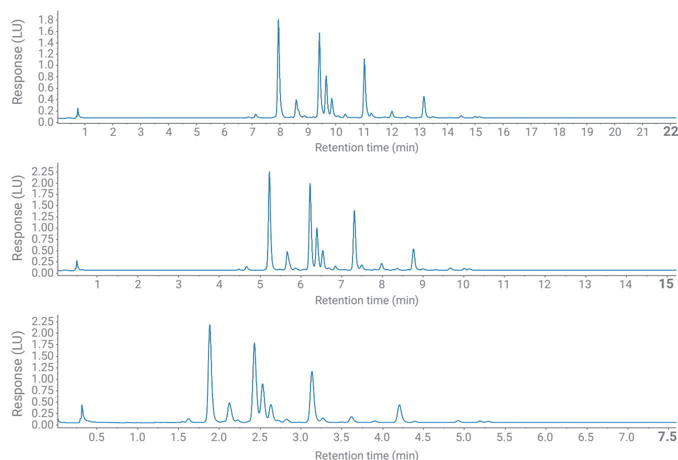
Application Note

Biopharma

Agilent

Trusted Answers

Resolution and Speed in the Separation of Glycans



Author

Edgar Naegele

Agilent Technologies, Inc.

Abstract

This application note demonstrates the reliable separation of 2-AB- and InstantPC-labeled glycans with the Agilent 1290 Infinity II Bio LC System under resolution- and speed-optimized conditions. The reliability is showcased by calculation of performance values such as retention time precision, area precision, and resolution, all of which obtained excellent values. For detection, the Agilent 1260 Infinity II Fluorescence Detector (FLD) with bio-inert flow cell was used. The 1290 Infinity II Bio LC System has low internal volume and the binary pump provides highly precise solvent compositions and flow rates, which enables method transfers from high-resolution to speed-optimized methods with comparable results.

Introduction

Today, monoclonal antibodies (mAbs) are the most important class of modern biotherapeutic compounds. All therapeutic mAbs belong to the class of immunoglobulins G (IgGs).[1] On their surface, the IgGs have an N-linked glycosylation site on each of their heavy chains, resulting in a mixture of up to 30 glycoforms.[2] Because the glycosylation pattern is important for the therapeutic efficacy of the mAbs, its analysis is a crucial part of the QA/QC process. Moreover, glycosylation is one of the most important critical quality attributes (CQA) for mAbs,

To cope with a larger number of samples, it is necessary to have not only fast sample preparation methods, but also fast analytical methods with sufficient resolution of complex mixtures. Typically, glycans are labeled with fluorescent compounds for their detection with an FLD after chromatographic separation. The labeling methods were accelerated by means of modern labeling compounds such as InstantPC. This label in particular shows a higher fluorescent sensitivity compared to the classical labels, and a good ionization for mass spectrometric detection.[3] The subsequent chromatographic separation was performed on a HILIC column, which is the standard method for the separation of glycans nowadays.[4]

This application note will demonstrate the high-resolution and high-speed separation of a fluorescent-labeled glycan ladder and a fluorescent-labeled human IgG N-glycan library by means of the 1290 Infinity II Bio LC equipped with Agilent AdvanceBio glycan mapping HILIC columns. Typical performance parameters, such as area and retention time RSD, and resolution, will be shown for a 150 and a 100 mm column. As fluorescent labels, the classical 2-AB label and the modern InstantPC label will be compared for the IgG library to show that identical elution patterns can be obtained for the high-resolution and high-speed methods.

Experimental

Instrument

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) including integrated Sample Thermostat (#101)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116B) with biocompatible Heat Exchanger
- Agilent 1290 Infinity II Fluorescence Detector (FLD) (G7162B), equipped with bioinert standard FLD flow cell (G5615-60005)

Columns

1. Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (859700-913)
2. Agilent AdvanceBio Glycan Mapping, 2.1 × 100 mm, 1.8 µm (858700-913)

Software

Agilent OpenLab version 2.5 and GPC/SEC add-on software V. 1.2

Samples

- AdvanceBio 2-AB Human IgG N-Glycan Library (part number GKSB-005)
- AdvanceBio InstantPC Human IgG N-Glycan Library (part number GKPC-005)
- AdvanceBio 2-AB Glucose Homopolymer Standard (part number GKSB-503)

Chemicals

- Acetonitrile, HPLC gradient grade
- Ammonium formate

Chemicals were purchased from VWR, Germany. Fresh ultrapure water was obtained from a Milli-Q integral system equipped with LC-Pak polisher and a 0.22 µm membrane point-of-use cartridge (Millipak).

General method settings

Parameter	Value
Binary Pump	
Solvents	A) water + 100 mM ammonium formate, pH 4.5 B) ACN
MCT	
Column Temperature	60 °C
FLD	
Wavelength (2AB)	Ex. 260 nm, Em. 430 nm
Wavelength (InstantPC)	Ex. 285 nm, Em. 345 nm
Data Rate	18.75 Hz (high-resolution methods)
Data Rate	39.75 Hz (fast-separation method)
Sampler	
Injection	1 µL
Needle Wash	3 s water/ACN 30/70
High-Resolution Method, 150 mm Column	
Binary Pump	
Flow Rate	0.5 mL/in
Gradient	0 min—80% B; 22 min—55% B; 22.25 min—40% B; 24.25 min—40% B; 24.5 min—80% B; stop time: 24.5 min; post time: 7 min
MCT	
	Column 1
High-Resolution Method, 100 mm Column	
Binary Pump	
Flow Rate	0.5 mL/in
Gradient	0 min—80% B; 15 min—55% B; 15.25 min—40% B; 16.75 min—40% B; 17.0 min—80% B; stop time: 17.0 min; post time: 5 min
MCT	
	Column 2
High-Speed Method, 100 mm Column	
Binary Pump	
Flow Rate	0.75 mL/in
Gradient	0 min—75% B; 7.5 min—65% B; 7.75 min—40% B; 8.75 min—40% B; 9.0 min—75% B; stop time: 9.0 min; post time: 3 min
MCT	
	Column 2

Results and discussion

For the initial development of a high-resolution method on a 150 mm AdvanceBio glycan mapping column, a glucose-based glycan ladder with 2-AB fluorescent label was used. The initial solvent ratio, the end-point solvent ratio, and the run time were determined to have good resolution between residual 2-AB label (0.735 minutes) and glucose unit 1 (GU1, 0.934 minutes) (Figure 1). Glucose homopolymers were separated and detected up to GU18.

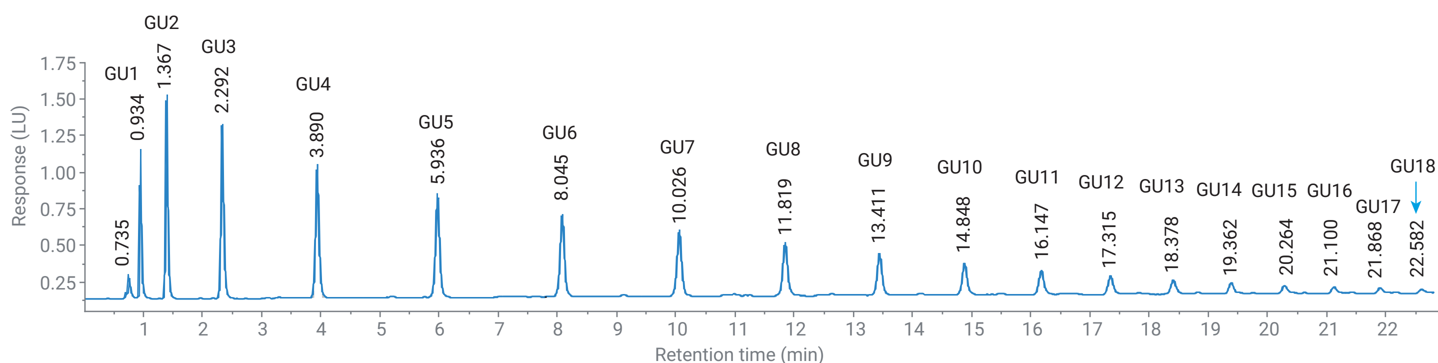


Figure 1. High-resolution separation of a glycan ladder on the 150 mm column, adjusted for baseline separation at the beginning for residual 2-AB and GU1 with a generic linear gradient.

The initial resolution of residual 2-AB and GU1 was 4.23, which is clearly baseline separation. The resolution increases up to GU6 at 8.045 minutes. For the later-eluting glucose homopolymers, the resolution declines as the peak width increases. Up to GU5, the area RSD is below 0.65%, up to GU13 between 1.17% and 3.4%. Later-eluting homopolymers show a higher area RSD but correspondingly low peak areas. The retention time RSD is typically below 0.08% for all compounds (Table 1).

The initial high-resolution method was adapted from the 150 mm column to a 100 mm column by decreasing the separation gradient to 15 minutes accordingly. Flushing and regeneration times were adapted to the lower column volume of the 100 mm column. In the obtained separation, the residual 2-AB was still separated from GU1 and the 18 glucose homopolymers were still baseline separated in the gradient run time (Figure 2).

Table 1. Performance results of the glycan ladder separation on the 150 mm column.

GU	Area RSD (%)	RT (min)	RT RSD (%)	Resol. USP	Width 50% (min)
1	0.59	0.934	0.06	4.23	0.028
2	0.47	1.367	0.08	8.29	0.034
3	0.64	2.292	0.09	14.03	0.044
4	0.33	3.890	0.08	18.27	0.057
5	0.61	5.936	0.06	18.59	0.069
6	1.23	8.045	0.09	16.89	0.075
7	1.20	10.026	0.08	14.81	0.079
8	1.17	11.819	0.05	12.85	0.082
9	1.50	13.411	0.06	11.17	0.082
10	1.77	14.848	0.05	9.92	0.084
11	2.65	16.147	0.05	8.84	0.085
12	3.40	17.315	0.05	7.92	0.085
13	2.79	18.378	0.05	7.15	0.085
14	6.65	19.362	0.04	6.53	0.089
15	6.44	20.264	0.04	5.99	0.090
16	6.76	21.100	0.03	5.39	0.094
17	6.77	21.868	0.04	4.77	0.093
18	5.57	22.582	0.04	4.52	0.093

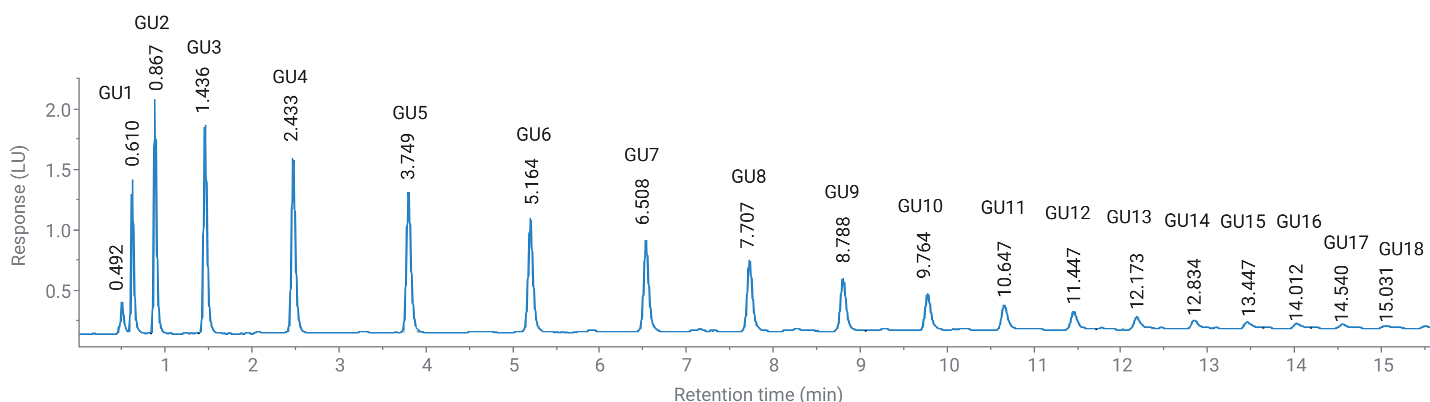


Figure 2. High-resolution separation of a glycan ladder on the 100 mm column, transferred from 150 mm column by adjusting the gradient to the shorter column. Residual 2AB and GU1 are still well separated.

The resolution of residual 2-AB and GU1 was 2.16. The resolution also increases up to GU5 eluting at 3.749 minutes. Up to GU4, the area RSD is below 1.00%, up to GU14 between 1.09% and 3.5%. Later-eluting homopolymers show a higher area RSD but correspondingly low peak areas. The retention time RSD is typically below 0.09% for all compounds (Table 2).

By comparison (Tables 1 and 2), the area RSDs and retention time RSDs obtained on the 150 mm and the 100 mm column are in the same order. Resolution, of course, declines when using shorter 100 mm column, but is still high enough for baseline separation of all peaks.

Both high-resolving methods, developed for the 100 mm AdvanceBio glycan mapping column and the 150 mm AdvanceBio glycan mapping column, were applied for the separation of a glycan sample, a fluorescence-labeled N-glycan library of human IgG (Figures 3 and 4). The separations were done with a 2-AB-labeled IgG N-glycan library (Figures 3A and 3B) and an InstantPC-labeled IgG N-glycan library (Figures 4A and 4B). The separation of the 2-AB-labeled glycan library shows a comparable elution pattern for the 100 mm and the 150 mm column (Figures 3A and 3B), with the gradient run time being around one third shorter for the 100 mm column. In both cases, the same low abundance peaks could be observed, and the three peaks that elute in the middle of the pattern were separated with good resolution.

Table 2. Performance results of glycan ladder separation on the 100 mm column.

GU	Area RSD (%)	RT (min)	RT RSD (%)	Resol. USP	Width 50% (min)
1	0.86	0.610	0.13	2.16	0.031
2	0.82	0.867	0.18	4.87	0.032
3	0.95	1.436	0.19	9.23	0.040
4	0.97	2.433	0.09	13.35	0.048
5	1.09	3.749	0.06	15.02	0.054
6	1.13	5.164	0.03	14.41	0.058
7	1.41	6.508	0.08	12.94	0.061
8	1.24	7.707	0.08	11.11	0.063
9	1.57	8.788	0.05	9.59	0.066
10	2.30	9.764	0.04	8.33	0.069
11	3.46	10.647	0.03	7.28	0.071
12	1.82	11.447	0.02	6.32	0.074
13	1.50	12.173	0.03	5.50	0.078
14	3.25	12.834	0.02	4.87	0.079
15	5.92	13.447	0.03	4.25	0.087
16	3.75	14.012	0.03	3.64	0.097
17	5.38	14.540	0.02	3.06	0.105
18	6.17	15.031	0.04	2.82	0.106

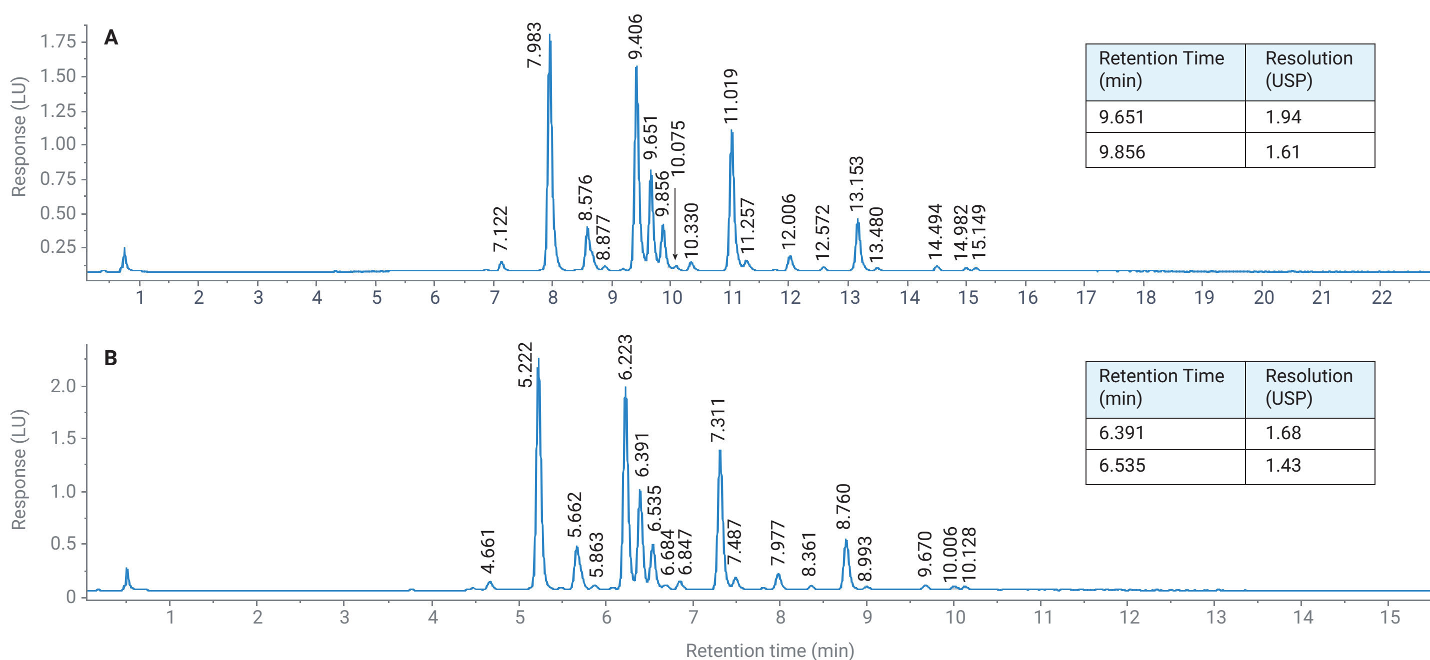


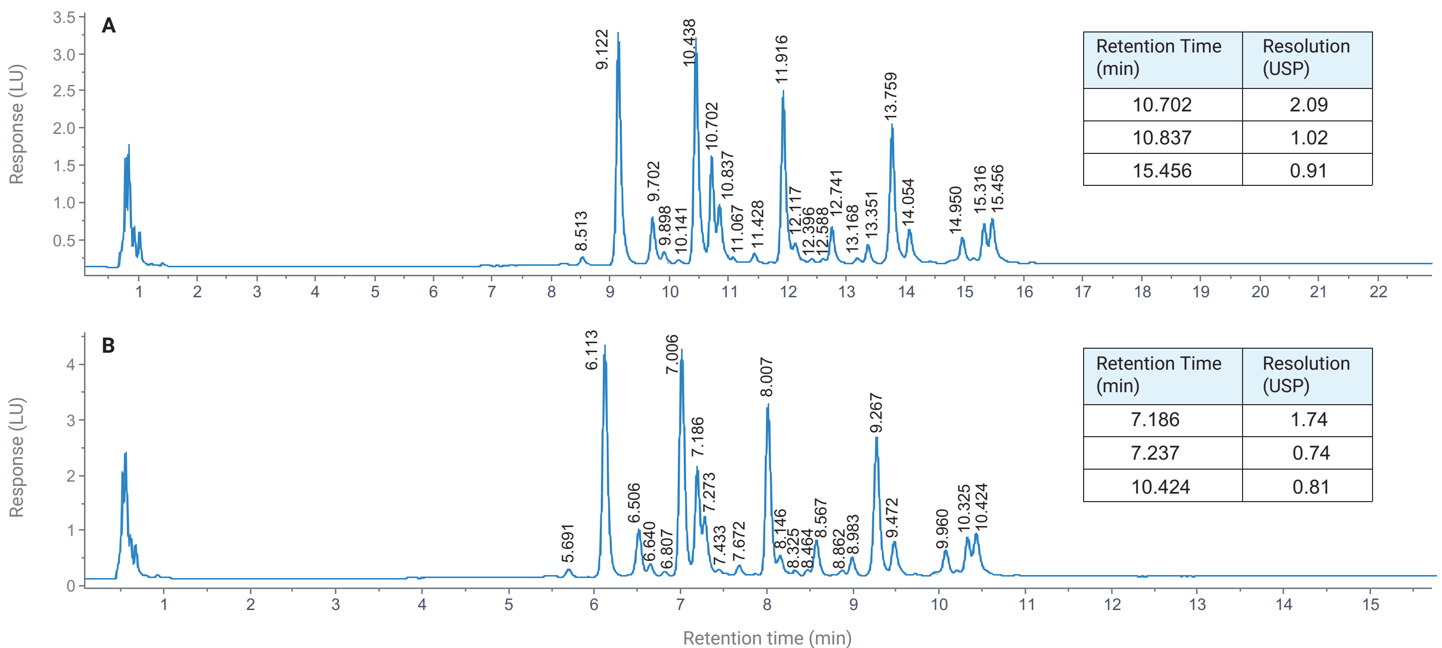
Figure 3. High-resolution separation of a 2-AB-labeled IgG N-glycan library on a 150 mm Agilent AdvanceBio glycan mapping column (A) and a 100 mm AdvanceBio glycan mapping column (B).

For instance, the peak eluting at 9.651 minutes from the 150 mm column had a resolution value of 1.94 from the peak eluting at 9.408 minutes. On the 100 mm column, this peak elutes at 6.391 minutes with a resolution of 1.68.

The fluorescent label InstantPC is a more recent development with the advantage of higher fluorescent sensitivity and the capability to be ionized for mass spectrometric detection.[3] The elution patterns obtained from the 150 and 100 mm columns were comparable (Figures 4A and 4B). They provided the same information about the low-level glycans and a comparable resolution for the peaks. In the case of the 100 mm column, the gradient run time was shortened by about one third. In the peak pattern in the middle of the run time, the peak that eluted at 10.702 minutes from the 150 mm column showed a resolution from the peak at 10.438 minutes of 2.09 (Figure 4A). On the 100 mm column, the same peak eluting at 7.186 showed a resolution of 1.74 (Figure 4B).

An identical behavior could be observed for the peaks eluting from the 150 mm and the 100 mm column at 10.837 and 7.237 minutes and for the peaks at 15.456 and 10.424, respectively.

Comparing both applied labels, the InstantPC-labeled separation of the glycan library showed more low-abundance peaks due to its higher fluorescence sensitivity compared to the label 2.



Retention Time (min)	Resolution (USP)
10.702	2.09
10.837	1.02
15.456	0.91

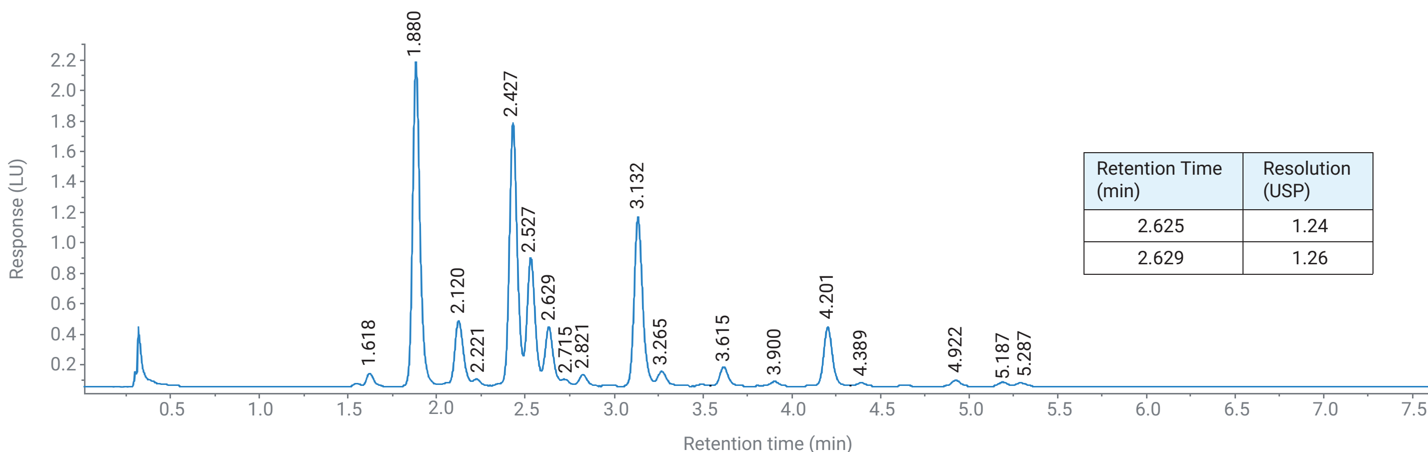
Retention Time (min)	Resolution (USP)
7.186	1.74
7.237	0.74
10.424	0.81

Figure 4. High-resolution separation of an InstantPC-labeled IgG N-glycan library on a 150 mm Agilent AdvanceBio glycan mapping column (A) and a 100 mm AdvanceBio glycan mapping column (B).

Since there were improvements in sample preparation, which made it much faster and so accelerated throughput, fast analytical methods were required.[3,4] For the development of a fast glycan separation method, the high-resolution method developed for the 100 mm column was used as the basis. To achieve a shorter run time while retaining the already known elution pattern with sufficient resolution, the flow rate was increased incrementally while the gradient time was decreased accordingly. This led to a method with a gradient run time only half as long as before, at a 50% higher flow rate. With this method, the separation of the 2-AB-labeled and InstantPC-labeled IgG N-glycan library was compared (Figures 5 and 6).

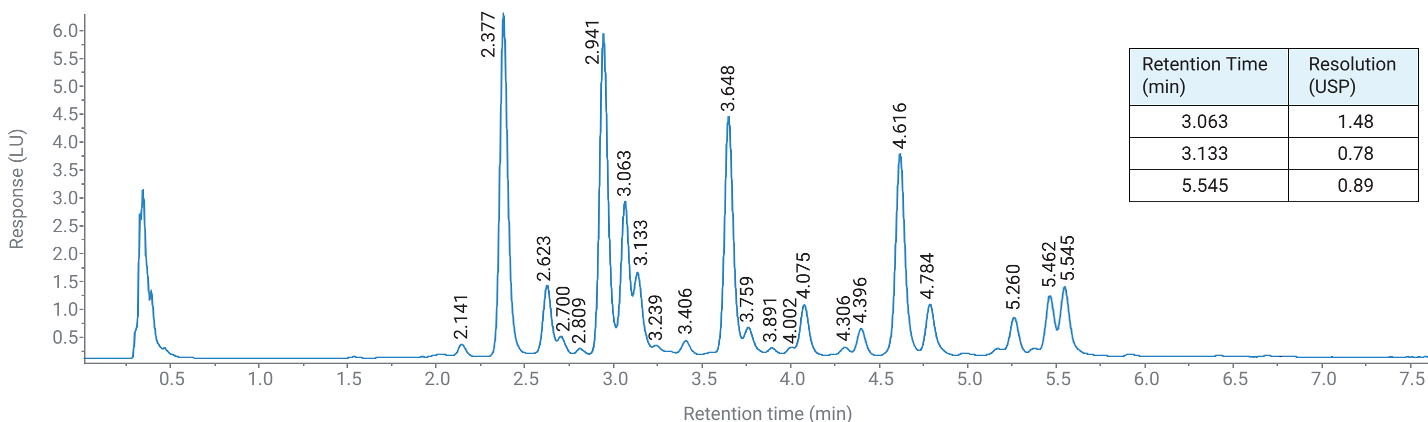
The comparison of the elution pattern obtained for the fast separation of the 2-AB-labeled glycans (Figure 5) with the separation obtained for the high-resolution method (Figure 3B) displays identical elution patterns. Even the resolution obtained for the glycans eluting in the middle of the pattern is sufficient for their visual identification and quantification. The peak that elutes at 2.527 minutes has a sufficient resolution of 1.24 from the previously eluting peak, while resolution was 1.68 for the high-resolution method.

Comparison of the fast separation of InstantPC-labeled glycans (Figure 6) with the high-resolution separation (Figure 4B) also shows comparable elution patterns. The peak eluting at 3.063 minutes had a resolution of 1.48 compared to a resolution of 1.74 obtained for the high-resolution method. Comparing Figures 5 and 6 also shows that the InstantPC-labeled sample provided more information about low abundance glycans as already seen for the high-resolution separations. Due to different chromatographic behavior, the resolution of InstantPC-labeled compounds is compromised in some cases compared to 2-AB-labeled glycans.



Retention Time (min)	Resolution (USP)
2.625	1.24
2.629	1.26

Figure 5. Separation of the 2-AB-labeled IgG N-glycan library by means of the developed fast separation method achieved on the 100 mm Agilent AdvanceBio glycan mapping column.



Retention Time (min)	Resolution (USP)
3.063	1.48
3.133	0.78
5.545	0.89

Figure 6. Separation of the InstantPC-labeled IgG N-glycan library by means of the developed fast separation method achieved on the 100 mm Agilent AdvanceBio glycan mapping column.

Conclusion

This application note demonstrates the use of the 1290 Infinity II Bio LC for the separation of fluorescent-labeled glycans. The biocompatible UHPLC system enables analyses of biological compounds of high complexity like glycans without the danger of losing lower abundance compounds due to unspecific adsorption on active surfaces in the system.

Comparable results can be obtained for method transfers from high-resolution methods to speed-optimized methods due to the low internal delay volume of the 1290 Infinity II Bio LC System, and the highly precise solvent mixing and flow rate provided by the 1290 Infinity II Bio High-Speed Pump. All calculated performance values like retention time precision, area precision, and resolution were obtained with excellent values.

References

1. Jefferis, R. Glycosylation of Recombinant Antibody Therapeutics. *Biotechnol. Prog*. **2005**, *21(1)*, 11–16.
2. Arnold, J. N. *et al.* Human Immunoglobulin glycosylation and the Lectin Pathway of Complement Activation. *Adv. Exp. Med. Biol.* **2005**, *564*, 27–43.
3. Yan, J.; Jones, A. Streamlined Workflows for N-Glycan Analysis of Biotherapeutics using Agilent AdvanceBio Gly-X InstantPC and 2-AB Express Sample Preparation with FLD/MS. *Agilent Technologies application note*, publication number 5994-1348EN, **2019**.
4. Marosella, J. *et al.* A Novel HILIC Column for High Speed N-linked Glycan Analysis. *Agilent Technologies application note*, publication number 5991-4886EN, **2017**.

www.agilent.com/chem

DE44251.1113541667

This information is subject to change without notice.

© Agilent Technologies, Inc. 2021

Printed in the USA, May 11, 2021
5994-3130EN

Agilent
Trusted Answers