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Amide-Bonded BEH HILIC Columns for High Resolution, HPLC-Compatible Separations of N-Glycans

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Abstract

In the following work, we demonstrate that BEH Glycan Columns packed with 1.7, 2.5, and 3.5 µm particle sizes afford scalability between glycan separations performed under UPLC and HPLC-compatible conditions.

Benefits

- Proven stationary phase for labeled glycan separations available in HPLC-compatible particle sizes (2.5 µm *XP* and 3.5 µm) and column diameters (2.1 and 4.6 mm ID)
- Noteworthy resolution with HPLC, albeit with analysis times longer than UPLC
- Consistent glycan profiles and GU values upon LC method transfer between different particle size columns
- BEH Glycan stationary phase is QC tested via separation of human-like, 2-AB labeled N-glycans to ensure consistent batch-to-batch column performance

Introduction

With little dispute, it is recognized that the glycosylation of biotherapeutics must be thoroughly and routinely characterized, since changes in glycan profiles can affect efficacy and immunogenicity or be indicative of manufacturing process instability.[1] In a common characterization approach, N-linked glycans from glycoproteins are released enzymatically, labeled with a fluorescent tag, subsequently separated by hydrophilic interaction chromatography (HILIC), and detected via fluorescence (FLR).[2-9]

The ACQUITY UPLC BEH Glycan Column, packed with sub-2-µm particles, has proven to be an enabling technology for the HILIC-based separation of these labeled glycans. With an optimized amide-bonded ethylene bridged hybrid (BEH) HILIC stationary phase, this column provides faster and higher resolution separations than alternative HPLC-based columns.[10]

However, many laboratories have not yet fully transitioned to UPLC technology. To enable them access to this amide-bonded BEH Technology, HPLC-compatible particle size (2.5 µm *XP* and 3.5 µm) columns are now available with the proven BEH glycan stationary phase.

In the following work, we demonstrate that BEH Glycan Columns packed with 1.7, 2.5, and 3.5 µm particle sizes afford scalability between glycan separations performed under UPLC and HPLC-compatible conditions. Using standard LC method transfer principles accounting for differences in particle diameter (d_p), we additionally show that noteworthy resolution can be achieved with the larger particle size columns at HPLC-compatible pressures, albeit with an increase in analysis time. Lastly, the following work demonstrates that highly similar glycan profiles and experimental glucose unit (GU) values are obtained, no matter the particle size or LC system employed.

Experimental

Method conditions (unless otherwise noted)

LC conditions

LC systems:	ACQUITY UPLC H-Class Bio System/Alliance 2695 HPLC
Detection:	ACQUITY UPLC FLR Detector/2475 FLR Detector
Excitation:	330 nm
Emission:	420 nm
Scan rate:	10 Hz
Time const.:	0.2 sec
Gain:	1.00
Column:	ACQUITY UPLC BEH Glycan, 1.7 µm, 130Å, 2.1 x 150 mm p/n 186004742) XBridge BEH Glycan, 2.5 µm *XP*, 130Å, 2.1 x 150 mm (p/n 186007265) XBridge BEH Glycan, 3.5 µm, 130Å, 2.1 x 150 mm (p/n 186007504) 100% Silica-based, HILIC Amide Bonded, 3 µm, 80 Å, 2.0 x 150 mm (Competitor product)
Column temp.:	60 °C
Sample temp.:	15 °C
Injection volume:	2.5 µL (Glycan Performance Test Standard + A3 Mixture), 2.0 µL (Glycan Performance Test

LC conditions	
	Standard), 1.0 µL (Dextran Calibration Ladder Standard)
Flow rate:	0.5 mL/min (0.25 mL/min for the highly aqueous regeneration step in the gradient)
Mobile phase A:	100 mM ammonium formate, pH 4.4
Mobile phase B:	Acetonitrile (ACN)
Vials:	LC/GC Certified Clear Glass 12 x 32 mm Screw Neck Qsert Vial (p/n 186001126C)
Gradient*:	Curves styles were linear.
Data Management:	UNIFI (v1.6) Waters Empower Pro (v2)

For a 1.7 µm, 2.1 x 150 mm column

Time(min)	%A	%B	Flow rate(mL/min)
0	22.0	78.0	0.5
38.5	44.1	55.9	0.5
39.5	80.0	20.0	0.25
44.5	80.0	20.0	0.25

Time(min)	%A	%B	Flow rate(mL/min)
46.5	22.0	78.0	0.5
50	22.0	78.0	0.5

**This gradient should not be used when aiming to assign identifications by means of the NIBRT GlycoBase.[6] For best agreement between experimental and database GU values, the method NIBRT has used for generating GU values should be employed.*

For a 2.5 µm, 2.1 x 150 mm XP column

Time(min)	%A	%B	Flow rate(mL/min)
0	22.0	78.0	0.34
56.62	44.1	55.9	0.34
58.09	80.0	20.0	0.17
65.44	80.0	20.0	0.17
68.38	22.0	78.0	0.34
73.53	22.0	78.0	0.34

For a 3.5 µm, 2.1 mm x 150 mm column

Time(min)	%A	%B	Flow rate(mL/min)
0	22.0	78.0	0.24
79.26	44.1	55.9	0.24
81.32	80.0	20.0	0.12
91.62	80.0	20.0	0.12
95.74	22.0	78.0	0.24
102.94	22.0	78.0	0.24

Sample description

Glycan Performance Test Standard (p/n 186006349) was mixed with 2-AB (2-aminobenzamide) labeled trisialylated A3 glycans (ProZyme) in water to make a solution of 3 pmol/µL. Aliquots (10 µL) of this mixture were diluted with 15 µL of ACN prior to injection.

For the evaluation of method transfer from a UPLC to an HPLC, Glycan Performance Test Standard (p/n 186006349) was reconstituted in 200 µL of 50:50 mobile phase A/mobile phase B to make a solution of 1.14 pmol/µL.

Dextran Calibration Ladder Standard (p/n 186006841) was reconstituted in 100 µL of Milli-Q water. This aliquot was then mixed with an equal volume of ACN prior to injection.

Calculations

Peak capacities for each separation were calculated by measuring peak widths for species 1 through 16. Species 8 and 9 co-eluted in some separations and were therefore not included in the calculations. Peak widths were measured from the FLR chromatograms at peak half-height ($W_{half\text{-}height}$), averaged, and converted to peak capacities according to the following equation. To be rigorous among the comparisons, the gradient time in

these calculations was defined as the difference in retention time of the first and last eluting component of the studied glycan mixture. Peak capacities were also calculated, in a similar manner, based on species 1 through 14 for the evaluation of method transfer from UPLC to HPLC instrumentation.

$$P^{*}_{c,half\text{-}height} = 1 + \left[\left(\frac{\Delta RT_{1,16}}{W_{half\text{-}height,avg}}\right)\right]$$

Results and Discussion

Performance of HILIC glycan columns varying in particle size at the same linear velocity

The performance of columns packed with BEH-based, amide-bonded HILIC stationary phase of varying particle sizes was evaluated for separations of released and fluorescently labeled N-glycans. A test mixture for this purpose was prepared by combining the 2-AB (aminobenzamide) labeled N-glycans of the Glycan Performance Test Standard, which contains 2-AB labeled N-glycans derived from pooled human serum IgG spiked with high mannose glycans (Man5 and Man6), with 2-AB labeled trisialylated A3 glycans. This is a mixture with complexity extending beyond that commonly found on human or human-like IgGs, making it challenging to fully resolve and thus an excellent probe to measure column performance.

In order to evaluate the separation capabilities of the noted columns in an unbiased manner, we first evaluated performance on a low dispersion, Waters H-Class Bio UPLC System capable of operating under both UPLC and HPLC pressures. A typical chromatogram obtained for this sample with an ACQUITY UPLC BEH Glycan 1.7 µm column and a method optimized for high resolving power is shown at the top of Figure 1. In this example UPLC separation, column pressures of up to 8700 psi were observed during the column wash step. This method could, as a result, not be implemented on an HPLC or most UHPLC instruments, given that such chromatographs are often limited to operating pressures of approximately 5,000–6,000 and 8,000–9,000 psi, respectively.

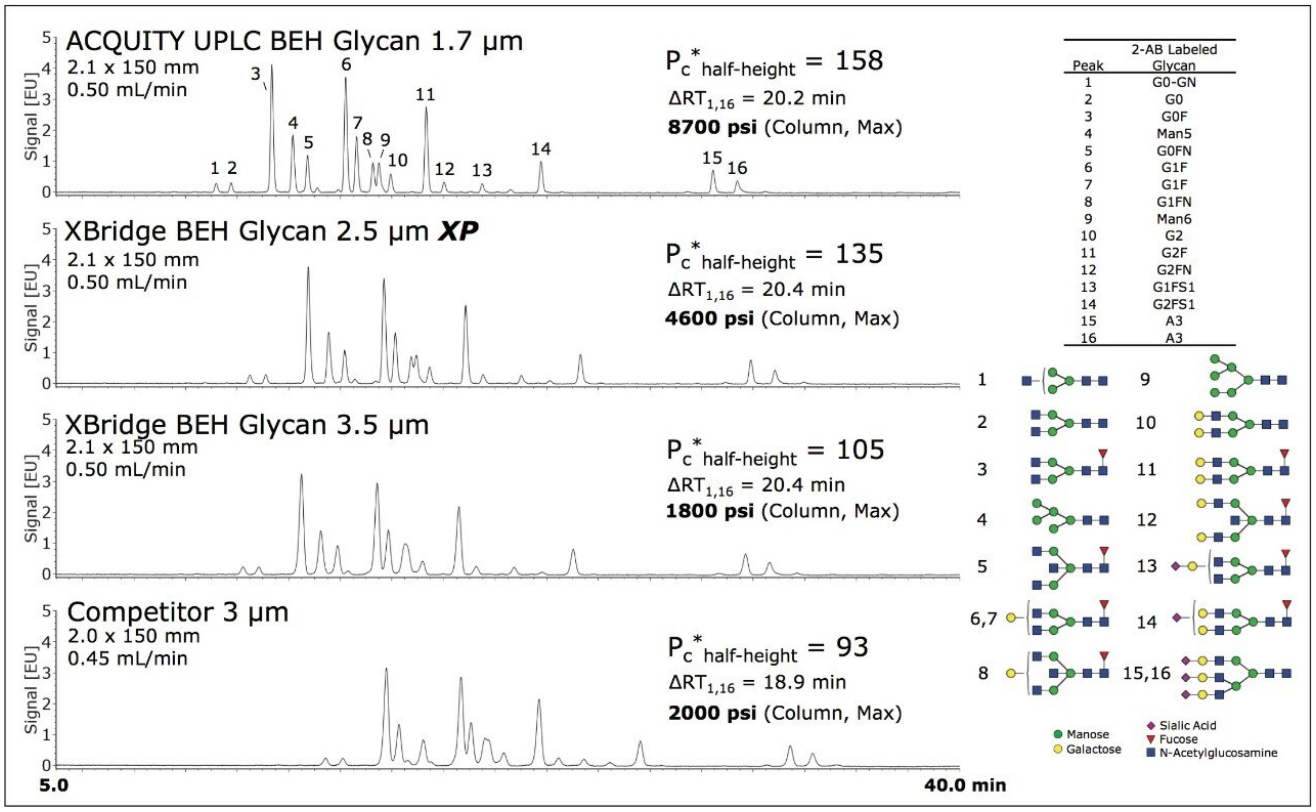

Figure 1. HILIC-FLR analysis of 2-AB labeled Glycan Performance Test Standard and trisialylated A3 glycans with columns packed with BEH-based, amide-bonded 1.7, 2.5, and 3.5 µm versus competitor's 100% silica, amide-bonded 3 µm particles. Separations were performed with an ACQUITY UPLC H-Class Bio using the same linear velocity with 3 pmol of sample injected in a volume of 2.5 µL. Peak capacities measured for each separation are shown as well as the maximum column pressures observed during column wash step.

Column pressure is inversely proportional to particle diameter.[11] HPLC-compatible methods can thereby be obtained through the use of larger particle columns, for instance those packed with 2.5 and 3.5 µm particles. Separations achieved with such columns are also shown in Figure 1. Both columns exhibited significantly lower pressures, namely 4600 and 1800 psi, making them compatible with modern-HPLC systems. At the same linear velocity, the 1.7 µm particle column yielded superior peak capacity, as a result of the enhanced kinetic efficiencies that coincide with sub-2-µm particle diameters. Peak capacities for the different BEH Glycan Columns ranged from 158 to 105, decreasing with increasing particle diameter, as would be expected. It is, however, noteworthy that all three of these BEH Glycan Columns afforded peak capacities superior to those obtained with a commonly-used, 100% silica-based, HPLC amide 3 µm particle column (competitor). Further, the

3.5 µm BEH Glycan column provides comparable, if not better, performance at a lower pressure compared to the competitor column.

In all, the peak capacities and separations demonstrated in Figure 1 underscore the advantage of being able to use and fully exploit the performance of columns packed with sub-2-µm particles. Higher peak capacities can be achieved with faster analysis times. Kinetic efficiency and amenability to faster linear velocities accompany decreases in particle diameter. Because of this, it is widely understood that in order to achieve similar peak capacities with columns packed with larger particle size diameters, it is necessary to decrease the linear velocity of the mobile phase, thus increasing the analysis time.

Scalability and HPLC-compatible separations

The highly resolving method developed for the UPLC column (1.7 µm) was scaled to methods suitable for the HPLC columns (2.5 µm *XP* and 3.5 µm) using the ACQUITY UPLC Column Calculator.[12] Linear velocities were decreased and gradient times increased by the calculated percentage difference between the differing particle size diameters (column dimensions were identical). For example, in going from the 1.7 µm to the 2.5 µm *XP* column, flow rate was decreased 1.5 fold, while the gradient was increased by the same factor, such that the change in acetonitrile per column volume remained constant. Figure 2 presents chromatograms obtained with these transferred methods. For the HPLC particle size separations, there were noticeable, up to 20%, increases in calculated peak capacities with the lower, properly scaled flow rates compared to data reported in Figure 1. Moreover, there were significant decreases in column pressure, further confirming the compatibility of the 2.5 µm *XP* and 3.5 µm columns with HPLC instrumentation.

It is worth noting that the 2.5 µm *XP* column, upon method transfer, was able to approach the peak capacity of the UPLC method (Pc 155 vs 158). As with the UPLC method, the HPLC-compatible method with the 2.5 µm *XP* column yielded significant resolution (R_S>1) between species 8 (G1FN) and 9 (Man6), which can often pose a challenge for most HILIC separations given the highly similar hydrophilicities of the two glycans. So, the 2.5 µm *XP* column, upon appropriate method scaling, is capable of noteworthy resolving power, albeit at the cost of increased analysis time when compared to true UPLC with a sub-2-µm column. As can be seen in comparing Figures 1 and 2, the 3.5 µm column also exhibited increases in peak capacity upon method transfer. The peak capacity of this column was lower than that of the 1.7 and 2.5 µm *XP* Columns (as expected), but was significantly higher than the peak capacity observed for the competitor's, silica-based, amide column containing slightly smaller, 3 µm particles (Figure 2).

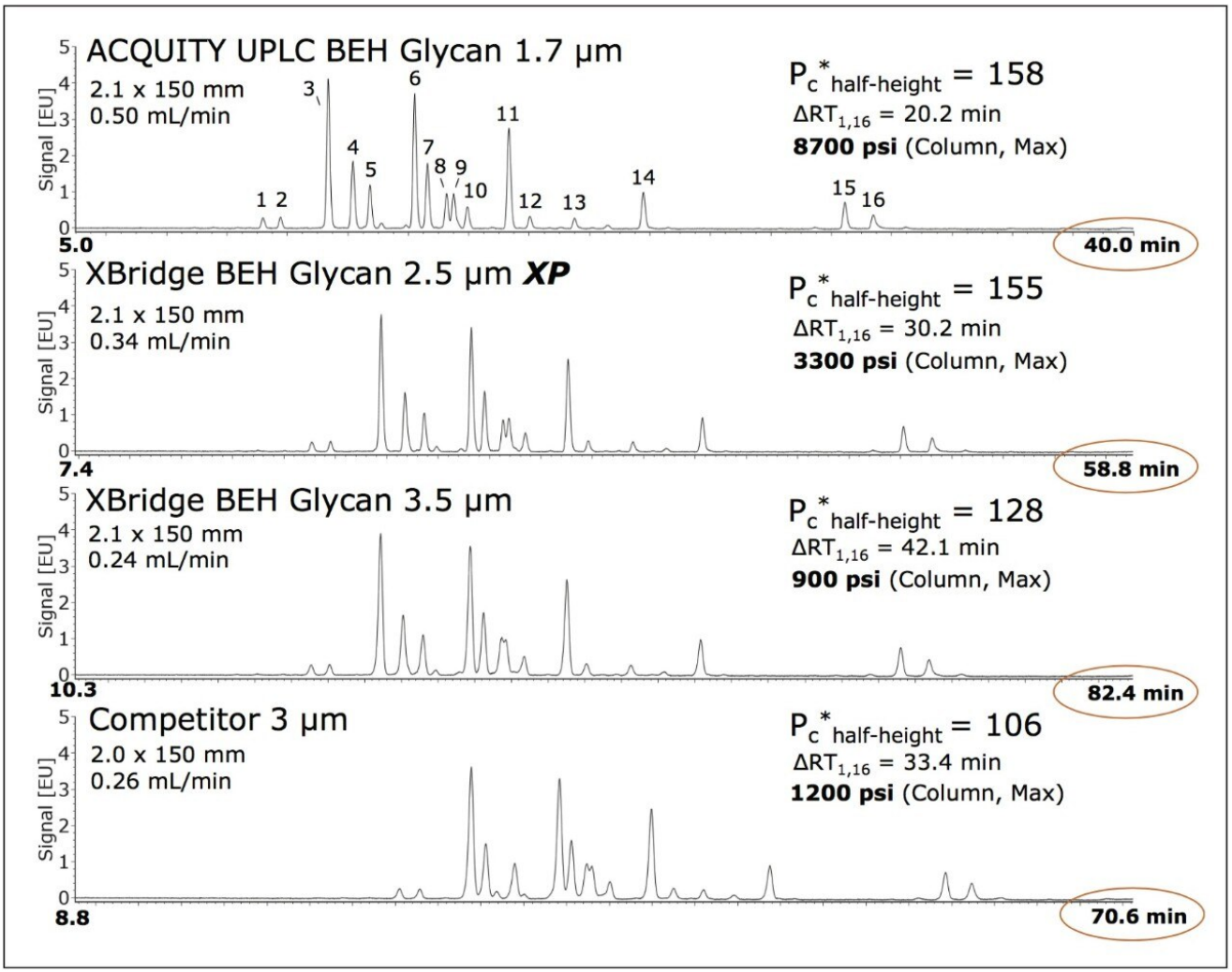

Figure 2. HILIC-FLR analysis of 2-AB labeled Glycan Performance Test Standard and trisialylated A3 glycans using an ACQUITY UPLC H-Class Bio System and Columns packed with 1.7, 2.5, 3, and 3.5 µm particles. Linear velocities and gradient times were scaled according to the change in dp according to the ACQUITY UPLC Column Calculator.

Successful method transfer among different particle sizes relies not only on obtaining similar resolving power but also maintaining comparable chromatographic selectivity. This is of utmost significance, as altered elution orders and changing chromatographic profiles would make it infeasible to develop any correspondence between data. For this reason, the separations with the different BEH Glycan Columns, shown in Figure 2, were additionally assessed in terms of comparability of chromatographic selectivity. Selectivity factors for three different sets of

peaks spread across the profile of the glycan test mixture were determined and found to be comparable for the various methods and particle sizes (Table 1).

	1.7 µm, 0.50 mL/min	2.5 µm *XP* 0.34 mL/min	3.5 µm 0.24 mL/min
α3,6	1.22	1.20	1.20
α6,11	1.20	1.18	1.18
α11,15	1.59	1.54	1.53

Table 1. Selectivity factors for the separations shown in Figure 2 that were obtained using appropriately scaled flow rates and gradient durations.
Note: Peak IDs are shown in Figure 1.

Glycan profiles and GU values

Consistency in the data derived from separations with BEH Glycan Columns packed with different particle sizes is of importance in biopharmaceutical laboratories. Most critically, these columns and their associated methods should deliver consistent determinations of a glycan profile. Figure 3 presents relative abundances determined from the separations shown in Figure 2. Very similar abundances for the separated glycan species were determined, regardless of which column had been used. The largest discrepancy in the glycan abundance values corresponded to labeled glycan species 8 and 9, where there were approximately 20% deviations in calculated results generated with columns containing the 3.5 µm versus 1.7 and 2.5 µm particles. In absolute terms, these were differences of 4.8% versus ~4.1% (peak 8) and 3.6% versus ~4.5% (peak 9). Reviewing the separations in Figure 2 gives a clear explanation for this discrepancy; resolution of species 8 and 9 was significantly poorer on the 3.5 µm column, which in turn impaired the accuracy of determining the abundances of the two species. In contrast, all other relative abundance determinations were in agreement to within 10%, indicating that this newly expanded suite of BEH Glycan Columns is indeed capable of yielding highly consistent analyses of a 2-AB labeled glycans.

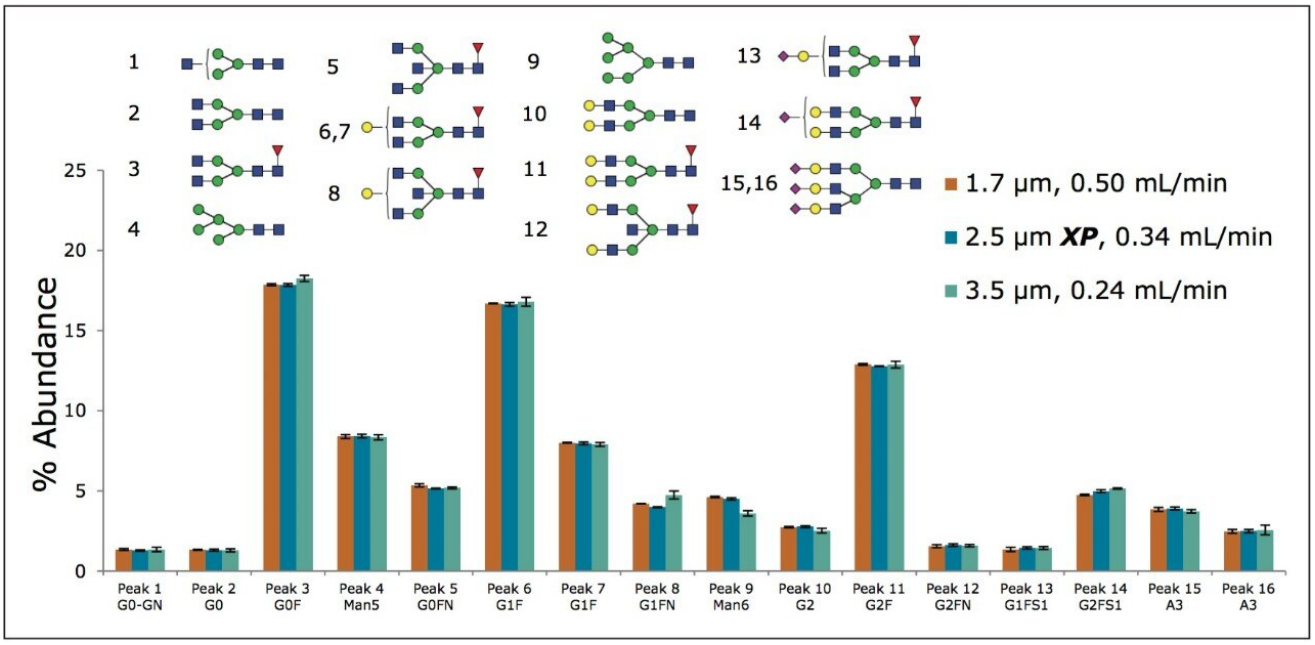

Figure 3. Relative abundances of 2-AB labeled glycans in the test mixture as determined using the 1.7 µm column and the 2.5 XP and 3.5 µm columns with methods scaled for d_p. Values shown were determined based on the three replicate analyses. Note that resolution of peaks 8 and 9 was poor with the 3.5 µm column, leading to a slight discrepancy in their abundances.

Separations with these BEH Glycan Columns may also be used in conjunction with glucose unit (GU) values. The concept of GU values was developed as a means to calibrate HILIC-based glycan separations. In essence, its use minimizes subtle retention time variations of separated glycans between runs by expressing the results in terms of standardized GU values.[13] To assign GU values, a dextran ladder (comprised of glucose multimers of increasing length) is used as an external calibrant. The retention times of the separated glycans are then converted to GU values via use of a software-calculated, GU Ladder calibration table. This process, represented in Figure 4, helps to address variability in retention times due to a method being run on different instruments and in different laboratories. It also has value for use with BEH Glycan separations transferred between different particle size columns. The GU values derived from the various columns and methods were compared (Figure 5) and found to be highly similar. Nevertheless, an average increase in GU values of 0.03 and 0.05 was observed in transition from the 1.7 µm column to the 2.5 µm *XP* and 3.5 µm columns, respectively. Yet, the magnitude of these increases is no greater than the experimental error in database GU values.[13]

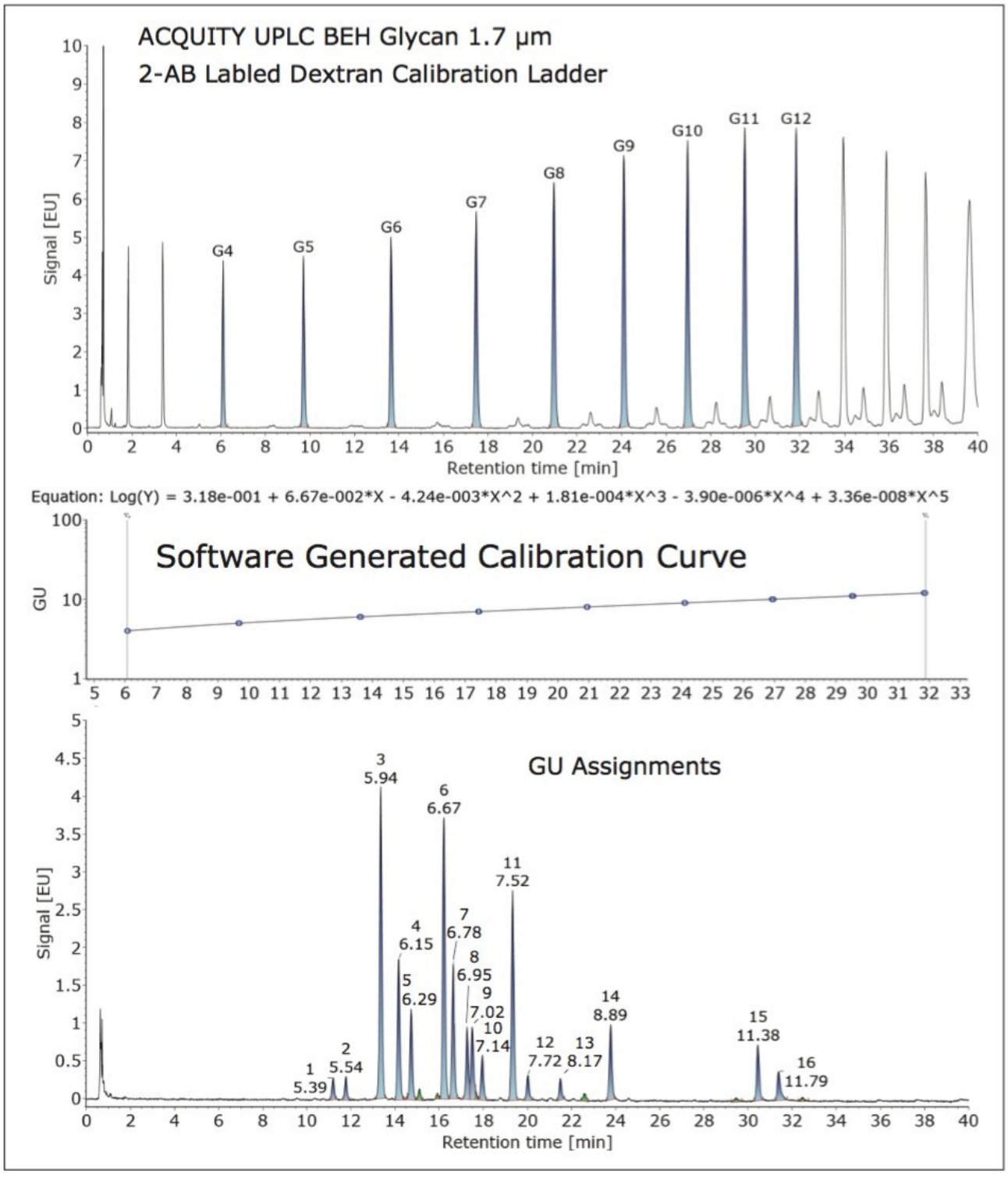

Figure 4. Workflow for the application of the 2-AB Labeled Dextran Calibration Ladder Standard and the

assignment of GU values from Figure 2.

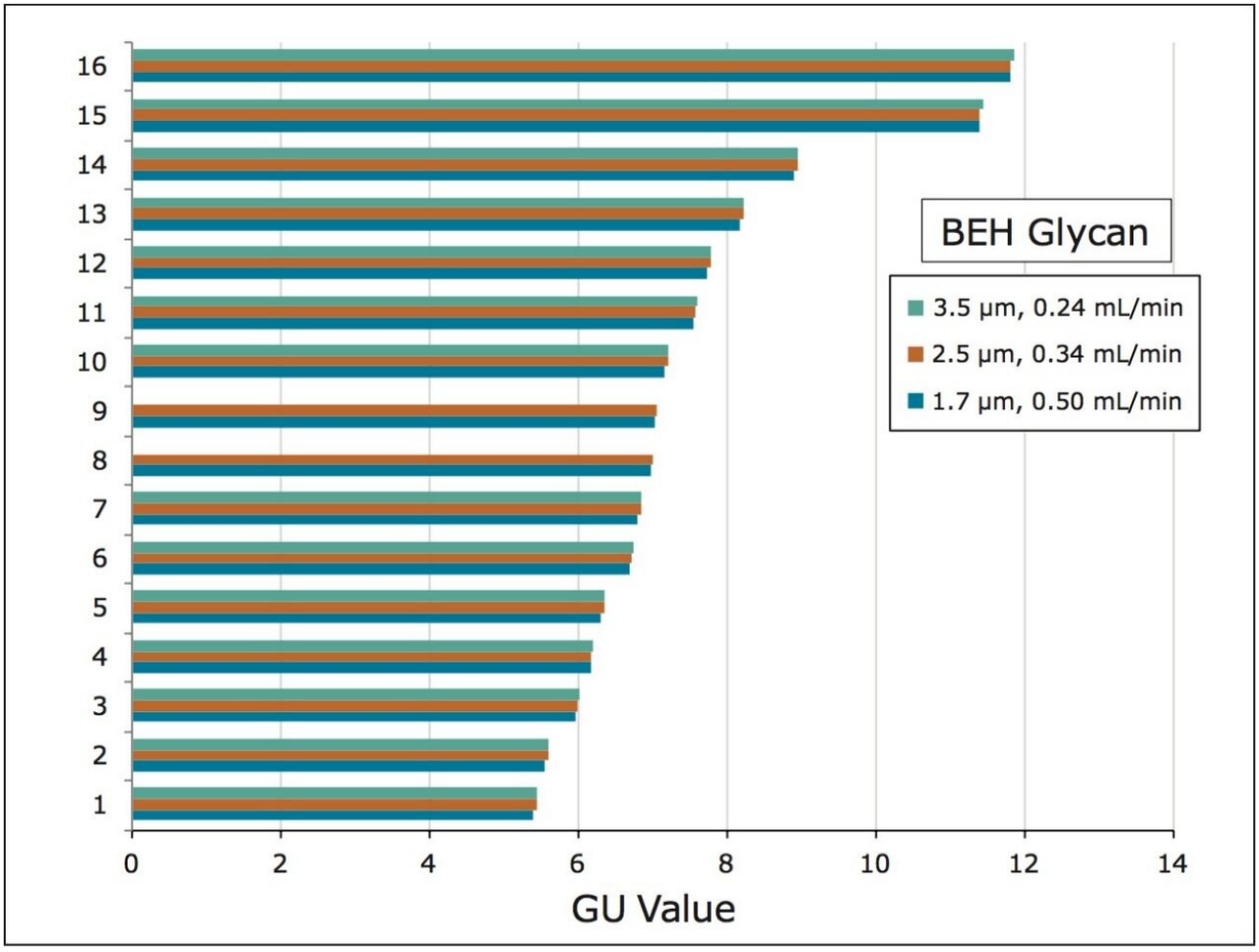

Figure 5. GU values assigned to components of the 2-AB labeled Glycan Performance Test Standard and trisialylated A3 glycans when using the UPLC BEH Glycan 1.7 µm column and the two, HPLC BEH Glycan (2.5 µm XP and 3.5 µm) columns with the scaled methods (all separations performed with an ACQUITY UPLC H-Class Bio). Values were derived from three replicate runs and a separation calibration based on two bracketing analyses of the Dextran Calibration Ladder Standard. Note: to generate GU values that can be searched against the NIBRT GlycoBase, different method conditions should be used (see experimental).

HPLC Instrumentation

As shown with the preceding results, the BEH Glycan Columns afford excellent scalability of a glycan separation across different UPLC and HPLC-based particle sizes. A more challenging proposition, however, is having the desired scalability and transferability across both different particle size columns and UPLC/HPLC instrumentation. In pursuit of this, we evaluated the transferability of a glycan separation from a UPLC and 1.7 µm column to an HPLC and a 2.5 µm *XP* column. Chromatograms obtained under these conditions for the 2-AB labeled Glycan Performance Test Standard (without sialylated A3 glycans) are displayed in Figure 6. Peak capacities observed across the 14 identified peaks were measured and found to be 110 with the UPLC separation and 78 with the HPLC separation. This corresponds to a 29% decrease in performance for the HPLC-based analysis. This metric of relative performance is shown for this and other separations in Table 2. What becomes clear from these data is that although the 2.5 µm *XP* column is capable of producing near UPLC resolving power with a properly scaled method and a UPLC (Figure 2), such performance may not be easily attained with HPLC instrumentation due to additional system dispersion (A band spread of 30 µL was measured for the Alliance HPLC, while 6 µL was measured for the ACQUITY UPLC H-Class Bio). Nonetheless, it is worth noting that the resolving power achieved with the 2.5 µm *XP* column and an Alliance HPLC is greater than that accessible with a competitor's 3 µm amide HILIC column, even when used in combination with a low dispersion UPLC.

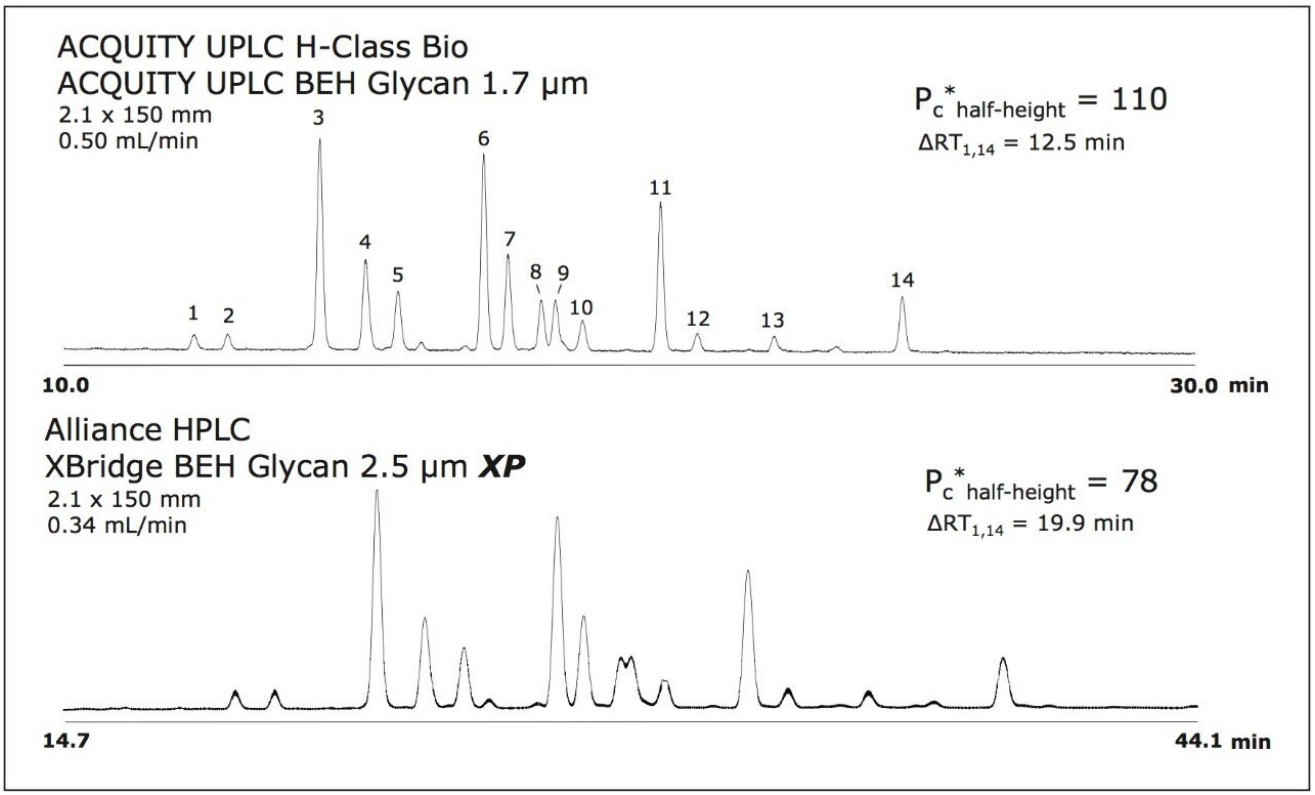

Figure 6. Comparison of HILIC-FLR chromatograms for 2-AB labeled Glycan Performance Test Standard as obtained with an ACQUITY UPLC H-Class Bio and an ACQUITY UPLC BEH Glycan 1.7 µm column versus an Alliance HPLC and an XBridge BEH Glycan 2.5 µm XP column. Separations were performed with ca. 2.3 pmol of sample injected in a volume of 2 µL.

P_c^* half-height / $\Delta RT_{1,14}$	BEH Glycan			
	1.7 µm	2.5 µm *XP*	3.5 µm	Amide-bonded Silica 3 µm
ACQUITY UPLCH-Class Bio Generated	110 (0%)	100 (-9%)	82 (-25%)	70 (-36%)
Alliance HPLC Generated	not possible	78 (-29%)	55 (-50%)	

Table 2. Peak capacities (half-height, ΔRT1,14) and percent decreases in peak capacity relative to a UPLC separation with an ACQUITY UPLC BEH Glycan 1.7 µm column. Larger particle amide columns were used in combination with scaled methods and either UPLC or HPLC instrumentation. Note: see Figure 1 for Peak IDs.

The transfer of the BEH Glycan separations from UPLC to HPLC was also evaluated in terms of GU value assignment. Figure 7 displays GU values assigned using the UPLC method versus the HPLC method. It can be seen that GU values were highly similar no matter the employed method. The largest difference in a GU value assignment was in fact only 0.02, which again, is no greater than the typical experimental error in database GU values.

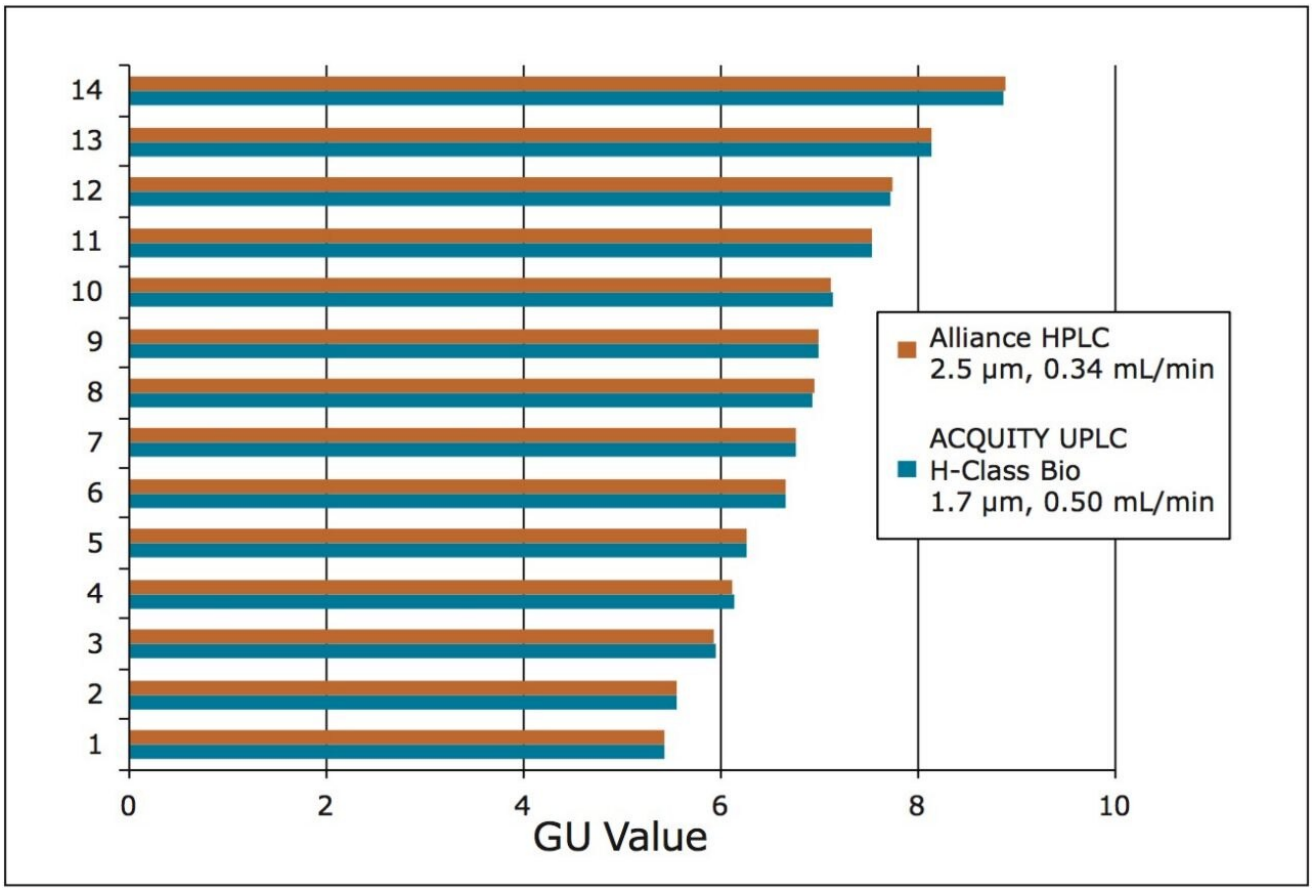

Figure 7. GU values assigned to components of the 2-AB labeled Glycan Performance Test Standard when using an ACQUITY UPLC H-Class Bio with an ACQUITY UPLC BEH Glycan 1.7 µm column versus an Alliance HPLC with an XBridge BEH Glycan 2.5 µm XP column and scaled methods.

Conclusion

In this application note, we have demonstrated that BEH Glycan Columns packed with 1.7, 2.5, and 3.5 µm particles indeed afford scalability between 2-AB labeled glycan separations performed under UPLC and HPLC-compatible conditions. With method transfer accounting for differences in particle diameter (d_p), we additionally show that noteworthy resolution can be achieved with columns packed with larger particles at HPLC-compatible pressures, albeit with an increase in analysis time compared to UPLC-based separations using BEH Glycan 1.7 µm columns. Lastly, this study demonstrates that highly similar glycan profiles and experimental glucose unit (GU) values are obtained, independent of the particle size or LC system (ACQUITY UPLC or Alliance HPLC) employed for a separation. The availability of these various BEH Glycan Columns should be of interest to laboratories looking for flexibility during method development or a facile means of transferring glycan separation methods between UPLC and HPLC instrumentation.

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