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Mass Spectrometry-based Identification of Transferrin Glycosylation Variants for Diagnosis of Congenital Disorders of Glycosylation

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ABSTRACT

Congenital disorders of glycosylation (CDG) are a group of metabolic conditions resulting from impaired glycosylation of proteins and/or lipids. Transferrin, a vital glycoprotein present in blood plasma, plays a significant role in our research. We aim to tackle the diagnostic challenges associated with CDG by focusing on transferrin as a key protein for study. Several techniques are used for the quantification of human transferrin, each offering valuable insights into protein levels. While these methods can be highly sensitive, they often fall short in terms of specificity and reproducibility. In contrast, Liquid Chromatography-Mass Spectrometry (LC-MS/MS) offers a more reliable and precise approach, even at low concentrations, making it a superior tool for transferrin analysis in CDG research.

In this study, we present: (i) an efficient and rapid method for purifying transferrin from human serum using rivanol, (ii) a comparative analysis of various techniques for screening CDG samples, including Isoelectric Focusing (IEF), 2D gel electrophoresis, MALDI, and LC-MS/MS, with LC-MS/MS proving to be the most reliable method, and (iii) an LC-MS/MS-based proteomics assay to quantify transferrin isoform levels in human serum, utilizing specific glycosylated peptides. We identified significant biomarker peptides with glycosylation variants, and their relative levels were used for confirmatory diagnosis of CDG. Early and accurate diagnosis of CDG is crucial for the timely initiation of appropriate therapies, which can greatly enhance clinical outcomes. We aim to validate these findings further in our ongoing research using a larger cohort of samples to develop a fast and reliable diagnostic test for CDG.

Keywords: *Transferrin; Congenital Disorders of Glycosylation; Iso-Electric Focusing; MALDI-TOF; LCMSMS*

ABBREVIATIONS:

CDG: Congenital Disorders of Glycosylation; DTT: Dithiothreitol; IDA: Iodoacetamide; IEF: Iso-Electric Focusing; IPG: Immobilized pH Gradient; LC-MS/MS: Liquid chromatography-tandem mass spectrometry; MALDI-TOF: Matrix Assisted Laser Desorption/Ionization Time-of-Flight; PTM: Post Translational Modifications; SDS-PAGE: Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis; TF: Transferrin

1. INTRODUCTION

Congenital disorders of glycosylation (CDG), previously referred to as carbohydrate-deficient glycoprotein syndrome, encompass a spectrum of over 130 inherited metabolic disorders (1). These disorders affect various stages of the glycosylation pathway involved in modifying proteins. Consequently, abnormal glycoproteins are formed, impairing their structure and metabolic functions (2). Many proteins undergo post-translational modifications (PTMs) to regulate their structure and function, glycosylation emerges as a pivotal PTM influencing diverse physiological processes and associated phenotypes. Moreover, glycosylation is increasingly recognized as a promising biomarker (3).

Transferrin, a vital glycoprotein present in blood plasma, plays a significant role in our research. Transferrin typically carries tetra-antennary carbohydrate chains at two sites N-432 and N-630 (Fig.1), each terminated with four sialic acid residues (15). The least sialylated isoforms of TF, categorized as carbohydrate-deficient transferrin, include those with zero (asialoTF), one

(monosialoTF), and two (disialoTF) and three (TrisialoTF) sialic acids (1). Diagnostically, CDG Type-I is typified by an increase in di- and/or asialotransferrin, while CDG Type-II is characterized by elevated levels of tri-, di-, mono-, and/or asialotransferrin.

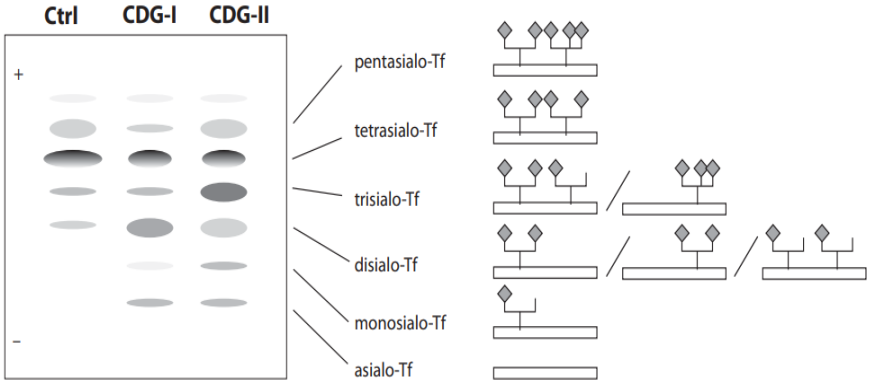


Figure 1: Isoforms of transferrin (Sandra Supraha Goreta et al)

Isoelectric focusing (IEF) of transferrin (Tf) remains one of the simplest and most commonly used methods for screening CDG (1). The advent of high-throughput technology platforms has enabled the identification of molecular alterations associated with CDG. Mass spectrometry-based proteomics stands out for its ability to globally identify and quantify proteins and glycoproteins in an unbiased manner. IEF provides clarity and accuracy (4) but by employing a sensitive and reliable mass spectrometry assay, (5) we endeavor to develop validated diagnostic markers for different types of CDG. This approach holds promise for enhancing the precision and effectiveness of CDG diagnosis, ultimately improving patient outcomes.

2. MATERIALS AND METHODS:

This prospective study involves four serum samples from human participants and it has been approved by the S2J Independent Ethics Committee (S2J IEC – ECR/284/Indt/AP/2017/RR-20) and with the informed consent of the participants.

2.1: Purification of Transferrin:

In modification to the purification protocol from *H Arefanian et al* (6) and *Walter E. Roop et al* (7), we have developed an efficient method for the purification of transferrin. To 100ul of serum, 3% rivanol was added. This was mixed gently and incubated at 4°C for 10min. Centrifuged at 10,000rpm for 10min at 4°C and supernatant (Sup-1) was collected into a tube containing 25mg of NaCl. This was incubated at 4°C for 10min and centrifuged at 10,000rpm for 10min at 4°C. To supernatant (Sup-2) equal volumes of saturated ammonium sulphate solution was added. Incubation was done at RT for 10 minutes and centrifuged at 10,000rpm for 10 minutes. The supernatant (Sup-3) was collected and TCA-acetone precipitation was carried out. The protein pellet was dissolved in 0.1% SDS and 50 mM NH_4HCO_3. The purified transferrin was loaded onto SDS-PAGE gel. The gel was silver stained to observe the bands.

Two-dimensional gel electrophoresis (8) was also carried out to check the purity of transferrin and glycosylation pattern. 300µg protein was added to the rehydration buffer and loaded onto an 18cm, pH 3-10 IPG Strip. After the first dimension, the strip was loaded onto SDS-PAGE for separation according to the molecular weight. The gels were coomassie stained and silver stained to observe the spots (9).

2.2: MALDI-TOF for identification of Transferrin

The purified transferrin gel band from SDS-PAGE and 2D spots was destained and treated with dithiothreitol (DTT) and iodoacetamide (IDA) for reduction and alkylation. Trypsin digestion was carried out overnight at 37^0C. Extraction of peptides was carried out and was analyzed on a MALDI-TOF-TOF mass spectrometer (Ultraflex III, Bruker Daltonics) to obtain a Peptide Mass Fingerprint (PMF). The sample spectrum (m/z) obtained was analyzed using flex control software (Bruker Daltonics). The processed spectrum was used for protein identification using MASCOT database search.

2.3: Electrophoresis of the purified Transferrin

To check the glycosylation pattern, the serum was loaded onto the IEF strips and IEF gels (10, 11). Neuraminidase-treated normal serum was used as a marker. After the IEF run, the gels were incubated with Polyclonal Rabbit Anti-Human Transferrin Antibody (DAKO Cat# A0061) (12) and were further silver stained to observe the bands.

2.4: LCMS Analysis for Identification of Specific Glycosylated Peptides

LCMSMS analysis was carried out to check the glycosylation pattern of transferrin (5, 13, 14). 100µg of the sample was

taken for digestion. The sample was diluted with 50mM NH4HCO3 and treated with 100mM dithiothreitol (DTT) at 95°C for 1hr followed by 250mM iodoacetamide (IDA) at room temperature in dark for 45min. The sample was then digested with trypsin (protein: trypsin = 20:1) and incubated overnight at 37˚C. The resulting sample was vacuum-dried and dissolved in 50µl of 0.1% formic acid in water. After desalting, 10µL of each sample was injected into the Waters BEH C18 UPLC column to separate peptides with buffer-A as 0.1% formic acid in water and buffer-B as 0.1% formic acid in Acetonitrile. The chromatographic conditions for an 80-minute run include a flow rate of 0.3ml/min, column temperature of 40°C, and gradient with initial B%-2, 45min B%-50, 70min B%-80, and 80min B%-2. The peptides separated on the column were directed to the Waters Xevo-G2 XS Q-TOF instrument for MS and MSMS analysis. The raw data was processed using MassLynx software version 4.1 from WATERS. The individual peptides' MSMS spectra were matched to the database sequence for protein identification on PLGS software, WATERS. Bioinformatic analysis was carried out with peptide tolerance of 10ppm, fragment tolerance of 20ppm, carbamidomethyl C as fixed modification, oxidation M and N-Glycosylation as variable modifications, and missed cleavages were allowed up to 1. The glycosylation pattern at **432** and **630** sites was checked for each sample. Extracted ion chromatograms were taken for glycosylated peptide masses for asialo, monosialo, and disialo and checked for area and intensity.

Expected aminoacid sequence after trypsin digestion:

432: CGLVPVLAENYNK

630: QQQHLFGSNVTDCSGNFCLFR

3. RESULTS AND DISCUSSIONS:

3.1: Purification of Transferrin

3.1.1: SDS-PAGE:

A single band of purified transferrin was observed at 80KDa (Fig.2 and Fig. 3).

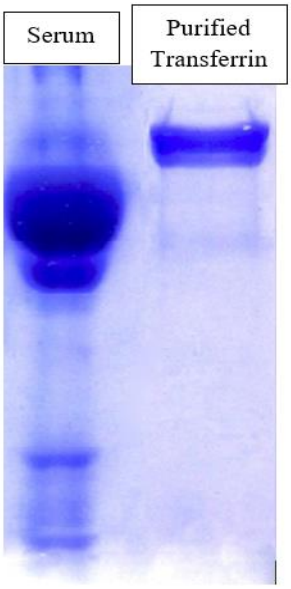


Figure 2: Coomassie-stained gel image showing profiles of normal serum and purified transferrin

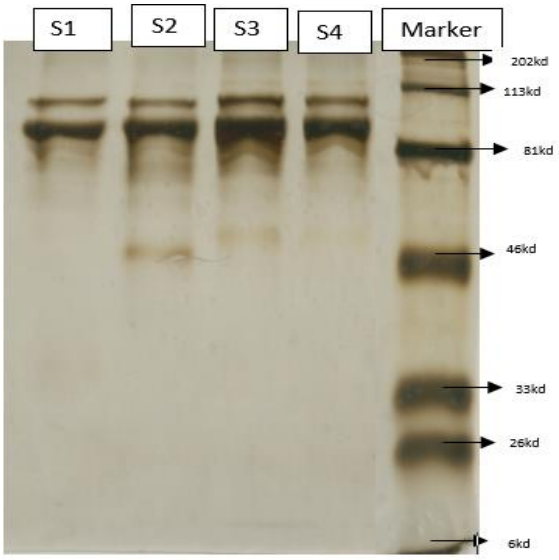


Figure 3: Silver stained gel image of purified transferrin profile of 4 samples

3.1.2 2D Gel Electrophoresis:

The difference in 2D gel profiles before and after the purification of transferrin protein from serum shows the efficiency of the purification procedure. (Fig. 4)

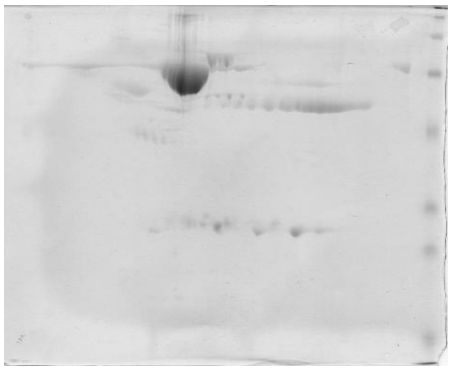
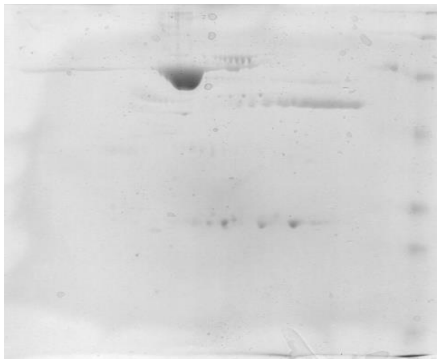
Normal sample before purification	Suspected sample before purification
	
Normal sample after transferrin purification	**Suspected sample after transferrin purification**
	

Figure 4: 2D gels of normal and suspected serum before and after transferrin purification

3.2: MALDI for Identification of Transferrin Protein

MALDI PMF (Fig. 5) was performed to confirm the purification of transferrin protein. The identification was confirmed by MASCOT search (Fig. 6)

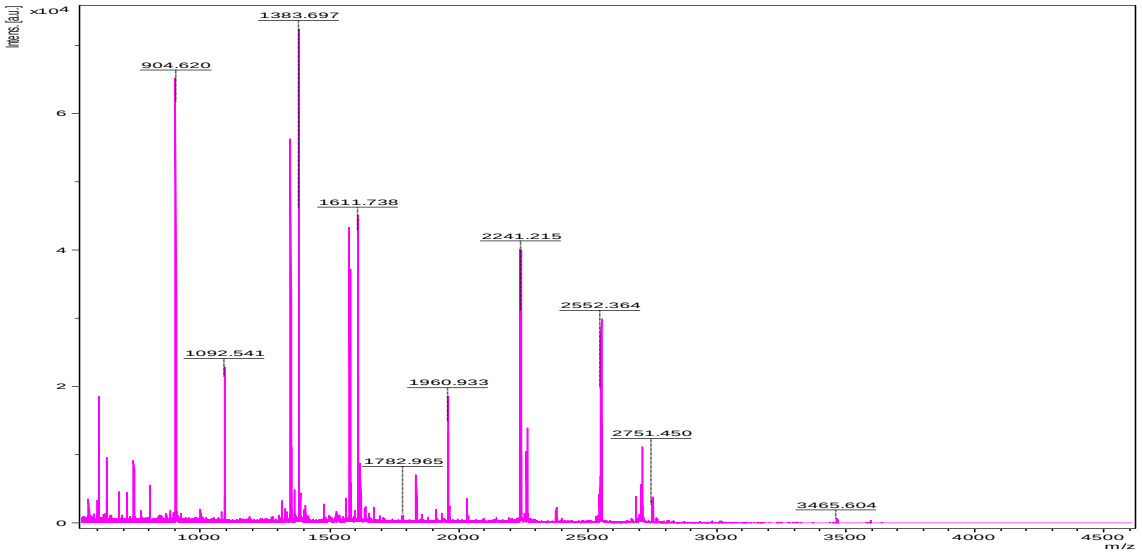


Figure 5: MALDI PMF Spectrum

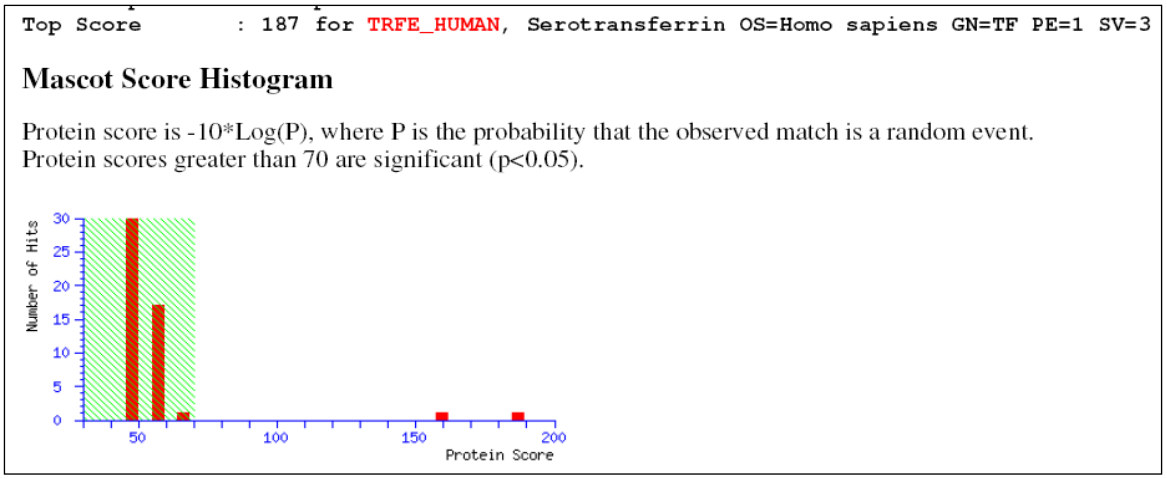


Figure-6: Mascot Search Page

3.3: Iso Electric Focusing

3.3.1: Separation on IPG Strip

Isoelectric focusing of the purified transferrin samples showed bands in the range of pH-5.5 to 6.5 and differences in the intensity of bands of various glycoforms (Fig. 7)

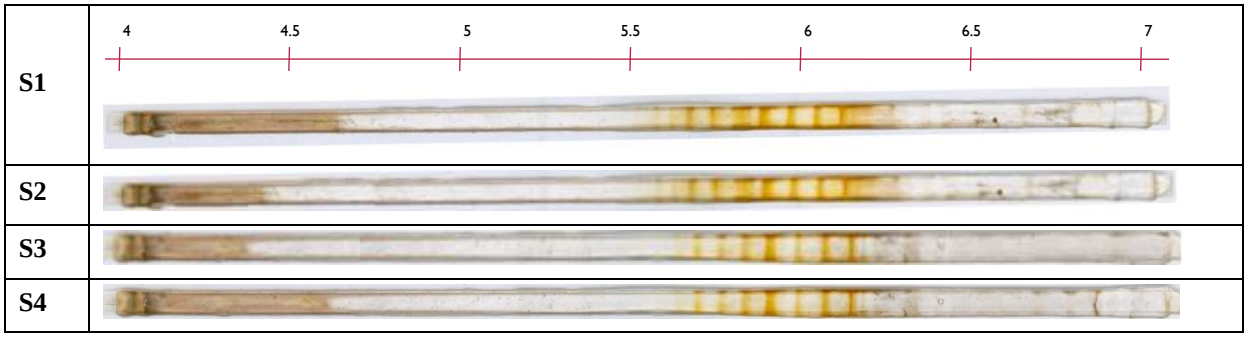


Figure-7: IEF Profile on 4-7pH gradient Strips-Silver Stained

3.3.2: Separation on IEF Gels

The profile of 4 samples in IEF gels showed a difference in the pattern of glycoforms (Fig. 8).

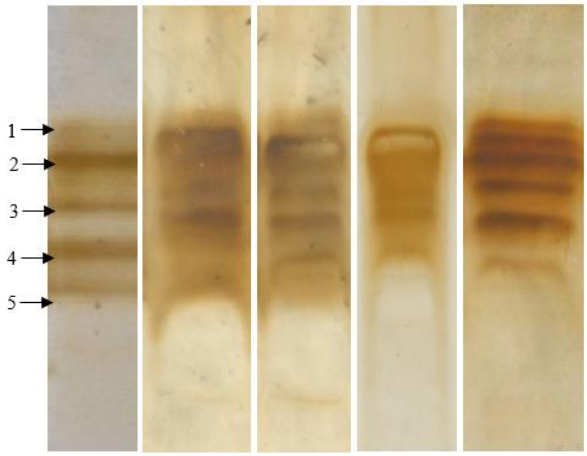


Figure-8: Profile of 4 samples on IEF Gels-Silver stained

Lane-1: Marker; Lane-2: Sample-1; Lane-3: Sample-2; Lane-4: Sample-3; Lane-5: Sample-4

3.4: LCMSMS

The identification of transferrin protein was carried out using the raw data generated by LC-MS/MS. The identification showed transferrin protein for tryptic peptides as shown in Fig. 9. The representative profiles of the LCMS Chromatogram (Fig. 10), MS Spectrum (Fig. 11), and MSMS Spectrum (Fig. 12) were shown. Relative quantification of the glycosylated peptides was carried out.

Accession	Entry	Description	mW (Da)	pI (pH)	PLGS Score	Peptides
P02787	TRFE_HUMAN	Serotransferrin OS=Homo sapiens OX=9606 GN=TF PE=1 SV=4	76999	6.8	20560.7	262

Figure 9: Protein Identification using PLGS software (Waters)

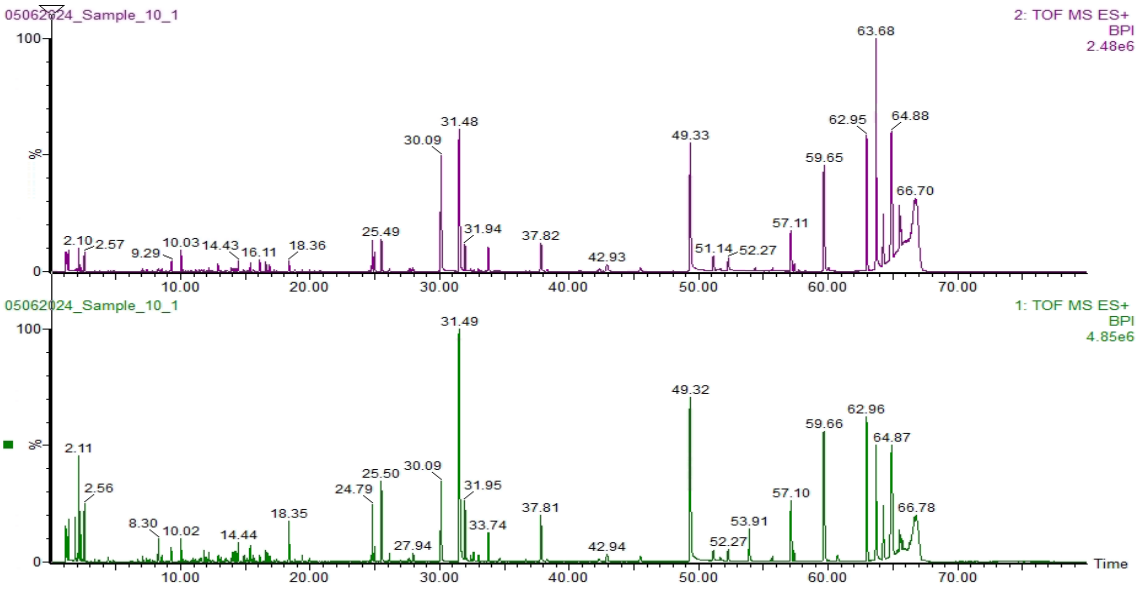


Figure 10: Representative Chromatogram of Purified Transferrin

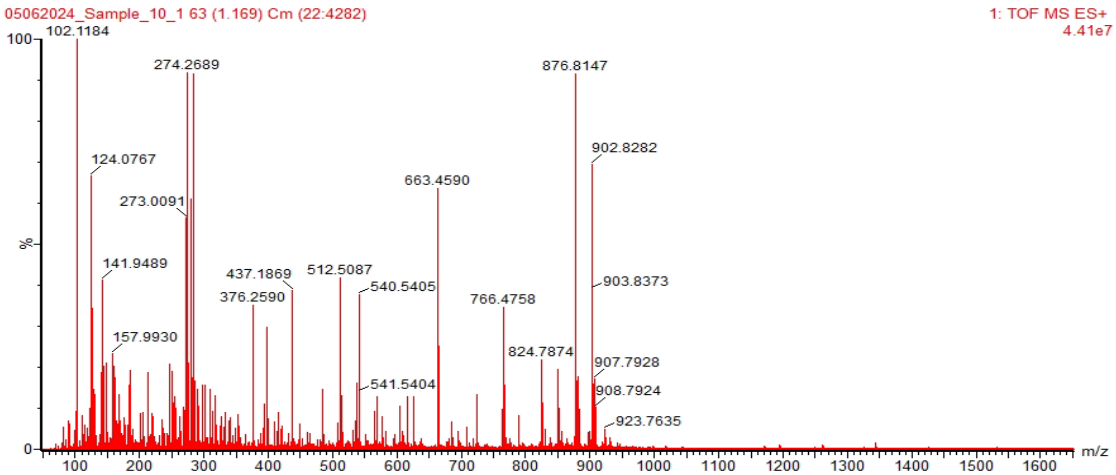


Figure 11: MS Spectrum

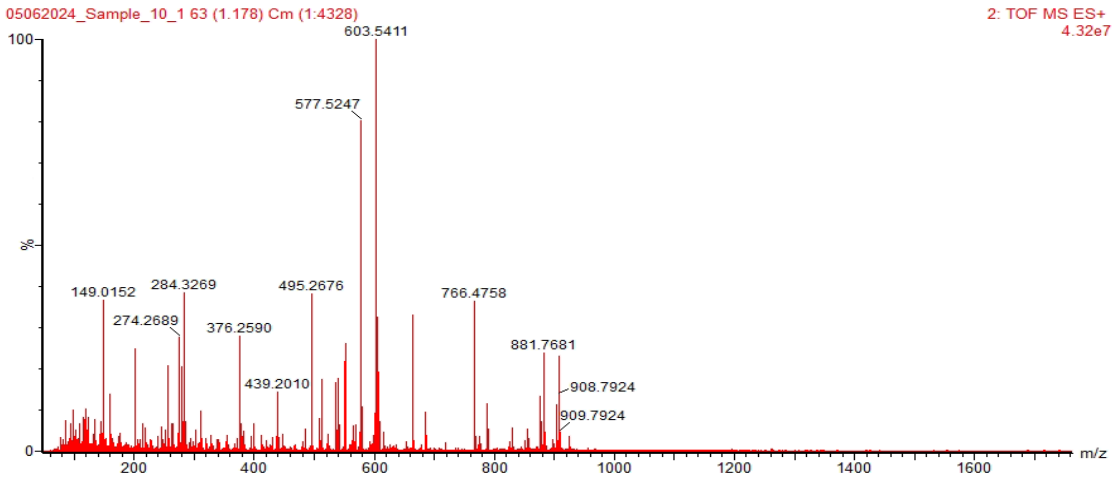
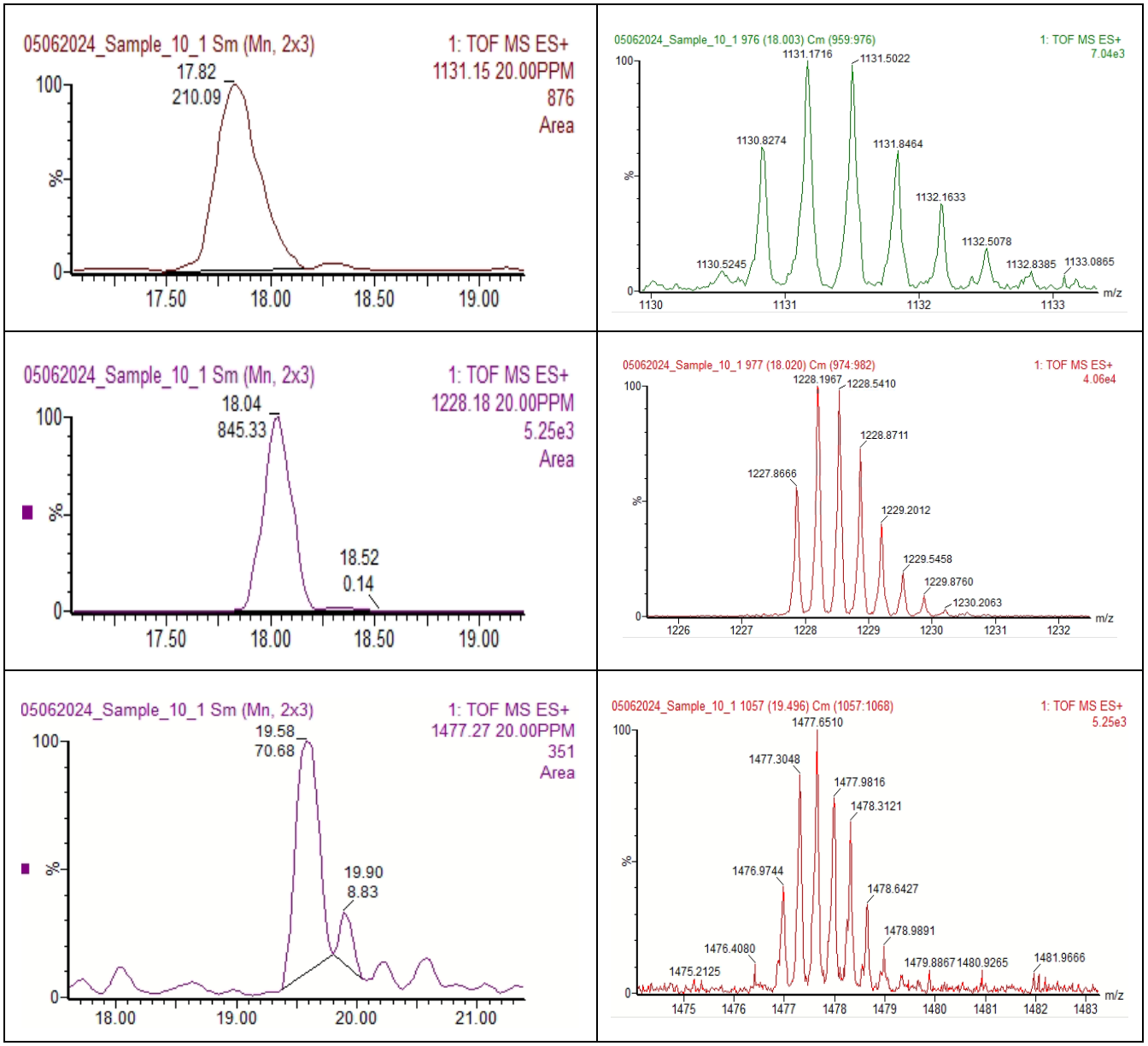


Figure 12: MSMS Spectrum

QTOF- PTM Analysis



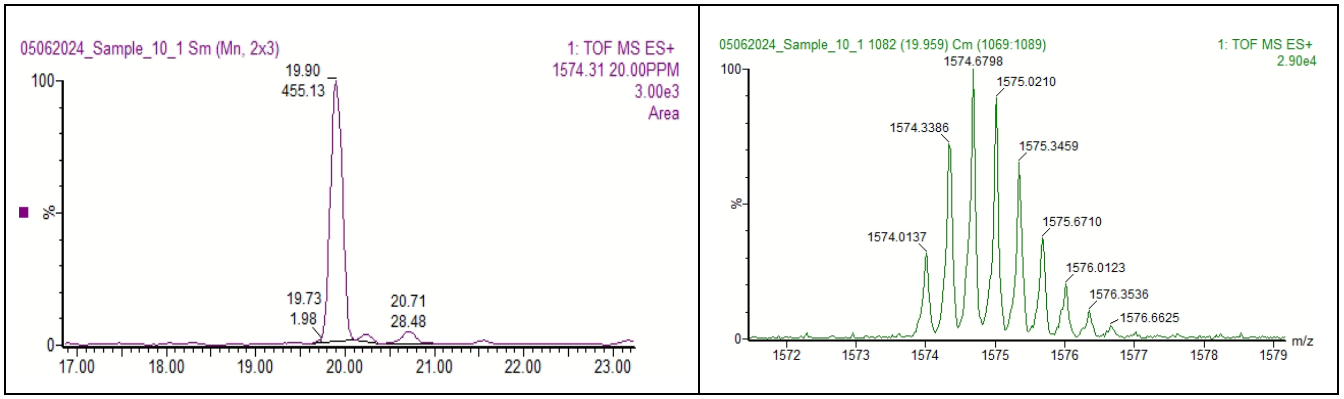
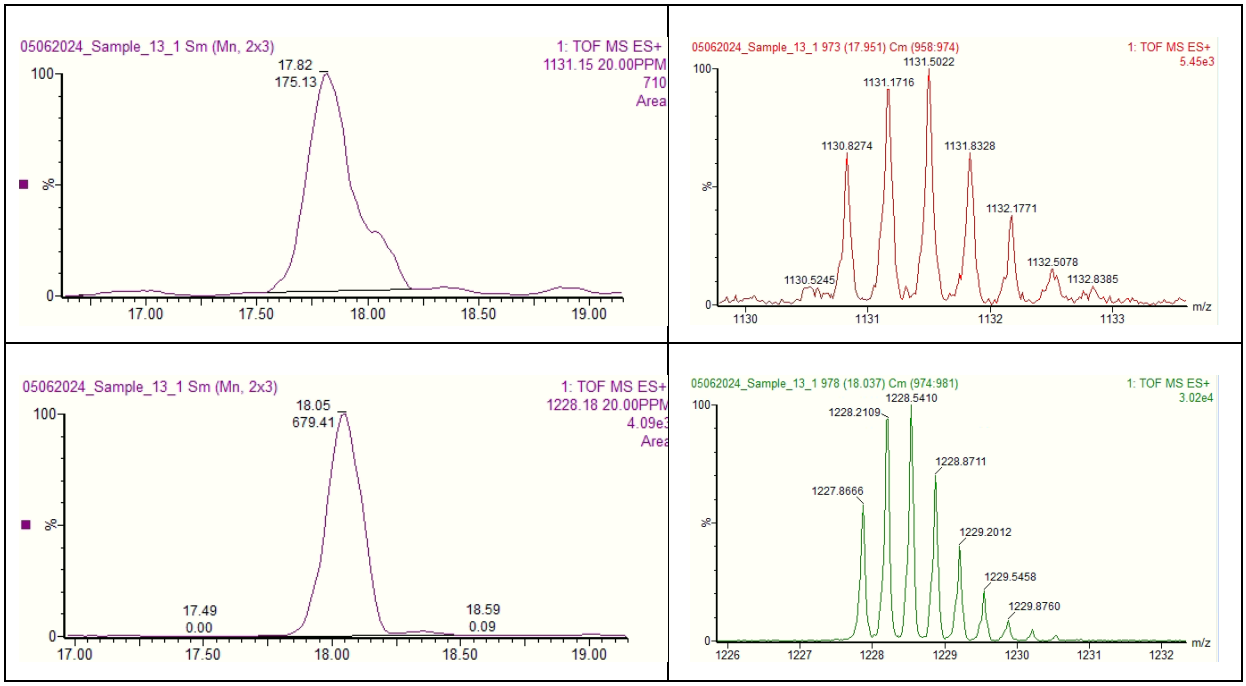


Fig. 13 shows the extracted ion chromatogram and corresponding MS spectrum of the glycopeptides in sample 1.

Table 1 depicts the RT, area, and intensity of different glycoforms.

Glycosylation	m/z under study	RT	Area	Intensity
432 - Asialo	--	--	--	--
432 - Monosialo	1131.15	17.82	210.09	866
432 - Disialo	1228.18	18.04	845.33	5243
630 - Asialo	--	--	--	--
630 - Monosialo	1477.27	19.58	70.682	317
630 - Disialo	1574.31	19.90	455.13	2959



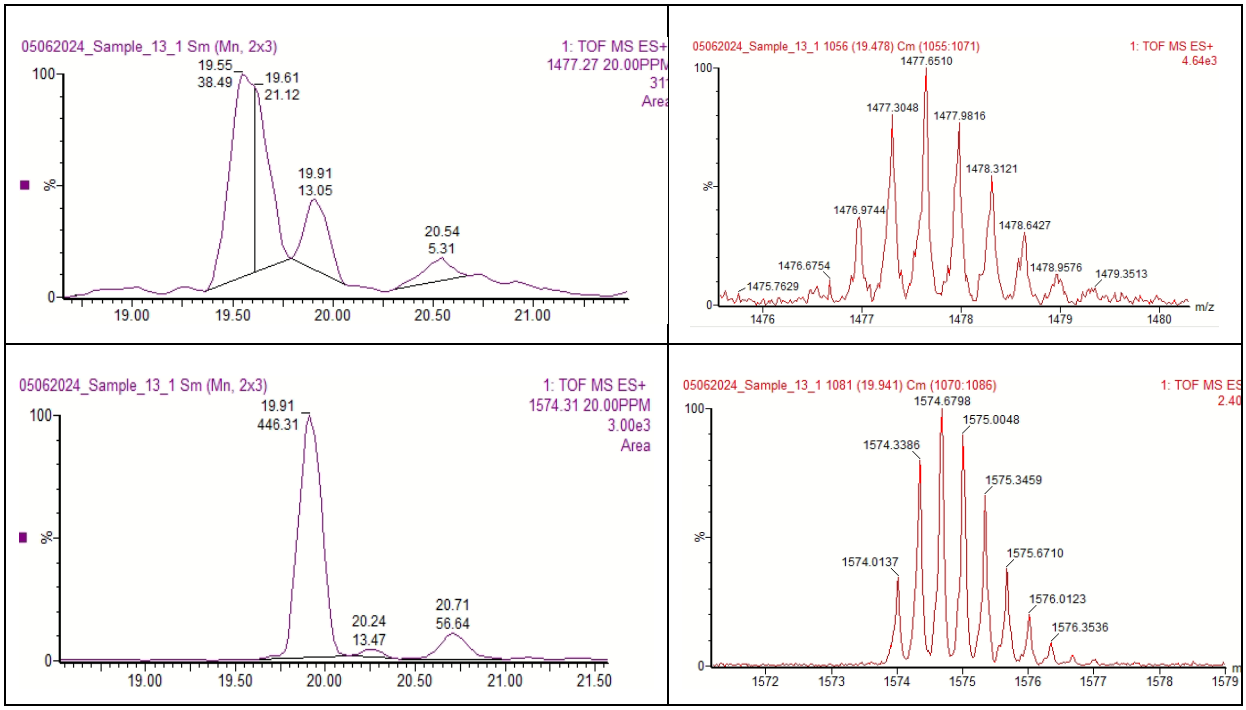
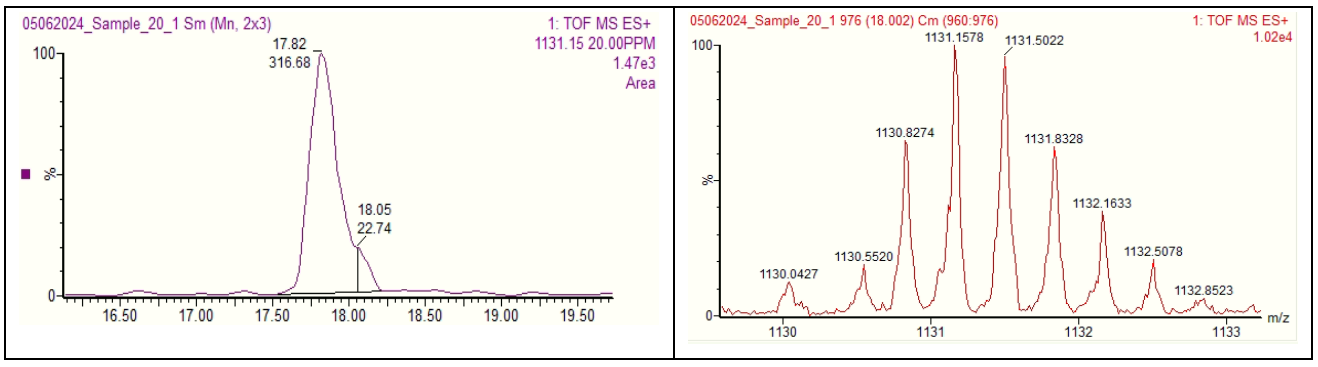


Fig. 14 shows the extracted ion chromatogram and corresponding MS spectrum of the glycopeptides in sample 2.

Table 2 depicts the RT, area, and intensity of different glycoforms.

Glycosylation	m/z under study	RT	Area	Intensity
432 - Asialo	--	--	--	--
432 - Monosialo	1131.15	17.82	175.13	694
432 - Disialo	1228.18	18.05	679.41	4084
630 - Asialo	--	--	--	--
630 - Monosialo	1477.27	19.55	38.49	284
630 - Disialo	1574.31	19.91	446.31	2957



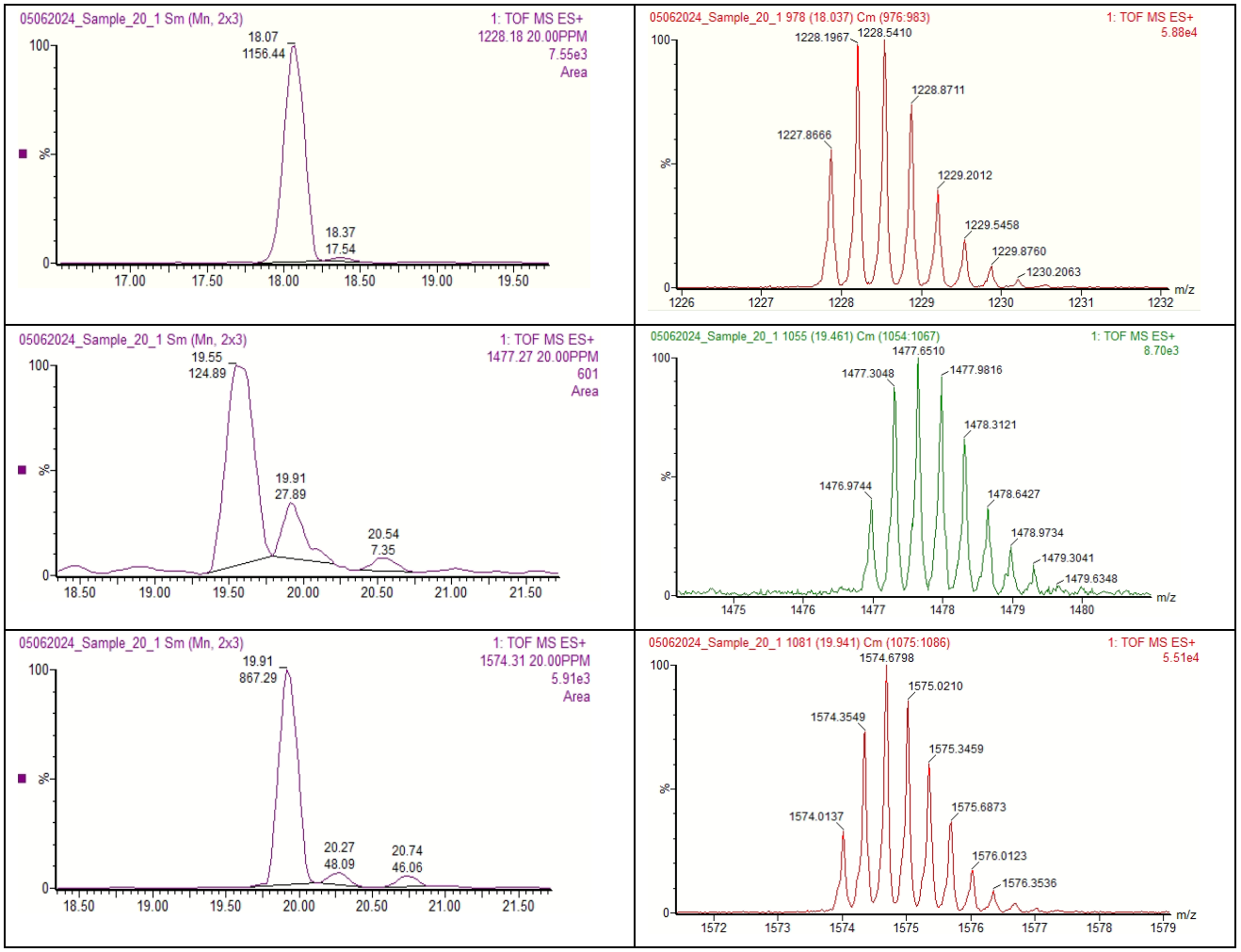


Fig. 15 shows the extracted ion chromatogram and corresponding MS spectrum of the glycopeptides in sample 3.

Table 3 depicts the RT, area, and intensity of different glycoforms.

Glycosylation	m/z under study	RT	Area	Intensity
432 - Asialo	--	--	--	--
432 - Monosialo	1131.15	17.82	316.68	1455
432 - Disialo	1228.18	18.07	1156.44	7496
630 - Asialo	--	--	--	--
630 - Monosialo	1477.27	19.55	124.89	574
630 - Disialo	1574.31	19.91	867.29	5817

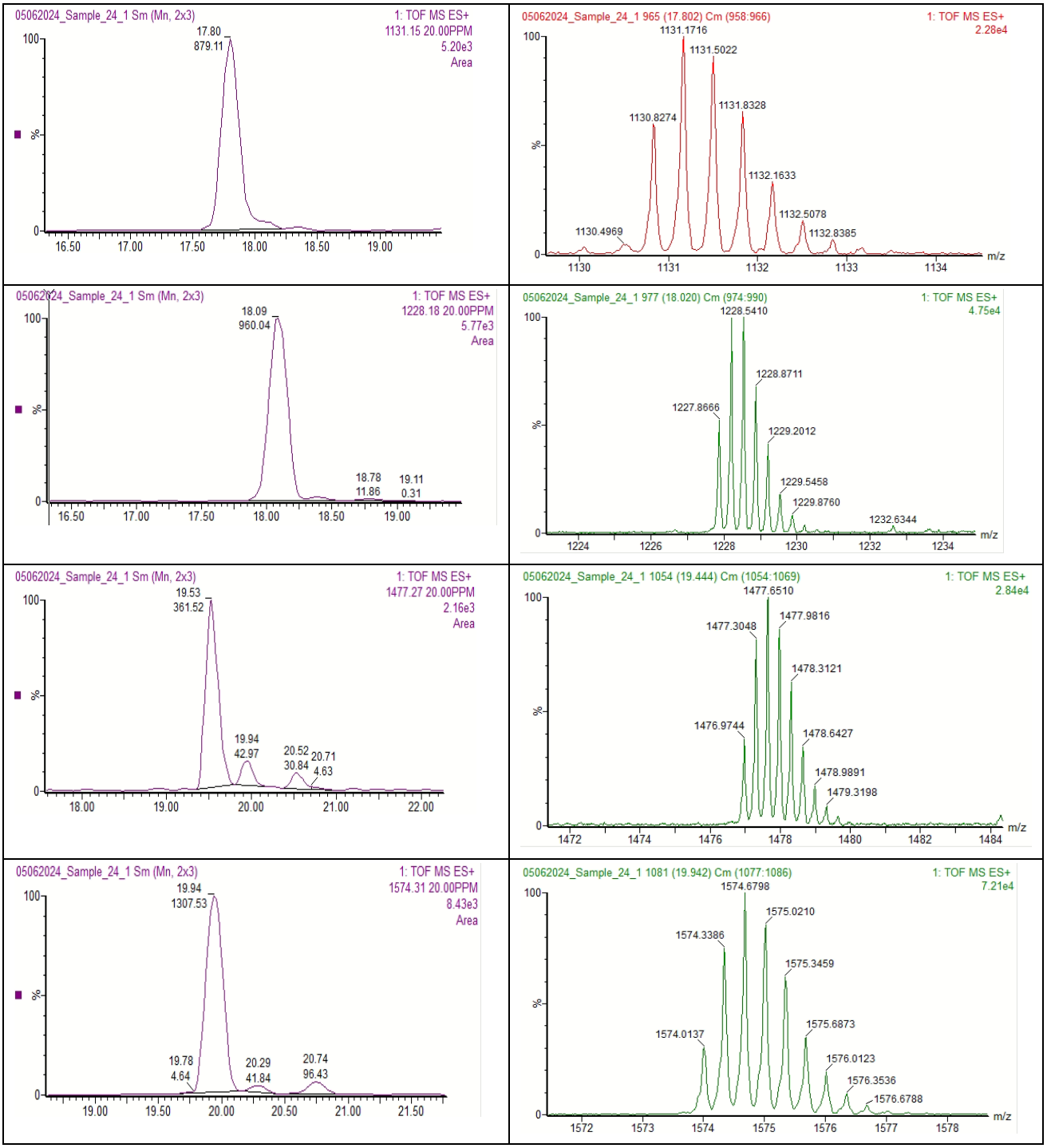


Fig.16 shows the extracted ion chromatogram and corresponding MS spectrum of the glycopeptides in sample 4.

Table 4 depicts the RT, area, and intensity of different glycoforms.

Glycosylation	m/z under study	RT	Area	Intensity
432 - Asialo	--	--	--	--
432 - Monosialo	1131.15	17.80	879.11	5164
432 - Disialo	1228.18	18.09	960.04	5743
630 - Asialo	--	--	--	--

630 - Monosialo	1477.27	19.53	361.53	2113
630 - Disialo	1574.31	19.94	1307.53	8313

Observations from the tables

1. In sample-1, 2 disialo form was found to be more abundant as compared to other glycan isoforms at both the glycosylation sites in transferrin protein indicating a normal profile.
2. In sample-3, along with the disialo form; the monosialo form is also abundant at 432 sites indicating an abnormality.
3. In sample-4, along with the disialo form; the monosialo form is adequately abundant at both sites indicating an abnormality.

4. CONCLUSIONS:

- A rapid and efficient method for isolating transferrin from serum has been optimized.
- The purification process was confirmed using SDS-PAGE, 2D gel electrophoresis, and MALDI analysis.
- Differences in glycosylation patterns were observed on both IEF strips and gels, though the sensitivity was low, and the results were somewhat unclear.
- Transferrin protein was identified using LC-MS, with specific glycosylation patterns analyzed at sites 432 and 630. Abnormal samples were detected based on the distribution of transferrin isoforms, which helped confirm the presence of CDG.
- Our objective is to validate this method with a larger cohort and identify specific biomarkers associated with various glycosylation patterns through MRM analysis.
- MRM analysis will be employed to develop a rapid, accurate, and reliable diagnostic method for CDG testing.

5. ACKNOWLEDGEMENTS:

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