

Simple and fast LC-free N-glycome profiling using neofleX benchtop MALDI-TOF/TOF and BioPharma Compass software

neofleX™ benchtop MALDI-TOF/TOF in combination with BioPharma Compass® software allows for simple and fast LC-free N-glycome profiling in a wide range of life sciences applications such as biomarker discovery and biologics characterization.

Abstract

- A simple and fast LC-free workflow for N-glycome profiling of biological samples is presented integrating the neofleX benchtop MALDI-TOF/TOF with BioPharma Compass software.
- The neofleX based workflow generates information-rich, easy-to-analyze N-glycome profiles at high throughput, taking advantage of the instrument's superior speed and analytical performance previously unseen in a benchtop MALDI-TOF instrument.
- neofleX is capable of detecting N-glycosylation patterns at both glycan and glycopeptide level as demonstrated for total human serum and IgGs purified from human serum, respectively.
- Instant confirmation of glycan composition and glycopeptide sequence by means of high-quality MALDI-MS/MS data adds to the confidence of MALDI-TOF based profiling results.
- BioPharma Compass software enables the straightforward analysis of N-glycan and -glycopeptide data supporting various targeted and untargeted application workflows ranging from biomarker discovery to characterization of glycosylated biologics such as therapeutic antibodies.

Keywords:
N-glycosylation,
MALDI-TOF, profiling,
glycans, glycopeptides,
glycomics, biomarker,
pharma, biopharma,
MS/MS, neofleX,
BioPharma Compass

Introduction

N-glycosylation is one of the most frequently occurring modifications in proteins and is involved in many biochemical pathways in nature. At the same time, N-glycosylation is the most complex protein modification due to its tremendous site-specific heterogeneity, which makes its analytical characterization particularly challenging.

Mass spectrometry (MS) based analysis of protein N-glycosylation can be performed at various levels. Enzymatic release of N-glycans from glycoproteins using PNGase F allows for integral profiling of the N-glycome in a sample. As an alternative to this glycan-centric approach, analysis of N-glycopeptides resulting from enzymatic digestion of glycoproteins enables protein- and site-specific profiling of N-glycan heterogeneity.

Various human diseases, including cancer, are known to be associated with changes in the N-glycosylation pattern of total serum and total IgG proteins, respectively. MS derived N-glycan profiles can, therefore, serve as a clinical biomarker offering a higher level of specificity compared to what was previously reported for classical biomarkers, for example cancer-antigen 125 (CA-125) in epithelial ovarian cancer (EOC) [1]. When performed at glycopeptide level, N-glycome profiling is capable of monitoring disease-specific alterations of N-glycan patterns originating from individual IgG subclasses [2].

In this work, we present an LC-free MALDI-MS solution for simple and fast N-glycome profiling of complex biological samples utilizing the new neoflex benchtop MALDI-TOF/TOF and BioPharma Compass software. Key features of the solution are as follows:

- Simple LC-free MALDI-TOF workflow generating information-rich, easy-to-analyze N-glycome profiles.
- Workflow can be performed at both released glycan and glycopeptide level.
- Unparalleled analysis speed enabling high sample throughput.
- Instant confirmational analysis of glycan compositions and glycopeptide sequences by means of MALDI-MS/MS.
- Seamless interpretation of N-glycan and N-glycopeptide MS and MS/MS data using BioPharma Compass software supporting various non-targeted (identification) and targeted (profile comparison) analysis workflows.
- The workflow can be employed in a variety of applications, ranging from clinical biomarker discovery to characterization of glycosylated biologics.

We demonstrate the neoflex workflow outlined above on the example of human blood serum N-glycome profiling. When analyzing PNGase F released N-glycans from total serum, 54 glycan compositions were detected. Profiling of Protein A affinity-purified, trypsin digested serum IgG N-glycopeptides yielded subclass-specific glycosylation patterns comprising up to 18 glycan compositions. The identity of selected glycans and glycopeptides was confirmed by means of direct MALDI-MS/MS analysis.

Results presented here illustrate the unique capabilities of neoflex MALDI-TOF/TOF in rapid-turnaround profiling analyses taking advantage of the speed and analytical performance now available from this benchtop MALDI instrument.

Experimental

Sample preparation

Human blood serum samples were prepared as reported in [1] and [2], respectively. In brief, N-linked glycans were cleaved off enzymatically using PNGase F. Released glycans were separated from the total serum sample matrix using a C18 stationary phase and were desalted on a graphitized carbon column. In a final step, the desalted glycan pool was permethylated to achieve comparable MS signal response for all neutral and acidic glycan species.

Preparation of samples for serum IgG N-glycome profiling at glycopeptide level included affinity purification of IgGs on Protein A Sepharose beads followed by tryptic digestion and HILIC purification of resulting N-glycopeptides.

MALDI-TOF/TOF analysis

All samples were prepared on a Bruker MTP AnchorChip 384 BC MALDI target plate.

Released, permethylated N-glycan samples were prepared on the MALDI plate using SDHB (10 g/L in 50:50 ACN:H₂O, v:v) as a MALDI matrix containing a final concentration of 5 mM NaCl to promote the formation of [M+Na]⁺ as the dominating ion species.

N-glycopeptide samples were prepared using HCCA matrix (1.4 g/L in 90:10 ACN:H₂O, v:v) spiked with 1 mM ammonium phosphate monobasic and 0.1% TFA.

All MS and MS/MS data were acquired on a Bruker neoflex benchtop MALDI-TOF/TOF instrument equipped with 10 kHz smartbeam 3D laser and controlled by flexControl 5.0 software.

Profile spectra of permethylated total serum N-glycans were acquired in positive reflector mode using the default application method "BioPharma_peptides_R" without any further tuning. Spectra were accumulated by adding 12,000 laser shots using the laser application profile "Thin Layer" and setting the *m/z* detection range to 1000–5000.

N-glycopeptide profiles were acquired in both positive and negative reflector mode. In positive ion mode, the same default application method was used as described for N-glycans. In negative ion mode, the generic instrument method "700–3500" was utilized. Spectra were accumulated by adding 8000 laser shots using the laser application profile "Dried Droplet" and detecting ions throughout the *m/z* range 1000-4000.

External *m/z* calibration was performed using a dextran reference standard and Bruker Peptide Calibration Standard II, respectively. MALDI-MS spectra were internally recalibrated using selected glycan or glycopeptide signals of known identity as calibrants.

MS/MS spectra of selected glycans and glycopeptides were acquired in positive ion mode using the default MS/MS method "500–5000 *m/z*".

Data analysis

MS and MS/MS spectra were processed in flexAnalysis 5.0 software (peak finding, optional smoothing and baseline correction).

Processed spectra were uploaded to BioPharma Compass (BPC) 2025 for further analysis. For analysis of glycan and glycopeptide MS data, BPC's GlycoQuest search function was used (CarbBank database). In case of glycopeptides, the peptide moiety was defined in the GlycoQuest search as a fixed offset mass.

For interpretation of glycan MS/MS spectra, the GlycoQuest MS/MS search function was utilized (CarbBank database).

MS/MS spectra of N-glycopeptides were analyzed applying BPC's Classification algorithm for pinpointing glycan and peptide moiety masses, GlycoQuest search function for assignment of glycan fragments and Theoretical Digest function for assignment of peptide fragments.

Results

Total serum N-glycome profiling

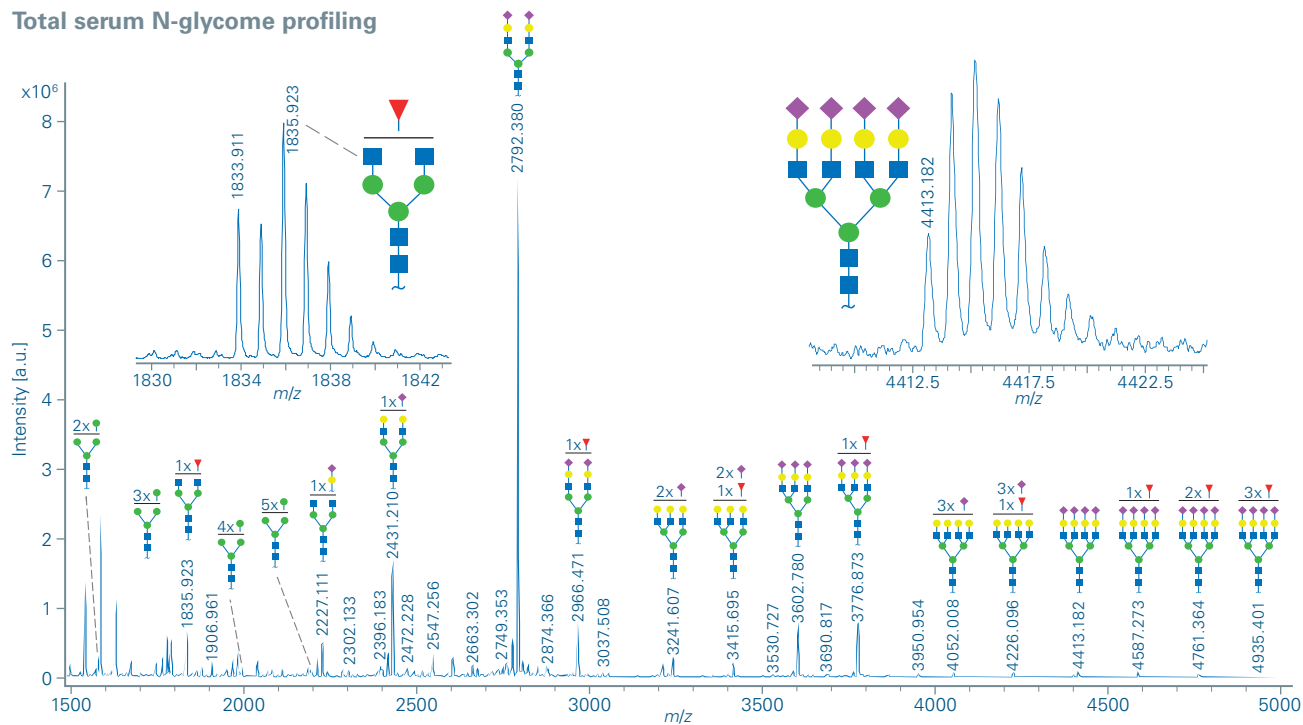


Figure 1

(+)MALDI-TOF spectrum of PNGase F released, permethylated N-glycan pool from total human blood serum.

Structures annotated in the spectrum have been assigned in accordance with [1]. A complete list of all detected glycan compositions is given in Table 1. Overall, 54 compositions of high-mannose-, complex- and hybrid-type N-glycans were detected, including various species that have been reported to show disease-state dependent regulation in ovarian cancer and, therefore, could serve as biomarkers. [1]

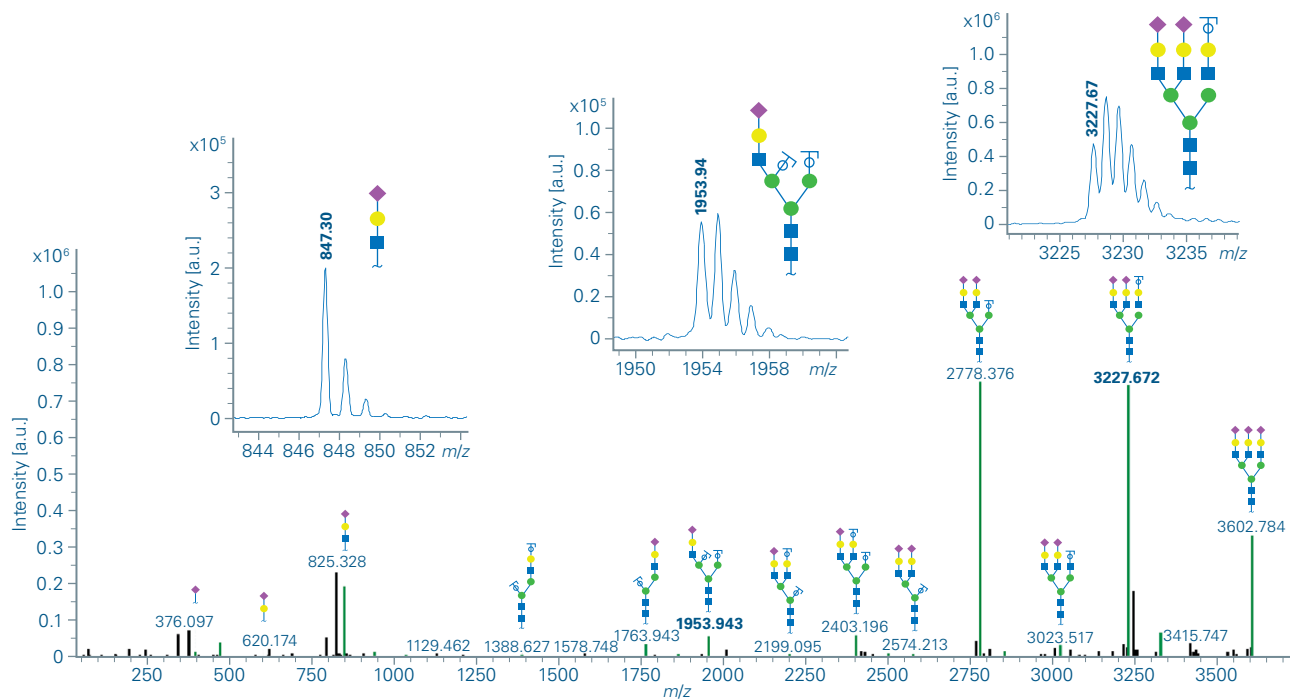


Figure 2

Verification of identity of selected N-glycans by means of MS/MS analysis.

The figure displays the (+)MALDI-TOF/TOF spectrum of precursor ion at m/z 3602.78. The result obtained from a GlycoQuest MS/MS search against Carbbank database confidently confirmed glycan composition Hex6HexNAc5NeuAc3. **Note:** Structures assigned to fragment ion signals in the spectrum may represent one out of multiple possible isomeric structures. To illustrate the outstanding neoflex MS/MS data quality, insets show zoomed views on the raw MS/MS data. Even for large precursor ions, here m/z 3602, fragment ions appear isotopically resolved across the entire m/z range, from the largest B- or Y-ions down to the smallest oxonium ions. Mass deviation averaged over all assigned fragment ions was 0.06 Da.

Table 1. The list of N-glycan compositions detected in the (+)MALDI-TOF profile spectrum of PNGase F released, permethylated N-glycans from total human blood serum. Average mass deviation was 3 ppm.

Composition	m/z meas.	m/z calc.	Δ MH+ [Da]	Δ MH+ [ppm]	Int. [a.u.]
Hex3HexNAc2	1171.5617	1171.5831	-0.0214	-18.22	34931
Hex4HexNAc2	1375.6882	1375.6828	0.0053	3.88	38140
Hex3HexNAc3	1416.7113	1416.7094	0.0020	1.39	109689
Hex5HexNAc2	1579.7831	1579.7826	0.0005	0.30	265773
Hex3HexNAc3dHex1	1590.7980	1590.7986	-0.0005	-0.34	50264
Hex4HexNAc3	1620.8036	1620.8091	-0.0055	-3.41	61636
Hex3HexNAc4	1661.8314	1661.8357	-0.0043	-2.58	55772
Hex6HexNAc2	1783.8817	1783.8824	-0.0007	-0.40	292885
Hex3HexNAc4dHex1	1835.9233	1835.9249	-0.0016	-0.87	669210
Hex4HexNAc4	1865.9316	1865.9355	-0.0039	-2.06	80350
Hex3HexNAc5	1906.9607	1906.9620	-0.0013	-0.70	214531
Hex4HexNAc3NeuAc1	1981.9873	1981.9828	0.0045	2.27	360305
Hex7HexNAc2	1987.9835	1987.9821	0.0014	0.70	83681
Hex4HexNAc4dHex1	2040.0258	2040.0247	0.0012	0.56	252556
Hex5HexNAc4	2070.0383	2070.0352	0.0030	1.46	95870
Hex3HexNAc5dHex1	2081.0426	2081.0512	-0.0086	-4.13	63698
Hex4HexNAc5	2111.0602	2111.0618	-0.0016	-0.76	122908
Hex4HexNAc3NeuAc1dHex1	2156.0693	2156.0720	-0.0027	-1.24	76284
Hex5HexNAc3NeuAc1	2186.0856	2186.0826	0.0030	1.39	144464
Hex8HexNAc2	2192.0843	2192.0819	0.0024	1.09	122126
Hex4HexNAc4NeuAc1	2227.1109	2227.1091	0.0018	0.80	480721
Hex5HexNAc4dHex1	2244.1351	2244.1245	0.0107	4.75	50427
Hex4HexNAc5dHex1	2285.1318	2285.1510	-0.0192	-8.38	116133
Hex5HexNAc5	2315.1420	2315.1616	-0.0196	-8.46	36110
Hex6HexNAc3NeuAc1	2390.1849	2390.1824	0.0025	1.05	97162
Hex9HexNAc2	2396.1828	2396.1817	0.0011	0.47	152818
Hex4HexNAc4NeuAc1dHex1	2401.1854	2401.1983	-0.0129	-5.38	107794
Hex5HexNAc4NeuAc1	2431.2097	2431.2089	0.0008	0.33	1635059
Hex5HexNAc5dHex1	2489.2298	2489.2508	-0.0210	-8.43	54154
Hex4HexNAc4NeuAc2	2588.2716	2588.2828	-0.0112	-4.32	40767
Hex5HexNAc4NeuAc1dHex1	2605.2950	2605.2981	-0.0031	-1.20	286106
Hex6HexNAc4NeuAc1	2635.2962	2635.3087	-0.0125	-4.73	44450
Hex4HexNAc5NeuAc1dHex1	2646.2990	2646.3247	-0.0257	-9.71	62945
Hex5HexNAc5dHex2	2663.3019	2663.3400	-0.0381	-14.29	168667
Hex5HexNAc5NeuAc1	2676.3244	2676.3352	-0.0108	-4.04	130633
Hex9HexNAc2Pen1dHex1	2730.3374	2730.3445	-0.0071	-2.59	55172
Hex5HexNAc4Neu2	2736.3568	2736.3927	-0.0359	-13.12	66084
Hex5HexNAc4NeuAc2	2792.3805	2792.3826	-0.0021	-0.75	7190128
Hex6HexNAc5NeuAc1	2880.4255	2880.4350	-0.0095	-3.29	113483
Hex5HexNAc4NeuAc2dHex1	2966.4714	2966.4718	-0.0004	-0.13	755931
Hex5HexNAc5NeuAc2	3037.5077	3037.5089	-0.0012	-0.40	89912
Hex6HexNAc5NeuAc1dHex1	3054.4763	3054.5242	-0.0479	-15.69	62317
Hex5HexNAc5NeuAc2dHex1	3211.5941	3211.5981	-0.0040	-1.24	187828
Hex6HexNAc5NeuAc2	3241.6072	3241.6087	-0.0015	-0.47	266975
Hex6HexNAc5NeuAc2dHex1	3415.6954	3415.6979	-0.0025	-0.73	195540
Hex6HexNAc5NeuAc3	3602.7801	3602.7823	-0.0022	-0.62	758103
Hex7HexNAc6NeuAc2	3690.8175	3690.8348	-0.0173	-4.69	39733
Hex6HexNAc5NeuAc3dHex1	3776.8725	3776.8716	0.0010	0.25	776823
Hex7HexNAc6NeuAc3	4052.0075	4052.0084	-0.0009	-0.23	81510
Hex7HexNAc6NeuAc3dHex1	4226.0960	4226.0976	-0.0017	-0.39	75094
Hex7HexNAc6NeuAc4	4413.1818	4413.1821	-0.0003	-0.07	105888
Hex7HexNAc6NeuAc4dHex1	4587.2731	4587.2713	0.0018	0.40	110266
Hex7HexNAc6NeuAc4dHex2	4761.3636	4761.3605	0.0031	0.65	49327
Hex7HexNAc6NeuAc4dHex3	4935.4013	4935.4497	-0.0484	9.81	13465

Subclass-specific serum IgG N-glycome profiling

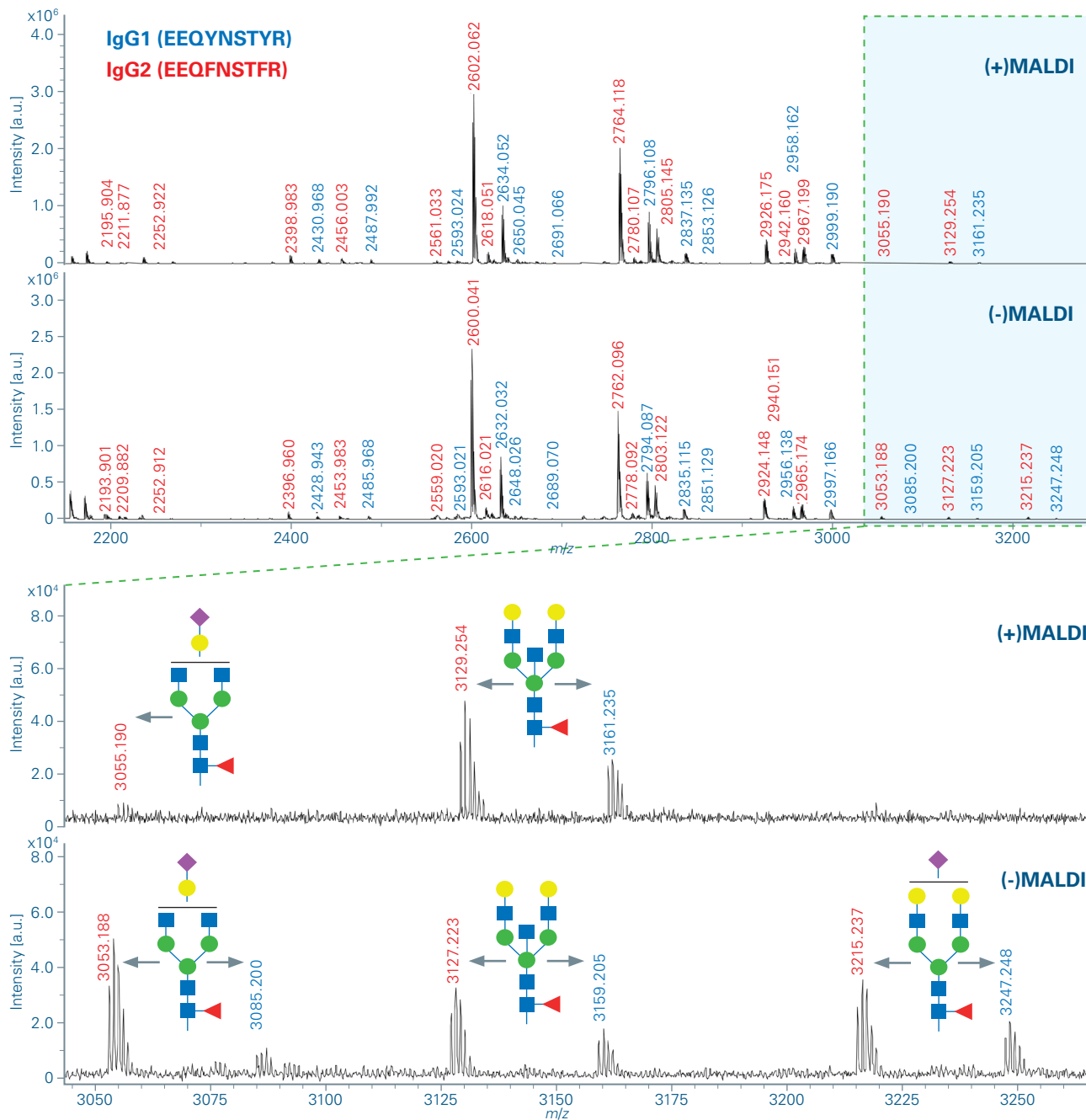


Figure 3

MALDI-TOF spectra of N-glycopeptides obtained from Protein A affinity-purified, trypsin digested human serum IgGs.

The complete list of detected N-glycopeptides is given in Table 2. Panels A and B display overview spectra acquired in positive and negative ion mode, respectively. The spectra provide IgG subclass-specific N-glycosylation profiles in the shape of glycopeptides EEQYNSTYR (IgG1) and EEQFNSTFR (IgG2), respectively. Panels C and D present zoomed views of m/z range 3000 – 3300 in positive and negative ion mode, respectively. Glycan structures have been annotated in accordance with [2]. For both IgG subclasses 1 and 2, negative ion mode detected a larger number of N-glycan compositions, which is explained by enhanced ionization efficiency of N-glycopeptides carrying acidic glycan moieties.

Table 2. The list of N-glycan compositions of human serum IgG1 and IgG2 detected in the shape of N-glycopeptides EEQYNSTYR and EEQFNSTFR, respectively. Average mass deviation was 2.5 ppm in both, positive and negative ion mode.

	Composition		Positive ion mode [M+H] ⁺				Negative ion mode [M+H] ⁻			
			m/z meas.	m/z calc.	Δ [ppm]	Int.	m/z meas.	m/z calc.	Δ [ppm]	Int.
IgG1	1	Hex3HexNAc3dHex1-EEQYNSTYR	2.430.968	2.430.967	0.7	77192	2.428.943	2.428.952	-3.7	39543
	2	Hex3HexNAc4-EEQYNSTYR	2.487.992	2.487.988	1.4	74645	2.485.968	2.485.973	-2.3	50524
	3	Hex4HexNAc3dHex1-EEQYNSTYR	2.593.024	2.593.019	1.6	28345	2.591.021	2.591.005	6.1	25780
	4	Hex3HexNAc4dHex1-EEQYNSTYR	2.634.052	2.634.046	2.3	1042797	2.632.032	2.632.031	0.4	806163
	5	Hex4HexNAc4-EEQYNSTYR	2.650.045	2.650.041	1.5	72564	2.648.026	2.648.026	0.1	44793
	6	Hex3HexNAc5-EEQYNSTYR	2.691.066	2.691.067	-0.4	16632	2.689.070	2.689.053	6.5	12660
	7	Hex4HexNAc4dHex1-EEQYNSTYR	2.796.108	2.796.099	3.5	880594	2.794.087	2.794.084	1.1	647197
	8	Hex3HexNAc5dHex1-EEQYNSTYR	2.837.135	2.837.125	3.4	219663	2.835.115	2.835.111	1.7	173723
	9	Hex4HexNAc5-EEQYNSTYR	2.853.126	2.853.120	2.2	16802	2.851.129	2.851.106	8.1	12507
	10	Hex5HexNAc4dHex1-EEQYNSTYR	2.958.162	2.958.152	3.6	244948	2.956.138	2.956.137	0.4	168912
	11	Hex4HexNAc5dHex1-EEQYNSTYR	2.999.190	2.999.178	3.8	220768	2.997.166	2.997.164	1.0	163679
	12	Hex4HexNAc4NeuAc1dHex1-EEQYNSTYR				n.d.	3.085.200	3.085.180	6.6	8593
	13	Hex5HexNAc5dHex1-EEQYNSTYR	3.161.235	3.161.231	1.2	24474	3.159.205	3.159.216	-3.7	13693
	14	Hex5HexNAc4NeuAc1dHex1-EEQYNSTYR				n.d.	3.247.248	3.247.232	4.8	14462
IgG2	1	Hex3HexNAc2dHex1-EEQFNSTFR	2.195.904	2.195.897	3.0	37013	2.193.901	2.193.883	8.5	128022
	2	Hex4HexNAc2-EEQFNSTFR	2.211.877	2.211.892	-6.7	22622	2.209.882	2.209.878	2.0	55521
	3	Hex3HexNAc3-EEQFNSTFR	2.252.922	2.252.919	1.5	16560	2.250.912	2.250.904	3.7	11121
	4	Hex3HexNAc3dHex1-EEQFNSTFR	2.398.983	2.398.977	2.6	197395	2.396.961	2.396.962	-0.7	110346
	5	Hex3HexNAc4-EEQFNSTFR	2.456.003	2.455.998	1.8	92529	2.453.983	2.453.984	-0.3	53227
	6	Hex4HexNAc3dHex1-EEQFNSTFR	2.561.033	2.561.029	1.5	63558	2.559.020	2.559.015	2.1	33983
	7	Hex3HexNAc4dHex1-EEQFNSTFR	2.602.062	2.602.056	2.4	2976748	2.600.042	2.600.042	0.0	2303949
	8	Hex4HexNAc4-EEQFNSTFR	2.618.051	2.618.051	0.0	188663	2.616.021	2.616.036	-6.0	134267
	9	Hex4HexNAc4dHex1-EEQFNSTFR	2.764.118	2.764.109	3.3	2038664	2.762.096	2.762.094	0.5	1474168
	10	Hex5HexNAc4-EEQFNSTFR	2.780.107	2.780.104	1.3	99803	2.778.092	2.778.089	1.0	69202
	11	Hex3HexNAc5dHex1-EEQFNSTFR	2.805.145	2.805.135	3.3	567466	2.803.122	2.803.121	0.3	427979
	12	Hex4HexNAc5-EEQFNSTFR	2.821.127	2.821.130	-1.0	53584	2.819.120	2.819.116	1.6	46228
	13	Hex5HexNAc4dHex1-EEQFNSTFR	2.926.175	2.926.162	4.4	463842	2.924.148	2.924.147	0.4	316110
	14	Hex6HexNAc4-EEQFNSTFR	2.942.160	2.942.157	1.2	20505	2.940.151	2.940.142	3.1	20580
	15	Hex4HexNAc5dHex1-EEQFNSTFR	2.967.199	2.967.188	3.8	318368	2.965.174	2.965.174	0.2	229444
	16	Hex4HexNAc4NeuAc1dHex1-EEQFNSTFR	3.055.190	3.055.204	-4.8	8748	3.053.188	3.053.190	-0.6	44898
	17	Hex5HexNAc5dHex1-EEQFNSTFR	3.129.254	3.129.241	4.1	44539	3.127.223	3.127.227	-1.2	29954
	18	Hex5HexNAc4NeuAc1dHex1-EEQFNSTFR				n.d.	3.215.237	3.215.243	-1.7	32489

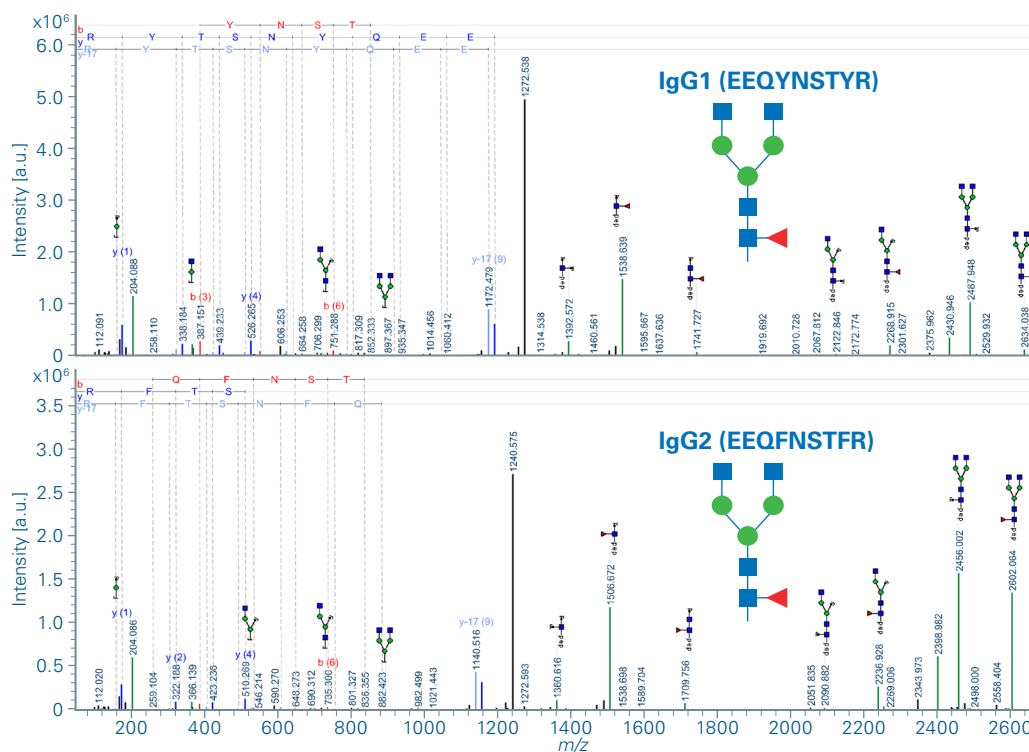


Figure 4
 (+)MALDI-MS/MS spectra of most abundant species within the IgG1 and IgG2 specific N-glycopeptide patterns. BioPharma Compass software allowed for annotation of both glycan and peptide backbone fragment ions confirming glycan composition G0F and peptide sequences EEQYNSTYR (IgG1) and EEQFNSTFR (IgG2), respectively.

Conclusions

- neoflex benchtop MALDI-TOF/TOF is an ideally suited platform for LC-free, rapid-turnaround N-glycosylation analysis.
- By integrating neoflex with BioPharma Compass software for data interpretation, a complete analysis workflow is provided, enabling simple and fast N-glycome profiling of biological samples, as demonstrated here for total human serum and affinity-purified serum IgGs.
- The method benefits from neoflex's unparalleled data acquisition speed and analytical performance and is, therefore, capable of generating information-rich N-glycome profiles at high sample throughput.
- neoflex's seamless TOF/TOF capability enables instant confirmation of glycan compositions and glycopeptide sequences based on high-quality MS/MS data.
- The workflow introduced here can be adapted without major changes to applications beyond biomarker discovery, for example, characterization of N-glycosylation patterns in therapeutic glycoproteins such as monoclonal antibodies.

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Bruker Switzerland AG

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