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Application News

Sialic Acid Stabilizing Kit for Linkage Isomer Discrimination: SialoCapper™-ID Kit

SialoCapper-ID Kit : A Novel Derivatization Tool for Glycan Analysis by Mass Spectrometry

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User Benefits

- Sialic acid linkage types scan be discriminated using only MS analysis without comparison to standard glycan analysis results, which can contribute to laboratory labor-savings.
- Sialoglycan sensitivity is improved, enabling analysis of even trace quantities without wasting precious samples.
- The kit is highly compatible with conventional glycan analysis methods and can be flexibly incorporated into existing experimental procedures.

■ Introduction

Known as the "third chain of life," glycans exist in various forms in the body. Glycosylation of proteins is closely related to the activities of hormones and biopharmaceuticals, as well as viral infections and diseases such as cancer. Consequently analyzing the structure of glycans is essential for understanding biological phenomena and the beneficial effects of pharmaceuticals.

Sialic acids (SA) refer collectively to the family of acidic monosaccharides that are usually the outermost component of a glycan. There are many biological phenomena related to sialic acids. For example, sialic acids play a central role in the influenza virus infection process.

HPLC analysis with fluorescent labeling previously was the gold standard for glycan analysis, but in recent years, highly sensitive and high-throughput mass spectrometry (MS) has been widely used. However, the analysis of acidic glycans with sialic acid residues is more difficult than that of neutral glycans.

This article describes a novel derivatization method and a corresponding new SialoCapper-ID reagent kit for facilitating the MS analysis of glycans (sialoglycans) that comprise sialic acids.

■ Technical Problems in MS Analysis of Sialoglycans

Sialic acid is an acidic monosaccharide with a carboxyl group that generally reduces sensitivity in MS analysis. Since sialic acids are often detached from glycans by decomposition during MS analysis, it is also difficult to accurately determine the number of sialic acids attached. In addition, the carboxyl group often leads to multiple salt formations with multiple *m/z* peaks. As a result, spectra containing sialoglycans with multiple sialic acids become complicated, making it very difficult to interpret quantitative spectra.

Furthermore, sialic acids with the α2,3-/α2,6-linkage isomers have identical mass. Due to the many biological phenomena associated with the difference in linkage isomers, such as the infection mechanism of influenza virus, the discrimination of the linkage isomer is often required in glycan analysis. The discrimination of isomers with the same mass is one of the challenging issues in MS.

■ Sialic Acid Linkage-Specific Derivatization

The neutralization of sialic acids by chemical derivatization solves the problems caused by the presence of sialic acids. By neutralizing carboxyl groups, the loss of sialic acids and multiple peaks caused by multiple alkali metal adduction are suppressed, improving sensitivity and quantification.

We have developed a novel chemical derivatization method that enables not only neutralization of sialic acids, but also the discrimination of the linkage types. Our patented sialic acid linkage-specific alkylamidation technology (SALSA method) reliably neutralizes sialic acids and changes their mass depending on the linkage type, thereby enabling differentiation of sialic acid linkage isomers by MS.[1,2] The SialoCapper-ID Kit is a reagent kit for glycan pretreatment that simplifies the SALSA procedure.

The SALSA method involving the SialoCapper-ID Kit consists of two reaction steps, but the second reaction is very fast and robust, so no additional reaction time and no purification step between the first and second reactions are required. Eventually, α2,6-linked sialic acids are isopropylamidated whereas α2,3-linked sialic acids are methylamidated, so that α2,3-/α2,6-linked sialic acids can be distinguished by a difference of 28 Da (Fig. 1).

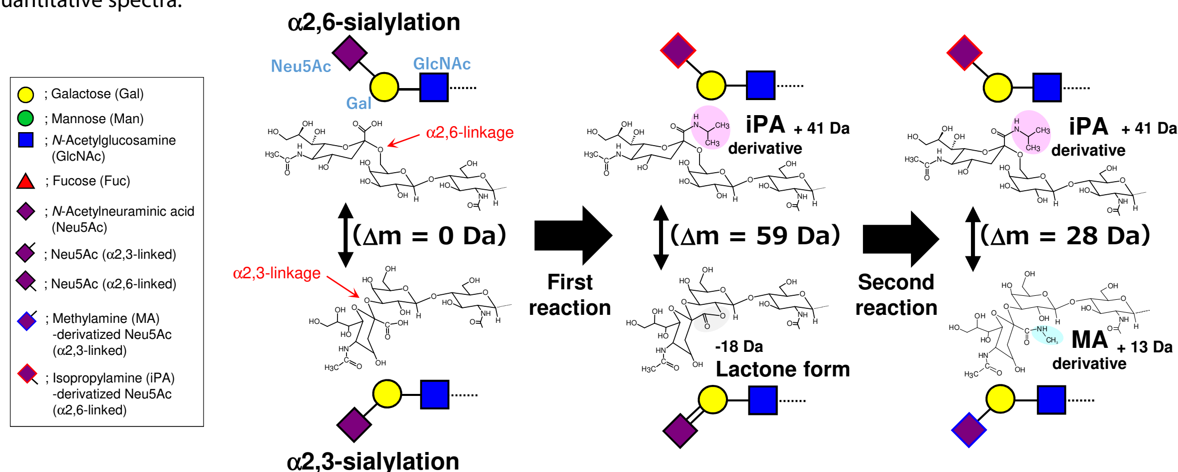


Fig. 1 Derivatization scheme of the SALSA method
The SALSA method that involves using the SialoCapper-ID Kit was developed by Shimadzu Corporation and improved in cooperation with Prof. Jun-Ichi Furukawa and Dr. Hisatoshi Hanamatsu at Hokkaido University.

■ Features of SialoCapper-ID Kit

The SialoCapper-ID Kit has three key features:

A. Simple analysis of sialic acid linkage isomers

B. Improved sialoglycan sensitivity

C. Versatility and scalability

A. and B. relate to the effectiveness of the kit, and C. relates to the usage of the kit.

■ Simple Analysis of Sialic Acid Linkage Isomers

By using the kit, a 28 Da mass difference in α2,3-/α2,6-linked sialic acids allows discrimination of the linkage isomers by MS. Fig. 2 shows the mass spectra of labeled disialyl glycans derivatized by the kit. Since these four glycans are isomers, their original *m/z* values are all the same ([M+Na]$^+$, *m/z* 2323.8), but after the derivatization, each isomer is detected at different *m/z* values depending on the type and number of linkages. The reaction conditions have been optimized so that there are no "unreacted" or "misconverted" forms that could degrade the α2,3-/α2,6-discrimination performance.

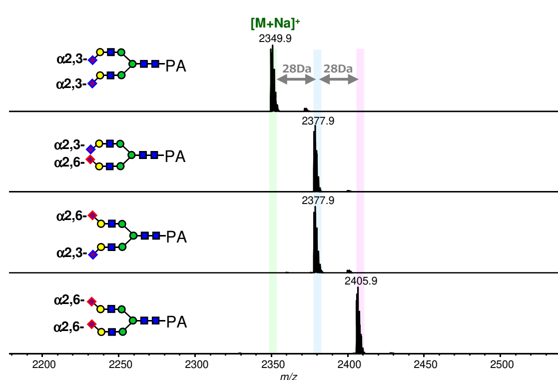


Fig. 2 Mass spectra of labeled glycan standards derivatized by the SialoCapper-ID Kit

Conventional methods for discriminating between sialic acid linkage isomers include 1) preparing a glycan standard material with a known sialic acid linkage type, performing LC/(MS) analysis, matching the LC retention times between the standard and samples, and 2) comparing the analysis results before and after specific sialidase treatments.

Since the SialoCapper-ID Kit is based on chemical reactions, it does not require a glycan standard with a known sialic acid linkage type. This is particularly beneficial when glycan standard materials are difficult to obtain, such as highly complicated glycans with multiple sialic acid residues.

■ Improved Sialoglycan Sensitivity

The neutralization of sialic acids prevents the loss of sialic acid residues during MS analysis and multiple salt addition to carboxyl groups. Consequently, the sensitivity of sialoglycan is improved, the mass spectrum is simplified, and spectral interpretation becomes easier. Quantitativity is also improved, with both neutral and sialylated glycans detected in the same spectrum with similar ionization efficiency.

Fig. 3 compares the mass spectra of *N*-linked glycans (*N*-glycans) derived from bovine fetuin before and after derivatization by the SialoCapper-ID Kit. After the derivatization, the sensitivity is improved and the mass spectrum is simplified by suppressing multiple salt formations.

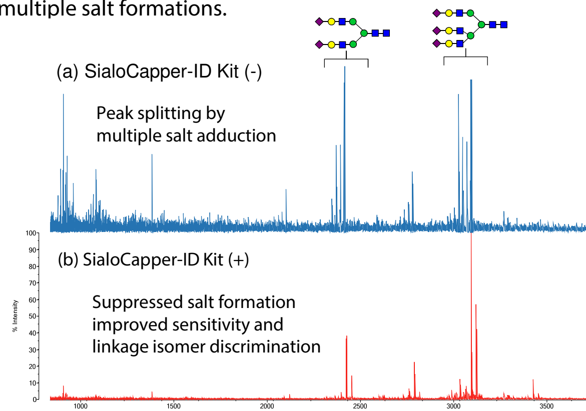


Fig. 3 Mass spectra of *N*-glycans derived from bovine fetuin (a) before and (b) after derivatization by the SialoCapper-ID Kit

■ Versatility and Scalability

The following steps are generally required for MS analysis of glycans attached to proteins.

1) Glycan release

2) Glycan labeling (If necessary)

3) Mass spectrometry

Proper purification may be required between each step. There may be an established protocol in laboratories already involved in analyzing glycans. The SialoCapper-ID Kit supports both liquid-phase and solid-phase reactions in a single kit, allowing labs with established protocols to easily integrate the kit into their analysis protocols for discriminating sialic acid linkage isomers. Because it completely stabilizes both α2,3-/α2,6-linked sialic acids, the kit can also be combined with other enzymatic and chemical reactions. Of course, the kit can be used in combination with the glycan reducing-end labeling commonly used in glycan analysis. Fig. 4 illustrates the general workflow of pretreatment for glycan analysis and the timing of derivatization with the kit.

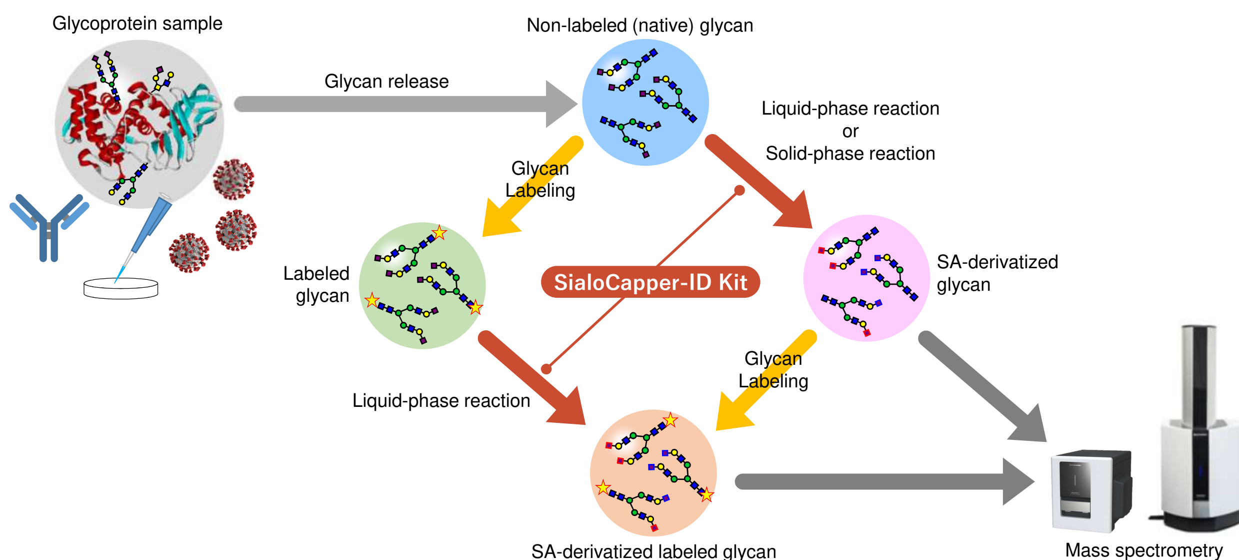


Fig. 4 Workflow of pretreatment for glycan mass spectrometry using the SialoCapper-ID Kit

Fig. 5 Appearance and contents of SialoCapper™-ID Kit

■ Using the SialoCapper-ID Kit

Using the kit (Fig. 5), sialic acid linkage-specific derivatization can be carried out by the following two methods (Fig. 6).

I. Liquid-phase reaction

for derivatizing glycans in a tube

II. Solid-phase reaction

for derivatizing glycans bound to solid supports

The timing of processing with this kit can be flexibly changed according to the condition of the glycan sample and the workflow of glycan analysis.

■ Liquid-Phase Reaction

The kit is normally used for liquid-phase reaction of a dried sample in a tube or dissolved in a small amount of water. It supports sialic acid derivatization of both reducing end labeled and unlabeled glycans. Reagents are dissolved and successively added to the tube according to the instruction manual. There is no need to remove excess reagents between the first and second reactions. In addition, the second reaction is completed instantaneously, so the total reaction time required for all derivatization steps is about 1 hour.

After the derivatization using the kit, purification using an HILIC carrier is needed to remove excess reagents. This carrier is not included in the kit, but any familiar HILIC chip or column may be used. Please refer to the instruction manual for the recommended purification protocol.

■ Solid-Phase Reaction

By combining this kit with glycan purification beads, glycan purification from crude samples and sialic acid derivatization can be performed simultaneously. In addition, it also facilitates the removal of excess reagents, simplifying the experimental workflow and contributing to experiment labor-savings.

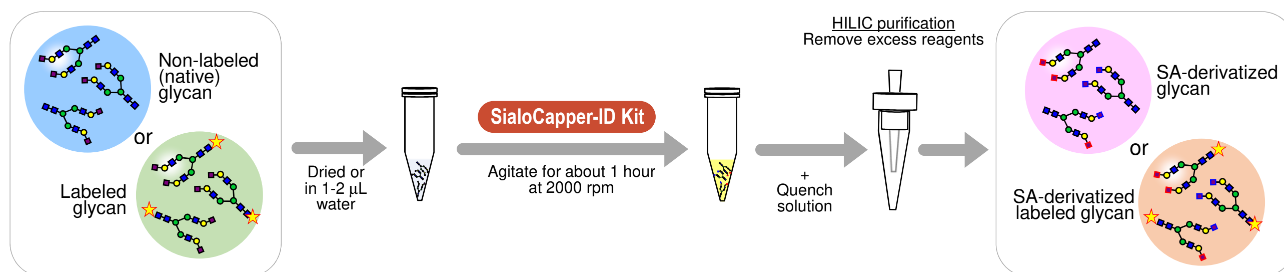
BlotGlyco® (Sumitomo Bakelite Co., Ltd.) beads are a glycan purification beads that can selectively bind to the reducing end of glycans. Sialic acid linkage-specific derivatization using this kit works well for the glycans captured on the beads. After releasing glycans from BlotGlyco® beads, any reducing end derivatization method can be carried out before MS analysis.

Since BlotGlyco® beads selectively bind to the reducing end of glycans, the solid-phase reaction cannot be applied to glycans that are already labeled, but it provides a highly robust solution for unlabeled (native) glycans. BlotGlyco® beads are highly selective for glycans, so contaminants can be effectively removed before the sialic acid derivatization. That is why solid-phase reaction is "highly robust."

■ Required but Not Included in the Kit

The kit contains only the reagent chemicals for the sialic acid derivatization. It does not include HILIC carriers for removing excess reagents, glycan purification beads (BlotGlyco®), or solutions required for the purification process. Be sure to prepare such necessary supplies separately. For details, refer to the instruction manual.

□ Liquid-phase reaction



□ Solid-phase reaction

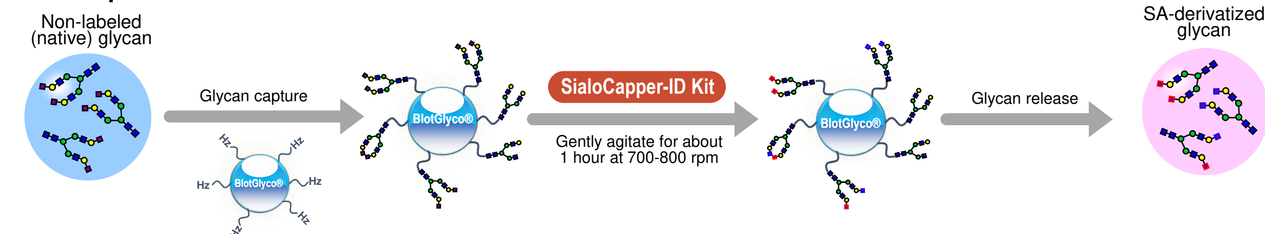


Fig. 6 Scheme of the liquid-phase/solid-phase reaction using the SialoCapper-ID kit

[Precautions for liquid-phase reaction]

- Before derivatization, it is recommended to dry up glycan samples in a tube. Glycan samples dissolved in a small amount of water are also acceptable, but may lead to unreacted and/or misconverted forms. Acceptable range: 1 to 2 µL (max. 5 µL) of water per tube.
- Contamination by nucleophiles such as ammonia, methylamine, or methanol can cause side reactions. If there is a risk of contamination, it is recommended to desalt and purify glycans with a glycan purification chip/column such as carbon or HILIC carrier, prior to the sialic acid derivatization.
- The SialoCapper-ID Kit also amidates the –COOH group on the labeled moiety of anthranilic acid (AA)-labeled glycans. To obtain 2AA-labeled glycans, please derivatize glycans with a SialoCapper-ID kit before 2AA-labeling.

[Number of samples]

- The kit contains reagents sufficient for 10 experiments. The 10 tubes of Reagent B are single-use and cannot be stored after dissolution. Since 1 tube of Reagent B can process up to 50 samples for liquid-phase reaction and 10 samples for solid-phase reaction, 1 kit can process up to 500 samples for liquid-phase and 100 samples for solid-phase reaction.

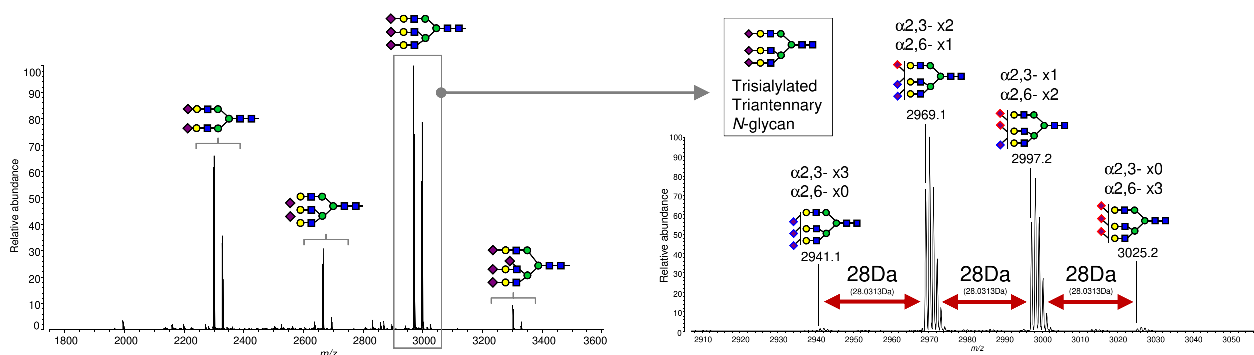


Fig. 7 Mass spectra of *N*-glycans derived from bovine fetuin derivatized with the SialoCapper-ID Kit

■ Mass Spectrometry

For the measurements of glycans treated with the SialoCapper-ID Kit, any type of mass spectrometer can be used. Because the α2,3-/α2,6-linkage types can be discriminated based on mass, chromatographic separation is not necessary. Therefore, samples can be analyzed by matrix-assisted laser desorption /ionization mass spectrometry (MALDI-MS), which does not usually involve chromatographic separation.

■ Example and Interpretation of Mass Spectra

Fig. 7 shows a typical example of a mass spectrum obtained with the kit (*N*-glycans derived from bovine fetuin). Fetuin has many sialic acids with both α2,3-/α2,6-linkages.

When a glycan has multiple sialic acids with α2,3-/α2,6-linkages, glycan peaks with differences of 28 Da can appear as clusters originating from a group of sialic acid linked isomers. In the case of triantennary glycans with three sialic acids, four peaks may be detected from the combination of α2,3-/α2,6-linkages (Fig. 7, right). The peak intensity ratio of these 28 Da intervals in the mass spectrum reflects the α2,3-/α2,6-ratio of sialic acids.

■ Software for Analyzing Mass Spectra

The first step in analyzing glycan mass spectra is to estimate the monosaccharide composition of glycans from the obtained m/z values. However, derivatization using the SialoCapper-ID Kit changes the mass of the sialic acid residues and also generates mass variations according to the linkage type. Therefore conventional software cannot support results of such sialic acid linkage-specific derivatizaion.

The Supporting Tool for SialoCapper-ID Kit software developed is glycan composition estimation software that can deal with the mass changes of sialic acid residues by sialic acid derivatization.

The glycan composition candidates for each *m/z* (monoisotopic) are calculated and presented by a brute-force search based on input parameters (tolerance, upper and lower limits of each monosaccharide, pretreatment method, detectable ion species, etc.) (Fig. 8).

■ Example: Serum Glycan Analysis by MALDImini™-1

The following is an example of analyzing *N*-glycans derived from serum glycoproteins. The *N*-glycans were derivatized with the SialoCapper-ID Kit and detected with a Shimadzu MALDImini-1 compact MALDI digital ion trap (MALDI-DIT) mass spectrometer (Fig. 9).

The blood serum used in this study is a commercial product for research purposes. First, 5 µL of the serum was denatured and reduced with SDS (sodium dodecyl sulfate) and DTT (dithiothreitol). After adding NP-40 (Nonidet P-40), PNGaseF (peptide-N-glycosidase F) was added, and *N*-glycans were released from the glycoprotein by reacting the solution at 37 °C for 18 hours.

Of the solution of *N*-glycans released from the glycol-proteins, 4 µL was mixed directly with reaction solution from the SialoCapper-ID Kit (liquid-phase reaction). The excess reagents were then removed using GL-Tip Amide (GL Sciences Inc.).

After sialic acid linkage-specific derivatization, the reducing end of the glycans was labeled with 2-aminobenzamide (AB) and the excess reagents were removed with GL-Tip Amide.

The sample solution (0.5 µL) was placed on a MALDI target plate and dried by overlaying the matrix solution (0.5 µL). Then MS^n analysis was carried out with the MALDImini-1. The matrix used in this example was CHCA (α-cyano-4-hydroxycinnamic acid) with added sodium chloride. For details concerning MALDImini-1, refer to Application News No. B100.

Fig. 9 Appearance of MALDImini™-1 Compact MALDI-DIT mass spectrometer

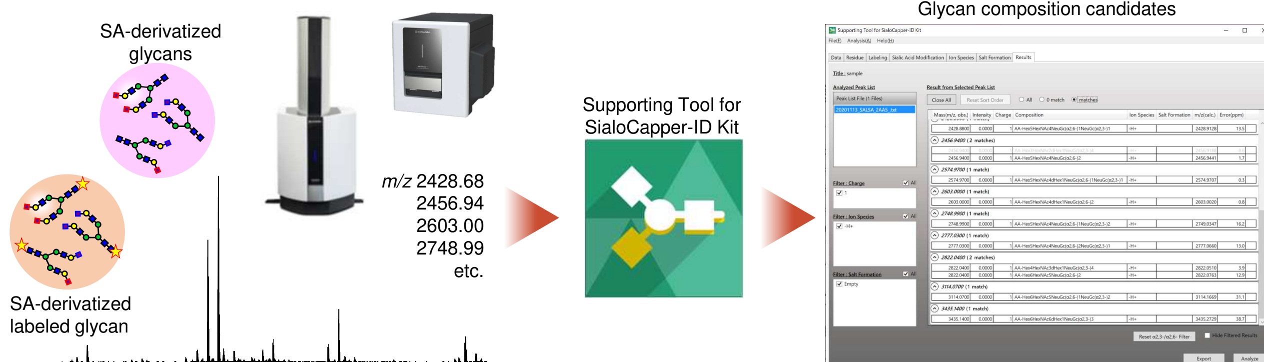


Fig. 8 Process flow for mass spectral analysis using the Supporting Tool for SialoCapper-ID Kit

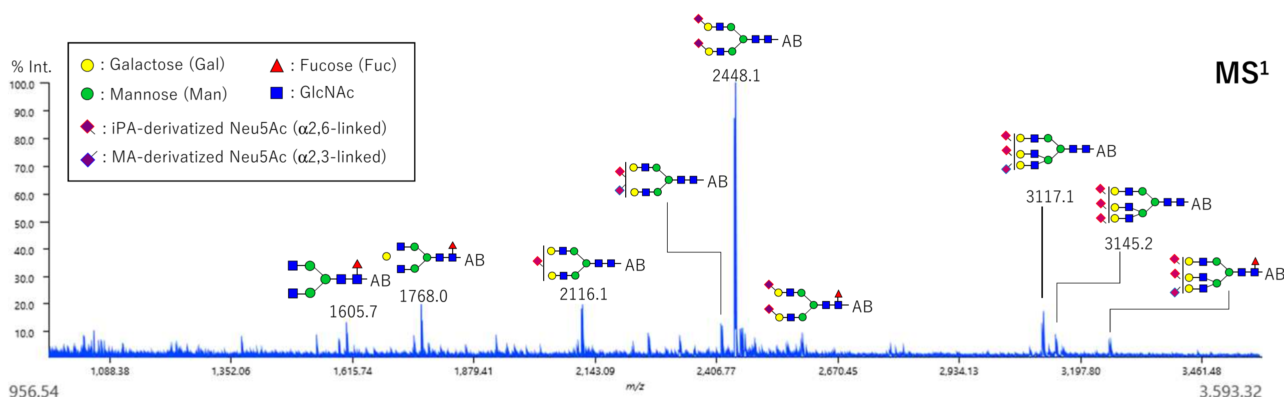


Fig. 10 MALDImini-1 mass spectrum (MS^1) of glycoprotein-derived *N*-glycans obtained from blood serum

■ MS^1 and MS^2 Analysis of Blood Serum *N*-Glycans

Various types of *N*-glycans, including bi- and tri-antennary *N*-glycans, were detected from blood serum glycoproteins (Fig. 10).

Since the two *N*-glycans detected at *m/z* 3117.1 and 3145.2 have a difference of 28 Da, they are presumably linkage isomers that only differ in terms of α2,3-/α2,6-linkages. According to the analysis results from the Supporting Tool for SialoCapper-ID Kit software (Table 1), the glycan composition of *m/z* 3117.1 was uniquely determined, whereas two candidates, A and B were calculated for *m/z* 3145.2. Considering the biosynthetic pathways of *N*-glycans, candidate B was more plausible. To support this assumption we obtained MS^2 mass spectra (Fig. 11). MS^2 experiments confirmed the sialic acid linkage types contained in the glycans based on the mass of the neutral losses corresponding to the derivatized sialic acid residues. We found that *m/z* 3117.1 corresponds to a mixture of α2,3-/α2,6-linkages and *m/z* 3145.2 only to α2,6-linkages (candidate B).

Supporting Tool for SialoCapper-ID Kit software has a unique filtering function named α2,3-/α2,6-Filter. The filter uses a constraint condition focused on the number of sialic acid residues and their linkage types. Using the α2,3-/α2,6-Filter, we were able to exclude A as a candidate.

Table 1 Monosaccharide composition candidates of serum *N*-glycans calculated using Supporting Tool for SialoCapper-ID Kit.

m/z (obs.)	*m/z* (calc.)	Glycan Composition	
1605.7	1605.602	AB-Hex3HexNAc4dHex1	
1768.0	1767.655	AB-Hex4HexNAc4dHex1	
2116.1	2115.808	AB-Hex5HexNAc4NeuAc(α2,6-)1	
2420.1	2419.935	AB-Hex5HexNAc4NeuAc(α2,6-)1NeuAc(α2,3-)1	
2448.1	2447.967	AB-Hex5HexNAc4NeuAc(α2,6-)2	
2594.1	2593.914	AB-Hex10HexNAc4	
2594.1	2593.999	AB-Hex5HexNAc2NeuAc(α2,3-)4	
2594.1	2594.025	AB-Hex5HexNAc4dHex1NeuAc(α2,6-)2	
3117.1	3117.226	AB-Hex6HexNAc5NeuAc(α2,6-)2NeuAc(α2,3-)1	
3145.2	3145.147	AB-Hex10HexNAc3dHex1NeuAc(α2,3-)2	A
3145.2	3145.257	AB-Hex6HexNAc5NeuAc(α2,6-)3	B
3263.1	3263.284	AB-Hex6HexNAc5dHex1NeuAc(α2,6-)2NeuAc(α2,3-)1	

SialoCapper is a trademark of Shimadzu Corporation.
BlotGlyco is a trademark of Sumitomo Bakelite Co., Ltd.

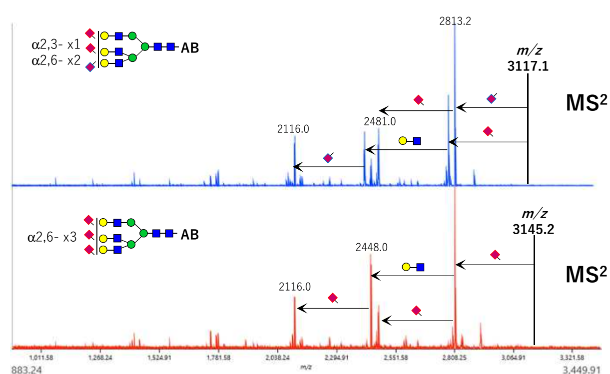


Fig. 11 Comparison of MS^2 mass spectra of triantennary glycans detected at *m/z* 3117.1 and *m/z* 3145.2 in MS^1 mass spectrum. The linkage types can be discriminated based on the mass of the neutral loss of derivatized sialic acid residues.

■ Conclusion

The SialoCapper-ID kit is a sialic acid linkage-specific derivatization kit for MS analysis of glycans that supports both liquid-phase and solid-phase reaction procedures. The kit enables the discrimination between different sialic acid linkage isomers by MS and also helps increase the sensitivity and improve the quality of mass spectra by stabilizing sialic acid residues. Due to the resulting ease of mass spectral interpretation, it is even beneficial when sialic acid linkage isomer discrimination is not required. We hope that the kit will be useful as a new derivatization tool for glycan MS analysis. For other application examples using this kit, refer to Application News (01-00109, 01-00110, 01-00111 etc.).

■ Acknowledgments

We would like to express our gratitude to Prof. Jun-Ichi Furukawa and Dr. Hisatoshi Hanamatsu at Hokkaido University, who have been involved in the improvement of the SALSA method.

<References>

1) Nishikaze T, et al. (2017) Differentiation of Sialyl Linkage Isomers by One-Pot Sialic Acid Derivatization for Mass Spectrometry-Based Glycan Profiling. *Anal Chem* 89: 2353-2360.
2) Hanamatsu H, et al. (2018) Silaic acid Linkage Specific Derivatization of Glycosphingolipid Glycans by Ring-Opening Aminolysis of Lactones. *Anal Chem* 90: 13193-13199.

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