

## Quantification of Mono and Disaccharides in Foods

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### Abstract

This Application note demonstrates accurate and reliable determination of sugar content in food and animal feed samples.

### Benefits

- Separation of galactose and glucose
  - Clean chromatogram with minimal interference from sample matrix
  - Accurate analysis of sugars with 25 min per injection cycle
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### Introduction

Monosaccharides fructose, galactose, and glucose, and disaccharides sucrose, lactose, and maltose are common sugar ingredients in foods. With the increasing concerns of obesity and diabetes in many countries, the need to monitor sugar intake has grown in recent years. Consequently, now there are requirements to provide accurate information about added sugar content on food product

labels in order to comply with current FDA food labeling regulation.<sup>1</sup>

High performance liquid chromatography (HPLC) is the method of choice for the analysis of sugars. However, the HPLC analysis of sugars is not a simple task. The main concern is the co-eluting compounds that may interfere with sugar quantification. For example, galactose often co-elutes with glucose. Recently a new method has been developed in which the six common sugars were separated on an XBridge BEH Amide XP Column (2.5  $\mu\text{m}$ , 3.0  $\times$  150 mm), and were quantified with an ACQUITY QDa Detector.<sup>2</sup> In this work, we improved the method and applied it to a wide range of foods, including a chicken feed sample. Figure 1 shows the SIR (Single Ion Recording) chromatograms of a sugar mixture solution. All six sugars are baseline separated.

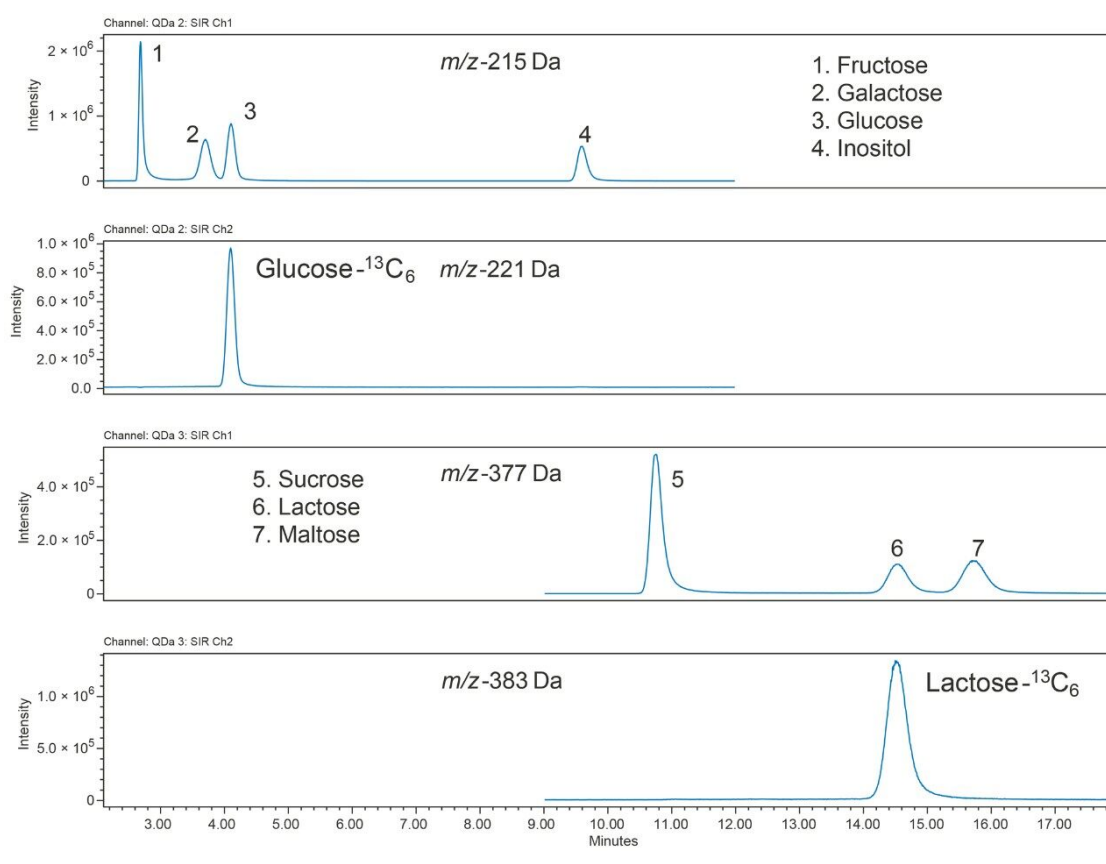


Figure 1. SIR chromatograms of sugar standard mix solution and the internal reference standards.<sup>1</sup>

## Experimental

## Sample description

### Standard preparation

A stock solution of (six) mono and disaccharide standard mixture was prepared at 500 mg/L. An internal reference standard solution of the stable isotope labeled glucose- $^{13}\text{C}_6$  and lactose- $^{13}\text{C}_6$  was prepared at 1000 mg/L. Standard solutions at seven levels (0.2, 0.5, 2, 5, 20, 50, and 100 mg/L) were prepared by serial dilution of stock solutions. The internal reference standards were added and kept at 20 mg/L in all standard solutions and sample solutions. The solvent used in all standard and sample solutions was 1:1 (v/v) acetonitrile:water.

## Sample preparation

Samples of non-fat dry milk powder, milk based infant formula (IF), soy based IF, oatmeal, lactose-free milk, chicken feeds, and cocoa powder were prepared as follows based on the procedure that was described elsewhere:<sup>2,3</sup>

- Add approximately 0.2 to 0.5 g sample to a 25 mL volumetric flask
- Add 10 mL 1:1 ethanol:water; for milk or liquid sample, add 5 mL ethanol
- Mix well, sonicate, and keep in a water bath (60 °C) for 25 min
- Add 500  $\mu\text{L}$  Carrez 1\* reagent, vortex for 1 min
- Add 500  $\mu\text{L}$  Carrez 2\*\* reagent, vortex for 1 min
- Add 5 mL acetonitrile, mix well
- Make to 25 mL with 1:1 ethanol:water
- Mix well and transfer to a 50 mL centrifuge tube
- Centrifuge at a rpm of 1900 rcf for 5 min
- Filter supernatant through a 0.2  $\mu\text{m}$  PVDF filter

\* Carrez 1 reagent - dissolve 0.36 g  $\text{K}_4[\text{Fe}(\text{CN})_6]\cdot 3\text{H}_2\text{O}$  in 10 mL water

\*\*Carrez 2 reagent - dissolve 0.72 g  $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$  in 10 mL water

The supernatant was then diluted with water:acetonitrile mixture at various ratios to fit into the calibration range. Aliquot of the internal reference standard mix solution (glucose- $^{13}\text{C}_6$  and lactose- $^{13}\text{C}_6$ ) was added to all sample solutions at 20 mg/L.

### Method conditions

## LC conditions

System:	ACQUITY Arc System
Runtime:	25 min
Column:	XBridge BEH Amide <i>XP</i> , 2.5 $\mu$ m, 3.0 $\times$ 150 mm (p/n: 186006725)
Column temp.:	90 $^{\circ}$ C
Mobile phase:	90:6:4 acetonitrile:water:methanol with 0.05 v/v% diethylamine and 0.5 mg/L guanidine hydrochloride
Flow rate:	0.8 mL/min
Injection volume:	1.0 $\mu$ L

## Column conditioning

New columns need to be properly conditioned to ensure optimal chromatographic performance. This is especially required for hydrophilic interaction liquid chromatography (HILIC) columns, for which a careful and thorough column conditioning prior to the initial use is recommended.<sup>4</sup> All new XBridge BEH Amide *XP* Columns used in this study were flushed with 50 column volumes of 80:20 acetonitrile:water, followed by 100 column volumes of the 90:6:4 acetonitrile:water:methanol mixture with 0.05 v/v% diethylamine and a higher guanidine hydrochloride concentration of 5 mg/L. Once the new column was conditioned, no further conditioning was conducted.

## MS conditions

System:	ACQUITY QDa (Performance)
Ionization mode:	ESI-
Capillary voltage:	0.8 kV

Cone voltage:	5.0 V
Probe temp.:	600 °C
Acquisition rate:	2 Hz
Full scan:	100–500 <i>m/z</i>
Curve fit:	Quadratic, 1/x weighting
Smoothing:	Mean, level 7
SIR [M+Cl] <sup>-</sup> :	215.1 Da for fructose, glucose, galactose 221.1 Da for glucose- <sup>13</sup> C <sub>6</sub> 377.1 Da for sucrose, lactose, maltose 383.1 Da for lactose- <sup>13</sup> C <sub>6</sub>

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## Results and Discussion

### Method selectivity

The main issue in the sugar analysis is co-elution interference from sample matrix and from sugar isomers. Compare to refractive index (RI) detection and evaporative light scattering (ELS) detection, the ACQUITY QDa Mass Detector, a single quadrupole mass spectrometer, is a highly selective detector. It has significantly less background signal noise from co-eluting food matrix.

The co-elution issue from sugar isomers was evaluated by examining the isomers' retention factors (*k'*) under the same LC conditions. The common mono and disaccharides' *k'* and their monoisotopic masses are shown in Table 1. Some sugars did elute closely to the sugars of interest. For example, lactulose partially co-eluted with sucrose, mannose partially co-eluted with galactose, and cellobiose co-eluted with lactose (chromatograms not shown). Fortunately, these sugar pairs rarely co-exist in the same samples.<sup>5</sup>

Compound	$k'$	Monoisotopic mass (Da)
Fructose	2.38	180.1
Mannose	3.44	180.1
Galactose	3.68	180.1
Glucose	4.15	180.1
Sucrose	12.25	342.1
Epi-lactose	12.55	342.1
Lactulose	12.63	342.1
Isomaltulose	14.00	342.1
Cellobiose	16.75	342.1
Lactose	16.78	342.1
Maltose	18.01	342.1
Trehalose	26.88	342.1
Isomaltose	28.83	342.1
Allo-lactose	33.55	342.1

*Table 1. Retention factors ( $k'$ ) of mono and disaccharides and their monoisotopic mass. The retention factors are measured under the conditions shown in the Experimental section.*

## Effect of salt

Foods may contain high levels of salts. It is well known that salt can cause issues in the peak shape and the separation of some early eluting sugars in HPLC. The effect of salt was evaluated, and it was found that even at high concentrations of 450 and 500 mM in sample solutions, sodium chloride (NaCl), ammonium formate (HCOONH<sub>4</sub>), and potassium chloride (KCl) did not cause any noticeable change in chromatograms (Figure 2). The effect of salt on the sugar separation in this method is minimal.

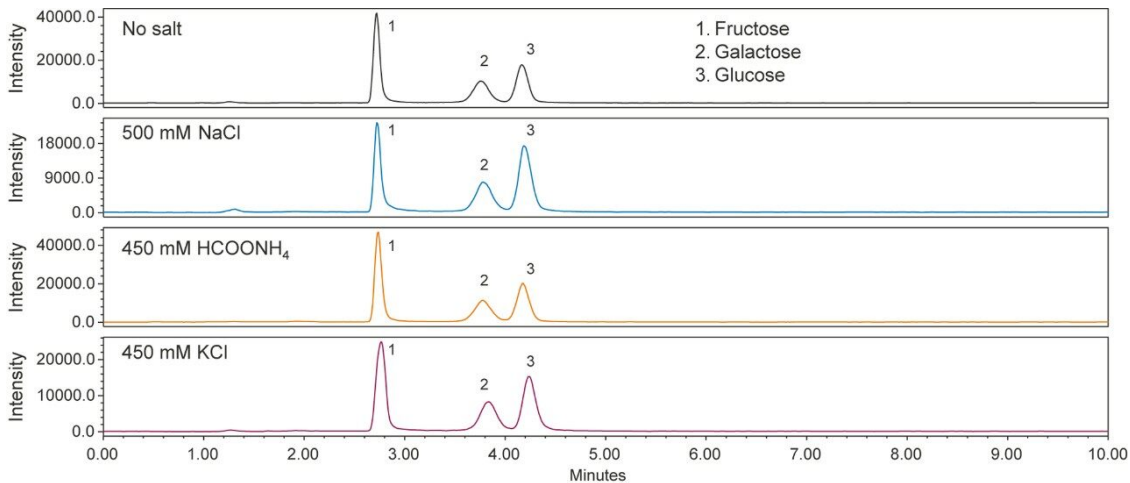


Figure 2. Comparison of SIR chromatograms of early eluting sugars (fructose, galactose, and glucose) with and without the presence of high concentration of different salts in sample solutions. No effect of salt on the peak shape and resolution was observed.

## Reference standards

It is a common practice in mass spectrometry (MS) to use stable isotope labeled standards as references to normalize the variation in signal intensity. In this method, the  $^{13}\text{C}$ -labeled glucose- $^{13}\text{C}_6$  and the lactose- $^{13}\text{C}_6$  were used as references. The glucose- $^{13}\text{C}_6$  was used as the reference for fructose, galactose, and glucose, and the lactose- $^{13}\text{C}_6$  was used for sucrose, lactose, and maltose. Figure 3 shows typical calibration curves for these sugars. These calibration curves were obtained by the least square regression to quadratic models with  $1/x$  weighting. The  $R^2$  values of at least 0.995 were obtained.

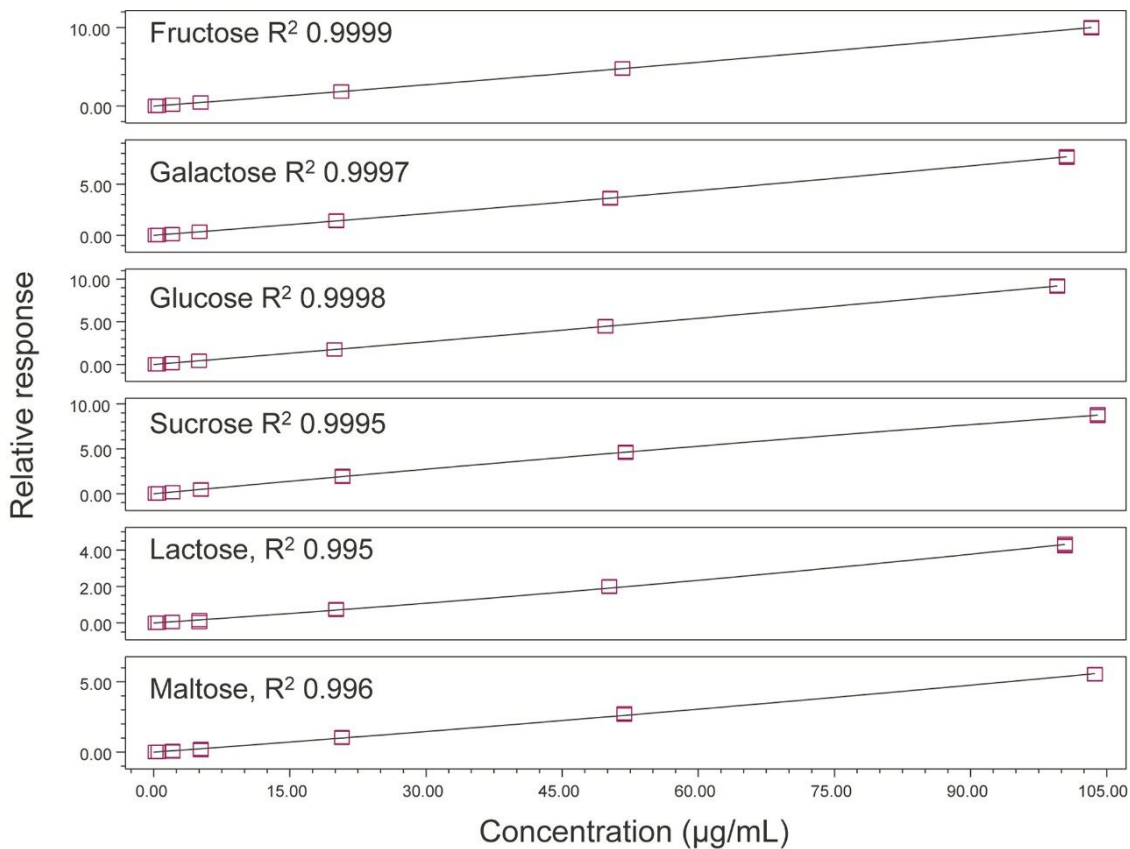


Figure 3. Calibration curves for six sugars using a quadratic fitting model and  $1/x$  weighting. The  $R^2$  values for each compound are shown on the plots. The concentration range in standard solutions are from about 0.5 to 100 ppm.

### Accuracy and reproducibility

Table 2 shows the recovery results for the sugar analysis in spiking experiments on IF and soy-based IF samples. The spiking levels in these samples were from 3 to 7 mg/g. Recovery values were between 90% to 125%. Measurement of the NIST standard reference material (SRM) 1849a showed a lactose (lactose monohydrate) concentration of 519 mg/g, which was 109% of the SRM reference value (Table 4).

Table 3 shows repeated measurement results on the sugar content in samples. The measurements were conducted on two different days with replicated measurements on each day. Relative standard deviation (RSD) values of less than 5.8% were obtained.



Sample	Analyte	Endogenous level (mg/g)	Spiked level (mg/g)	Measurement result (mg/g)	Recovery (%)
Infant formula	Glucose	7.50	5.89	13.07	94.6%
	Sucrose	0.00	6.93	8.09	116.7%
Infant formula (soy-based)	Fructose	0.65	4.10	5.78	125.1%
	Galactose	0.00	4.20	4.20	100.2%
	Lactose	0.00	3.66	3.35	91.7%

Table 2. Recovery results for sugars in spiking experiments with infant formula (IF) and soy-based IF samples.

Analyte	Concentration (mg/g)	RSD (%)	Sample matrix
Galactose	2.62	5.8	IF
Glucose	22.3	0.8	IF (soy-based)
Sucrose	11.2	5.0	Oatmeal
Sucrose	138	4.0	IF (soy based)
Lactose	532	3.0	IF
Maltose	26.1	1.6	IF (soy-based)

Table 3. Measurement results for sugars in selected samples.

Measurements were conducted on two different days with replicated measurements each day (n=4).

## Quantification of food samples

Table 4 shows the sugar analysis results for various samples. The samples include oatmeal, non-fat milk powder, IF, milk, chicken feeds, cocoa powder, and NIST IF standard reference material (NIST 1849a). It was found that the non-fat dry milk (powder) and IF samples contained high levels of lactose, while the lactose-free milk (liquid) contained relatively high levels of galactose and glucose. Sucrose was the main sugar in oatmeal, soy-based IF, chicken feed, and cocoa powder. Figure 4 shows the SIR chromatograms of selected food samples.

Sample	Content (mg/g)					
	Fructose	Galactose	Glucose	Sucrose	Lactose	Maltose
Oatmeal	ND	ND	ND	10.00	ND	ND
Non-fat dry milk	ND	0.94	0.50	ND	572	ND
Infant formula	ND	2.64	7.30	1.29	556	ND
Infant formula (soy-based)	0.28	ND	24.97	122.77	ND	22.51
Lactose-free milk	0.08	32.70	31.56	ND	ND	ND
Chicken feed	1.21	0.25	1.39	18.41	ND	ND
Coca powder	0.09	ND	ND	4.82	ND	ND
NIST IF SRM 1849a		0.62	0.23	0.78	519	–

Table 4. Sugar analysis results for various samples.

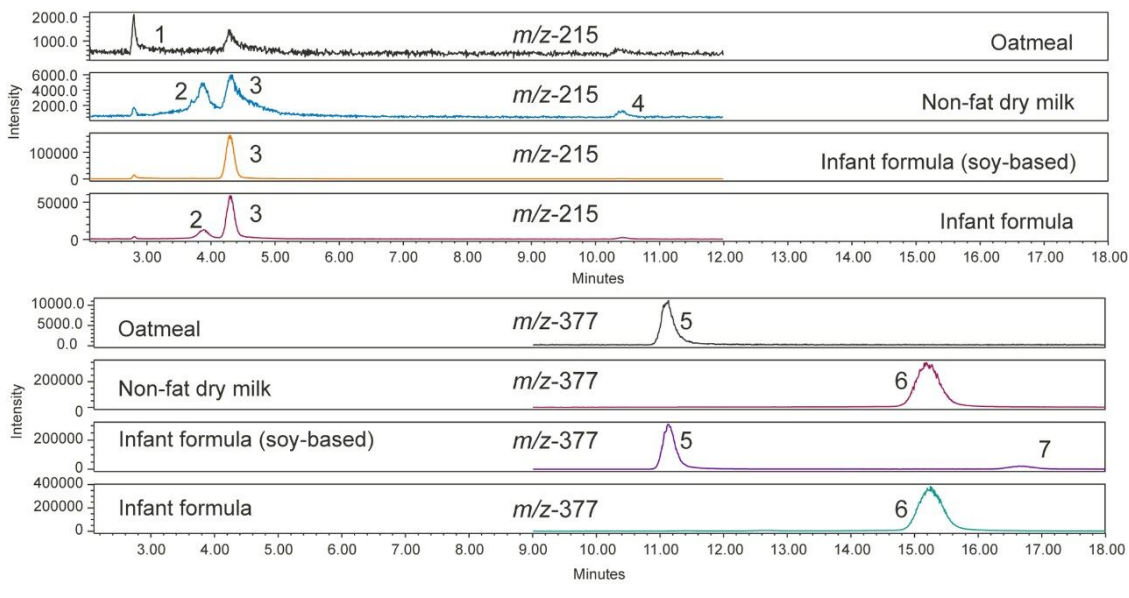


Figure 4. SIR chromatograms of selected samples at  $m/z$  215 Da and  $m/z$  377 Da. The peak IDs are the same as in Figure 1.

## Conclusion

This sugar analysis method offers scientists an accurate and reliable way to determine the content of fructose, galactose, glucose, sucrose, lactose, and maltose in food and animal feed samples.

The key features of this method include:

- Separation of the often co-eluting galactose and glucose so that an accurate quantification of these two sugars is possible.
- Sensitive and clean chromatogram with little background noise from sample matrix.
- Comparing to high performance anion exchange chromatography (HPAEC), this method takes only 25 min for an injection cycle, which is about half of the time that it usually takes in HPAEC.

This method could be a suitable method for the routine sugar analysis in foods and animal feeds.

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## References

1. Revision of the Nutrition and Supplement Facts Labels, Food and Drug Administration, May 27, 2016 CFR: 21 CFR Part 101.
2. Benvenuti, M.; Cleland, G.; Burgess, J. Profiling mono and disaccharides in mild and infant formula using the ACQUITY Arc System and ACQUITY QDa Detector. Waters Application Note 720005767EN, 2016.
3. Chavez-Servin, J L; et al. Analysis of Mono and Disaccharides in Milk Based Formulae by High Performance Liquid Chromatography with Refractive Index Detection. *J. Chromatogr., A.* 2004, 1043 (2), 211–215.
4. XBridge XP 2.5  $\mu\text{m}$  Columns. Waters Care and Use Manual 720004162EN, March 2012.
5. Lactulose is a man-made sugar. Mannose often exist in fruits and some berries, but galactose exists in dairy products. The cellobiose can be found in pine needles, maize stems, and in the sap of certain trees, while the lactose is found only in milk and dairy products. The chance for the lactulose and sucrose, the mannose and galactose, and the cellobiose and lactose to exist in same samples is rare.

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