

# Application News

## No. B79

### MALDI-TOF Mass Spectrometry

## A Study on a Method for Evaluating Glycans in Biopharmaceuticals - Part 2

### - Comparison of various pretreatment methods for *N*-Glycan Analysis -

Many protein-based biopharmaceutical products are synthesized in cultured cells derived from eukaryotes. For this reason, the synthesized proteins are mainly glycoproteins which comprise proteins with glycans linked to them. The glycans in these glycoproteins are broadly divided into *N*-linked glycans (*N*-glycans) and *O*-linked glycans (*O*-glycans), each having diverse and complex branching structures.

The structure of the glycan is known to affect the function and stability of the glycoprotein. Therefore, if the structure of the glycan in a synthesized glycoprotein changes, due to changes in the culture environment for example, there may be unexpected changes in the function and stability of the glycoprotein itself. This possibility can lead to serious problems in the development and manufacture of biopharmaceutical products, and therefore monitoring whether the glycan structure has changed or not is a primary element in managing quality.

It is important to correctly analyze and evaluate changes in glycan structure, but there are a variety of pretreatment methods for glycan analysis and they are not standardized, so the result of analysis of the same glycoprotein may differ if different pretreatment methods are used.

This article introduces the results of comparing some of the pretreatment methods widely used in *N*-glycan analysis and investigating how they affect the analysis results.

S. Nakaya

### ■ Releasing *N*-Glycans from Glycoproteins

Three types of glycoprotein were used for the comparison of pretreatment methods: commercially available IgG, RNase B, and  $\alpha$ 1-acid glycoprotein. First, each sample was desalted using a PD-10 column (GE Healthcare) and freeze dried, then re-dissolved in water to achieve 10 mg/mL, and 5  $\mu$ L of this solution was used. To this, 200 mM sodium phosphate buffer solution (pH 7.2, 2.5  $\mu$ L), 5 % SDS solution (1.25  $\mu$ L), 0.5 M DTT solution (1.25  $\mu$ L), and water (6  $\mu$ L) were added and, after stirring well, the glycoprotein was denatured by warming for 10 minutes at 65 °C. After that the sample solution was ice cooled, then 5 % NP-40 solution (5  $\mu$ L) was added and it was stirred, after which PNGaseF (0.5 mU/ $\mu$ L, 4  $\mu$ L, Takara Bio) was added. The solution was left to react at 37 °C for 18 hours to release *N*-glycans from the glycoprotein.

### ■ Comparison of Free *N*-Glycan Purification Methods

The reaction solution after releasing *N*-glycans contains impurities such as the enzyme used, buffer solution components and other low molecular weight compounds (SDS and DTT), and it is usually necessary to remove these impurities. Therefore, in this study we compared the major methods for free glycan purification in current use: 1. reversed phase solid-phase extraction, 2. graphitized carbon solid-phase extraction, 3. ultrafiltration, 4. ethanol precipitation, and 5. no purification. The procedures for each method of purification are shown in Tables 1 to 5.

**Table 1 Purification by Reversed Phase Solid-Phase Extraction**

Step	Procedure
1	Prepare an Oasis <sup>®</sup> HLB 1 cc cartridge or a Supel <sup>™</sup> -Select HLB 1 cc cartridge with methanol (1 mL) and water (1 mL).
2	Add water (200 $\mu$ L) to the reaction solution (25 $\mu$ L), and stir well.
3	Load the solution from step 2 into the column.
4	Rinse out the tube in which the reaction solution was contained with 10 % methanol (200 $\mu$ L) and then load the solution into the column. Collect the eluate in a new tube.
5	Repeat step 4.
6	Load 10 % methanol (500 $\mu$ L) into the column.
7	Elute all the solution from the column and collect it.
8	Mix all of the solution collected in steps 4 through 7, and dry it by centrifugal evaporator at room temperature.

**Table 2 Purification by Graphitized Carbon Solid-Phase Extraction**

Step	Procedure
1	Prepare a Supelclean <sup>™</sup> ENVI-Carb <sup>™</sup> SPE bed wt.100 mg with acetonitrile (1 mL) and water (3 mL).
2	Load the reaction solution (25 $\mu$ L) into the column.
3	Rinse out the tube in which the reaction solution was contained with water (1 mL) and then load the solution into the column.
4	Wash the column with water (2 mL).
5	Fit a 0.22 $\mu$ m Millex <sup>™</sup> -LG filter at the outlet of the column.
6	Elute with 50 % acetonitrile (1 mL) containing 20 mM triethylamine acetate and collect it.
7	Dry the eluate by centrifugal evaporator at room temperature.

**Table 3 Purification by Ultrafiltration**

Step	Procedure
1	Add water (0.5 mL) to the sample chamber [Thermo Scientific™ Pierce™ Protein Concentrator PES, 10K MWCO, 0.5 mL (for RNase B and $\alpha$ 1-acid glycoprotein) or 30K MWCO, 0.5 mL (for IgG)] then centrifuge at 15,000 × g for 10 minutes to wash the filter membrane.
2	Discard all of the solution that has passed through and the solution that remains in the sample chamber.
3	Add water (400 $\mu$ L) and the reaction solution (25 $\mu$ L) to the sample chamber.
4	Rinse out the tube that contained the reaction solution with water (100 $\mu$ L) and add the solution to the sample chamber.
5	Centrifuge at 15,000 × g for 15 minutes and collect the solution that has passed through the membrane.
6	Add water (200 $\mu$ L) to the sample chamber, centrifuge at 15,000 × g for 15 minutes, and collect the solution that has passed through the membrane.
7	Add water (200 $\mu$ L) to the sample chamber, centrifuge at 15,000 × g for 10 minutes, and collect the solution that has passed through the membrane.
8	Mix all of the solution collected in steps 5 through 7, and dry it by centrifugal evaporator at room temperature.

**Table 4 Purification by Ethanol Precipitation**

Step	Procedure
1	Cool ethanol to $-20$ °C.
2	Add the cooled ethanol (75 $\mu$ L) to the reaction solution (25 $\mu$ L), gently stir it, then leave it at $-20$ °C for three hours.
3	Centrifuge at 15,000 × g for 10 minutes and collect the supernatant.
4	Dry the collected solution by centrifugal evaporator at room temperature.

**Table 5 No Purification**

Step	Procedure
1	Dry the reaction solution (25 $\mu$ L) by centrifugal evaporator at room temperature.

Glycan-labeling solution was added to the free *N*-glycans obtained through the procedures in Tables 1 to 5 for fluorescent labeling. The labeling solution was prepared by first adding 300  $\mu$ L of dimethyl sulfoxide/acetic acid (7 : 3) solution to 15 mg of 2-aminobenzamide (2-AB, Sigma-Aldrich), then adding 12 mg of sodium cyanoborohydride to 200  $\mu$ L of this solution and stirring well. For the labeling reaction, the labeling solution (5  $\mu$ L) was added to the glycan sample and left to react for 18 hours at 37 °C.

The *N*-glycans labeled with 2-AB were then purified by solid-phase extraction using the procedure in Table 6 and then re-dissolved by adding 50  $\mu$ L of 50 % acetonitrile solution to finally obtain the analysis sample. MALDI-TOF mass spectrometry (MS) and liquid chromatography (LC) were used for the analysis.

**Table 6 Purification of 2-AB-Labeled *N*-Glycans by Hydrophilic Interaction Solid-Phase Extraction**

Step	Procedure
1	Prepare an Oasis® HLB 1 cc cartridge with 95 % acetonitrile (1 mL).
2	Add acetonitrile (95 $\mu$ L) to the reaction solution (5 $\mu$ L), stir well, and load it into the column.
3	Rinse out the tube that contained the reaction solution with 95 % acetonitrile (0.5 mL) and then load the solution into the column.
4	Wash the column with 95 % acetonitrile (2 mL).
5	Elute the 2-AB labeled <i>N</i> -glycan with 20 % acetonitrile (0.5 mL).
6	Dry the eluted solution by centrifugal evaporator at room temperature.

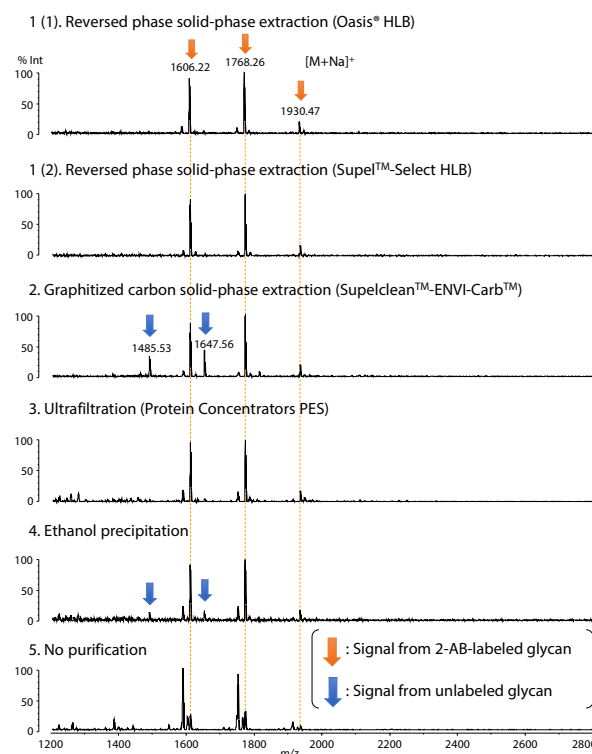
## MALDI-TOF MS Analysis of 2-AB-Labeled *N*-Glycans Obtained Using Various Free Glycan Purification Methods

The analysis sample solution (0.5  $\mu$ L) was placed on the MALDI target plate. Then 0.5  $\mu$ L of matrix solution was overlaid on the placed sample solution. After drying, the sample was measured using the benchtop MALDI-TOF mass spectrometer "MALDI-8020" (Fig. 1). The matrix solution was prepared by dissolving 5 mg of 2,5-dihydroxybenzoic acid (DHB, Shimadzu GLC) in 50 % acetonitrile/0.05 % TFA solution (500  $\mu$ L).



**Fig. 1 Appearance of Benchtop MALDI-TOF MS "MALDI-8020"**

When IgG after each pretreatment method was analyzed, 2-AB-labeled *N*-glycans were detected as  $\text{Na}^+$  adduct ions with the reversed phase solid-phase extraction and ultrafiltration methods. However, with the graphitized carbon solid-phase extraction and ethanol precipitation methods, unlabeled *N*-glycans with aminated reducing ends were strongly detected in addition to labeled *N*-glycans. Where no purification of free glycans was performed, the detected ion was 2-AB-labeled *N*-glycan, but the main ion was the  $\text{H}^+$  adduct ion (Fig. 2).



**Fig. 2 MALDI-TOF Mass Spectra of IgG-Derived *N*-Glycans**

As for IgG, unlabeled glycan ions were strongly detected for RNase B too with the graphitized carbon solid-phase extraction and ethanol precipitation methods (Fig. 3).

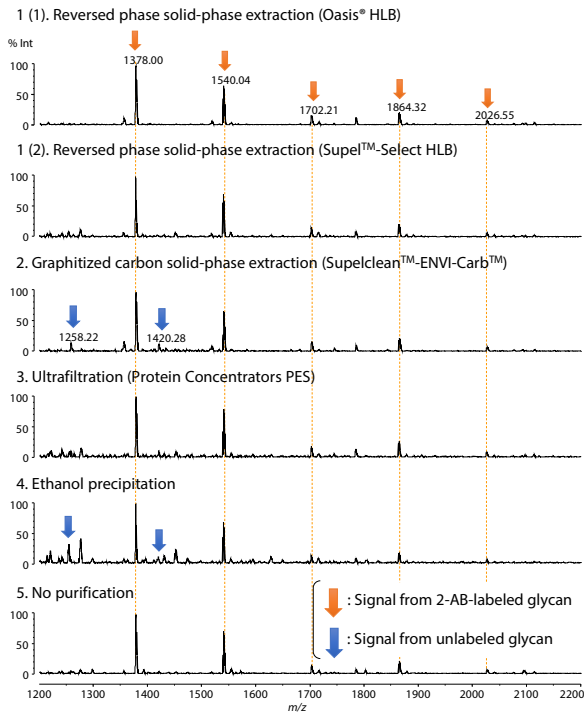


Fig. 3 MALDI-TOF Mass Spectra of RNase B-Derived N-Glycans

For  $\alpha$ 1-acid glycoprotein, no signals from unlabeled glycans could be confirmed, but with the ultrafiltration method, the relative intensity value of 3-branch sialylated N-glycan ions became weaker. In addition, many signals with a mass difference of 22, expected to be due to the substitution of the -OH of the carboxyl group of sialic acid with -ONa, were detected (Fig. 4).

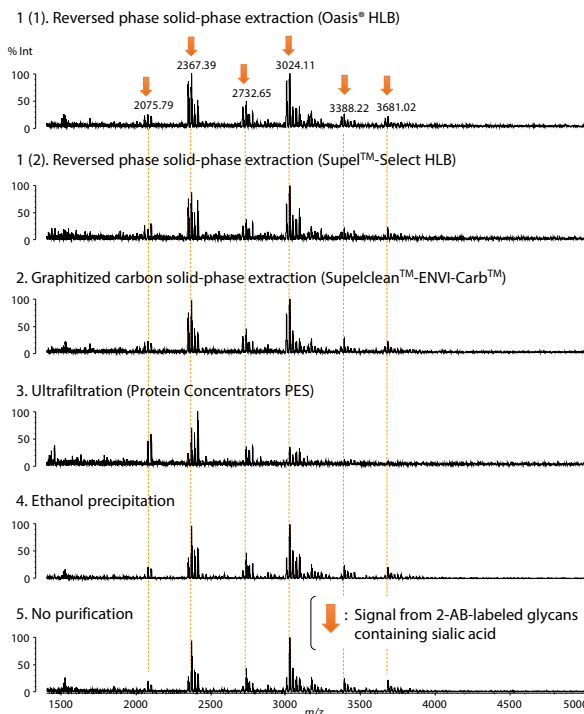


Fig. 4 MALDI-TOF Mass Spectra of  $\alpha$ 1-Acid Glycoprotein-Derived N-Glycans

### ■ LC Analysis of 2-AB-Labeled N-Glycans Obtained Using Various Free Glycan Purification Methods

LC analysis was performed using 2  $\mu$ L of each analysis sample solution under the conditions in Table 7 using an HILIC-amide column.

Table 7 LC Analysis Conditions for Comparison of Free Glycan Purification Methods

Instrument	: Nexera™ X2
Column	: TSKgel® Amide-80, 2.0 $\mu$ m 150 mm L 2 mm I.D. (Tosoh)
Mobile phase A	: 100 mM Ammonium formate (pH 4.5)
Mobile phase B	: Acetonitrile
Total flow rate	: 0.25 mL/min
Concentration of mobile phase B	: 0 to 2 min: 75 %, 2 to 50 min: 75 $\rightarrow$ 50 %
Column temp.	: 45 °C
Detection	: Fluorescence (Ex 330 nm, Em 420 nm)

In LC analysis, no major differences were observed among the chromatograms by each pretreatment method for IgG and RNase B, but in the case of  $\alpha$ 1-acid glycoprotein which contains multiple sialic acids, a chromatogram differing greatly from those by other methods was obtained with the ultrafiltration method. A graph comparing the areas of the chromatogram peaks obtained with each pretreatment method is shown in Figs. 5 to 7.

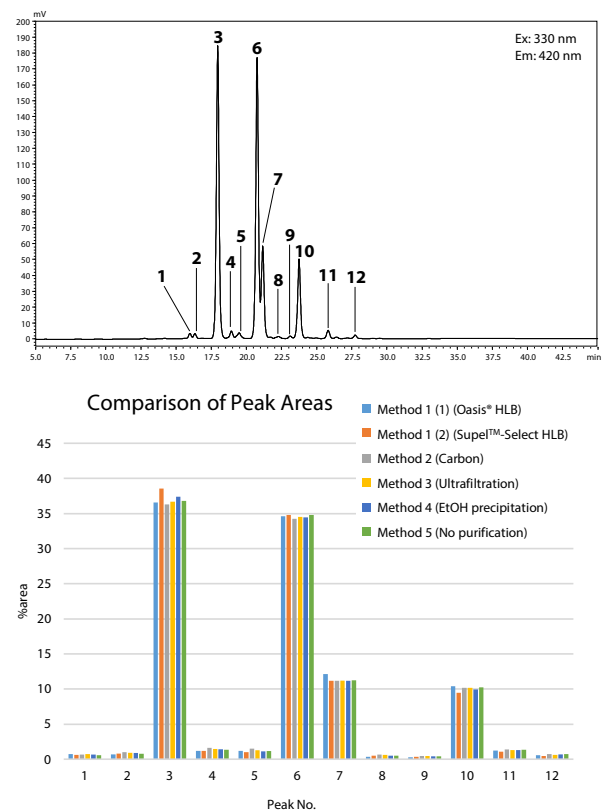
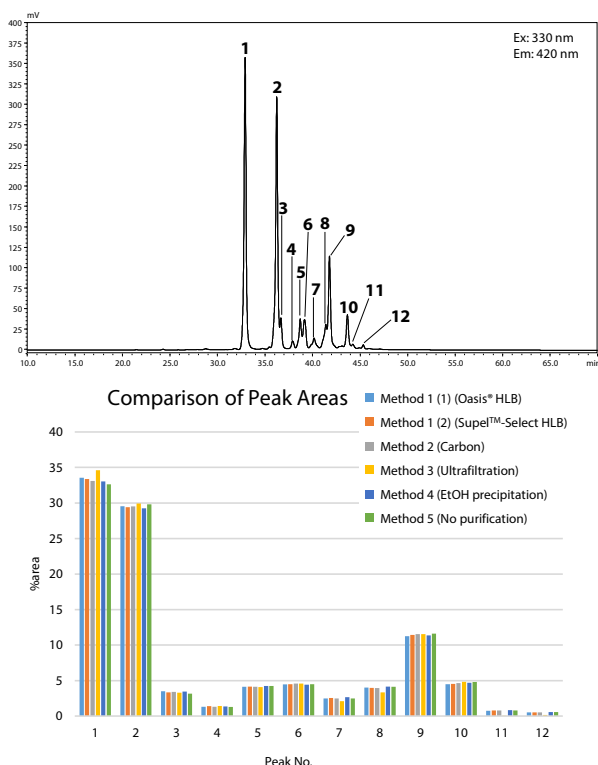
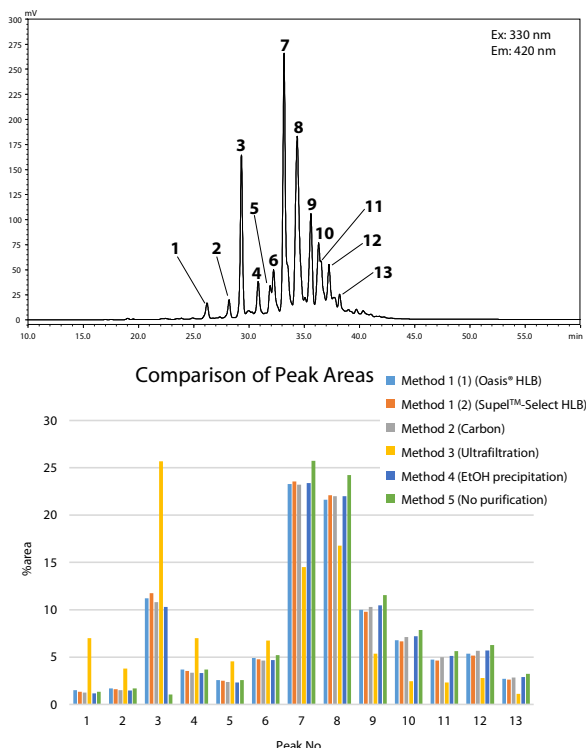


Fig. 5 Example Fluorescence Chromatogram of IgG-Derived N-Glycans (Top) and Peak Area Comparisons for Free Glycan Purification Methods (Bottom)



**Fig. 6 Example Fluorescence Chromatogram of RNase B-Derived N-Glycans (Top) and Peak Area Comparisons for Free Glycan Purification Methods (Bottom)**



**Fig. 7 Example Fluorescence Chromatogram of α1-Acid Glycoprotein-Derived N-Glycan (Top) and Peak Area Comparisons for Free Glycan Purification Methods (Bottom)**

The results from comparing the free glycan purification methods show that when *N*-glycans with no sialic acid are released from the glycoprotein, the same glycan profile is obtained whether the employed purification method for the released glycan is solid-phase extraction by reversed phase or graphitized carbon, or it is ultrafiltration or ethanol precipitation.

On the other hand, when *N*-glycans of a high molecular weight contain multiple sialic acids, as in the case of α1-acid glycoprotein, it was found that it is sometimes not possible to completely extract these high molecular weight *N*-glycans by using the ultrafiltration method.

It was also found that with the amount of buffer, surfactant, or reducing agent used for the release of glycans in this study, if the glycoprotein is desalted in advance with PD-10 for example, there is no need for purification after releasing glycans.

### ■ Comparison of Conditions for Derivatization of N-Glycans

*N*-glycans that are released from glycoproteins are difficult to analyze as is due to factors such as sensitivity, so that it is needed to derivatize the glycans by using fluorescent or another useful reagent to increase sensitivity. However, this kind of derivatization is often carried out in a high temperature environment, so care must be taken about issues such as decomposition of the glycans during the reaction process.

In light of this situation, we next compared two fluorescent reagents, 2-aminobenzamide (2-AB, Sigma-Aldrich) and 2-aminobenzoic acid (2-AA, Sigma-Aldrich), which are widely used for derivatization (labeling), to see the extent of decomposition of glycans according to differences in reaction conditions. The conditions for the 2-AB and 2-AA labeling that were compared this time are shown in Tables 8 to 10.

The *N*-glycans released from the glycoproteins were first purified by solid-phase extraction using an Oasis® HLB 1 cc cartridge and then derivatized. The derivatized glycans were then purified using the solid-phase extraction method indicated in Table 6 earlier. However, for the 2-AA labeling method using boric acid (Table 10), it proved impossible to use a solid-phase extraction cartridge for purification of derivatized glycans because the cartridge clogged, so the gel filtration method indicated in Table 11 was used.

**Table 8 Conditions for 2-AB Labeling of N-Glycans**

2-AB labeling (65 °C, 3 hrs)	
2-AB labeling (37 °C, 18 hrs)	
Step	Procedure
1	Add dimethyl sulfoxide/acetic acid (7 : 3) solution (300 μL) to 2-AB (15 mg) and heat to dissolve the reagent.
2	Add the solution from step 1 (200 μL) to sodium cyanoborohydride (12 mg).
3	Add the labeling solution from step 2 (5 μL) to the dried free glycans, mix well, and allow to react for 18 hours at 37 °C or 3 hours at 65 °C.

**Table 9 Conditions for 2-AA Labeling of N-Glycans**

2-AA labeling (65 °C, 3 hrs)	
2-AA labeling (37 °C, 18 hrs)	
Step	Procedure
1	Add dimethyl sulfoxide/acetic acid (7 : 3) solution (300 μL) to 2-AA (15 mg) to dissolve the reagent.
2	Add the solution from step 1 (200 μL) to sodium cyanoborohydride (12 mg).
3	Add the labeling solution from step 2 (5 μL) to the dried free glycans, mix well, and allow to react for 18 hours at 37 °C or 3 hours at 65 °C.

**Table 10 Conditions for an Alternative 2-AA Labeling Method of *N*-Glycans**

Alternative 2-AA labeling (80 °C, 1 hr) Alternative 2-AA labeling (37 °C, 18 hrs)	
Step	Procedure
1	Add sodium acetate trihydrate (4 g) and boric acid (2 g) to methanol (100 mL).
2	Add 2-AA (30 mg) and sodium cyanoborohydride (30 mg) to the reagent solution from step 1 (1 mL).
3	Add the labeling solution from step 2 (100 µL) to the dried free glycans, mix well, and allow to react for 18 hours at 37 °C or 1 hour at 80 °C.

**Table 11 Purification of Derivatized *N*-Glycans After the Alternative 2-AA Labeling Method**

Step	Procedure
1	Add water (10 mL) into the gel filtration column (PD MiniTrap G-10).
2	After adding water (50 µL) to the derivatized glycan solution and mixing, load it into the column.
3	Rinse out the tube for the derivatized glycan solution with water (a volume to make 700 µL when combined with the volume of liquid in step 2), and load the solution into the column.
4	Add water (0.5 mL) into the column and collect the eluted solution.
5	Dry the collected solution by centrifugal evaporator at room temperature.

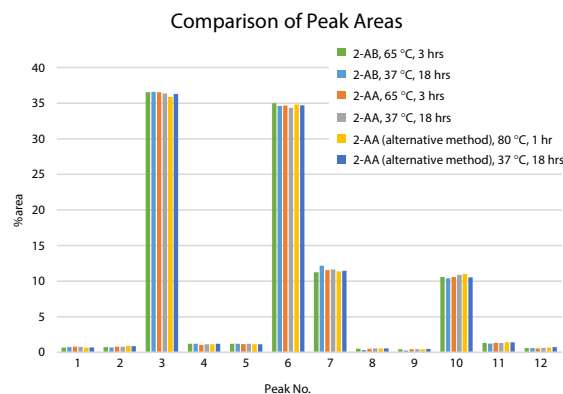
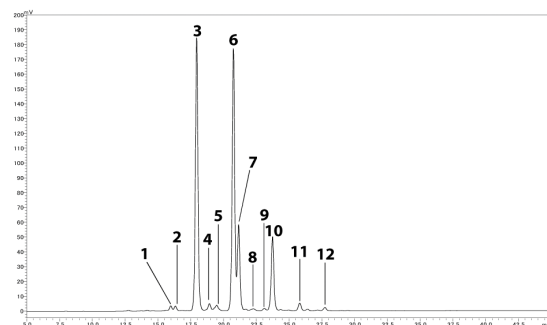
### LC Analysis of *N*-Glycans Labeled by Each Derivatization Condition

Both 2-AB-labeled *N*-glycans and 2-AA labeled *N*-glycans obtained under standard derivatization conditions (65 °C for 3 hrs or 80 °C for 1 hr) and those obtained by derivatization at 37 °C for 18 hours were each dissolved in a 50 % acetonitrile solution and then 2 µL of the solution was analyzed using an HILIC-amide column under the conditions in Table 12.

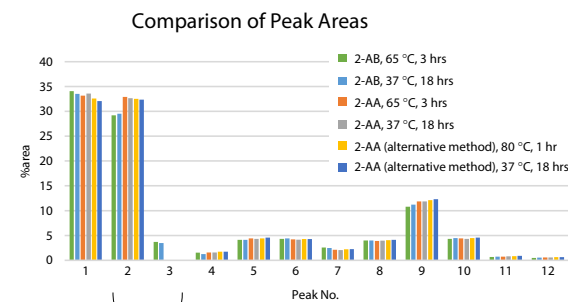
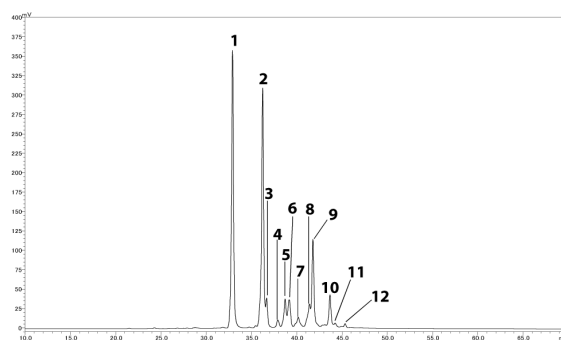
**Table 12 LC Analysis Conditions for Comparison of Derivatization Conditions**

Instrument	: Nexera™ X2
Column	: TSKgel Amide-80, 2.0 µm 150 mm L 2 mm I.D. (Tosoh)
Mobile phase A	: 100 mM Ammonium formate (pH 4.5)
Mobile phase B	: Acetonitrile
Total flow rate	: 0.25 mL/min
Concentration of mobile phase B	: 0 to 2 min: 75 %, 2 to 50 min: 75 → 50 %
Column temp.	: 45 °C
Detection	: Fluorescence Ex 330 nm, Em 420 nm (2-AB) Ex 350 nm, Em 425 nm (2-AA)

As a result of analyzing each of the 2-AB-labeled and 2-AA-labeled samples, for IgG (Fig. 8) and RNase B (Fig. 9), which have mainly neutral *N*-glycans, differences in glycan profile depending on the reaction conditions were only subtle. However, in the case of α1-acid glycoprotein (Fig. 10), which has mainly acidic *N*-glycans containing multiple sialic acids, it was found that in the reactions at 65 °C (2-AB/2-AA labeling) and at 80 °C (2-AA labeling), sialic acids frequently become partially detached as indicated by chromatogram peaks No. 7 and No. 10, unlike the reaction at 37 °C.

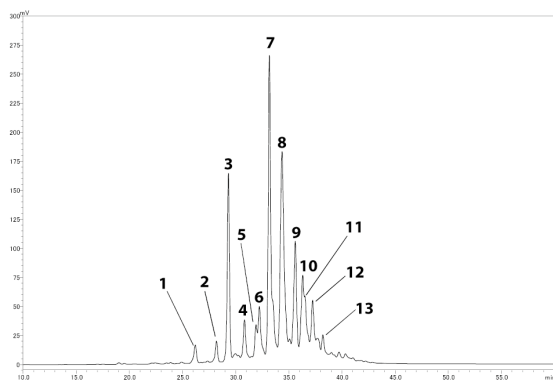


**Fig. 8 Example Fluorescence Chromatogram of IgG-Derived *N*-Glycans (Top) and Comparison of Glycan Peak Areas for Derivatization Conditions (Bottom)**

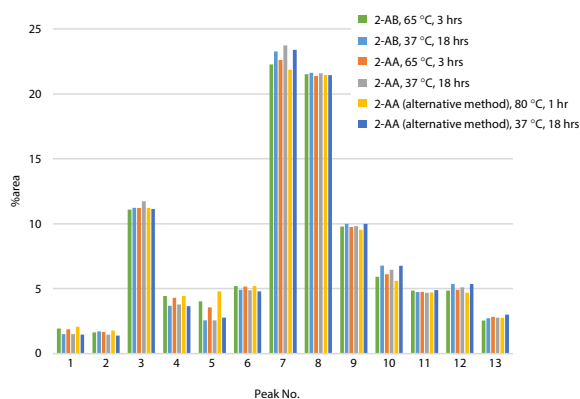


Peaks did not separate when 2AA-labeled *N*-glycans were analyzed using an HILIC-amide column.

**Fig. 9 Example Fluorescence Chromatogram of RNase B-Derived *N*-Glycans (Top) and Comparison of Glycan Peak Areas for Derivatization Conditions (Bottom)**



Comparison of Peak Areas



**Fig. 10 Example Fluorescence Chromatogram of  $\alpha$ 1-Acid Glycoprotein-Derived *N*-Glycans (Top) and Comparison of Glycan Peak Areas for Derivatization Conditions (Bottom)**

### Comparison of Derivatized *N*-Glycan Purification Methods

When derivatizing *N*-glycans such as with a fluorescent labeling reagent, a considerable excess of the labeling reagent and other reagents are used. Therefore, these excess reagents are generally removed after the derivatization reaction. The methods currently used to remove them include solid-phase extraction using hydrophilic interaction, gel filtration and acetone precipitation.

Accordingly, we compared these methods and checked whether the same glycan profile could be obtained with different purification methods.

For this comparison, *N*-glycans released from glycoproteins were purified by solid-phase extraction using an Oasis® HLB 1 cc cartridge and then labeled with 2-AB in a reaction for 18 hours at 37 °C. The methods that were compared are: 1) solid-phase extraction, 2) gel filtration, and 3) acetone precipitation, which have been described above, along with the case where no purification is performed.

The procedures for each purification method are described in Tables 13 to 16.

**Table 13 Excess Reagent Removal by Hydrophilic Interaction Solid-Phase Extraction**

Step	Procedure
1	Prepare an Oasis® HLB 1 cc cartridge with 95 % acetonitrile (1 mL).
2	Add acetonitrile (95 $\mu$ L) to the derivatized glycan solution (5 $\mu$ L), stir well (final conc.: 95 % acetonitrile) and load it into the column.
3	Rinse out the tube in which the derivatized solution was contained with 95 % acetonitrile (0.5 mL) and then load the solution into the column.
4	Wash the column with 95 % acetonitrile (2 mL).
5	Elute the derivatized <i>N</i> -glycan with 20 % acetonitrile (0.5 mL).
6	Dry the eluted solution by centrifugal evaporator at room temperature.

**Table 14 Excess Reagent Removal by Gel Filtration**

Step	Procedure
1	Add water (10 mL) into the PD MiniTrap G-10.
2	After adding water (50 $\mu$ L) to the derivatized glycan solution and mixing, load it into the column.
3	Rinse out the tube for the derivatized glycan solution with water (a volume to make 700 $\mu$ L when combined with the volume of liquid in step 2), and load the solution into the column.
4	Add water (0.5 mL) into the column and collect the eluted solution.
5	Dry the collected solution by centrifugal evaporator at room temperature.

**Table 15 Excess Reagent Removal by Acetone Precipitation**

Step	Procedure
1	Add acetone (1 mL) to the derivatized glycan solution and mix well.
2	Centrifuge at 15,000 $\times$ g for 10 minutes
3	Remove the supernatant by decantation and dry the pellet.

**Table 16 No Removal of Excess Reagent**

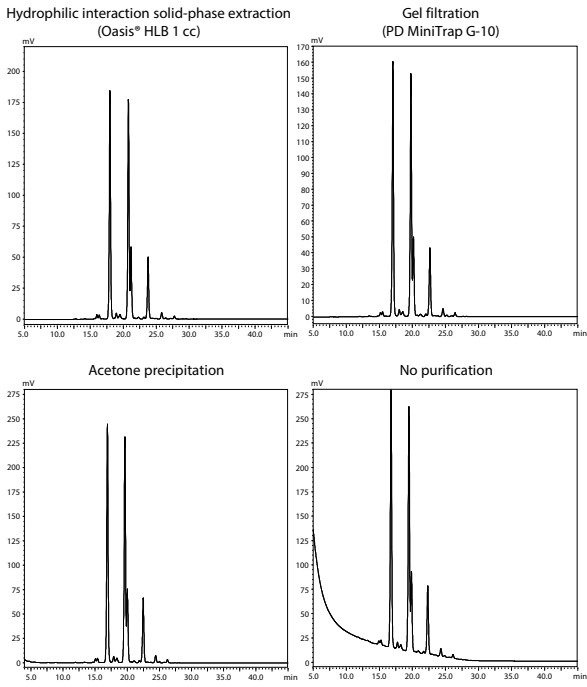
Step	Procedure
1	Dry the derivatized glycan solution by centrifugal evaporator at room temperature.

### LC Analysis of 2-AB-Labeled *N*-Glycans Obtained by Each Derivatized Glycan Purification Method

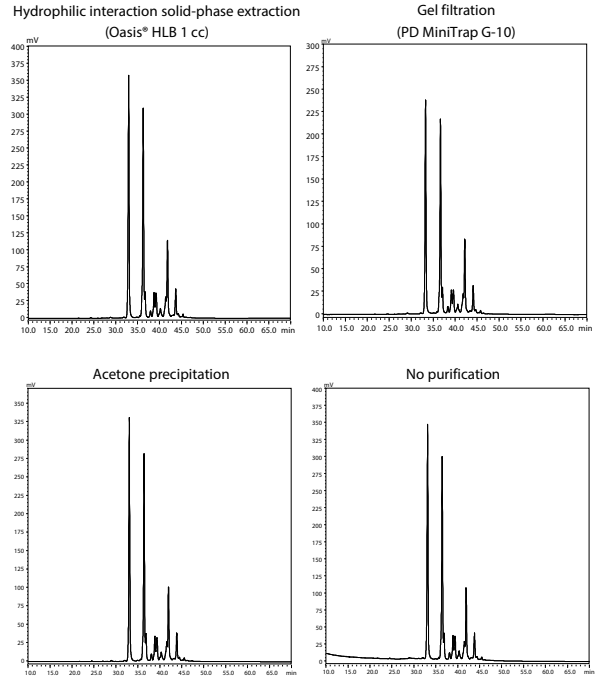
2-AB-labeled *N*-glycans purified through the various derivatized glycan purification methods and unpurified 2-AB-labeled *N*-glycans were each dissolved in a 50 % acetonitrile solution, and 2  $\mu$ L of each sample was subjected to LC analysis under the conditions indicated in Table 17 using an HILIC-amide column (Figs. 11 to 16).

**Table 17 LC Analysis Conditions for Comparison of Derivatization Conditions**

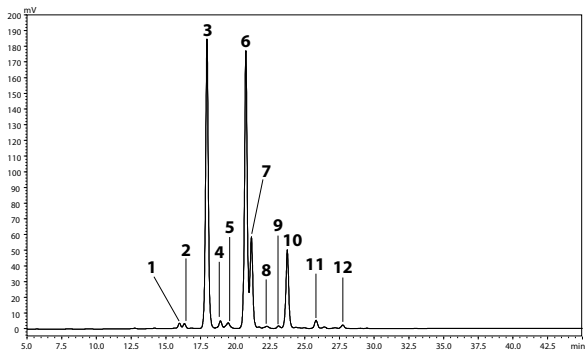
Instrument	: Nexera™ X2
Column	: TSKgel Amide-80, 2.0 $\mu$ m 150 mm L 2 mm I.D. (Tosoh)
Mobile phase A	: 100 mM Ammonium formate (pH 4.5)
Mobile phase B	: Acetonitrile
Total flow rate	: 0.25 mL/min
Concentration of mobile phase B	: 0 to 2 min: 75 %, 2 to 50 min: 75 $\rightarrow$ 50 %
Column temp.	: 45 °C
Detection	: Fluorescence (Ex 330 nm, Em 420 nm)



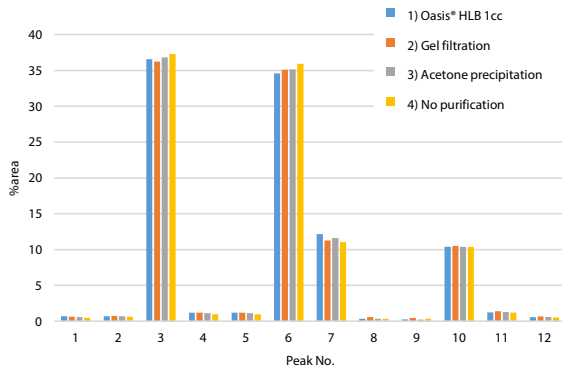
**Fig. 11** Fluorescence Chromatograms of IgG-Derived *N*-Glycans Obtained by Each Purification Method



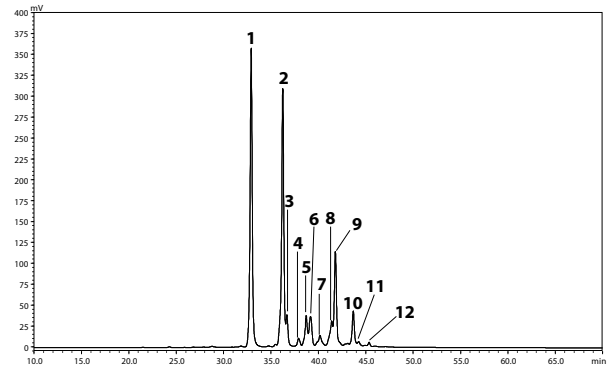
**Fig. 13** Fluorescence Chromatograms of RNase B-Derived *N*-Glycans Obtained by Each Purification Method



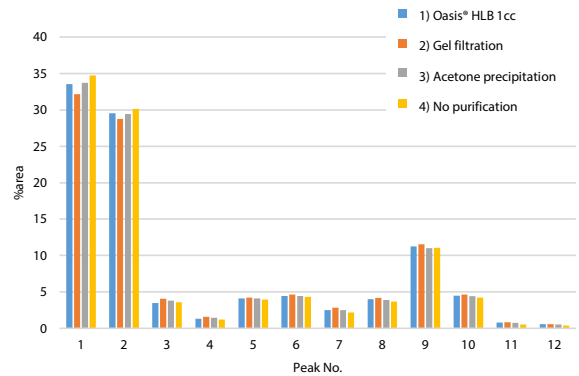
Comparison of Peak Areas



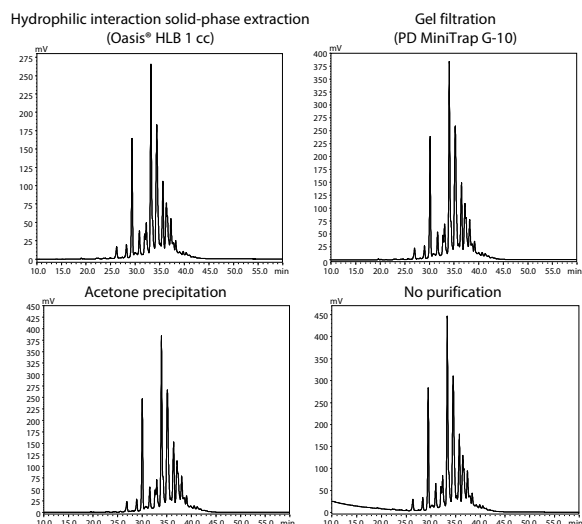
**Fig. 12** Comparison of Glycan Peak Areas for IgG-Derived *N*-Glycans Obtained by Each Purification Method



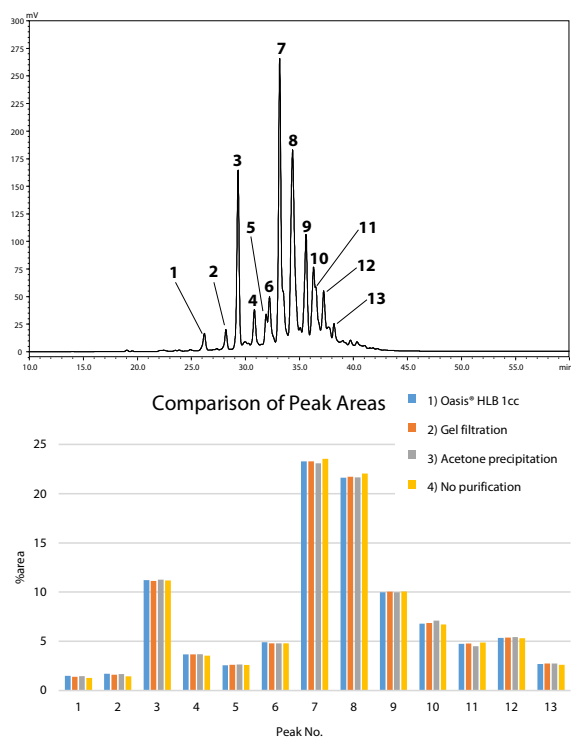
Comparison of Peak Areas



**Fig. 14** Comparison of Glycan Peak Areas for RNase B-Derived *N*-Glycans Obtained by Each Purification Method



**Fig. 15 Fluorescence Chromatograms of  $\alpha$ 1-Acid Glycoprotein-Derived N-Glycans Obtained by Each Purification Method**



**Fig. 16 Comparison of Glycan Peak Areas for  $\alpha$ 1-Acid Glycoprotein-Derived N-Glycans Obtained by Each Purification Method**

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TSKgel is a registered trademark of Tosoh Corporation.

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As a result of analyzing the samples purified using each method, it was found that almost the same glycan profiles were obtained whichever method was used.

#### Conclusion

Three processes are necessary in order to analyze N-glycans released from glycoproteins: purification of the released glycans, derivatization of the glycans, and purification of the derivatized glycans.

Based on the comparison of pretreatment methods undertaken in this study, it is considered that solid-phase extraction using HLB cartridges is the most suitable method, particularly when performing mass spectrometry, since it can avoid the issue of unlabeled glycans being left after derivatization. However, where the glycoprotein sample is desalted in advance, it is likely that purification of free glycans can be omitted without any problem occurring in fluorescent labeling for derivatization. In cases where ultrafiltration has to be used because no other methods can be used for some reason, care must be taken to determine whether the analysis results are influenced by the pretreatment, by running parallel pretreatments using ultrafiltration membranes with different pore sizes, for example.

As for the next process of derivatizing glycans, it is considered possible to obtain the requisite glycan profile while minimizing the elimination of sugars such as sialic acid that are easily lost in pretreatment, by carrying out a reaction over a long time in a low-temperature environment in which derivatization reaction is possible. On the other hand, when handling samples that contain no acidic glycans, the glycan profile will not be significantly affected by a high reaction temperature, so in this case it is also possible to save on the time required for pretreatment by carrying out the reaction over a short time at a high temperature.

Regarding the final process of purifying the derivatized glycans, if a high-density sample is available and the amount of reagent used for the labeling reaction is about the same as that used in this study, analysis is possible without purification. However, it is considered that purification should be carried out if possible due to the likelihood of problems such as baseline drifts in LC analysis. Hydrophilic interaction solid-phase extraction is likely to be the most reliable purification method, but acetone precipitation can also be used as a simple, low-cost purification method.