

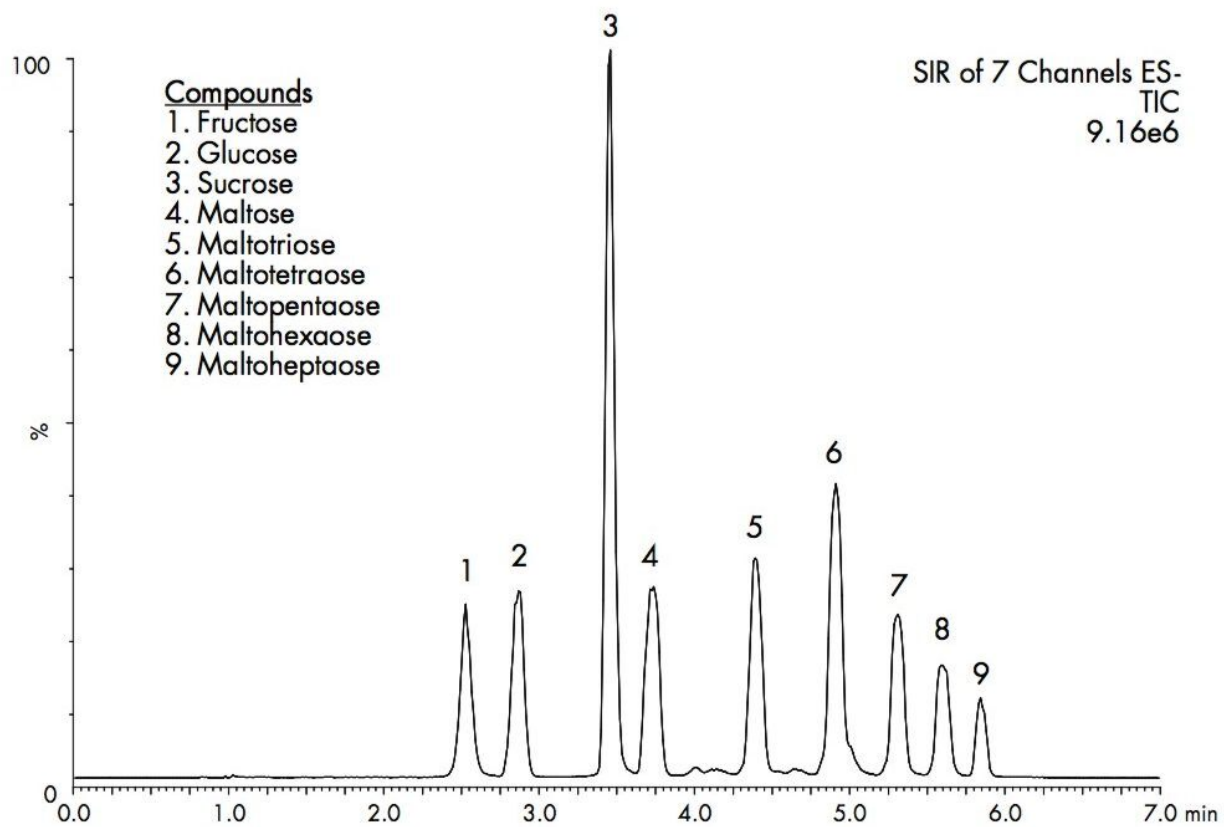


Figure 3. UPLC-MS separation of mono-, di-, and oligosaccharides. Mobile phase A is 80/20 ACN/H<sub>2</sub>O with 0.1% NH<sub>4</sub>OH. Mobile phase B is 30/70 ACN/H<sub>2</sub>O with 0.1% NH<sub>4</sub>OH. Gradient from 0 to 60 % B in 5 min, reset and equilibrate for 10 min. The flow rate is 0.17 mL/min, the column temperature is 35 °C, and the injection volume is 0.7 μL. The concentration of each analyte is 10 μg/mL in 50/50 ACN/H<sub>2</sub>O. The column dimensions are 2.1 x 50 mm, 1.7 μm.



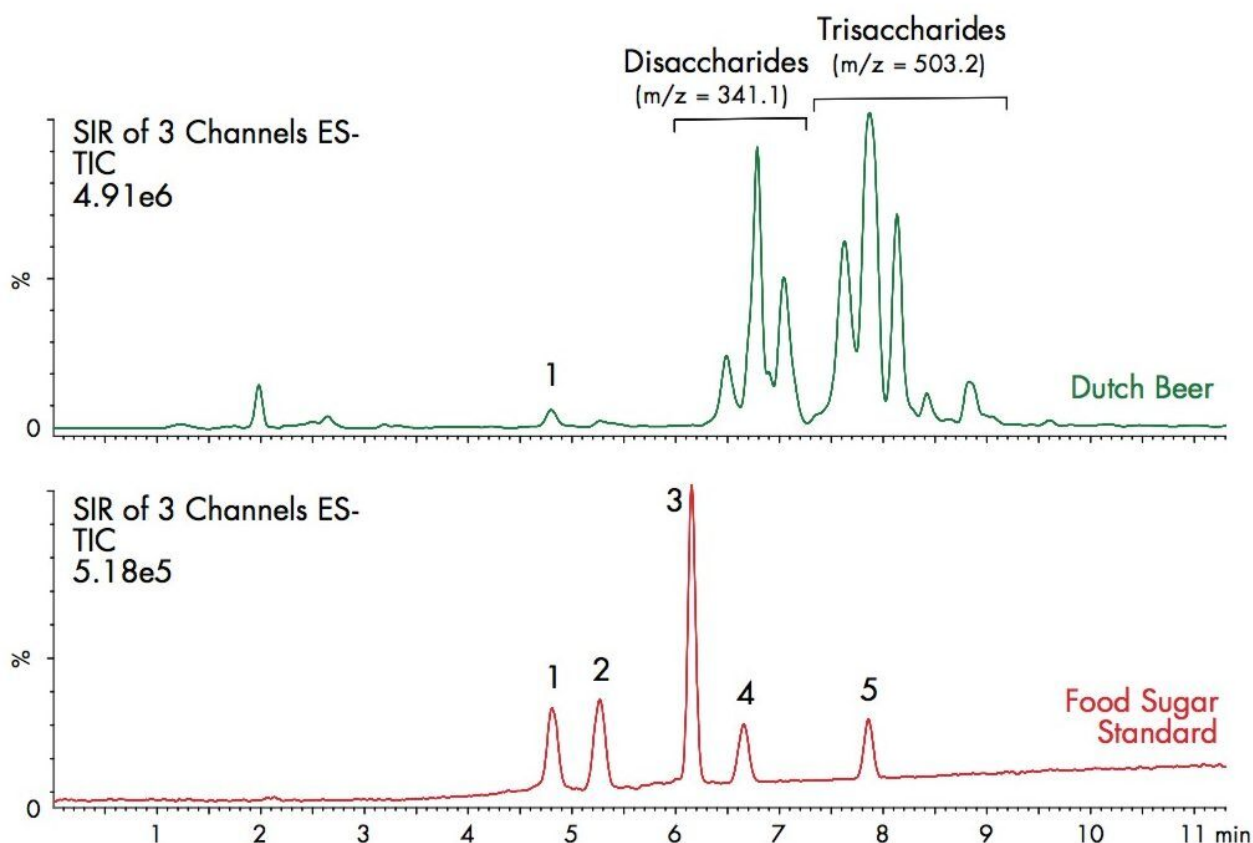


Figure 4. UPLC-MS analysis of carbohydrates in beer. Mobile phases are identical to Figure 3. Gradient from 10 to 70 % B in 10 min, reset and equilibrate for 25 min. The flow rate is 0.13 mL/min, the column temperature is 35 °C, and the injection volume is 2  $\mu$ L. The concentration of each analyte in the standard is 10  $\mu$ g/mL in 50/50 ACN/H<sub>2</sub>O. The column dimensions are 2.1 x 100 mm, 1.7  $\mu$ m. Peaks: (1) fructose, (2) glucose, (3) sucrose, (4) maltose, and (5) maltotriose.

This particular separation was performed under gradient conditions due to the presence of both simple and complex carbohydrates. The UPLC-MS analysis of the beer sample shows the presence of many disaccharide and trisaccharide isomers at significant levels. While most of the simple sugars (e.g., glucose and sucrose) are not observed, there is still a trace amount of fructose. This example shows the ability of UPLC-MS to identify the sugars in finished beverage products. The same method can also be used for in-process analysis of fermented beverages, as well as for raw materials.

UPLC-MS was also used to compare many commercially-available maple syrups. There are many different processing techniques that influence the carbohydrate content of the final product. Currently, there is a shortage of maple syrup, and manufacturers may add "filler" syrups to stretch the short supply of pure maple

syrup. A comparison of 5 different syrups is shown in Figure 5. The primary component of authentic maple syrup is sucrose, as is indicated on the figure. However, it is clear from UPLC-MS analysis that not all of the commercial syrups are pure maple syrup, indicating the use of “fillers” such as corn syrup to enhance the sweetness and stretch the supply of the pure maple syrup. Differences between the light syrup and other brands can also be detected, especially in the region containing oligosaccharides (> 4 min).

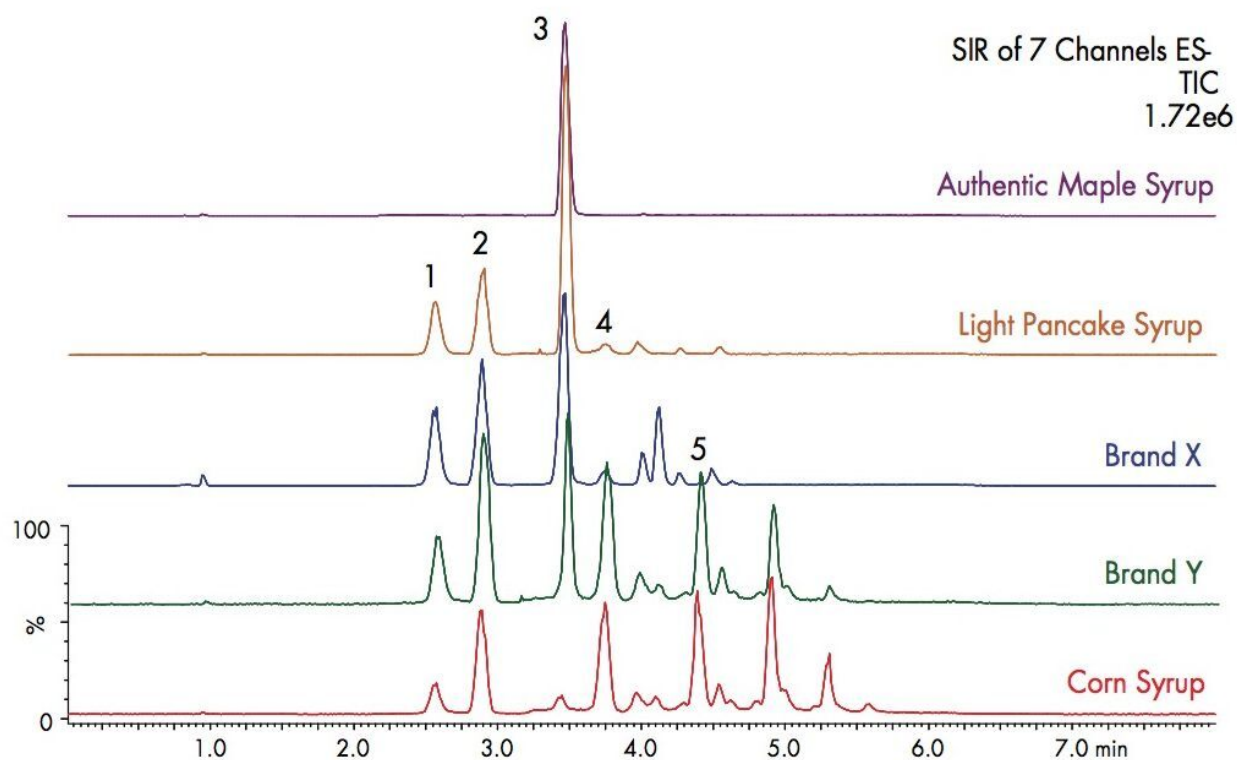


Figure 5. UPLC-MS analysis of carbohydrates in different maple syrups. Conditions are identical to Figure 3. Peaks: (1) fructose, (2) glucose, (3) sucrose, (4) maltose, and (5) maltotriose.

Finally, UPLC-MS was used to analyze the content of stevia-related compounds and erythritol (sugar alcohol) in natural sweetening products (Figure 6). Stevia is a genus of about 240 species of herbs and shrubs native to South America and Central America. Extracts from stevia leaves have approximately 300 times the sweetness of sugar, and therefore many countries have used them as low-carbohydrate, low sugar sweetener alternatives. The sweetness of stevia is attributed to many of the steviol glyco-sides contained in the plant.

Until recently, the US FDA banned the use of stevia compounds as sweeteners, but now that the ban has

been lifted, many stevia-related sweeteners are finding their way to market. Figure 6 shows the comparison of two commercially-available stevia-related sweeteners. UPLC-MS analysis shows that one commercial sweetener (Brand Y) contains the expected steviol glycosides. The other, Brand X, is primarily composed of erythritol, which is a sugar alcohol (also a natural sweetener). Careful examination of the baseline indicates that there is a small quantity (< 1%) of rebaudioside A (Reb-A) in the Brand X product, which is associated with the stevia plant.

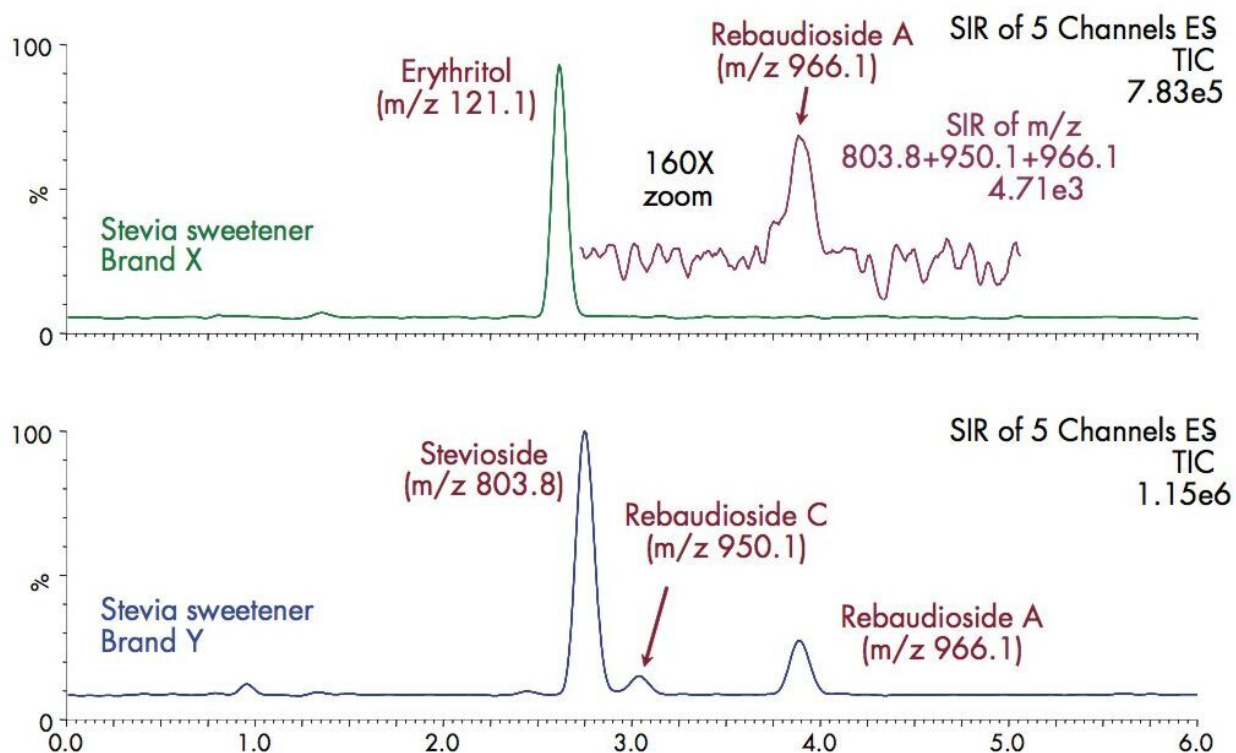


Figure 6. UPLC-MS analysis of two commercially-available stevia-related sweeteners. Isocratic mobile phase of 77.5/22.5 ACN/H<sub>2</sub>O with 0.1% NH<sub>4</sub>OH. The flow rate is 0.2 mL/min, the column temperature is 35 °C, and the injection volume is 1.3 µL. The concentration of the Brand Y product is 50 µg/mL each in 50/50 ACN/H<sub>2</sub>O. The Brand X product is prepared at 10 µg/mL in 50/50 ACN/H<sub>2</sub>O. The column dimensions are 2.1 x 100 mm, 1.7 µm.

## Conclusion

- The ACQUITY UPLC BEH Amide Column was used to separate simple and complex carbohydrates in commercial food and beverage products.
- The presence of a high pH modifier (ammonium hydroxide) in the mobile phase allows for anomer collapse, which simplifies the chromatography and allows for more accurate carbohydrate identification.
- The use of a high pH mobile phase on the BEH Amide column increases ionization efficiency of the saccharides, which enables direct detection of carbohydrates with electrospray ionization mass spectrometry.
- Acetone can be used in place of ACN without changes in separation selectivity.
- UPLC-MS can be successfully used for confirming the mass of different carbohydrates found in commercial beverages, as well as identifying process differences for commercial food products.
- The BEH Amide column can also be used to separate steviol glycosides and sugar alcohols in order to characterize natural sweetening products.

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