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FOCUS: MASS SPECTROMETRY IN GLYCOBIOLOGY AND RELATED FIELDS: RESEARCH ARTICLE

Absolute Quantitation of Glycoforms of Two Human IgG Subclasses Using Synthetic Fc Peptides and Glycopeptides

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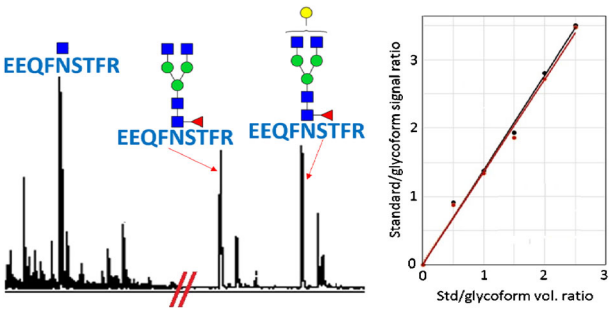

Abstract. Immunoglobulins, such as immunoglobulin G (IgG), are of prime importance in the immune system. Polyclonal human IgG comprises four subclasses, of which IgG1 and IgG2 are the most abundant in healthy individuals. In an effort to develop an absolute MALDI-ToF-MS quantitative method for these subclasses and their Fc *N*-glycoforms, (glyco)peptides were synthesized using a solid-phase approach and used as internal standards. Tryptic digest glycopeptides from monoclonal IgG1 and IgG2 samples were first quantified using EEQYN(GlcNAc)STYR and EEQFN(GlcNAc)STFR standards, respectively. For IgG1, a similar glycopeptide where tyrosine (Y) was isotopically labelled was used to quantify monoclonal IgG1 that had been treated with the enzyme Endo-F2, i.e., yielding tryptic glycopeptide EEQYN(GlcNAc)STYR. The next step was to quantify single subclasses within polyclonal human IgG samples. Although ion abundances in the MALDI spectra often showed higher signals for IgG2 than IgG1, depending on the spotting solvent used, determination of amounts using the newly developed quantitative method allowed to obtain accurate concentrations where IgG1 species were predominant. It was observed that simultaneous analysis of IgG1 and IgG2 yielded non-quantitative results and that more success was obtained when subclasses were quantified one by one. More experiments served to assess the respective extraction and ionization efficiencies of EEQYNSTYR/EEQFNSTFR and EEQYN(GlcNAc)STYR/EEQFN(GlcNAc)STFR mixtures under different solvent and concentration conditions.

Keywords: Immunoglobulins, Glycopeptides, MALDI-MS, Quantitative analysis, Internal standard, Glycoproteomics, MALDI-ToF-MS, Absolute quantitation, IgG tryptic digests

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Introduction

One of the most abundant proteins in human serum is immunoglobulin G (IgG). Healthy adult serum contains ca. 76% of IgG in the total immunoglobulin protein composition (with other classes IgA, IgE, IgD, IgM being present) [1]. Back in 1964, heavy-chain subclasses of human IgG were identified [2, 3]. IgG is therefore not a single type of molecule

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but rather a class of immunoglobulins, which are divided in four subclasses (IgG1 to IgG4). These are named according to their decreasing average natural abundances, IgG1 being the most abundant and IgG4, the least [4, 5]. In healthy individuals, the relative proportions of each subclass have been found in relatively narrow ranges. There is 60–71% of IgG1, 19–31% of IgG2, 5–8% of IgG3, and 1–4% of IgG4 [1]. These ranges vary slightly in other reports [6–8]. Human IgG subclasses have more than 90% amino acid sequence homology. However, each subclass has different properties such as their antigen binding ability, immune complexes formation, complement activation, half-life, effector cell activation, and transport into the placenta [5].

During immune response, soluble protein antigens and membrane protein antigens induce mostly IgG1, although low quantities of the other subclasses are also involved [9]. Some conditions characterized by antibody deficiencies may occur, for instance, low levels of IgG1, which are linked to the recurrence of infections [6]. On the other hand, the serum of patients afflicted with monoclonal gammopathy of unknown significance (MGUS) has been shown to contain high levels of IgG1 [10]. In either instance, it is important to enable the rapid monitoring of subclass proportion changes. Traditionally, IgG subclass levels have been determined by specific immunoassay methods involving monoclonal antibodies [1]. With the availability of mass spectrometry (MS) and ionization methods adapted to the analysis of proteins, it is useful and practical to propose the development of a quantitative method for human IgG subclasses. The specificity of mass spectrometry (MS) allows amino acid sequencing as well as convenient *N*-glycosylation analysis of the gamma chains of particular subclasses. There have been reports of quantitative analysis of IgG subclasses by selecting subclass specific tryptic peptides, in which liquid-chromatography/electrospray ionization (ESI)-MS/multiple reaction monitoring (LC-MS-MRM) was utilized to detect key peptide and glycopeptide fragments [11, 12]. The present report relies on matrix-assisted laser ionization/desorption (MALDI) MS as the main detection method.

All four human IgG subclasses have a conserved *N*-glycosylation site at asparagine-297 of the heavy chains, and the antigen binding domains (Fab) of IgGs are also glycosylated in about 10–20% of the cases [5]. Human IgG *N*-glycosylation has been documented extensively, as described in recent review articles, e.g., [13]. The *N*-glycans have a common core structure composed of $(GlcNAc)_2(Man)_3$, where GlcNAc = *N*-acetylglucosamine and Man = mannose. This core is usually extended by the presence of more GlcNac residues, fucose (Fuc), galactose (Gal), and *N*-acetylneuraminic acid (Neu5Ac). Healthy serum IgGs have been found to contain several glycoforms, with the dominant species being biantennary composed of one Fuc and 0, 1, or 2 Gal residues and sometimes a Neu5Ac group [14, 15].

The characterization of IgG *N*-glycosylation by MS has for long been conducted using glycomic approaches, where *N*-glycans are detached with an enzyme such as peptide *N*-glycosidase F (PNGase F), labelled and analyzed separately from the polypeptide chain [16, 17]. The profiling of released *N*-glycans can be complemented or replaced by a glycoproteomic approach, where IgGs are digested with a proteolytic enzyme (most often trypsin), and *N*-glycopeptides are characterized. This allows accurate determination of glycosylation sites and glycan compositions [14, 15, 18].

In glycoproteomic studies of human IgG, it is possible to take advantage of the different amino acid sequences of the heavy chain's tryptic *N*-glycopeptides EEQYNSTYR (IgG1), EEQFNSTFR (IgG2), EEQYNSTFR (IgG3), and EEQFNSTYR (IgG4) [18], although the two latter are isobaric. It is thus possible to elaborate a quantitative method for IgG1 and IgG2 based on synthetic glycopeptides with their respective sequences. It is well established that similar size peptides with different amino acid compositions or sequences yield different MS detection responses. Therefore, it is important that the amount of each different IgG *N*-glycopeptide be determined independently based on a synthetic peptide of same amino sequence.

A quantitative and comparative study of this type was conducted by Stavenhagen and co-workers [19]. Several versions/variations of a tryptic *N*-glycopeptide and non-glycosylated peptide from human protein C (EVFVHPNYSK) were synthesized in order to compare signal intensities with different ionization methods: ESI and MALDI on a range of analyzers: ion trap, quadrupole, time-of-flight (ToF), and Fourier-transform ion cyclotron resonance (FT-ICR). They observed that glycopeptide MALDI signals tended to be lower than non-glycosylated peptides. The bias varied according to the technique and instrument used [19]. These observations emphasize the need for a rigorous calibration method when different peptide backbones are to be investigated and compared on a specific instrument. Many quantitative methods developed for glycopeptides are relative, i.e., they allow the comparison of structures with the same amino acid backbone between two or more samples (duplex to multiplex) [20, 21].

There is not a large variety of glycopeptides available commercially that can be used as internal standards for MS or other techniques. Custom synthesis is thus required. Specifically for human IgG subclasses IgG1 and IgG2, it is potentially very convenient to use some EEQYNSTYR and EEQFNSTFR (glycosylated and non-glycosylated) standards for absolute quantitation. While these two may be differentiated by mass, IgG3 (EEQYNSTFR) and IgG4 (EEQFNSTYR) are isobaric. Although IgG3 and IgG4 can be separated on protein A or protein G columns [18], it may be much simpler to find or make labelled standards corresponding to other tryptic peptides unique to these two subclasses. Examples are CPEPK for IgG3 (UniprotKB entry P01860) and GLPSSIEK for IgG4 (P01861) (www.uniprotkb.com).

The present report describes the development of an absolute quantitative method for human IgG1 and IgG2, the two most abundant forms of IgG in healthy individuals [5]. This method utilizes synthetic EEQYNSTYR and EEQFNSTFR (glyco)peptides as internal standards in

mixtures of glycopeptides enriched from human IgG tryptic digest mixtures for analysis by MALDI-ToF-MS without MS/MS or MRM. The main topics explored herein are as follows: absolute quantitation of mAbs IgG1 and IgG2 when present as sole species, absolute quantitation of IgG1 and IgG2 independently in polyclonal human IgG, and possibility of quantification of IgG1 and IgG2 when mixed, one by one or all in one pot. The different sensitivities of the two respective tryptic peptides under MALDI conditions will then be discussed.

Experimental

Materials

All 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, with either an *O-tert*-butyl (OtBu), trityl (Trt), or *tert*-butyl (tBu) side-chain protecting groups, were obtained from Bachem (Torrance, CA). Wang resin, to which N_{α}-9-fluorenylmethoxycarbonyl-N_{ω}-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl)-L-arginine was already immobilized, was also purchased from Bachem (Torrance, CA). The resin had an average particle size of 100–200 mesh and a loading capacity range of 0.4–0.8 mmol/g. L-Asparagine containing a peracetylated *N*-acetylglucosamine (GlcNAc) attached to its side chain was used to synthesized the glycopeptide, and therefore, Fmoc-L-Asn(GlcNAc(Ac)$_3$-β-OH) was purchased from Carbosynth (San Diego, CA). Isotopically labelled Fmoc-Tyr(tBu)-OH containing 9× ^{13}C and 1× ^{15}N was obtained from Cambridge Isotopes Laboratories (Andover, MA, USA). Mass spectrometry-grade Trypsin Gold™ was purchased from Promega (Madison, WI, USA). Reverse-phase C18 solid-phase extraction (SPE) cartridges (3 mL, 100 mg) were obtained from Biotage (Uppsala, Sweden). Starta™ × 33 μm polymeric reverse-phase C18, (3 mL, 60 mg) cartridges acquired from Phenomenex (Torrance, CA) were also used.

Polyclonal human IgG was purchased from Sigma, and the Herceptin mAb, modified with enzyme endoglycosidase-F (Her2FS, MW 150 kDa), was obtained by cell culture [22] from J. Rini's laboratory at the University of Toronto. A fully glycosylated hybrid human-camelid mAb sample (Eg2hFc, MW 80 kDa) was obtained from M. Butler's laboratory (University of Manitoba) [23, 24].

N,N-dimethylformamide (DMF), dichloromethane (DCM), *N,N*-diisopropylethylamine (DIPEA), *N*-methylmorpholine, piperidine, *p*-chloranil (tetrachloro-1,4-benzoquinone), trifluoroacetic acid (TFA), potassium carbonate (K_2CO_3), 30% ammonium hydroxide solution, and 2,5-dihydroxybenzoic acid (DHB) were all purchased from Sigma (St. Louis, MO). The peptide coupling reagent *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (TBTU) was purchased from AK Scientific Inc. (Union City, CA). HPLC-grade methanol, acetonitrile, and acetic acid were obtained from Fisher Scientific (Nepean, ON). Acetic acid was also purchased from Fisher Scientific. Milli-Q water was used for all experiments. Solvent evaporation was completed using a Savant SC110 Speed Vac (San Diego, CA), equipped with a Welch Duoseal Vacuum Pump (Prospect, IL).

SPPS of Peptides and Glycopeptides on Wang Resin

Synthesis of EEQYNSTYR and EEQFNSTFR

These peptides, which represent the backbone of tryptic *N*-glycopeptides of the human IgG1 Fc portion, were prepared following a standard SPPS protocol based on Fmoc protecting group strategy on Wang resin [25–27]. The synthesis proceeded in a linear fashion from the *C*- to the *N*-terminus. Initially, 150 mg of Wang resin (with Fmoc-protected arginine attached) was swelled in DMF for 10–15 min. The Fmoc group was removed under basic conditions (4:1 DMF/piperidine). The Fmoc-protected amino acid was then coupled to the free amine using TBTU and *N*-methylmorpholine in DMF. A repeating cycle of deprotection and coupling yielded the desired peptide fixed on the solid support. To ensure reaction completion at each step, a Chloranil test was performed by transferring a small amount of resin to an Eppendorf tube and adding *p*-chloranil (2% *w/v*) in DMF. A stained bead (red or blue) indicated the presence of free amine.

Once the peptide sequence completed, the resin was subjected to 95:5 TFA/H_2O for 2 h 45 min to cleave the peptide from the solid support and remove side-chain protecting groups. The peptide solution was carefully neutralized using ammonium hydroxide followed by drying in vacuo. The dried peptides were stored at − 20 °C.

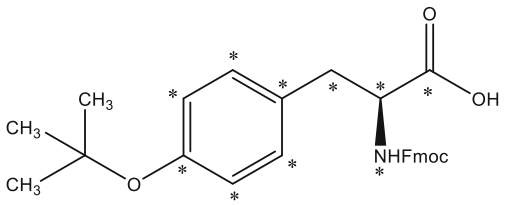
For cleanup and desalting, C18 SPE cartridges were conditioned with 5× (1 mL ACN + 0.1% TFA), then with 5× (1 mL of water + 0.1% of TFA). The peptide crude mixture was loaded and rinsed with 3× (1 mL of water). The desired peptides were eluted with a manual gradient of ACN:H_2O + 0.1% TFA (ACN/H_2O proportions varied from 10:90 to 30:70). Fractions were collected and concentrated by centrifugal Speedvac™ evaporation. Samples were resuspended in 4 μL of 30:70 ACN/H_2O + 0.1% TFA (TA 30) in preparation for MALDI-ToF-MS analysis.

Synthesis of EEQYN(GlcNAc)STYR and EEQFN(GlcNAc)STFR

The same protocol was followed, and Fmoc-L-Asn(β-D-GlcNAc(Ac)$_3$)OH was used instead of Fmoc-L-Asn(Trt)-OH. For the removal of acetyl groups, the glycopeptide dry powder was first dissolved in methanol and potassium carbonate in solution (30%) was added at room temperature. The solution was allowed to stir vigorously overnight, and then it was neutralized using acetic acid. The solvent was then evaporated in vacuo. C18 cartridge cleanup was used as described above.

*Synthesis of Isotopically L Glycopeptide EEQYN(GlcNAc)STY*R*

The same procedure was performed, except that Fmoc-Tyr(tBu)-OH was replaced by the isotopically-labelled reagent shown in Scheme 1.



Scheme 1. Structure of labelled Fmoc-Tyr(tBu)-OH, where each asterisk denotes a ^{13}C or a ^{15}N

Tryptic Digestions of Antibodies

Antibodies (endo-F treated mAb, Her2FS (22 µg), fully glycosylated Eg2-hFc (400 µg), polyclonal human IgG from Sigma (100 µg), and human IgG from healthy donors tested during a MGUS study [28] were reconstituted in 50 mM ammonium bicarbonate (pH 7.8). The samples were then digested by Trypsin Gold™ in a 20:1 *w/w* antibody to trypsin ratio. Tryptic digestion was allowed to take place at 37 °C for 18–20 h. Digestion was stopped by freezing the sample mixtures at − 20 °C for 30 min. The samples were then either cleaned using a C18 reverse-phase cartridge using the procedure described above or treated with Proteoglycan™ glycopeptide enrichment kits (EMD-Millipore, Etobicoke, ON) according to the manufacturer's protocol. Glycopeptides fractions were dried and reconstituted in 4 µL of 30:70 acetonitrile-water with 0.1% TFA (TA30) for quantitative analysis.

Quantitative Analysis

A series of standard addition experiments were performed as listed in Table 1. For all experiments, four or five spots of saturated DHB in TA30 (0.5 µL) were deposited onto a MALDI target, and on top of each, 0.5 µL of the sample solution (in TA30) was added. Then, increasing volumes of the standard solutions (in 0.1% TFA in water) were added to the spots as specified in Table 1. Samples were allowed to dry for MALDI-ToF-MS analysis. The standard addition experiments were divided in three different categories:

- Absolute quantitation of IgG1 in mAbs and polyclonal human IgG.
- Absolute quantitation of IgG2 in mAbs and polyclonal human IgG.
- Simultaneous quantitation of IgG1 and IgG2 in polyclonal human IgG.

Mass Spectrometric Analysis

An UltraFleXtreme MALDI-TOF-MS instrument (Bruker Inc., Billerica, MA) was used for most MS experiments, using a N_2 laser system (337 nm) with adjustable power. Samples were analyzed in the positive reflector mode. The instrument was

Table 1. Summary of Standard Addition Experiments for the Quantitative Analysis of Glycopeptides and Peptides Specific to Antibodies

Figure no.	Standard (concentration)	Analyte (conc. or est'd. amount digested)	Est'd total glycopeptide conc.[a]	Vol. used for standard additions (µL)	Glycopeptide concentration measured[b]
1a	EEQYN(GlcNAc)STY*R (22 mM)	EEQYN(GlcNAc)STYR (23 mM)	23 mM	0.15, 0.25, 0.50, 0.75, 1.00	23 mM
1b	EEQYN(GlcNAc)STYR (1.32 µM)	EEQYN(GlcNAc)STY*R (1.31 µM)	1.32 µM	0.25, 0.50, 0.75, 1.00, 1.25	1.32 µM
1c	EEQYN(GlcNAc)STY*R (3.32 mM)	Her2FS glycopeptides (~22 µg)	5.9 mM	0.15, 0.50, 0.75, 1.00	7.8 mM
1d	EEQYN(GlcNAc)STY*R (0.38 µM)	Her2FS glycopeptides (~2.5 ng)	0.67 µM	0.50, 0.75, 1.00, 1.25	0.31 µM
2a	EEQYN(GlcNAc)STYR (5.75 mM)	Eg2hFc glycopeptides (~400 µg)	20 mM	0.15, 0.25, 0.75, 1.00	12 mM
2b	EEQYN(GlcNAc)STYR (0.079 mM)	hIgG IgG1 glycopeptides (~100 µg)	2.66 mM	0.25, 0.50, 0.75, 1.00, 1.25	0.58 mM (+0.17 mM)
S3a	EEQYNSTYR (8.41 mM)	Eg2hFc glycopeptides (~400 µg)	20 mM	0.25, 0.50, 1.00, 1.25	127 mM
S3b	EEQYNSTYR (1.05 mM)	hIgG IgG1 glycopeptides (~100 µg)	2.66 mM	0.5, 1.0, 2.0, 2.5	1.6 mM (+3.6 mM)
4a	EEQYN(GlcNAc)STYR (875 µM)	EEQYNSTYR (841 µM)	N/A	0.25, 0.5, 0.75, 1.0, 1.25	N/A
4b	EEQYN(GlcNAc)STYR (101 mM)	EEQYNSTYR (100 mM)	N/A	0.25, 0.5, 0.75, 1.0, 1.25	N/A
5a	EEQFN(GlcNAc)STFR (0.053 mM)	IgG2 glycopeptide Sample 1	Unknown	0.5, 0.75, 1.0, 1.25	0.11 mM
5b	EEQFN(GlcNAc)STFR (0.101 mM)	hIgG IgG2 glycopeptides (~100 µg)	2.66 mM	0.25, 0.5, 0.75, 1.0, 1.25	0.17 mM (+0.58 mM)
S4a	EEQFNSTFR (1.01 mM)	IgG2 glycopeptide Sample 2	Unknown	0.25, 0.5, 0.75, 1.0, 1.25	0.55 mM
S4b	EEQFNSTFR (1.07 mM)	hIgG IgG2 glycopeptides (~100 µg)	2.66 mM	0.25, 0.5, 0.75, 1.0, 1.25	3.6 mM (+1.6 mM)
7	EEQYN(GlcNAc)STYR (0.984 µM) EEQFN(GlcNAc)STFR (0.0632 µM)	hIgG IgG1 and IgG2 glycopeptides (~100 µg)	14 µM	0.25, 0.5, 0.75, 1.25	1.5 µM

1b: In this case, additions of unknown were made to a constant amount of standard
N/A not available
[a]Concentration estimated from sample provider before analysis from amount of Ab digested, if known
[b]Total glycopeptide concentrations measured; for more detail on each glycoform, see Tables 2 and 3. Numbers in brackets (column 6) refer to the other subclass concentration for polyclonal human IgG

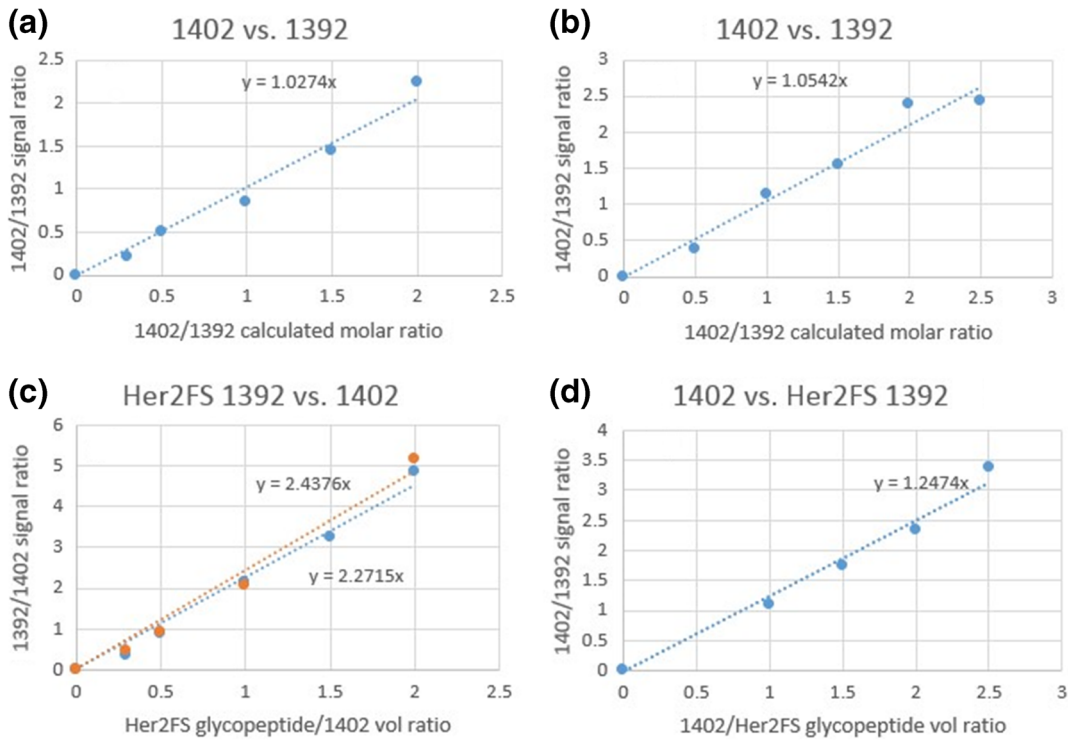
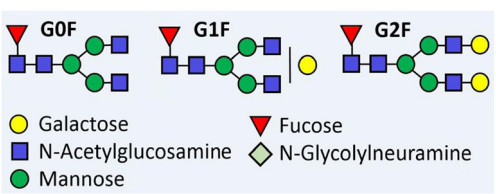


Figure 1. (**a**) Additions of incremental volumes of EEQYN(GlcNAc)STY*R (1402, 22 mM) to a fixed amount of EEQYN(GlcNAc)STYR (1392, 0.5 µL of 23 mM solution) on the MALDI target and measurements of relative areas of $[M+H]^+$ peaks. (**b**) Same, with lower concentrations (1402 1.31 µM, 1392 1.32 µM). (**c**) Additions of increasing volumes of a solution of EEQYN(GlcNAc)STYR from HeR2SF mAb onto constant volumes (0.5 µL) of 1402 at 3.32 mM. (**d**) Additions of increasing volumes of 1402 standard at 0.38 µM to constant volumes (0.5 µL) of EEQYN(GlcNAc)STYR from HER2SF mAb

equipped with LID-LIFT™ technology for tandem MS experiments. DHB (20 mg/mL in TA30, 0.5 µL) was first spotted onto the MALDI target and allowed to dry. The samples in TA30 were applied directly onto the matrix (0.5 µL or otherwise specified). A standard calibration mixture composed of eight well-characterized peptides with masses ranging from 700 to 5000 Da was used (American Peptide Company, Vista, CA). ESI-MS experiments were performed on a TripleTOF5600 mass spectrometer (Sciex, Concord, ON). MS spectra were acquired each at 250 ms on a *m/z* range of 370–1500 as described elsewhere [29].

Results and Discussion

Internal standard (glyco)peptides were synthesized: EEQYNSTYR, EEQYN(GlcNAc)STYR, EEQYN(GlcNAc)STY*R, EEQFNSTFR, and EEQFN(GlcNAc)STFR (* = isotopically labelled). The first and second compounds were applied to the quantitation of IgG1 mAb and human IgG glycopeptides forms G0F, G1F, and G2F. EEQYN(GlcNAc)STY*R was used for the analysis of EEQYN(GlcNAc)STYR in monoclonal antibody (mAb) Herceptin samples that had been treated with endoglycosidase F (http://www.sigmaaldrich.com/technical-documents/articles/biology/glycobiology/endoglycosidases.html), HeR2SF. As for EEQFNSTFR and EEQFN(GlcNAc)STFR standards, they were spiked in digested samples of human IgG containing only IgG2 and in glycopeptides samples from polyclonal human IgG. Lastly, IgG1 and IgG2 peptide glycoforms were analyzed as mixtures and quantitation was attempted. It was assumed that for all antibodies the Fc portion was glycosylated on asparagine-297 at 100%.



Scheme 2. The three most abundant glycoforms of EEQYNSTYR (IgG1) and EEQFNSTYR (IgG2). The glycans are attached at the N site at the reducing end of fucosylated *N*-acetylglucosamine

Absolute Quantitation of IgG1 Abs

In the first section of the discussion, it will be demonstrated that for monoclonal antibody (mAb) samples, it is possible to use synthetic EEQYN(GlcNAc)STYR and EEQYNSTYR as internal standards for the quantitation of IgG1, the former being more reliable than the latter.

The first experiment was to ensure that synthetic EEQYN(GlcNAc)STYR ($[M+H]^+$ ions at *m/z* 1392) and EEQYN(GlcNAc)STY*R (*m/z* 1402) deposited in mM-range concentrations yielded equivalent signals in MALDI-MS.

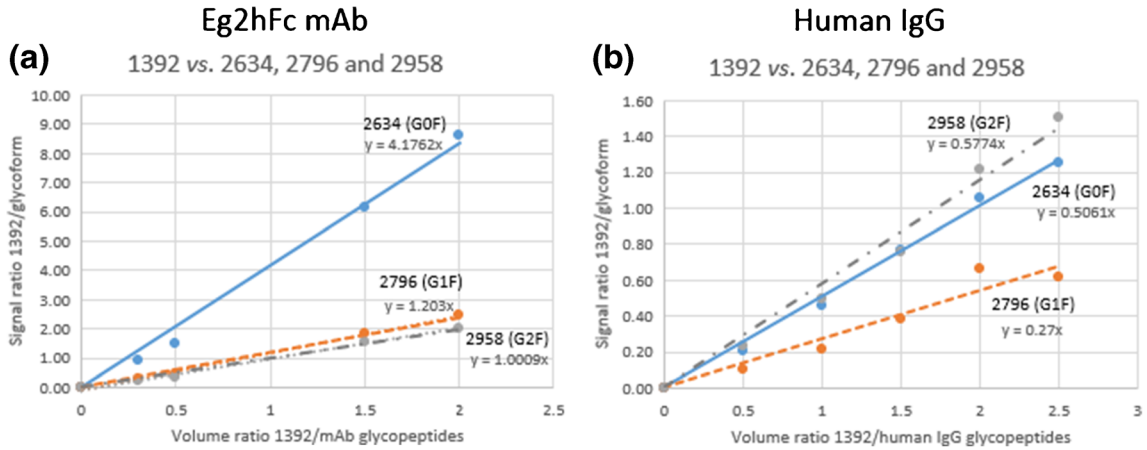


Figure 2. Use of glycopeptide EEQYN(GlcNAc)STYR (1392) as internal standard for the dosage of solutions of tryptic glycopeptides from (**a**) Eg2hFc mAb and (**b**) human IgG. Concentration of standard: 5.75 mM for the mAb and 79.0 μM for human IgG. Results are reported in Table 2

Increasing amounts of the labelled standard were added to a fixed amount of the non-labelled glycopeptide (Fig. 1a). The slope is close to 1, showing that both compounds yield equivalent signals for $[M+H]^+$ ions. In Fig. 1b, standard additions were effected over a lower μM concentration range (see Table 1). A slope close to 1 was also obtained. It was important to perform these experiments over different concentration ranges to assess the reliability of the MALDI-MS response. The concentrations of samples received from users are often under- or overestimated, and it is important that the method be adaptable to these situations where a wide range of concentrations is possible.

The next step was to measure the concentration of a monoclonal antibody (mAb) *N*-glycosylated with only one core GlcNAc, Her2SF. Its tryptic *N*-glycopeptide was thus EEQYN(GlcNAc)STYR, producing $[M+H]^+$ ions at *m/z* 1392. Synthetic EEQYN(GlcNAc)STY*R was used as an internal standard with $[M+H]^+$ ions at *m/z* 1402. In Fig. 1c (duplicate experiment), the volume ratio of an unknown concentration solution of 1392 was increased stepwise from 0 to 2 over that of a 3.32-mM solution of 1402. As from Fig. 1a, b, it can be assumed that the signal ratio is equivalent to the molar ratio, the average slope of the graph represents the concentration factor between the two solutions, standard and unknown. If concentrations were equal, the slope would be 1. Therefore, the unknown solution spiked onto the target has a concentration of 3.32 mM × average slope (2.35) = 7.8 mM. In Fig. 1d, the same experiment was repeated at much lower concentration values, with the 1402 standard solution at 0.38 μM. Solutions were made so that the spotting of equal volumes of standard and unknown would yield MALDI signals within the same range. The unknown solution needed to be diluted accordingly, and this time, the volume of sample solution was kept constant while increasing volumes of added standard. The unknown's concentration was determined to be 0.31 μM.

Another method to calculate the unknown's concentrations was to plot a standard additions graph, where the fixed volume compound's signal was normalized to 100 arbitrary units in all spectra and other peak areas were recalculated accordingly. The graphs obtained corresponding to datasets of Fig. 1c, d are shown in Fig. S1. The *x*-intercepts from equations featured on the graphs allowed to calculate the molar amount of unknown in the 0.5 μL. Concentrations found were equivalent to those found above using the "slopes" method.

In a next set of experiments, the synthetic glycopeptide 1392 was used against a fraction of Eg2-hFc mAb [15, 24] tryptic digest glycopeptide fraction. The purpose was to evaluate the linearity and consistency of the response factors between 1392 and higher glycoforms G0F, G1F, and G2F of EEQYNSTYR (Scheme 2). Synthetic EEQYN(GlcNAc)STYR was prepared at 5.75 mM. Results are shown in Fig. 2a.

In the determination of glycoform concentrations, a parameter of interest is the relationship between response ratios and mixed solution volume ratios. The relative abundances of G0F, G1F and G2F $[M+H]^+$ increase in this order in Fig. 3a (sample spectrum), and the slopes in Fig. 2a go decreasing from top to bottom. From MALDI spectra of the glycoforms (e.g., Fig. 3a), average peak area ratios are 11.55, 40.06, and 48.39%, while $(\text{slope})^{-1}$ ratios are 11.57, 40.16, and 48.27%. It is important to note that slope values depend on the signal of the standard, whereas spectral ratios are calculated independently from the standard. This good agreement of values indicates that MALDI-MS responses for the three glycoforms are even relative to that of EEQYN(GlcNAc)STYR. Concentrations of G0F, G1F, and G2F glycoforms of EEQYNSTYR are obtained by dividing 5.75 mM by the slopes in Fig. 2 and are listed in Table 2, column 2. Calculations using the standard additions method (see Fig. S2 for graphs) yielded the same values.

The same experiment was repeated with a glycopeptide mixture from a tryptic digest of commercial polyclonal human IgG (Fig. 2b). The concentration of 1392 standard was 79.0 μM. Calculated concentrations for the three glycoforms of interest are given in Table 2, column 4. The relative proportions of reverse slopes are 26.66, 49.97, and 23.37%. These

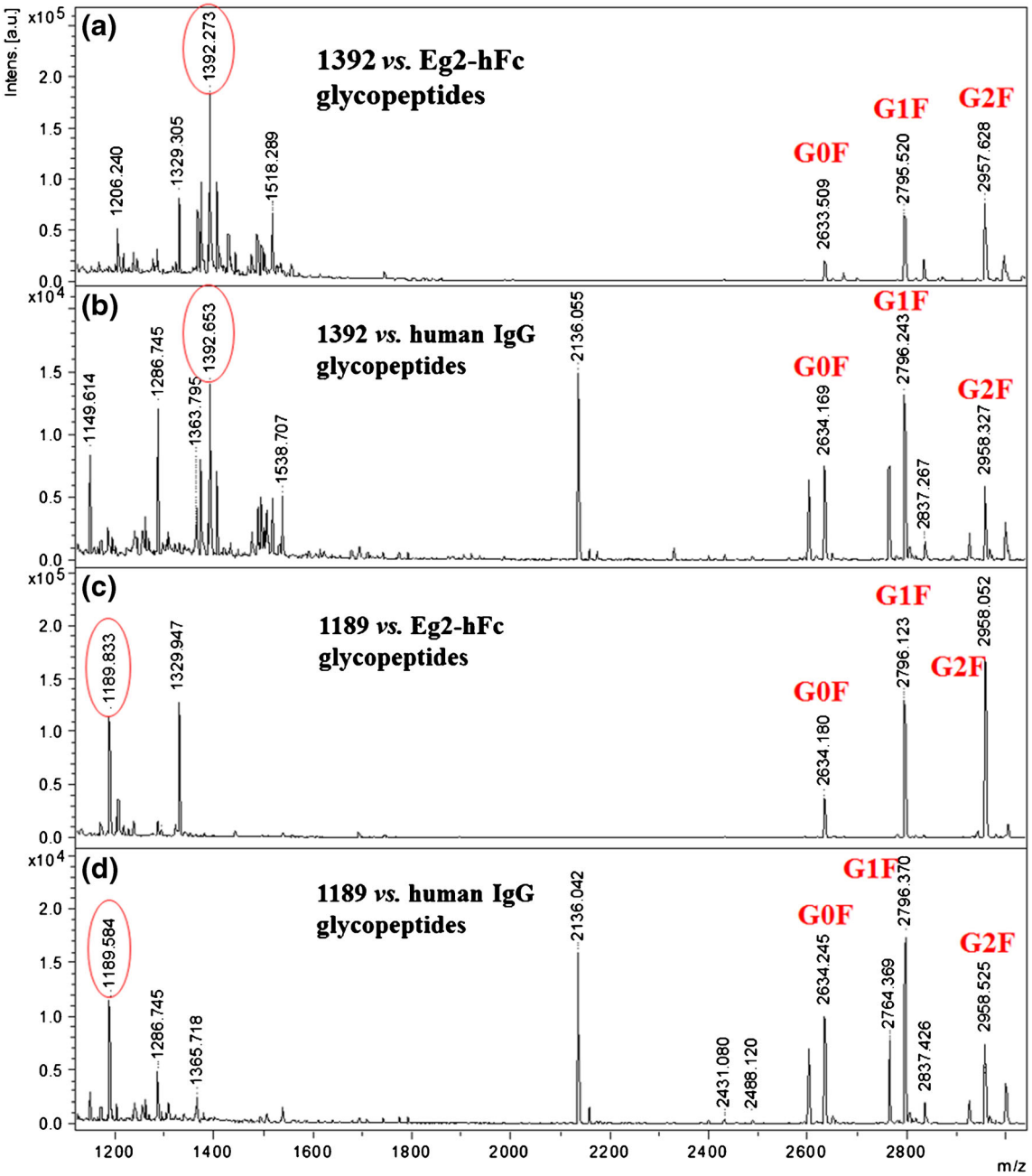


Figure 3. Typical MALDI mass spectra of tryptic glycopeptide fractions, analyzed with internal standards EEQYN(GlcNAc)STYR (*m/z* 1392, **a**, **b**) and EEQYNSTYR (*m/z* 1189, **c**, **d**). The G0F, G1F, and G2F glycans in these glycoforms are shown in Scheme 2. Peaks corresponding to the internal standard are circled in red

compare well with relative abundances observed in the spectrum of human IgG glycopeptide spectra, e.g., Figure 3b (26.09, 50.40, and 23.51%). Standard addition graphs are featured in Fig. S2b, and this method yielded the same values as the "slopes" method.

The synthetic peptide EEQYNSTYR (*m/z* 1189 for $[M+H]^+$ ions) was also used as a potential internal standard in the dosages of Eg2-hFc mAb and human IgG glycosylated analogs. Typical MALDI spectra are shown in Fig. 3c, d. Figure S3 shows the correlations. If a 1:1 response is assumed, the concentrations calculated using the slopes are featured in Table 2. Linearity was not satisfactory for the mAb, and the *y*-origin was much above zero. The plot was forced through the (0,0) origin, and slopes were obtained. The reverse values of these slopes were 9.45, 37.44, and 53.11% in proportion vs. 9.38, 37.43, and 53.19% on average in the MALDI spectra for G0F, G1F and G2F (e.g., Fig. 3c). For IgG1 in polyclonal human IgG, the results were 27.68, 51.38, and 20.94% (slopes) vs. 26.12, 50.17, and 23.73% (spectra, e.g., Fig. 3d).

The calculated mAb glycoform concentrations are reported in Table 2, column 3. These are one order of magnitude higher than those of column 2 with the glycopeptide standard. The

Table 2. Calculated EEQYNSTYR Glycoform Initial Concentrations of Solutions Deposited on the MALDI Target, Then Spiked with (Glyco)peptide Standard Solutions

Sample → Glycoform of EEQYNSTYR (*m/z*)	Eg2hFc mAb 1392 standard	Eg2hFc mAb 1189 standard	Human IgG 1392 standard	Human IgG 1189 standard
G0F (2634)	1.4 mM	12 mM	160 μM	450 μM
G1F (2796)	4.8 mM	48 mM	290 μM	820 μM
G2F (2958)	5.7 mM	67 mM	140 μM	340 μM
Total IgG1	12 mM	127 mM	0.58 mM	1.6 mM

column 3 values are too large to be realistic, according to Table 1, as they overshoot the user's estimated concentration. Also, values in column 5 are almost three times higher than those in column 4.

Overall, the 1189 peptide did not yield as much signal as its 1392 counterpart. Figure 4 was plotted to illustrate this feature, where same concentration solutions of 1392 and 1189 were spotted on target with incremental volumes of 1392 over 1189. This was studied over two concentration ranges, ~ 850 μM (a) and ~ 100 mM (b). Both cases showed that enhanced sensitivity was obtained with 1392. Overall, 1392 was considered a better internal standard than 1189 for the absolute quantitation of higher glycoforms of the peptide EEQYNSTYR.

Absolute Quantitation of IgG2 Abs

The same approach was used for the quantitation of IgG2 in SPE fractions containing only this subclass of human IgG tryptic glycopeptides [28] and in polyclonal human IgG glycopeptide samples. Two internal standards were used, synthetic EEQFN(GlcNAc)STFR (1360) and EEQFNSTFR (1157). Figure 5 shows the plots obtained for the attempts in quantifying the G0F, G1F and G2F glycoforms of EEQFNSTFR with EEQFN(GlcNAc)STFR in (a) IgG2 *N*-glycopeptides and (b) human IgG *N*-glycopeptides. In (a), slope^{-1} values reflected well the relative glycoform abundances observed in the spectra: 48.41, 41.26, 10.33 (slopes) vs. 49.82, 39.51, and 10.67 (areas). The concentrations of IgG2 peptide glycoforms deposited on target were determined and are reported in Table 3, column 2.

The graphs of Fig. 5b represent the quantitation of IgG2 glycopeptides in a polyclonal human IgG sample obtained commercially. The relative reverse slopes (42.36, 43.30, 14.34%) matched the average peak areas in the mass spectra (42.03, 43.24, 14.73%) suggesting even responses between the standard and analyte glycoforms. Concentrations of EEQFNSTFR glycoforms were calculated and reported in Table 3, column 4.

The use of non-glycosylated synthetic EEQFNSTFR (1157) as internal standard was also investigated for the analysis of IgG2 peptide glycoforms (Fig. S4, sample spectra in Fig. S5). Figure S4a corresponds to IgG2 fractions from human IgG, and reverse slope and peak area proportions showed relatively good matching values (36.68, 46.72, 16.60 for the slopes vs. 35.99, 46.17, and 17.84 for peak areas). Glycoform concentrations are listed in Table 3, column 3.

For the analysis of IgG2 in polyclonal human IgG, results are shown in Fig. S4b. The reversed slopes (42.39, 43.22, 14.87%) vs. the relative peak areas (42.06, 43.07, 14.87%) offered a better match than in the case discussed above. Glycoform concentrations for IgG2 EEQFNSTFR were calculated as indicated in Table 3, column 5. Once again for human IgG, the 1157 non-glycosylated standard exhibited less ionization efficiency than the 1360 glycosylated peptide, such that sample concentrations yielded by 1189 were much higher than with 1360. A total of 5.6 mM in glycopeptide concentration (1.6 mM IgG1 + 3.6 mM IgG2, Table 1) was deemed too high for these Ab samples. Moreover, IgG2 glycopeptide concentration exceeded IgG1's. The glycosylated peptide standard 1360 was decidedly a better internal standard for IgG2 than the non-glycosylated peptide 1157.

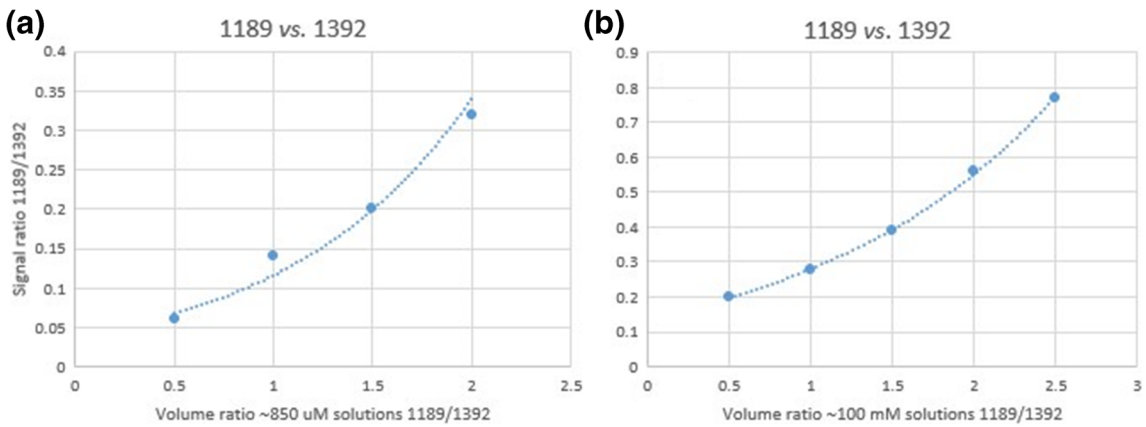


Figure 4. Mixture of equimolar amounts of EEQYNSTYR and EEQYN(GlcNAc)STYR on the MALDI target and measurements of relative areas of $[M+H]^+$ peaks. Concentrations of 1189 and 1392: (**a**) 875 and 841 μM; (**b**) 101 and 100 mM

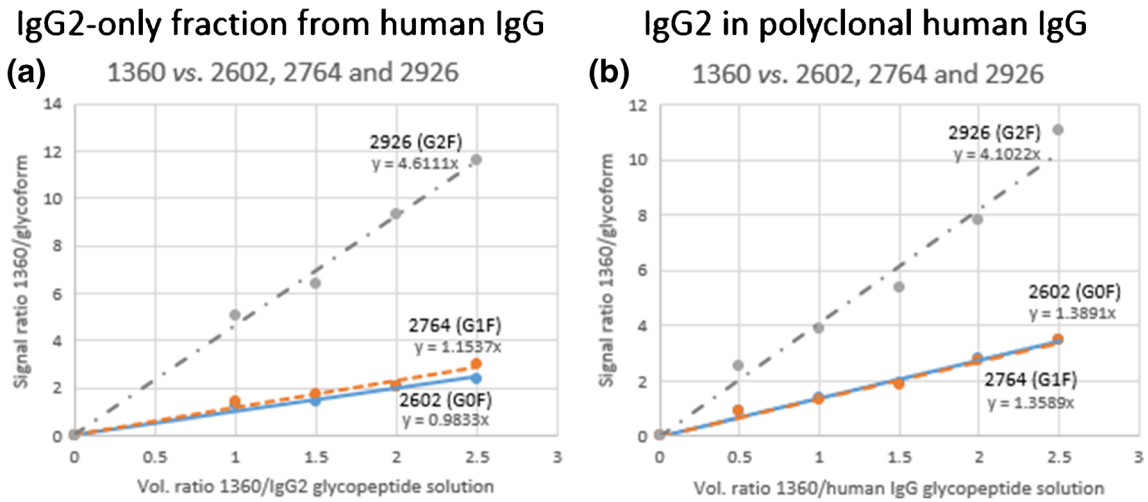


Figure 5. Use of EEQFN(GlcNAc)STFR (1360) as internal standard for the dosage of solutions of tryptic glycopeptides from (**a**) human IgG2 and (**b**) IgG2 in polyclonal human IgG. Concentration of standard: (**a**) 0.052 mM and (**b**) 101 μM

For human polyclonal IgG samples, Figs. 2b and 5b suggest that the presence of another peptide subclass does not interfere significantly in the independent analysis of either IgG1 or IgG2 glycopeptides.

Comparison of IgG1 and IgG2 Signals When Respective (Glyco)peptides Are Mixed on-Target: Attempts at Quantification

Intact human IgG1 and IgG2 have very similar molecular weights, i.e., 146 kDa on average [5]. These antibodies upon comparison not only have many analogies in their sequences, but also count many differences, as represented in Fig. S6 by the conserved gamma sequences of the heavy chains (UniProtKB—P01857 (IGHG1_HUMAN), P01859 (IGHG2_HUMAN)) (www.uniprotkb.com).

It is difficult to measure the subclass components of intact polyclonal IgG directly by ESI-MS or MALDI-MS. Even for very high-resolution mass spectrometers, the variation of molecular weights due to the sequences and possible glycosylation of the variable portions of each subclass renders the exercise laborious unless very specific IgGs, i.e., with consistent variable domains, are being investigated. Most studies on mass measurements of intact antibodies have been conducted on monoclonal species [30, 31].

Knowing that abundant ratios in healthy individuals should be around 60–71 (IgG1) to 19–31 (IgG2) [1], i.e., that the average IgG1/IgG2 ratio should be around 262%, it was interesting to observe that previous measurements of Proteoglycan™-enriched tryptic glycopeptides by MALDI-TOFMS indicated abundance ratios with various trends, where the signal due to the IgG2 peptide was always predominant over that of the IgG1 peptide. These measurements were repeated by ESI-MS and the results corroborated the MALDI-MS observations. Table 4 gives ion abundance ratios obtained by both ionization techniques for glycopeptides from tryptic digests of healthy human IgG1 enriched with the EMD Proteoglycan™ kit.

A few possible reasons were investigated to explain these observations: (i) In MALDI and ESI-MS, the IgG2 peptide has better ionization efficiency than the IgG1 peptide; (ii) the composition of the MALDI spotting solvent has an impact on ionization; (iii) the Proteoglycan™ resin has more affinity for EEQFNSTFR than EEQYNSTYR; and (iv) trypsin digests IgG2 more efficiently than IgG1.

Relative ionization efficiencies of IgG1 and IgG2 glycopeptides

Relative sensitivity measurements were conducted on both synthetic glycopeptides under same solvent conditions (TA30). In the analysis of glycopeptides from human polyclonal antibodies by MALDI-ToF-MS, the presence of both IgG1 and IgG2 species on the same sample target cannot be quantified by a direct comparison of their molecular ions' abundances as shown in Table 4.

Figure 6a shows that the increase of EEQFN(GlcNAc)STFR (1360) amount vs. EEQYN(GlcNAc)STYR (1392) does not

Table 3. Calculated EEQFNSTFR Glycoform Concentrations of Solutions to Which Peptide and Glycopeptide Internal Standards Were Added. Note: Both IgG2 Fractions Are Not from the Same Human IgG Sample and Cannot Be Compared Directly

Sample → Glycoform of EEQFNSTFR (*m/z*)	IgG2 fraction sample 1 Glycopeptide standard	IgG2 fraction sample 2 Peptide standard	Human IgG Glycopeptide standard	Human IgG Peptide standard
G0F (2602)	0.053 mM	0.20 mM	72 μM	1.5 mM
G1F (2764)	0.045 mM	0.26 mM	74 μM	1.6 mM
G2F (2926)	0.011 mM	0.093 mM	24 μM	0.52 mM
Total IgG2	0.11 mM	0.55 mM	0.17 mM	3.6 mM

Table 4. Ion Abundance Ratios Obtained for the Fc *N*-Glycopeptides of IgG1/IgG2

Sample (Proteoglycan™-enriched tryptic digest)	Ionization method	Signal ratio EEQYNSTYR/EEQFNSTFR (%)
Healthy serum IgG no. 1	MALDI	18
Healthy serum IgG no. 2	MALDI	35
Healthy serum IgG no. 3	MALDI	32
Healthy serum IgG no. 4	MALDI	10
Healthy serum IgG no. 5	MALDI	20
Healthy serum IgG no. 6	MALDI	42
Human IgG (Sigma)	MALDI (Fig. 3)	165
Human IgG (Sigma)	MALDI (Fig. S5)	91
Human IgG (Sigma)	ESI (HPLC)	58
Human IgG (Sigma)	ESI (HPLC) (no EMD)	53

produce a linear plot. Instead, the figure suggests a greater MALDI-MS sensitivity for 1360 than 1392 through a competitive ionization mechanism, as each aliquot of 1360 added is detrimental to the 1392 signal, resulting in a quasi-exponential curve. In Fig. 6b, two solutions of glycopeptides from unique subclasses, IgG1 and IgG2, were mixed in different volume ratios. Signal ratios were compiled for each pair of analogous glycoforms, e.g., EEQFN(G0F)STFR/EEQYN(G0F)STYR, and plotted against the volume ratios. The same trend as seen in Fig. 6a was observed, and exponential fits best represented the plots. Overall, these experiments suggest that simultaneous quantitation of IgG1 and IgG2 glycoforms with both 1360 and 1392 standards on a MALDI target would be very difficult to achieve. As will be shown further, it is preferable to perform the analysis of one subclass at a time using its related glycopeptide standard.

Using values reported in Tables 2 and 3, both in column 4, it was possible to calculate the proportions of IgG1 (76%) to IgG2 (24%) in the polyclonal sample. These values fit very well within the established IgG subclass range for healthy patients, i.e., 60.3–71.5% IgG1 to 19.4–31.0% IgG2 [1], normalized to 66.1–77.8% IgG1 to 21.3–34.1% IgG2 when not including IgG3 and IgG4.

Lastly, quantitative analysis of IgG1 and IgG2 glycoforms with their respective glycopeptide standards was attempted with all compounds being present on target. The analytes were human IgG glycopeptides. To a constant amount of analyte, both standards were added in identical increasing amounts. Plots were obtained for IgG1 and IgG2 species separately. The IgG1 plots (Fig. 7a) showed a linear relationship, although the intercept clearly did not go through zero. For IgG2 (Fig. 7b), the situation was similar although less pronounced. Human IgG glycopeptides concentrations were calculated using the unmodified slopes (Fig. 7a, b) and also using slopes obtained by forcing the plots through the 0,0 origin. Results are given in Table 5. IgG2/IgG1 ratios (either 0.10/1.4 or 0.11/1.7) are below the predicted values of 0.3–0.4 [1]. Thus, the method of using one internal standard at a time provided more reliability that the "all in one pot" method.

Effect of the composition of MALDI spotting solvents on ionization Phenylalanine has a noticeably greater solubility in water than tyrosine (29.6 vs. 0.45 mg/mL at 25 °C (https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/Product_Information_Sheet/t3754pis.pdf; https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/Product_Information_Sheet/p2126pis.pdf), which is likely to influence the relative solubilities of EEQFNSTYR and EEQYNSTYR. Solubility effects were studied with the following experiments. An equimolar mixture of EEQYNSTYR and EEQFNSTFR was prepared in water (0.1% TFA) and DHB matrix onto a MALDI target. The mixture was dried and analyzed (Fig. S7a). Then, a drop of water was added onto the dry sample, and pipetted

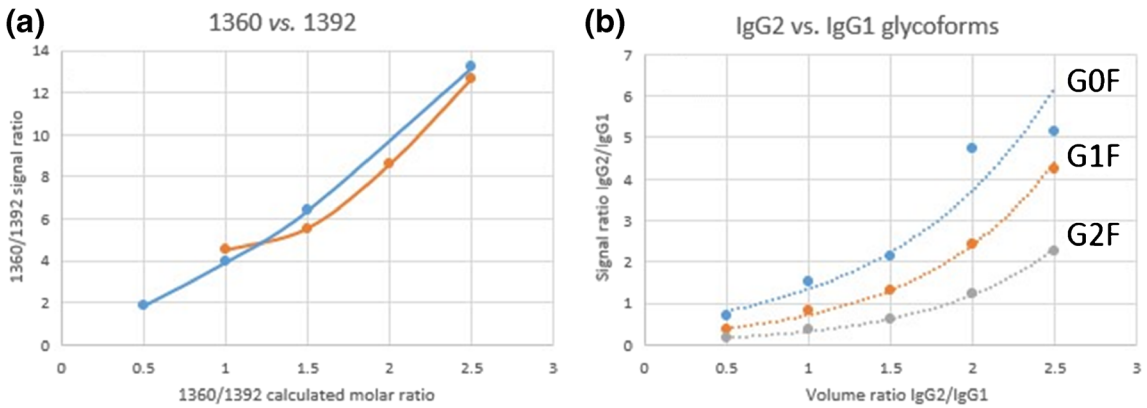


Figure 6. (**a**) Variation of signal ratios vs. molar ratios for EEQFN(GlcNAc)STFR/EEQYN(GlcNAc)STYR solution mixtures. (**b**) Variation of signal ratios according to volume ratios of IgG2/IgG1 glycopeptide solutions from IgG2 and IgG1 mAbs

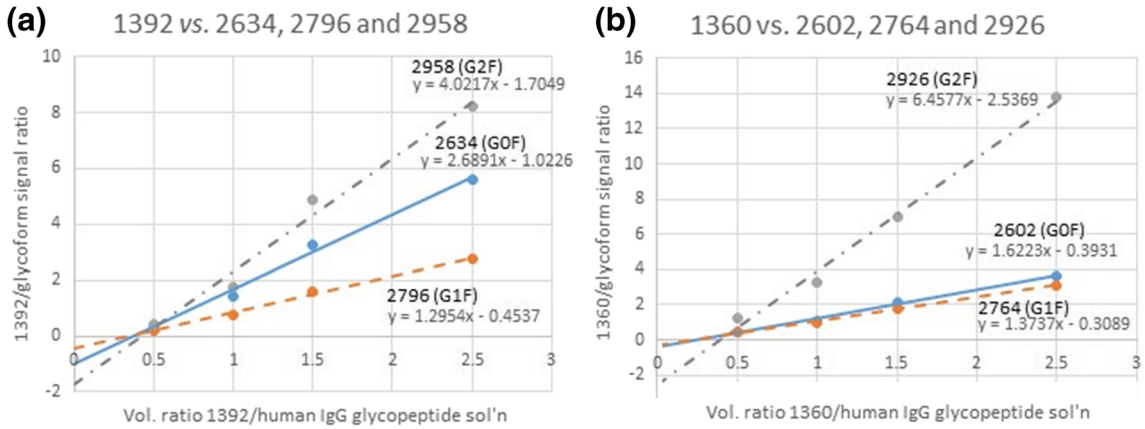


Figure 7. Simultaneous quantitation of IgG1 and IgG2 glycopeptides in human IgG with standard additions of EEQYN(GlcNAc)STYR (1392, IgG1, 0.95 μM) and EEQFN(GlcNAc)STFR (1360, IgG2, 0.063 μM). Each spot analyzed contained 0.5 μM of human IgG glycopeptides

out 90 s later. The sample was dried again and re-analyzed: the spectrum showed a decrease in abundance of EEQYNSTYR relative to EEQFNSTFR (Fig. S7b). The pipetted water drop was also deposited with DHB matrix and analyzed by MALDI, confirming the previous result: the *m/z* 1189 signal was higher than the *m/z* 1157 signal (Fig. S7c). These findings suggest higher water solubility for EEQYNSTYR vs. EEQFNSTYR, which goes against the relative solubilities of individual Y and F in water. On a hydrophobicity scale [32], EEQYNSTYR is more hydrophilic than EEQFNSTFR, such that in reversed-phase HPLC, a larger percentage of acetonitrile in water is needed to elute EEQFNSTFR than EEQYNSTYR.

With an equimolar solution of the synthetic peptides prepared in water and in an aqueous acetonitrile solution, it was observed that relative ion abundances in MALDI-ToF-MS had some important variations (Fig. 8). Figure 8b shows the results obtained for solutions prepared in TA30, which is the solvent normally used for matrix and MALDI sample preparation in the authors' laboratory. In Fig. 8a, water was used with 0.1% TFA. The most representative peak area ratios (vs. molar ratios) were obtained when the solution was prepared and spotted in water (0.1% TFA).

The conditions used in Fig. 8b are commonly used as they represent well the composition of C-18 HPLC or SPE fractions collected for glycopeptides. TA30 was used as spotting solvent for all of this work unless specified.

Relative affinity of the Proteoglycan™ enrichment material EEQFNSTYR and EEQYNSTYR It has been observed that the Proteoglycan™ enrichment kit retains mostly glycopeptides, but also a low amount of non-glycosylated peptides [15, 33, 34]. There is thus a good probability that synthetic non-glycosylated EEQYNSTYR and EEQFNSTFR will be retained by EMD, and relative affinities can be estimated. An experiment was designed where a solution yielding even signals for EEQYNSTYR and EEQFNSTFR (Fig. S8) was subjected to EMD enrichment. The binding and wash fractions were in turn analyzed by MALDI-ToF-MS (wash fraction; Fig. S8b). Nothing significant was observed in the binding fraction (not shown). It was observed that EEQYNSTYR has more affinity for the resin than EEQFNSTFR, i.e., upon washing the beads it was easy to release EEQFNSTFR, whereas EEQYNSTYR would remain bound.

This result was reasonable, given the tyrosine's hydroxyls in EEQYNSTYR and the hydrophilic nature of the resin. This suggests that in a workflow involving Proteoglycan™ enrichment of glycopeptides, a larger proportion of EEQFN(glycan)STFR than EEQYN(glycan)STYR could be obtained in solution.

Digestion efficiency of IgG1 vs. IgG2 Whether DTT reduction was used or not prior to tryptic digestion, there was always a higher EEQFNSTFR/ EEQYNSTYR glycoform peak area proportion in the MALDI spectra for healthy human IgG glycopeptides. Logically, the structure of intact IgG2 with two

Table 5. Calculated EEQYNSTYR (IgG1) and EEQFNSTFR (IgG2) Glycoform Concentrations of a Solution of Internal Standards EEQYN(GlcNAc)STYR and EEQFN(GlcNAc)STFR Were Added Simultaneously at Increasing Volume Ratios. Analytes: Human IgG Glycopeptides

Glycoform	IgG1		IgG2	
	Calc. conc. (μM) (original slope)	Calc. conc. (μM) (through origin)	Calc. conc. (μM) (original slope)	Calc. conc. (μM) (through origin)
G0F	0.36	0.46	0.040	0.045
G1F	0.76	0.94	0.046	0.056
G2F	0.24	0.32	0.010	0.012
Total	1.4	1.7	0.10	0.11

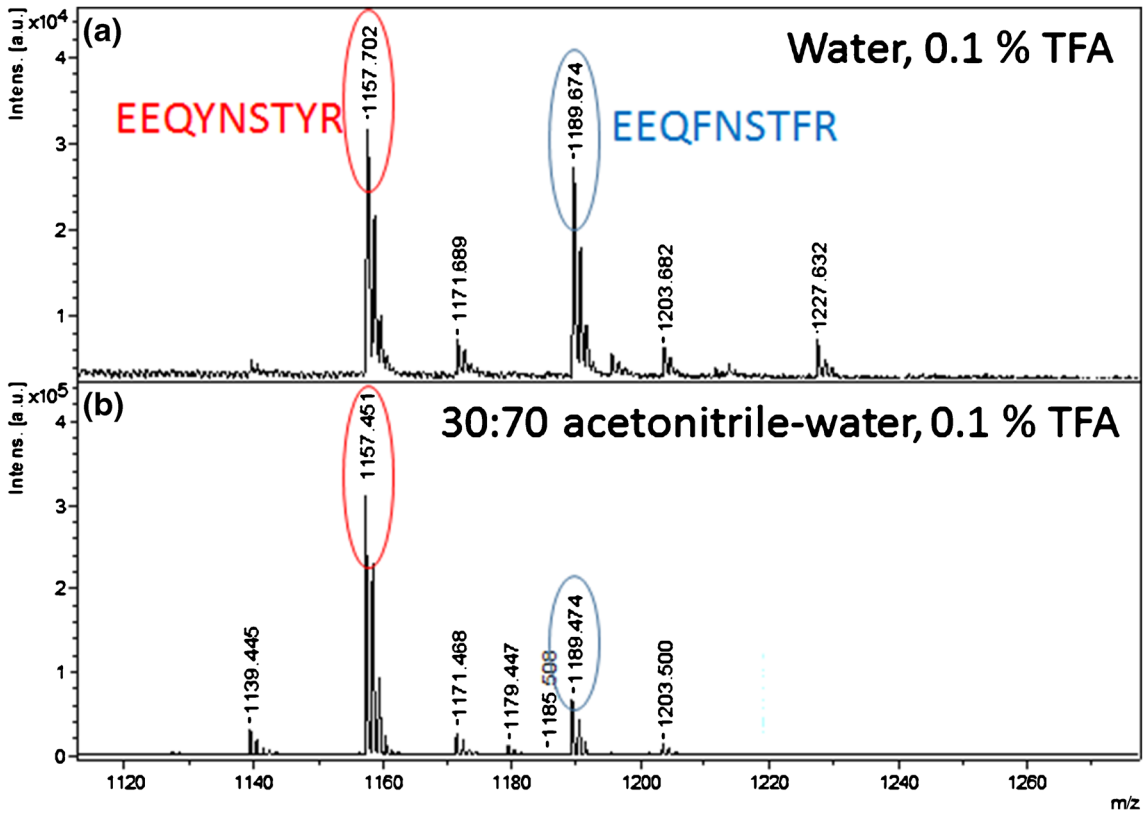


Figure 8. MALDI-MS spectra of equimolar quantities of synthetic peptides EEQFNSTFR (*m/z* 1157) and EEQYNSTYR (*m/z* 1189) acquired after dissolving and spotting using different aqueous solvents. **a**) 0.1 % TFA in water, **b**) TA30. TA30 was used for all of this work unless specified

disulfide bonds in the hinge region should be more resistant to reduction than that of IgG1, which only has one disulfide bond in that region [5]. The observation of more ions for the IgG2 peptide therefore goes against the predicted effect of this distinct feature of the antibodies. Another indication of reduction performance is the abundance of missed cleavage peptides TKPREEQFNSTFR and TKPREEQYNSTYR. In the samples listed in Table 4, MALDI-ToF-MS did not detect significant quantities of these peptides. With HPLC-ESI-MS of the enriched and non-enriched samples, missed cleavage peptides were observed and their signals still showed predominance of IgG2 over IgG1 (not shown). Overall, there does not seem to be a detectable difference in the efficiency of tryptic digestion between IgG1 and IgG2.

Conclusion

The synthesis of peptide internal standards corresponding to Fc tryptic fragments of human IgGs has allowed absolute quantitative analysis of mAb and polyclonal samples. Mainly, it was possible to perform the quantitation of one subclass glycopeptide at a time, to which we demonstrated on a mAb or a polyclonal sample. More specifically, IgG1 glycoforms of EEQYNSTYR were best quantified by the EEQYN(GlcNAc)STYR standard than by the bare peptide EEQYNSTYR. Whether or not IgG2 was present or not in the sample did not seem to influence the linearity of the standard additions plots significantly with respect to IgG1. It was also possible to quantify IgG2 with EEQFN(GlcNAc)STFR. For human IgG, the ratio of IgG2/IgG1 obtained using this method was within the predicted range in healthy donors. However, it was shown that the IgG2 peptide backbone EEQFNSTFR has a higher ionization efficiency than EEQYNSTYR (IgG1) and that in the instance of a mixture, the presence of the IgG2 peptide may have a competitive effect on the ionization of IgG1's and alter the analysis. It was also shown that the spotting solvent has a strong influence on the relative abundance of IgG2 vs. IgG1 peptides. This helps to explain some inconsistencies in the signals of human IgG glycoforms from difference subclasses observed in this work and in the literature. This work highlights the importance of consistency in sample preparation for MALDI-MS analysis. IgG1 and IgG2 glycopeptides have different ionization efficiencies and can be quantified preferentially one by one, as it is not possible to perform quantitative analysis in an "all in one pot" method.

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