UHPLC Analysis of 2-Aminobenzamide-Labeled Glycans with the Vanquish Flex System

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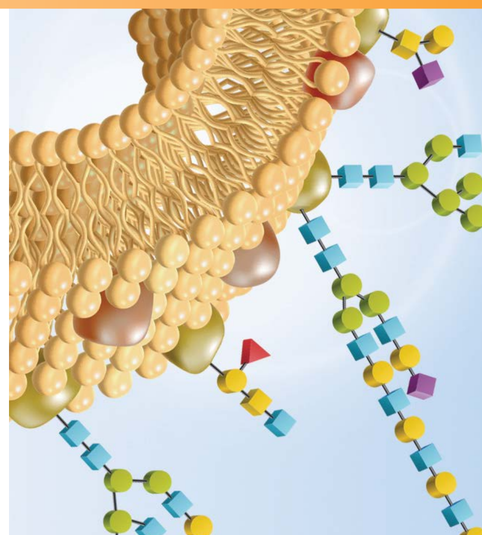
Technical Note 164

Key Words

Biosimilars, Biotherapeutics, Bovine Fetuin, Fluorescence Detector, Glycan Analysis, IgG

Goal

To demonstrate successful separation and detection of 2-aminobenzamide (2-AB)-labeled complex glycan libraries on a Thermo Scientific™ Vanquish™ Flex UHPLC system equipped with a Vanquish Fluorescence Detector F. Retention time precision for these glycan libraries as well as for a 2-AB-labeled single glycan standard are reported. Area precision and linearity are evaluated for three labeled glycan standards. Further, recommendations for the selection of the right detector flow cell are given.



Introduction

Glycans are involved in many biological and physiological processes, such as cell and pathogen recognition, gene expression, or cellular immunity, communication, growth, and development. In biological systems, glycans can be found in a "free state" or in conjugated forms like glycoproteins, glycolipids, or proteoglycans. *N*-linked glycans are considered an important species in therapeutic protein drug development because there is strong evidence that bioactivity and efficiency of proteins depend on glycosylation. Examples for glycosylated protein-based drugs are recombinant proteins and monoclonal antibodies. The function of these proteins often depends on the structure and type of glycans attached to them.[1] Further, glycans can serve as biomarkers for severe diseases such as cancer.[2,3,4] Thus, glycan analysis is of tremendous interest, especially in the biopharmaceutical industry.

In protein glycosylation studies, typically the glycans are analyzed qualitatively and quantitatively after enzymatical or chemical release from the protein. This approach often leads to quite complex and heterogeneous mixtures of oligosaccharides that require high-efficiency separation techniques prior to characterization, usually done by mass spectrometry (MS). Characterization cannot be accomplished by ultraviolet (UV) detection because glycans lack chromophores; this requires a modification to the detection strategy.

A common approach is to label the glycans with fluorescent tags, separate them with high performance liquid chromatography (HPLC), and detect them with fluorescence detectors (FLD). Even though the tagging procedure and sample cleanup are time consuming, no further sample treatment prior to MS-analysis is needed. Consequently, an HPLC system with a fluorescence detector can be coupled directly to an MS. In addition, fluorescently labeled glycans generally provide better and more MS/MS fragmentation peaks.[5] Therefore, HPLC with fluorescence detection is a commonly used approach for glycan analysis, especially when sensitivity is critical and analysis by MS is also required.

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This technical note shows the separation of two 2-AB-labeled glycan libraries and linearity data for three different 2-AB-labeled glycan standards on a Thermo Scientific Vanquish Flex system equipped with a Vanquish Fluorescence Detector F. The system, including two different flow cells, is fully biocompatible. Measurements were conducted with two different columns, the Thermo Scientific™ GlycanPac™ AXR-1 column and the Thermo Scientific™ Accucore™ 150 Amide HILIC column (hydrophilic interaction chromatography).

Experimental

Instrumentation

Vanquish Flex UHPLC system (Figure 1), equipped with:

- System Base (P/N VF-S01-A)
- Quarternary Pump F (P/N VF-P20-A)
- Split Sampler FT with 25 µL sample loop (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A) with active preheater (P/N 6732.0110)
- Fluorescence Detector F (P/N VH-D51-A) equipped with either 2 µL flow cell (P/N 6079.4330) or 8 µL flow cell (P/N 6079.4230)

Reagents and Materials

- Deionized (DI) water 18 MΩ×cm resistivity or higher
- Acetonitrile, Optima™ LC/MS grade (Fisher Scientific P/N 10001334)
- Ammonium formate, Optima LC/MS grade (Fisher Scientific P/N 11377490)
- Formic acid, Optima LC/MS grade (Fisher Scientific P/N 10596814)

Standards

- 2-AB-labeled *N*-glycan library from bovine fetuin (Prozyme P/N GKSB-002)
- 2-AB-labeled *N*-glycan Immunoglobulin G (IgG) library (Ludger P/N CAB-IGG-01)
- 2-AB labeled NA2 glycan (Ludger P/N CAB-NA2-01)
- 2-AB labeled A1 glycan (Ludger P/N CAB-A1-01)
- 2-AB labeled A2 glycan (Ludger P/N CAB-A2-01)

List of Abbreviations

2-AB-NA2:	2-AB-labeled asialo, galactosylated biantennary glycan
2-AB-A1:	2-AB-labeled mono-sialylated, galactosylated biantennary glycan
2-AB-A2:	2-AB-labeled di-sialylated, galactosylated biantennary glycan
ACN:	Acetonitrile
FLD:	Fluorescence detector
PMT:	Photomultiplier tube
RSD:	Relative standard deviation

Preparation of Ammonium Formate Buffers

Dissolve 6.30 g (100 mM) or 3.15 g (50 mM) of ammonium formate in 1 L of DI water, respectively. Adjust pH to 4.4 by carefully adding formic acid.

Settings for the Vanquish Fluorescence Detector F

The Vanquish Fluorescence Detector F was used with two different excitation/emission wavelength pairs. Sensitivity was set to 8 unless stated otherwise. The lamp mode was set to HighPower during data acquisition and to LongLife during re-equilibration.

The emission signal stray light can be suppressed by setting the appropriate cut-off filter-wheel. Several settings are available for the emission filter in the Vanquish Fluorescence Detector F: Auto, 280, 370, 435, and 530 nm. With the Auto setting for the filter wheel, the most suitable cut-off filter is selected automatically, which was 370 nm for the experiments described here.

Figure 1. Vanquish Flex UHPLC System with Fluorescence detector.

Chromatographic Conditions I

Column:	GlycanPac AXR-1, 2.1 × 250 mm, 1.9 µm (P/N 088135)		
Mixer volume:	350 µL + 50 µL		
Mobile phase A:	Acetonitrile		
Mobile phase B:	DI water		
Mobile phase C:	Ammonium formate, 100 mM, pH 4.4		
Gradient:	Time (min)	%B	%C
	-5.0	93	7
	0	93	7
	60	31	50
	60.5	0	100
	65.5	0	100
	75	93	7
	100	93	7
Flow rate:	0.40 mL/min		
Temperature:	Column compartment:	30 °C	
	Detector flow cell:	35 °C	
Detection parameters:	Excitation wavelength:	250 nm	
	Emission wavelength:	430 nm	
	Lamp mode:	HighPower	
	Sensitivity:	7	
	Data collection rate:	10 Hz	
	Response time:	0.2 s	
Sample:	1 nmol/mL 2-AB-labeled *N*-glycan library from bovine fetuin Sample was dissolved in DI water		

Chromatographic Conditions II

Column:	Accucore 150-Amide-HILIC, 2.1 × 150 mm, 2.6 µm (P/N 16726-152130)	
Mixer volume:	350 µL + 50 µL	
Mobile phase A:	Acetonitrile	
Mobile phase B:	Ammonium formate, 50 mM, pH 4.4	
Gradient:	Time (min)	%B
	0	20
	30	42
	30.5	50
	32	50
	32.5	20
	60	20
Flow rate:	0.40 mL/min	
Temperature:	Column compartment:	50 °C
	Detector flow cell:	45 °C
Detection parameters:	Excitation wavelength:	320 nm
	Emission wavelength:	420 nm
	Lamp mode:	HighPower
	Sensitivity:	8
	Data collection rate:	5 Hz
	Response time:	1 s
Samples:	1 nmol/mL 2-AB labeled *N*-glycan IgG library 1 nmol/mL 2-AB labeled NA2 glycan 1 nmol/mL 2-AB labeled A1 glycan 1 nmol/mL 2-AB labeled A2 glycan Samples were dissolved with appropriate amounts of 82/18 acetonitrile/ammonium formate 50 mM pH 4.2.	

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, version 7.2 SR 3

Results and Discussion

Separation of 2-AB-Labeled Glycan Library Samples

The *N*-linked glycans from bovine fetuin are heterogeneous in terms of charge, as their sialic acid content can vary between 0 and 5. Here, we show that these rather diverse glycans samples can be successfully separated and detected with the Vanquish Flex system equipped with a Vanquish Fluorescence Detector F. Over 60 peaks were detected in the sample of 2-AB-labeled *N*-fetuin glycans. All peaks eluted within 50 min in a ternary gradient separation (Figure 2).

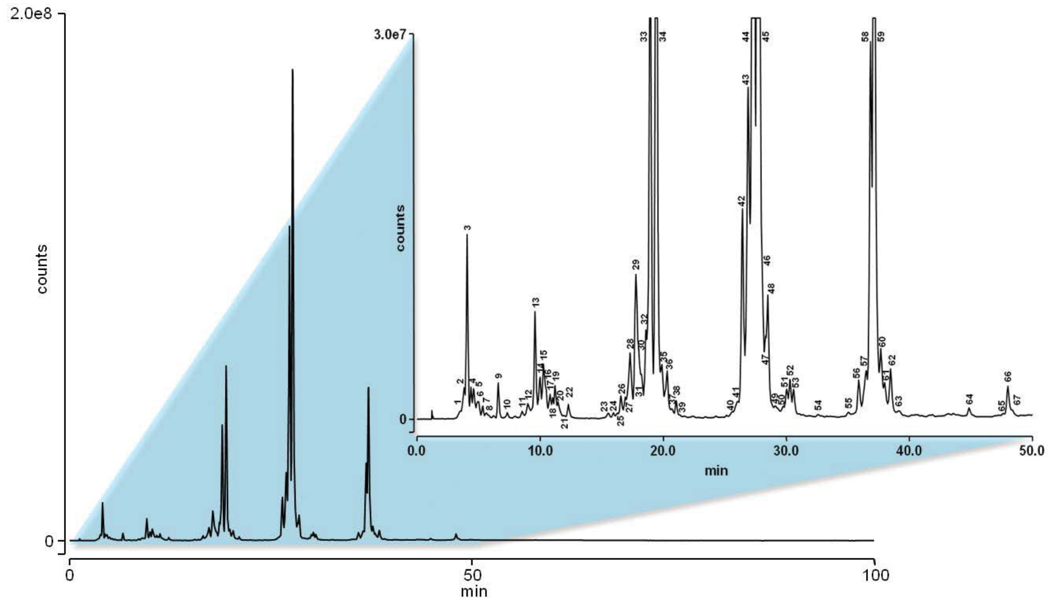

Figure 2. Separation of 50 pmol bovine fetuin 2-AB-*N*-glycans measured with the Vanquish FLD F using a 2 µL flow cell. Chromatographic conditions correspond to those described in the table “Chromatographic Conditions I”.

The IgG sample is less heterogeneous, consisting primarily of neutral and mono-sialylated glycans. The IgG 2-AB-*N*-glycans could be separated on an Accucore 150-Amide-HILIC column, separating the sample by size, delivering over 20 peaks within 30 minutes (Figure 3).

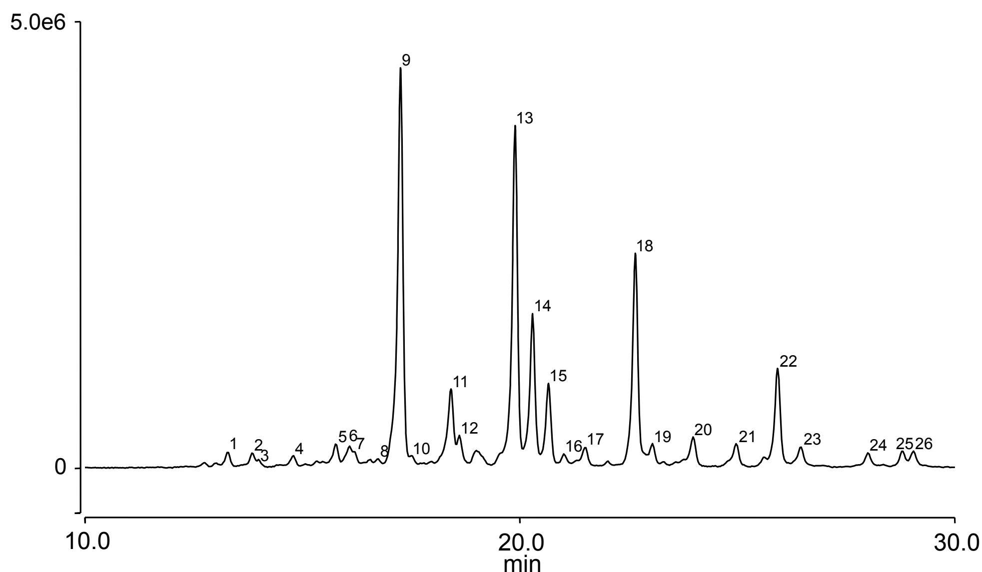

Figure 3. Separation of 5 pmol IgG 2-AB-*N*-glycans measured with the Vanquish FLD F using an 8 µL flow cell. Chromatographic conditions correspond to those described in the table “Chromatographic Conditions II”.

Retention Time Precision

Retention time precision (RSD) is essential in labeled glycan analysis for peak identification. This applies particularly to cases where the relative retention times are expressed based on the retention time of glucose oligomers, with the purpose of structure assignment based on a database.[6] A method with repeatable retention times allows the injection of the unknown sample and the glucose ladder standard in separated runs, and subsequent peak identification by comparison of the chromatograms. This avoids the practice of spiking the sample with the glucose standard, hence eliminating the risk of co-elution between glucose oligomers and analyte peaks.

Retention time precision was measured for repeated injections of the bovine fetuin 2-AB-*N*-glycans and the IgG 2-AB-*N*-glycans, as well as for a single standard of 2-AB-NA2 glycan

For the bovine fetuin 2-AB-*N*-glycan separation with a ternary gradient shown in Figure 2, 10 arbitrarily selected peaks were evaluated. The retention time precision for these peaks is shown in Figure 4. The RSD for this separation is generally ≤ 0.1%, which is excellent for a ternary gradient separation.

For the IgG 2-AB-*N*-glycan separation shown in Figure 3, 10 arbitrarily selected peaks were evaluated. The retention time precision for these peaks is shown in Figure 5. For this separation, the retention time RSD is even better and < 0.04% for all peaks.

For all peaks shown in this technical note, the RSD was far below the value 0.3% that is commonly reported.[7] In addition, retention time precision was evaluated for six consecutive injections of the single standard 2-AB-NA2; an RSD of 0.008% was measured in this case. Retention time precision data can be found in Table 1.

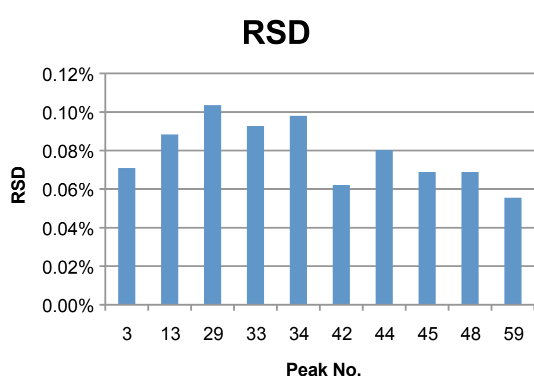

Figure 4. Retention time precision for 10 arbitrarily selected peaks from the bovine fetuin 2-AB-*N*-glycan separation shown in Figure 2.

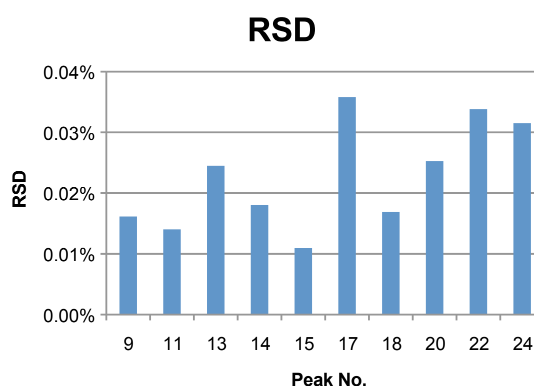

Figure 5. Retention time precision for 10 arbitrarily selected peaks from the IgG 2-AB-*N*-glycan separation shown in Figure 3.

Table 1. Retention time precision (n=6). Fetuin glycans from Figure 2, RSD% represents average of 10 peaks; IgG glycans from Figure 3, RSD% represents average of 10 peaks; 2-AB-NA2 standard, measured with "Chromatographic Conditions II".

	Fetuin 2-AB-*N*-glycans	IgG 2-AB-*N*-glycans	2-AB-NA2
Retention time precision (RSD%)	0.079	0.023	0.008

Area Precision and Linearity

Area precision was evaluated for five consecutive injections of 1 pmol 2-AB-NA2, and the measured RSD was 0.92%. Linearity was evaluated for three different glycan standards (2-AB-NA2, 2-AB-A1, and 2-AB-A2) and two different flow cells (2 µL and 8 µL), respectively. Linearity was excellent in all cases and interpolation resulted in r^2 well above 0.99 (Table 2).

Table 2. Linearity obtained for 3 different glycan standards with different sample amounts (n ≥ 5) in two different flow cells, respectively. Same chromatographic conditions as in Figure 3.

Sample	Range (pmol)	r^2 in 2 µL flow cell	r^2 in 8 µL flow cell
2-AB-NA2	0.05 – 5.00	0.9998	0.9999
2-AB-A1	0.50 – 10.00	0.9976	0.9976
2-AB-A2	0.25 – 5.00	0.9996	0.9976

Conclusions

The Vanquish Flex system equipped with the Vanquish Fluorescence Detector F is a powerful tool for the analysis of 2-AB-labeled glycans. The retention time precision of the system is excellent. Hence, this system can be effectively used for separation of labeled glycans and peak recognition by customized or public databases.

Linearity of the method was excellent for detection of samples in the range spanning from low femtomole to low picomole, independent of the flow cell. Most likely the linearity range stretches to much higher concentrations, however this aspect is outside the purposes of this study and was not investigated.

The Vanquish Fluorescence Detector F can be equipped with two different flow cells, an 8 µL standard flow cell and a 2 µL micro flow cell for minimized extra-column band dispersion, both biocompatible. Figure 6 shows an overlay of measurements obtained by use of these two different flow cells.

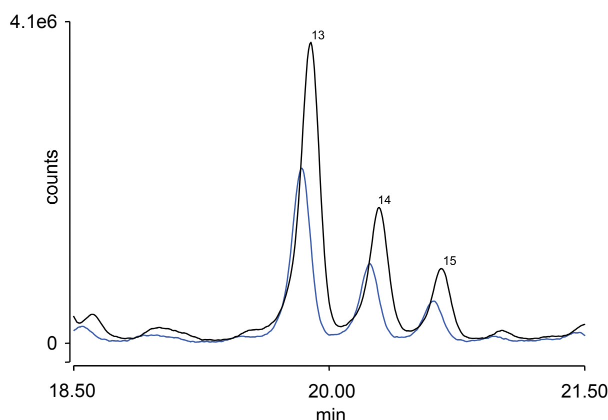

Figure 6. Overlay of three peaks from Figure 3, measured using an 8 µL flow cell (black), with peaks detected with an identical experiment but a 2 µL flow cell (blue).

Although the size of the flow cell is not critical for this application, with the 8 µL flow cell peaks were higher and broader, compared to those measured with the 2 µL flow cell. The increase in peak height leads to lower limits of detection while the increased peak width impairs resolution. Consequently, the 8 µL flow cell should be the preferred choice for methods with very low amounts of sample and very well resolved peaks (as in Figure 3), whereas the 2 µL flow cell should be chosen when higher resolving power is required (as in Figure 2).[8]

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