Site-Specific Protein N- and O-Glycosylation Analysis by a C18-Porous Graphitized Carbon-Liquid Chromatography-Electrospray Ionization Mass Spectrometry Approach Using Pronase Treated Glycopeptides

Supplementary information

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Experimental section

SDS-PAGE and in-gel proteolytic digest

Five to ten microgram of protein were denatured in nonreducing Laemmli buffer for 10 min at 70°C prior to loading onto a 4-12% Bis-tris sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel (NuPAGE; Life Technology, Carlsbad, CA, USA), including a precision plus protein standard (Biorad, Hercules, CA). Proteins were separated for 50 min at 200 V and stained using Coomassie staining (SimplyBlue; Life Technology) before bands were excised and cut into small pieces.

After washing the gel pieces with 25 mM ammonium bicarbonate (AmBiC), followed by acetonitrile (ACN), the proteins were reduced by adding 10 mM dithiothreitol (DTT) in 25 mM AmBiC for 30 min at 60°C. The gel pieces were then washed with ACN before alkylation of the cysteine bonds by incubation with 55 mM iodacetamide (IAA) in 25 mM AmBiC for 20 min in dark. After that the gel pieces were washed again by 2 cycles of 25 mM AmBiC followed by ACN and a final step of drying in a vacuum concentrator.

For trypsin digestion 30 µl with 0.005 µg/µl trypsin in 25 mM AmBiC was added to the sample and pronase digestion was performed by adding the enzyme in a ratio of 3:1 (protein: enzyme, by weigh) in 25 mM AmBiC. The gel pieces were incubated for 45 min on ice to allow hydration of the gel pieces and incubation overnight at 37°C.

The next day the supernatant, containing peptides and glycopeptides, was collected and a second extraction with 30 µl 25 mM AmBic was performed for 1 h on a shaker. The supernatant was again removed and combined with the first one.

Cotton HILIC-solid phase extraction

The samples of human fibrinogen α-subunit needed a further enrichment step using cotton HILIC-solid phase extraction (SPE)[1] before LC-MS analysis. In detail, 4 µl of pronase in-gel digest were mixed with 36 µl of ACN so that the resulting ratio was 90% ACN. A small amount of cotton was positioned in a 10 µl pipet tip [1] and washed three times with 10 µl water and conditioned with three times 10 µl 90% ACN/ 10% water containing 0.1% TFA. The sample was applied by pipetting up and down 20 times, followed by a washing step of three times 10 µl 90% ACN/ 10% water containing 0.1% TFA and three times 90% ACN. The glycopeptides were eluted in 20 µl of water and stored at -20°C until further use.

LC-MS (glyco)peptide analysis

Tryptic and trypsin-AspN treated peptides were C18-RP-LC-separated as follows: approximately 25 ng of tryptic peptide and 36 ng of trypsin-AspN peptide sample were loaded onto a C18 µ-pre

column (C18 PepMap 100, 300 µm x 5 mm, 5 µm, 100 Å, Dionex/Thermo Scientific, Breda, The Netherlands) with 10 µl/min of loading solvent (99% water/ 1% ACN/ 0.05% TFA) for 5 min. The analytes were then separated on a C18 analytical column (Acclaim PepMap RSLC, 75 µm × 15 cm, 2 µm, 100 Å, Dionex/Thermo Scientific) at 45°C column oven temperature. Tryptic peptides were eluted at a flow rate of 0.7 µl/min with solvent A (water containing 0.1% FA (v/v)) and solvent B (80% acetonitrile/ 20% water containing 0.1% FA (v/v)). A linear gradient of 3–40% solvent B in 15 min was applied followed by column washing and reconditioning. For trypsin-AspN treated peptides elution was performed at a flow rate of 0.5 µl/min and a linear gradient of 1–53.5% solvent B in 31.5 min followed by column washing and reconditioning.

MALDI-MS analysis of released glycans

Twenty µg of IgG3 in PBS was incubated with CaptureSelect IgG-Fc (Hu) beads (Invitrogen Europe, Bleiswijk, The Netherlands) on a 96-well filter plate. After washing, the captured IgG3 samples were incubated in 35 µl of 50 mM Na_2HPO_4/150 mM NaCl at 37°C overnight. The IgG3 was then washed with PBS and water, eluted in 100 mM formic acid and dried.

Glycan release was performed overnight at 37°C with 0.25 µl N-glycosidase F (Roche Diagnostics, Mannheim, Germany) in 1.2x PBS, 0.8% SDS, 0.8% NP-40 in a total volume of 25 µl. The glycans were derivatized by ethyl esterification of α2,6-linked sialic acids was done as described by Reiding et al.[2] Purification of the derivatized glycans was achieved with an adjusted version of HILIC purification[1]: the released glycans in organic solution (67.6% ACN, 27% ethanol) were loaded onto an AcroPrep 96-well filter plate with a hydrophilic GHP membrane to which the glycans adhered. The glycans were washed with 96% ACN and eluted in H_2O.

The samples were then spotted on an AnchorChip plate together with 1 µl 5 mg/ml super-DHB (Sigma-Aldrich) in 100% ACN containing 1 mM NaOH. After drying the sample was analyzed in reflectron positive mode in an UltrafleXtreme MALDI-TOF-MS (Bruker Daltonics).

Results

C18-PGC-LC-ESI-QTOF-MS/MS method

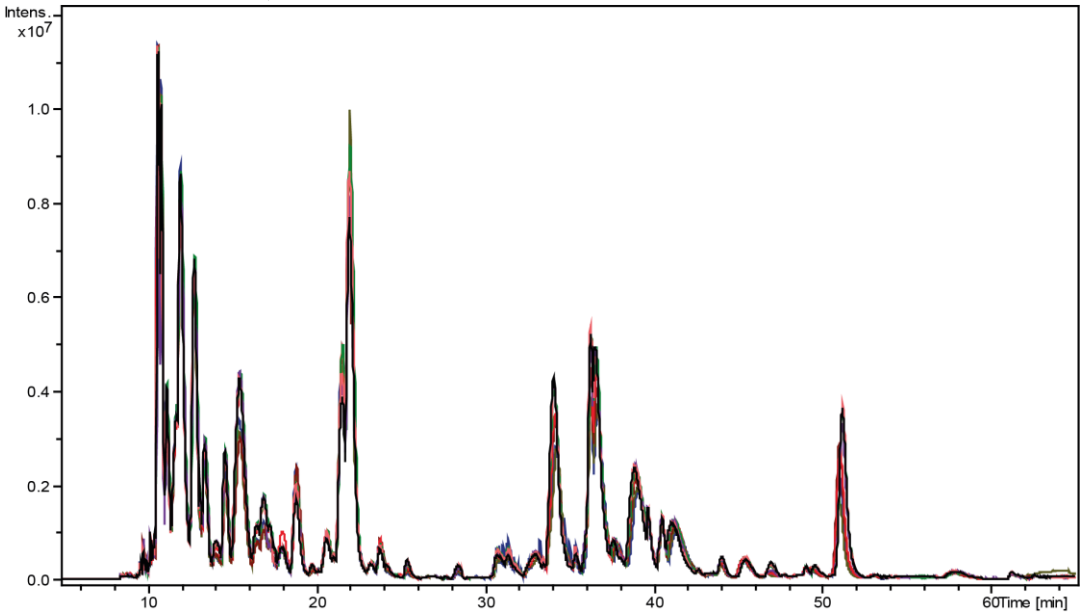

Figure S-1: Base peak chromatogram of eight consecutive C18-PGC-LC-ESI-MS/MS runs of Pronase treated IgG3 (glyco)peptides.

Glycopeptide identification by combined lower- and enhanced-energy CID

The spectra in Figure S-2 to S-9 and the Table S-1 show that a combined lower- and enhanced energy CID results in more diagnostic ions for glycopeptide identification than individual analysis by lower- or enhanced-energy CID. Using lower-energy CID specific glycan-derived fragment ions are observed allowing partial structural elucidation of the glycans. However, hardly any peptide backbone fragmentation could be observed. On the other side, the application of enhanced-energy CID only resulted in peptide backbone fragmentation for peptide sequence identification. Due to the elevated energy less glycan-derived ions, in particular Y-ions, were observed which are necessary to identify for example the presence of a bisecting GlcNAc. (Supplementary Figure S-7A, S-9A). Thus, the combined-energy CID mode was used to allow more detailed glycopeptide identification.

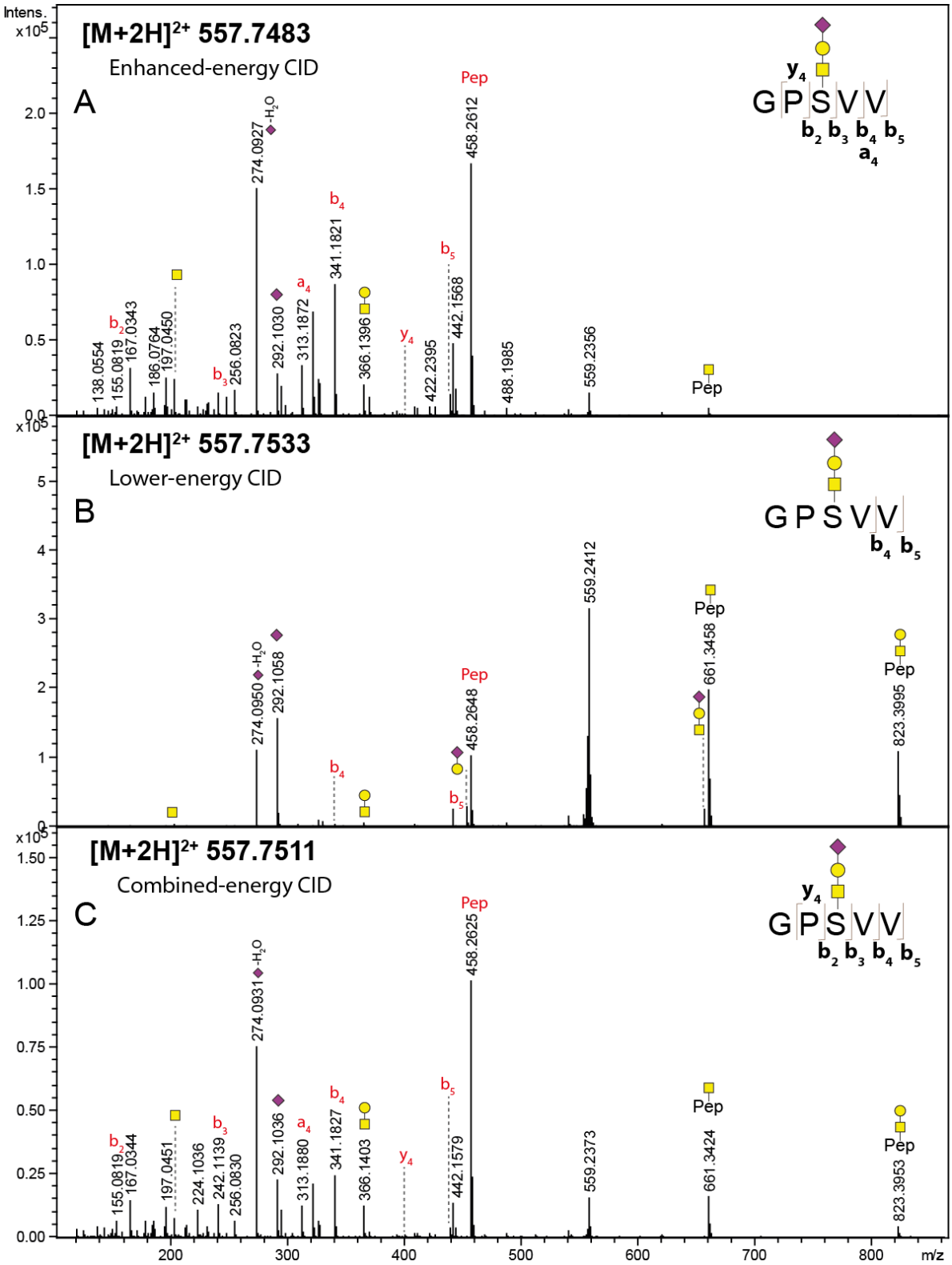

Figure S-2: MS/MS fragmentation spectra of a bovine fetuin O-glycopeptide with the peptide sequence GPSVV and a monosialylated core 1 structure by (A) enhanced-energy CID using 100% of the collision energy without stepping and (B) lower-energy CID using 45% of the collision energy without stepping. (C) Combined lower- and enhanced-energy CID using 80-140% of the collision energy in stepping mode.

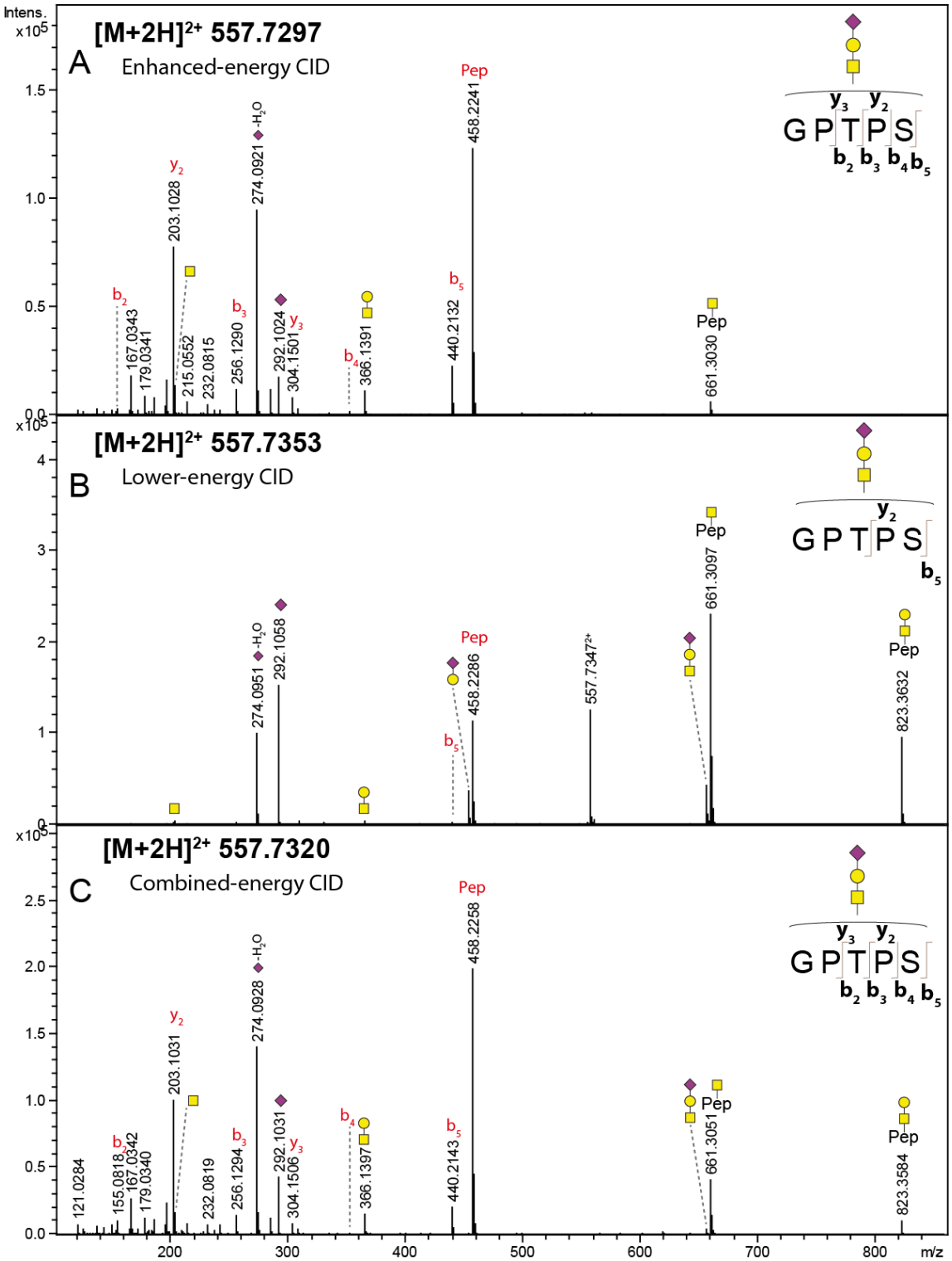

Figure S-3: MS/MS fragmentation spectra of a bovine fetuin O-glycopeptide with the peptide sequence GPTPS and a monosialylated core 1 structure by (A) enhanced-energy CID using 100% of the collision energy without stepping and (B) lower-energy CID using 45% of the collision energy without stepping. (C) Combined lower- and enhanced-energy CID using 80-140% of the collision energy in stepping mode.

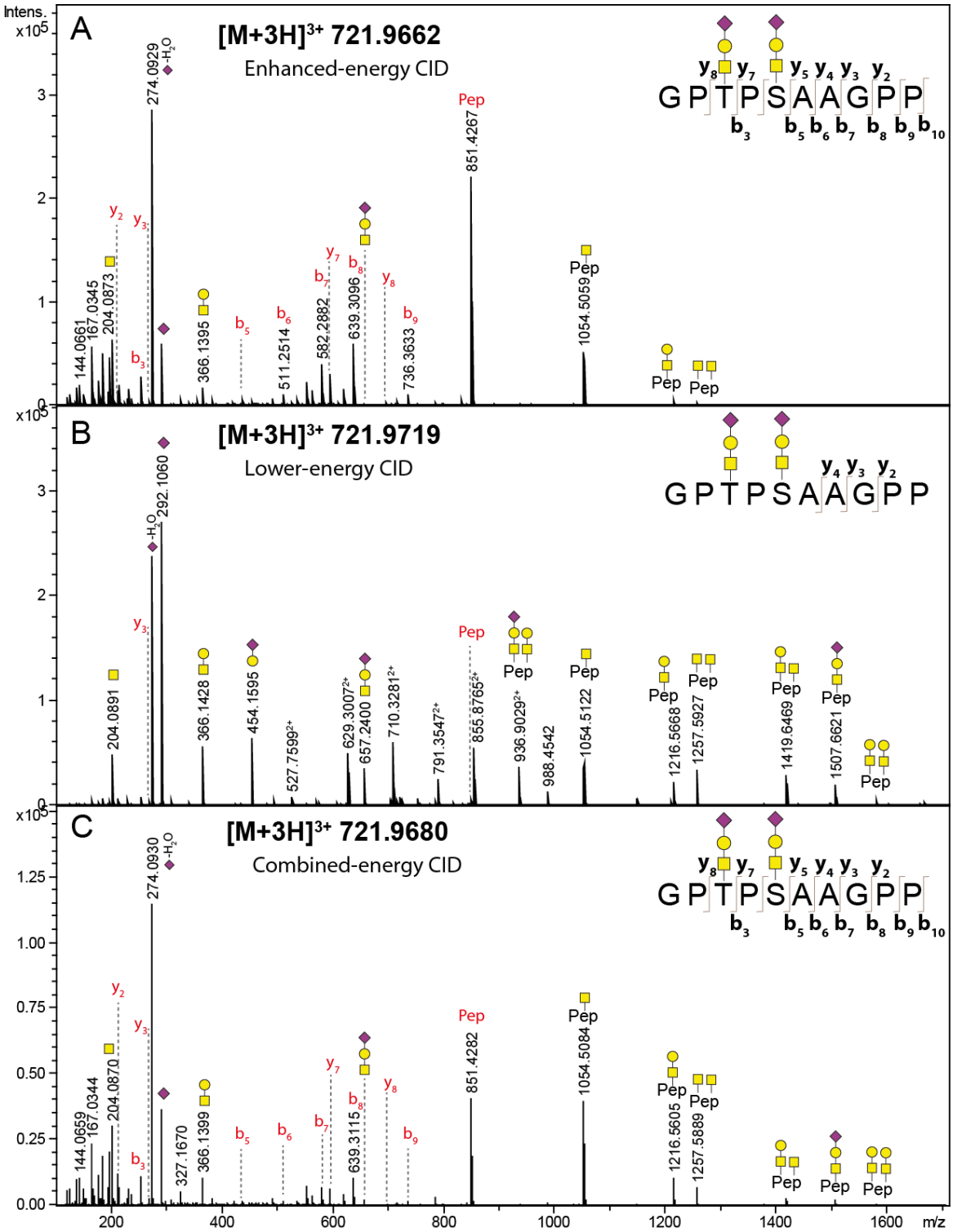

Figure S-4: MS/MS fragmentation spectra of a bovine fetuin O-glycopeptide with the peptide sequence GPTPSAAGPP and two monosialylated core 1 structures by (A) enhanced-energy CID using 100% of the collision energy without stepping and (B) lower-energy CID using 45% of the collision energy without stepping. (C) Combined lower- and enhanced-energy CID using 80-140% of the collision energy in stepping mode.

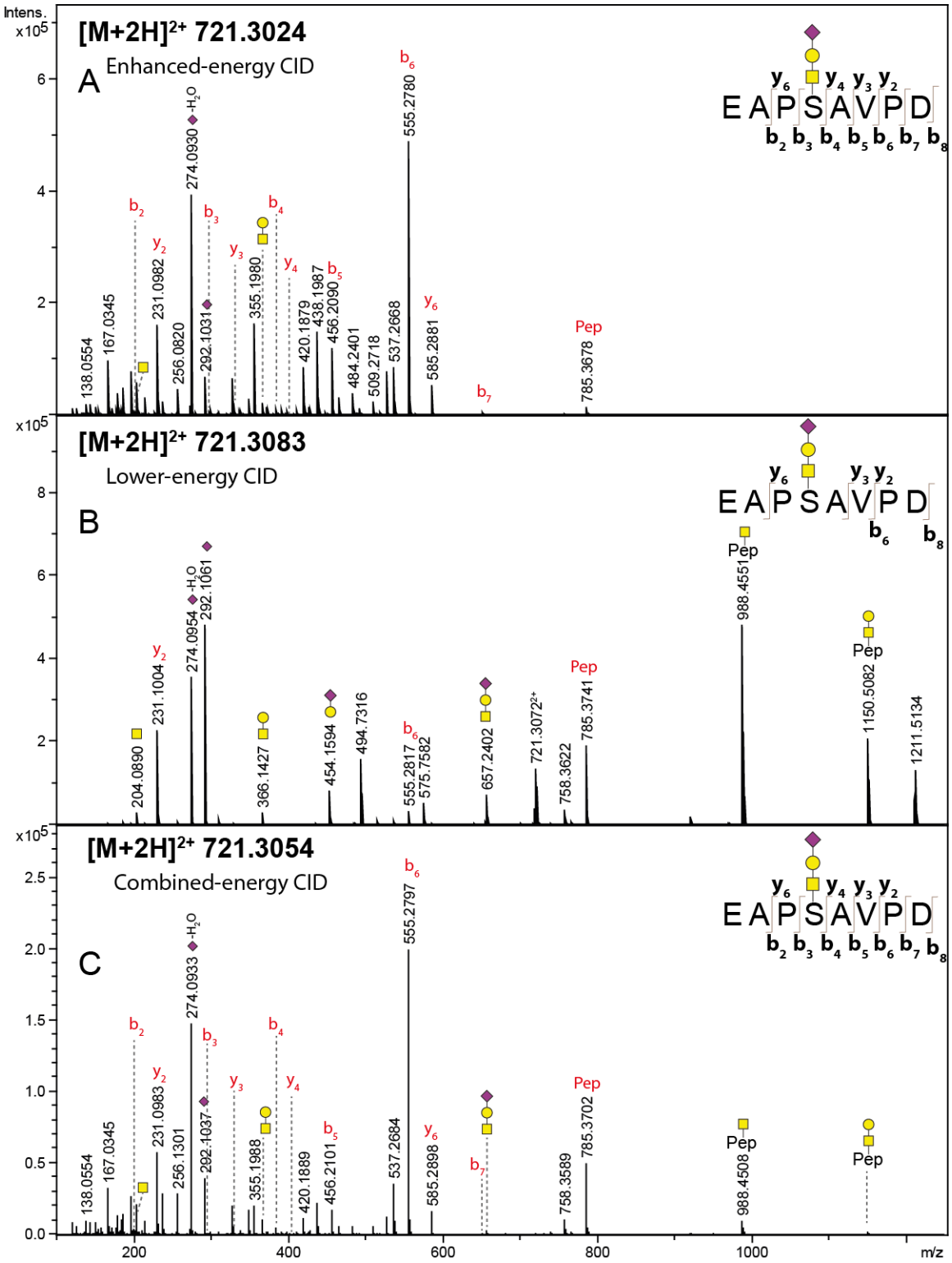

Figure S-5: MS/MS fragmentation spectra of a bovine fetuin O-glycopeptide with the peptide sequence EAPSAVPD and a monosialylated core 1 structure by (A) enhanced-energy CID using 100% of the collision energy without stepping and (B) lower-energy CID using 45% of the collision energy without stepping. (C) Combined lower- and enhanced-energy CID using 80-140% of the collision energy in stepping mode.

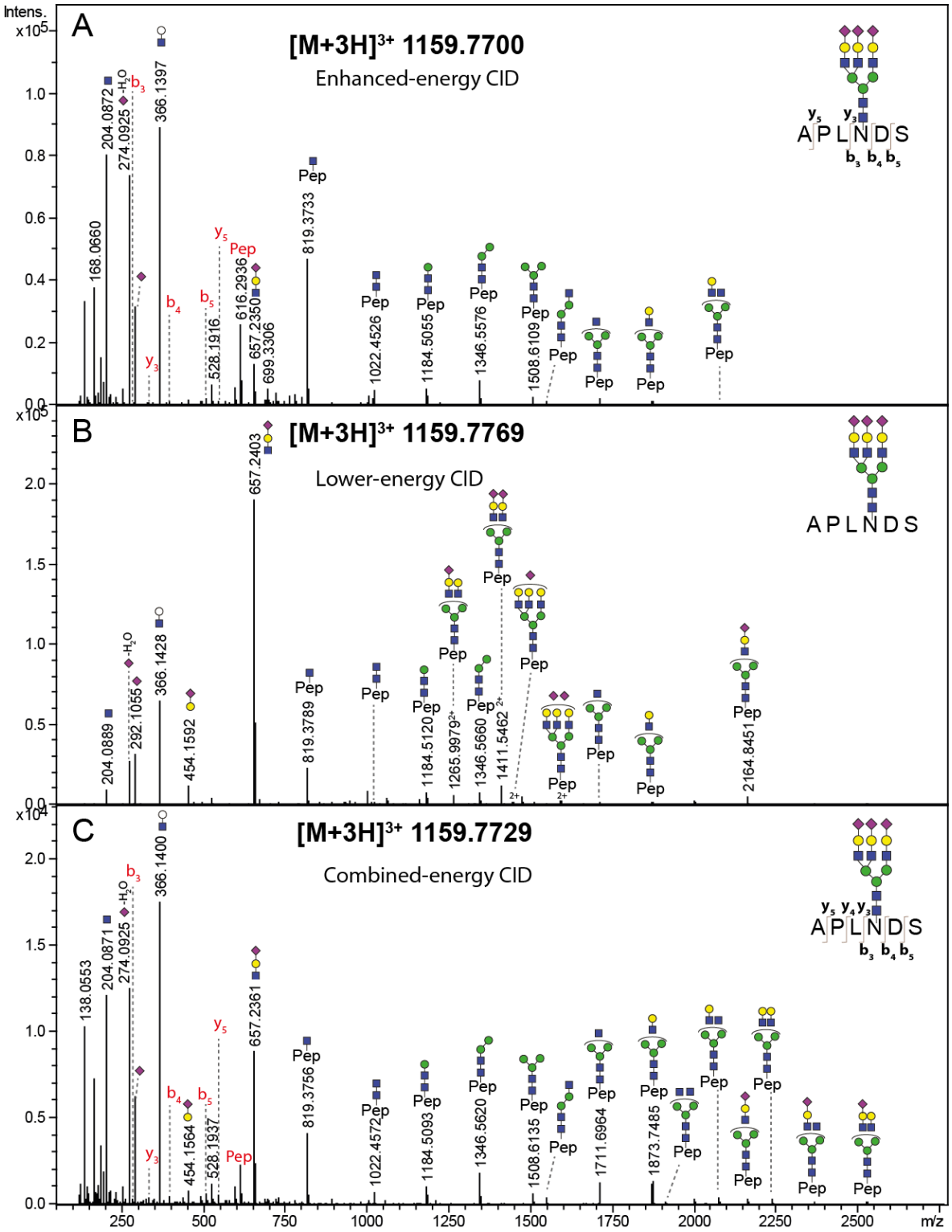

Figure S-6: MS/MS fragmentation spectra of a bovine fetuin N-glycopeptide with the peptide sequence APLNDS and a triantennary trisialylated N-glycan by (A) enhanced-energy CID using 100% of the collision energy without stepping and (B) lower-energy CID using 45% of the collision energy without stepping. (C) Combined lower- and enhanced-energy CID using 80-140% of the collision energy in stepping mode.

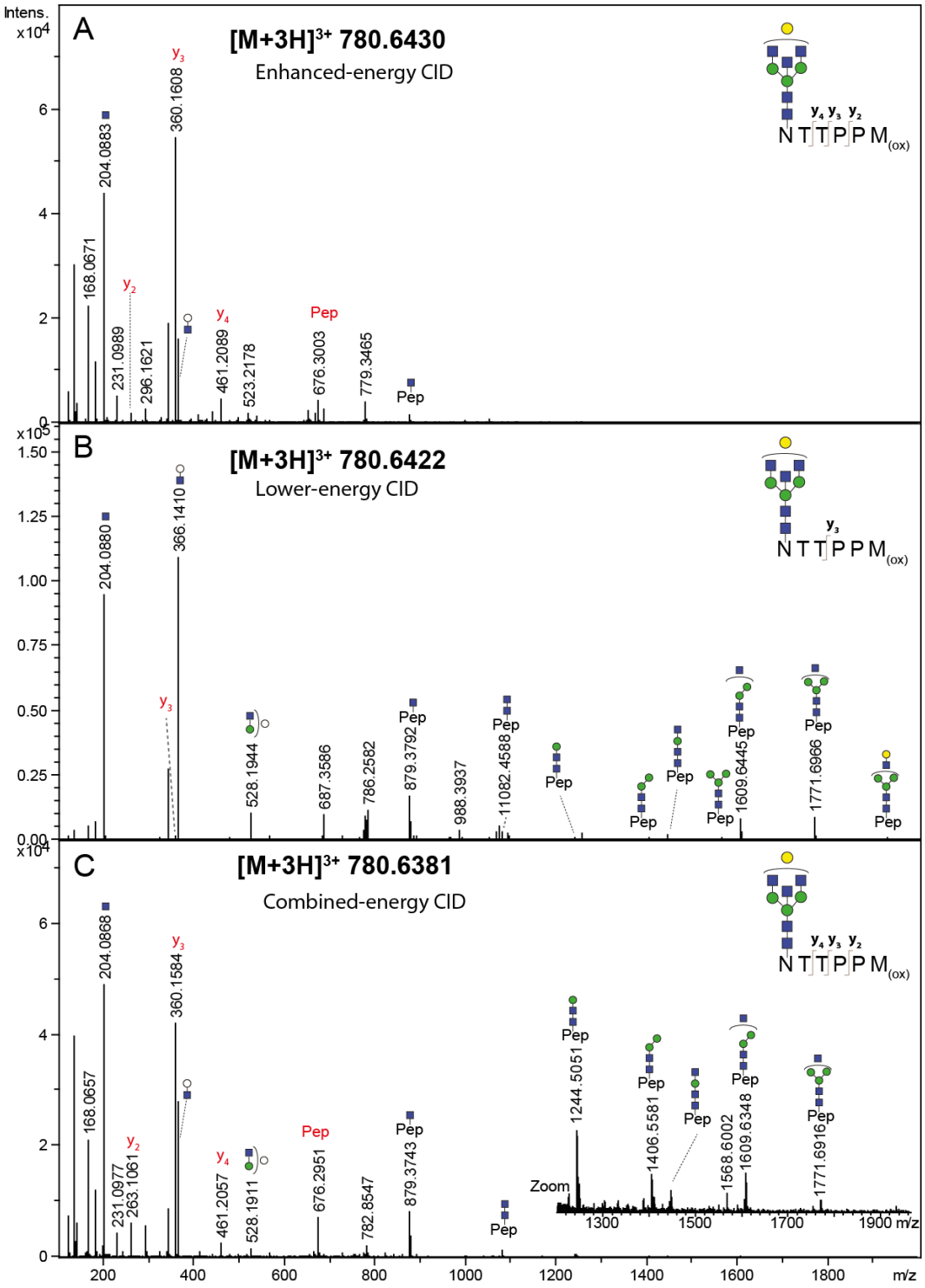

Figure S-7: MS/MS fragmentation spectra of a human IgG3 N-glycopeptide with the peptide sequence NTTPPM(ox) and a diantennary monogalactosylated glycan with a bisecting GlcNAc by (A) enhanced-energy CID using 100% of the collision energy without stepping and (B) lower-energy CID using 45% of the collision energy without stepping. (C) Combined lower- and enhanced-energy CID using 80-140% of the collision energy in stepping mode.

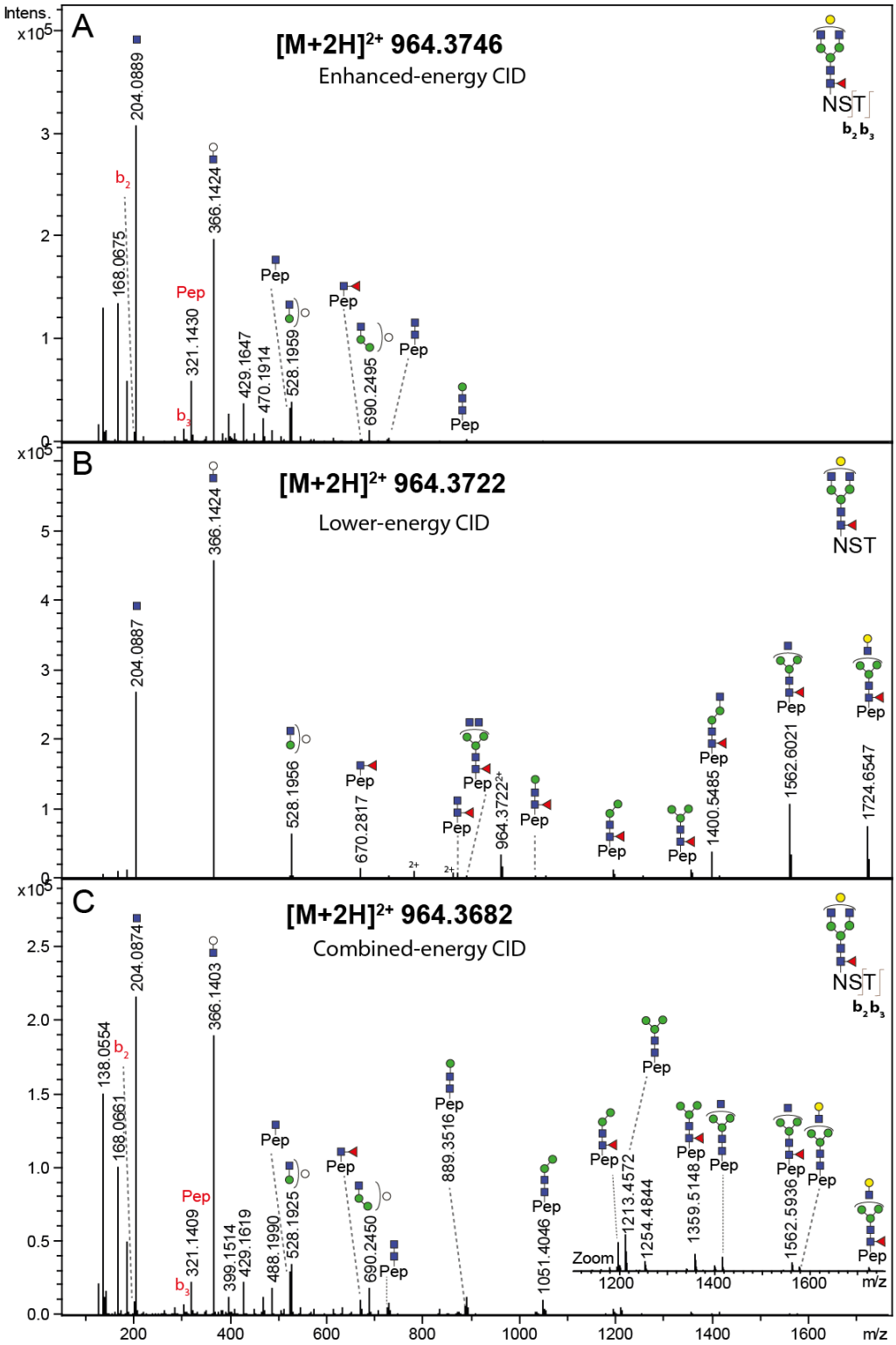

Figure S-8: MS/MS fragmentation spectra of a human IgG3 N-glycopeptide with the peptide sequence NST and a diantennary monogalactosylated glycan by (A) enhanced-energy CID using 100% of the collision energy without stepping and (B) lower-energy CID using 45% of the collision energy without stepping. (C) Combined lower- and enhanced-energy CID using 80-140% of the collision energy in stepping mode.

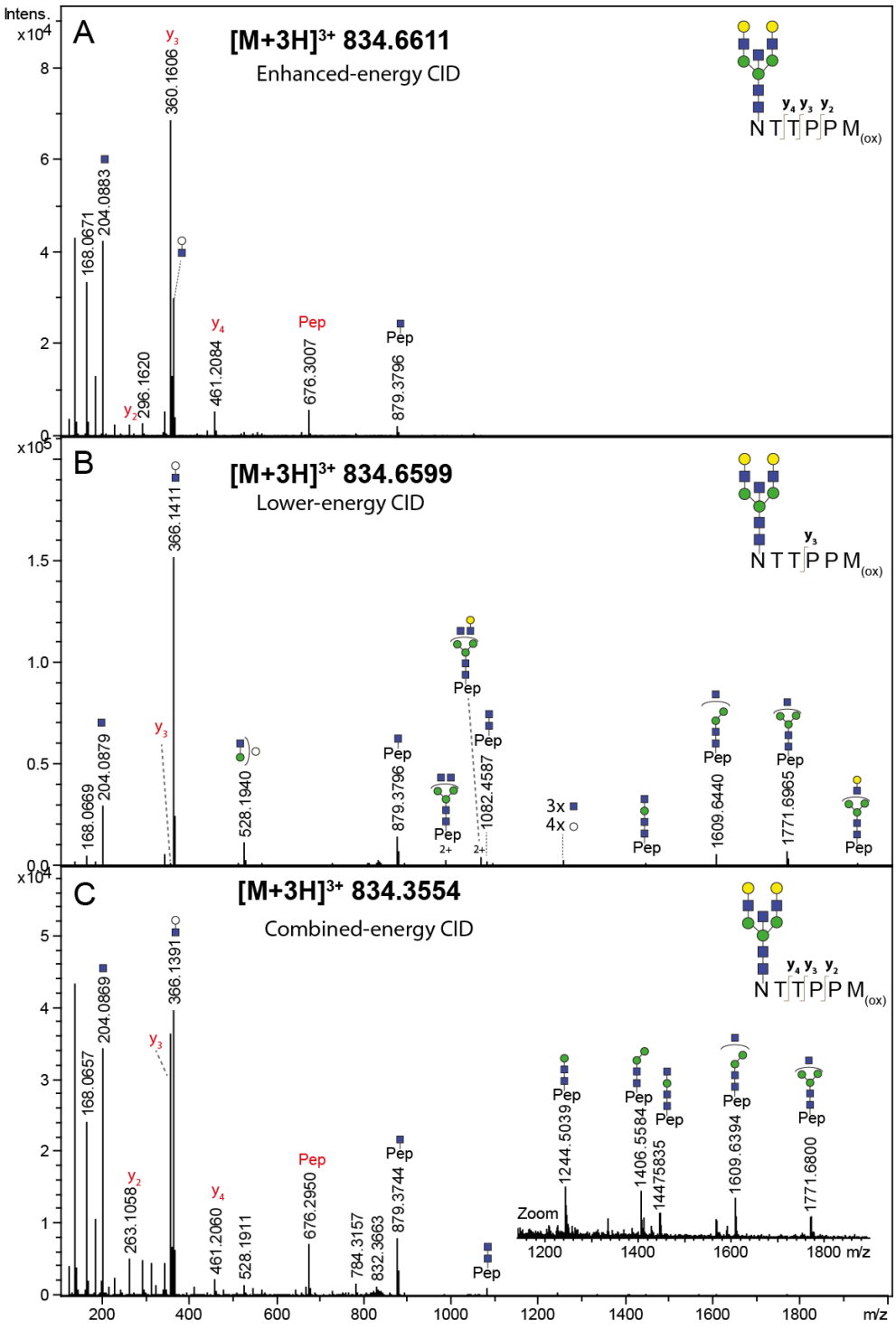

Figure S-9: MS/MS fragmentation spectra of a human IgG3 N-glycopeptide with the peptide sequence NTTPPM(ox) and a diantennary digalactosylated glycan with a bisecting GlcNAc by (A) enhanced-energy CID using 100% of the collision energy without stepping and (B) lower-energy CID using 45% of the collision energy without stepping. (C) Combined lower- and enhanced-energy CID using 80-140% of the collision energy in stepping mode.

Table S-1: MS/MS fragment ions of N- and O-glycopeptides from bovine fetuin and human IgG3 with lower-energy CID using 45% of the collision energy without stepping (lower), enhanced-energy CID using 100% of the collision energy without stepping (enhanced) and combined lower- and enhanced-energy CID using 80-140% of the collision energy in stepping mode (combined). All detected peptide fragments, oxonium ions and glycan derived Y-type ions are listed. In column seven and eight 'N' represents N-acetylhexosamin, 'H' hexose, 'S' sialic acid. Table continues on next page.

CID energy mode	Peptide sequence	Observed glycopeptide mass	observed peptide mass [M+H]+	Glycan composition	Peptide fragments	Oxonium ions	Glycan derived Y-ions
enhanced	GP**S**VV	557.7483^{2+}	458.2612	NHS	b2 155.0819, b3 242.1139, b4 341.1821, b5 440.2502, y4 401.2391, a4 313.1872, pep 458.2612	N 204.0871, S-H2O 274.0927, S 292.1030, NH 366.1396, NHS 657.2354	Pep+N 661.3401
lower	GP**S**VV	557.7533^{2+}	458.2648	NHS	b4 341.1853, b5 440.2545, pep 458.2648	N 204.0891, S-H2O 274.0950, S 292.1058, NH 366.1427, HS 454.1593, NHS 657.2399	Pep+N 661.3458, Pep+NH 823.3995
combined	GP**S**VV	557.7511^{2+}	458.2625	NHS	b2 155.0819, b3 242.1139, b4 341.1827, b5 440.2516, y4 401.2400, a4 313.1880, y5 458.2625, pep 458.2625	N 204.0872, S-H2O 274.0931, S 292.1036, NH 366.1403, NHS 657.2368	Pep+N 661.3424, Pep+NH 823.3953
enhanced	GP**T**P**S**	557.7297^{2+}	458.2241	NHS	b2 155.0821, b3 256.1290, b4 353.1808, b5 440.2132, y2 203.1028, y3 304.1501, pep 458.2241	N 204.0867, S-H2O 274.0921, S 292.1024, NH 366.1391, NHS 657.2359	Pep+N 661.3030, Pep+NH 823.3575
lower	GP**T**P**S**	557.7353^{2+}	458.2286	NHS	b5 440.2179, y2 203.1049, pep 458.2286	N 204.0888, S-H2O 274.0951, S 292.1058, NH 366.1431, HS 454.1595, NHS 657.2400	Pep+N 661.3097, Pep+NH 823.3632
combined	GP**T**P**S**	557.732^{2+}	458.2258	NHS	b2 155.0818, b3 256.1294, b4 353.1819, b5 440.2143, y2 203.1031, y3 304.1506, pep 458.2258	N 204.0869, S-H2O 274.0928, S 292.1031, NH 366.1397, NHS 657.2358	Pep+N 661.3051, Pep+NH 823.3584
enhanced	EAP**S**AVPD	721.3024^{2+}	785.3678	NHS	b2 201.0875, b3 298.1402, b4 385.1717, b5 456.2090, b6 555.2780, b7 652.3303, b8 767.3567, y2 231.0982, y3 330.1661, y4 401.2030, y6 585.2881, pep 785.3678	N 204.0872, S-H2O 274.0930, S 292.1031, NH 366.1396, NHS 657.2354	Pep+N 988.4543
lower	EAP**S**AVPD	721.3083^{2+}	785.3741	NHS	b6 555.2817, b8 767.3627, y2 231.1004, y3 330.1692, y6 585.2928, pep 785.3741	N 204.0890, S-H2O 274.0954, S 292.1061, NH 366.1427, HS 454.1594, NHS 657.2402	Pep+N 988.4551, Pep+NH 1150.5082
combined	EAP**S**AVPD	721.3054^{2+}	785.3702	NHS	b2 201.0874, b3 298.1408, b4 385.1727, b5 456.2101, b6 555.2797, b7 652.3316, b8 767.3584, y2 231.0983, y3 330.1668, y4 401.2035, y6 585.2898, pep 785.3702	N 204.0871, S-H2O 274.0933, S 292.1037, NH 366.1404, NHS 657.2366	Pep+N 988.4508, Pep+NH 1150.5046
enhanced	GP**T**P**S**AAGPP	721.9662^{3+}	851.4267	2xNHS	b2 155.0822, b3 256.1291, b5 440.2138, b6 511.2514, b7 582.2882, b8 639.3096, b9 736.3633, b10 833.4163, y2 213.1240, y3 270.1454, y4 341.1823, y5 412.2186, y7 596.3040, y8 697.3521, pep 851.4267	N 204.0873, S-H2O 274.0929, S 292.1031, NH 366.1395, NHS 657.2352	Pep+N 1054.5059, Pep+NH 1216.5591, Pep+N2 1257.5858, Pep+N2H 1419.6387
lower	GP**T**P**S**AAGPP	721.9719^{3+}	851.4307	2xNHS	y2 213.1257, y3 270.1477, y4 341.1848, pep 851.4307	N 204.0891, S-H2O 274.0953, S 292.1060, NH 366.1428, HS 454.1595, NHS 657.2400	Pep+N 1054.5122, Pep+NH 1216.5668, Pep+N2 1257.5927, Pep+N2H 1419.6469, Pep+NHS 1507.6621, Pep+N2H2 1581.6989, Pep+N, Pep+N2HS^{2+} 936.9029, Pep+N2HS^{2+} 855.8765, Pep+NHS^{2+} 754.3360, Pep+NH^{2+} 608.7874, Pep+N2H2^{2+} 791.3547, Pep+N2H^{2+} 710.3281, Pep+N2^{2+} 629.3007, Pep+N^{2+} 527.7599
combined	GP**T**P**S**AAGPP	721.968^{3+}	851.4282	2xNHS	b2 155.0820, b3 256.1295, b5 440.2137, b6 511.2533, b7 582.2894, b8 639.3115, b9 736.3638, b10 833.4186, y2 213.1239, y3 270.1455, y4 341.1829, y5 412.2185, y7 596.3053, y8 697.3545, pep 851.4282	N 204.0870, S-H2O 274.0930, S 292.1034, NH 366.1399, NHS 657.2369	Pep+N 1054.5084, Pep+NH 1216.5605, Pep+N2 1257.5889, Pep+N2H 1419.6386, Pep+NHS 1507.6621, Pep+N2H2 1581.6983

CID energy mode	Peptide sequence	Observed glycopeptide mass	observed peptide mass [M+H]+	Glycan composition	Peptide fragments	Oxonium ions	Glycan derived Y-ions
enhanced	APL**N**DS	1159.77^{3+}	616.2936	N5H6S3	b3 282.1827, b4 396.2243, b5 511.2509, y3 335.1199, y5 545.2572, pep 616.2936	N 204.0872, S-H2O 274.0925, S 292.1029, HS 454.1561, NH2 528.1916, NHS 657.2350, N2H2 731.2718	Pep+N 819.3733, Pep+N2 1022.4526, Pep+N2H 1184.5055, Pep+N2H2 1346.5576, Pep+N2H3 1508.6109, Pep+N3H2 1549.6381, Pep+N3H3 1711.6885, Pep+N3H4 1873.7411, Pep+N4H3 1914.7648, Pep+N3H3S 2002.8039, Pep+N4H4 2076.8275, Pep+N3H4S 2164.8429, Pep+N4H5 2238.8731
lower	APL**N**DS	1159.7769^{3+}	Pep+N 819.3789	N5H6S3		N 204.0889, S-H2O 274.0949, S 292.1055, NH 366.1428, HS 454.1592, NH2 528.1965, NHS 657.2403, N2H2 731.2757, NH2S 819.2931, N2H3S 1184.4272, N2H3S2 1475.5246	Pep+N 819.3789, Pep+N2 1022.4584, Pep+N2H 1184.5120, Pep+N2H2 1346.5660, Pep+N2H3 1508.6110, Pep+N3H2 1549.6474, Pep+N3H3 1711.7022, Pep+N3H4 1873.7491, Pep+N4H3 1914.7845, Pep+N3H3S 2002.7916, Pep+N4H4 2076.8291, Pep+N3H4S 2164.8451, Pep+N4H5 2238.8774, Pep+N4H4S 2367.9205, Pep+N4H5S 2529.9796, Pep+N3H3^{2+} 856.3547, Pep+N3H3S^{2+} 1001.9038, Pep+N3H4S^{2+} 1082.9275, Pep+N4H5S^{2+} 1265.4965, Pep+N5H4S2^{2+} 1329.9716, Pep+N5H5S^{2+} 1367.0368, Pep+N4H5S2^{2+} 1411.0443, Pep+N5H6S^{2+} 1448.0624, Pep+N5H5S $^{2+}$ 1512.5812, Pep+N5H6S2^{2+} 1593.6097
combined	APL**N**DS	1159.7729^{3+}	616.2951	N5H6S3	b3 282.1821, b4 396.2247, b5 511.2522, y3 335.1219, y5 545.2583, pep 616.2951	N 204.0871, S-H2O 274.0925, S 292.1032, NH 366.1400, HS 454.1564, NH2 528.1937, NHS 657.2361, N2H2 731.2747, NH2S 819.2886, N2H2S 1184.4191	Pep+N 819.3756, Pep+N2 1022.4572, Pep+N2H 1184.5093, Pep+N2H2 1346.5620, Pep+N2H3 1508.6135, Pep+N3H2 1549.6376, Pep+N3H3 1711.6964, Pep+N3H4 1873.7485, Pep+N4H3 1914.7763, Pep+N3H3S 2002.7973,Pep+N4H4 2076.8343, Pep+N3H4S 2164.8448, Pep+N4H5 2238.8893, Pep+N4H4S 2367.9210, Pep+N4H5S 2529.9720
enhanced	**N**ST	964.3746^{2+}	321.143	N4H3F	b2 202.0842, b3 303.1322, pep 321.1430	N 204.0889, NH 366.1424, NH2 528.1959, NH3 690.2495, N2H3 893.3299, N2H4 1055.3837	Pep+N 524.2235, Pep+NF 670.2822, Pep+N2 727.3038, Pep+N2F 873.3599, Pep+N2H 889.3569, Pep+N2HF 1035.4137, Pep+N2H2 1051.4110, Pep+N2H2F 1197.4702, Pep+N2H3 1213.4621, Pep+N3H2 1254.4908, Pep+N2H3F 1359.5197, Pep+N3H2F 1400.5499, Pep+N3H3 1416.5425, Pep+N3H3F 1562.6019, Pep+N3H4 1578.6095, Pep+N3H4F 1724.6551
lower	**N**ST	964.3722^{2+}	321.1418	N4H3F	pep 321.1418	N 204.0887, NH 366.1424, NH2 528.1956, NH3 690.2491, N2H3 893.3298, N2H4 1055.3828, N3H4 1258.4624	Pep+N 524.2231, Pep+NF 670.2817, Pep+N2 727.3034, Pep+N2F 873.3618, Pep+N2H 889.3583, Pep+N2HF 1035.4150, Pep+N2H2 1051.4098, Pep+N2H2F 1197.4686, Pep+N2H3 1213.4635, Pep+N3H2 1254.4901 , Pep+N2H3F 1359.5215, Pep+N3H2F 1400.5485, Pep+N3H3 1416.5429, Pep+N3H3F 1562.6021, Pep+N3H4 1578.5959, Pep+N3H4F 1724.6547
combined	**N**ST	964.3682^{2+}	321.1409	N4H3F	b2 202.0827, b3 303.1304, pep 321.1409	N 204.0874, NH 366.1403, NH2 528.1925, NH3 690.2450, N2H3 893,3242, N2H4 1055.3772	Pep+N 524.2201, Pep+NF 670.2776, Pep+N2 727.2992, Pep+N2F 873.3566, Pep+N2H 889.3516, Pep+N2HF 1035.4084, Pep+N2H2 1051.4046, Pep+N2H2F 1197.4620, Pep+N2H3 1213.4572, PepN3H2 1254.4844, Pep+N2H3F 1359.5148, Pep+N3H2F 1400.5410, Pep+N3H3 1416.5367, Pep+N3H3F 1562.5936, Pep+N3H4 1578.5886, Pep+N3H4F 1724.6477

CID energy mode	Peptide sequence	Observed glycopeptide mass	observed peptide mass [M+H]+	Glycan composition	Peptide fragments	Oxonium ions	Glycan derived Y-ions
enhanced	**N**TTPPM(ox)	780.643^{3+}	676.3003	N5H4	y2 263.1073, y3 360.1608, y4 461.2089, pep 676.3003	N 204.0883, NH 366.1415	Pep+N 879.3818
lower	**N**TTPPM(ox)	780.6422^{3+}	676.2986	N5H4	y3 360.1601, pep 676.2986	N 204.0880, NH 366.1410, NH2 528.1944, N2H2 731.2728, N2H3 893.3286, N2H4 1055.3807, N3H4 1258.4600	Pep+N 879.3792, Pep+N2 1082.4588, Pep+N2H 1244.5118, Pep+N2H2 1406.5659, Pep+N3H 1447.5924, Pep+N2H3 1568.6173, Pep+N3H2 1609.6445, Pep+N3H3 1771.6966, Pep+N3H4 1933.7492, Pep+N4H4^{2+} 1068.9197, Pep+N4H3^{2+} 987.8933, Pep+N3H4^{2+} 967.3778, Pep+N4H2^{2+} 906.8662, Pep+N3H3^{2+} 886.3536, Pep+N3H2^{2+} 805.3266
combined	**N**TTPPM(ox)	780.6381^{3+}	676.2951	N5H4	y2 263.1061, y3 360.1584, y4 461.2057, pep 676.2951	N 204.0868, NH 366.1392, NH2 528.1911, N2H2 731.2700, N2H3 893.3239	Pep+N 879.3743, Pep+N2 1082.4541, Pep+N2H 1244.5051, Pep+N2H2 1406.5581, Pep+N3H 1447.5804, Pep+N2H3 1568.6002, Pep+N3H2 1609.6348, Pep+N3H3 1771.6916
enhanced	**N**TTPPM(ox)	834.6611^{3+}	676.3007	N5H5	y3 263.1081, y3 360.1606, y4 461.2084, Pep 676.3007	N 204.0883, NH 366.1414, NH2 528.1946	Pep+N 879.3796, Pep+N2 1082.4512
lower	**N**TTPPM(ox)	834.6599^{3+}	676.3015	N5H5	y3 360.1597, Pep 676.3015	N 204.0879, NH 366.1411, NH2 528.1940, NH3 690.2450, N2H3 893.3285, N2H4 1055.3798, N3H4 1258.4597, N3H5 1420.5122	Pep+N 879.3796, Pep+N2 1082.4587, Pep+N2H 1244.5083, Pep+N2H2 1406.5700, Pep+N3H 1447.5948, Pep+N2H3 1568.6189, Pep+N3H2 1609.6440, Pep+N3H3 1771.6965, Pep+N3H4 1933.7519, Pep+N4H4^{2+} 1068.9195, Pep+N4H3^{2+} 987.8932, Pep+N3H4^{2+} 967.3787, Pep+N4H2^{2+} 906.8673, Pep+N3H3^{2+} 886.3542
combined	**N**TTPPM(ox)	834.3554^{3+}	676.295	N5H5	y3 263.1058, y3 360.1585, y4 461.2060, Pep 676.2950	N 204.0869, NH 366.1391, NH2 528.1911, NH3 690.2441, N2H3 893.3250	Pep+N 879.3744, Pep+N2 1082.4536, Pep+N2H 1244.5039, Pep+N2H2 1406.5584, Pep+N3H 1447.5835, Pep+N2H3 1568.6096, Pep+N3H2 1609.6394, Pep+N3H3 1771.6800

Application to the standard proteins bovine fetuin and human fibrinogen

Table S-2: N- and O-glycopeptides of bovine fetuin detected by C18-PGC-LC-ESI-MS/MS. Glycoproteins were subjected to in-gel Pronase treatment.

							Experimental [M+H]+		Experimental [M+2H]2+		Experimental [M+3H]3+		Experimental [M+4H]4+		Peptide mass extracted from MS2 spectrum		Glycan composition				Theoretical glycopeptide mass			
Peptide sequence (neighboring amino acid)	Peptide sequence numbering	Glycosylation site	Theoretical peptide mass [M]	Glycan composition	Elution time (min)	Column	Observed m/z	Mass deviation [ppm]	observed m/z	Mass deviation [ppm]	observed m/z	Mass deviation [ppm]	observed m/z	Mass deviation [ppm]	observed m/z pep	Mass deviation [ppm]	HexNAc	Hex	Sia	Fuc	[M+H]+	[M+2H]2+	[M+3H]3+	[M+4H]4+
Bovine fetuin N-glycans																								
(L)ANCS(V)	98-101	Asn99	450.1533	HexNAc5Hex6Sia3	13.2	C18			1656.5827	-0.7	1104.7280	2.7	828.7984	3.4	451.1609	0.7	5	6	3		3312.1606	1656.5839	1104.7250	828.7956
(L)ANCS(V)	98-101	Asn99	450.1533	HexNAc4Hex5Sia2	58.9	PGC			1328.4720	1.4	885.9838	1.5			451.1618	2.7	4	5	2		2655.9330	1328.4701	885.9825	664.7387
(L)ANCS(V)	98-101	Asn99	450.1533	HexNAc5Hex6Sia2	11.9/59.5	C18/PGC					1007.6949	1.6					5	6	2		3021.0652	1511.0362	1007.6932	756.0218
(L)ANCS(V)	98-101	Asn99	450.1533	HexNAc4Hex5Sia1	48.2	PGC					788.9518	1.4					4	5	1		2364.8376	1182.9224	788.9507	591.9649
(L)APLNDSR(V)	153-159	Asn156	771.3876	HexNAc4Hex5Sia2	14.6	C18			1489.0927	3.7	993.0641	3.5	745.0497	3.3	772.3966	2.3	4	5	2		2977.1672	1489.0873	993.0606	745.0473
(L)APLNDSR(V)	153-159	Asn156	771.3876	HexNAc5Hex6Sia3	16.8	C18			1817.2103	5.1	1211.8077	3.8	909.1075	3.7	772.3971	2.9	5	6	3		3633.3948	1817.2011	1211.8031	909.1042
(L)APLNDSR(V)	153-159	Asn156	771.3876	HexNAc5Hex6Sia4	20.2	C18					1308.8390	3.1	981.8813	3.4	772.3981	4.2	5	6	4		3924.4902	1962.7488	1308.8349	981.8780
(L)APLNDSR(V)	153-159	Asn156	771.3876	HexNAc5Hex5Sia2	14.5	C18					1114.7755	3.7	836.3333	3.6	772.3965	2.2	5	6	2		3342.2994	1671.6534	1114.7713	836.3303
(L)NDSR(V)	156-159	Asn156	490.2136	HexNAc5Hex6Sia3	11.1	C18					1118.0775	-0.9			491.2252	8.8	5	6	3		3352.2209	1676.6141	1118.0785	838.8107
(L)APLNDS(R)	153-158	Asn156	615.2865	HexNAc5Hex6Sia2	17.3	C18					1062.7408	3.0					5	6	2		3186.1983	1593.6028	1062.7376	797.3050
(L)APLNDS(R)	153-158	Asn156	615.2865	HexNAc4Hex5Sia2	17.5	C18			1411.0425	4.1	941.0301	3.4			616.2957	3.2	4	5	2		2821.0661	1411.0367	941.0269	706.0220
(L)APLNDS(R)	153-158	Asn156	615.2865	HexNAc5Hex6Sia3	22.7	C18			1739.1587	4.7	1159.7726	2.7	870.0812	2.7	616.2953	2.6	5	6	3		3477.2937	1739.1505	1159.7694	870.0789
(N)AESNG(S)	173-177	Asn176	476.1867	HexNAc5Hex6Sia3	14.2	C18					1113.4003	-2.3					5	6	3		3338.1940	1669.6006	1113.4029	835.3040
(A)ESNGS(Y)	173-177	Asn176	492.1817	HexNAc5Hex6Sia4	14.3	C18					1215.7711	4.0					5	6	4		3645.2843	1823.1458	1215.7663	912.0765
(A)ESNGS(Y)	173-177	Asn176	492.1817	HexNAc5Hex6Sia2	12.9	C18					1021.7039	1.2					5	6	2		3063.0935	1532.0504	1021.7027	766.5288
Bovine fetuin O-glycans																								
(A)EAPSAVPD(A)	268-275	Ser271	784.3603	HexNAc1Hex1Sia1	17.6	C18	1441.6009	3.9	721.3053	5.6					785.3703	3.4	1	1	1		1441.5952	721.3012	481.2033	361.1543
(A)EAPSAVPD(A)	268-275	Ser271	784.3603	HexNAc1Hex1Sia2	20.2	C18	1732.6935	1.7	866.8525	4.1					785.3706	3.8	1	1	2		1732.6906	866.8489	578.2351	433.9281
(A)EAPSAVPD(A)	268-275	Ser271	784.3603	HexNAc2Hex2Sia2	20.3	C18			1049.4185	3.3	699.9480	3.2			785.3696	2.5	2	2	2		2097.8228	1049.4150	699.9458	525.2112
(E)APSAVPD(A)	269-275	Ser271	655.3178	HexNAc1Hex1Sia2	19.5	C18	1603.6449	-1.9	802.3343	8.3					656.3267	2.6	1	1	2		1603.6480	802.3276	535.2209	401.6675
(E)APSAVPD(A)	269-275	Ser271	655.3178	HexNAc1Hex1Sia1	16.6	C18	1312.5568	3.2	656.7828	4.3					656.3271	3.2	1	1	1		1312.5526	656.7799	438.1891	328.8936
(A)GPTPS(A)	278-282	Thr280/Ser282	457.2173	HexNAc1Hex1Sia1	44.5	C18/PGC	1114.4545	2.1	557.7319	3.9					458.2257	2.4	1	1	1		1114.4522	557.7297	372.1556	279.3685
(A)GPTPS(A)	278-282	Thr280/Ser282	457.2173	HexNAc1	44.6	PGC	661.3052	1.8							458.2247	0.3	1				661.3040	331.1556	221.1062	166.0814
(A)GPTPS(A)	278-282	Thr280/Ser282	457.2173	HexNAc1Hex1Sia2	13.6	C18			703.2796	3.1					458.2257	2.4	1	1	2		1405.5476	703.2774	469.1874	352.1423
(A)GPTPSAAGPP(V)	278-287	Thr280/Ser282	850.4185	HexNAc1Hex1Sia1 + HexNAc1Hex+1	16	C18			936.8995	3.3	624.9355	3.4			851.4286	3.3	2	2	1		1872.7856	936.8964	624.9334	468.9519
(A)GPTPSAAGPP(V)	278-287	Thr280/Ser282	850.4185	2x HexNAc1Hex1Sia1	19	C18			1082.4482	3.8	721.9680	3.9			851.4283	2.9	2	2	2		2163.8810	1082.4441	721.9652	541.7257
(A)GPTPSAAGPP(V)	278-287	Thr280/Ser282	850.4185	HexNAc1Hex1Sia2 + HexNAc1Hex1Sia1	23.4	C18			1227.9953	2.8	818.9992	2.7			851.4290	3.8	2	2	3		2454.9764	1227.9918	818.9970	614.4996
(A)GPTPSAAGPP(V)	278-287	Thr280/Ser282	850.4185	HexNAc1Hex2Sia2 + HexNAc1Hex1Sia1	23.4	C18			1410.5618	2.7	940.7108	3.3			851.4284	3.1	3	3	3		2820.1086	1410.5579	940.7077	705.7826
(A)GPTPSAAGPPVAS(V)	278-290	Thr280/Ser282	1107.5561	2x HexNAc1Hex1Sia1	20.4	C18			1211.0172	3.5	807.6804	3.3			1108.5669	3.2	2	2	2		2421.0186	1211.0129	807.6777	606.0101
(A)GPTPSAAGPPVAS(V)	278-290	Thr280/Ser282	1107.5561	HexNAc1Hex1Sia2 + HexNAc1Hex1Sia1	25.3	C18			1356.5666	4.4	904.7111	1.8			1108.5653	1.8	2	2	3		2712.1140	1356.5606	904.7095	678.7839
(A)GPTPSAAGPPV(A)	278-288	Thr280/Ser282	949.4869	2x HexNAc1Hex1Sia1	21.9	C18			1131.9816	2.9	754.9904	3.2			950.4971	3.0	2	2	2		2262.9494	1131.9783	754.9880	566.4928
(V)GPSVV(A)	294-298	Ser296	457.2537	HexNAc1Hex1Sia1	17.3	C18			557.7507	5.0					458.2625	3.4	1	1	1		1114.4886	557.7479	372.1677	279.3776
(V)GPSVV(A)	294-298	Ser296	457.2537	HexNAc1Hex1Sia2	21.2	C18			703.2975	2.7					458.2622	2.7	1	1	2		1405.5840	703.2956	469.1995	352.1514

Table S-3: N- and O-glycopeptides of human fibrinogen α-, β- and γ-subunit detected by C18-PGC-LC-ESI-MS/MS. Glycoproteins were subjected to in-gel Pronase treatment.

							Experimental [M+H]+		Experimental [M+2H]2+		Experimental [M+3H]3+		Experimental [M+4H]4+		Peptide mass extracted from MS2 spectrum		Glycan composition				Theoretical glycopeptide mass			
Peptide sequence (neighboring amino acid)	Peptide sequence numbering	Glycosylation site	Theoretical peptide mass [M]	Glycan composition	Elution time (min)	Column	Observed m/z	Mass deviation [ppm]	observed m/z	Mass deviation [ppm]	observed m/z	Mass deviation [ppm]	observed m/z	Mass deviation [ppm]	observed m/z pep	Mass deviation [ppm]	HexNAc	Hex	Sia	Fuc	[M+H]+	[M+2H]2+	[M+3H]3+	[M+4H]4+
Fibrinogen alpha O-glycans																								
(G)SETESPRNPS(S)	281-290	281-290	1102.4891	HexNAc1Hex1Sia1	12.8	C18			880.3663	0.8	587.2467	0.9			1103.4983	1.7	1	1	1		1759.7240	880.3656	587.2462	440.6865
(S)ETESPRNPS(S)	282-290	282-290	1015.4571	HexNAc1Hex1Sia1	12.7	C18			836.8500	0.5	558.2357	0.4			1016.4644	0.0	1	1	1		1672.6920	836.8496	558.2355	418.9284
(T)ATWKPG(S)	319-324	Thr320	658.3439	HexNAc1Hex1Sia1	15.5	C18			658.2934	0.6					659.3519	1.1	1	1	1		1315.5788	658.2930	439.1978	329.6501
(A)TWKPG(S)	320-324	Thr320	587.3068	HexNAc1Hex1Sia1	15	C18			622.7754	1.5					588.3147	1.1	1	1	1		1244.5417	622.7745	415.5187	311.8909
(Q)NPGSPRPG(S)	348-355	Ser351	780.3879	HexNAc1Hex1Sia1	12.2	C18			719.3173	3.2	479.8797	1.3			781.3961	1.2	1	1	1		1437.6228	719.3150	479.8791	360.1611
(A)STGKT(F)	524-528	524-528	492.2544	HexNAc1Hex1Sia1	43.2	PGC			575.2496	2.3					493.2611	-1.2	1	1	1		1149.4893	575.2483	383.8346	288.1278
(E)SSSHHPGIA€	560-568	560-568	891.4199	HexNAc1Hex1Sia1	15.6	C18			774.8318	1.0					892.4273	0.1	1	1	1		1548.6548	774.8310	516.8898	387.9192
Fibrinogen beta glycans																								
(M)GEN(R)	392-394	Asn394	318.1176	HexNAc4Hex5	41.7	PGC			971.3603	3.5	647.9096	4.0			319.1277	8.9	4	5			1941.7065	971.3569	647.9070	486.1821
(M)GEN(R)	392-394	Asn394	318.1176	HexNAc4Hex5Sia1	46.4	PGC			1116.9095	4.4	744.9422	4.6			319.1262	4.2	4	5	1		2232.8019	1116.9046	744.9388	558.9559
(M)GEN(R)	392-394	Asn394	318.1176	HexNAc4Hex5Sia2	53.2	PGC			1262.4565	3.4	841.9739	3.9			319.1266	5.5	4	5	2		2523.8973	1262.4523	841.9706	631.7298
(G)ENR(T)	393-395	Asn394	417.1972	HexNAc4Hex5Sia1	57.8	PGC			1166.4478	2.9	777.9682	3.7			418.2037	-1.9	4	5	1		2331.8815	1166.4444	777.9654	583.7258
(G)EN(R)	393-394	Asn394	261.0961	HexNAc4Hex5	40.5	PGC			942.8487	2.7	628.9018	3.1					4	5			1884.6850	942.8461	628.8998	471.9267
(G)EN(R)	393-394	Asn394	261.0961	HexNAc4Hex5Sia1	44.9	PGC			1088.3983	4.1	725.9349	4.5			262.1041	2.7	4	5	1		2175.7804	1088.3938	725.9316	544.7006
(G)EN(R)	393-394	Asn394	261.0961	HexNAc4Hex5Sia2	51.6	PGC			1233.9447	2.6	822.9660	3.1			262.1057	8.8	4	5	2		2466.8758	1233.9415	822.9634	617.4744
(R)EEAPS(L)	53-57	Ser57	531.2177	HexNAc1Hex1Sia1	12.8/48.0	C18			594.7293	-1.0					532.2247	-0.5	1	1	1		1188.4526	594.7299	396.8224	297.8686
Fibrinogen gamma N-glycans																								
(V)EN(K)	77-78	Asn78	261.0961	HexNAc4Hex4	39.9	PGC			861.8229	3.7							4	4			1722.6322	861.8197	574.8822	431.4135
(V)EN(K)	77-78	Asn78	261.0961	HexNAc4Hex5	40.7	PGC			942.8504	4.5	628.9024	4.1			262.1053	7.2	4	5			1884.6850	942.8461	628.8998	471.9267
(V)EN(K)	77-78	Asn78	261.0961	HexNAc4Hex4Sia1	44.1	PGC			1007.3731	5.6	671.9172	4.7			262.1042	3.1	4	4	1		2013.7276	1007.3674	671.9140	504.1874
(V)EN(K)	77-78	Asn78	261.0961	HexNAc4Hex5Sia1	44.7	PGC			1088.4008	6.4	725.9362	6.3			262.1047	5.0	4	5	1		2175.7804	1088.3938	725.9316	544.7006
(V)EN(K)	77-78	Asn78	261.0961	HexNAc4Hex5Sia2	50.4	PGC			1233.9473	4.7	822.9682	5.8			262.1042	3.1	4	5	2		2466.8758	1233.9415	822.9634	617.4744

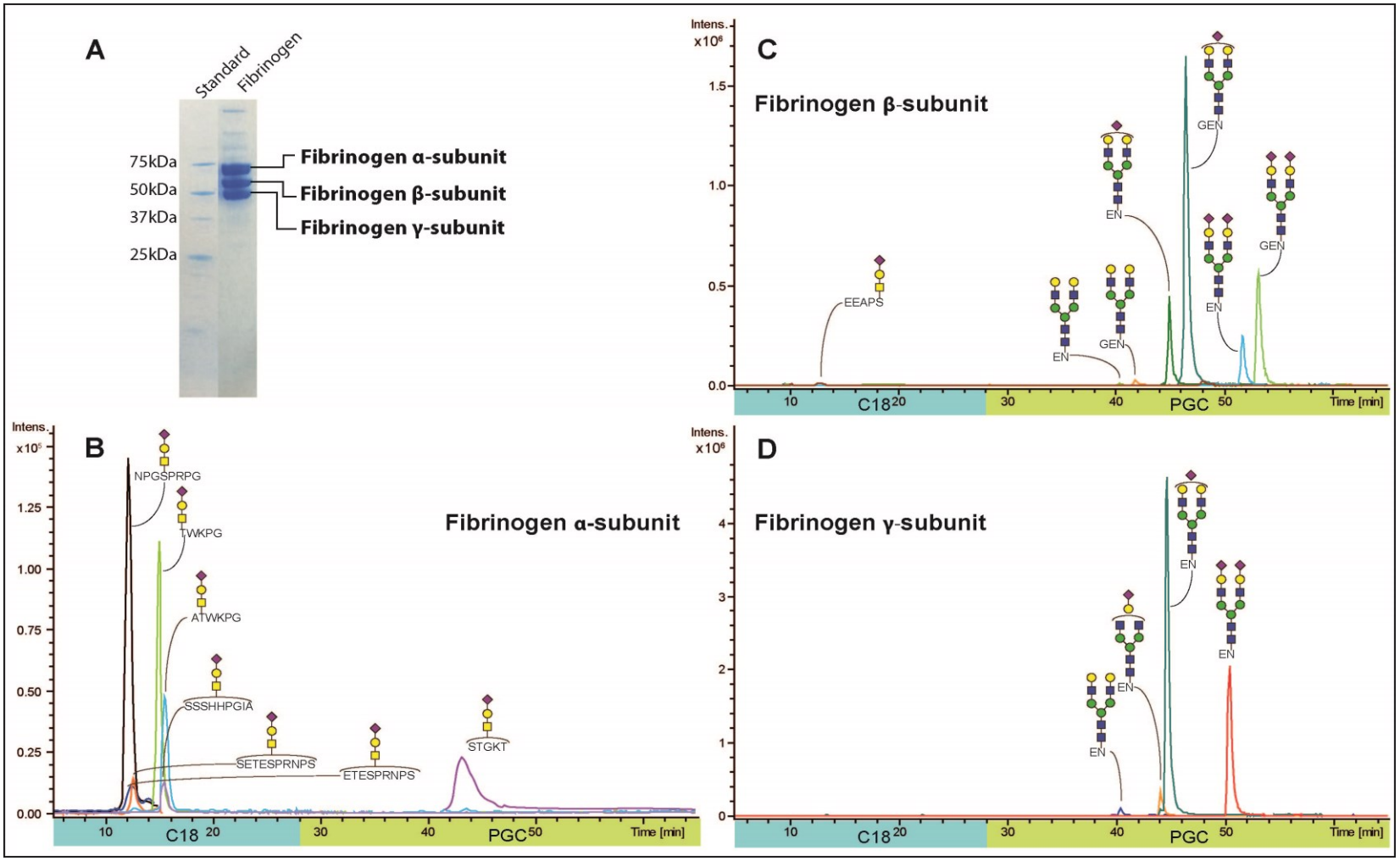

Figure S-10: C18-PGC-LC-ESI-QTOF-MS/MS analysis of human fibrinogen α-, β- and γ-subunit N- and O-glycopeptides. The fibrinogen mixture was separated on a SDS-PAGE and digested in-gel using Pronase (A). All three subunits were then analyzed by C18-PGC-ESI-QTOF-MS/MS and the EICs (± 0.02 *m/z*) of all detected N-glycopeptides are depicted for α-subunit (A), β-subunit (B) and γ-subunit (C). Glycopeptides elute from the C18 column (blue section) as well as from the PGC column (green section). (Blue square = N-acetylglucosamine, yellow square = N-acetylgalactosamine, green circle = mannose, yellow circle = galactose, pink diamond = sialic acid, red triangle = fucose).

Site-specific glycosylation analysis of IgG3 assisted by C18-PGC-LC-ESI-QTOF-MS/MS

Table S-4: N- and O-glycopeptides of human IgG3 detected by C18-PGC-LC-ESI-MS/MS. Glycoproteins were subjected to in-gel Pronase treatment. The IgG3 hinge region contains three repeating units including a possible glycosylation site each. The numbering of the amino acid sequence will be as followed: HX-Y, with X indicating the exon number (1-4) and Y the amino acid number within this exon (1-17)[3]. The table is continued on the next page.

							Experimental [M+2H]2+		Experimental [M+3H]3+		Peptide mass extracted from MS2 spectrum		Glycan composition				Theoretical glycopeptide mass	
Peptide (neighboring amino acid)	Peptide sequence	Glycosylation site	Theoretical peptide mass [M]	Glycan composition	Elution time (min)	Column	observed m/z	Mass deviation [ppm]	observed m/z	Mass deviation [ppm]	observed m/z pep	Mass deviation [ppm]	HexNAc	Hex	Sia	Fuc	[M+2H]2+	[M+3H]3+
N-glycans CH3 domain																		
(Y)NTTPPM(L)	392-397	Asn392	659.2949	HexNAc5Hex5	14.7	C18	1243.4908	4.5	829.3292	4.0	660.3034	1.8	5	5			1243.4852	829.3259
(Y)NTTPPM(L)	392-397	Asn392	659.2949	HexNAc5Hex4	14.7	C18	1162.4638	4.3	775.3119	4.6	660.3038	2.5	5	4			1162.4588	775.3083
(Y)NTTPPM(L)	392-397	Asn392	659.2949	HexNAc2Hex5	14.5	C18	938.8700	4.1			660.3046	3.7	2	5			938.8661	626.2465
(Y)NTTPPM(L)	392-397	Asn392	659.2949	HexNAc5Hex3	14.9	C18	1081.4367	4.0	721.2934	3.7	660.3058	5.5	5	3			1081.4324	721.2907
(Y)NTTPPM(L)	392-397	Asn392	659.2949	HexNAc4Hex5Sia1	16.7	C18	1287.5038	8.2	858.6684	4.5	660.3045	3.5	4	5	1		1287.4932	858.6646
(Y)NTTPPM(L)	392-397	Asn392	659.2949	HexNAc5Hex4Sia1	17.1	C18			872.3426	2.9			5	4	1		1308.0065	872.3401
(Y)NTTPPM(L)	392-397	Asn392	659.2949	HexNAc5Hex5Sia1	16.7	C18	1389.0461	9.5	926.3617	4.3	660.3064	6.4	5	5	1		1389.0329	926.3577
(Y)NTTPPM(L)	392-397	Asn392	659.2949	HexNAc4Hex5	14.5	C18	1141.9487	2.8	761.6358	4.0	660.3022	0.0	4	5			1141.9455	761.6328
(Y)NTTPPM(L)	392-397	Asn392	659.2949	HexNAc4Hex4	14.7	C18	1060.9228	3.5					4	4			1060.9191	707.6152
(Y)NTTPPMox(L)	392-397	Asn392	675.2898	HexNAc4Hex5	12.3	C18	1149.9471	3.6	766.9677	4.3			4	5			1149.9430	766.9644
(Y)NTTPPMox(L)	392-397	Asn392	675.2898	HexNAc4Hex5Sia1	13.6	C18	1295.4986	6.1	863.9999	4.3			4	5	1		1295.4907	863.9962
(Y)NTTPPMox(L)	392-397	Asn392	675.2898	HexNAc5Hex3	12.5	C18	1089.4344	4.1	726.6250	3.7			5	3			1089.4299	726.6223
(Y)NTTPPMox(L)	392-397	Asn392	675.2898	HexNAc5Hex4	12.5	C18	1170.4613	4.3	780.6432	4.2	676.2978	1.0	5	4			1170.4563	780.6399
(Y)NTTPPMox(L)	392-397	Asn392	675.2898	HexNAc5Hex5	12.5	C18	1251.4854	2.2	834.6609	4.0	676.3005	5.0	5	5			1251.4827	834.6575
(Y)NTTPPMox(L)	392-397	Asn392	675.2898	HexNAc5Hex4Sia1	13.8	C18			877.6740	2.6			5	4	1		1316.0040	877.6717
(Y)NTTPPMox(L)	392-397	Asn392	675.2898	HexNAc5Hex5Sia1	13.6	C18			931.6930	3.9			5	5	1		1397.0304	931.6893
(Y)NTTPPMox(L)	392-397	Asn392	675.2898	HexNAc2Hex5	12.1	C18	946.8672	3.8			676.3002	4.6	2	5			946.8636	631.5781
Hinge-region O-glycosylation																		
(C)DTPPPCPR(C)	H2-(6-13), H3-(6-13), H4-(6-13)	H2-7, H3-7, H4-7	938.4280	HexNAc1Hex1	12.6	C18	652.7905	4.8			939.4392	4.1	1	1			652.7874	435.5274
(C)DTPPPCPR(C)	H2-(6-13), H3-(6-13), H4-(6-13)	H2-7, H3-7, H4-7	938.4280	HexNAc1Hex1Sia1	13.9	C18	798.3373	2.8	532.5616	4.6	939.4393	4.2	1	1	1		798.3351	532.5592
(C)DTPPPCPR(C)	H2-(6-13), H3-(6-13), H4-(6-13)	H2-7, H3-7, H4-7	938.4280	HexNAc1Hex1Sia2	16	C18			629.5932	3.6			1	1	2		943.8828	629.5910

							Experimental [M+2H]2+		Experimental [M+3H]3+		Peptide mass extracted from MS2 spectrum		Glycan composition				Theoretical glycopeptide mass	
Peptide (neighboring amino acid)	Peptide sequence	Glycosylation site	Theoretical peptide mass [M]	Glycan composition	Elution time (min)	Column	observed m/z	Mass deviation [ppm]	observed m/z	Mass deviation [ppm]	observed m/z pep	Mass deviation [ppm]	HexNAc	Hex	Sia	Fuc	[M+2H]2+	[M+3H]3+
N-glycans CH2 domain																		
(Y)NST(F)	297-299	Asn297	320.1332	HexNAc4Hex3Fuc1	39.6	PGC	883.3457	5.5	589.2325	4.8	321.1415	3.1	4	3		1	883.3408	589.2297
(Y)NST(F)	297-299	Asn297	320.1332	HexNAc4Hex4Fuc1	40.3	PGC	964.3729	5.9	643.2510	5.8	321.1416	3.4	4	4		1	964.3672	643.2473
(Y)NST(F)	297-299	Asn297	320.1332	HexNAc4Hex5Fuc1	41.2	PGC	1045.3987	4.8	697.2685	5.2	321.1412	2.1	4	5		1	1045.3936	697.2649
(Y)NST(F)	297-299	Asn297	320.1332	HexNAc4Hex4Fuc1Sia1	46.3	PGC	1109.9200	4.6	740.2821	4.1	321.1414	2.8	4	4	1	1	1109.9149	740.2791
(Y)NST(F)	297-299	Asn297	320.1332	HexNAc4Hex5S1Fuc1	46.6	PGC	1190.9467	4.5	794.2998	4.0	321.1419	4.3	4	5	1	1	1190.9413	794.2967
(Y)NST(F)	297-299	Asn297	320.1332	HexNAc5Hex3Fuc1	38.5	PGC	984.8844	3.9	656.9254	4.0	321.1409	1.2	5	3		1	984.8805	656.9228
(Y)NST(F)	297-299	Asn297	320.1332	HexNAc5Hex4Fuc1	39	PGC	1065.9112	4.0	710.9435	4.4	321.1413	2.5	5	4		1	1065.9069	710.9404
(Y)NST(F)	297-299	Asn297	320.1332	HexNAc5Hex5Fuc1	39.3	PGC	1146.9376	3.7	764.9610	3.9	321.1415	3.1	5	5		1	1146.9333	764.9580
(Y)NST(F)	297-299	Asn297	320.1332	HexNAc5Hex5S1Fuc1	46	PGC			861.9943	5.2			5	5	1	1	1292.4810	861.9898
(Y)NST(F)	297-299	Asn297	320.1332	HexNAc4Hex3	37.8	PGC	810.3149	3.7			321.1414	2.8	4	3			810.3119	540.5437
(Y)NST(F)	297-299	Asn297	320.1332	HexNAc4Hex4	38.7	PGC	891.3419	4.1	594.5641	4.7	321.1411	1.8	4	4			891.3383	594.5613
(Y)NST(F)	297-299	Asn297	320.1332	HexNAc4Hex5	39.6	PGC	972.3682	3.6	648.5814	3.9	321.1407	0.6	4	5			972.3647	648.5789
(Y)NST(F)	297-299	Asn297	320.1332	HexNAc4Hex4Sia1	45.5	PGC	1036.8900	3.9					4	4	1		1036.8860	691.5931
(Y)NST(F)	297-299	Asn297	320.1332	HexNAc4Hex5Sia1	46	PGC	1117.9175	4.6	745.6140	4.4			4	5	1		1117.9124	745.6107
(Y)NST(F)	297-299	Asn297	320.1332	HexNAc5Hex3	36.7	PGC	911.8543	3.0			321.1408	0.9	5	3			911.8516	608.2368
(Y)NST(F)	297-299	Asn297	320.1332	HexNAc5Hex4	37.1	PGC	992.8820	4.0	662.2566	3.3			5	4			992.8780	662.2544
(Y)NST(F)	297-299	Asn297	320.1332	HexNAc5Hex5	37.6	PGC	1073.9080	3.4	716.2739	2.6	321.1412	2.1	5	5			1073.9044	716.2720
(Y)N(S)	297	Asn297	132.0535	HexNAc4Hex3Fuc1	39.6	PGC	789.3038	3.6	526.5384	3.8	133.0613	3.7	4	3		1	789.3010	526.5364
(Y)N(S)	297	Asn297	132.0535	HexNAc4Hex4Fuc1	40.4	PGC	870.3312	4.4	580.5566	4.4	133.0612	2.9	4	4		1	870.3274	580.5540
(Y)N(S)	297	Asn297	132.0535	HexNAc4Hex5Fuc1	41.1	PGC	951.3569	3.3	634.5740	3.8	133.0614	4.4	4	5		1	951.3538	634.5716
(Y)N(S)	297	Asn297	132.0535	HexNAc4Hex4S1Fuc1	47.2	PGC	1015.8785	3.4	677.5890	4.7	133.0621	9.7	4	4	1	1	1015.8751	677.5858
(Y)N(S)	297	Asn297	132.0535	HexNAc4Hex5S1Fuc1	47.7	PGC	1096.9056	3.7	731.6061	3.7	133.0615	5.2	4	5	1	1	1096.9015	731.6034
(Y)N(S)	297	Asn297	132.0535	HexNAc5Hex3Fuc1	38.5	PGC	890.8438	3.5	594.2315	3.3	133.0615	5.2	5	3		1	890.8407	594.2296
(Y)N(S)	297	Asn297	132.0535	HexNAc5Hex4Fuc1	38.7	PGC	971.8701	3.1	648.2492	3.2			5	4		1	971.8671	648.2472
(Y)N(S)	297	Asn297	132.0535	HexNAc5Hex5Fuc1	39.1	PGC	1052.8973	3.6	702.2670	3.2			5	5		1	1052.8935	702.2648
(Y)N(S)	297	Asn297	132.0535	HexNAc4Hex3	37.6	PGC	716.2730	1.3			133.0608	-0.1	4	3			716.2720	477.8505
(Y)N(S)	297	Asn297	132.0535	HexNAc4Hex4	38.7	PGC	797.3007	2.8					4	4			797.2984	531.8681
(Y)N(S)	297	Asn297	132.0535	HexNAc4Hex5	39.3	PGC	878.3276	3.1	585.8877	3.5			4	5			878.3248	585.8857
(Y)N(S)	297	Asn297	132.0535	HexNAc4Hex4Sia1	46	PGC	942.8486	2.6					4	4	1		942.8461	628.8999
(Y)N(S)	297	Asn297	132.0535	HexNAc4Hex5Sia1	46.7	PGC	1023.8776	4.9	682.9198	3.4			4	5	1		1023.8725	682.9175
(Y)N(S)	297	Asn297	132.0535	HexNAc5Hex3	36.4	PGC	817.8152	4.2					5	3			817.8117	545.5436
(Y)N(S)	297	Asn297	132.0535	HexNAc5Hex4	36.7	PGC	898.8407	2.8	599.5631	3.2	133.0609	0.7	5	4			898.8381	599.5612
(Y)N(S)	297	Asn297	132.0535	HexNAc5Hex5	37.4	PGC	979.8679	3.4	653.5812	3.7			5	5			979.8645	653.5788
(Y)N(S)	297	Asn297	132.0535	HexNAc5Hex5Sia1	45.5	PGC			750.6134	3.8			5	5	1		1125.4122	750.6106

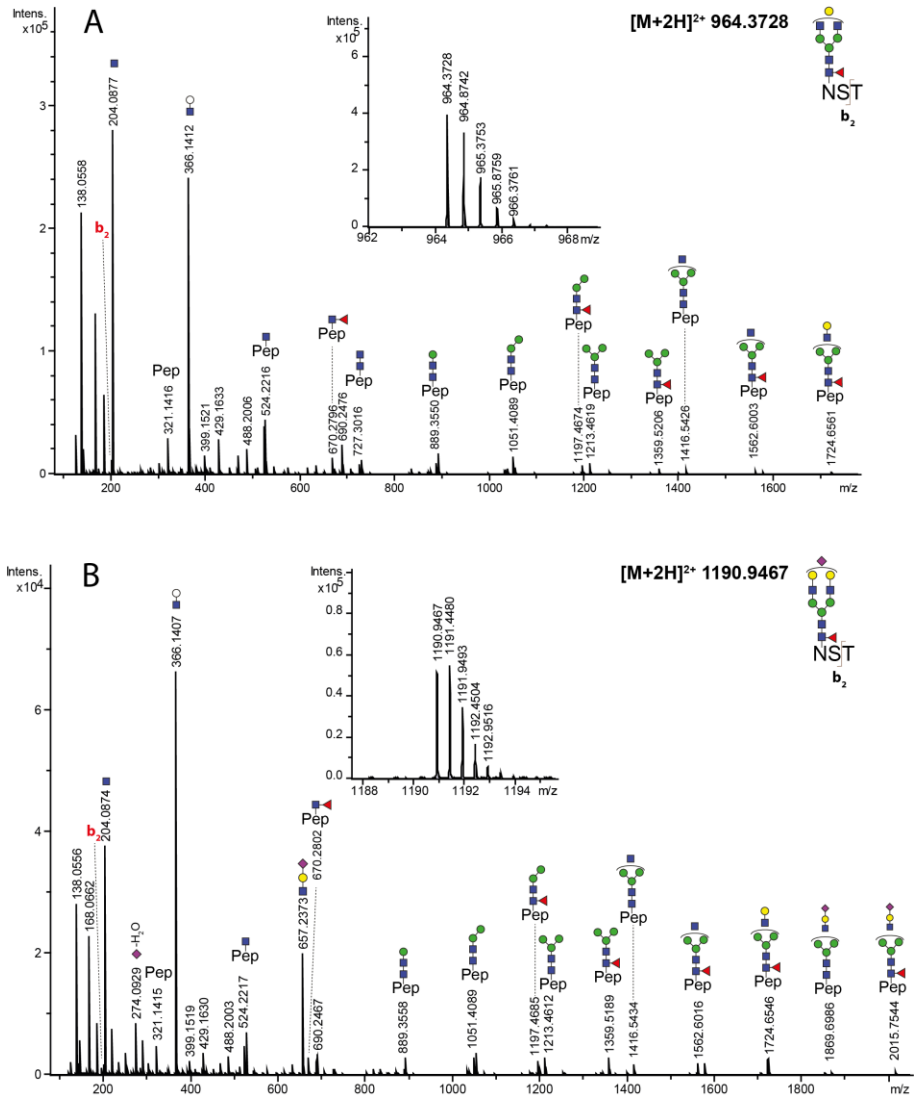

Figure S-11: MS/MS fragmentation spectra of two representative IgG3 C_H2 domain N-glycopeptides (A) $[M+2H]^{2+}$ at *m/z* 964.3728 and (B) $[M+2H]^{2+}$ at *m/z* 1190.9467 by combined lower- and elevated-collision energy CID.

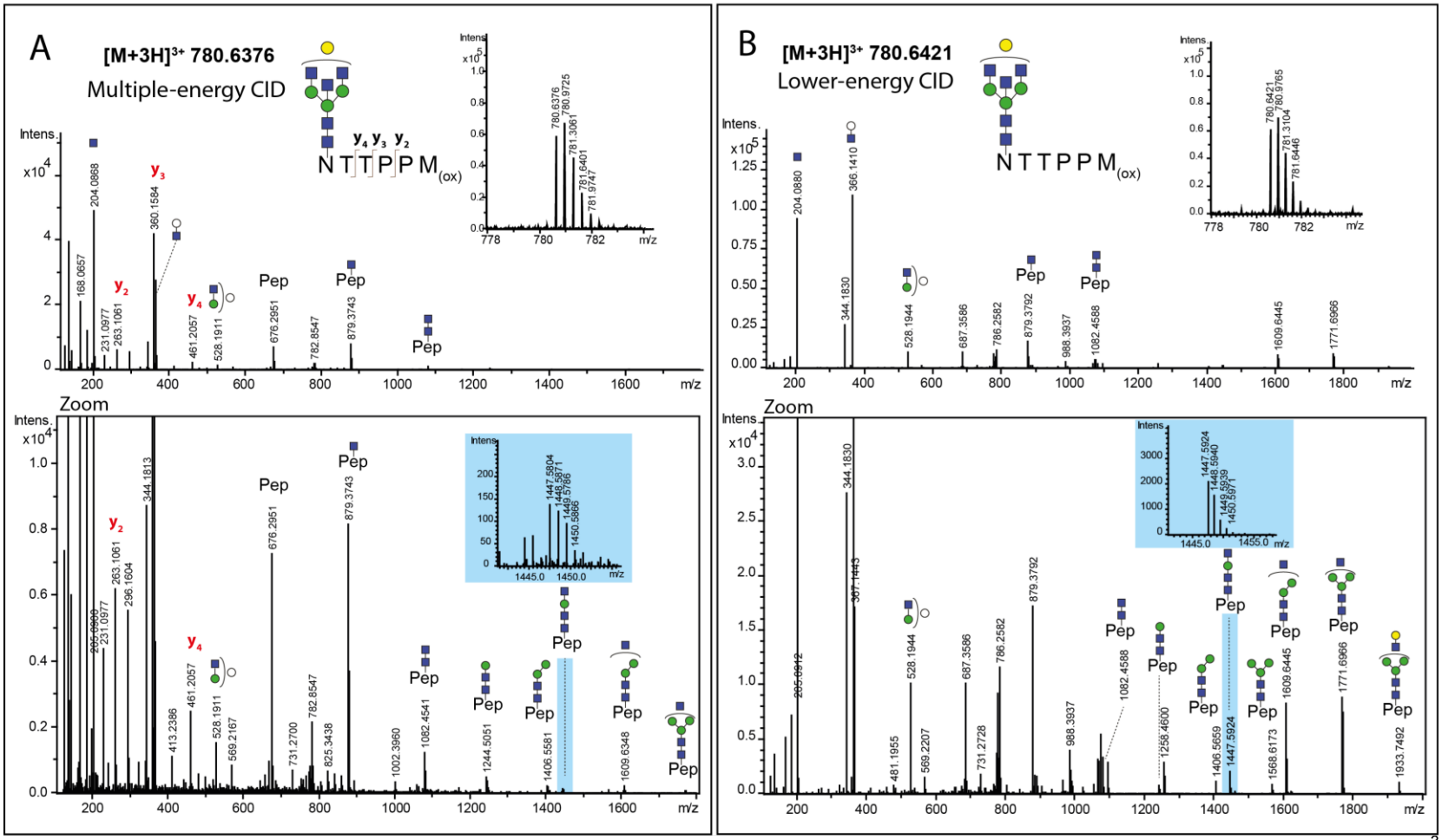

Figure S-12: MS/MS fragmentation spectra of a diantennary IgG3 C_H3 domain N-glycopeptide with a bisecting GlcNAc $[M+3H]^{3+}$ at *m/z* 780.6376 by (A) combined lower- and elevated-collision energy CID (lower panel: zoom in). (B) Lower-energy CID (45% of the collision energy without stepping) was applied to investigate the presence of a bisecting GlcNAc resulting in a more intense peak at *m/z* 1447.5924 (lower panel: zoom in).

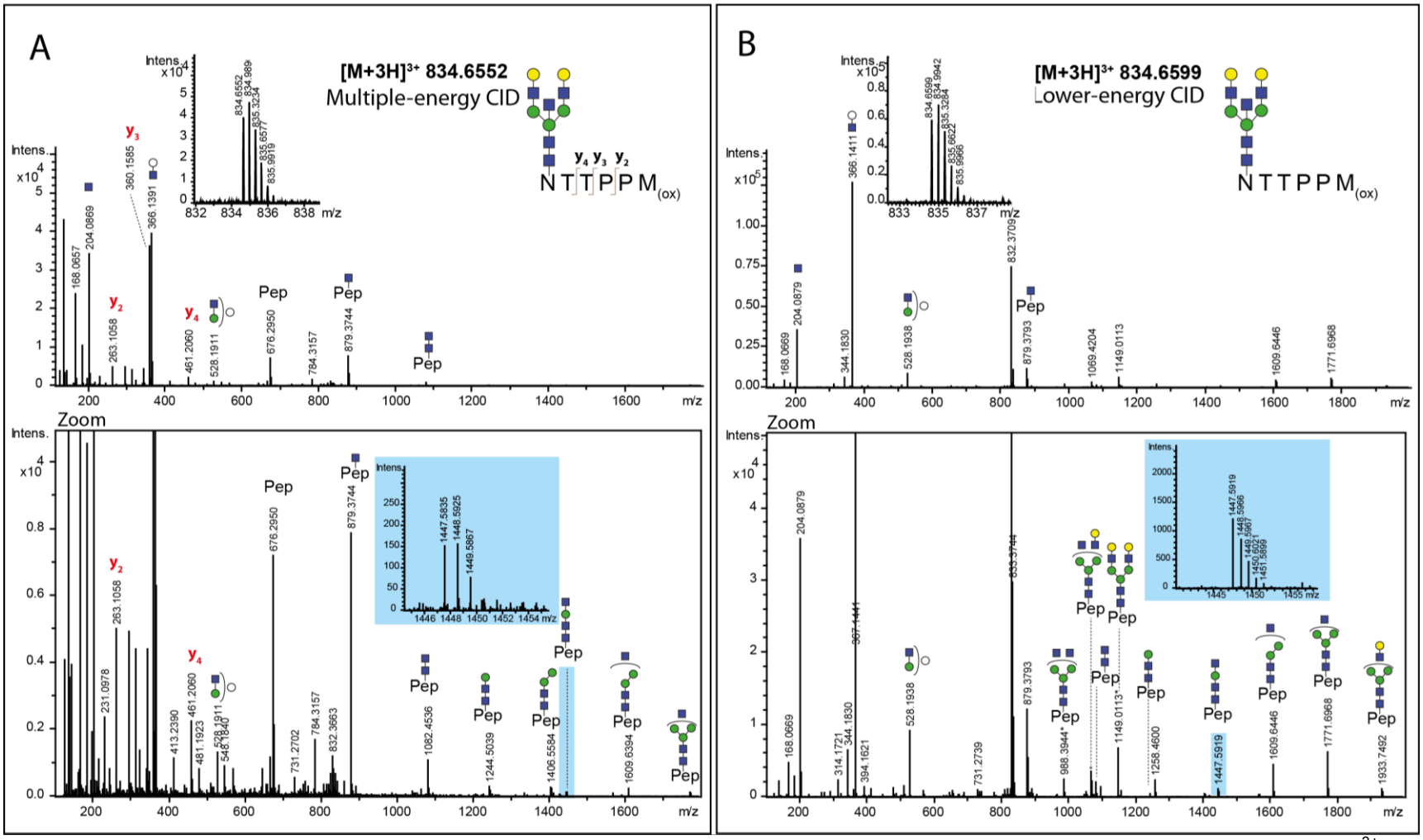

Figure S-13: MS/MS fragmentation spectra of a diantennary IgG3 C_H3 domain N-glycopeptide with a bisecting GlcNAc $[M+3H]^{3+}$ at *m/z* 834.6552 by (A) combined lower- and elevated-collision energy CID (lower panel: zoom in). (B) Lower-energy CID (45% of the collision energy without stepping) was applied to investigate the presence of a bisecting GlcNAc resulting in a more intense peak at *m/z* 1447.5919 (lower panel: zoom in). * Doubly charged ions.

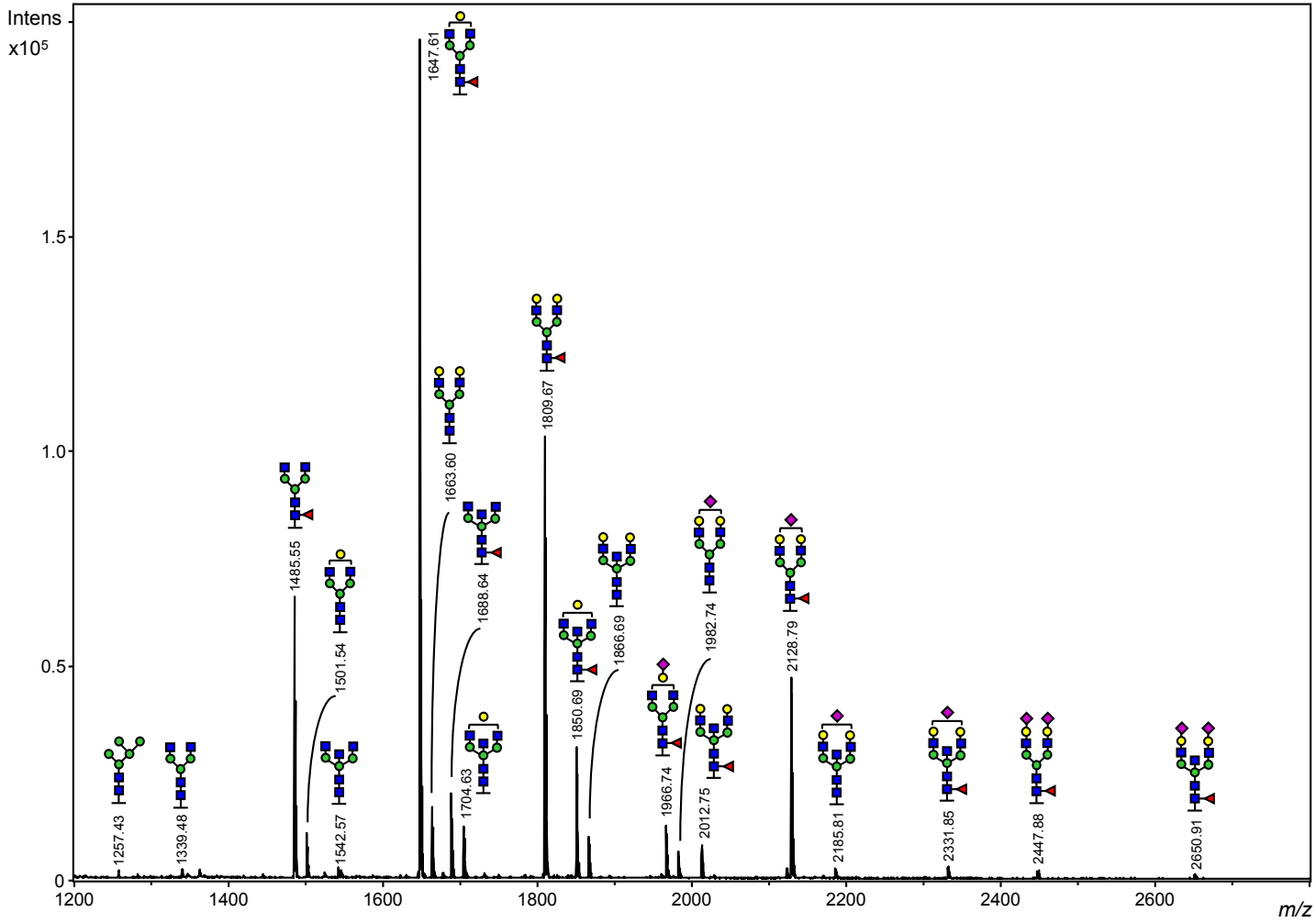

Figure S-14: Reflectron positive MALDI-TOF-MS spectrum showing released glycans from IgG3 (of both the Fc and the Fab part). The glycans have been derivatized with ethyl esterification, which allows identification of the N-acetylneuraminic acid linkage (the derivatized N-acetylneuraminic acids in this spectrum exhibit a mass of 319.13 Da, indicating an α2,6 linkage).

Table S-5: Relative quantitation of tryptic glycopeptides (C_H2 domain N-glycopeptides and hinge region O-glycopeptides) and trypsin-AspN digested glycopeptides (C_H3 domain N-glycopeptides). Glycopeptides were analyzed by C18-RP-ESI-QTOF-MS/MS. Signal intensities were extracted within a retention time window of 1 min and the intensities were normalized on the total intensity of all glycopeptides.

Glycan composition	CH2 relative intensities of glycopeptides (293EEQYNSTFR301)			CH3 relative intensities of glycopeptides (276DIAVEWESSGQPENNYNTTPPML398)			Hinge-region H2-(4-13), H3-(4-13), H4-(4-13) relative intensities of glycopeptides (SCDTPPPCPR)			CH2 relative intensities		CH3 relative intensities		Hinge-region relative intensities	
	I	II	III	I	II	III	I	II	III	average	stdev	average	stdev	average	stdev
HexNAc4Hex3Fuc1	15.00	15.29	15.38							15.22	0.20				
HexNAc4Hex4Fuc1	27.36	28.52	28.87							28.25	0.79				
HexNAc4Hex5Fuc1	16.93	17.25	17.25							17.14	0.18				
HexNAc4Hex4Fuc1Sia1	2.02	1.98	1.94							1.98	0.04				
HexNAc4Hex5S1Fuc1	17.63	16.81	16.85							17.10	0.46				
HexNAc5Hex3Fuc1	3.71	3.60	3.67							3.66	0.06				
HexNAc5Hex4Fuc1	6.06	5.81	6.00							5.95	0.13				
HexNAc5Hex5Fuc1	2.04	1.87	1.87							1.93	0.10				
HexNAc5Hex5Sia1Fuc1	0.35	0.32	0.31							0.32	0.02				
HexNAc4Hex3	1.09	0.96	0.95							1.00	0.07				
HexNAc4Hex4	2.80	2.58	2.53	1.24	1.15	1.18				2.63	0.14	1.19	0.05		
HexNAc4Hex5	2.23	2.01	2.00	10.20	9.99	10.01				2.08	0.13	10.07	0.12		
HexNAc4Hex4Sia1										n.q.					
HexNAc4Hex5Sia1	1.38	1.17	1.16	11.80	11.74	11.67				1.24	0.13	11.74	0.07		
HexNAc5Hex3	0.28	0.93	0.34	9.57	9.32	9.50				0.52	0.36	9.46	0.13		
HexNAc5Hex4	0.79	0.60	0.59	21.89	22.04	21.92				0.66	0.11	21.95	0.08		
HexNAc5Hex5	0.34	0.30	0.30	21.69	21.74	21.35				0.31	0.02	21.59	0.22		
HexNAc5Hex4Sia1				2.11	3.15	3.09						2.78	0.59		
HexNAc5Hex5Sia1				12.85	12.41	12.61				n.q.		12.62	0.22		
HexNAc2Hex5				8.65	8.46	8.67						8.59	0.11		
HexNAc1Hex1							34.19	29.25	28.74					30.73	3.01
HexNAc1Hex1Sia1							48.41	56.07	57.10					53.86	4.75
HexNAc1Hex1Sia2							17.40	14.68	14.16					15.41	1.74

n.q. = not quantified

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