thermoscientific

Accurate and Precise Quantification of mAb-released *N-glycans* with an Amide HILIC Column

Stacy Tremintin, Xin Zhang; Thermo Fisher Scientific, Chromatography Columns and Consumables, 1228 Titan Way, Sunnyvale, CA, 94085, USA

INTRODUCTION

More than two-thirds of recombinant biopharmaceutical products on the market are glycoproteins, and every stage of their manufacturing is carefully monitored and tested to ensure consistency in quality, safety, and effectiveness.[1] Glycosylation is one of the key critical quality attributes (CQAs) of monoclonal antibody(mAb)-based biotherapeutics. Robust, information-rich, and reproducible methods for glycan analysis must be included in regulatory filings for glycoprotein-based biotherapeutics to ensure accuracy and consistency.[2]

Current glycan analysis methods involve sample preparation, followed by high-performance capillary electrophoresis (HPCE) or high-performance liquid chromatography (HPLC) separation. Sample preparation takes anywhere from a few hours to multiple days for a 96-well plate. Techniques include protein denaturation, enzymatic deglycosylation, dye labeling, and excess matrix removal.

Methods coupling liquid chromatography to fluorescence detection (LC-FLD) have been extensively used for glycan analysis, affording relative quantification of glycans in a sample with good reproducibility.[3] HILIC using amide-based stationary phases is a well-established, robust technique employed by many laboratories to obtain high-resolution separation of N-linked glycans released from glycoproteins. Tagging the glycans with a fluorescent label such as 2-AB (2-aminobenzamide), 2-AA (2- anthranilic acid), or APTS (8-aminopyrene-1,3,6-trisulfonic acid trisodium salt) allows the sugars to be detected at femtomole levels.

Here, the Accucore™150-Amide HILIC column (2.1 × 150 mm) demonstrated great inter- and intra- column reproducibility, as well as excellent column lifetime for protein glycan analysis. Furthermore, superb accuracy and precision were obtained for the relative quantification of human IgG *N-glycans*, which is critical for quality control and risk management in biopharmaceutical manufacturing.

MATERIALS AND METHODS

Sample Preparation

APT labeled NIST mAb *N-glycans* were prepared with Thermo Fisher Applied Biosystems™ GlycanAssure™ HyPerformance APTS Kit.

Instrumentation

Vanquish Horizon UHPLC system with Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) 7.2.5

LC Method	
Column	Accucore™ 150-Amide-HILIC 2.1 × 150 mm, 2.6 µm (P/N 16726-152130)
Mobile phase A	ACN 100 %
Mobile phase B	Ammonium formate 100 mM, pH 4.4
Flow rate	0.45 mL/min
Column temp.	50 °C
Sample volume	1–10 µL
LC gradient	0–10min, 30%B – 32%B 10–45min, 32%B – 60%B 45–50min, 60%B – 30%B

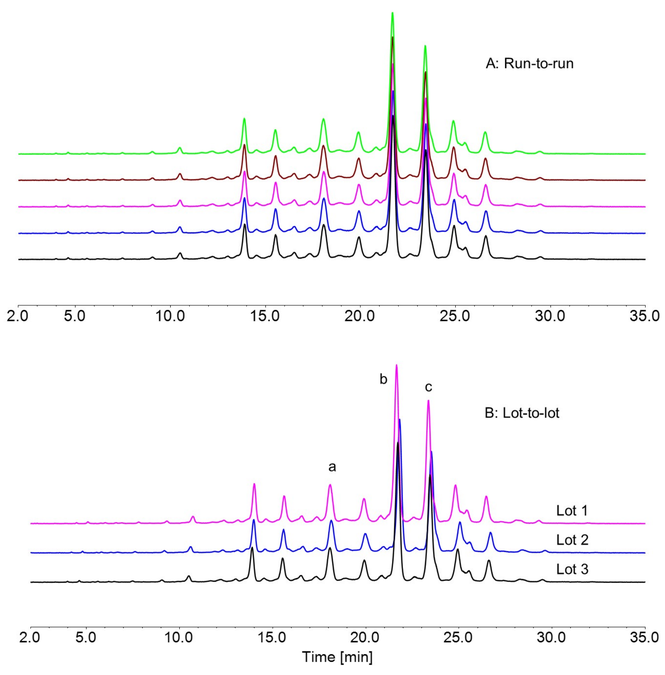
RESULTS AND DISCUSSIONS

Intra and Inter Column Reproducibility

Consistent intra- and inter-column performance was achieved (Figure A and B) with compelling retention time, peak asymmetry, and efficiency for the 2-AB labeled fetuin glycans A3G3S2 (peak A), A3G3S3 (peak B), and A3G2S4 (peak C). The lot-to-lot % RSD for retention time, peak asymmetry, and efficiency are less than 2%, 6.5%, and 3%, respectively.

The backpressure was also monitored and was very moderate (170 bar/2500 psi). Reproducible pressure traces were observed with column-to-column % RSD < 3% (figures not shown).

Overall lot-to-lot reproducibility % RSD < 4% (except Peak A asymmetry at 6.4%), with protein glycan separations indicating excellent column reproducibility with this HILIC phase.

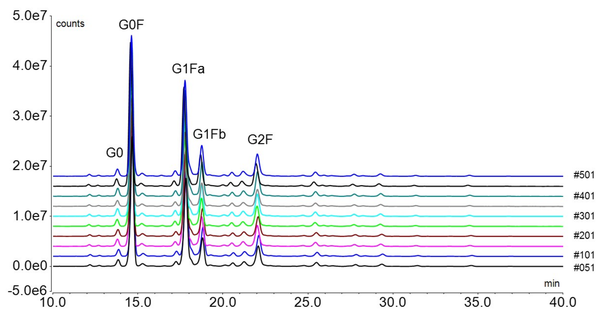


Column Lot-to-Lot Reproducibility (%RSD)			
Peak name	**RT**	**Peak Asym.**	**Efficiency (EP)**
Peak a: A3G3S2	1.83%	6.39%	3.18%
Peak b: A3G3S3	1.71%	1.09%	2.65%
Peak c: A3G2S4	1.61%	1.64%	1.41%

Column Lifetime – Robustness Test

The column lifetime was evaluated with 500 continuous runs at a 10 µL injection volume with APTS labeled protein glycan/maltotriose across nine days. The retention time of major glycan components in this mAb were calculated and <0.5% RSD was obtained.The G1Fa and G1Fb isomer peaks were well resolved; USP resolution was measured from 2.07 to 2.26 with % RSD at 2.76%.

The quantification consistency was evaluated with relative area % for these five major components. The relative area % for each component (ranging from 2% to 45%) was almost identical from run-to-run with the RSD of all the major peaks at 1% with one low abundant peak at 3%.

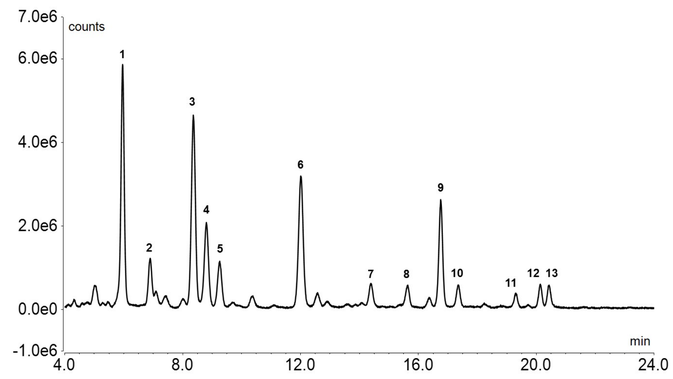


Column Lifetime: Retention Time and Resolution Reproducibility				
501 injections	G0	G0F	G2F	Resolution G1Fa and G1Fb
Average	13.825	14.65	22.05	2.18
RSD	0.48%	0.46%	0.41%	2.76

Quantification Consistency: Major Peaks % Relative Area Count					
Rel.Area %	G0	G0F	G1Fa	G1Fb	G2F
NIST 1	2.20	45.15	33.45	10.61	8.59
NIST 51	2.20	44.39	33.92	10.63	8.86
NIST 101	2.20	44.28	34.10	10.66	8.76
NIST 151	2.27	44.12	33.91	10.60	8.68
NIST 201	2.27	44.28	34.13	10.60	8.72
NIST 251	2.28	44.12	34.28	10.58	8.74
NIST 301	2.29	43.84	34.47	10.65	8.75
NIST 351	2.29	43.80	34.64	10.66	8.61
NIST 401	2.12	43.75	34.50	10.77	8.86
NIST 451	2.13	43.75	34.63	10.63	8.85
NIST 501	2.13	44.09	34.61	10.36	8.82
Average	2.22	44.14	34.24	10.61	8.75
RSD	3.0%	0.9%	1.1%	0.9%	1.1%

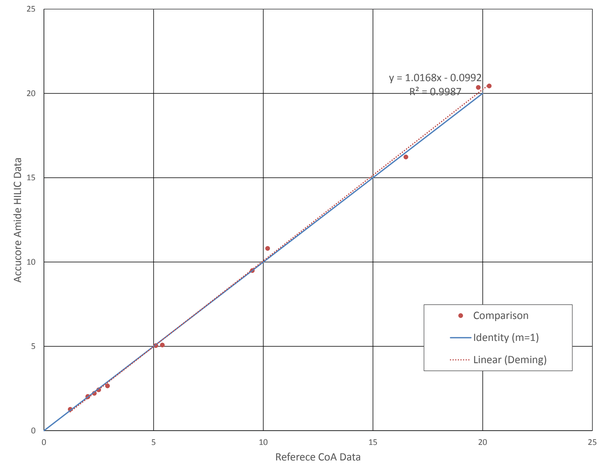
Quantification of 2-AB Labeled Human IgG *N-glycan*

Excellent separation and quantification of 2-AB labeled human IgG *N-glycans* were obtained with the Accucore 150-Amide-HILIC column. Thirteen well-resolved major glycans were detected within 22 minutes. The % relative peak area of each component glycan was calculated with average % RSDs < 4% (n=5). The quantitative results were compared to the 2-AB labeled human IgG *N-glycan* standard manufacturers' data. An absolute average % bias of ±3% indicates that the test data is highly consistent with the manufacturer's data.



Accucore Amide HILIC Column (Tested) vs. Reference (Manufacturer Reported)						
Peak ID	**Full Name**	**Short Name**	**Structure**	**% Relative Area (reported)**	**% Relative Area (Tested)**	**%BIAS**
1	F(6)A2	FA2		19.8	20.4	-3%
2	F(6)A2B	FA2B		5.4	5.1	6%
3	F(6)A2[6]G(4)1	FA2G1		20.3	20.4	-1%
4	F(6)A2[3]G(4)1	FA2G1		9.5	9.5	0%
5	F(6)A2[6]BG(4)1	FA2BG1		5.1	5.0	1%
	F(6)A2[3]BG(4)1	FA2BG1				
6	F(6)A2G(4)2	FA2G2		16.5	16.2	2%
7	F(6)A2BG(4)2	FA2BG2		2.9	2.7	9%
8	A2G(4)2S1	A2G2S1		2.5	2.4	3%
9	F(6)A2G(4)2S1	FA2G2S1		10.2	10.8	-6%
10	F(6)A2BG(4)2S1	FA2BG2S1		2.3	2.2	4%
11	A2G(4)2S2	A2G2S2		1.2	1.3	-5%
12	F(6)A2G(4)2S2	FA2G2S2		2	2.0	-2%
13	F(6)A2BG(4)2S2	FA2BG2S2		2	2.0	0%

Deming regression (Figure below) has a slope of 1.017, demonstrating parallelism between the manufacture methods and the Accucore 150-Amide-HILIC based analysis. A correlation (R^2) of > 0.99 indicates an excellent correlation between the results obtained in the two analyses.



CONCLUSIONS

- The Accucore 150-Amide-HILIC columns coupled to a Vanquish UHPLC system formed a robust platform and an excellent choice for glycan separations.
- The superb lot-to-lot column reproducibility and column lifetime demonstrate the consistency and robustness of the column.
- The accurate and precise quantification of *N-glycan* of human IgG illustrates the impressive correlation with a fully validated manufacturing quantification method, providing a robust and confident reference for biotherapeutics QbD approach and quality control validation.

REFERENCES

1. Walsh, G. *Nat. Biotechnol.* **2010,** *28* (9), 917–924.
2. Fournier, *J. BioPharm. International* **2015,** *28* (10), 32–37.
3. Sanda, M.; Pompach, P.; Brnakova, Z.; Wu, J.; Makambi, K.; Goldman, R. *Mol. Cell. Proteomics* **2013,** *12,* 1294–1305.

ACKNOWLEDGEMENTS

We thank our Thermo Fisher Scientific colleagues Jim Thayer and Anahita Eckardfor gererous assistance with glycan samples. We thank Jonathan Bones, Principal Investgator, NIBRT for comments that greatly improved the manuscript.

TRADEMARKS/LICENSING

ThermoFisher SCIENTIFIC

PO21857-EN 0718S