Rapid and sensitive methods for the analysis and identification of O-glycans from glycoproteins

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Chapter 5

Comparison of procainamide and 2-aminobenzamide labelling for profiling and identification of glycans by LC-FLR-ESI-MS.

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Abstract

One of the most widely used methods for glycan analysis is fluorescent labelling of released glycans followed by HILIC-(U)HPLC. Here, we compare the data obtained by (U)HPLC-FLR coupled to ESI-MS for procainamide and 2-AB labelled N-glycans released from human IgG. Fluorescence profiles from procainamide show comparable chromatographic separation to those obtained for 2-AB but gave higher fluorescence intensity as well as significantly improved ESI ionization efficiency (up to 30 times that of 2-AB). Thus, labelling with procainamide increases the ability to identify minor glycan species which may have significant biological activity.

Introduction

Protein glycosylation is a post-translational modification that influences many protein functions.[1,2] Detailed monitoring and controlling glycosylation is essential in biopharmaceutical development and quality control of drugs that are glycosylated (e.g., erythropoietin, EPO).[3,4]

A number of analytical techniques have been developed for glycan characterization including high-performance liquid chromatography (HPLC), mass spectrometry (MS) and high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Each of these techniques can provide different information and a combination of these techniques in an LC-MS system can greatly improve full glycan characterization.[5]

HPLC analysis of released and fluorescently labelled (via reductive amination) N-glycans is one of the most common methods; the stoichiometric attachment of one label per glycan allows the relative quantitation of different glycan species based on fluorescence or UV-absorbance intensity.[6] This approach, combined with specific enzyme digestions can help to assign glycan structures more fully.

MS and MS/MS can provide composition and sequence data, however samples containing isomeric and isobaric glycan structures might not be distinguished by this technique.

HPLC-FLR coupled to ESI-MS (electrospray ionization) system can greatly improve glycan analysis by providing specific structural information.

There are several fluorescent labels that have been used for the reductive amination of N-glycans, such as 2-aminobenzamide (2-AB), 2-aminobenzoic acid (2-AA), 1-aminopyrene-3,6,8-trisulfonic acid (APTS), 2-aminopyridine (AP), procaine or

procainamide.[7] 2-AB is the most widely used label providing relative quantitation of N-glycan structures through efficient labelling and the production of highly stable labelled glycans. However, a major drawback of 2-AB is poor ionization efficiency when analyzing N-glycan structures by ESI-MS.[5]

Yoshino et al.[8] and Takao et al.[9] reported a 1000-fold increase in sensitivity over free oligosaccharides for glycans labelled with procaine (2-(diethylamino)ethyl 4-aminobenzoate) in matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) and ESI-MS. However, Pabst et al.[7] found that the performance of procaine in NP–HPLC was disappointing. The considerable differences between elution times and separation of glycans labelled with procaine and those labelled with 2-AB were observed.

Here we present data comparing two fluorescent labels for quantitative N-glycan profiling and identification by (U)HPLC-FLR coupled to ESI-MS and MS/MS. N-glycans from human polyclonal IgG were released, 2-AB labelled or procainamide (4-amino-N-(2-diethylaminoethyl) benzamide) labelled and purified on a liquid-handling robotic platform.

Materials and Methods

Materials

All reagents and kits for release, N-glycan labelling and clean-up were from Ludger Ltd. (Oxford, UK). Acetonitrile (Romil; 190 SpS for UV/ gradient quality) was obtained from Charlton Scientific (Charlton, Oxon, UK). Human IgG was from Ludger

Ltd (Oxford, UK) and all other reagents were obtained from Sigma-Aldrich (Dorset, UK).

Glycan release, 2-AB labelling, procainamide labelling and labelled glycan purification were performed on a Hamilton Microlab STARLet liquid-handling robotic platform.[10]

Release of N-glycans

Samples were denatured by heating for 7 min at 99°C in 2% SDD/1M β-mercaptoethanol, before overnight incubation at 37°C with PNGase F (Ludger Ltd.) in the presence of Triton X-100. The released N-glycans were converted to aldoses with 0.1% formic acid,[11] filtered through a protein binding plate (Ludger Ltd.) and dried.

Fluorescent labelling

Released N-glycans were fluorescently labelled by reductive amination in 10 µL of water with either 10 µL of 2-AB labelling solution as per the Ludger 2-AB glycan labelling kit containing 2-picoline borane or 10 µL of procainamide labelling solution as per Ludger procainamide glycan labelling kit containing 2-picoline borane. Samples were incubated at 65°C for 1 hour.

Purification of 2-AB labelled glycans

The 2-AB labelled N-glycans were cleaned up using Ludger Clean T1 Cartridges. 2-AB labelled N-glycans were eluted with water (1ml). The samples were dried by vacuum centrifugation and re-suspended in water (100 µL) for further analysis.

Purification of Procainamide labelled glycans

The removal of unreacted procainamide dye was performed using a Ludger Clean plate for cleanup of procainamide labelled glycans. The procainamide labelled N-glycans were eluted with water (200 µL). The samples were dried by vacuum centrifugation and re-suspended in water (100 µL) for further analysis.

LC-ESI-MS and MS/MS analysis

2-AB and procainamide labelled samples were analysed by LC-ESI-MS. 25 µL of each sample was injected onto an ACQUITY UPLC® BEH-Glycan 1.7 µm, 2.1 x 150 mm column at 40°C on the Dionex Ultimate 3000 UHPLC instrument with a fluorescence detector (λex = 250nm, λem = 428nm for 2-AB and λex = 310nm, λem = 370nm for procainamide), attached to a Bruker Amazon Speed ETD. The running conditions used were: Solvent A was 50 mM ammonium formate pH 4.4 made from Ludger Stock Buffer (Ludger), and solvent B was acetonitrile. Gradient conditions were: 0 to 38.5 min, 76% to 58 % B; 38.5 to 40.5 min, 58 to 40.5 % B at flow rate 0.4mL/min. 40.5 to 42.5 min, 40 % B at a flow rate of 0.25 mL/min; 42.5 to 44.5 min, 40 to 76 % B at a flow rate of 0.25 mL/min; 44.5 to 50.5 min, 76% B at a flow rate of 0.25 mL/min. 50.5 to 51.5 min, 76% B at a flow rate 0.25 mL/min; 51.5 to 55 min, 76% B at a flow rate 0.4 mL/min. The Amazon Speed settings used were: source temperature 250 °C, gas flow 10 L/min; Capillary voltage 4500 V; ICC target 200,000; max accu time 50.00 ms; rolling average 2; number of precursors ions selected 3, release after 0.2 min; Positive ion mode; Scan mode: enhanced resolution; mass range scanned, 200-1500; Target mass, 900. A glucose homopolymer ladder (Ludger), labelled with either 2AB or procainamide was used as

a system suitability standard as well as an external calibration standard for GU allocation.[11]

Results and Discussion

Release and recovery of N-glycans with free reducing termini is important for structural and functional analysis. Using human IgG as a model glycoprotein, we compared two fluorescent labels, 2-aminobenzamide (2-AB) and procainamide. The N-glycans from human IgG were released, labelled and purified using a liquid-handling robotic platform.

The profiles obtained by (U)HPLC-FLR coupled to ESI-MS from the human IgG samples labelled with 2-AB and procainamide were compared in Figure 1. Comparable N-glycan profiles were obtained for each sample, labelled with 2-AB and procainamide (Figure 1a and 1d), as described before.[12] The peak intensity of the fluorescent profiles from the samples labelled with procainamide (the emission and excitation wavelengths were set to λex 310nm and λem 370nm) were higher than those from 2-AB labelled (λex 250nm and λem 428nm) allowing better integration of the lower intensity peaks.

The base peak chromatogram (BPC) in positive mode ESI-MS for procainamide labelled human IgG N-glycans showed up to 30 times higher signal intensity compared to 2-AB (Figure 1b and 1e). The extracted ion chromatogram (EIC) profiles for the 2-AB labelled (Figure 1c) and procainamide labelled (Figure 1f) A2BG2S2 glycan (Table 1, Figure 1a and 1d, peak 17) showed significantly higher ion intensity for procainamide derivative, enabling identification of low level glycan species not mass detected using 2-AB. This high sensitivity in positive mode ESI-MS

can be explained because of the high proton affinity of the procainamide basic tail (2-(diethylamino)ethyl group).

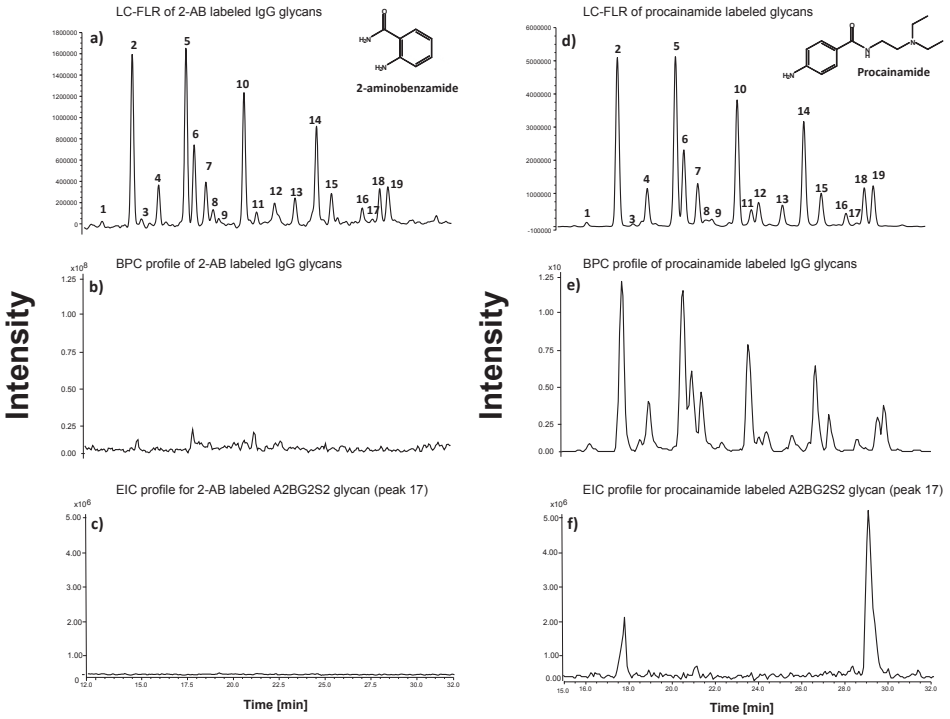

Figure 1. Comparison of fluorescently labelled human IgG glycans. (a) LC-FLR of 2-AB labelled IgG glycans, (b) LC-ESI-MS BPC profile of 2-AB labelled IgG glycans, (c) LC-ESI-MS EIC for 2-AB labelled A2BG2S2 glycan (peak 17 on LC-FLR trace), (d) LC-FLR of procainamide labelled glycans, (e) LC-ESI-MS BPC profile of procainamide labelled IgG glycans, (f) LC-ESI-MS EIC for procainamide labelled A2BG2S2 glycan (peak 17 on LC-FLR trace).

All of the 19 peaks that were detected by (U)HPLC-FLR were identified by MS and MS/MS (Table 1). One, two or three charge states were detected for the procainamide derivatives. In some cases, when the MS/MS analysis was performed on singly charged precursor ions, a loss of 73 Da was observed. This observation

indicated degradation of the procainamide label during MS/MS fragmentation and corresponds to loss of a diethylamine ion.

In summary, the comparison of the two fluorescent labels presented here demonstrates that procainamide derivatization can be used as a standard method for manual and automated glycan characterization. The procainamide labelled glycans are suitable for both (U)HPLC-FLR analysis, providing good chromatographic separation for relative quantitation and ESI-MS analysis, providing more efficient ionization for glycan identification.

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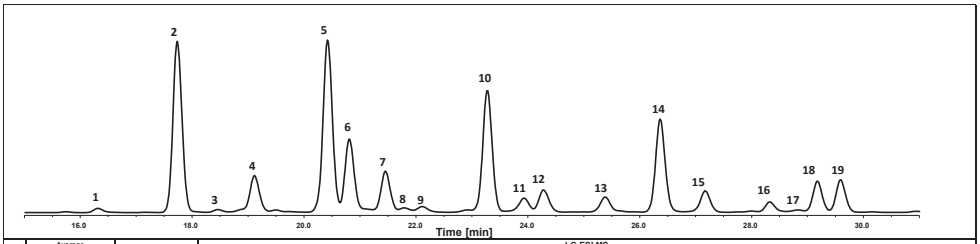
Supplementary information



HPLC Peak Id	Average GU (Procainamide)	% Area	Structure	Composition: Hex (H)	HexNAc (N)	Fuc (F)	NeuSAc (S)	[M/Z]+ calculated	[M/Z]2+ calculated	[M/Z]3+ calculated	LC-ESI-MS: [M/Z]+ registered	[M/Z]2+ registered	[M/Z]3+ registered	[M/Z] characteristic fragment ions (composition)
1	5.29	0.50		3	4	0	0	1536.67	768.84	512.89	1536.87	768.96	nd	1463.62 (H3N4-PROC)* 968.45 (H2N2-PROC) 1333.68 (H3N3-PROC)* 895.46 (H2N2-PROC)* 1260.59 (H3N3-PROC)* 806.46 (H1N2-PROC) 1171.60 (H2N3-PROC) 644.41 (N2-PROC) 1130.57 (H2N2-PROC)* 441.30 (N1-PROC)
2	5.64	18.62		3	4	1	0	1682.73	841.87	561.58	1682.80	841.99	nd	1609.75 (H3N4F1-PROC)* 1317.84 (H2N3F1-PROC) 952.70 (H1N2F1-PROC) 587.53 (N1F1-PROC) 1536.86 (H3N4-PROC) 1276.79 (H3N2F1-PROC) 880.69 (H1N2F1-PROC)* 571.43 (N2-PROC)* 1480.09 (H3N3F1-PROC) 1171.77 (H2N3-PROC) 790.66 (N2F1-PROC) 514.41 (N1F1-PROC)* 1406.78 (H3N3F1-PROC)* 1114.76(H2N2F1-PROC) 717.51 (N2F1-PROC)* 441.42 (N1-PROC) 1333.80 (H3N3-PROC) 1042.75(H2N2F1-PROC)* 644.49 (N2-PROC)
3	5.83	0.33		5	2	0	0	1453.61	727.81	485.54	1454.67	727.92	nd	1014.41 (H5N1) 1014.41 (H5N1) [646.90]2+ (H4N2-PROC) [403.84]3+ (H1N2-PROC) 852.34 (H4N1) 852.34 (H4N1) 528.28 (H2N1) 366.27 (H1N1) 806.46 (H1N2-PROC) 806.46 (H1N2-PROC) [565.88]2+ (H3N2-PROC) [322.81]3+ (N2-PROC) 690.27 (H3N1) 690.27 (H3N1) [484.85]2+ (H2N2-PROC) 204.11 (N1) 644.44 (N2-PROC) 644.44 (N2-PROC) 441.40 (N1-PROC)
4	6.01	4.73		3	5	1	0	1885.80	943.41	629.27	nd	943.52	nd	1682.85 (H3N4F1-PROC) 1317.71 (H2N3F1-PROC) [740.40]2+ (H3N3F1-PROC) 441.40 (N1-PROC) 1536.72 (H3N4-PROC) 1299.53 (H3N4) 644.47 (N2-PROC) 366.18 (H1N1) 1520.74 (H2N4F1-PROC) 1098.45 (H3N3) [659.30]2+ (H2N3F1-PROC) 350.25 (N1F1) 1479.74 (H3N3F1-PROC) [841.92]2+ (H3N4F1-PROC) 587.42 (N1F1-PROC) 204.08 (N1) 1333.78 (H3N3-PROC) 790.41 (N2F1-PROC) 569.25 (H2N1)
				4	4	0	0	1698.72	849.86	566.91	nd	849.95	nd	1495.77 (H4N3-PROC) 1055.47 (H4N2) 528.31 (H2N1) 1333.73 (H3N3-PROC) 969.55 (H2N2-PROC) 441.33 (N1-PROC) 1258.60 (H4N3) [748.42]2+ (H4N3-PROC) 366.19 (H1N1) 1171.84 (H2N3-PROC) [667.37]2+ (H3N3-PROC) 204.07 (N1) 1130.63 (H3N2-PROC) 644.38 (N2-PROC)
5	6.37	19.07		4	4	1	0	1844.78	922.89	615.60	nd	922.97	nd	1641.79 (H4N3F1-PROC) 1276.61 (H3N2F1-PROC) 952.59 (H2N3) 528.22 (H2N1) 1495.72 (H4N3-PROC) 1276.61 (H4N3) 893.41 (H3N2) 441.32 (N1-PROC) 1479.73 (H3N3F1-PROC) 1132.56 (H3N2-PROC) 690.28 (H3N1) 366.13 (H1N1) 1334.63 (H3N3-PROC) 1114.57 (H2N2F1-PROC) 644.56 (N2-PROC) 204.08 (N1) 1317.64 (H2N3F1-PROC) 1114.57 (H3N3) 587.34 (N1F1-PROC)
				4	5	0	0	1901.80	951.40	634.60	nd	951.50	nd	1698.80 (H4N4-PROC) 1258.47 (H4N3) 644.34 (N2-PROC) 1536.76 (H3N4-PROC) 1171.61 (H2N3-PROC) 528.19 (H2N1) 1461.64 (H4N4) [849.90]2+ (H4N4-PROC) 441.28 (N1-PROC) 1374. 76 (H2N4-PROC) [768.80]2+ (H3N4-PROC) 366.13 (H1N1) 1338.68 (H3N3-PROC) 690.31 (H3N1) 204.06 (N1)
6	6.48	8.58		4	4	1	0	1844.78	922.90	615.60	nd	922.97	nd	1642.79 (H4N3F1-PROC) 1276.61 (H3N2F1-PROC) 969.53 (H2N2-PROC) 528.20 (H2N1) 1495.69 (H4N3-PROC) 1276.61 (H4N3) 952.56 (H2N3) 441.22 (N1-PROC) 1479.74 (H3N3F1-PROC) 1130.66 (H3N2-PROC) 893.38 (H3N2) 366.15 (H1N1) 1334.81 (H3N3-PROC) 1130.66 (H2N2F1-PROC) 644.56 (N2-PROC) 204.03 (N1) 1317.73 (H2N3F1-PROC) 1114.60 (H3N3) 587.34 (N1F1-PROC)
7	6.67	4.73		4	5	1	0	2047.86	1024.43	683.29	nd	1024.51	nd	1845.87 (H4N4F1-PROC) 1461.63 (H4N4) 441.32 (N1-PROC) 1682.83 (H3N4F1-PROC) 1317.70 (H2N3F1-PROC) 366.16 (H1N1) 1642.79 (H4N3F1-PROC) 1259.48 (H4N3) 1520.76 (H2N4F1-PROC) 587.40 (N1F1-PROC) 1479.74 (H3N3F1-PROC) 528.08 (H2N1)
8	6.77	0.56		6	2	0	0	1616.67	808.84	539.56	nd	808.93	nd	1292.63 (H4N2-PROC) [727.92]2+ (H5N2-PROC) 528.28 (H2N1) 1176.46 (H6N1) 690.28 (H3N1) 441.37 (N1-PROC) 1130.63 (H3N2-PROC) [846.88]3+ (H4N2-PROC) 366.20 (H1N1) 1014.40 (H5N1) 644.44 (N2-PROC) [322.76]3+ (N2-PROC) 852.35 (H4N1) [565.88]2+ (H3N2-PROC) 204.13 (N1)
9	6.87	0.79		5	4	0	0	1860.77	930.89	620.93	nd	931.00	621.05	1657.78 (H5N3-PROC) 1130.59 (H3N2-PROC) 441.33 (N1-PROC) 1495.82 (H4N3-PROC) 1056.22 (H4N2 366.17 (H1N1) 1420.61 (H5N3) 968.54 (H2N2-PROC) 204.06 (N1) 1333.72 (H3N3-PROC) 644.36 (N2-PROC) 1258.53 (H4N3) 528.26 (H2N1)

Supplementary Table 1. Structural characterization of procainamide labelled IgG glycans. Human IgG glycans were released and labelled on the liquid-handling workstation as described in the Methods Section, followed by analysis by LC-ESI-MS/MS.The following symbols are used to depict glycan structures:[13] ◊, galactose; ♦, *N*-acetylgalactosamine; ○, mannose; ◈, fucose; ■, *N*-acetylglucosamine; ★, *N*-acetylneuraminic acid; dashed line, α-linkage; solid line, β-linkage.

Glycan compositions are given in the terms of hexose (H), *N*-acetylhexosamine (N), deoxyhexose (F), *N*-acetylneuraminic acid (S). * Mass corresponds to o loss of diethylamine ion (73 Da).

10	**7.22**	**14.04**		5	4	1	0	2006.83	1003.40	669.62	nd	1003.99	nd	1641.88 (H4N3F1-PROC) 1495.72 (H4N3-PROC) 1479.75 (H3N3F1-PROC) 1420.59 (H5N3) 1333.63 (H3N3-PROC)	1317.78 (H2N3F1-PROC) 1276.76 (H3N2F1-PROC) 1218.50 (H5N2) 1114.63 (H2N2F1-PROC) 952.83 (H1N2F1-PROC)	790.48 (N2F1-PROC) 587.42 (N1F1-PROC) 528.18 (H2N1) 441.31 (N1-PROC) 366.16 (H1N1)		
11	**7.43**	**1.80**		5	5	1	0	2209.91	1105.46	737.31	nd	1105.57	737.44	1845.85 (H4N4F1-PROC) 1698.81 (H4N4-PROC) 1682.81 (H3N4F1-PROC) 1641.75 (H4N3F1-PROC) 1624.65 (H5N4)	1539.69 (H3N4-PROC) 1479.78 (H3N3F1-PROC) 1420.63 (H5N3) 1333.59 (H3N3-PROC) 1258.51 (H4N3)	1318.75 (H2N3F1-PROC) 1157.47 (H1N3F1-PROC) 952.72 (H1N2F1-PROC) 790.47 (N2F1-PROC) 587.40 (N1F1-PROC)	528.16 (H2N1) 441.30 (N1-PROC) 366.15 (H1N1)	
12	**7.54**	**2.66**		4	4	1	1	2135.87	1068.44	712.63	nd	1068.54	713.07	1642.74 (H4N3F1-proc) 1479.71 (H3N3F1-PROC) 1334.72 (H3N3)-PROC0 1317.68 (H2N3F1-PROC) 1277.59 (H3N2F1-PROC)	1131.53 (H3N2-PROC) 1114.60 (H2N2F1-PROC) [867.48]$^{[2]}$ (H4N3F1S1-PROC [813.94]$^{[2]}$ (H4N4F1-PROC) [886.4]$^{[2]}$ (H3N3F1S1-PROC	819.37 (H2N1S1) 657.30 (H1N1S1) 644.38 (N2-PROC) 587.38 (N1F1-PROC) 441.33 (N1-PROC)	366.17 (H1N1)	
13	**7.90**	**1.84**		5	4	0	1	2151.87	1076.44	717.96	nd	1076.54	718.09	1786.84 (H4N3S1-PROC) 1729.85 (H5N3S1) 1549.66 (H4N3S1) 1495.75 (H4N3-PROC) 1420.64 (H5N3)	1333.68 (H3N3-PROC) 1130.56 (H3N2-PROC) 968.53 (H2N2-PROC) 819.33 (H2N1S1) 657.31 (H1N1S1)	644.44 (N2-PROC) 528.28 (H2N1) 441.30 (N1-PROC) 366.19 (H1N1)		
14	**8.23**	**10.51**		5	4	1	1	2297.93	1149.47	766.65	nd	1149.55	nd	1932.89 (H4N3F1S1-PRO 1787.78 (H4N3S1-PROC) 1770.72 (H3N3F1S1-PRO 1711.67 (H5N3S1) 1641.78 (H4N3F1-PROC)	1508.69 (H5N2S1) 1479.75 (H3N3F1-PROC) 1333.80 (H3N3-PROC) 1317.56 (H2N3F1-PROC) 1276.68 (H3N2F1-PROC)	1184.47 (H3N2S1) 1130.91 (H3N2-PROC) 1114.53 (H2N2F1-PROC) [1077.05]$^{[2]}$ (H5N4S1-PROC 981.31 (H3N1S1)	[967.45]$^{[2]}$ (H4N3F1S1-PRO 952.34 (H1N2F1-PROC) [923.44]$^{[2]}$ (H4N4F1-PROC) 819.28 (H2N1S1) 790.31 (N2F1-PROC)	657.30 (H1N1S1) 587.39 (N1F1-PROC) 441.27 (N1-PROC) 366.17 (H1N1)
15	**8.52**	**2.71**		5	5	1	1	2501.01	1251.01	864.34	nd	1251.05	nd	2136.01 (H4N4F1S1-PRO 1989.88 (H4N4S1-PROC) 1974.89 (H3N4F1S1-PRO 1932.83 (H4N3F1S1-PRO 1844.87 (H4N4F1-PROC)	1770.38 (H3N3F1S1-PROC) 1712.64 (H5N3S1) 1682.84 (H3N4F1-PROC) 1642.77 (H4N3F1-PROC) 1536.56 (H3N4-PROC)	1479.73 (H3N3F1-PROC) 1317.75 (H2N3F1-PROC) 1277.69 (H3N2F1-PROC) 1114.91 (H2N2F1-PROC) 819.28 (H2N1S1)	790.38 (N2F1-PROC) 657.26 (H1N1S1) 587.35 (N1F1-PROC) 441.18 (N1-PROC) 366.12 (H1N1)	
16	**8.94**	**1.16**		5	4	0	2	2442.96	1221.99	814.99	nd	1222.02	nd	2002.76 (H5N3S2) 1786.87 (H4N3S1-PROC) 1624.76 (H3N3S1-PROC) 1496.73 (H4N3-PROC) 1333.65 (H3N3-PROC)	[1077.00]$^{[2]}$ (H5N4S1-PROC) 819.35 (H2N1S1) 657.28 (H1N1S1) 644.44 (N2-PROC) 441.32 (N1-PROC)	366.12 (H1N1)		
17	**9.13**	**0.30**		5	5	0	2	2646.04	1323.53	882.69	nd	nd	882.78	1989.89 (H4N4S1-PROC) 1828.13 (H3N4S1-PROC) 1786.91 (H4N3S1-PROC) 1711.66 (H5N3S1) 1699.82 (H4N4-PROC)	1549.61 (H4N3S1) 1536.56 (H3N4-PROC) 1495.68 (H4N3-PROC) 1387.55 (H3N3S1) 1333.68 (H3N3-PROC)	[1222.54]$^{[2]}$ (H5N4S2-P) [1103.42]$^{[2]}$ (H5N4S2) 1171.64 (H2N3-PROC) [996.96]$^{[2]}$ (H4N4S1-PROC) 969.59 (H2N2-PROC)	[914.94]$^{[2]}$ (H3N4S1-PROC) 819.34 (H2N1S1) [769.39]$^{[2]}$ (H3N4-PROC) 657.30 (H1N1S1) 644.44 (N2-PROC)	528.28 (H2N1) 441.35 (N1-PROC) 366.18 (H1N1) 292.11 (S1) 204.05 (N1)
18	**9.26**	**3.42**		5	4	1	2	2589.02	1295.01	863.68	nd	1295.06	864.13	1933.91 (H4N3F1S1-PRO 1787.91 (H4N3S1-PROC) 1770.85 (H3N3F1S1-PRO 1712.66 (H5N3S1) 1641.79 (H4N3F1-PROC)	1508.66 (H5N3S1) 1479.75 (H3N3F1-PROC) 1276.68 (H3N2F1-PROC) [1222.50]$^{[2]}$ (H5N4S2-PROC) [1140.58]$^{[2]}$ (H5N4F1S1-PRO	[1088.54]$^{[2]}$ (H4N4F1S1-PRO [867.94]$^{[2]}$ (H4N3F1S1-PRO 819.34 (H2N1S1) 790.44 (N2F1-PROC) 657.29 (H1N1S1)	644.56 (N2-PROC) 587.44 (N1F1-PROC) 441.26 (N1-PROC) 366.14 (H1N1)	
19	**9.43**	**6.35**		5	5	1	2	2792.10	1396.55	931.37	nd	nd	931.43	1549.55 (H4N3S1) 1479.76 (H3N3F1-PROC) 1388.56 (H3N3S1) 1333.66 (H3N3-PROC) 1317.69 (H2N3F1-PROC)	[1295.57]$^{[2]}$ (H5NS2-PROC) [1251.07]$^{[2]}$ (H5N5F1S1-PRO 1171.33 (H2N3-PROC) [1149.48]$^{[2]}$ (H5N5S1-PROC) [1088.54]$^{[2]}$ (H4N4F1S1-PRO	[887.98]$^{[2]}$ (H3N4F1S1-PRO 819.35 (H2N1S1) 790.53 (N2F1-PROC) 657.31 (H1N1S1) 644.47 (N2-PROC)	587.40 (N1F1-PROC) 441.42 (N1-PROC) 366.18 (H1N1) 292.15 (S1) 204.09 (N1)	

Supplementary Table 1. (Continued).