Identification and accurate quantitation of biological oligosaccharide mixtures

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Supplemental Eq. 1

$$\frac{H}{D} = \frac{E_1}{E_2 - \left(\frac{E_1 \cdot T_2}{T_1}\right)}$$

$E_1 = experimental\ first\ isotope\ intensity$

$E_2 = experimental\ second\ isotope\ intensity$

$T_1 = theoretical\ first\ isotope\ intensity$

$T_2 = theoretical\ second\ isotope\ intensity$

Supplemental Eq. 2

$$\left(\frac{H}{D}\right)_{adjusted} = \frac{mass_{dil}}{mass_{con}} \cdot \left({}^{H}/_{D}\right)_{dil}$$

$mass_{con} = amount\ of\ deuterated\ standard\ added\ to\ reference\ sample\ (at\ zero\ time - point)$

$mass_{dil} = amount\ of\ deuterated\ standard\ added\ to\ catabolized\ sample$

$\left({}^{H}/_{D}\right)_{dil} = ratio\ of\ catabolized\ sample$

Supplemental Eq. 3

$$\%\ consumption = \frac{\left({}^{H}/_{D}\right)_{or} - \left(\left({}^{H}/_{D}\right)_{adjusted}\right)_{cat}}{\left({}^{H}/_{D}\right)_{or}} * 100$$

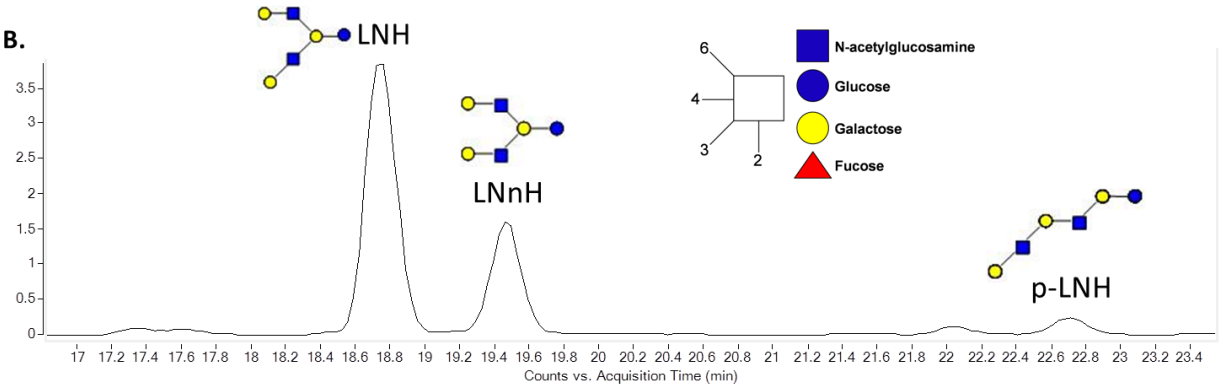
$\left({}^{H}/_{D}\right)_{or} = ratio\ of\ original\ sample$

$\left(\left({}^{H}/_{D}\right)_{adjusted}\right)_{cat} = ratio\ of\ adjusted\ catabolized\ sample$

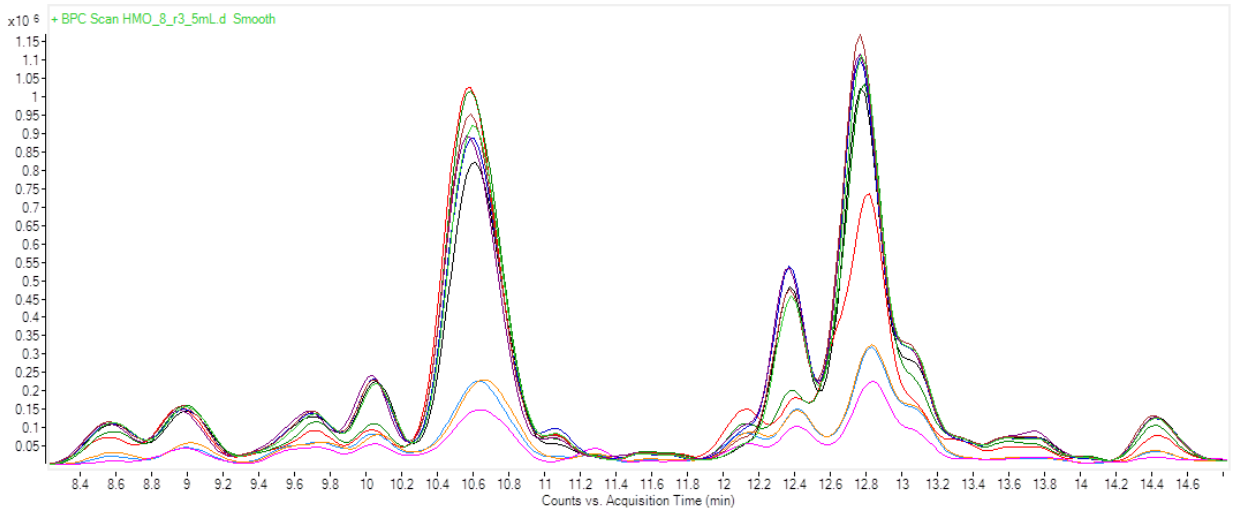
A.

Name	RT (min)	Hex	HexNAc	Dxyh	NeuAc	A+1mass	A+1int	A+2mass	A+2int
LNH	18.75	4	2	0	0	1074.3962	100	1075.3996	45.9
LNnH	19.45	4	2	0	0	1074.3962	100	1075.3996	45.9
p-LNH	22.70	4	2	0	0	1074.3962	100	1075.3996	45.9

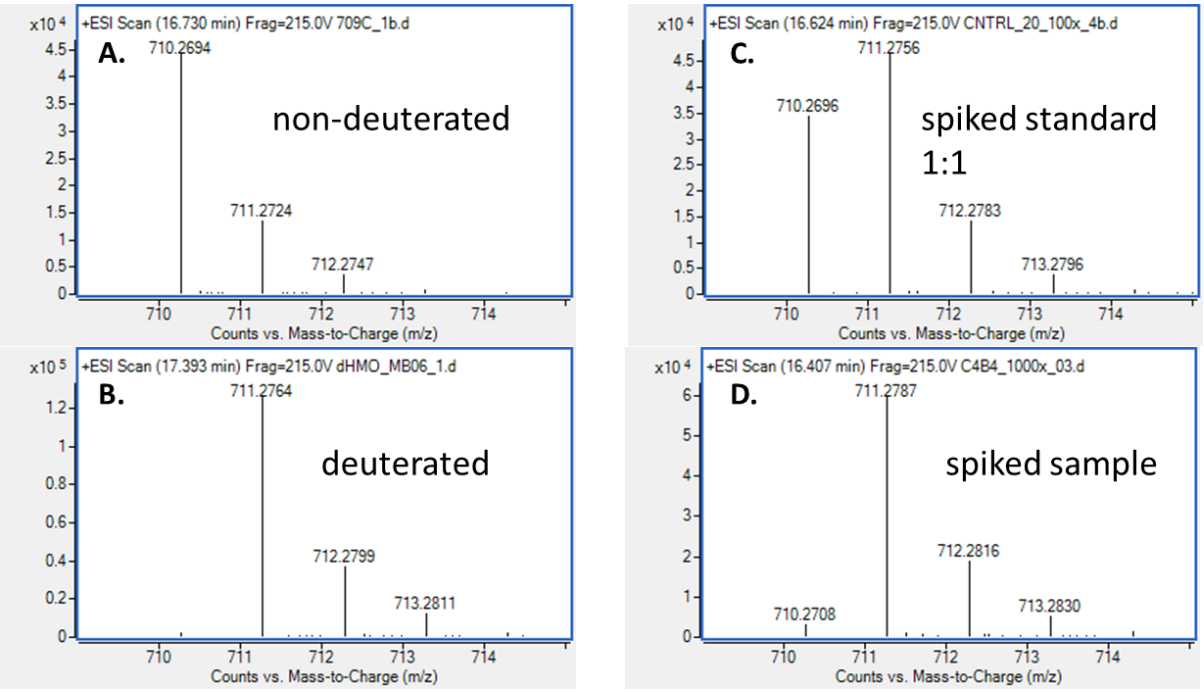
B.



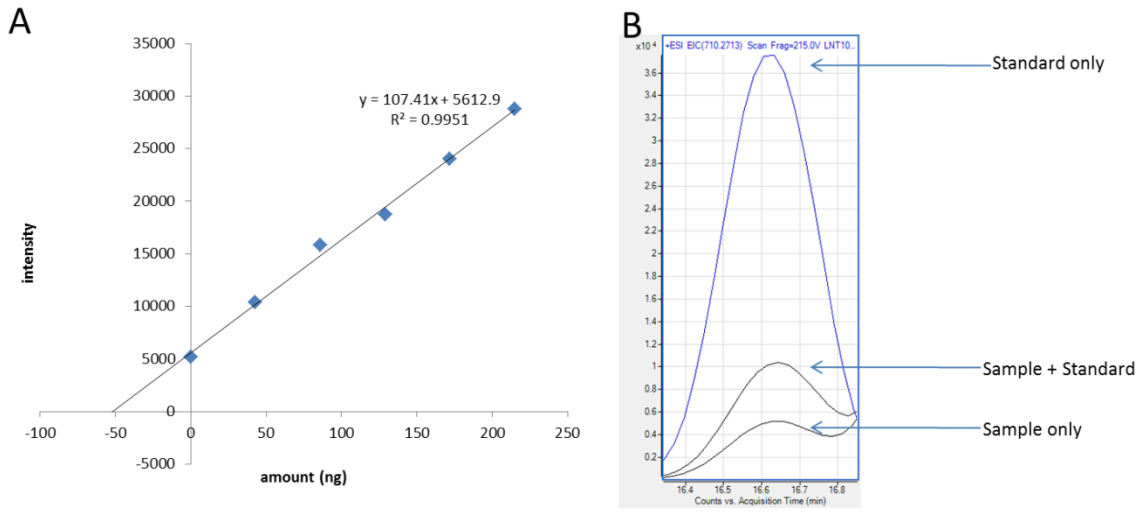
Supplemental Figure 1. A. Excerpt from HMO structure library for three isomers. B. EIC showing the separation and detection of three HMO isomers with HPLC-Chip/TOF-MS.



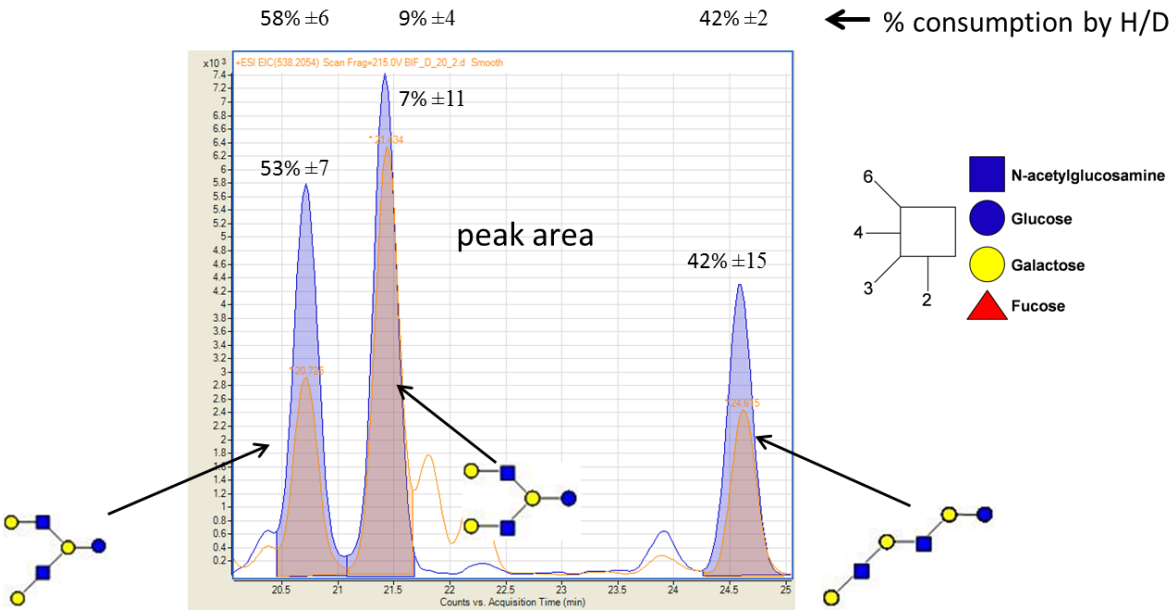
Supplemental Figure 2. Demonstration of highly reproducible retention times for a range of concentrations and composition profiles of HMO mixtures.



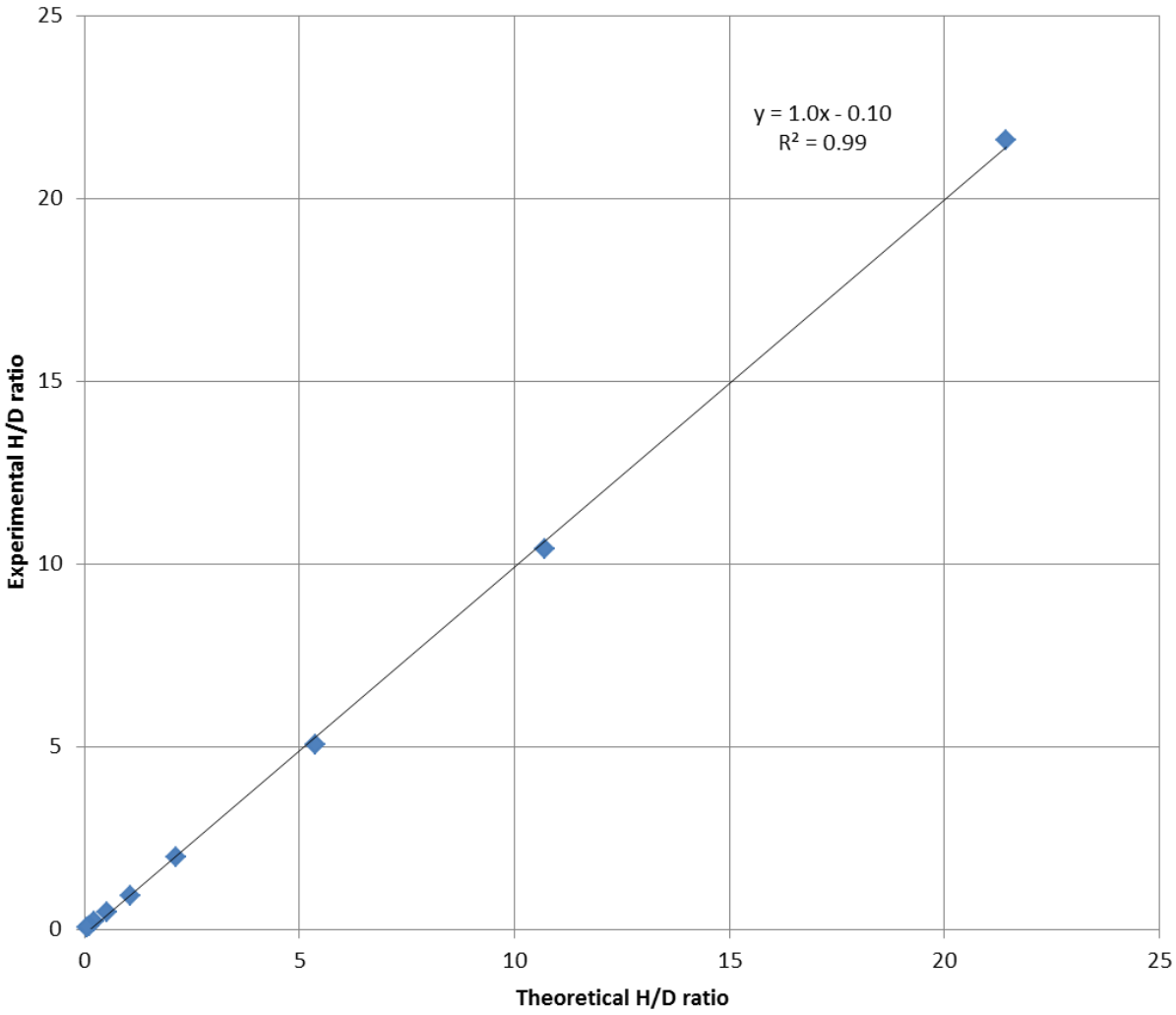
Supplemental Figure 3. Demonstration of isotope distributions of known standards (A-C) and a sample (D).



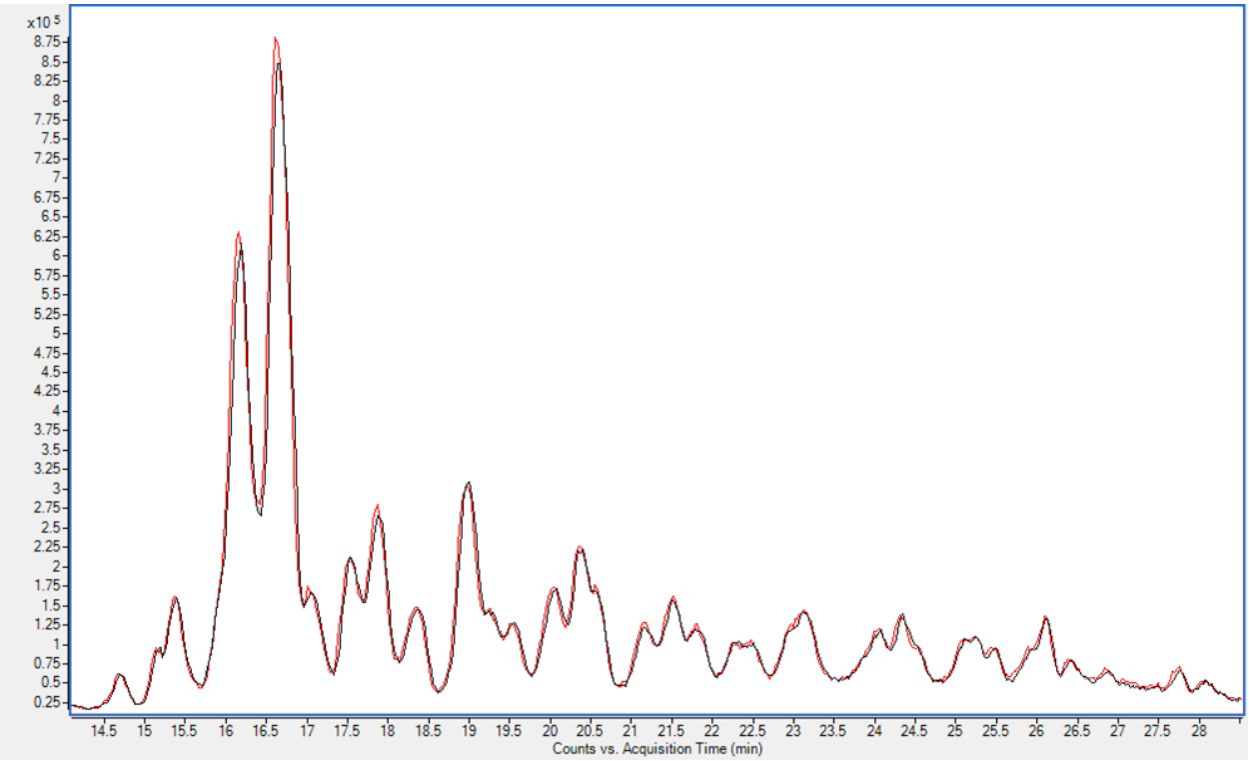
Supplemental Figure 4. A. Standard addition quantitation for LNT. B. Comparison of HMO ionization efficiency in two different chemical environments demonstrates the need to quantify with an internal standard. The two lower peaks are the first and second points on the standard addition plot in A. The larger peak is the measurement of the amount of standard that was used for each addition. The fact that the difference in intensity between the two spikes into the matrix is less than the intensity of the spike analyzed without the matrix reveals the problem of matrix effects for this analysis.



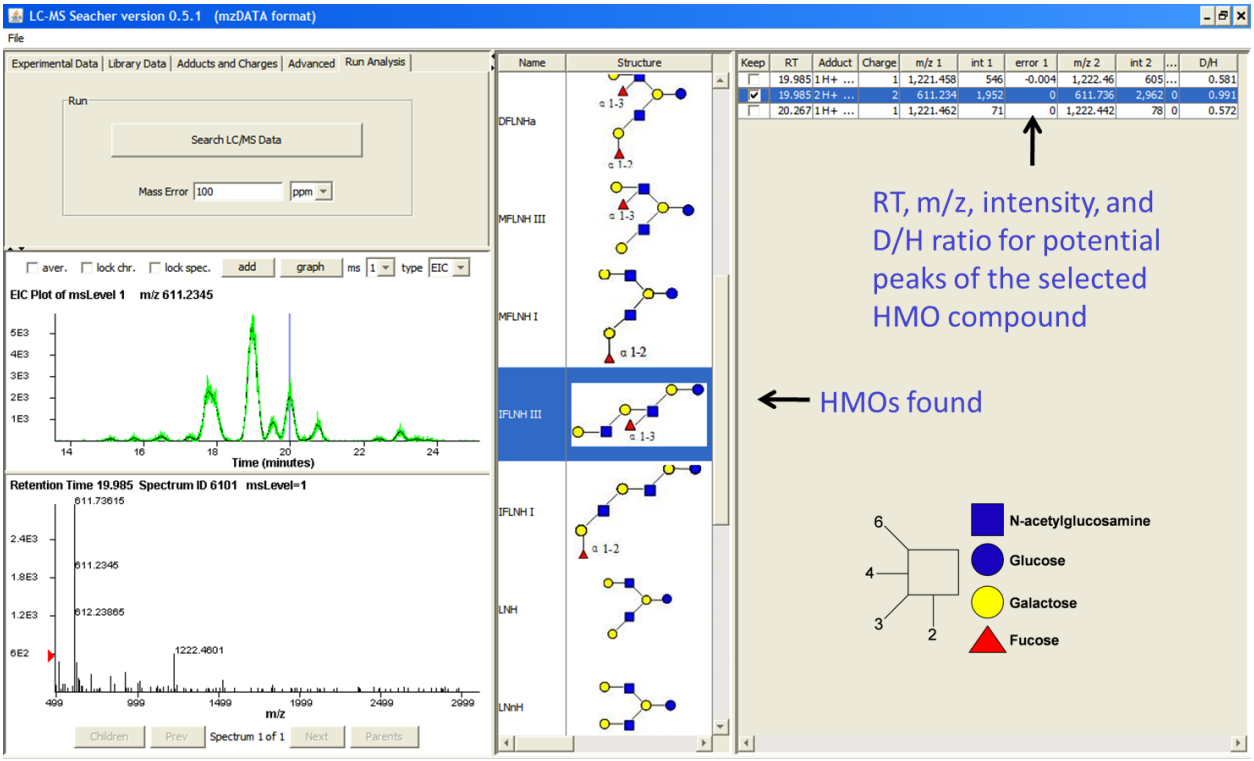
Supplemental Figure 5. Comparison of quantitation with H/D ratio versus absolute peak intensity. Blue peaks are EICs for m/z 538.2064 from a chromatographic separation of HMOs *prior* to consumption. Orange peaks are EICs for m/z 538.2064 from a chromatographic separation of HMOs *after* consumption by *B. infantis.* The method employing H/D ratio corresponds well to that employing peak area. The advantage of H/D ratios is that overlaps are not a problem and peak shapes do not have to be perfectly Gaussian. It is also significantly simpler to automate the calculation of H/D ratio than peak areas.



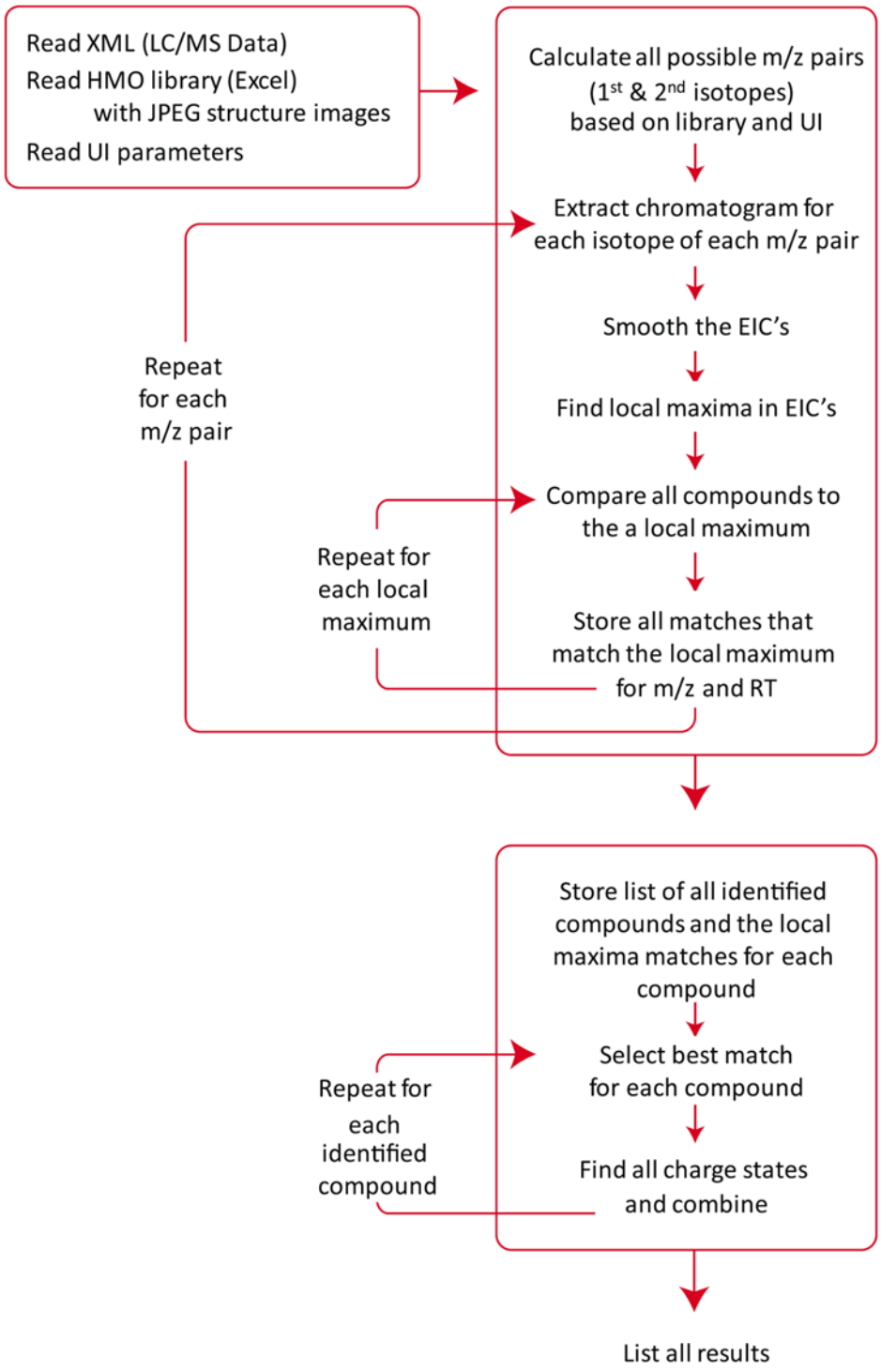
Supplemental Figure 6. Demonstration of the accuracy and linearity of expected and observed H/D ratios obtained from spiked standards. The best fit is obtained by accounting for any systematic bias in the theoretical vs. empirical isotopic distributions.



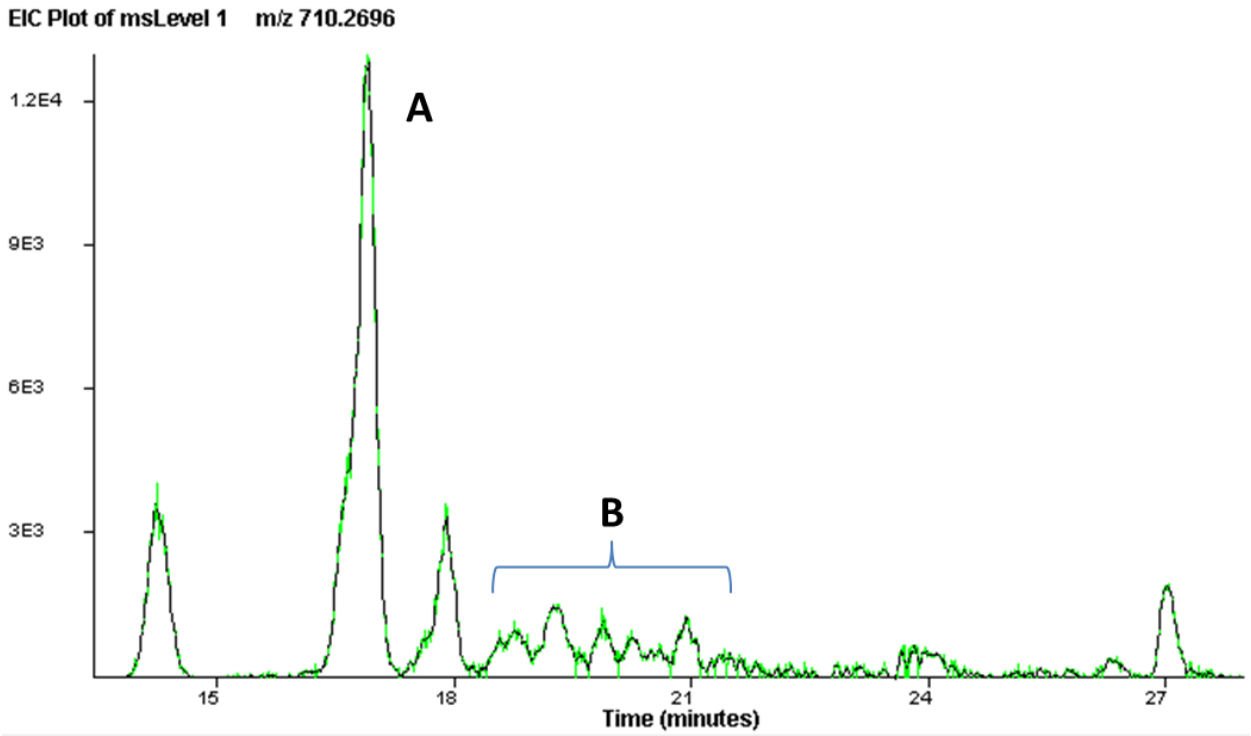
Supplemental Figure 7. The deuterated and non-deuterated HMOs show virtually identical ionization efficiencies. The black trace is a non-deuterated HMO pool. The red trace is the deuterated version of the same pool.



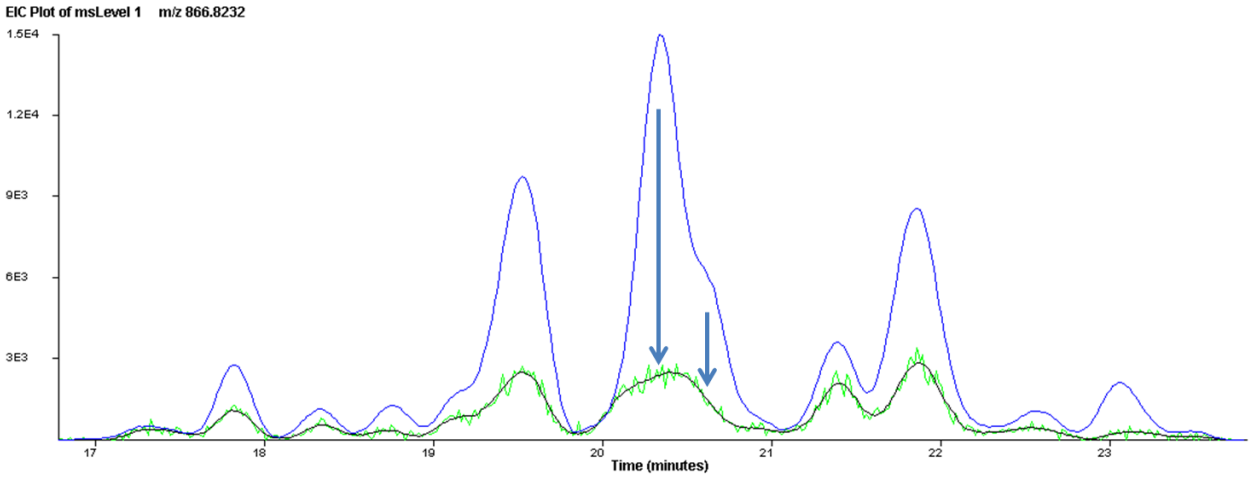
Supplemental Figure 8. User interface for in-house software that automatically identifies, and quantifies HMO structures from LC-MS data and a structure library. LC/MS visualization tools originate from *Babraham Bioinformatics*; mzViewer code available to the public at http://www.bioinformatics.bbsrc.ac.uk/projects/mzviewer



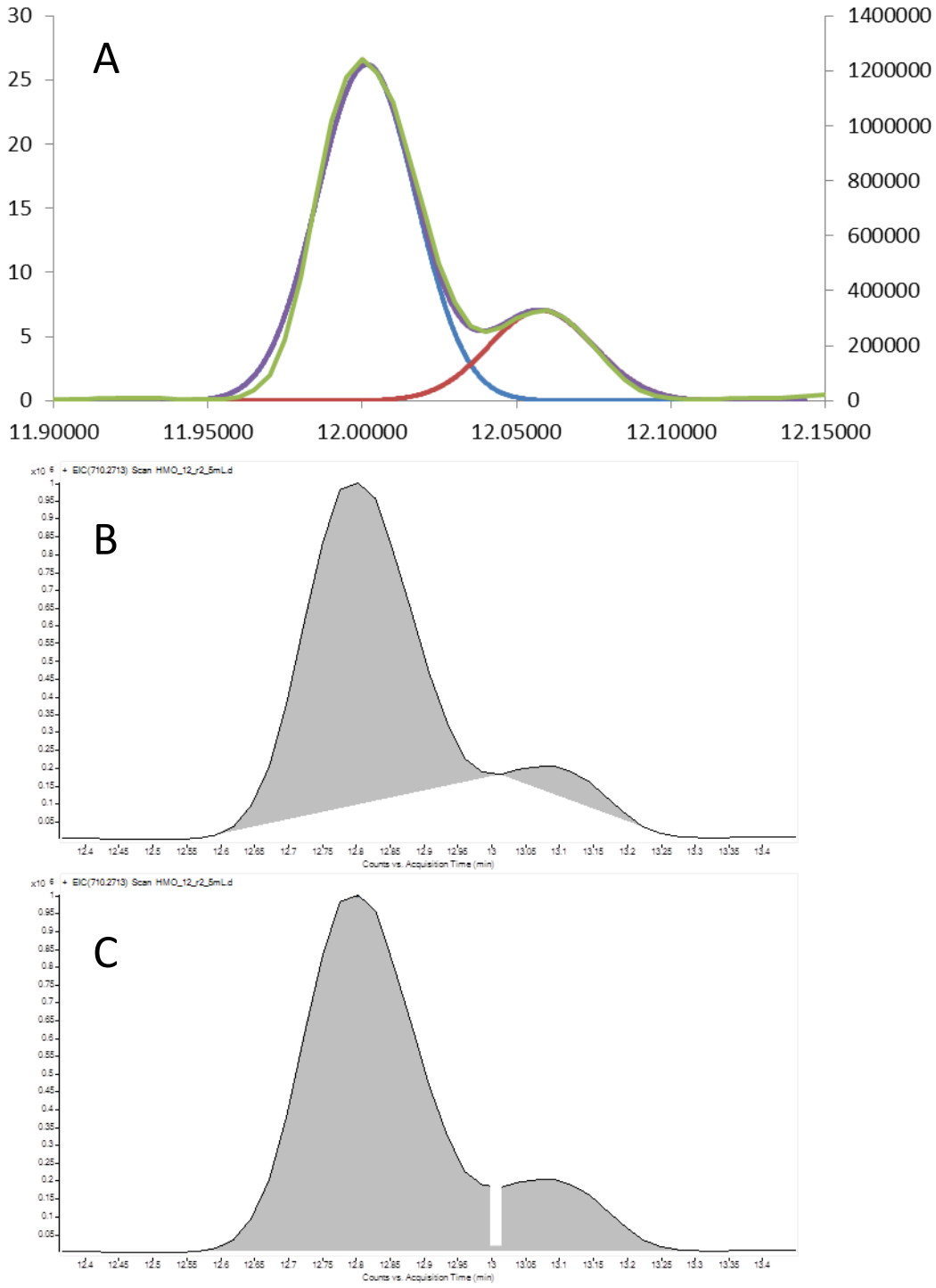
Supplemental Figure 9. Diagram of the compound-finding algorithm.



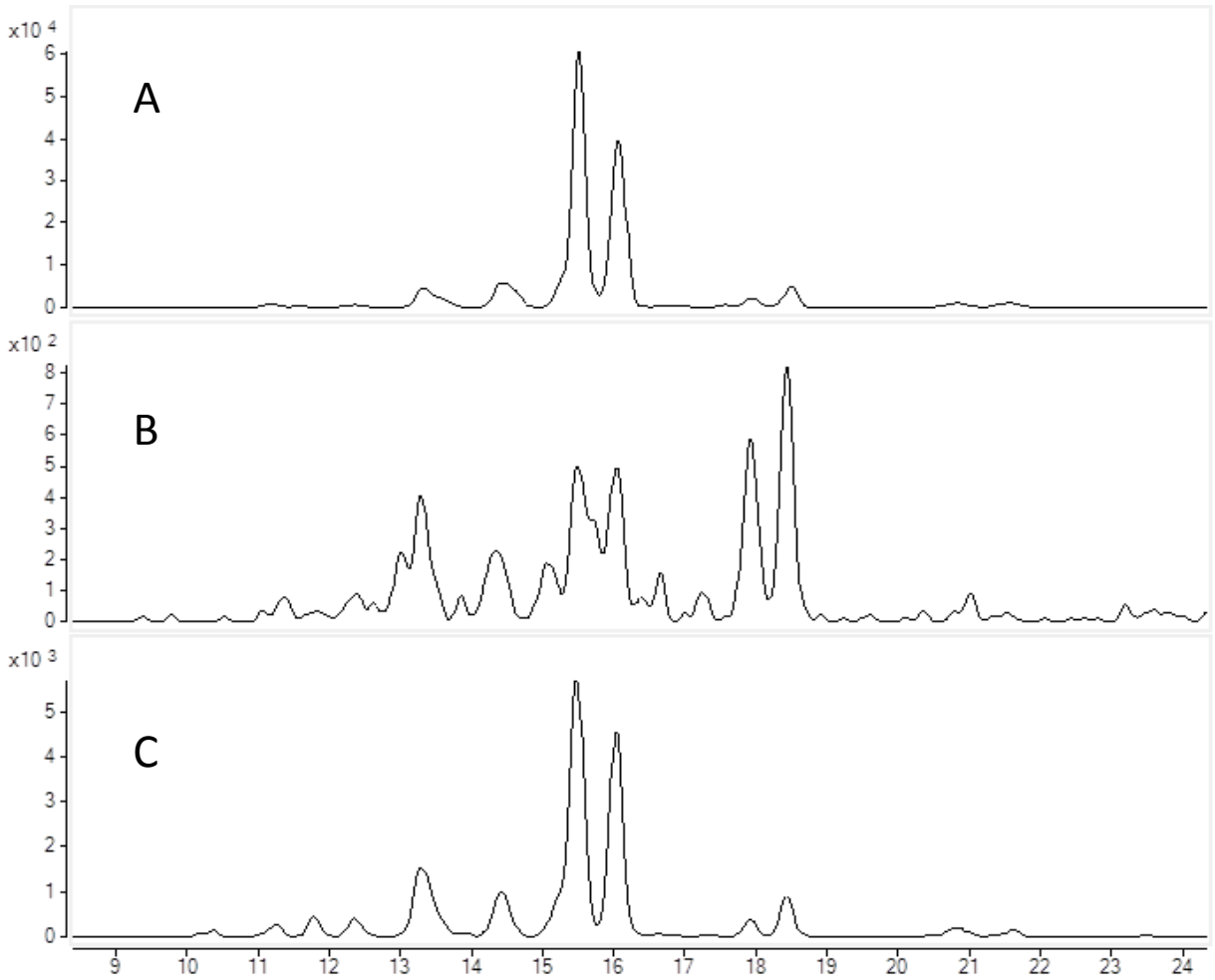
Supplemental Figure 10. Peak A has a well-defined shape and is a good candidate for chromatographic deconvolution. The peaks labeled under B are poor candidates due to uncertainty regarding the cause of the unexpected peak shapes. The cause could be overlapping peaks or simply poor signal quality. The green line is the raw EIC and the black line is the smoothed profile.



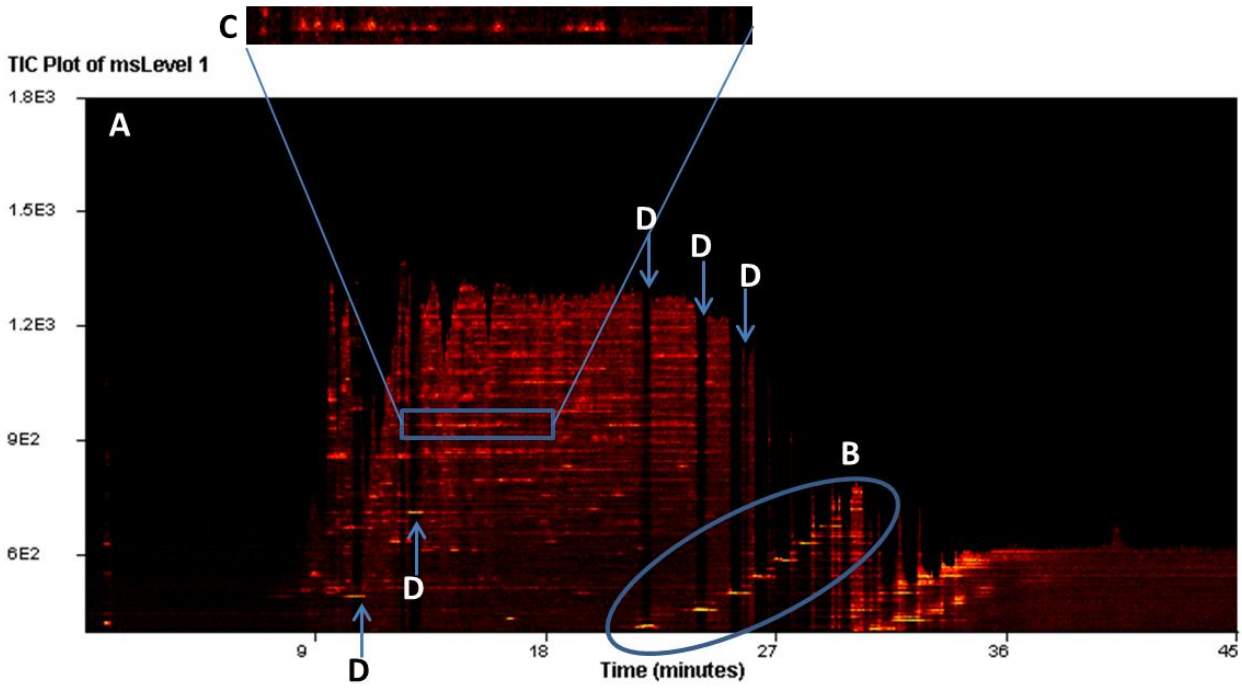
Supplemental Figure 11. The top EIC (blue) is the deuterated profile and helps identify the expected retention times of two poorly unresolved catabolized HMOs in the bottom EIC.



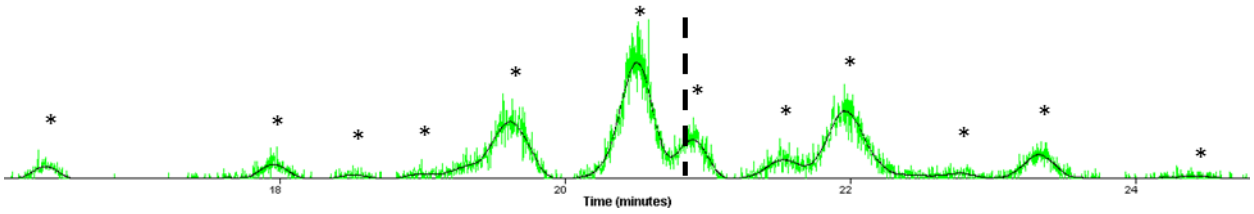
Supplemental Figure 12. Visualization of three methods for determining peak area: A) chromatographic deconvolution, B) shifting baseline assumption, and C) steady baseline assumption.



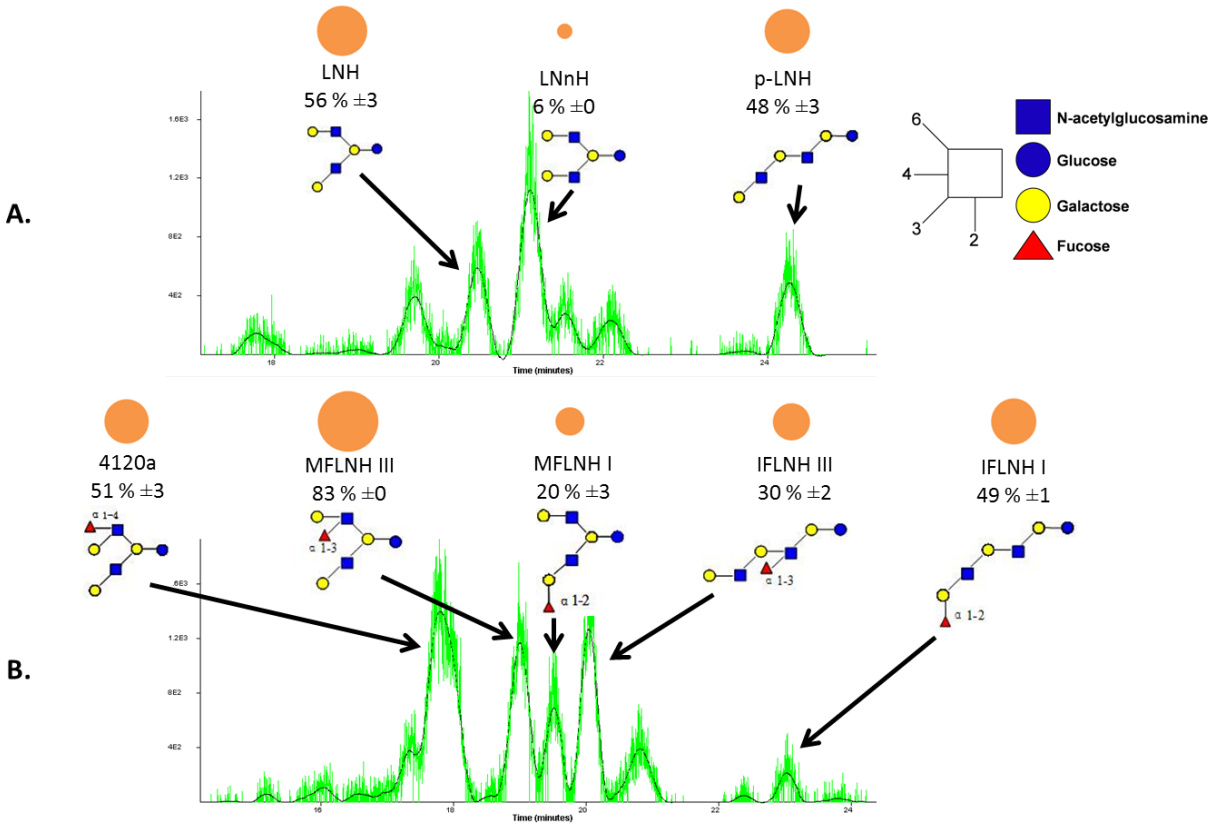
Supplemental Figure 13. Demonstration of the change in profile of HMOs following catabolism and the utility of the unchanged profiles of the monodeuterated internal standards. A. EIC profile of m/z 538.2054 (monoisotope) from reference HMO pool. B. EIC profile of m/z 538.2054 (monoisotope) from catabolized HMO sample. C. EIC profile of m/z 538.7115 (second isotope + deuterated monoisotope) from catabolized HMO sample. Demonstration of how the profile of the deuterated species (second isotope) allows accurate determination of correct retention times. The problem with using the non-deuterated ion (first isotope) is that after *in vitro* HMO catabolism, the profile is no longer comparable to the HMO pool data.



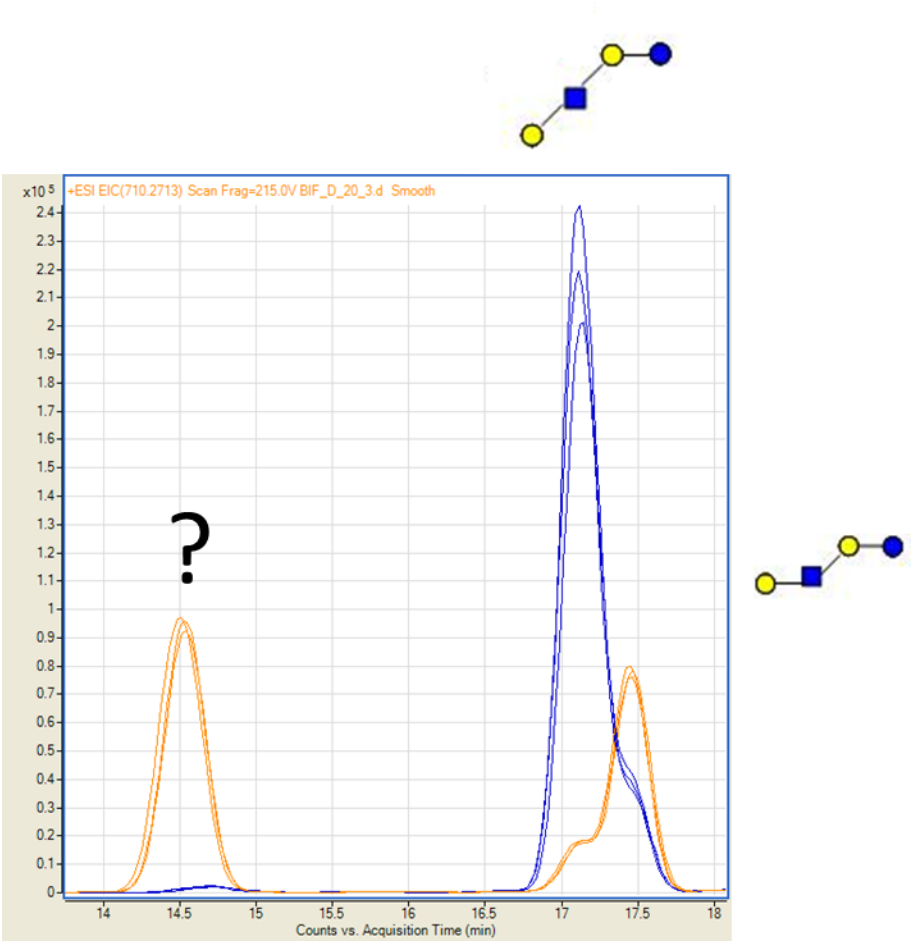
Supplemental Figure 14. Demonstration of the isomer profile with a heat map and how the heat map can efficiently reveal information about the quality of the data. A. Heat map. B. Polymer impurities. C. Zoomed-in view of a family of isomers. D. Corresponding bright spots and the shadows cast across the mass range, revealing ion suppression.



Supplemental Figure 15. Raw (green) and smoothed (black) EIC of a family of HMO isomers. The asterisks show local maxima and the dotted vertical line shows the theoretical retention time of an HMO structure for the structure-library.



Supplemental Figure 16. Percent consumption of selected HMO structures by *B. infantis.* The area of the orange circles represents the percent consumption of each HMO structure as calculated by Supplemental Eq. 3. The green trace is the raw EIC data and the black trace is the smoothed version. A. Results from the EIC of m/z 538.2064 B. Results from the EIC of m/z 611.2387.



Supplemental Figure 17. Although the majority of the HMOs decreased in concentration, certain HMOs increased; the unexpected increase is probably the result of larger HMOs that have been degraded. The blue trace shows three repeated analyses of the HMOs prior to catabolism (EIC of monoisotope). The orange trace shows three replicates of the catabolized sample (EIC of monoisotope). The difference in the abundances of the non-deuterated forms clearly shows that one structure has decreased while the other two have increased. Both the unknown HMO at 14.5 minutes and LNnT at 17.45 minutes increased in abundance during the exponential bacterial growth; whereas LNT at 17 minutes decreased significantly.