Electronic Supplementary Material (ESI) for Analyst.

Comparative Analysis of Monoclonal Antibody *N*-Glycosylation using Stable Isotope Tagging and UPLC-Fluorescence-MS

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Supplementary Information

Materials and Methods:

Chemicals and reagents:

Standard *N*-glycans (A2F, NA2F, Man5, NA2) and peptide-N-glycosidase F (PNGase F) were purchased from Prozyme (San Leandro, CA). $^{12}C_6$ 2-aminobenzoic acid, $^{13}C_6$ 2-aminobenzoic acid, dimethyl sulfoxide (DMSO), acetic acid and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Sodium cyanoborohydride (Across Organics), water and acetonitrile (LC-MS Optima) were purchased from Fisher Scientific (Dublin, Ireland). Nanosep 10 kDa centrifugal devices were obtained from Pall (Port Washington, NY). Normal-phase tips (PhyTip, 10 µL bed volume) were sourced from PhyNexus Inc. (San Jose, CA). Different commercial lots of the chimeric IgG1 monoclonal antibody (mAb) for analysis were generously provided by San Cecilio University Hospital (Granada, Spain).

Glycoprotein deglycosylation:

500 µg aliquots of commercial chimeric IgG1 were deglycosylated overnight (16 h) at 37 °C in 20 mM sodium bicarbonate buffer pH 7.0. The deglycosylation mixture was then centrifuged through a 10 kDa molecular weight cut-off (MWCO) to remove the protein. The filtrate containing the released glycans was reduced to dryness *via* vacuum centrifugation. Once dry, the released glycans were treated with 50 µL of 1% v/v aqueous formic acid to ensure complete conversion to the reducing sugar form prior to derivatization.

Derivatization of free glycans with $^{12}C_6$/ $^{13}C_6$ 2-aminobenzoic acid:

Glycan samples were derivatized with either light or heavy 2-AA by reductive amination in the presence of sodium cyanoborohydride. 10 µl of a freshly prepared solution of 0.37 M $^{12}C_6$ 2-AA or 0.56 M $^{13}C_6$ 2-AA containing 1 M $NaCNBH_3$ prepared in DMSO/acetic acid (70:30), were added to the dried glycans and incubated for 5 hours at 65 °C. Following labelling the light and heavy samples were mixed together and made to a final volume of 1 mL of 90% v/v acetonitrile. The combined sample was then cleaned using PhyNexus normal phase tips as described previously[1]. The excess

label was removed by washing the PhyTips ten times with 95% v/v acetonitrile followed by elution using 5 x 200 µL of 20% v/v acetonitrile. Elutions were combined into a single tube and dried *via* vacuum centrifugation. Cleaned *N*-glycans were resuspended in 25 µL deionized water and stored at -40 °C until analysis.

Separation of 2AA-labeled glycans UPLC-FLR-MS:

Labelled *N*-glycans were separated using a Waters ACQUITY UPLC BEH Amide column (1.0 x 150 mm, 1.7 µm particle) connected to an ACQUITY UPLC equipped with online fluorescence detection (Waters Corporation, Milford, MA, USA) under the control of MassLynx 4.1 Software. The fluorescence excitation and emission wavelength parameters were 350 and 425 nm, respectively. The flow rate was 0.150 $mLmin^{-1}$ and column temperature was maintained at 60 °C. Solvent A was 50 mM ammonium formate pH 4.4 and solvent B was acetonitrile. A 40 minute linear gradient was used and was as follows: 28% A for 1 min, 28-43% A for 30 minutes, 43-70 % A for 1 minute, 70 % A for 3 min, 70-28% solvent A for 1 min and finally 28 % A for 4 minutes. Samples were diluted in 75% acetonitrile prior to analysis, the injection volume was 8 µL.

The outlet of the chromatographic system was coupled directly to a Waters Xevo G2 QToF mass spectrometer (Milford, MA, USA) equipped with an electrospray ionization interface. The instrument was operated in negative ion mode with a capillary voltage of 1.80 kV. The ion source and nitrogen desolvation gas temperatures were set at 120 °C and 400 °C, respectively. The desolvation gas flow rate was 600 L/h. The cone voltage was maintained at 50 V. Full-scan MS data was acquired over the range of 450 to 2500 m/z. Data collection and processing was controlled using MassLynx 4.1 (Waters Corporation, Milford, MA, USA). To avoid contamination of the instrument, the column flow was diverted to waste for the first 1.2 minutes and after 32 minutes of the chromatographic run.

LC-MS^E based glycosylation site occupancy analysis:

Proteolytic digestion was performed using an adaptation of the filter aided sample preparation procedure (FASP).[2] 100 µg of mAb was diluted in 200 µL of 50 mM Tris pH 8.0 containing 8 M urea (UA solution) and added to a 0.5 mL 10 kDa molecular weight cut off (MWCO) spin filter (Millipore, Carrigtwohill, Cork, Ireland). The filter unit was centrifuged at 14,000 x g for 10 minutes to remove formulation excipients. An additional two washes with UA solution were performed. The mAb was then reduced at room temperature for 30 minutes through the addition of 200 µL of UA containing 100 mM DTT. The reduction solution was removed by centrifugation, the reduced mAb washed with UA solution and subsequently alkylated by incubation with 50 mM IAA prepared in UA solution at room temperature in the dark for 60 minutes. Residual IAA was removed by washing three times with UA solution followed by buffer exchange into 50 mM ammonium bicarbonate (ABC) for either trypsin (1:50 w/w) or PNGase F (12.5 mU) followed by trypsin digestion. Resulting peptides were collected by centrifugation and reduced to dryness *via* vacuum centrifugation. The dried peptides were reconstituted in 20 µL 0.1% formic acid and analysed by LC-MS^E performed using a Waters Acquity I-Class UPLC instrument connected to a Waters Synapt G2 HDMS Mass Spectrometer. Peptide separations were performed using a Waters BEH130 C_{18} 2.1 X 150 mm column using a gradient of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Gradient conditions were as follows: 2% B initially for 2 minutes, increased to 10% B in three minutes with a further increase to 28% B over 40 minutes followed by an increase to 90% B in one minute with a two minute isocratic hold. Initial conditions were restored in 1 minute and held for an additional 10 minutes to ensure column re-equilibration. The column temperature was maintained at 40^{o}C throughout. The injection volume was 4 µL.

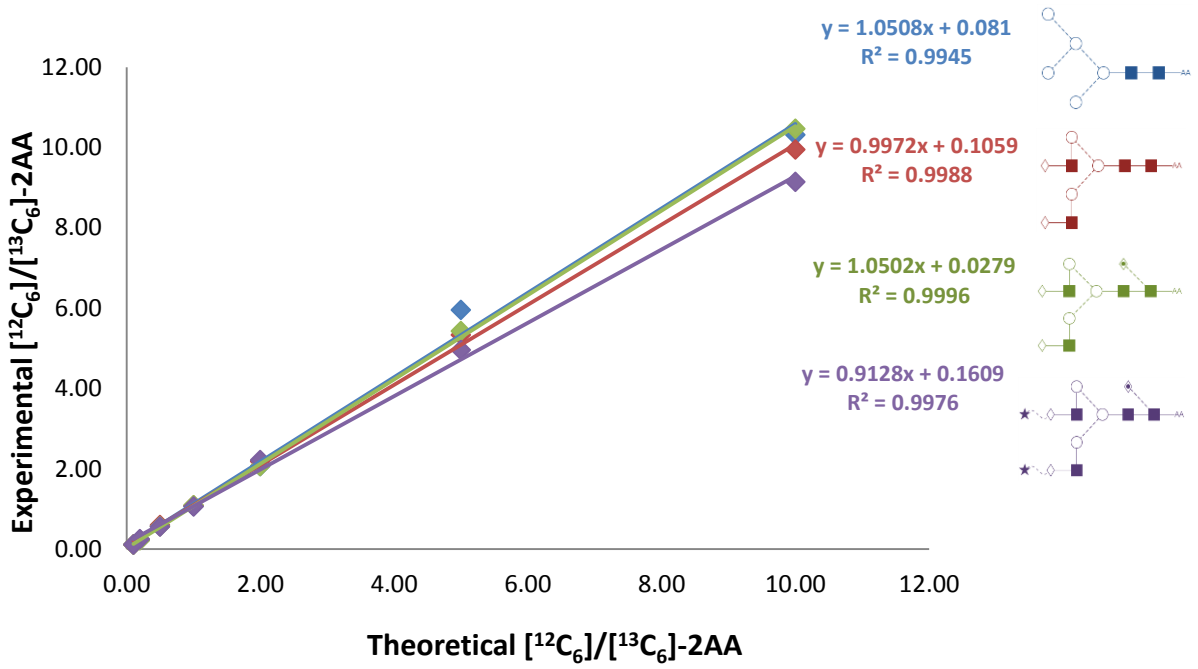
The mass spectrometer was operated in positive ion data independent analysis mode with 0.6 second sequential low and high energy scans in the range of 100 to 2,000 Da, the spray voltage was 3 kV. For the high energy function a collision energy ramp from 20 – 45 V was employed. Glu-fibrinopeptide (*m/z* 785.8427, *z*=2) was used as the lock mass solution on a 60 second interval.

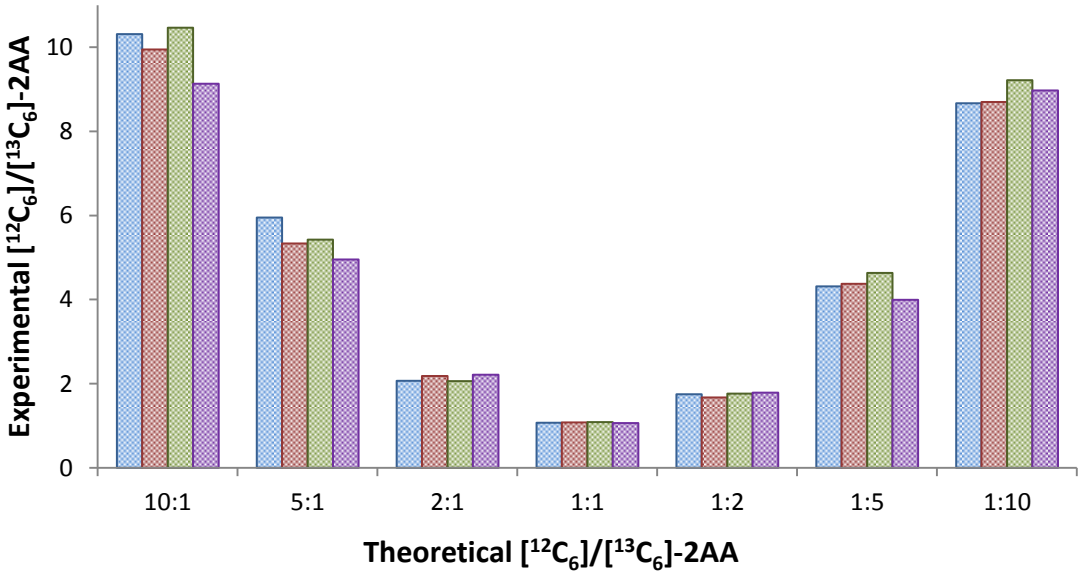
Resulting LC-MS^E data was processed using ProteinLynx Global Server Version 3.0.1 using the following work flow parameters: peptide and fragment tolerance calculated automatically with lock spray recalibration, digestion enzyme trypsin, maximum number of missed cleavages = 1, fixed modification = carbamidomethyl C, variable modifications = deamidation N,Q, oxidation M, minimum number of fragment ion matches per peptide = 3, minimum number of fragment ion matches per protein = 7, minimum number of unique peptides per protein = 2, false discover rate controlled at 1% FDR.

Liquid chromatography profiling of mAb critical quality attributes following forced degradation:

Replicate samples of the non-deglycosylated chimeric IgG1 mAb were subjected to forced degradation condition to determine the effect of stress on the stability of the mAb. Resulting modifications were determined using liquid chromatography profiling as described as follows. All analyses were performed using an Acquity H-Class Bio UPLC instrument with UV detection at 280 nm. Aggregates and higher order structures were determined using size exclusion chromatography (SEC) on a Waters BEH200 SEC column under isocratic conditions of 100 mM phosphate buffer, pH 6.8 at 0.3 $mLmin^{-1}$. Charge variant analysis was performed using cation exchange chromatography (CEX) on a Waters Protein Pak Hi-Res CM column. Variant peaks were eluted using a linear gradient of 0-10% 0.7 M NaCl in 20 mM sodium phosphate buffer pH 7.0 in 25 minutes. Oxidation products were separated using a linear gradient of 20-55% acetonitrile containing 0.1% v/v TFA on a BEH300 C_4 reversed-phase column, (Waters Corporation, Milford, MA).

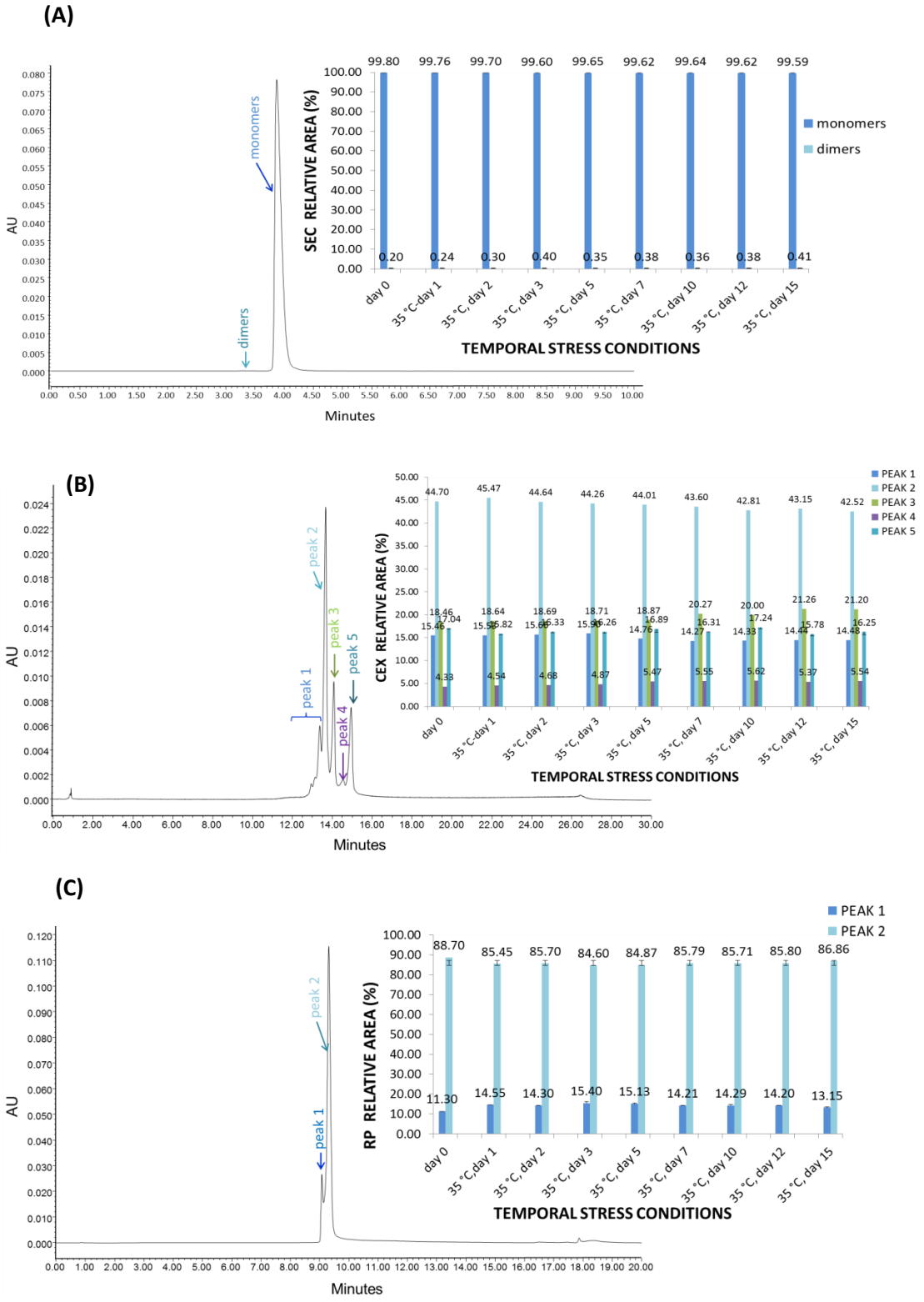
Supplementary Figure 1:





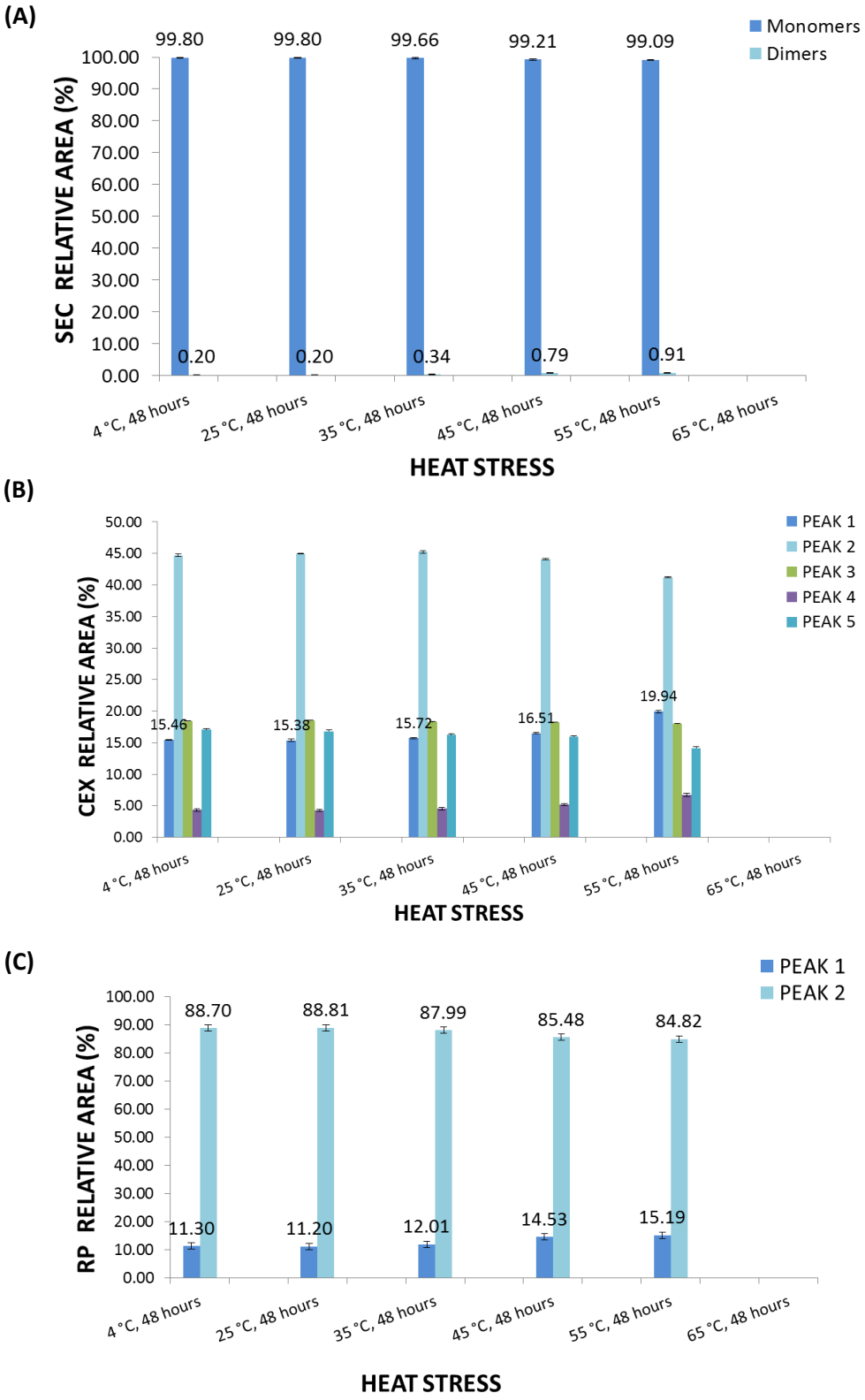
Quantitation linearity was evaluated using a variety of *N*-glycan standards (oligomannose, asialo bi-antennary with and without core fucose and antennary galactose residues) in both directions, i.e. increasing light to heavy and increasing heavy to light in theoretical molar ratios of 1:1, 2:1, 5:1 and 10:1. The bar chart displays the experimentally determined light to heavy ratios in each mixing direction. Values were transformed into linearity plots for correlation coefficient determination.

Supplementary Figure 2:



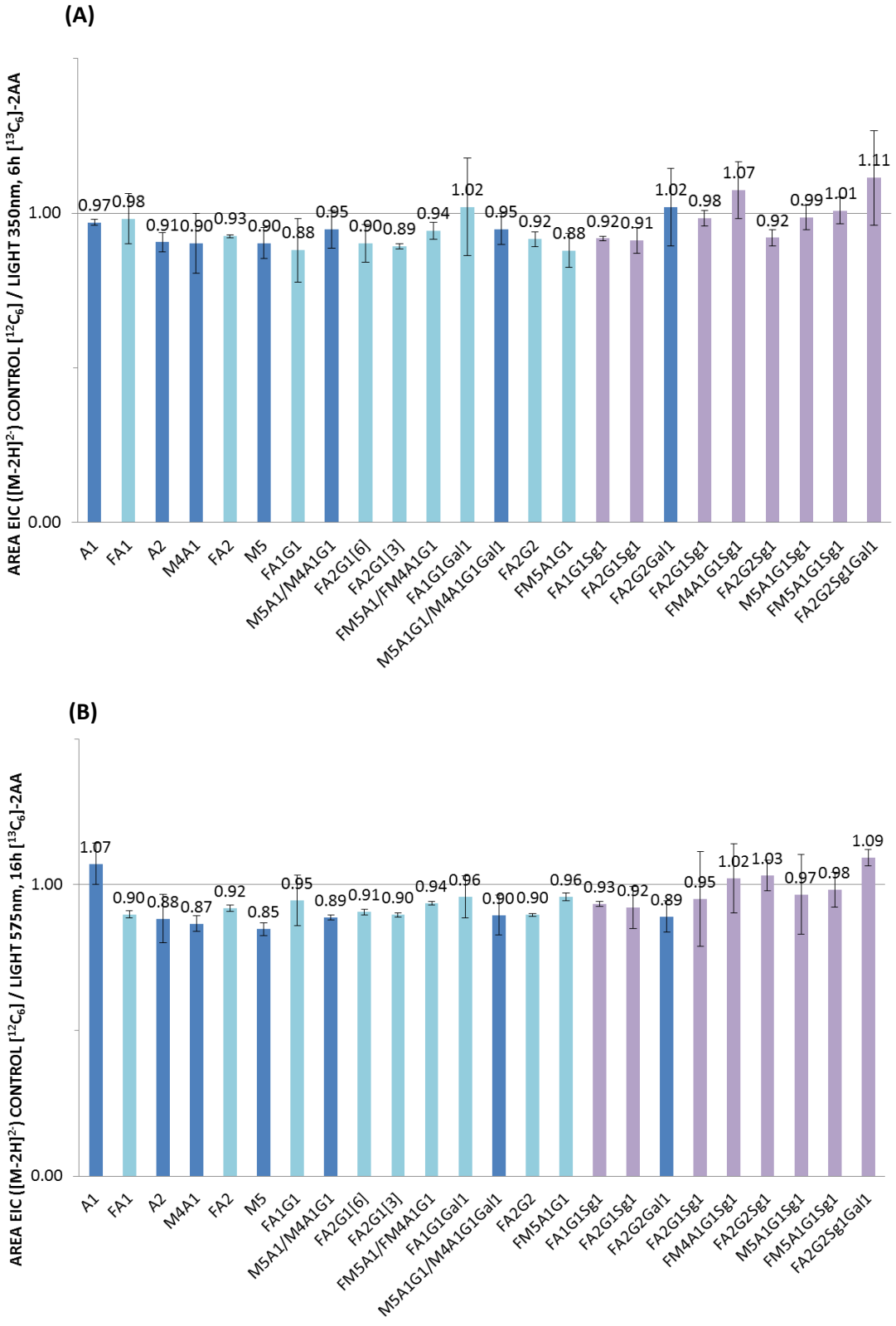
Stability of the intact non-deglycosylated mAb following temporal stress as detected using (A) size exclusion chromatography for aggregate analysis, (B) cation exchange chromatography for charged variant analysis and (C) C_4 reversed-phase chromatography for the determination of oxidized species.

Supplementary Figure 3:



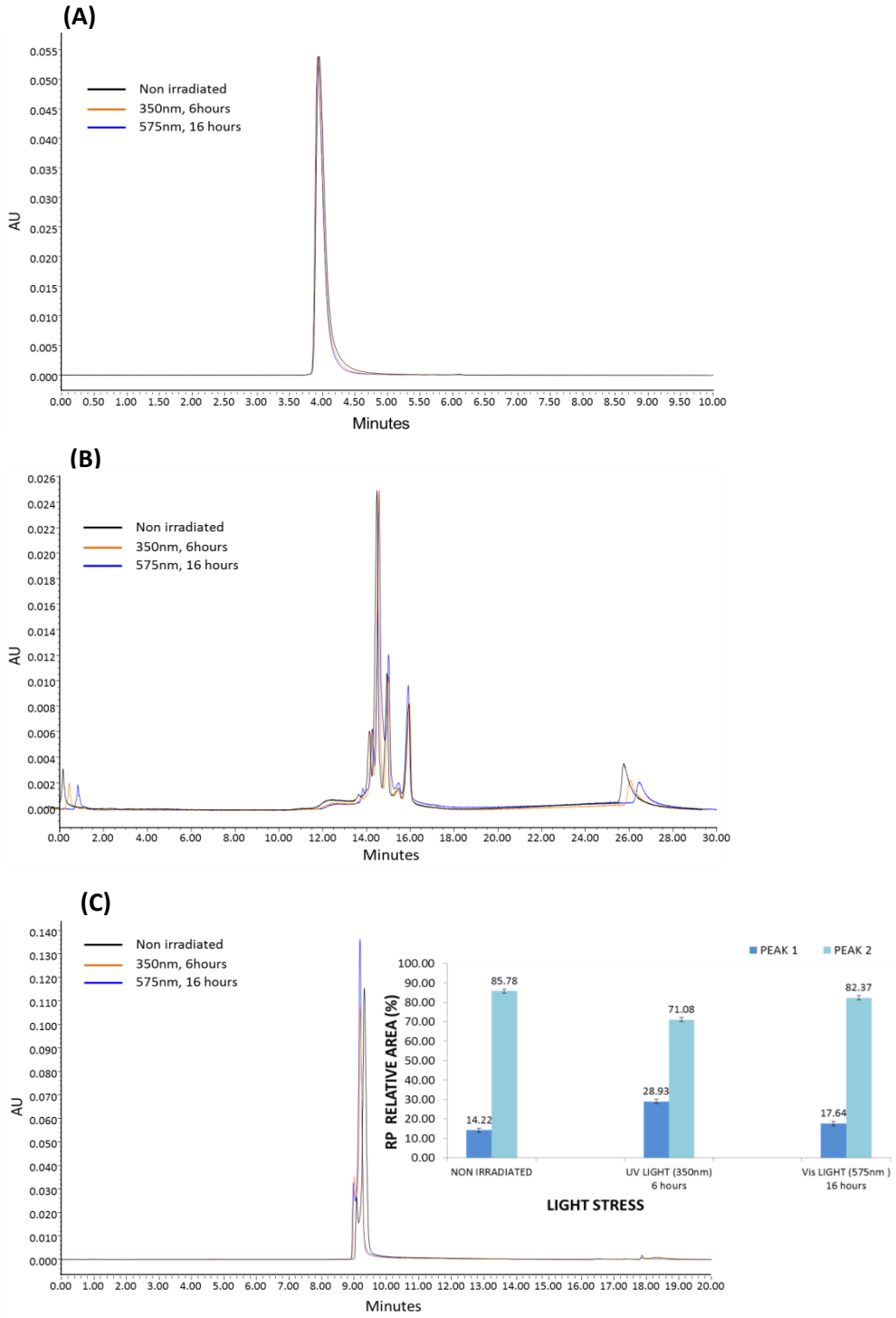
Stability of the intact non-deglycosylated mAb following thermal stress as detected using (A) size exclusion chromatography for aggregate analysis, (B) cation exchange chromatography for charged variant analysis and (C) C_4 reversed-phase chromatography for the determination of oxidized species.

Supplementary Figure 4:



Relative light to heavy ratios for the *N*-glycans present on the chimeric IgG1 mAb following exposure to (A) UV at 350 nm and (B) visible light at 575 nm. Irradiated samples were labelled with $^{13}C_6$ 2-AA, non-irradiated samples were labelled with $^{12}C_6$ 2-AA.

Supplementary Figure 5:



Stability of the intact non-deglycosylated mAb following UV and visible light irradiation as detected using (A) size exclusion chromatography for aggregate analysis, (B) cation exchange chromatography for charged variant analysis and (C) C_4 reversed-phase chromatography for the determination of oxidized species. No differences in the relative areas of peaks present in the SEC and CEX chromatograms were detected. A significant increase in oxidized species was detected following irradiation of the sample with UV light at 350 nm.

(1) Bones, J.; McLoughlin, N.; Hilliard, M.; Wynne, K.; Karger, B. L.; Rudd, P. M. *Analytical chemistry* 2011, *83*, 4154-4162.
(2) Ostasiewicz, P.; Zielinska, D. F.; Mann, M.; Wisniewski, J. R. *Journal of proteome research* 2010, *9*, 3688-3700.