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Characterization of an IgG1 Monoclonal Antibody and Related Sub-Structures by LC/ESI-Tof MS

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Abstract

In this study, we have developed two rapid, sensitive and efficient generic desalting/cleanup LC/electrospray time-of-flight (ESI-TOF) MS methods that can be used for the characterization of an intact antibody and its variants, deglycosylated forms of that antibody, resolution of constituent heavy and light chain structures and analysis of the common antibody fragments that are generated following papain cleavage.

Introduction

Monoclonal antibodies comprise a significant proportion of biotechnology-derived molecules used for diagnostic and therapeutic applications. The inherent heterogeneity of such products has dictated the need for thorough analytical characterization methodologies so that safe, effective and reproducible products can be produced. LC-MS has become a powerful tool as part of the standard analytical package used to characterize these important biomolecules.

IgG1s comprise a subclass of the antibodies developed as therapeutic and diagnostic agents. Specificity of interaction is produced by hypervariable regions of sequence present within a constant sequence backbone structure. Thus, while antibodies can have vastly differing binding selectivity, the overall structure is highly conserved between antibodies of the same class, and standard analytical methods can often be used as a starting point towards developing an optimized analytical strategy for an individual molecule.

Like many biotechnology-derived products, IgG1s have a complex heterogeneous structure, where multiple glycoforms and substoichiometric modifications produce a wide population of structures (variants) for a single antibody. The overall molecule (MW ~150 kD) comprises two identical heavy chains (HC, Figure 1, dark blue) linked together through two disulfide bonds, and two light chains (LC, Figure 1, light blue), each linked to a heavy chain by a single disulfide bridge.

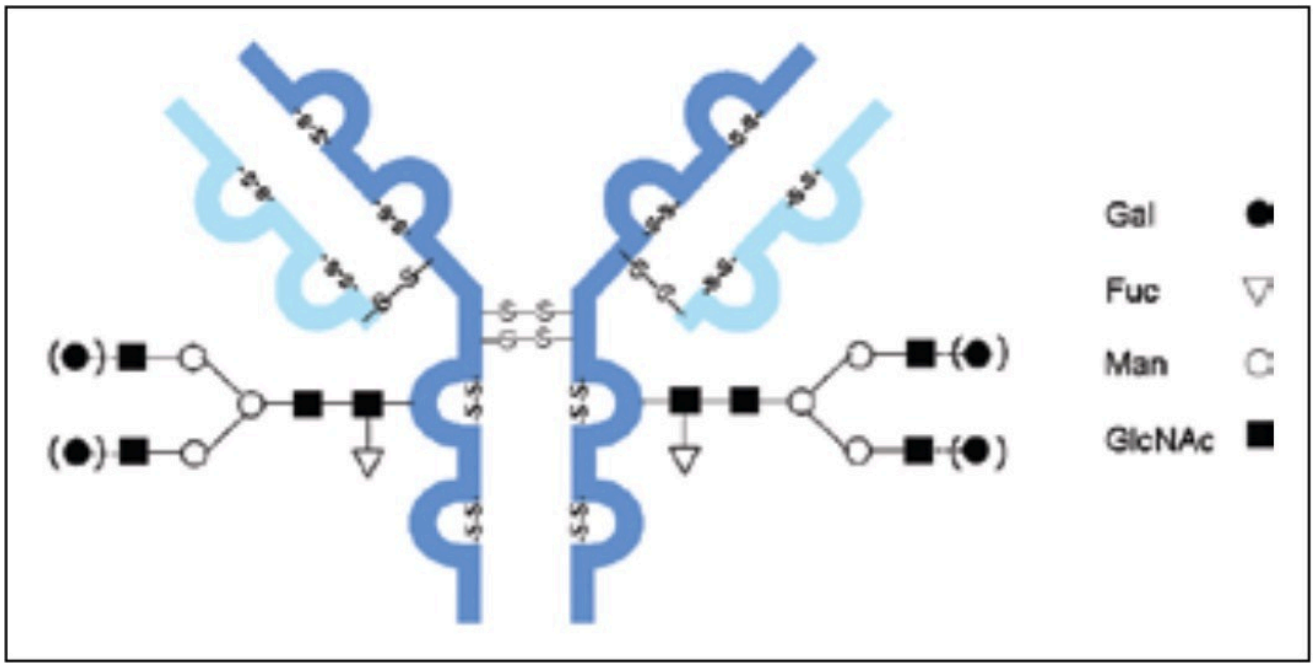

Figure 1. Structure of a monoclonal IgG1 antibody used in these studies. This particular IgG1 was found to contain N-linked biantennary carbohydrates linked each of the heavy chains. The major product variants that were observed correspond to glycan heterogeneity (0-4 galactose residues per antibody).

The typical IgG1 contains an additional 12 intra-chain disulfide bonds for a total of 16 disulfide bonds in the intact molecule1. Major product variants observed with IgG1s include the presence of a lysine on one or both C-termini

of the heavy chains, pyroglutamic acid modification of free N-termini containing glutamine and heterogeneous heavy chain glycosylation (e.g. in Figure 1 where an N-linked biantennary core glycan is shown with up to four terminal galactose residues). A variety of less specific modifications (e.g. deamidation, oxidation, etc.) can also appear at multiple sites on an antibody and levels of these modifications have been found to be altered by production and storage conditions.

The increased interest in recombinant monoclonal antibodies as therapeutic biomolecules has presented a challenge for the development of analytical methodologies required to permit their full characterization. High resolution mass spectrometry of the intact antibody and its variants provides a powerful approach for assessing batch-to-batch variation and for the study of antibody structural stability over time.

Additional insight has also been achieved by targeted processing of the intact antibody to generate smaller and less complicated antibody substructures. This has been achieved by chemical reduction of disulfides to produce free heavy and light chains, enzymatic or chemical deglycosylation and targeted cleavage of the antibody hinge region to generate characteristic antibody fragments. As with the intact antibody, the primary structure of these substructures will vary from antibody to antibody, but they will be sufficiently similar to permit analysis using a generic LC-MS methodology.

Most antibodies are stored in a matrix of biological buffers and non-volatile salts and stabilizers. Thus, one of the most significant challenges encountered during mass analysis of intact antibodies is processing of the sample to remove these agents, which often form non-covalent adducts that reduce MS response and further complicate the resulting mass spectral data.

Experimental

Materials

Protein A affinity purified mouse monoclonal antibody (IgG1,k) was obtained from VICAM Inc (Watertown, MA). Papain was purchased from Boehringer Mannheim (Indianapolis, IN). Dithiothreitol (DTT) and cysteine-HCl were obtained from Pierce (Rockford, IL). Sodium phosphate and EDTA were purchased from Sigma (St. Louis, MO). *Rapi*Gest SF and MassPREP MALDI Matrix, DHB were obtained from Waters Corporation (Milford, MA). Peptide N-Glycosidase F (PNGase F) was purchased from New England BioLabs (Ipswich, MA).

Preparation of Intact IgG1

Intact IgG1 stock (11.3 µg/µL, 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3) was diluted with 50 mM ammonium bicarbonate to achieve 1.0 µg/ µL IgG1. Lesser concentrations (0.1-0.5 µg/µL) were obtained by serial dilution with ammonium bicarbonate. LC-MS analyses were performed on 10 µL of diluted IgG1 samples.

Preparation of Reduced IgG1 (To Form Heavy and Light Chains)

Reduction of disulfides in the IgG1 (0.5 µg/µL) was accomplished using 20 mM DTT at 80 °C for 15 min. The reduced sample was injected onto the column for LC-MS analysis (10 µL) without further processing or storage.

Preparation of Deglycosylated Intact IgG1

PNGase F digestion of IgG1 was performed in IgG1 stock solution buffer (0.1 M sodium bicarbonate buffer, 0.5 M NaCl, pH 8.3). *Rapi*Gest SF (0.05% final) was added to the stock IgG1 solution (11.3 µg/µL) and heated at 60 °C for 30 min. The resulting solution was cooled to room temperature, and deglycosylation was initiated by the addition of PNGase F (11 U/µg IgG1), and incubated at 37 °C for 18 h. Following deglycosylation, the sample was diluted with 5% acetonitrile in 0.1% formic acid to 0.1 µg/µL, then treated with neat hydrochloric acid (2% v/v) for 1 h at 37 °C. LC-MS analysis was performed on a 10 µL of 0.1 µg/ µL processed sample.

Preparation of Deglycosylated and Reduced Intact IgG1

PNGase F digestion of IgG1 in the presence of DTT was performed in IgG1 stock solution buffer (0.1 M sodium bicarbonate buffer, 0.5 M NaCl, pH 8.3. *Rapi*Gest SF (0.05% final) was added to IgG1 solution (11.3 µg/µL) and heated at 60 °C for 5 mins. DTT was then added at a concentration of 20 mM and heated at 60 °C for 25 min. The resulting solution was cooled to room temperature, and deglycosylation was initiated by the addition of PNGase F (11 U/µg IgG1), and incubated at 37 °C for 18 h. Following deglycosylation, the sample was diluted with 5% acetonitrile in 0.1% formic acid to 0.5 µg/µL, then treated with neat hydrochloric acid (2% v/v) for 1 h at 37 °C. LC-MS analysis was performed on a 10 µL of 0.5 µg/µL processed sample.

Papain Digestion (No Cysteine)

Stock IgG1 was buffer exchanged against cysteine-free papain digestion buffer (1 mM EDTA, 50 mM sodium phosphate buffer, pH 6.3) by centrifugal ultrafiltration (VIVASPIN, 5000 MWCO, 11,000 x g, 5 °C). Papain was activated by adding one part papain suspension (10 mg/mL) to nine parts freshly prepared activation buffer (1 mM EDTA, 10 mM cysteine, 50 mM sodium phosphate buffer, pH 7.0), and incubating for 15 min at 37 °C. The

excess cysteine was removed by buffer exchange (against 6 vol. cysteine-free digestion buffer) using centrifugal ultra-filtration.

The activated papain was then diluted in cysteine-free digestion buffer (1 µg/µL), added to the IgG1 solution at an enzyme:antibody ratio of 1% (w/w), and incubated at 37 °C for 2 h. The papain digest was diluted with 5% acetonitrile in 0.1% formic acid to obtain 0.5 µg/µL, and used for LC-MS analysis (10 µL).

Papain Digestion (Addition of Cysteine)

Stock IgG1 was buffer exchanged against papain digestion buffer plus cysteine (10 mM cysteine, 1 mM EDTA, 50 mM sodium phosphate buffer, pH 7.0) by centrifugal ultra-filtration (VIVASPIN, 5000 MWCO, 11,000 x g, 5 °C).

Papain was activated by adding one part papain suspension (10 mg/mL) to nine parts freshly prepared activation buffer (1 mM EDTA, 10 mM cysteine, 50 mM sodium phosphate buffer, pH 7.0), and incubating for 15 min at 37 °C. The excess cysteine was removed by buffer exchange (against 6 vol. cysteine-free digestion buffer) using centrifugal ultra-filtration.

Papain digestion was carried out in digestion buffer plus cysteine at 37 °C overnight at an enzyme:antibody ratio of 1% w/w. The papain digest was diluted with 5% acetonitrile in 0.1% formic acid to obtain 0.5 µg/µL, and used for LC-MS analysis (10 µL).

HPLC Separations of Intact and Deglycosylated Intact Monoclonal Antibody

A Waters Alliance HPLC 2796 Bioseparations System or ACQUITY UPLC System was directly coupled to a Waters LCT Premier ESI-TOF Mass Spectrometer for all experiments. Reversed-phase separations of intact antibody and PNGase F treated antibody were performed on a MassPREP Desalting Cartridge (2.1 x 10 mm) using a 5 min gradient (5 to 90% B) at a flow rate 0.4 mL/min and a column temperature of 30 °C (see Tables 1 and 2). Mobile phase A was 0.1% formic acid in water, while mobile phase B contained 0.1% formic acid in acetonitrile. The column effluent was diverted from the MS source to waste using a 10-port valve for the first 2 min of the run to prevent salt contamination of the ESI source. Following each separation, the column was re-equilibrated with 5% B for 2 min.

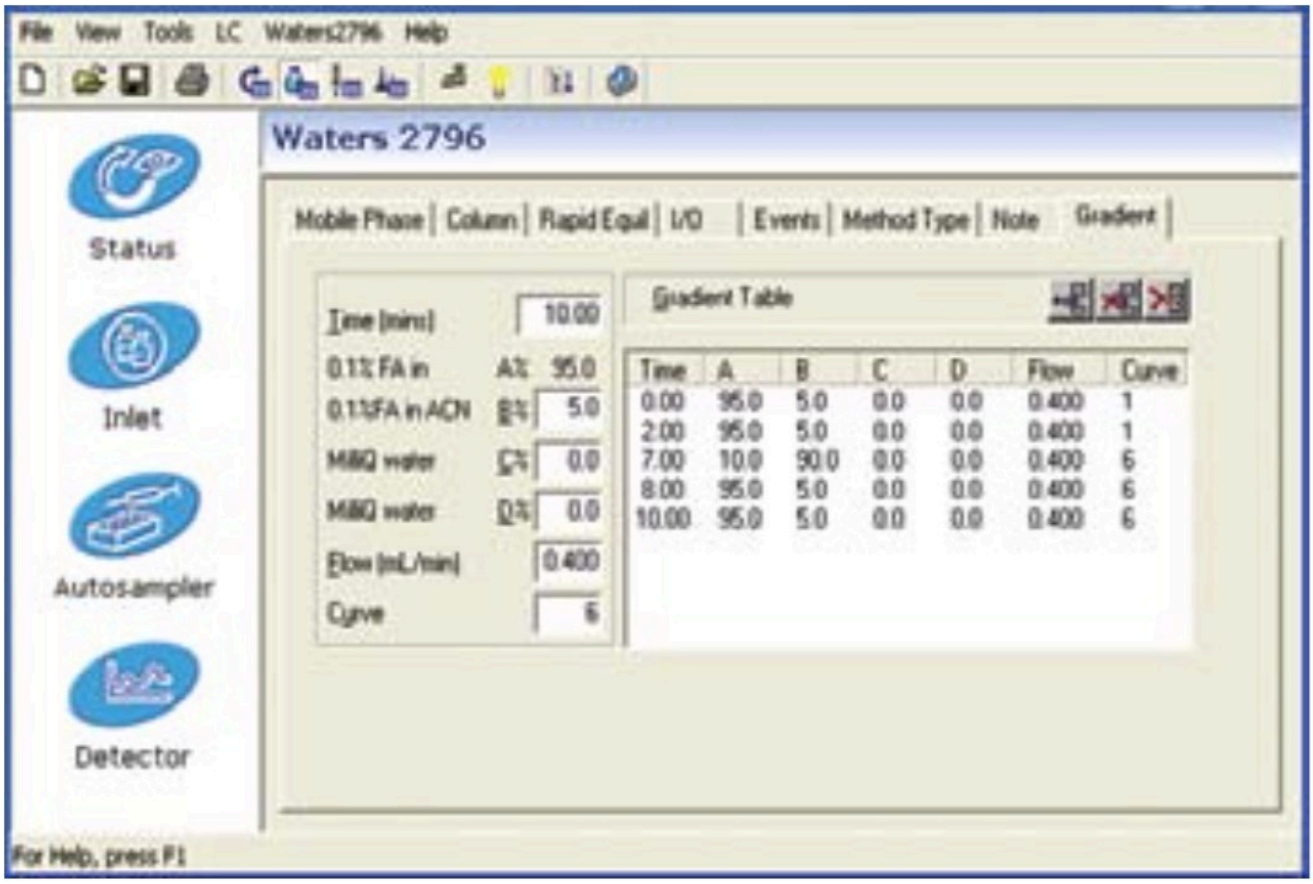

Table 1. Gradient profile used for the intact and deglycosylated IgG1 monoclonal antibody.

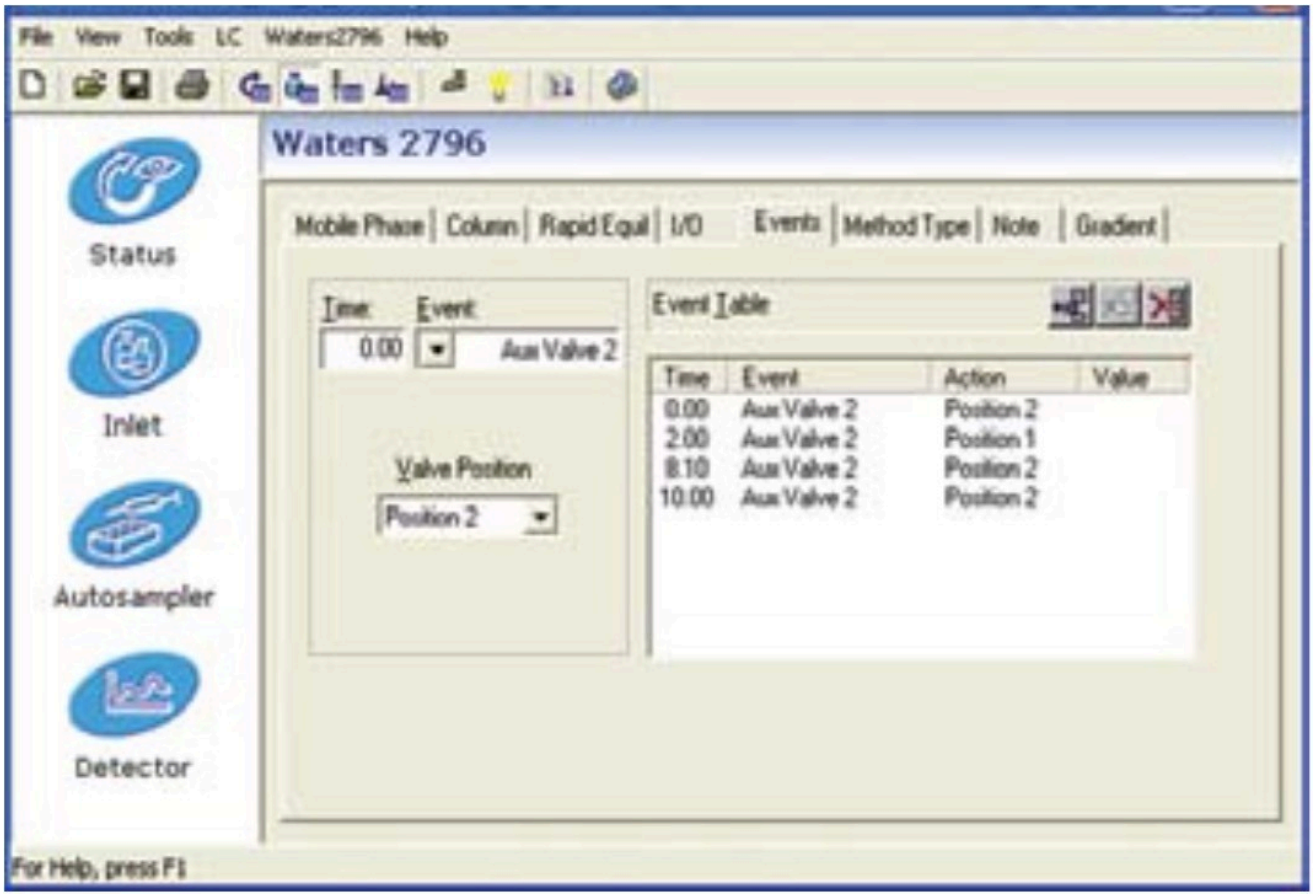

Table 2. Salt divert valve events used for the intact and deglycosylated IgG1 monoclonal antibody.

HPLC separations of reduced IgG1 (LC/HC), deglycosylated reduced IgG1 (LC/HC), and papain digested IgG1 fragments

Reversed-phase separations of reduced IgG1 (light and heavy chain fragments), deglycosylated LC and HC and papain digest fragments were performed on a MassPREP Desalting Cartridge (2.1 x 10 mm) using a 15 min gradient (5 to 90% B) unless otherwise stated. All other conditions for the separation were identical to those used for the intact antibody analysis. Tables 3 and 4 display the gradient table and salt divert valve events used for these experiments.

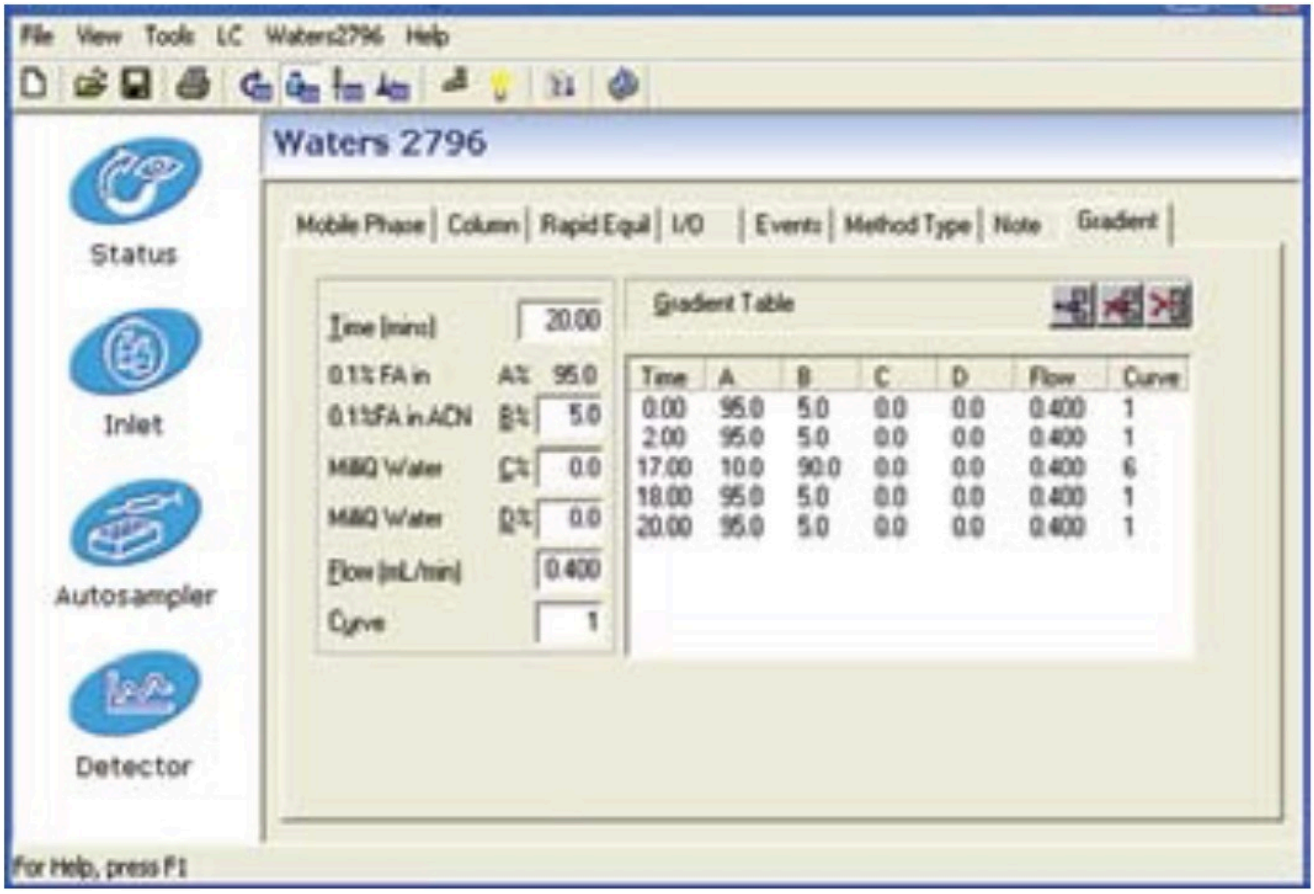

Table 3. Gradient profile used for reduced deglycosylated IgG1 (LC/HC) and papain digest fragments.

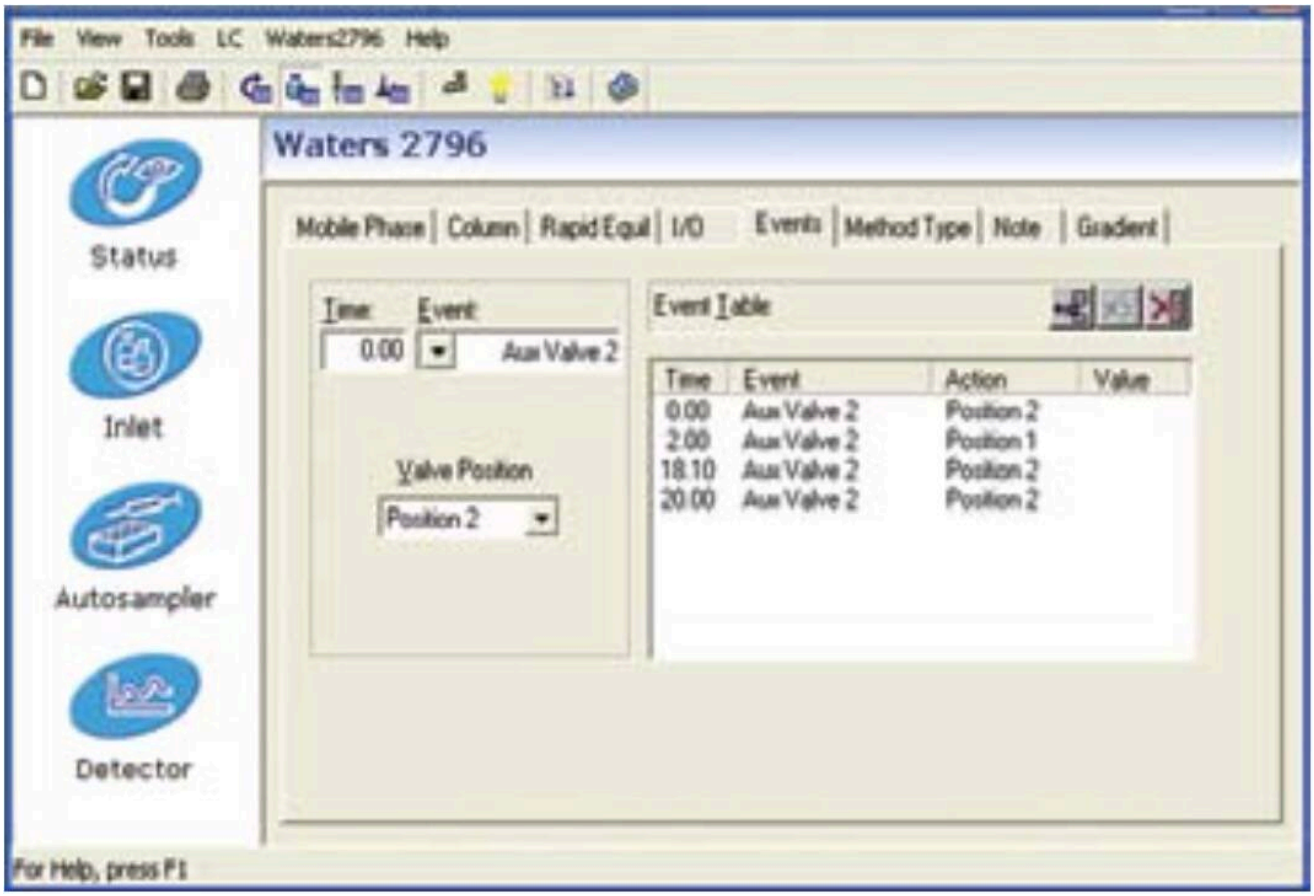

Table 4. Salt divert valve events used for reduced deglycosylated IgG1 (LC/HC) and papain digest fragments.

Purification of Released Glycans

Glycans released from the intact IgG1 by PNGase F treatment were enriched using the 96-well MassPREP HILIC µElution plate according to the Waters Care and Use Instructions and a published paper by Yu and colleagues.[2] Briefly, the method used to extract glycans was as follows:

- A µElution plate well was washed with 200 µL of Milli-Q water (Millipore, Billerica, MA), and equilibrated with 200 µL of 85% acetonitrile
- The deglycosylated IgG1 (9 µg/µL) that was acid-treated to break down RapiGest was diluted with acetonitrile to obtain the equivalent of 0.3 µg/µL IgG1 in 85% organic solvent
- 200 µL of diluted sample was loaded on the plate by gravity

- Deglycosylated IgG1 and salts were washed off with 200 µL of 85% acetonitrile
- Glycans were eluted with 10 mM ammonium citrate in 25% acetonitrite (50 µL, twice), and the collected sample was dried under vacuum (Labconco, Kansas City, MO)

Analysis by electrospray ionization mass spectrometry Protein mass information was obtained using the time-of-flight (TOF) Waters LCT Premier Mass Spectrometer operated in the positive ion W-mode. The electrospray (ESI) source was operated under the following conditions:

Source temp.:	150 °C
Desolvation temp.:	350 °C
Desolvation gas:	800 L/h
Cone voltage:	40 V
Capillary voltage:	3.2 kV
Ion guide 1:	100 V

The mass spectrometer was calibrated using an external multipoint calibration based on singly-charged CsI ions (2 mg/mL CsI dissolved in 50% isopropanol). Mass spectra were acquired in the *m/z* range of 600 to 5000. A 1 Hz scan rate with 0.1 sec interscan delay was used to acquire data over the entire analysis.

Analysis by MALDI Mass Spectrometry

Purified glycans were reconstituted in 30 µL of 25% acetonitrile. Sample (0.5 µL) and an equal volume of matrix (20 mg/mL DHB in 80% acetonitrile) were co-spotted on a stainless steel MALDI plate and allowed to dry at ambient temperature. Ethanol (0.5 µL) was overlaid on the dried well to form a more homogeneous crystalline sample.

Glycan masses, and tandem (MS/MS) spectra from the purified glycans, were determined using a Waters MALDI Q-Tof Premier Mass Spectrometer. The MALDI Q-Tof Premier was equipped with a N2 laser (337 nm) source

operated at 10 Hz laser firing rate for data acquisition. Calibration was accomplished using polyethylene glycol plus sodium iodide in 50% acetonitrile with CHCA matrix (MassPREP Calibration Mix-MALDI Reflectron) in the mass range of *m/z* 50 to 3000. Mass spectra were acquired in the positive ion mode with V-optics. Argon was used as the collision gas and the collision cell pressure was maintained at 5.30 10^{-3} mBar. Collision energy in MS mode was 10V and 500 laser shots were combined to generate an MS spectrum; glycan fragmentation spectra were acquired using collision energy of 125 V and combining 1,200 laser shots.

Data Processing

Summed electrospray mass spectra were generated by combining all spectra over chromatographic peaks as indicated. The resulting summed spectra (*m/z* regions as indicated in subsequent figures) were deconvoluted by the Waters MaxEnt1 algorithm to produce neutral mass information. MaxEnt1 processing parameters included an output bin size of 1 Da, a Gaussian damage model with a peak width of 1 Da (intact and deglycosylated IgG1) or 0.7 Da (reduced IgG1, reduced and deglycosylated IgG1, and papain fragments), minimum intensity ratio of 33% (right and left) and processing to model convergence.

Results and Discussion

It is essential that nonvolatile buffers, salts and stabilizing agents are removed prior to MS analysis, as they reduce MS response and produce noncovalent adducts that complicate interpretation of antibody mass spectra. Here we have utilized a robust largepore polymeric reversed phase trap column (MassPREP On-Line Desalting Cartridge) for sample cleanup and desalting prior to online mass analysis.

Although the key function of the column was to remove interfering substances for LC-MS analysis, sufficient separation capacity exists within the trap column to achieve partial resolution between heavy and light chains, deglycosylated heavy and light chains and the major fragments of papain digestion. Two LC-MS methods (differing primarily by length and slope of reversed phase gradient) are provided within this document to accomplish the desalting or desalting/separation functions described below.

Analysis of an intact IgG1

A fast and efficient LC/ESI-MS method was used to identify multiple structural variants of a model IgG1

molecule, with a total injection-to-injection cycle time of 10 min. The corresponding gradient table and salt divert valve event times are shown in Tables 1 and 2, respectively.

Figure 2 depicts the fluidic configuration used for LC-MS analysis, including a post-column salt diversion valve, and the direct flow of eluent into the mass spectrometer. Column effluent was diverted from the mass spectrometer to waste using a post-column 10-port two-position valve (in position 1) for the first 2 min, to prevent salt contamination of the ESI source. Eluent flow was restored to the mass spectrometer (valve position 2) and the IgG1 was eluted using a 5 min gradient of 5 to 90% acetonitrile in 0.1% FA, followed by a 2 min column regeneration period with 5% acetonitrile in 0.1% FA.

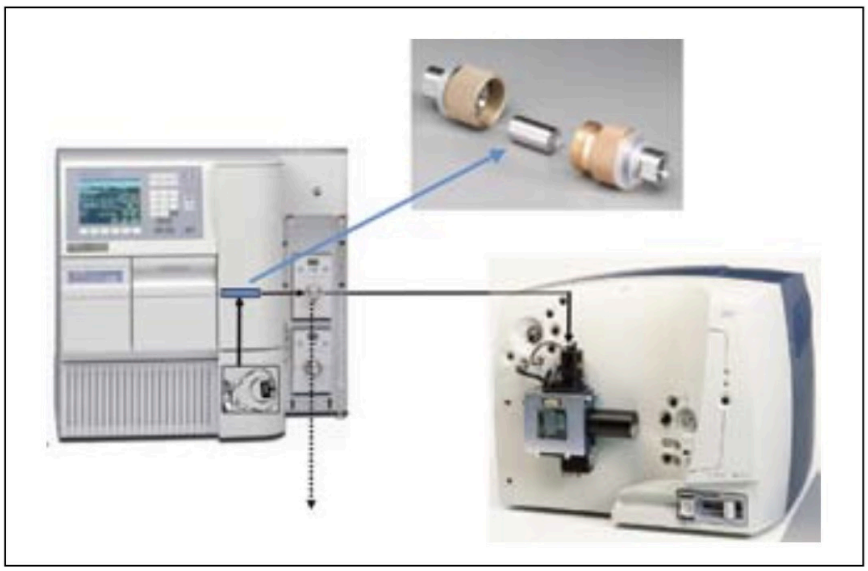

Figure 2. LC/ESI-Tof MS configuration. A Waters Alliance HPLC 2796 Bioseparations System was directly coupled to a Waters LCT Premier ESI-Tof MS for antibody mass analysis. Protein separation and desalting were accomplished using a 2.1 x 10 mm polymeric RP cartridge column. During the LC-MS analysis, the column effluent was diverted to waste for the first two minutes each run, using a post-column 10-port two-position valve, to prevent salt contamination of the ESI source.

Carryover and binding capacity of the trap column were briefly investigated by loading increasing amounts (1, 5, 10 µg) of intact antibody with intervening blank runs. The overlaid (y-axis linked) total ion current

chromatograms (TICs) for this experiment and the associated summed mass spectra are shown as Figures 3 and 4, respectively. The results show no detectable carryover in the blank following a 1 µg injection, and less than 4% carryover at the 5 µg and 10 µg loadings (Figure 4). An increase in MS response (max counts of the summed spectra) of ~3 fold was seen in the 5 µg loading compared to the 1 µg loading, whereas the 10 µg loading resulted in only a ~4 fold signal increase relative to the 1 µg loading (Figure 4).

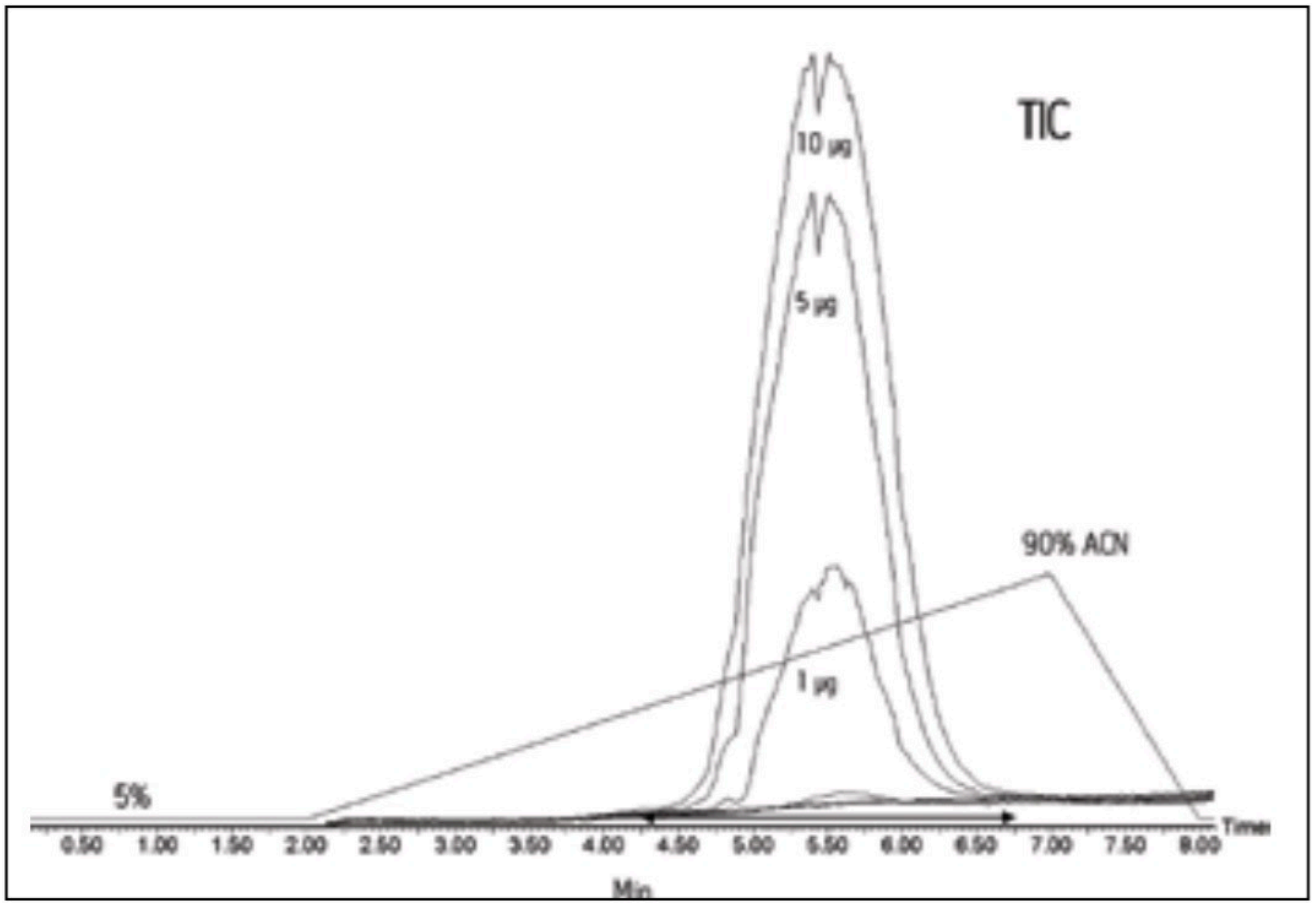

Figure 3. Total ion current chromatograms (TIC) from LC/ESI-MS analyses of an intact IgG1 loaded at varying amounts (1 to 10 µg). Blank LC-MS runs conducted between each of the samples were used to demonstrate the low amount of sample carryover for this method.

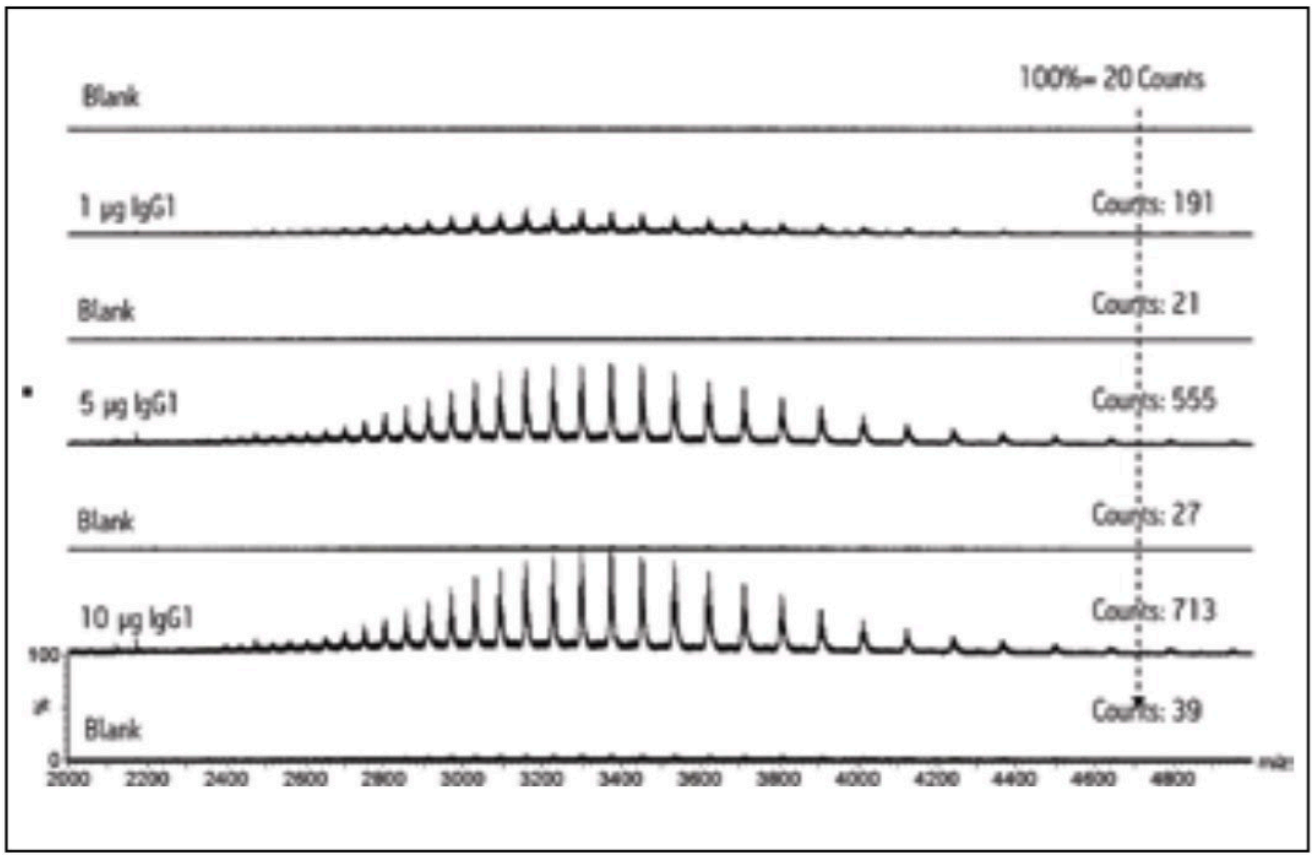

Figure 4. Combined ESI-Tof mass spectra of an intact IgG1 demonstrating the relative carryover at varying amounts (1 to 10 µg) from the intervening blank runs. There was no carryover detected with the 1 µg loading, and less than 5% carryover associated with the 5 and 10 µg loadings.

Monoclonal antibodies demonstrate significant structural heterogeneity (variants) due to complex patterns of post-translational modifications. The most common set of protein variants differ with respect to processing of terminal amino acids (N-terminal pyroglutamic acid formation, C-terminal Lysine processing of heavy chains), and heterogeneity in the glycoform structures present on each of the heavy chains. Other variants (e.g. those containing deamidation and oxidation) arise due to environmental conditions of growth, storage and processing.

The isotopic distribution of an intact antibody is sufficiently wide (~25 Da at half height for the neutral molecule) that lower mass modifications may not be spectrally resolved from the unmodified antibody, but patterns of glycosylation (140 to 200 Da) and lysine processing (128 Da) can be easily discerned. The presence of low mass modifications can be observed as partially resolved peaks, or as a mass shift for a peak that will be in direct

proportion to the relative levels of the species. The analysis of antibody substructures (e.g. light and heavy chains, or Fab and Fc fragments) or peptide mapping analysis is typically used to directly confirm the presence of these low mass modifications.

The summed ESI-Tof mass spectrum for our intact IgG1 (5 µg load) revealed a symmetrical charge state envelope over 2400 to 5000 *m/z* (Figure 5). The +44 charge state region, at the center of the envelope, has been enlarged (Figure 5, inset) to show greater detail, and reveals at least six major antibody variants, even before MaxEnt1 processing. The maximum entropy approach uses all MS information to discern the most likely deconvoluted spectrum to have given rise to the observed *m/z* spectrum. Because of this reliance on the "global view," the effective resolution achievable following MaxEnt1 deconvolution is greater than revealed from examination of any given charge state.

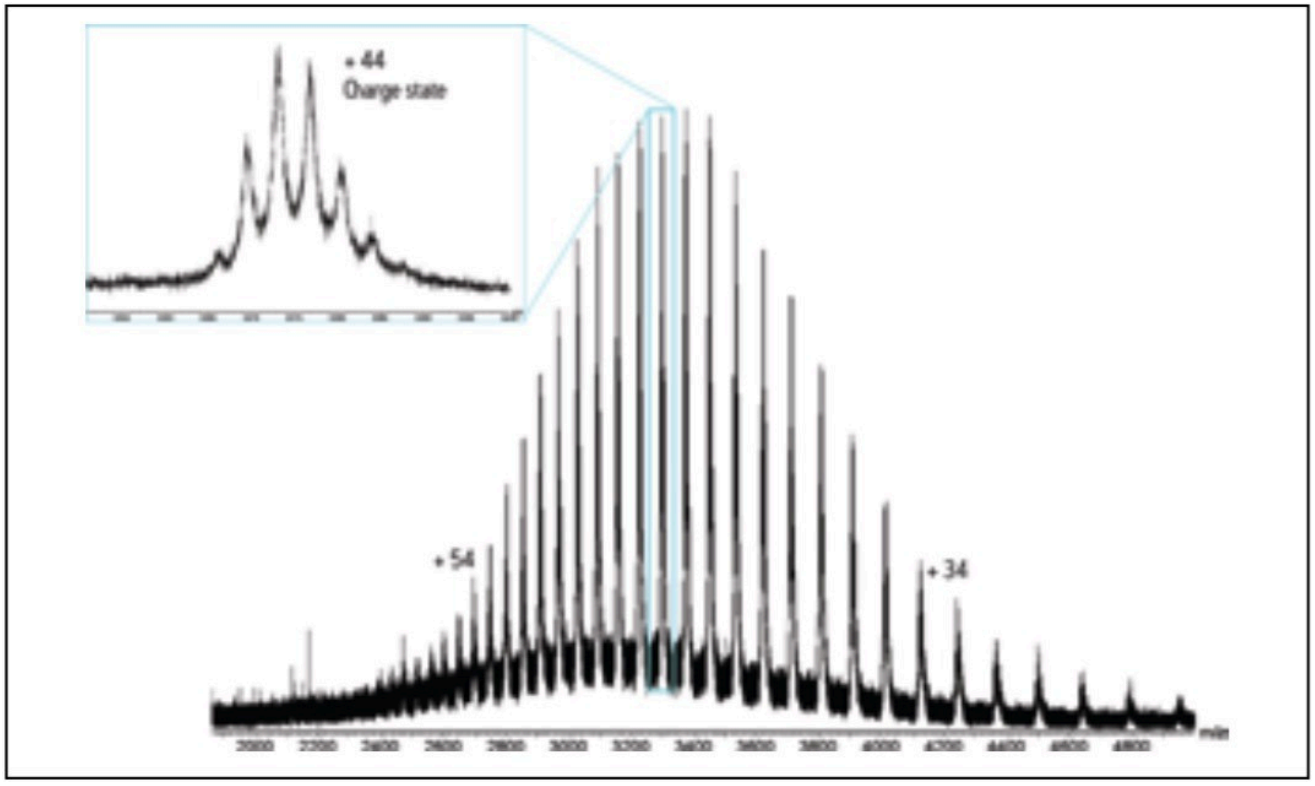

Figure 5. The summed ESI-MS mass spectrum over the TIC peak shown for the 5 µg IgG1 load. A charge envelope of 2400 to 4800 m/z was observed for the intact IgG1, centered on +44 charge state of the protein.

The MaxEnt1 deconvoluted spectrum (Figure 6) for the intact IgG clearly revealed heterogeneity in the

carbohydrate moieties attached to the two heavy chains. Five peaks demonstrate a characteristic sequential mass difference of ~162 Da consistent with extension of the two core glycan structures by up to four hexose (galactose) residues. An additional peak pair (148,214 Da and 148,068 Da) demonstrates a mass difference of 146 Da corresponding to incomplete occupancy of a fucosylation site on the core glycans.

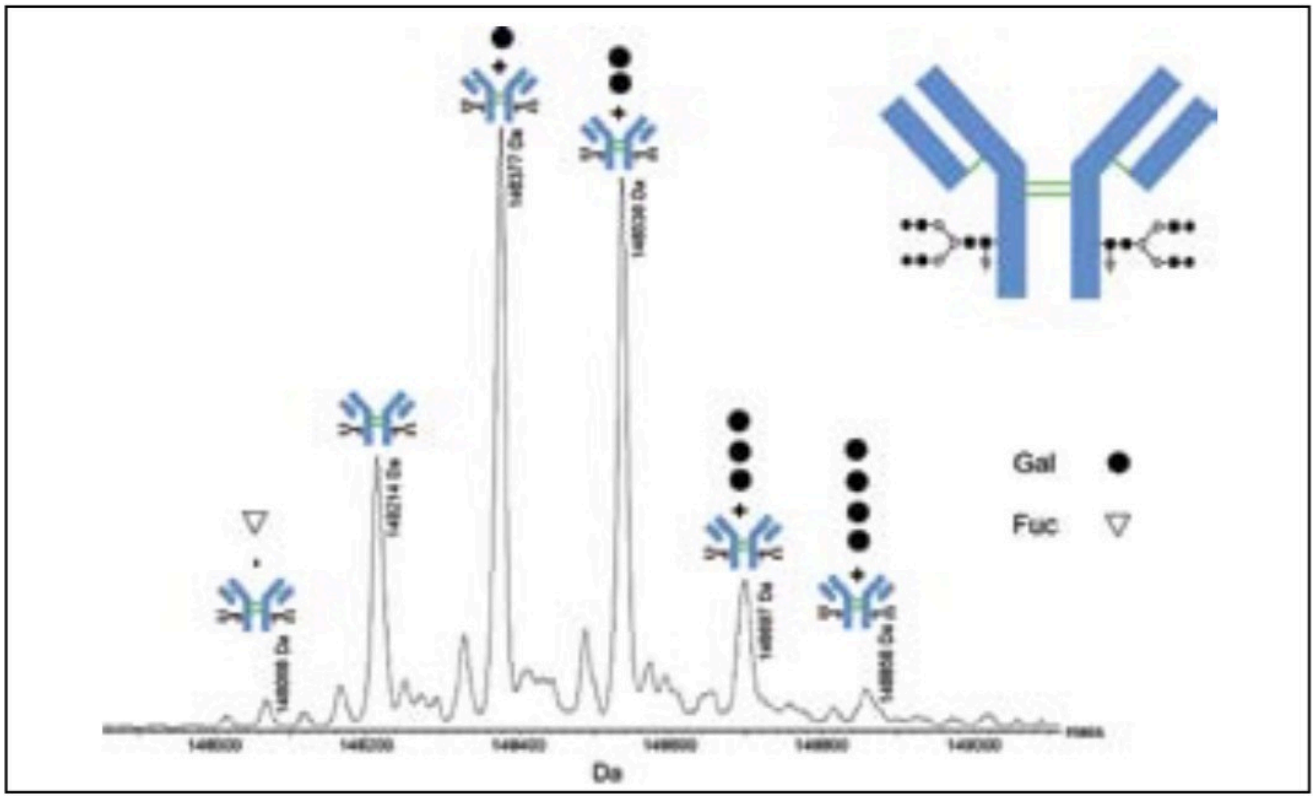

Figure 6. MaxEnt1 deconvoluted mass spectrum of the intact IgG1. Major variants observed were due to carbohydrate heterogeneity. The deconvoluted mass spectrum shows the intact IgG1 with up to four terminal galactose residues (▪), and slightly incomplete core glycan fucosylation (Δ). No C-terminal lysine processing variants were observed on this antibody.

MaxEnt1 produces a result that is quantitatively conserved with the raw spectral data, and for chemically similar analytes (as we expect IgG1 variants to be), the MaxEnt1 result can be used for relative quantitation for each of the identified species. There were no additional variants observed that would reveal incomplete lysine processing on one or both heavy chains.

Overall, the data demonstrates that a single IgG1 monoclonal antibody exists as a complex population of variants,

and that mass spectrometry on the intact antibody is capable of characterizing much of this heterogeneity.

Analysis of a Reduced IgG1

Reduction of the intact IgG1 with dithiothreitol (DTT) breaks intra-chain disulfide bonds, and inter-chain disulfide bonds that connect the light chains to the heavy chains and two heavy chains to one another. The resulting liberation of light and heavy chains provides the opportunity for more detailed structural studies on the individual subunits.

Finding proper conditions for this reduction can be somewhat challenging as the various IgG subtypes have differing susceptibility to reduction of the inter-chain bonds, and the intra-chain disulfides often require even more stringent conditions to achieve complete reduction. In some LC-MS studies, we have observed additional species and TIC peaks arising due to incomplete reduction of an antibody. For our IgG1 antibody, complete reduction of both intrachain and inter-chain disulfide bonds was achieved using elevated temperature and DTT concentration (20 mM, 15 min, and 80 °C) than may be typical (10 mM, 10 min, and 60 °C). The minimum effective treatment should be used, as heat and other environmental stresses have the potential to produce undesirable structural changes in proteins.

Separation of the heavy and light chains was obtained by applying a longer gradient (5 to 50% acetonitrile in 0.1% FA over 8 min) for an 11 min injection-injection cycle time. The first 1 min of a chromatographic run was diverted to waste for sample desalting, and the last 3 min of the run was utilized for column washing and regeneration. Gradient entries and salt divert valve timing for these methods are shown in Table 5.

Time (min)	%B	Flow (mL/min)	Curve	
0.00	5	0.2	Initial	Load/Wash -Divert Flow-
1.0	5	0.2	6	
1.01	10	0.2	6	Gradient
8.11	50	0.2	6	
8.5	90	0.5	6	Column Washing and Regeneration
8.6	5	0.5	6	
9.1	90	0.5	6	
9.6	5	0.5	6	
9.7	90	0.5	6	
9.8	5	0.5	6	
11	5	0.5	6	

Table 5. Gradient profile and salt divert timing used for a reduced IgG1 (LC/HC).

A post-column salt diversion valve (top-left corner of the LCT Premier) was used to divert buffers and non-volatile salts to waste during sample loading and column washing steps.

The TIC of the reduced IgG1 (heavy and light chain fragments) is displayed in Figure 7. In this chromatogram, the light chain is an earlier eluting peak, while the heavy chain elutes later. Figure 8 shows the summed mass spectrum (inset) and deconvoluted spectrum of the light chain, which reveals a single major peak at 24,199 Da. Minor peaks (sodium adduct and loss of water) are also visible in the deconvoluted spectrum.

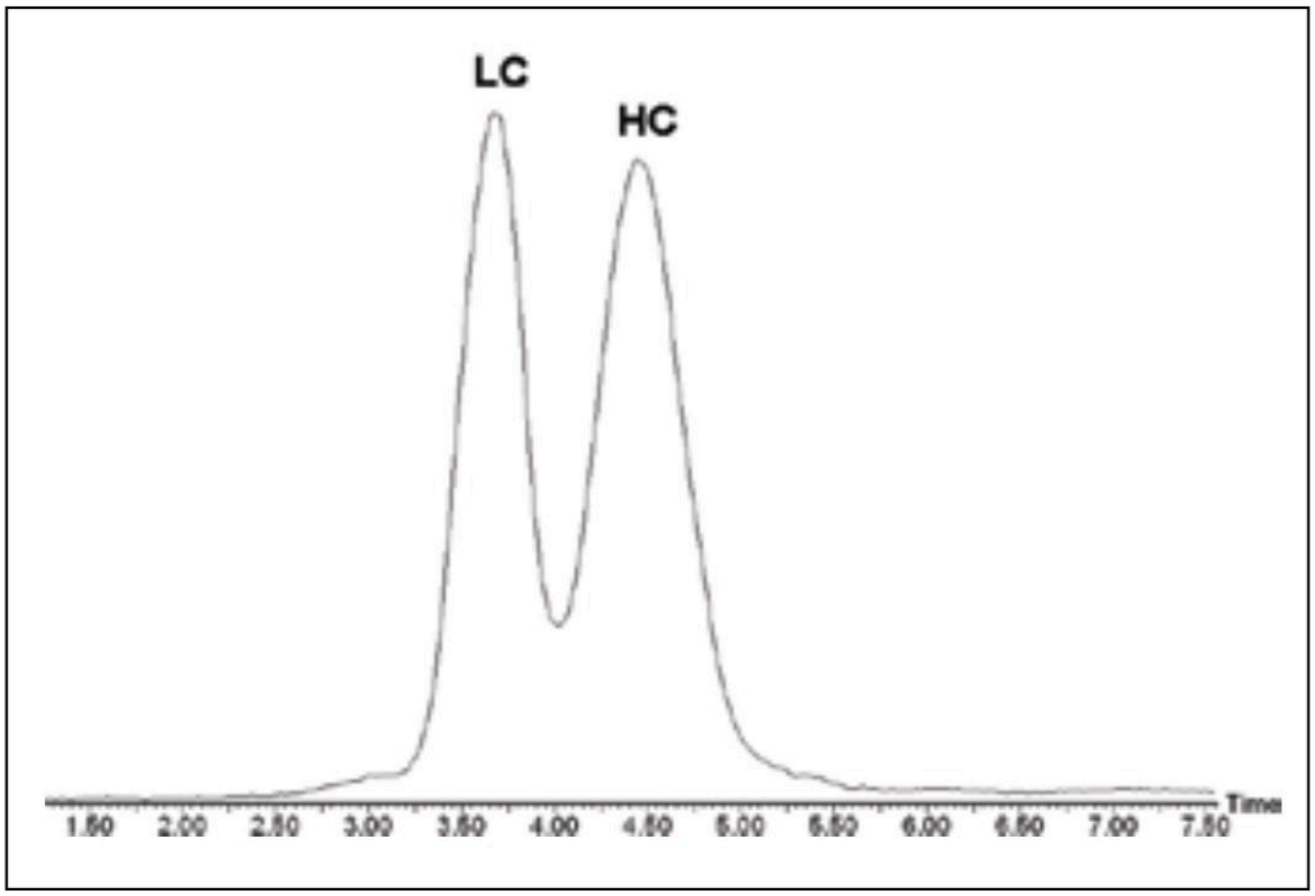

Figure 7. TIC chromatogram from ACQUITY UPLC-MS analysis of the reduced IgG1 antibody. Separation was achieved using an 8 min linear gradient (5 to 50%) of acetonitrile. The column temperature was maintained at 80 °C throughout the run.

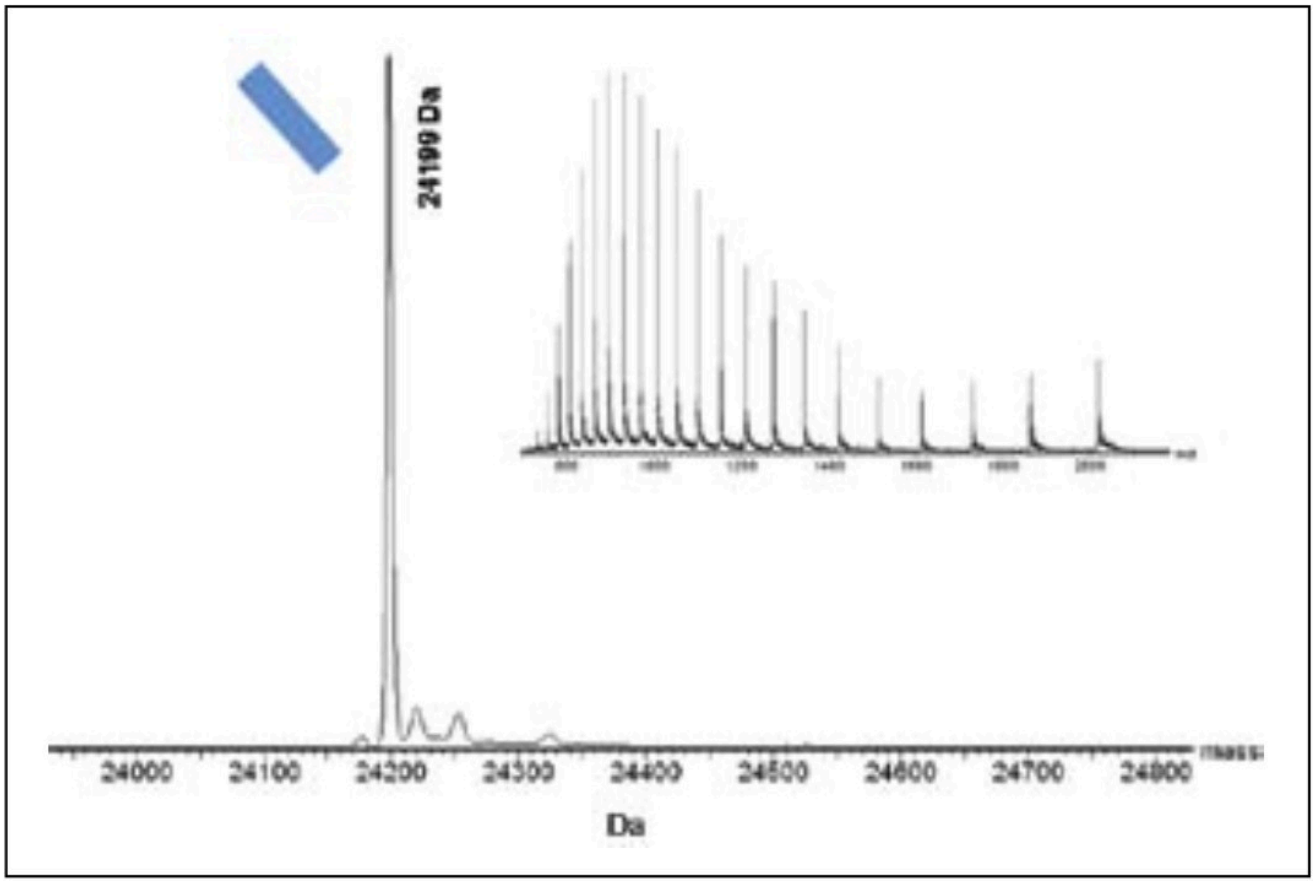

Figure 8. Combined mass spectrum (inset) and deconvoluted mass spectrum of the light chain derived from the IgG1. The complete reduction of intrachain and interchain disulfide bonds was achieved using the condition referenced in the methods section. The light chain mass was determined as 24,199 Da.

Figure 9 depicts the summed mass spectrum (inset) and deconvoluted mass spectrum of the glycosylated heavy chain. Three major peaks with a mass differential of ~162 Da correspond to the heavy chain containing the core glycan, and variants where the core glycan is extended by one or two galactose residues. This is in agreement with our previous findings for the intact IgG1 antibody, where up to four additional galactose residues were observed.

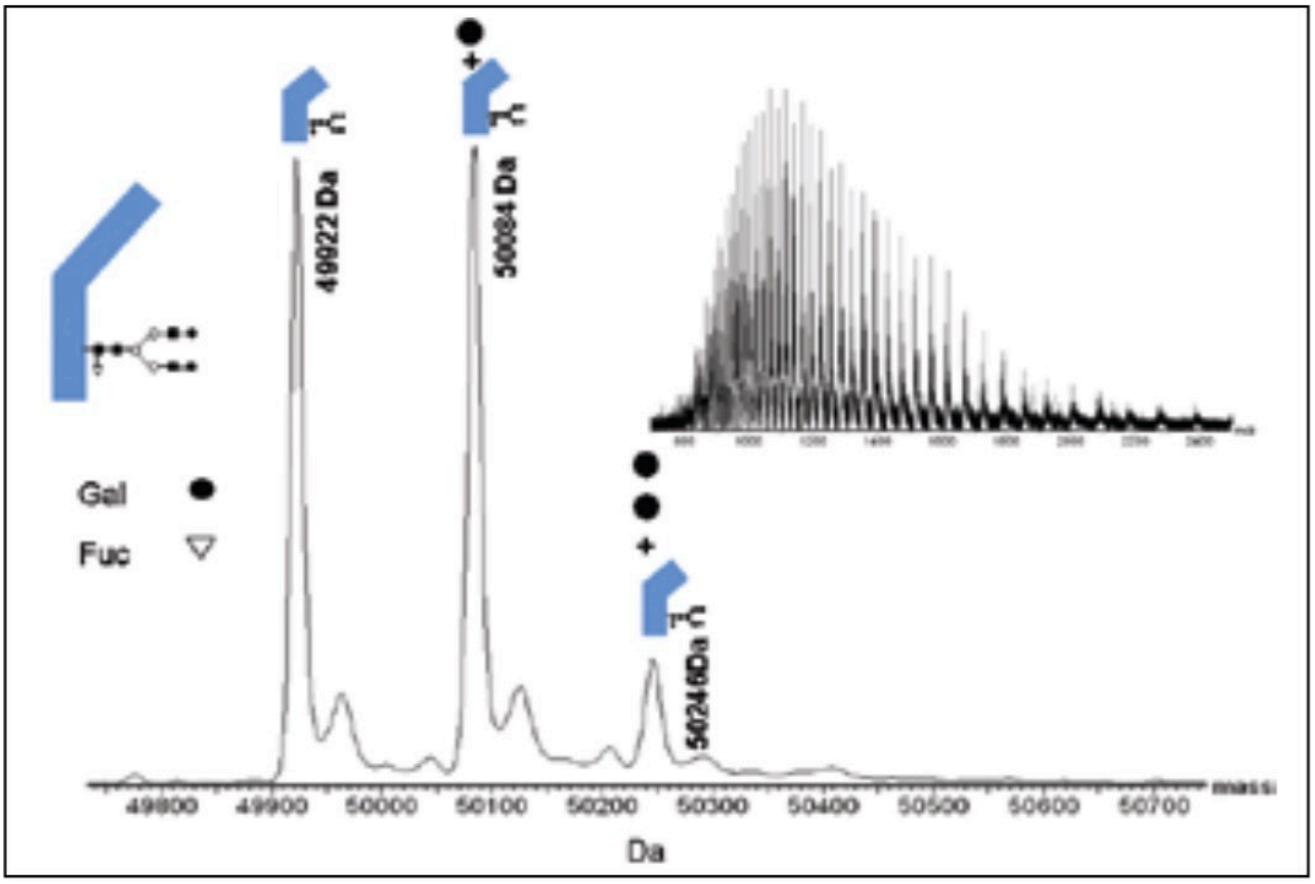

Figure 9. Combined mass spectrum (inset) and deconvoluted mass spectrum of the glycosylated heavy chain derived from the IgG1. Three major peaks differing by ~162 Da, corresponded to the heavy chain with no (Mass 49,922 Da), one (Mass 50,084 Da), and two (Mass 50,246 Da) terminal galactose residues.

In basic antibody math, the mass of the intact IgG1 can be calculated from the combined masses of two light chains, two heavy chains, and restoration of the 16 disulfide bonds (-32 Da). Based on our mass observations of the light chain and heavy chain (containing only the core glycan), we would predict an IgG1 mass of 148,210 Da. This prediction is only 4 Da (or 30 ppm) lighter than the observed intact IgG1 mass of 148,214 Da (LC-MS of intact IgG1 analysis). This result was obtained using a 1 Da precision to MaxEnt1 deconvolution, which potentially accounts for much of that mass difference.

This result confirms that LC/ESI-Tof/MS can produce a mass measurement for the intact IgG1 of similar precision to that determined for the much smaller constituent heavy and light chains. The main advantage of working with the reduced antibody becomes the ability to directly detect lower mass modifications such as

oxidation (+16 Da) that are obscured under the isotopic envelope of the intact antibody.

Analysis of a Deglycosylated IgG1

PNGase F is an glycosidase that releases the oligosaccharides attached to an asparagine residue (N-glycosylation sites) within a glycoprotein by cleaving the beta-aspartylglycosylamine linkage. In this process, the attachment site Asn is converted to Asp via a glycosidase-mediated hydrolysis reaction.[3] Consequently, the observed mass for a deglycosylated protein or peptide shifts upwards by one Da from the predicted value. Factoring in this small difference, the mass difference between the deglycosylated antibody and intact antibody is the combined mass of the two heavy chain glycans.

There are several types of core glycan structures that differ by composition and extent and location of branching points, but a thorough discussion is beyond the scope of this document. There are, however, several common core glycan structures for which the masses have been calculated4, 5, and are commonly deduced from mass analysis of an antibody with and without enzymatic deglycosylation.

The deglycosylated IgG1 was analyzed under identical LC-MS conditions to the intact antibody. As with the intact antibody, LC-MS of the PNGase F deglycosylated IgG1 reveals a single TIC peak (Figure 10), and there was no obvious shift in retention time between the intact and deglycosylated IgG1.

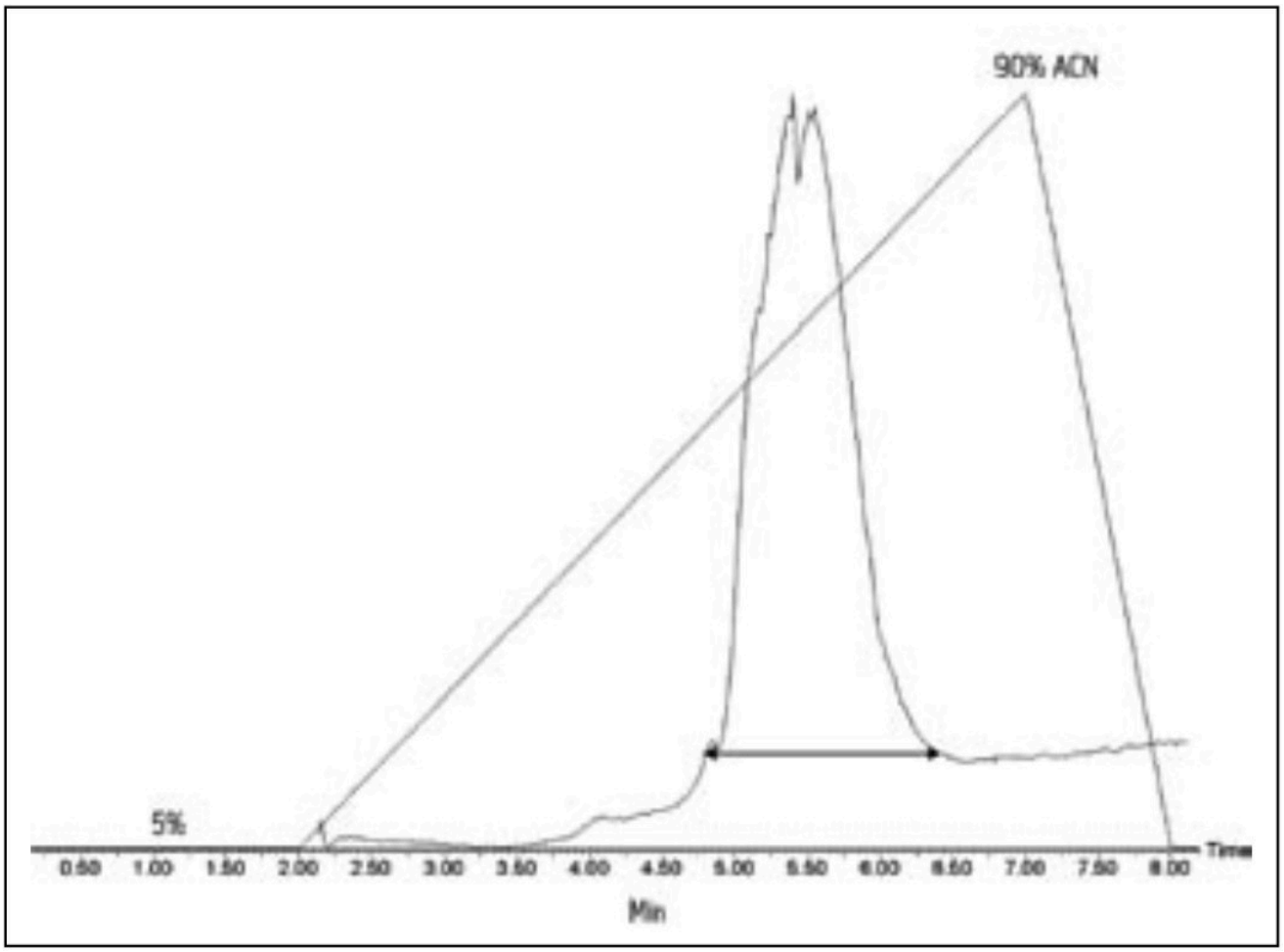

Figure 10. TIC chromatogram from LC/ESI-MS analysis of deglycosylated IgG1 produced by PNGase F digestion (37 °C, 18 h).

The combined ESI mass spectrum of the deglycosylated IgG1 (Figure 11, inset) resulted in a deconvoluted mass spectrum (Figure 11) containing one major component (deglycosylated IgG1 with a mass of 145,328 Da), and one minor component (Unidentified, mass of 147,508 Da). The mass difference between the intact IgG1 (containing 2 core glycans) of 148,214 and the deglycosylated IgG1 145,328 Da is 2,886 Da.

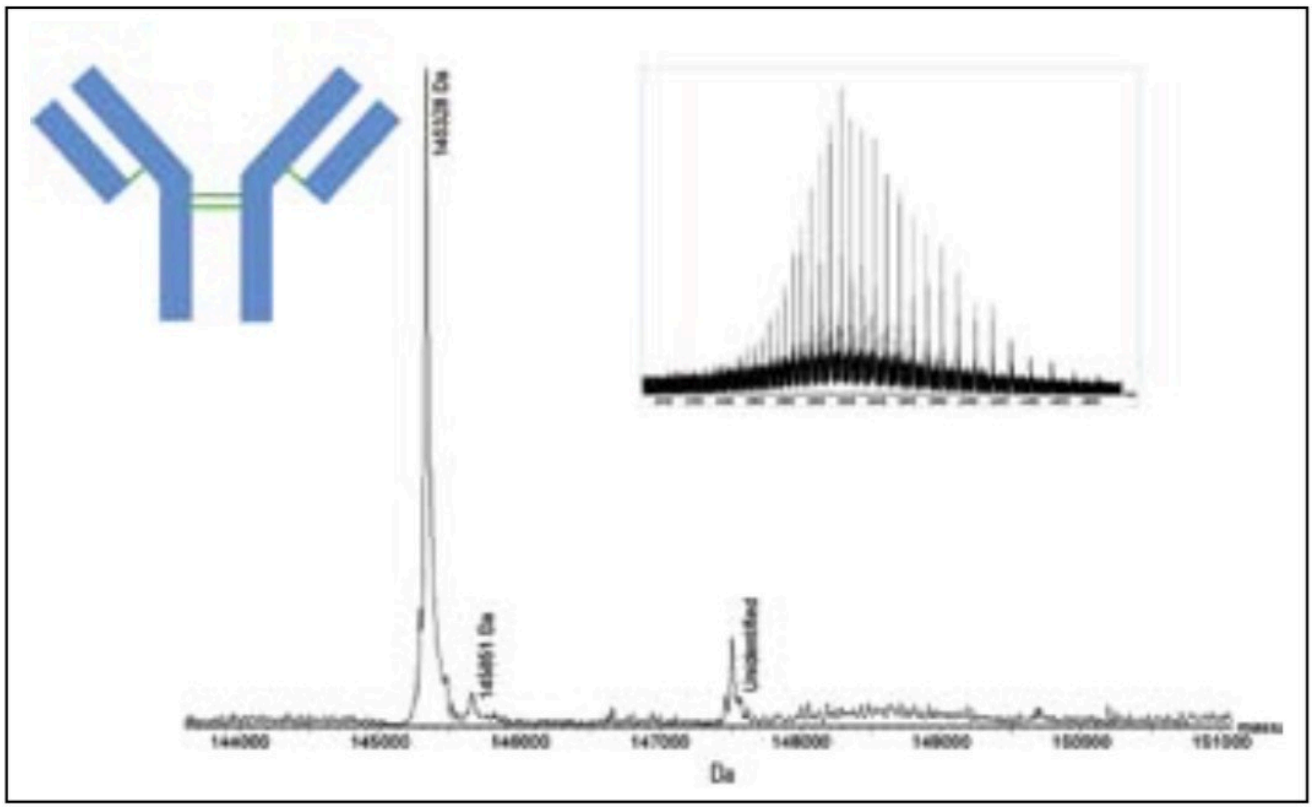

Figure 11. Combined ESI-TOF mass spectrum (inset) and deconvoluted mass spectrum of the deglycosylated IgG1 reveals a single major component with a mass of 145,328 Da.

Applying the correction factor of two Da for the N → D product of the enzymatic reaction produces a "real" mass difference of 2,888 Da or 1,444 Da per glycan chain. This mass is consistent with that arising from a common core fucosylated biantennary glycan structure (1,446 Da), as shown in Figure 1. This core structure has N-acetyl-glucosamine residues at the four nonreducing ends of the glycan branches, which are the common sites for extension by single galactose residues, as was observed to varying extent with the intact antibody.

Analysis of a Reduced and Deglycosylated IgG1

LC-MS data for the reduced and deglycosylated IgG1 was acquired under the same conditions as for the reduced IgG1, with partial resolution achieved between heavy and light chains using the extended gradient (Figure 12). The mass of the earlier eluting light chain (Figure 13) was determined to be 24,199 Da, in exact agreement with the mass determined for the reduced IgG without PNGase F processing.

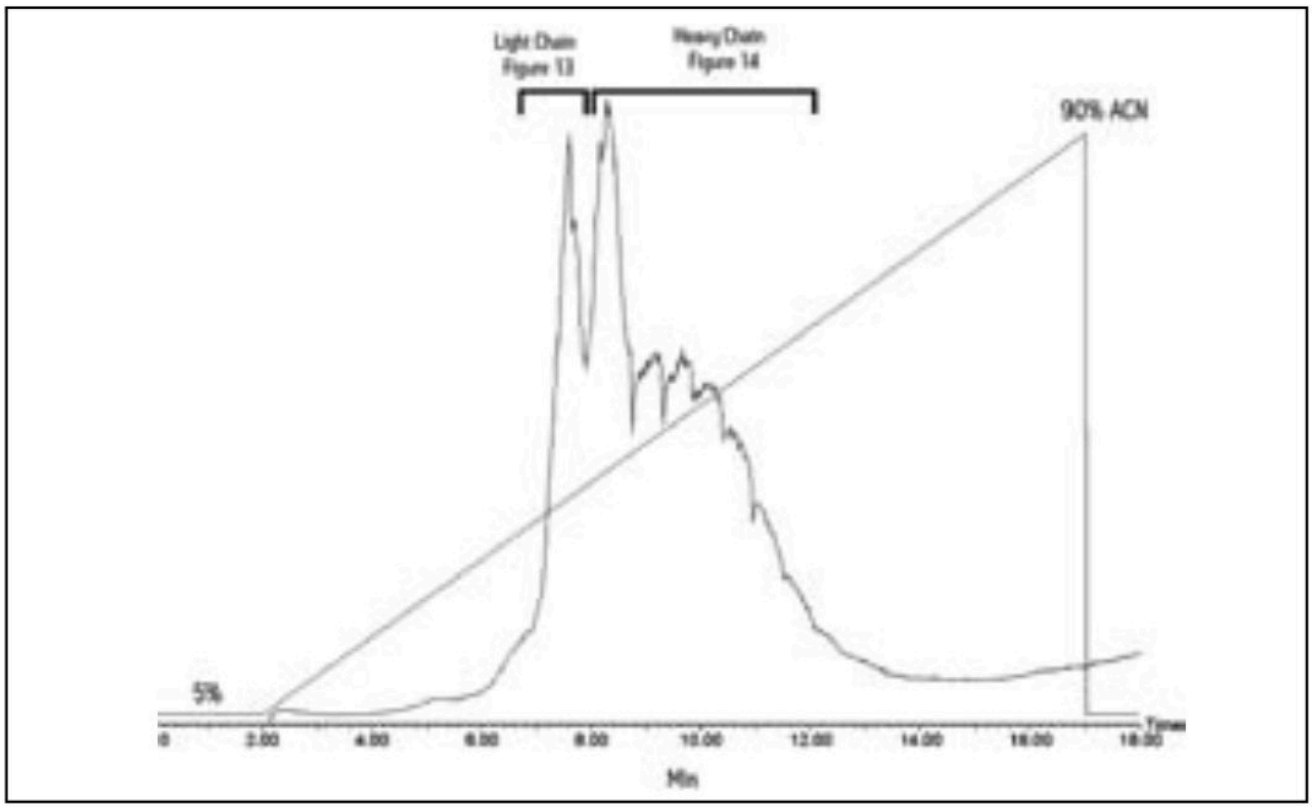

Figure 12. LC-MS TIC chromatogram of deglycosylated LC and HC generated from PNGase F deglycosylated IgG1 in the presence of 20 mM DTT. Partial separation of LC and HC was achieved using a 15 min (5 to 90%) gradient of acetonitrile.

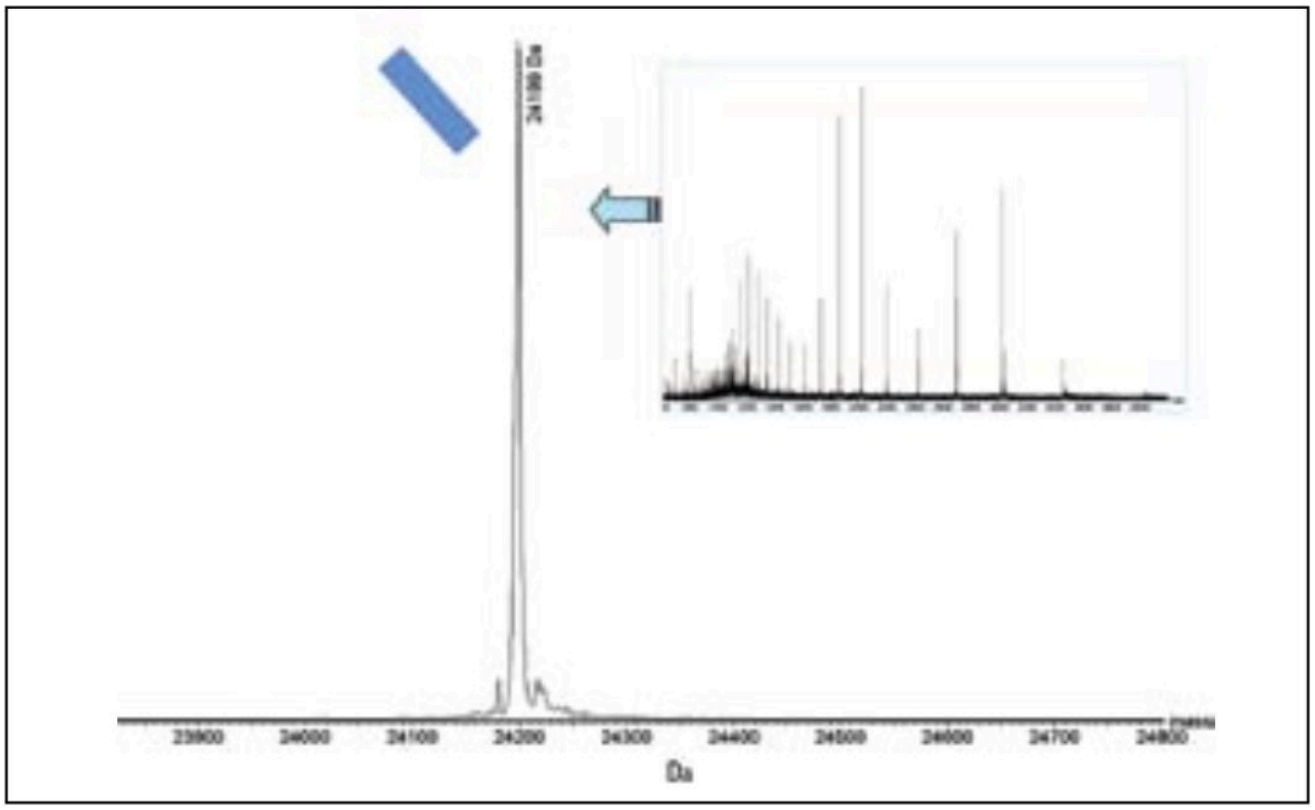

Figure 13. Combined (inset) and deconvoluted mass spectrum of the light chain (24,199 Da) derived from reduced deglycosylated IgG1. As expected, this mass corresponds to the mass of the light chain from the unprocessed antibody.

As would be expected, the later eluting heavy chain (Figure 14) is simplified to a single major species (48,476 Da) following deglycosylation. The mass difference between glycosylated (49,922 Da) and deglycosylated heavy chain was 1,447 Da, also accounting for the +1 Da N → D transformation of the glycosylation site. This measurement is once again consistent with an N-linked biantennary fucosylated carbohydrate core structure (1,446 Da) commonly found in the Fc region of IgG1 heavy chain.

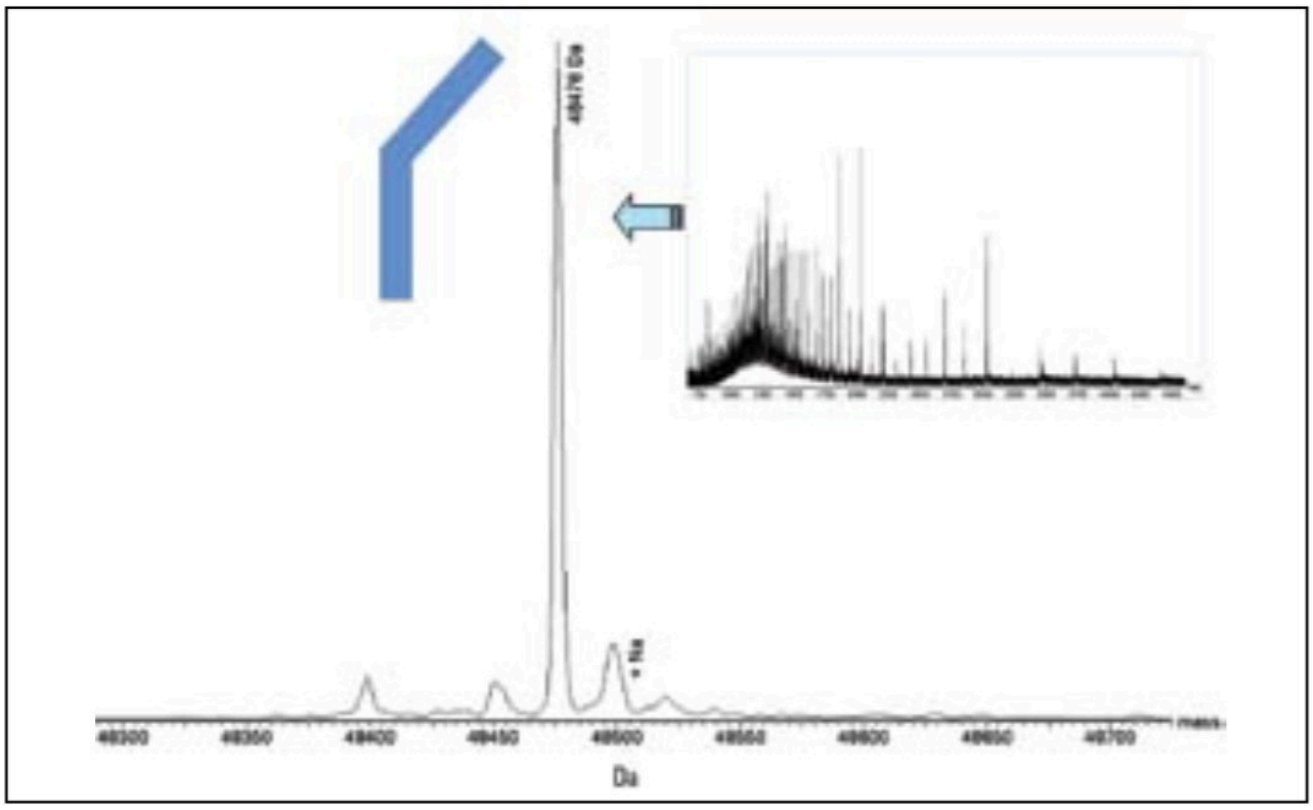

Figure 14. Combined (inset) and deconvoluted mass spectrum of the heavy chain (48,476 Da) derived from reduced deglycosylated IgG1. This mass corresponds to the mass of the heavy chain lacking the core glycan structure and related galactosylated variants.

PNGase F releases N-linked oligosaccharides as glycosylamines which are not retained by typical reversed phase columns used in these studies. The carbohydrates can be collected in the diverted flowthrough desalting fraction, and subjected to targeted methods for glycan sequencing to confirm the assigned glycan structures.

Analysis of an IgG1 Subjected to Papain Digestion

Fab fragments and other truncated antibody structures offer many potential advantages over intact antibodies as immunochemical tools, diagnostic agents and therapeutic molecules. They demonstrate improved pharmacokinetics, lower incidences of patient immunogenicity and eliminate non-specific binding interactions associated with the glycosylated Fc portion of the IgG.[4, 5]

Papain is a nonspecific, thiol-endopeptidase that preferentially cleaves antibodies in the hinge region of the

molecule. In an IgG1, the hinge region is proximal to the two disulfides that bridge the heavy chains. Depending on the location of papain cleavage within this region (above or below the disulfide bonds), processing can generate bivalent F(ab')2 fragments (~100 kDa) or univalent Fab fragments (~ 50 kDa), as well as their corresponding Fc fragments (~ 25 kDa). These papain fragment structures are illustrated in Figure 15.

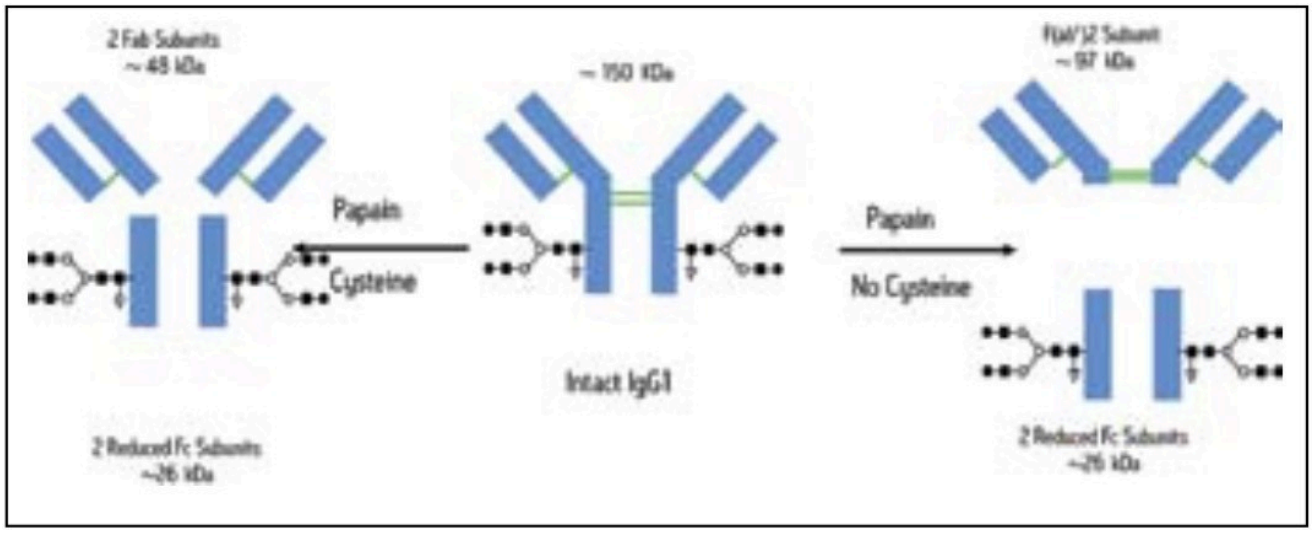

Figure 15. Papain processing of an IgG1 in the presence and absence of cysteine.

Interpreting these processing events by LC-MS can be complicated, as differential cleavage susceptibility of various IgG sub-classes and antibody source species creates some variability in processing results.[6, 7] In addition, the broad enzyme substrate specificity of papain may dictate the need for tight controls on reaction conditions (pH, time, enzyme concentration) to achieve consistently processed antibody fragments.

It has been reported[6] that the presence or absence of cysteine as well as solution pH can determine whether a Fab or F(ab')2 fragment is generated by papain. In that study, the production of F(ab')2 and Fc fragments was achieved by preactivating papain with cysteine, removal of excess cysteine following activation and subsequent digestion at pH 6.3. The TIC (Figure 16) of IgG1 digested under similar conditions reveals two peaks corresponding to these expected digestion products. This LC-MS analysis was accomplished using the more resolving LC-MS method that was previously used to analyze the reduced IgG1 sample.

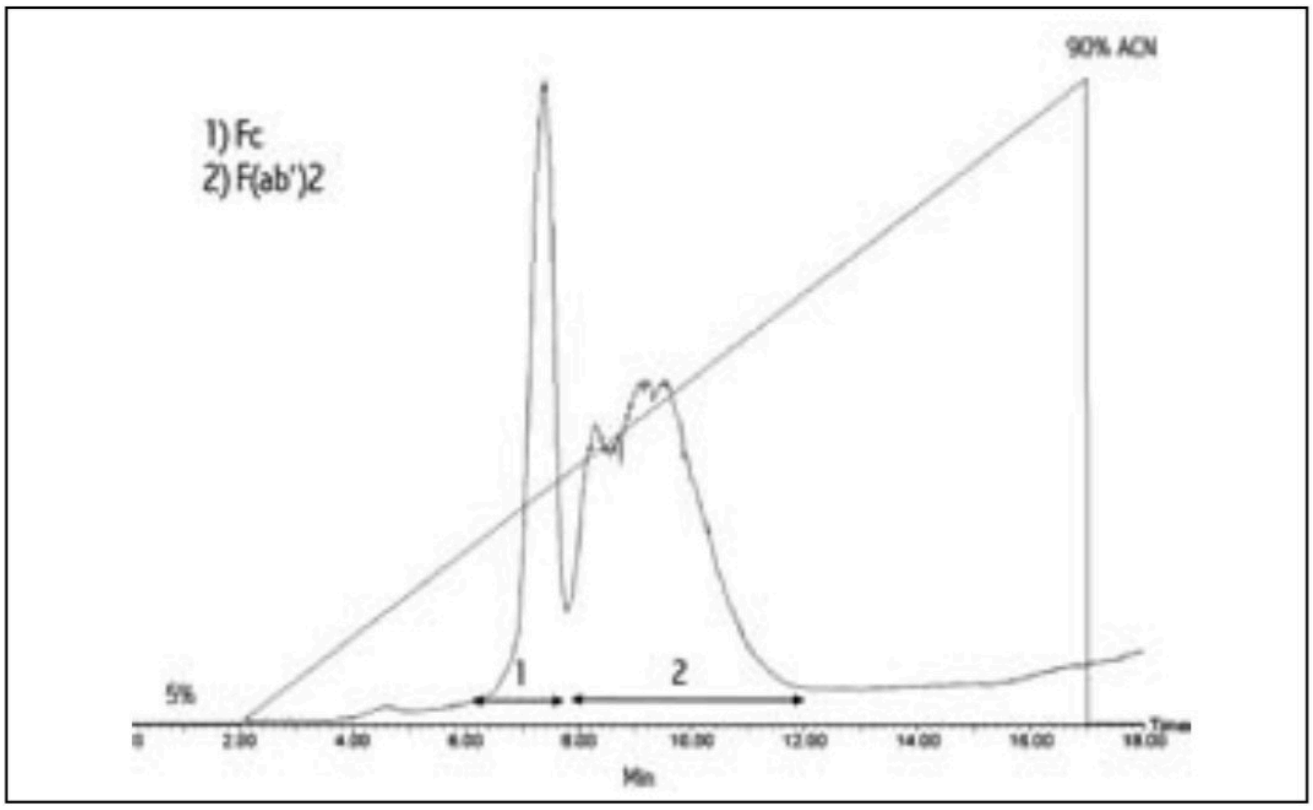

Figure 16. LC/MS TIC from the analysis of IgG1 papain digestion without cysteine in the digestion solution. Partial separation of the Fc (Peak 1) and F(ab')2 (Peak 2) fragments was achieved by using a 15 min linear gradient (5 to 90%) of acetonitrile.

The spectrum of the earlier eluting Fc fragment (Figure 17) demonstrates a wide biphasic charge state envelope (1200 to 4000 *m/z*), that reveals three major components (25,726 Da, 25,888 Da, and 26,050 Da) upon deconvolution with mass differentials of 162 Da; this corresponds to the Fc fragment with one or two galactose residues. The spectrum of the later eluting F(ab')2 fragment (Figure 18) reveals a single major component with a mass of 96,795 Da. Evidence for formation of any Fab fragments was not observed under cysteine free/pH 6.3 digestion conditions.

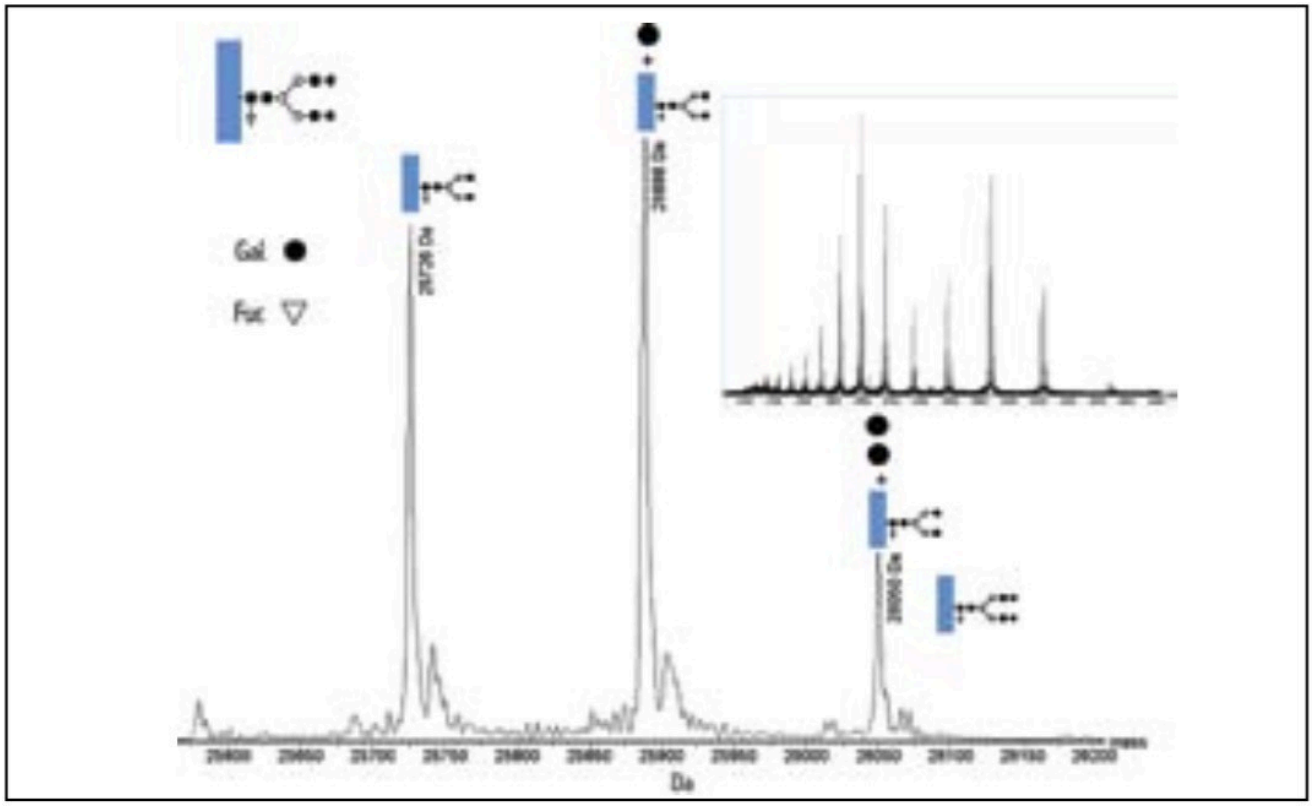

Figure 17. Combined mass spectrum (inset) of Fc fragment (peak 1) revealed a series of components upon MaxEnt1 deconvolution. Three components with a mass differences of 162 Da were observed that represented the Fc and core carbohydrate with no (25,726 Da), one (25,888 Da), or two (26,050 Da) terminal galactose residues.

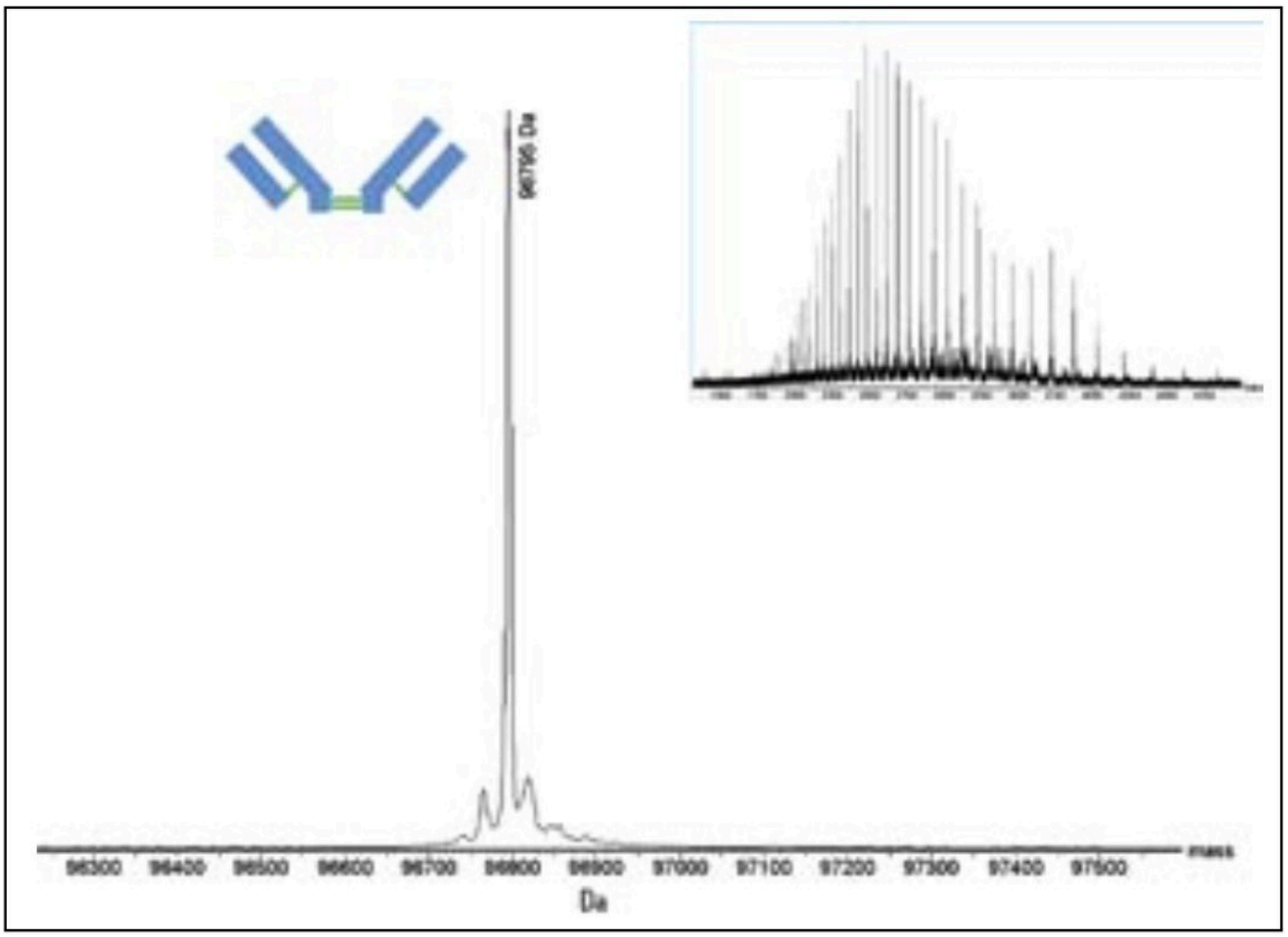

Figure 18. Combined (inset) and deconvoluted mass spectrum generated from the F(ab')2 fragment (peak 2) of the IgG1. A single major component with a mass of 96,795 Da was observed.

As with the other analyses, the masses observed correlate with the observed mass of the intact IgG1. In this case, the mass of the F(ab')2 plus two Fc (core glycan only) equals 148,247 Da, or 33 Da greater than the 148,214 Da observed for the intact IgG1. This is accounted for by the mass of two water molecules (18 Da each) added during hydrolytic cleavage of the two peptide bonds by papain.

The overnight digestion of IgG1 with papain (pH 7) in the presence of cysteine resulted in processing of the IgG1 to Fab and Fc fragments. Intermediates of this processing were observed when 2 h digestion was employed (not shown), but overnight papain digestion showed two partially resolved TIC peaks (Figure 19) that correspond to the expected products. The deconvoluted mass spectrum of the earlier eluting Fc fragment (Figure 20) again

showed three components (25,726 Da, 25,888 Da, and 26,050 Da) corresponding to the Fc fragment with one or two galactose addition variants.

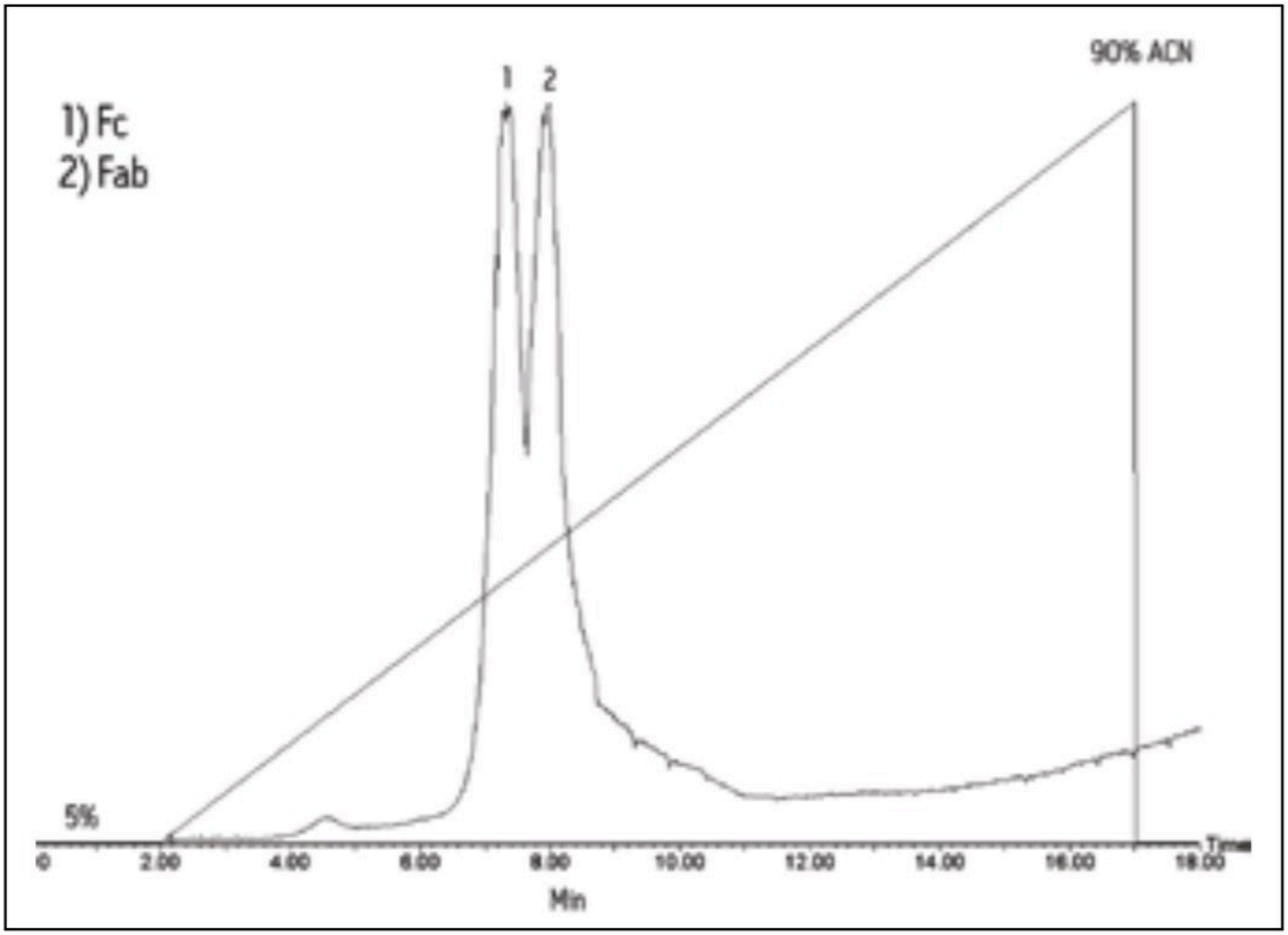

Figure 19. LC-MS TIC from the analysis of an IgG1 that was digested with papain at pH 7 in the presence of cysteine shows partial separation of the Fc (Peak 1) and Fab (Peak 2) fragments using a 15 min linear gradient (5 to 90%) of acetonitrile.

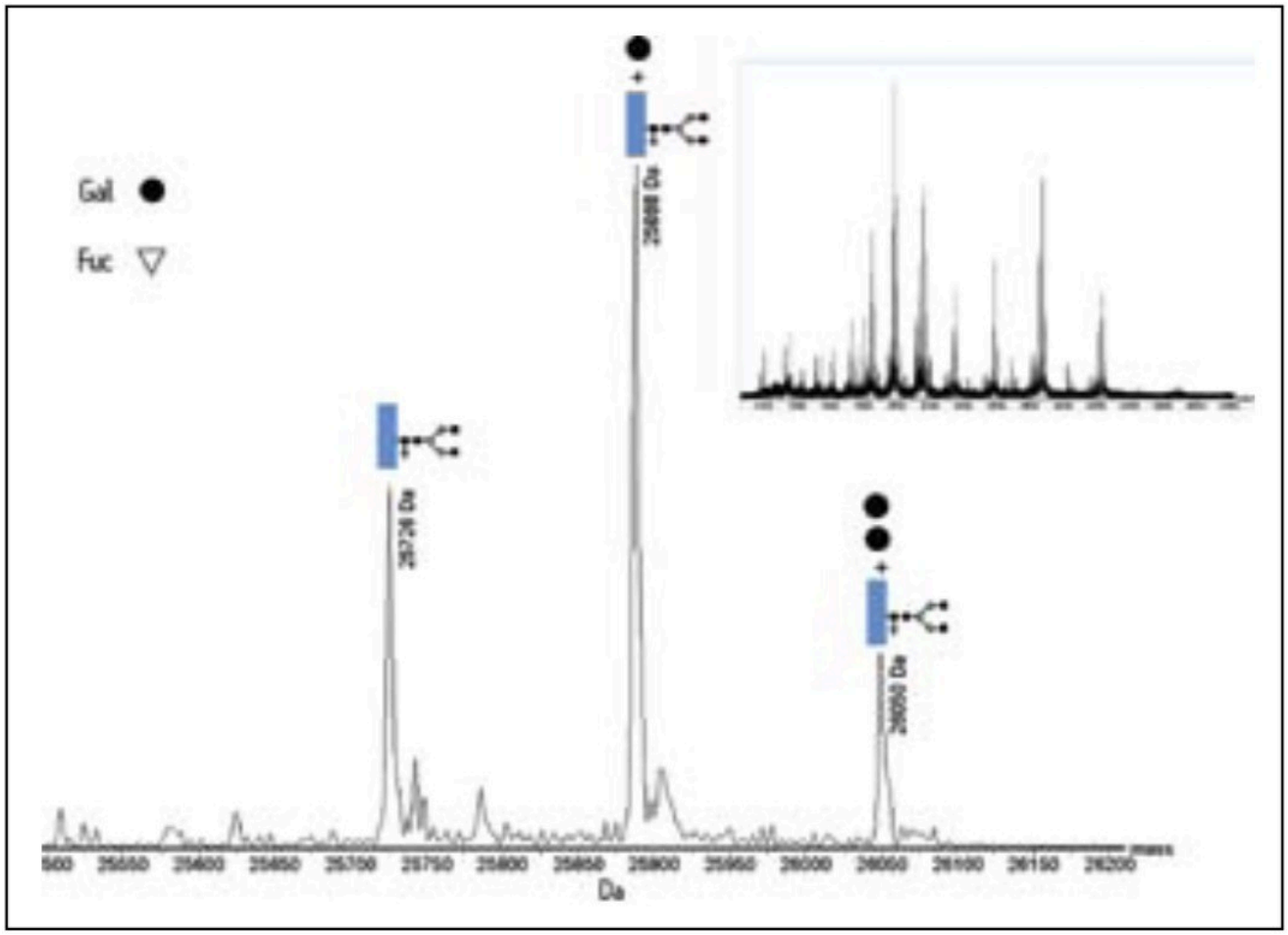

Figure 20. The combined mass spectrum (inset) of the Fc fragment generated by papain digestion in the presence of cysteine shows three components varying intensity differing by 162 Da. These correspond to the Fab fragment containing a core glycan with no (25,726 Da), one (25,888 Da), or two (26,050 Da) terminal galactose residues. This Fc fragment directly corresponds to the Fc generated using the cysteine free papain digestion conditions.

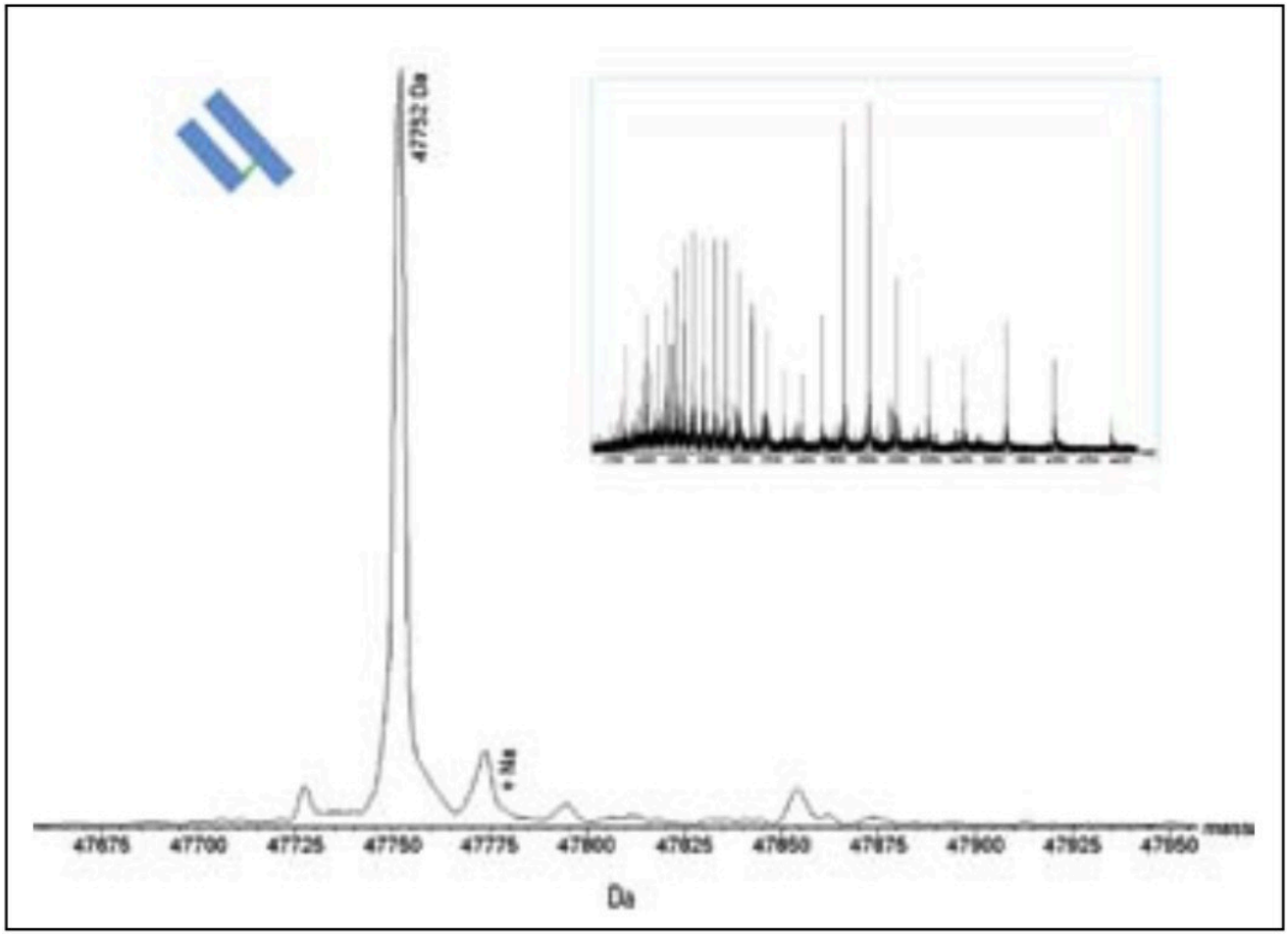

Figure 21. Combined (inset) and deconvoluted mass spectrum corresponding to the Fab fragment (peak 2) generated by papain digestion in the presence of cysteine. Complete processing of the F(ab')2 fragment to the Fab fragment (47,752 Da) was achieved under the optimized conditions.

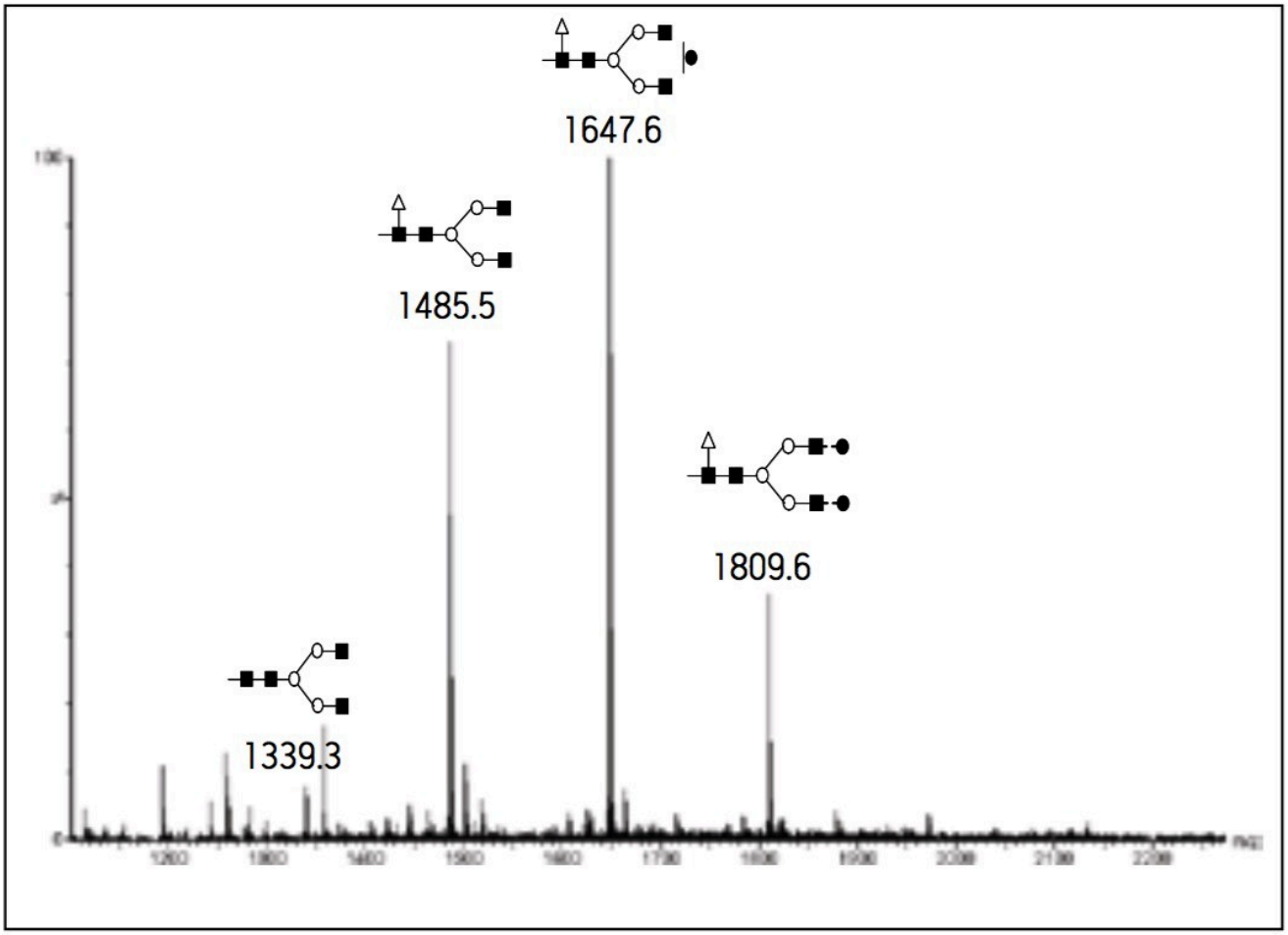

Figure 22. MALDI MS spectrum of purified glycans obtained from the intact antibody. Observed masses were consistent with the monosodiated adducts of the biantennary fucosylated glycan structures shown.

Confirmation of glycan structure

Data from the deglycosylation experiments on the intact and reduced antibody revealed PNGase F induced mass differences consistent with N-glycosylation with a fucosylated biantenerary core glycan. This result was validated by mass and tandem mass analysis of glycans released and purified from the intact antibody using the MALDI Q-Tof Premier. The MS spectrum of the purified glycans (Figure 22) revealed a pattern mirroring the glycan distribution observed with the heavy chain (Core glycan + 1 Galactose > Core glycan > Core glycan + 2 Galactose), all with masses consistent with the monosodium adducts of the expected glycan structures. The MS/MS spectrum of the sodiated core glycan (Precursor ion @ 1485.5 *m/z*) in Figure 23 provides additional high

coverage confirmation of the predicted glycan structure.

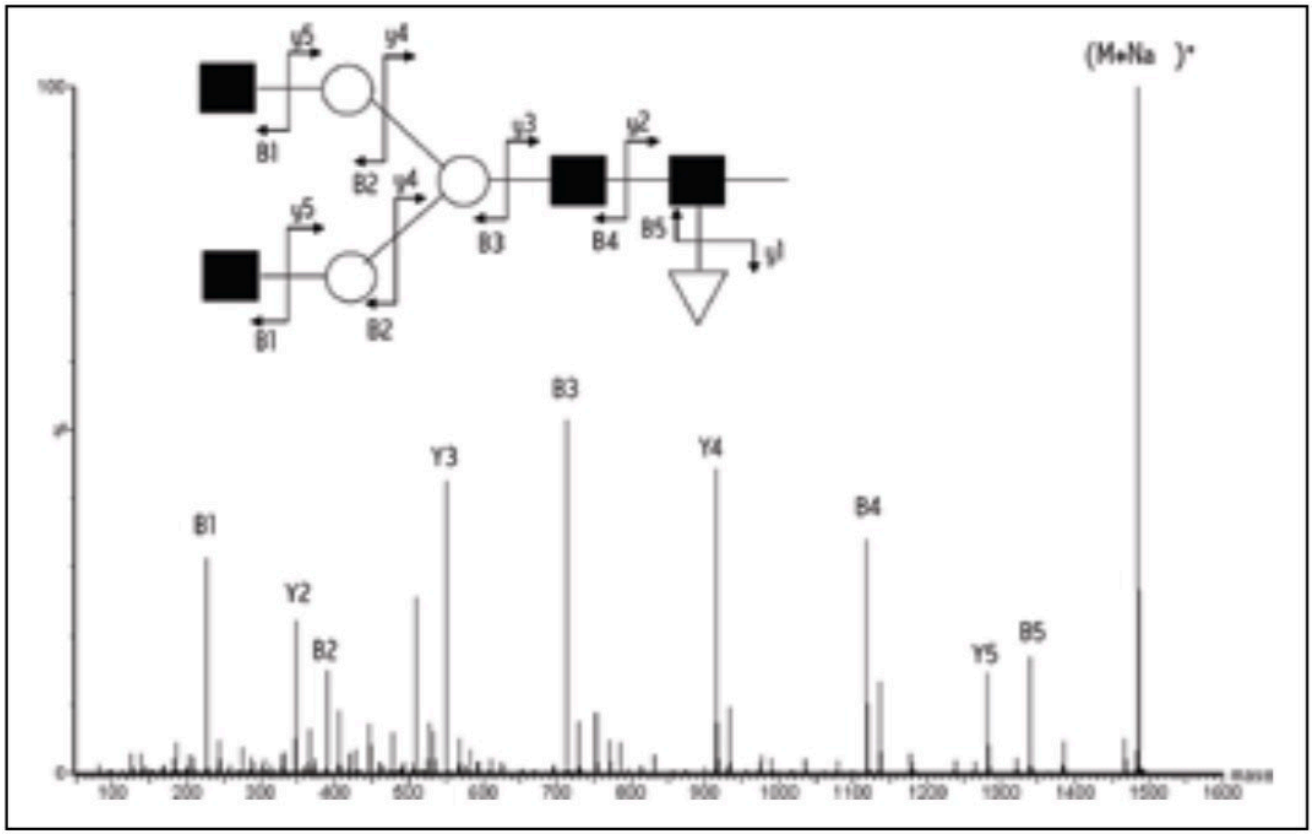

Figure 23. MALDI MS/MS of the sodiated non-glucosylated core glycan (Figure 22, Precursor ion 1485.5 m/z). High sequence coverage was obtained as detailed on the associated glycan structure.

Conclusion

In this work, we have strived to demonstrate that antibodies and a broad subset of common antibody sub-structures can be efficiently analyzed by LC-MS using a common instrument configuration and two generic separation methods.

The MassPrep Desalting Cartridge proved effective for LC-MS analysis of an IgG1 antibody and for partial resolution of the reduced (or digested) substructures of this antibody.

ESI-Tof MS analysis using the LCT Premier is capable of generating precise mass measurements (<30 ppm) over the wide range of antibody structures and substructures studied.

Using the MALDI Q-Tof Premier for MS and MS/MS analysis of released glycans can confirm the tentative assignments generated by comparing an intact antibody or substructure with the deglycosylated version.

The use of multiple antibody analysis conditions can generate a wealth of complementary - and entirely self-consistent LC-MS results.

Acknowlegements

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