BEST PRACTICES IN THE ANALYSIS OF *RAPI*FLUOR-MS LABELED GLYCANS USING THE ACQUITY QDa DETECTOR (PERFORMANCE MODEL)

The following document provides basic understanding and guidance in the configuration of an ACQUITY UPLC® System – specifically an H-Class Bio – equipped with an FLR and ACQUITY® QDa® Detector (Performance model) in the analysis of *Rapi*Fluor-MS™-labeled glycans. The fundamentals of mass detection theory, system configuration, and method settings are discussed. A best practices section is also provided as a diagnostic tool to qualitatively gauge the performance of the system when configured with an FLR and ACQUITY QDa Detector, and to offer guidance on improving system performance.

Reagents/Supplies Quick Reference Table			
Part	**Supplier**	**Website**	**Part number**
ACQUITY UPLC Glycan BEH Amide Column, 130Å, 1.7 µm, 2.1 mm x 150 mm	Waters	www.waters.com	186004742
*Rapi*Fluor-MS Glycan Performance Test Standard	Waters	www.waters.com	186007983
Waters Ammonium Formate Solution – Glycan Analysis	Waters	www.waters.com	186007081
LCMS Certified Total Recovery Vial	Waters	www.waters.com	600000750cv
N,N-Dimethylformamide *also provided in the RFMS GlycoWorks kit	Fisher Scientific	www.fishersci.com	AC61094-1000
Pierce™ Water	Pierce	www.thermofisher.com	51140
Pierce Acetonitrile	Pierce	www.thermofisher.com	51101

Table 1.

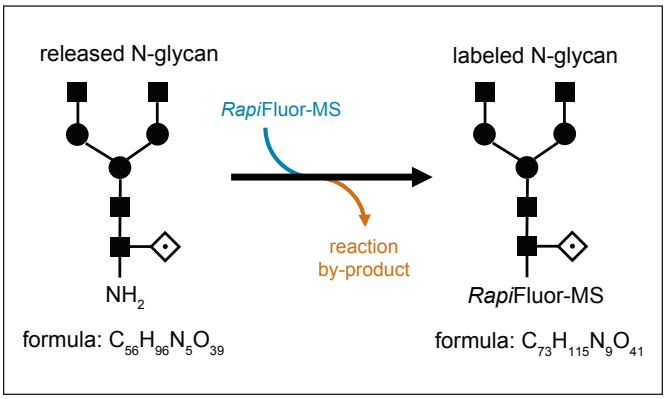


Figure 1.

Empirical formula	
Released N-glycan (FA2)	$C_{56}H_{96}N_5O_{39}$
*Rapi*Fluor-MS reagent	+ $C_{21}H_{25}N_5O_5$
Reaction by-product	- $C_4H_5N_1O_3$
Glycosylamine proton loss	- H
Labeled N-glycan (FA2)	= $C_{73}H_{115}N_9O_{41}$

Table 2.

Figure 1 and Table 2. The RapiFluor-MS labeled FA2 ($C_{73}H_{115}N_9O_{41}$) glycan is used as an example in this document to demonstrate some principles of MS fundamentals and spectrum interpretation. Information on nomenclature and structure symbols can be found at the following:

1. https://glycobase.nibrt.ie/glycobase/documents/abbreviations.pdf

2. Harvey, D. J., et al. (2009). "Proposal for a standard system for drawing structural diagrams of N- and O-linked carbohydrates and related compounds." Proteomics 9(15): 3796-3801. 2009.

3. Essentials of Glycobiology. A. Varki, R. D. Cummings, J. D. Esko et al. Cold Spring Harbor, NY.

Figure 2. ACQUITY UPLC H-Class Bio System

LC SYSTEM CONFIGURATION

The data presented in this document was acquired with an ACQUITY UPLC H-Class Bio System in a split stack configuration. Variations of results (e.g., retention time and peak width) may be observed due to differences in system configuration and should be considered when comparing results.

It is strongly recomended that existing lab systems that are being re-purposed for *Rapi*Fluor-MS labeled glycan analysis using the ACQUITY QDa be cleaned prior to use in order to remove residual contaminants such as alkali metal salts, which reduce mass detector sensitivity. For best performance, the recommended Waters® AAA cleaning protocol (P/N USRM134761474, pp 42–44) should be used and can be located on the Waters website.

- **Reservoirs:**
  Standard (1 L) borosilicate lab-approved glassware
  Cleaning protocol: Sonicate in LC-MS grade 50:50 IPA:H_2O with 0.1% formic acid for 20–30 min. Rinse with LC-MS grade organic (e.g., methanol or acetonitrile) and dry prior to use.
  Sinkers and tubing: Standard H-Class Bio titanium sinkers and tubing.
- **QSM pump:**
  Mixer: 100 µL mixer
  Tubing: Tubing is all standard that ships with H-Class Bio
- **Autosampler:**
  Needle type: flow through needle (FTN)
  Column connection: ACQUITY UPLC APH (Active pre-heater), MP35N 18.5" Long (P/N 205000775)
  Sample vials: LCMS Certified Total Recovery Vial (P/N 600000750cv)
- **Column heater:**
  The column manager (CM-A) in this configuration is being utilized as a column heater using the active pre-heater technology bypassing valve port use. Connection from column to optical detector (FLR) is Assy, Welded Tube, MP35N, 19.0 LG, LP (P/N 700005483)
- **Optical detector:**
  ACQUITY UPLC FLR Analytical Flow Cell Assy, 2 µL (P/N 700003711)
- **ACQUITY QDa:**
  Model: Performance model
  ESI Probe Assembly: 250 mm x 100 µm (P/N 700009641)

METHOD SETTINGS

- **Sample:**

Waters *Rapi*Fluor-MS Glycan Performance Test Standard (P/N 186007983).
Prepared in 22.5% H_2O, 25% DMF, 52.5% acetonitrile at a concentration of 10 pmol/µL.

Note: For the 400 pmol standard this would be (9 µL) H_2O, (10 µL) DMF, (21 µL) ACN. This should be sufficient for 5–6 injections using a 6 µL (60 pmol) injection volume. Smaller injection volumes (3 µL– 4 µL) can be used for diagnostic purposes.

- **FLR:**
  Mode: 2D
  λ Ex: 265, λ Em: 425
  Data rate: 2 pts/sec
- **ACQUITY QDa:**
  Full scan: 350–1250 *m/z*
  Mode: positive, centroid
  Probe: 400 °C
  Capillary: 1.5 kV
  Cone: 15 V
  Data rate: 5 pts/sec
  SIRs: Use when appropriate
- **Column:**
  ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7µm, 2.1 mm x 150 mm (P/N 186004742)
  Column/sample temp: 60 °C /10 °C
- **Mobile phase:**
  Mobile phase A: 50 mM NH_4HCO_2, pH 4.4 **(LC-MS grade)**
  Mobile phase B: acetonitrile **(LC-MS grade)**

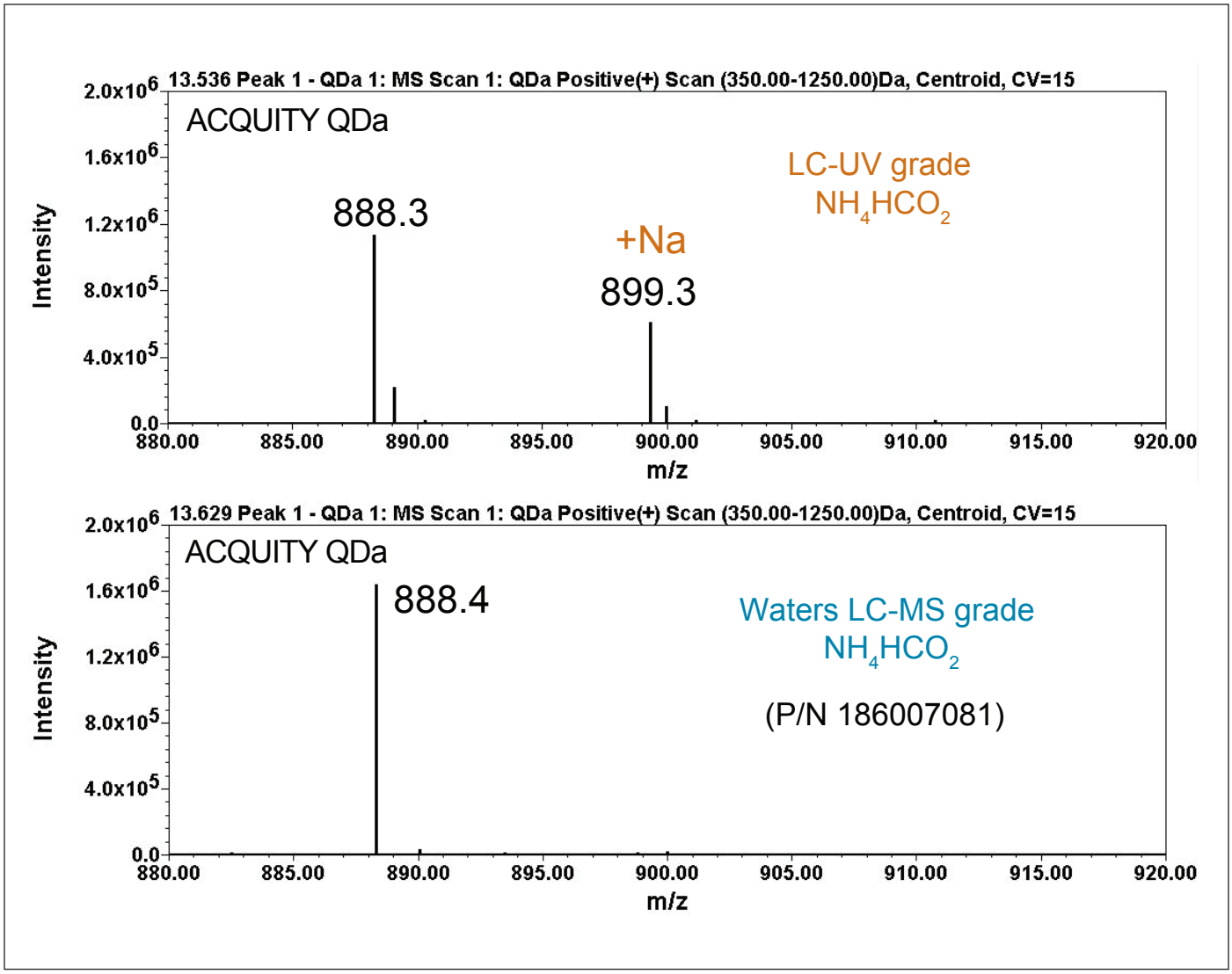


*Figure 3. Waters Ammonium Formate Concentrate. Purity of reagents is a key aspect in reducing adduct formation in MS-based analyses. MS-grade reagents and solvents should always be used for glycan analysis using the ACQUITY QDa. Waters is now providing a 10X solution of **LC-MS grade** ammonium formate (NH_4CO_2) concentrate (P/N 186007081) which is diluted in 1 L of LC-MS grade H_2O to provide optimal MS detector performance with negligible alkali metal adducts when compared to mobile phases prepared with commercially-available **LC-UV grade** ammonium formate.*

- **Mobile phase A preparation:**
  Add contents of Waters Ammonium Formate Concentrate Solution – Glycan Analysis (P/N 186007081) to 1 L Pierce-thermo water (LC-MS grade, Thermo Fisher, P/N 51140). If necessary, determine water amount by mass into an LC-MS grade bottle. Avoid using standard laboratory glassware.
  Note: After combining constituents, sonicate or manually mix solution.
- **Mobile phase B preparation:**
  1 L Pierce-thermo acetonitrile (LC-MS grade, Thermo Fisher, P/N 51101)

Gradient table

Time	Flow (mL/min)	%A	%B
Initial	0.400	25.0	75.0
35.00	0.400	46.0	54.0
36.50	0.200	100.0	0.0
39.50	0.200	100.0	0.0
43.10	0.200	25.0	75.0
47.60	0.400	25.0	75.0
55.00	0.400	25.0	75.0

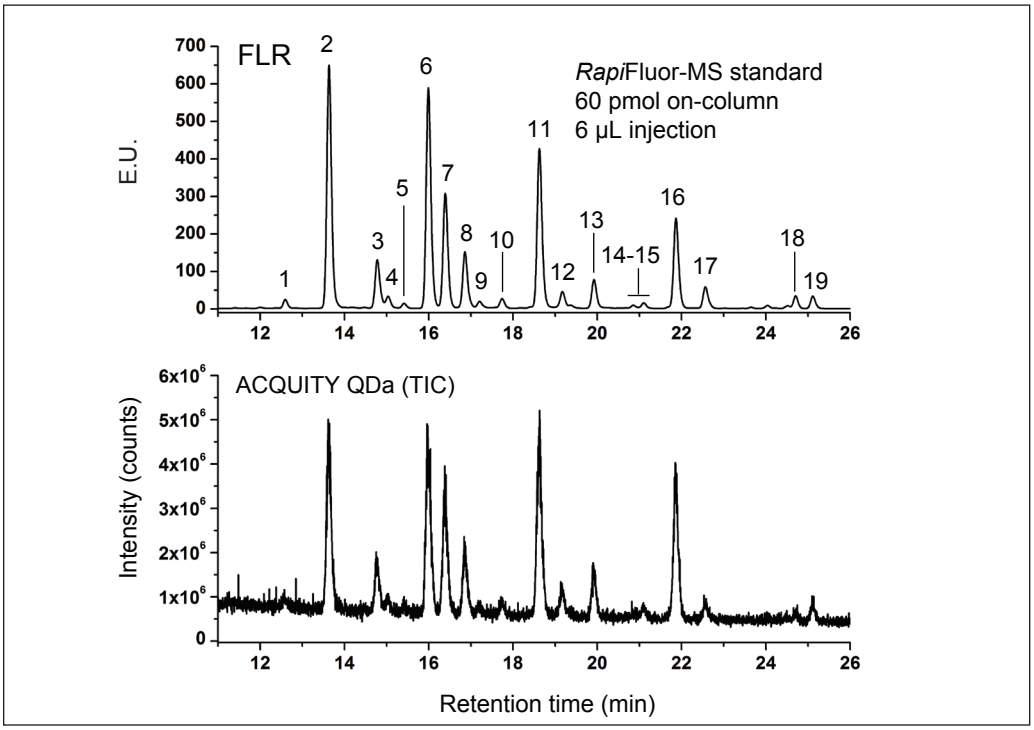


Figure 4. Representative Waters RapiFluor-MS standard chromatograms. A 6 µL injection of the Waters RapiFluor-MS standard provides MS data of sufficient quality for accurate mass assignment of the identified peaks.

					*Rapi*Fluor-Glycan average *m/z*		
Peak	Glycan	Formula (no label)	Formula (with label)	Average mass (with label)	$[M+1H]^{+1}$	$[M+2H]^{+2}$	$[M+3H]^{+3}$
1	A2	$C_{50}H_{86}N_5O_{35}$	$C_{67}H_{105}N_9O_{37}$	1628.6	1629.6	815.3	543.9
2	FA2	$C_{56}H_{96}N_5O_{39}$	$C_{73}H_{115}N_9O_{41}$	1774.7	1775.7	888.4	592.6
3	FA2B	$C_{64}H_{109}N_6O_{44}$	$C_{81}H_{128}N_{10}O_{46}$	1977.9	1978.9	990.0	660.3
4	A2G1a	$C_{56}H_{96}N_5O_{40}$	$C_{73}H_{115}N_9O_{42}$	1790.7	1791.7	896.4	597.9
5	A2G1b	$C_{56}H_{96}N_5O_{40}$	$C_{73}H_{115}N_9O_{42}$	1790.7	1791.7	896.4	597.9
6	FA2G1a	$C_{62}H_{106}N_5O_{44}$	$C_{79}H_{125}N_9O_{46}$	1936.9	1937.9	969.4	646.6
7	FA2G1b	$C_{62}H_{106}N_5O_{44}$	$C_{79}H1_{25}N_9O_{46}$	1936.9	1937.9	969.4	646.6
8	FA2BG1a	$C_{70}H_{119}N_6O_{49}$	$C_{87}H_{138}N_{10}O_{51}$	2140.1	2141.1	1071.0	714.4
9	FA2BG1b	$C_{70}H_{119}N_6O_{49}$	$C_{87}H_{138}N_{10}O_{51}$	2140.1	2141.1	1071.0	714.4
10	A2G2	$C_{62}H_{106}N_5O_{45}$	$C79H_{125}N_9O_{47}$	1952.9	1953.9	977.4	652.0
11	FA2G2	$C_{68}H1_{16}N_5O_{49}$	$C85H_{135}N_9O_{51}$	2099.0	2100.0	1050.5	700.7
12	FA2BG2	$C_{76}H_{129}N_6O_{54}$	$C93H_{148}N_{10}O_{56}$	2302.2	2303.2	1152.1	768.4
13	FA2G1S1	$C_{73}H_{123}N_6O_{52}$	$C90H_{142}N_{10}O_{54}$	2228.1	2229.1	1115.1	743.7
14	Unknown	~	~	~	~	~	~
15	Unknown	~	~	~	~	~	~
16	FA2G2S1	$C_{79}H_{133}N_6O_{57}$	$C_{96}H_{152}N_{10}O_{59}$	2390.3	2391.3	1196.1	797.8
17	FA2BG2S1	$C_{87}H_{146}N_7O_{62}$	$C_{104}H_{165}N_{11}O_{64}$	2593.5	2594.5	1297.7	865.5
18	FA2G2S2	$C_{90}H_{150}N_7O_{65}$	$C_{107}H_{169}N_{11}O_{67}$	2681.5	2682.5	1341.8	894.8
19	FA2BG2S2	$C_{98}H_{163}N_8O_{70}$	$C_{115}H_{182}N_{12}O_{72}$	2884.7	2885.7	1443.4	962.6

Table 3. Structural and mass information for RapiFluor-MS labeled glycan analysis standard.

*green = observed charge state using ACQUITY QDa

Mass detection fundamentals

Terminology	Definition
Monoisotopic mass	The sum of the lowest isotopic mass of the atoms in a molecule. For most small molecules, the monoisotopic ion is the most abundant (base peak) in the spectrum. For larger molecules, such as glycans and peptides, the base peak ion may be different.
Average mass	The mass obtained by summing the constituent atoms in a molecule using their average atomic mass. The average atomic mass can differ significantly from the monoisotopic mass. Factors such as instrument resolution and charge state will impact the MS profile.
Nominal mass	The monoisotopic mass rounded up or down to the nearest integer.
Mass-to-charge ratio (*m/z*)	The ACQUITY QDa mass scale is in m/z and not Daltons. For the doubly-charged monoisotopic ion $[M+2H]^{+2}$ of the *Rapi*Fluor-MS labeled FA2 glycan, m = 1774.7 Da and z = 2. Therefore, a peak will appear on the spectrum at [1774.7+2]/2 = 888.4 *m/z*.
$[M+H]^+$ ion	The positively charged form of a molecule that is created when one proton is added. The ion detected is one unit higher than the monoisotopic mass of the uncharged molecule.
$[M+H]^-$ ion	The negatively charged form of a molecule that is created when one proton is removed. The ion detected is one unit lower than the monoisotopic mass of the uncharged molecule.
Dalton	The unit of atomic mass.
Empirical formula consideration	The incorporation of labels such as *Rapi*Fluor-MS in the analysis of released glycans will alter the empirical formula used in calculating the monoisotopic or average mass. The empirical formula for the structure including the *Rapi*Fluor-MS label should be used for calculating average mass and charge states.
Capillary voltage	The voltage applied to the electrospray capillary to promote the ionization process.
Cone voltage	A focusing voltage applied to the ion guide, transferring ions generated in the source into the ACQUITY QDa.
Probe temperature	The temperature applied to the electrospray probe to promote mobile-phase evaporation as part of the ionization process.
MS scan (or full scan)	Records all of the masses within a user-defined range at each time point in the chromatogram to produce qualitative information. An MS scan can be performed in positive (where only positively charged ions are transmitted and detected) or negative ion mode. This is similar to 3D PDA data.
Single ion recording (SIR)	Records a single user-defined m/z value (positive or negative ion mode) at each time point in the chromatogram, with all other ions being rejected. This technique can introduce specificity and higher sensitivity into an analysis, and is most commonly used for quantitative analysis where lower limits of detection are required. This is similar to 2D PDA data.
Extracted ion chromatogram (XIC)	A chromatogram extracted from an MS scan that only displays the intensity of a selected mass.
Total ion chromatogram (TIC)	A chromatogram in which the intensity is displayed as a sum of all individual ion intensities.
Sampling rate or sampling frequency (Hz)	The number of data points per second acquired for each MS experiment function, such as SIR or MS scan, that you specify.
Mass scale calibration	The mass scale is calibrated against a series of known masses. The calibration is required to ensure that the theoretical and observed masses correspond. You can automatically configure a mass scale calibration check upon instrument start-up.

Table 4. Common terminology encountered in MS analyses.

Element	Isotopic masses (% abundance)	Atomic mass (average)	Atomic mass (monoisotopic)
C	12.0000 (98.90%) 13.0034 (1.10%)	12.0110	12.0000
H	1.0078 (99.99%) 2.0141 (0.015%	1.0079	1.0078
N	14.0031 (99.63%) 15.0001 (0.37%)	14.0067	14.0031
O	15.9949 (99.76%) 16.9991 (0.038%) 17.9992 (0.20%)	15.9994	15.9949
Sum (M or neutral molecule)	$C_{73}H_{115}N_9O_{41}$	1774.7472	1773.7158
Sum $[M+H]^+$	$C_{73}H_{115}N_9O_{41} + [H]^+$	1775.7551	1774.7236

Table 5. RapiFluor-MS labeled FA2 glycan average mass and monoisotopic mass comparison.

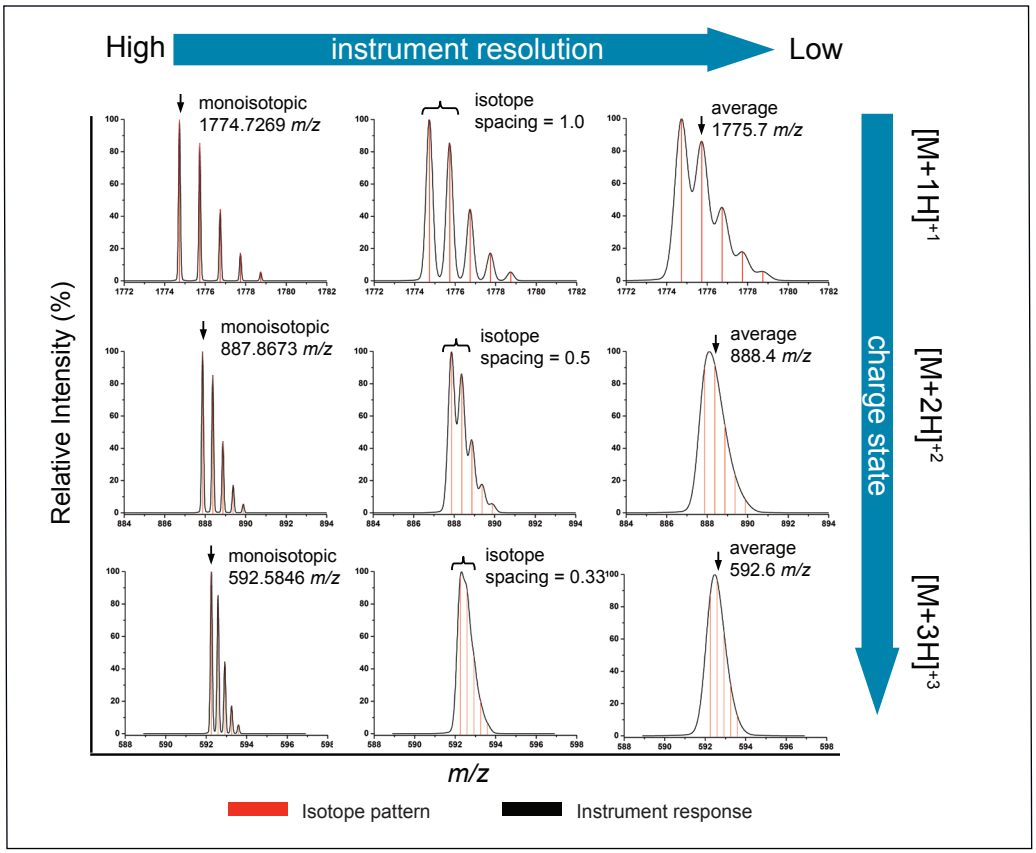
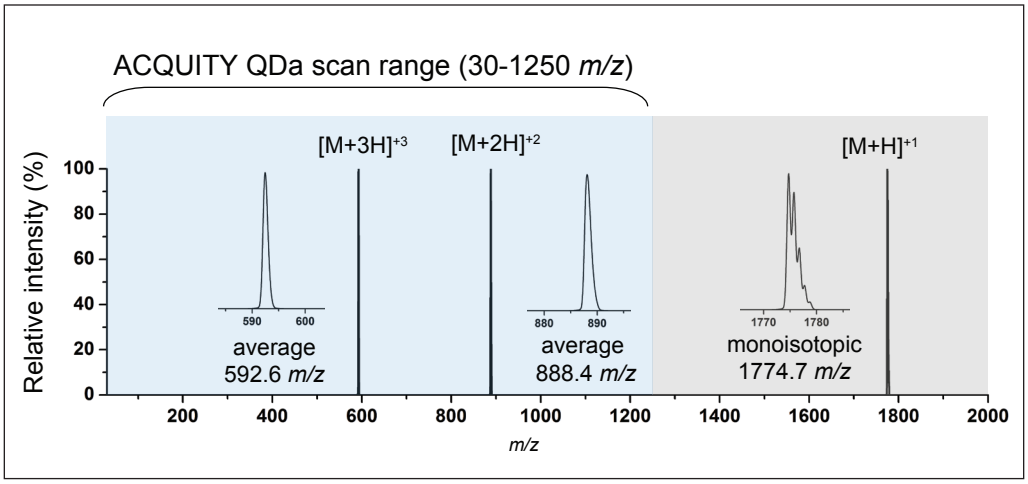


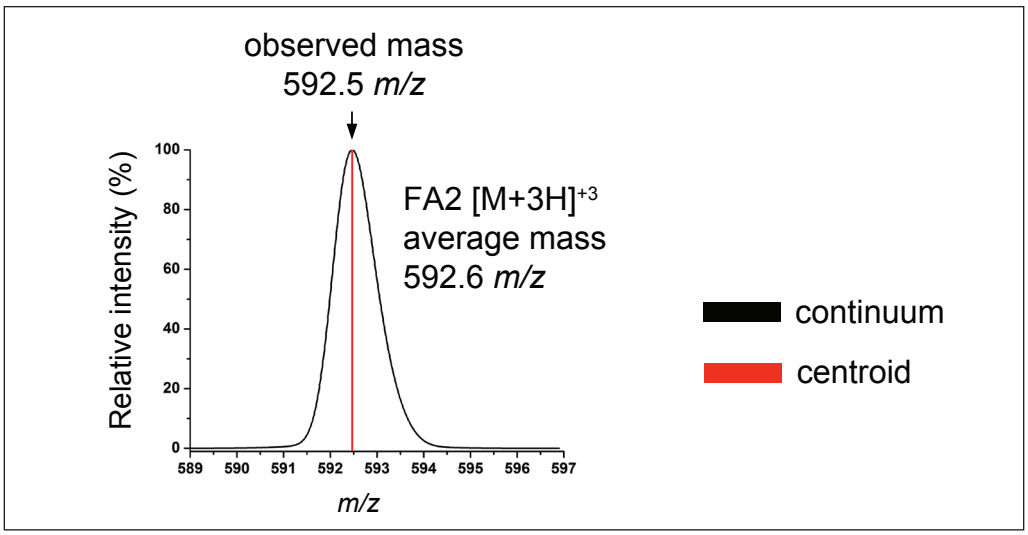
Figure 5. MS instrument trends. Factors such as instrument resolution and charge state will impact the MS profile. High-resolution instruments are capable of resolving isotopes of molecules such as the FA2 glycan with the observed m/z value based on the monoisotopic mass (or neutral mass) of the molecule, as illustrated in the first column of graphs. The ability to resolve isotopes is also affected by the charge state of the molecule. As shown in the second column of graphs, the isotope spacing is inversely proportional to the charge state. As a result large molecules such as peptides and glycans – which generally are observed with more than one charge state as illustrated in the third column of graphs (e.g. $[M+2H]^{+2}$ and $[M+3H]^{+3}$) – have an observed m/z value based on the average mass of the neutral molecule when using instruments with lower resolving power. The ACQUITY QDa is capable of detecting isotopes of molecules that are singly charged, $[M+H]^{+1}$, and that exhibit a mass within the scan range of the ACQUITY QDa.

Note: Data simulated for illustrative purposes.



Note: Multiple charge states were simulated with equal relative abundance for illustrative purposes.

Figure 6. Scan range, units, and charge state. The ACQUITY QDa mass scale is m/z and not Daltons. For singly-charged species ($[M+H]^{+1}$) that have an m/z value within the ACQUITY QDa scan range, the observed m/z value corresponds to the monoisotopic mass (highest peak in the scan). For larger molecules such as glycans, it is common to see higher charge states such as $[M+2H]^{+2}$ and $[M+3H]^{+3}$, therefore a series of progressively smaller m/z peaks can be observed corresponding to the same molecule with different charge states. Due to the reduced isotopic spacing in the $[M+2H]^{+2}$ and $[M+3H]^{+3}$ forms, isotopic resolution is not achieved and the observed m/z value is based on the average mass of the neutral molecule.



Note: Data simulated for illustrative purposes.

Figure 7. Continuum vs. centroid. Continuum data shows a full profile of the distribution of ions present in a spectrum. Centroid data (or stick data) is continuum data processed to display a single centered point for each distribution of ions in a spectrum. Large molecules such as glycans, which often are observed with more than one charge state, have an observed mass closer to the average mass.

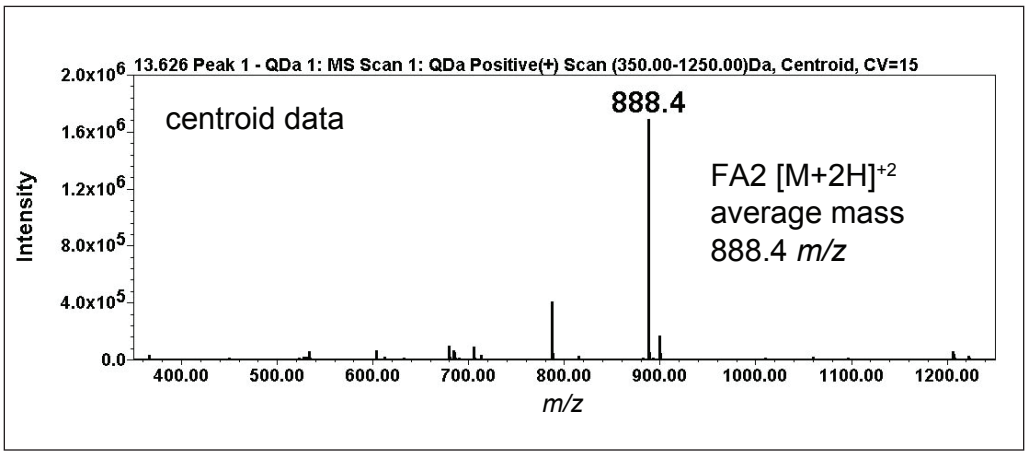


Figure 8. Centroid data of RapiFluor-MS labeled FA2 glycan. Factors such as the chemistry of the analyte and method conditions will impact relative abundance and the number of observed charge states for a given molecule.

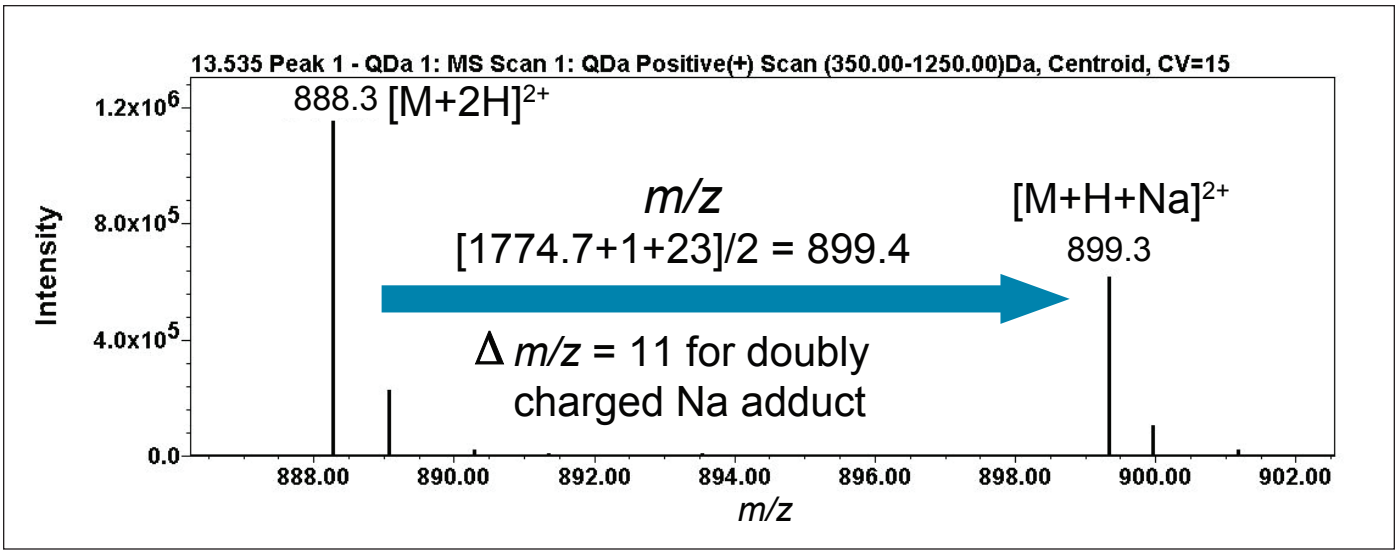


Figure 9. Adduct ions. Adduct ions are formed by the association of metal ions, or other species contained in the eluent, with analytes in the ESI source. The formation and abundance of the adduct species depends on the chemistry of the analyte and mobile phase components. Adduct formation can increase with lower quality solvents.

		m/z difference
Common positive ion adducts	*m/z* observed	$[M+H]^+$
$[M+H]^+$	M+1	0
$[M+NH_4]^+$	M+18	+17
$[M+Na]^+$	M+23	+22
$[M+K]^+$	M+39	+38
Common negative ion adducts	*m/z* observed	$[M-H]^-$
$[M-H]^-$	M-1	0
$[M+Cl]^-$	M+35	+36
$[M+HCOO]^-$ (formate)	M+45	+46
$[M+CH_3COO]^-$ (acetate)	M+59	+60

Table 6. Common adducts and where they exist in a spectrum. Predicting singly charged metal adduct m/z values for Rapifluor-MS labeled glycans when acquiring data in positive mode can be achieved by adding the adduct to the neutral peak, M. The m/z difference is calculated as ($[M + H]^+$ - $[M + adduct]^+$). For more complex adduct formations such as adduct(s) occurring on multiply charged species a derivative of the algebraic formula as shown in Figure 9 can be used to predict the m/z value.

Best practices for improving performance in glycan analyses using the ACQUITY QDa Detector

Successful performance of separations of *Rapi*Fluor-MS labeled N-linked glycans using the ACQUITY QDa relies on several key aspects including sample preparation, system settings, as well as quality of reagents and system cleanliness working in a synergistic manner. The following discussion points illustrate how to identify and improve potential areas that can impact data quality in *Rapi*Fluor-MS labeled glycan analysis using the ACQUITY QDa.

Labeling technology and standard preparation

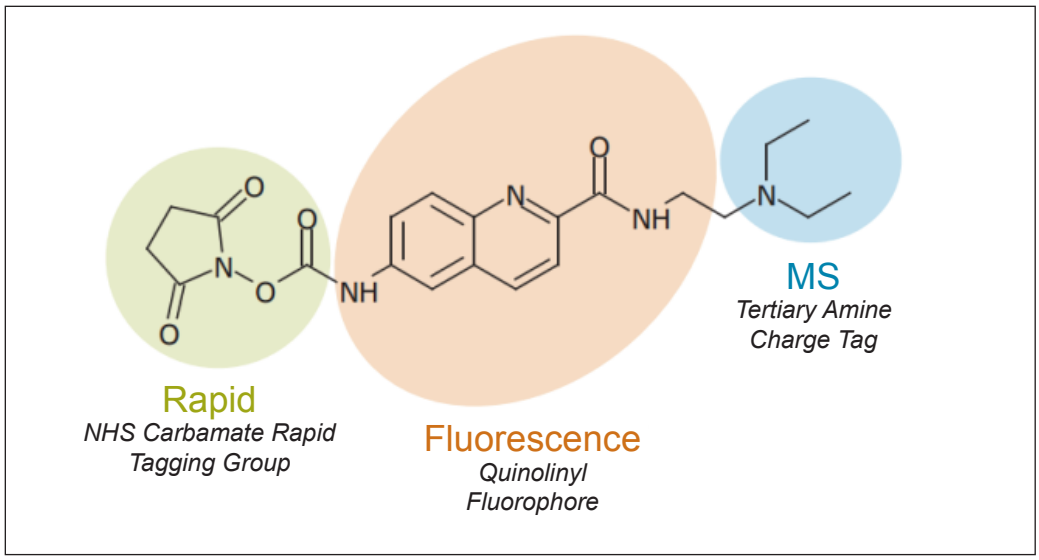


Figure 10. RapiFluor-MS molecular structure. Features of the chemical structure that enable rapid tagging, efficient fluorescence, and enhanced ionization efficiency are highlighted. This technology enables unprecedented fluorescent and mass spectrometric performance for glycan detection while also improving the throughput of N-glycan sample preparation. The tertiary amine charge tag enables labeled glycans to be observed in higher charge states when compared to alternative labeling technologies such as 2-AB, allowing for detection with the ACQUITY QDa (P/N 720005352EN). Detection of low abundant species such as the FA2BG2S2 species (peak 19, Figure 4) may require higher mass-loads on-column for adequate detection using the ACQUITY QDa. A mass load of 40 pmol is recommended as a starting point for method development of glycan monitoring assays using the ACQUITY QDa, however more or less may be used based on application needs. A calculation is provided for theoretical yields using the GlycoWorks™ RapiFluor-MS N-Glycan Kit (P/N 176003606):

$$1.5\ x\ 10^7 pg\ IgG \times \frac{1\ pmol}{150{,}000\ pg} \times \frac{2\ pmol\ glycan}{1\ pmol\ IgG} = 200\ pmol$$

Note: This calculation is based on the assumption that: the sample of Intact mAb was 15 µg, that the mAb has a molecular weight of 150 kDa, and that there are only two N-glycans per one mAb. For increased working concentration, samples may be pooled, dried down, and re-constituted in smaller volumes.

Chromatography considerations

Sample preparation can impact chromatography due to poor loading or inability of the analyte to adsorb efficiently onto the stationary phase at the head of the column upon injection. In addition, when using HILIC chromatography, it is imperative that the column is properly conditioned (P/N 715004903, p 9). When the column is first received, flush in 6% acetonitrile: 40% aqueous (or initial starting conditions) for 50-column volumes. Equilibrate with 20-column volumes of initial mobile-phase conditions before making first injection. Equilibrate with 8 to 10-column volumes between injections. Failure to appropriately equilibrate the column could result in drifting retention times. Furthermore, if the sample is sitting in the autosampler for too long, the organic may evaporate in the vial and one could then see solvent effects in the form of frontal tailing on peaks.

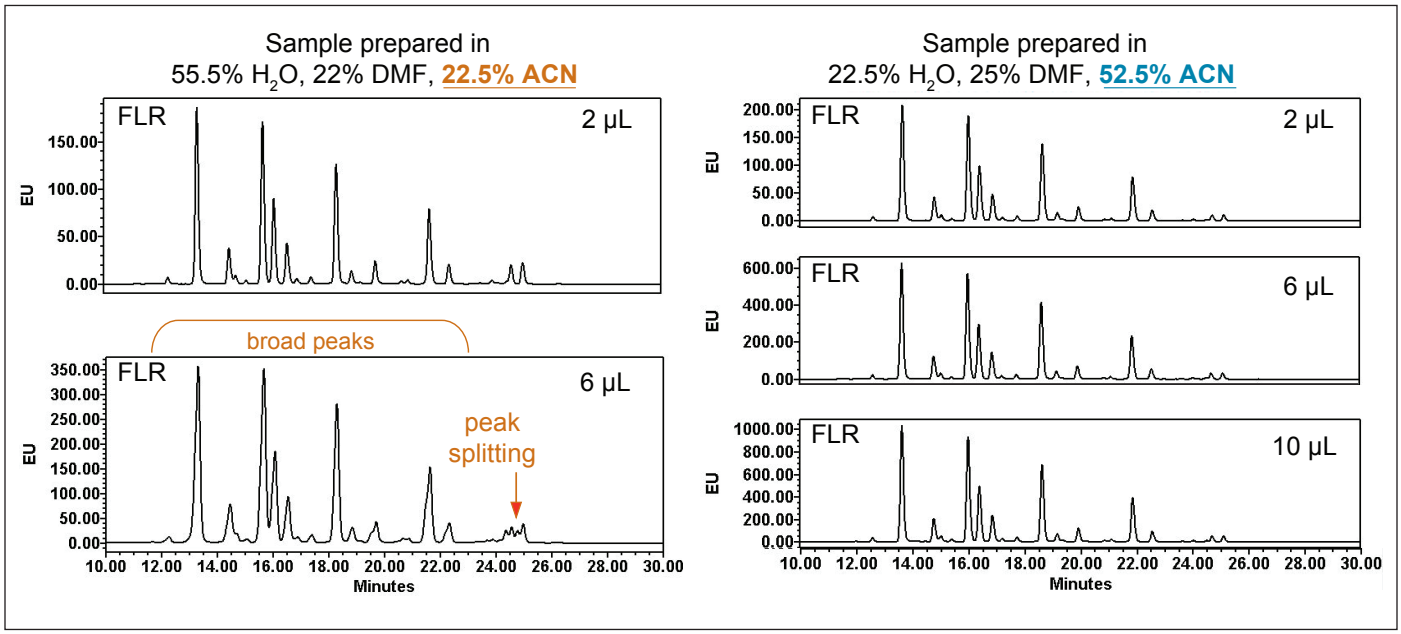


*Figure 11. Sample Composition. Glycan samples prepared in lower than the recommended organic composition (55.5% H_2O, 22% DMF, **22.5% ACN**) are not suitable for large injection volumes on low-dispersion systems due to insufficient time to allow for mixing of the injection plug and mobile phase. Reduced chromatographic performance such as broader peaks with lower detector response – and in extreme cases, peak splitting – are observed due to poor focusing on the column at injection. Samples prepared in the recommended 22.5% H_2O, 25% DMF, **52.5% ACN** indicate a high degree of method repeatability with negligible shifts in retention times and no peak splitting at higher injection volumes. The improved chromatographic performance afforded by optimal sample compositions allows for higher injection volumes for increased FLR and ACQUITY QDa Detector response.*

System performance

System performance, system cleanliness, and reagent quality can impact separation of *Rapi*Fluor-MS labeled glycans. System maintenance, reagent quality, and system history (e.g., salt use) are key factors to consider if non-ideal performance is observed with respect to method repeatability and salt adducts. FLR responses should be proportional to mass load; however, absolute emission units (EU) may vary based on calibration and should be kept in mind when comparing data.

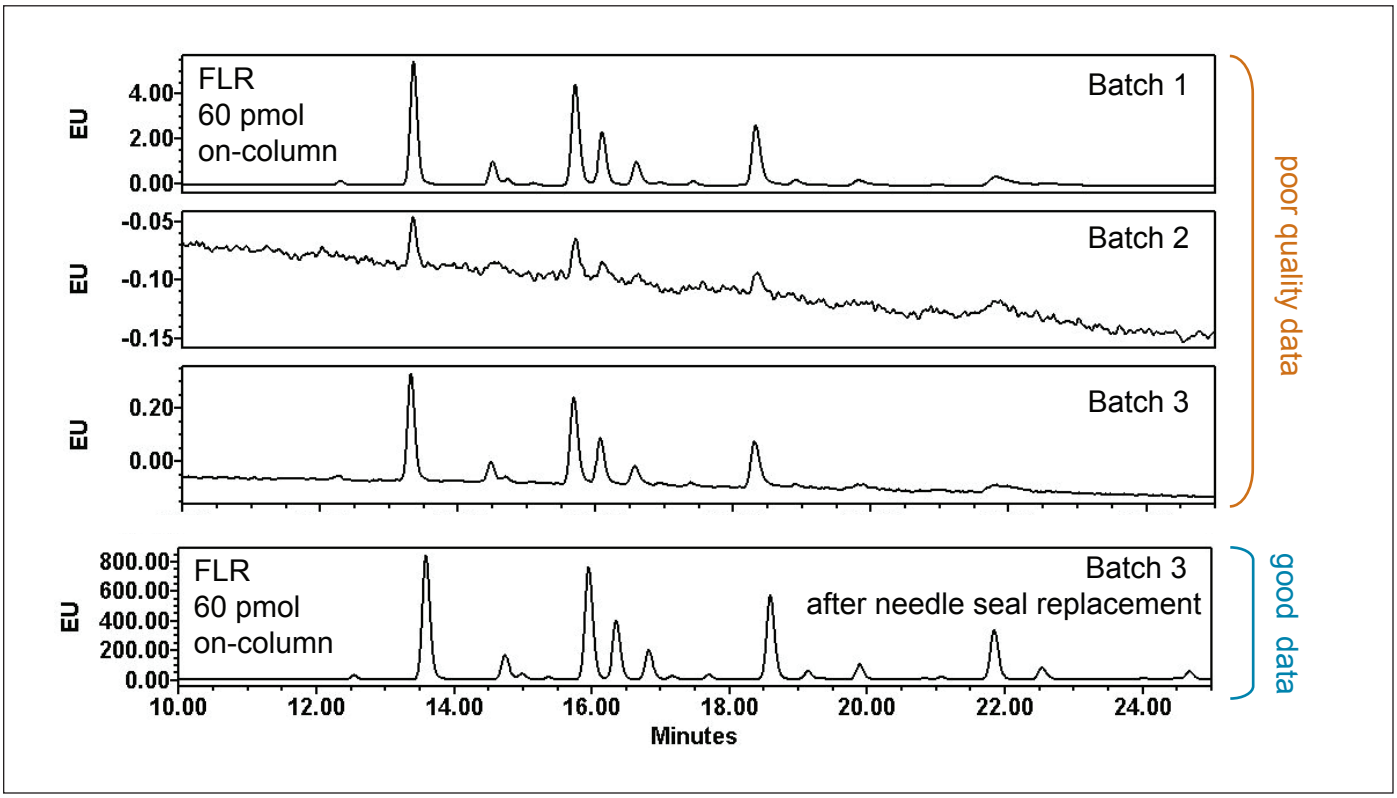


Figure 12. System performance – system readiness. Three different batches of the RapiFluor-MS standard were prepared with the recommended composition (22.5% H_2O, 25% DMF, 52.5% ACN). Serial injections with the same sample load were performed for each standard. The inconsistency in injection repeatability is apparent from the variation in FLR detector response and broad peaks in later eluting RapiFluor-MS labeled glycans. Seal tests were performed via the console for the pump and needle, the results of which indicated the needle seal was failing. After replacement of the needle and needle seal, the FLR response and chromatography performance increased to acceptable levels. Regular system maintenance is recommended for optimum performance and detection of RapiFluor-MS labeled glycans.

System contaminants

Liquid chromatography (LC) systems in a typical lab setting are often used interchangeably for different MS-compatible chromatography techniques such as RPLC and HILIC. In extreme cases, the same LC system may be used with optical detection only for techniques such as SEC and IEX that use aqueous-based separations incorporating MS-incompatible salt buffers. In the case of non-dedicated systems such as these, special attention has to be taken to clean the system properly in order to remove potential contaminants that can impact MS sensitivity when reconfiguring systems for a new method. The following table is adapted from the Waters LC-MS cleaning protocol document (P/N 715001307) to provide some recommended cleaning mixtures for the removal of contaminants.

	LC Mixture 1[a]	LC Mixture 2[a]	LC Mixture 3[a]
Purpose	General purpose solution for applications where use of high-pH mobile phase is not advisable	"Universal" wash solution for high background spectra	Strong acid wash
Caution			Use as a last resort Do not use in seal-wash lines Remove reservoir sinkers before cleaning with phosphoric acid After cleaning with acid (or base), flush with ultrapure water until the pH is neutral (~pH 7) before connecting to a detector
Mixture	100% 2-propanol (isopropyl alcohol, or IPA)	25% acetonitrile 25% methanol 25% 2-propanol 25% water 0.2% formic acid	30% phosphoric acid 70% water

a. Do not introduce any cleaning solutions into the MS system or column.

Table 7. Controlling contamination in LC-MS systems. To clean contamination in LC systems, use the highest purity solvent mixtures. Do not introduce any cleaning solutions into the MS system (ACQUITY QDa) or column. If you know what the contaminant is, use the mixture in which it is most soluble. Flush the system component with the selected cleaning mixture, rinse with 50% acetonitrile or mobile phase to remove the cleaning solution, test for contamination, and repeat the procedure until the background is down to an acceptable level. If you use mixture 3, pump ultrapure water through the system until the pH is neutral (about pH=7).

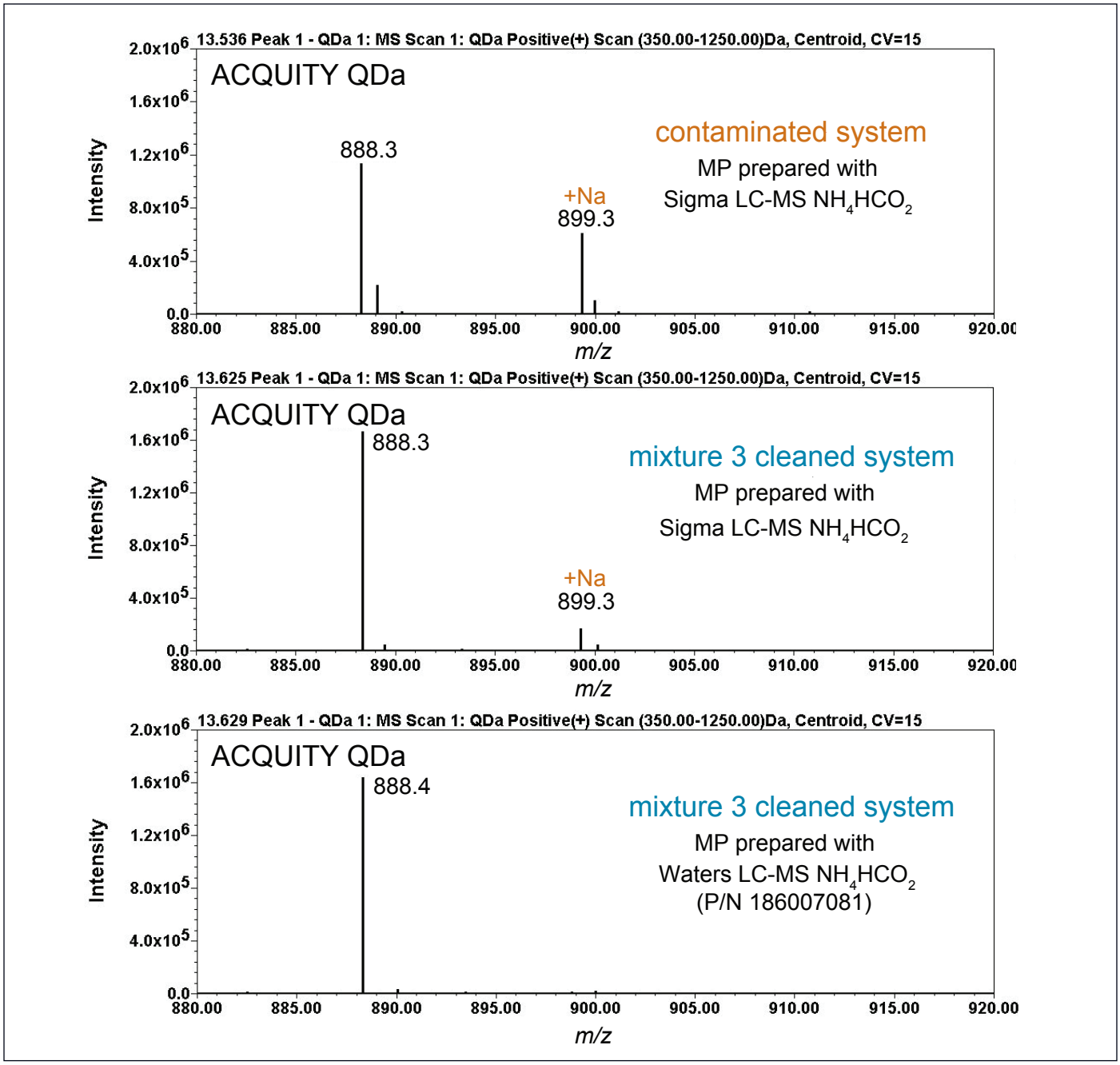


Figure 13. System performance – salt contamination. Adduct formation is commonly encountered in MS-based analyses. Salt adducts can reduce instrument sensitivity and impact reported m/z values. This is of particular importance when using SIRs because adducts will result in a degree of ions not being detected, reducing method sensitivity. In this example, the sample and mobile phase were prepared in LC-MS grade reagents, as per the protocol, ruling out the possibility of poor quality reagents. The system was previously utilized for aqueous-based techniques using buffers of modest ionic strength. Cleaning mixture 3 was implemented to remove residual alkali metal salts from the systems flow path. The Na^+ adduct is significantly reduced in the cleaned system using the same buffer. For difficult to remove contaminants, a more rigorous cleaning protocol can be found in the Amino Acid Analysis Systems guide located on the Waters website (P/N USRM134761474, p 42-44).

Purity of reagents is a key aspect in reducing adduct formation in MS-based analyses. MS grade reagents and solvents should always be used for glycan analysis using the ACQUITY QDa. Waters provides a convenient 10X solution of MS grade ammonium formate concentrate (P/N 186007081) with which transfer of solvent to additional glassware is not required. This concentrate provides equivalent performance when compared to mobile phases meticulously prepared with commercially available LC-MS grade ammonium formate as shown in Figure 13.

ACQUITY QDa settings (intensity and fragmentation)

For ease-of-use, the ACQUITY QDa has limited parameters for optimization. However, the three settings available for analysts to adjust (i.e. cone voltage, capillary voltage, and probe temperature) can impact MS spectrum quality, especially in the analysis of glycans. Method adjustment may be needed on an application basis based on instrument performance. The following observations of the ACQUITY QDa settings impact on the analysis of *Rapi*Fluor-MS labeled glycans are provided for reference.

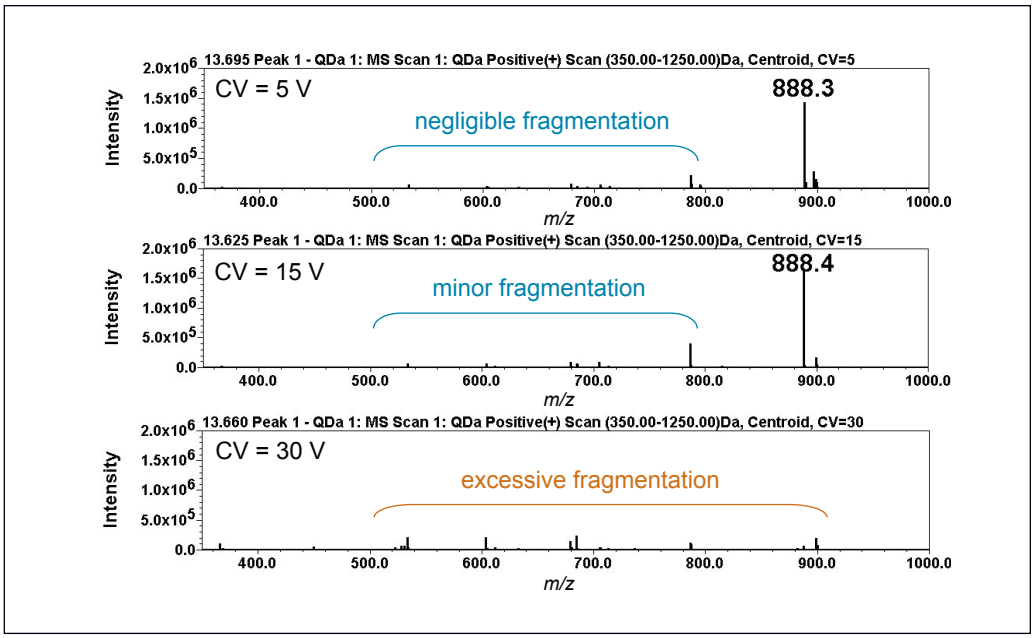


Figure 14. Cone voltage. Peaks observed at a lower m/z value to the analyte can correspond to fragments of the analyte generated in the source. Higher proportions of fragment ions are observed at higher cone voltages as more energetic collisions occur in the source. Excessive fragmentation can lead to reduced sensitivity of the method as shown in the signal decrease of the parent peak of the FA2 $[M+2H]^{+2}$ species (m/z = 888.4).

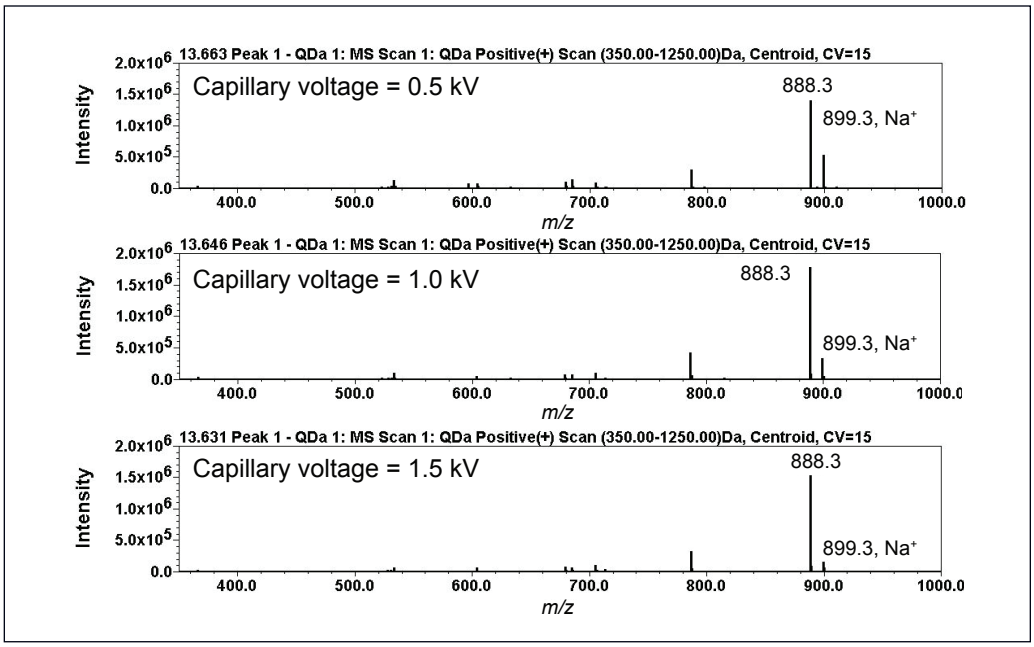


Figure 15. Capillary voltage. Capillary voltage impact on fragmentation is negligible. However, higher capillary voltages can reduce adduct formation as noted by the decrease in the FA2 $[M+1H+Na]^{+2}$ species (m/z = 899.3), therefore increasing signal intensity of the $[M+2H]^{+2}$ peak.

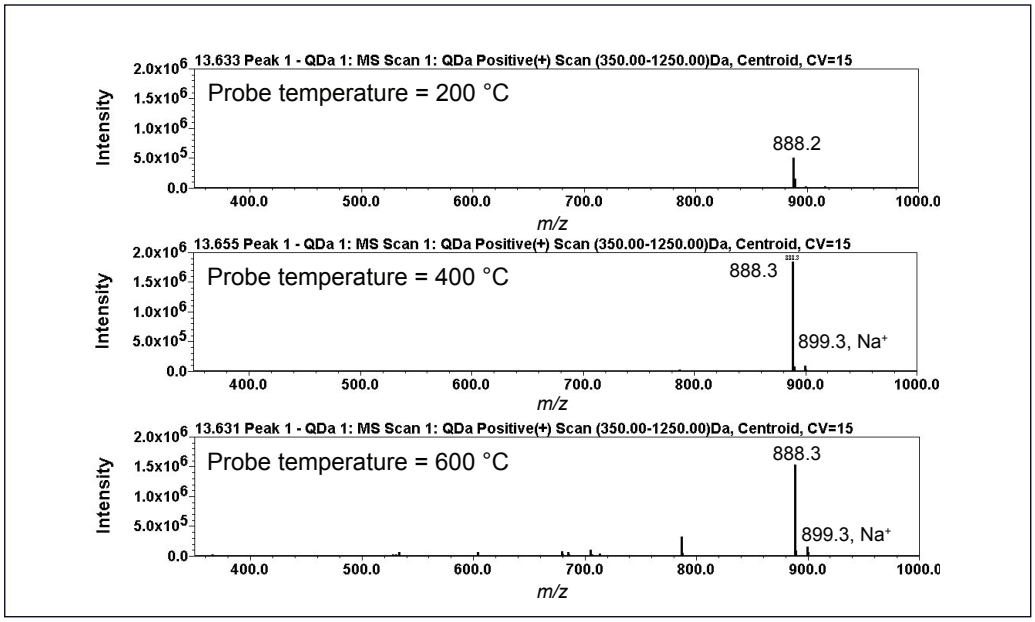


Figure 16. Probe temperature. Probe temperature can be adjusted to reduce fragmentation at the cost of sensitivity as noted by the lower intensity of the FA2[M+2H]$^{+2}$ (m/z = 888.3) with a lower probe temperature. Probe temperature has been observed to cause significant fragmentation in certain scenarios in the analysis of RapiFluor-MS labeled glycans using the ACQUITY QDa. It is recommended to use a probe temperature of 400 °C as a starting point and adjust the method accordingly if significant fragmentation is observed. Adduct formation for the FA2 [M+1H+Na]$^{+2}$ species (m/z = 899.3) was not observed to be significantly impacted by probe temperature.

Detection method:

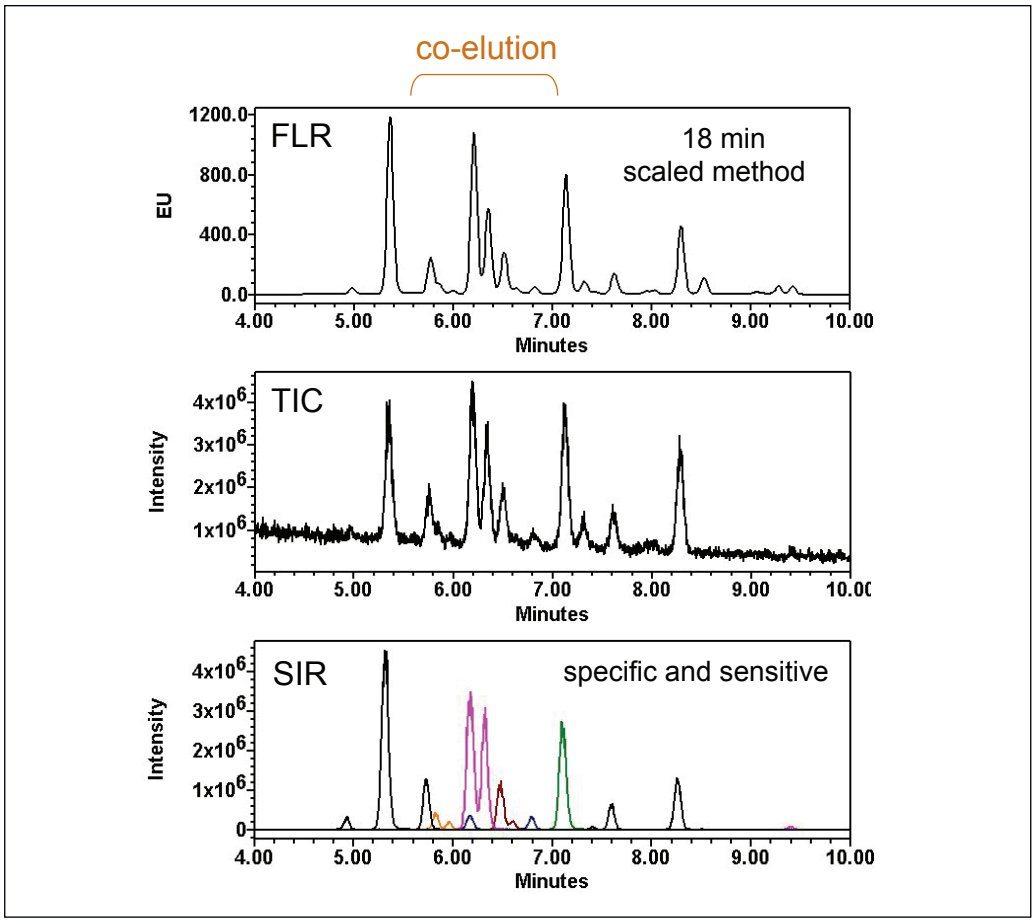


Figure 17. Detection method. Shorter columns can be used for higher throughput methods. Scaling the 55-minute method using the 150 mm column to an 18-minute method using a 50 mm column allows for shorter run times with minimum loss of resolution (P/N 720005438EN). Co-elution of peaks can be addressed through the use of SIRs. Inclusion of SIRs into methods allow for maximum specificity and sensitivity for accurate integration and identification of co-eluting or partially co-eluting peaks.

Data processing

Processing data and the type of processing can impact data quality. Processing data options, use of smoothing, as well as noise reduction processing should be considered with the application in mind when reviewing data or designing report templates. **It is highly recommended to use the FLR channel for integration and data review in the mass analysis window.** The following processing observations are provided for reference.

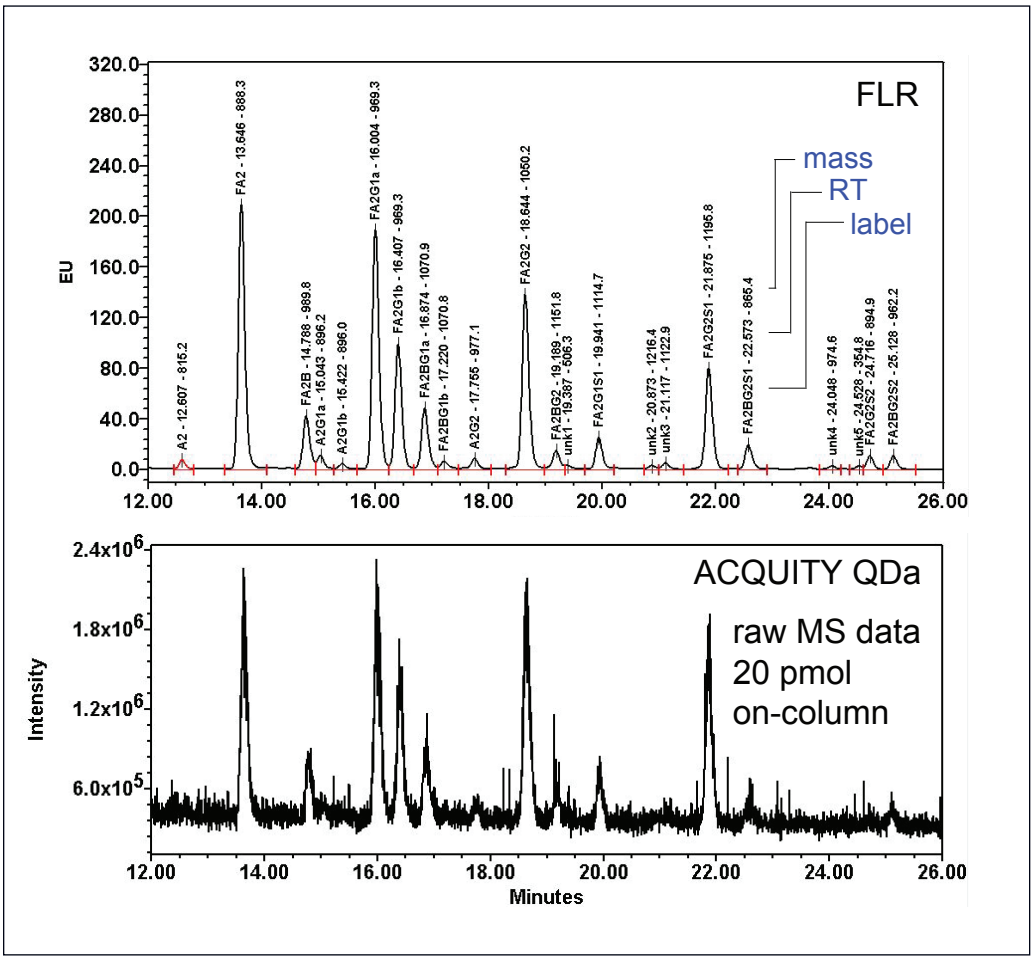


Figure 18. Processing method. A 20 pmol mass load was used to enhance observed noise in the MS spectrum. As recommended, the FLR channel was processed in the method for detection, identification, and mass assignment of previously characterized glycans (top panel). As shown, a 20 pmol mass load provided sufficient spectral quality for correct mass assignment for the RapiFluor-MS labeled glycans shown in the FLR chromatogram. The processing method combines mass spectrum corresponding to the FLR peak width in the raw MS channel to determine the mass of the RapiFluor-MS labeled glycan without the need for additional signal processing.

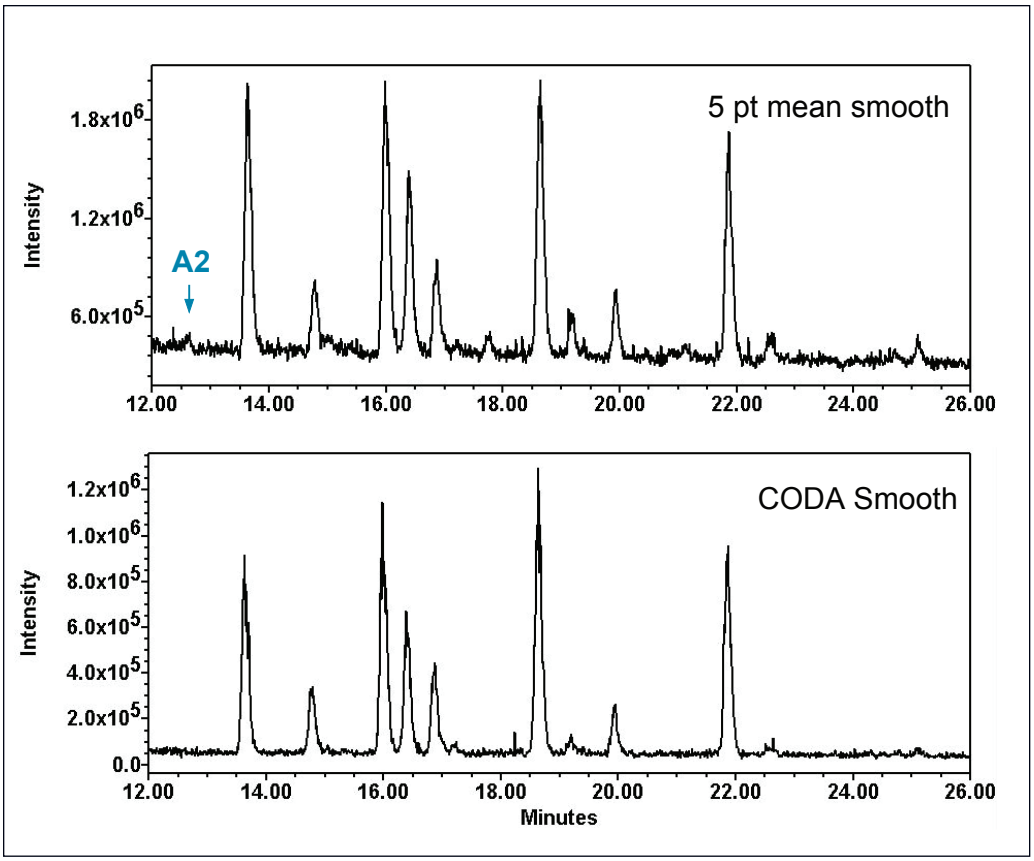


Figure 19. Chromatogram smoothing. Smoothing can impact the look and integrity of the MS spectrum depending on the type of smoothing used. Mean and Savitsky-Golay smooths are often used to reduce noise in the MS spectrum in order to facilitate improved integration while preserving MS raw spectrum data associated with the peak. A five-point mean smooth was used to smooth noise in the MS spectrum to improve integration of low SNR peaks as observed with the A2 peak (815.3 m/z) eluting at 12.60 minutes as shown in the top panel.

Noise reduction algorithms such as CODA (Component Detection Algorithm) – which can be activated when setting up a processing method within the Empower® Software project – filter for removal of background noise and spikes from chromatograms. As shown in the bottom panel of Figure 19, the noise in the spectrum is significantly reduced compared to the raw spectrum. However, signal intensity has dropped as data has been removed from the spectrum. This is more apparent for the A2 peak (815.3 *m/z*) eluting at 12.60 minutes where the peak has been "smoothed out" in the processed data. Alternatively, 3D subtraction can be applied to spectrums to improve SNR of low intensity peaks (data not shown). **Processing data options, use of smoothing, as well as noise reduction processing should be evaluated on an application-need basis.**

CONCLUSION

Sample preparation, system performance, quality of reagents, and MS settings work in a synergistic manner that can impact the analysis and monitoring of *Rapi*Fluor-MS labeled N-linked glycans using the ACQUITY QDa. A degree of method optimization is not unexpected when incorporating new technologies, and preferably can be quickly adapted and readily deployed to meet the demands of the biopharmaceutical industry. Identification and impact of key challenges in LC-MS-based analyses were demonstrated to offer guidance in efficient method development for improved productivity in the analysis of *Rapi*Fluor-MS labeled N-linked glycans using the ACQUITY QDa.

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