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Characterization of Whole and Fragmented Wild-Type Porcine IgG

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Abstract

Glycoproteomic analyses of tryptic (glyco)peptides from wild-type (WT) porcine IgG were performed. In a first protocol, intact antibody was digested with trypsin, followed by glycopeptide enrichment and liquid chromatography-tandem MS (HPLC–MS/MS). This procedure allowed to detect *N*-glycopeptides observed previously (Lopez, P. G. et al., *Glycoconj. J.* 2016, *33* (1), 79), plus other non-reported *N*-glycopeptides. The method provided useful information but did not allow to discern between Fab (antigen-binding region) and Fc (constant region, fragment crystallizable) peptides/glycopeptides. In a second scheme, glycoproteomic analysis was attempted for Fab and Fc fragments obtained by papain and Fabulous™ hydrolysis. Usually employed for milligram amounts of antibodies, the papain and Fabulous™ protocols were adapted to 200 μg of WT IgG. Fab and Fc fragments were separated by size-exclusion (SEC) HPLC. Fractions collected were reanalyzed by gel electrophoresis (SDS-PAGE). Bands were excised, and fragments digested in-gel, followed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS and HPLC/MS–MS. In the protocol no glyco-peptide enrichment was involved, that is, whole tryptic digests were analyzed. Fc *N*-glycopeptides were identified, and greater numbers of non-glycosylated peptides were tabulated. Very few peptides overlapped between Fc and Fab, as most peptides were clearly from Fc or Fab. HPLC-MS/MS detected more sialylated glycoforms than MALDI-TOF-MS. Sections of Fab and Fc were assigned de novo, through a database search or manually.

Keywords: porcine IgG, papain, enzymatic fragmentation, Fabulous™, glycoproteomics

1. Introduction

There have been reports on the mass spectrometric (MS) analysis of pig immu-noglobulins (IgG) in relationship with use in a xenotransplantation context [1–4]. These studies have explored the amino acid composition and glycosylation of pig IgG according to glycoproteomic [2, 3] and glycomic [1] workflows involving the enzymatic digestion of whole antibodies. Glycoproteomic workflows resulted in the

identification of many peptides that could be matched with the conserved gamma portion of the heavy chains of some of the 11 subtypes of pig IgG [5], including *N*-glycopeptides EEQFNSTYR and EAQFNSTYR [3]. No specific information was given on the variable portions of neither Fab nor Fc components, as most of such assignments would have had to be attributed *de novo*. The conserved Fc glycosylation site is often described as the only IgG site glycosylated at 100%, in spite of the fact that 10–15% of wild-type antibodies have glycosylation also in their variable region [6], and reports have shown that even higher glycosylation levels (up to 30–40%) can exist in the variable regions of polyclonal IgGs [7, 8].

For more specificity, the analysis of antibodies by MS can take great advantage of enzymatic fragmentation with papain [9, 10] or new enzymes produced by recombinant methods and available on the market [11]. This type of procedure has not been reported for the fragmentation of porcine IgG, to the authors' knowledge. For instance, procedures have been published for mouse [12, 13], chicken [14], rabbit [15], sheep [16], and human [17, 18] IgGs. The two groups of antibody fragments of primary interest are the antigen-binding fragments (Fab) and class-defining crystallizable fragments (Fc). The hinge region of immunoglobulins (IgGs) is readily accessible to proteolytic attack by enzymes [9, 10], and cleavage at that point produces F(ab')$_2$ or Fab fragments and the Fc fragment. Papain is a nonspecific thiol-endopeptidase and has a sulfhydryl group in its active site, which must be reduced for activity. When IgG molecules are incubated with papain in the presence of a reducing agent, one or more peptide bonds in the hinge region are split, producing three fragments of similar size: two Fab fragments and one Fc fragment [9, 10].

Fabulous™ enzyme is a recombinant cysteine protease that under reduced conditions digests in the hinge region of antibodies from many species and subclasses, including human, mouse, rat, and goat, yielding Fab and Fc fragments [11]. As a reducing agent is present during digestion, it is likely that interchain thiols will be reduced. Fabulous™ and papain have typically been used for the production of relatively large amounts of antibody fragments (10 mg of starting material), whereas methods adapted to MS require much less, about 50–200 μg. There is a need for downscaling these workflows, especially for porcine IgG, which has not been previously studied by fragmentation followed by MS.

Reports on post-fragmentation MS analyses of antibodies have demonstrated that detailed fragment characterization allows for the identification of more glycosylation sites than bottom-up approaches [19, 20]. It is also important to study amino acid sequences of the variable portions of IgG for therapeutic purposes [21], and the information obtained after fragmentation is much more specific than data generated through the tryptic digestion of whole intact antibodies.

The interest of the present study is to compare results from two main workflows aimed at characterizing wild-type porcine IgG's glycosylation and amino acid sequence features. In the first workflow, porcine IgG is digested with trypsin, followed by glycopeptide enrichment. Reversed-phase high-performance liquid chromatography (RPLC) coupled with electrospray ionization (ESI) MS and tandem MS (MS/MS) is used to characterize the glycopeptide-rich fraction. The second workflow involves subjecting porcine IgG to fragmentation by one of two enzymes, papain or Fabulous™. The steps necessary between fragmentation and MS included size-exclusion chromatography (SEC), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and tryptic digestion. This is the first attempt to characterize porcine IgGs in small amounts (sub-mg) using a combination of these methods. Two different MS techniques were used for the analysis of tryptic products of antibody fragments: matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS and ESI/MS–MS coupled with RPLC.

As the tryptic digestion products of whole wild-type porcine IgG antibodies have been characterized by MALDI-MS [2, 3] and ESI-MS and MS/MS (new results presented in this report), data from these different workflows serve as comparative benchmarks between intact and fragmented IgG samples. Overlaps and differences allow to identify peptides and glycopeptides as originating from either the Fc or Fab portions, and database searches [22] can verify if these sequences are already available in the literature or need to be determined *de novo*. Porcine IgG is a complex mixture of subtypes, and the complementarity of MALDI- and LC/ESI-MS–MS brings a considerable amount of information to document the identification of these IgG components.

2. Experimental

2.1 Materials

Wild-type porcine IgGs were obtained from Université de Nantes (Dr. Jean-Paul Soulillou's Laboratory). Trypsin Ultra™ was purchased from Promega (Wisconsin, USA). The Fabulous™ enzyme was kindly provided by Genovis (Cambridge, MA). Dihydroxybenzoic acid (DHB), sinapinic acid, ammonium bicarbonate, dithiothreitol (DTT), L-cysteine, iodoacetamide (IAA), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO). Mini-Protean TGX precast gels (4–15%), Precision Plus™ protein standard, 2-mercaptoethanol, and 4x Laemmli sample buffer were obtained from Bio-Rad (Hercules, CA). Imperial™ protein stain, tris base (2-amino-2-(hydroxymethyl)-1,3-propanediol) and immobilized papain-cross linked and 6% in beaded agarose supplied as 50% glycerol in sodium acetate pH 4.5 were purchased from Thermo Scientific (Rockford, IL). Strata-X C-18 cartridges were obtained from Phenomenex (Torrance, CA). Acetonitrile (ACN) was purchased from EMD-Millipore (Darmstadt, Germany). Sodium phosphate dibasic anhydrous was purchased from McArthur Chemical Co. Ltd. (Montreal, Canada). Hydrochloric acid was purchased from Anachemia (Vancouver, Canada) and deionized water was obtained from an adapted filtration system (Millipore).

2.2 Tryptic digestion of whole porcine IgG

Porcine IgG (200 μg) was dissolved in 100 mM ammonium bicarbonate (pH~8) and vortexed. A DTT solution (10 mM) was added to the sample, which was then vortexed and left to react at 56°C for 40 min, then cooled to room temperature. After 500 mM IA was added, and the sample was left to react in the dark for 45 min. The excess of IA was quenched with the addition of 100 mM DTT, and the sample was left to react for 10 min in the dark. Trypsin was added and proteolysis took place at 37°C for ~18 h. To deactivate trypsin, the sample was frozen and dried under vacuum. Glycopeptide enrichment was then performed using a ProteoExtract™ glycopeptide enrichment kit (Millipore-Sigma, Etobicoke, ON) according to the manufacturer's procedure [23].

2.3 Papain digestion of porcine IgG

Just before use, 20 mM sodium phosphate digestion buffer was prepared with a 10 mM cysteine content, and the pH was adjusted to 7 Bead-immobilized papain slurry (20 μL, 50%) was added to an Eppendorf™ tube. The beads were washed twice with 160 μL of digestion buffer and then re-suspended in the buffer. Porcine IgG (200 μg) was dissolved in the digestion buffer. This was added to the tube containing the washed immobilized papain and digestion buffer. The sample was

incubated for ~24 h at 37°C. Constant mixing of the bead slurry was maintained during the whole incubation. The bead-immobilized enzyme was separated from the digest by centrifugation and 20 μL of 10 mM Tris–HCl, pH 7.5 was added before centrifugation. The supernatant, which contained the IgG fragments, was removed.

2.4 Fabulous™ digestion for porcine IgG

The IgG sample (200 μg) was added to 200 units of Fabulous™ enzyme in 200 μL of 10 mM Tris, 50 mM cysteine buffer. The sample was vortexed and incubated at 37°C for 1 h.

2.5 Fractionation of IgG fragments by HPLC using a SEC column

The digestion mixtures were injected into a preconditioned SEC-300 4.6 × 300 mm silica-based column (Phenomenex, Torrance, CA). The mixtures were eluted with a mobile phase of 0.1% TFA, 0.1% formic acid in 20% ACN at a flow rate of 0.3 mL/min (manufacturer's recommendation). The HPLC system used was a Waters 1525 binary pump equipped with a Waters 2707 autosampler and a Waters 2998 photodiode array detector (Milford, MA). Fractions were collected, dried, and re-suspended for MALDI-MS analysis.

2.6 Separation of IgG fragments by SDS gel electrophoresis

Once fractionated by SEC, Fab and Fc components were separated on a Mini-Protean™ Tetra cell system (Bio-Rad). Bio-Rad TGX™ gels used had 10 wells and a density gradient of 4–15%. Wells were washed individually four times with running buffer (10 × tris-glycine-SDS buffer diluted 1 × with water) prior to the loading samples. Each sample fraction containing Fab, Fc, or both Fab and Fc had its own lane on the gel. Each gel was loaded with 15 μL of each fraction (in water) in 11.3 μL of 4 × Laemmli sample buffer, without adding 2-mercapto-ethanol. Well 1 was loaded with 10 μL of Precision Plus Protein Kaleidoscope™ standard. Intact-reduced IgG (15 μL, ~14 μg) was loaded into well 2. IgG fragments (~14 μg) were loaded in other lanes. Running buffer was poured in the cell system and the voltage was set at 150 V. Samples were allowed to migrate for 40 min, until the dye front reached the bottom of the gel. The gel was removed from the cell and was rinsed four times with Millipore water, and sufficient Imperial™ protein stain was added. IgG fragments absorbed the stain overnight, and the stain was decanted and replaced with Millipore water until gel bands became visible.

2.7 In-gel tryptic digest of IgG Fab and Fc fragments

Non-reduced Fab and Fc bands were excised from the gel. Tryptic digestion was performed on each single cut out band. Bands were cut into small pieces and placed into 1.5 mL Eppendorf tubes. The digestion buffer was 50 mM ammonium bicarbonate in water. Each tube contained one lane worth of gel. Gel pieces were washed with wash buffer (1:1 digestion buffer-ethanol) until all protein stain was removed. They were then incubated in absolute ethanol for 10 min. Gel pieces were then rewashed with digestion buffer for 20 min and then incubated again in absolute ethanol for 20 min, which was removed from the gel by vacuum centrifugation. Trypsin solution was added and the tubes were placed on ice where the gel was allowed to swell. Thereafter, excess trypsin solution was discarded. Gel pieces

were covered with digestion buffer and incubated at 37°C for ~18 h. Glycopeptides and peptides were extracted on C_{18} cartridges with buffer A, 0.5% acetic acid; extraction buffer B, 5:3 30%; ACN, 0.5% acetic acid; and extraction buffer C, 100% ACN. Samples were dried down for further analysis.

2.8 Peptide-*N*-glycosidase F (PNGase F) removal of glycans from trypsin digested Fab and Fc fragments

A solution of PNGase F (4 μL, 10 units/μL) was added to a solution of tryptic glycopeptides. The sample was vortexed and set at 37°C for ~18 h. After the digest, glycans were separated from the de-glycosylated peptides on a C_{18} cartridge. The cartridge was conditioned with 5 x 1 mL (ACN + 0.1% TFA), then 5 × 1 mL of (H_2O + 0.1% TFA). The sample was loaded onto the cartridge and allowed to equilibrate for about 1–2 min. Glycans were eluted with 3 mL H_2O + 0.1% TFA and collected in two fractions. Peptides were eluted with 1.5 mL of 50:50 ACN:H_2O + 0.1% TFA. Solvent was evaporated from the fractions.

2.9 Preparation of samples for MALDI-MS analysis

Glycopeptide fractions were mixed directly with 5 μL of DHB in 30:70 ACN: water. Samples (1 μL) were then spotted onto the stainless steel target and allowed to dry, for reflector positive mode MALDI-MS. For the Fab and Fc fragments from HPLC fractions, 5 μL of 0.1% TFA and 5 μL of sinapinic acid in 0.1% TFA, 30:70 ACN: water was added, and 1 μL was spotted onto the target already pre-spotted with 0.5 μL of sinapinic acid in ethanol. Spots were then allowed to dry for linear positive mode MALDI-MS.

2.10 MALDI-TOF-MS analysis

These analyses were performed on an UltraFleXtreme™ mass spectrometer (Bruker, Billerica, MA) equipped with LID-LIFT™ technology for tandem MS experiments. For positive ionization mode, a nine-peptide calibration mixture with masses ranging from 500 to 5000 Da was used. In linear positive mode, the instrument was calibrated using tryptic peptides of cytochrome C, mass ranging from 10,000 Da to 160,000 Da.

2.11 Preparation of samples for HPLC-ESI-MS analysis

For the Fab and Fc tryptic digest fragments, 100 μL of water was added to the pooled C_{18} cartridge fractions of each Fab and Fc tryptic digests. Samples were sonicated and then ready for HPLC–MS analysis.

2.12 RPLC-MS/MS analysis of Fab and Fc tryptic digests

Both digestion mixtures (50 μL) were in turn injected into a preconditioned Acquity BEH C_{18} (1.7 μm, 2.1 × 50 m) silica-based, reverse phase column, on a Waters Acquity UPLC system (Waters, Mississauga, ON). The flow rate was 0.25 mL/min, and a linear increase from zero to 28% ACN in water with 0.1% formic acid was used. Mass spectrometric detection was performed on a Waters G2 Synapt ESI-MS instrument. Positive ionization mode was used. The analyzer mode was set to high resolution, with a capillary voltage of 3.00 kV and a cone voltage of 25 V. The Progenesis™ software was used to handle and search databases for these HPLC-MS/MS analyses.

2.13 Analysis of whole porcine IgG tryptic digest by RPLC-MS/MS

Separations were conducted on a LC Ultra system (Eksigent, Dublin, CA). A 100 μm × 200 mm analytical column packed with 3 μm Luna C18 (Phenomenex, Torrance, CA) was used, at 500 nL/min flow rate. A 300 μm × 5 mm PepMap 100 trap-column (Thermo Fisher, San Jose, CA) was used to protect the analytical column. The elution gradient was as described above. A Triple TOF 5600 mass spectrometer (ABSciex, Concord, ON) was used in standard MS/MS data-dependent acquisition mode. Mass spectra (250 ms) were collected (m/z 370–1250) and followed by up to 20 MS/MS measurements on the most abundant parent ions (400 counts/s threshold, +2 to +5 charge state, m/z 100–1500 mass range for MS/MS, 100 ms each). Database search was performed using the Global Proteome Machine (GPM) proteomics data analysis system [24].

3. Results and discussion

3.1 Mass spectrometric analysis of tryptic digests of whole porcine IgG samples

In previous studies, wild-type porcine IgG was digested with trypsin, and glycopeptides/peptides were fractionated on a C18 cartridge. Glycopeptide fractions were analyzed by MALDI-MS/MS [2, 3, 30]. In a new separate experiment, the results of which are presented here, all tryptic products were then enriched for glycopeptides using an EMD-Millipore ProteoGlycan™ kit [23]. The glycopeptide-enriched fraction was injected into a RPLC-MS/MS system, using data-dependent MS/MS acquisition. **Figure 1a** shows the MS/MS total ion chromatogram (TIC) obtained from the elution, whereas **Figure 1b** represents the elution of peptides with m/z 204
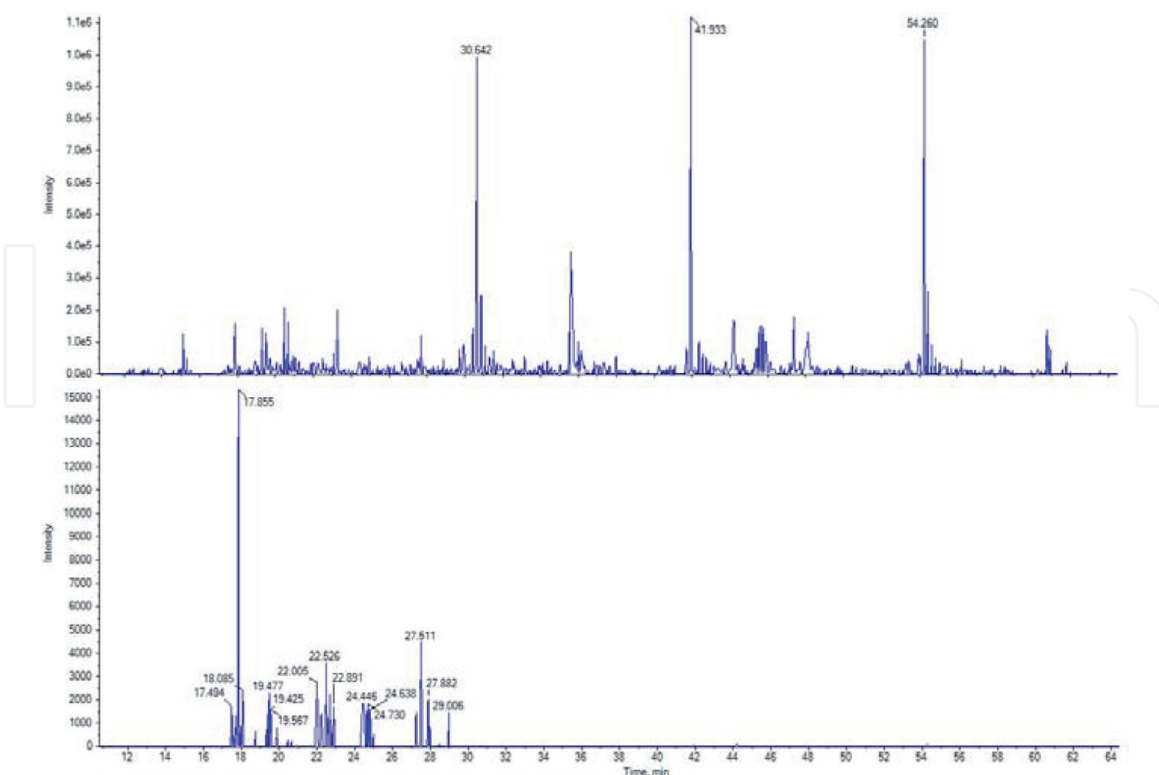

Figure 1.
(a) Total HPLC/MS ion chromatogram obtained from the MS/MS spectra of porcine IgG tryptic peptides and glycopeptides after enrichment on EMD-Millipore Proteoglycan™. (b) Selected MS/MS fragment ion chromatogram at m/z 204, indicating the elution times of glycopeptides [25].

ions as product in their MS/MS spectra, identified as glycopeptides [25]. Although enrichment was performed, there were still non-glycosylated peptides present in the sample. The m/z 204 trace shows that glycosylated peptides eluted early in the analysis and that the most abundant compounds eluting in (a) were most probably non-glycosylated peptides.

A database search using GPM [24] helped to identify some non-glycosylated peptides, while for glycopeptides the extracted m/z 204 chromatogram was used to manually identify as many glycopeptides as possible. **Table 1** gives a listing of all peptides detected and sequenced with GPM [24] or manually. For glycopeptide sequencing, MS/MS spectra were treated with CycloBranch [26] after removing abundant glycopeptide ions. As a few sequences are available in UniprotKB [22] for the Fc gamma portion of porcine IgG (K7ZLAZ, L8B1B0, L8B0S7, L8B0S2, L8B0Z4), and more are available [3] from previously published DNA sequences [5], some peptides show more than one identification source in **Table 1**. Only one entry was found for a porcine IgG Fab portion, P01846, which corresponds to the lambda (λ) constant region. **Figure A1** compares the published porcine IgG Fc heavy chain sequences relating to **Table 1** assignments.

Points of interest arising from this table are (i) the presence of a large number of non-glycosylated, even after enrichment, from the Fab and Fc portions of the antibody and (ii) the detection of seven distinct glycosylated peptides, some with complex glycoforms and one with high-mannose glycoforms, thus of the *N*-type. Indeed, all MS/MS spectra of these glycopeptides showed characteristic fragment ions corresponding to the $(M + H)^+$ ions of the bare peptides, next to the characteristic $(M + H + 83)^+$ and $(M + H + 203)^+$ ions [27], with the latter being predominant.

Figure 2 shows four MS/MS spectra of glycopeptides, starting with two of the complex G0F forms of peptides of (a) constant region EAQFNSTYR [3] and (b) a variable region sequence partially determined as H_2N-(300)-QNFSVFR by the CycloBranch software [26]. In these cases, the mass difference between the protonated precursors and $(M + H)^+$ bare peptide fragments is 1444. The m/z 204 and 366 fragment ions are predominant as glycopeptide signature. In **Figure 2c**, a sialylated complex glycoform is featured, the G1FS form of peptide EEQFNSTYR [3]. In previous studies of wild-type pig IgG, the presence of *N*-glycolyl neuraminic acid (NeuGc) is featured exclusively (i.e., no *N*-acetyl neuraminic acid (NeuAc)) has been reported [2, 3]. The fragmentation of NeuGc-containing species produces distinctive m/z 308 and 290 fragments, as observed in (c). **Figure 2d** shows the fragmentation of a high-mannose glycoform of a peptide of undetermined sequence. The bare peptide $(M + H)^+$ ions appear at m/z 1069, with +83 ions (m/z 1152) and + 203 ions (m/z 1272).

For known peptide sequences such as in **Figure 2a** and **c**, it is possible to find most bare peptide y and b ions, although they appear with very low abundance and are not accounted for by the search engine, due to the domination of all glycopeptide signature ions. There was an attempt by the authors to sequence all unknown glycopeptide sequences, with partial success, as indicated in red in **Table 1**.

Overall results suggest that *N*-glycosylation occurs in the Fc but also in the variable regions of the Fab and/or Fc domains of porcine IgG. Each glycosylated peptide detected indicated patterns linked to *N*-glycosylation, while there was no obvious detection of *O*-glycans. This may be due to the conditions used to enrich the glycopeptides with the EMD-Millipore Kit, which were optimized for *N*-glycosylated peptides [23, 28]. Results obtained with this first workflow will be compared with those generated with a more elaborated procedure involving fragmentation enzymes as discussed below.

Peptide sequence	m/z (M + H)+	Error (ppm)	m/z glycoform	Glycoform	Identification source[*]
Unknown	646.35				Unavailable
NFSTYR	**787.37**	0	2231.14 2392.28 2701.08 2863.10	G0F G1F G1FS G2FS	IgG1a-b,IgG2a-b,IgG4a-b,IgG5a-b, IgG6a-b,L8B0S7,L8B0S2,K7ZLA7,L8B180, L8B0Z4
LLVELIR	855.57	4.44			Unavailable
TVTPSECA	864.38	3.8			P01846
FSGAISGNK	880.45	2.72			P01846
DLPAPTIR	882.5	−4.88			IgG2ba-b,IgG4a-b,IgG6a-b,L8B180,L8B0S7,L8B0Z4
LLLDLFR	889.55	−0.56			Unavailable
LLNGYRR	891.52	−4.6			Unavailable
AGGTTVTQVE	962.48	1.14			P01846
LIYQATNR	978.54	3.37			IPR 007110 (Ig C1-set)
VDPALPLEK	981.56	−1.58			Unavailable
NRPTGVPSR	983.54	−1.9			Unavailable
TISKATGPSR	1017.57	1.28			IgG3
LSSPATLNSR	1045.56	−3.44			Unavailable
Unknown	**1069.55**		2933.2	M9	Unavailable
FQQTPGQPPP	1096.54	−2.09			P01846
EAQFNSTYR	**1115.49**	−19.4	2414.05 2560.12 2722.15 2884.24 3029.35 3191.50	G0 G0F G1F G2F G1FS G3F	IgG6a,L8B180,L8B0Z4
Unknown	**1154.53**		2599.1	G0F	Unavailable

Peptide sequence	m/z (M + H)+	Error (ppm)	m/z glycoform	Glycoform	Identification source*
EEQFNSTYR	**1173.5**	−14.6	2471.01 2618.09 2634.07 2780.13 3087.23 2942.19 3104.23 3249.28 2833.04	G0 G0F G1 G1F G1FS G2F G3F G2FS M9-N	IgG1a-b,IgG2a-b,IgG4a-b,IgG5ab,IgG6b, L8B0S7,L8B0S2,K7ZLA7
TNNRPTGVPSR	1198.63				Unavailable
H2N-300-QNFSVFR	**1212.13**		2656.09 2819.14 2981.20	G0F G1F G2F	Unavailable
SYLALSASDWK	1240.62	−0.64			P01846
DTNRPSGIPER	1241.62	−2.6			Unavailable
STNSRPTGVPSR	1258.65	0.16			P01846
FSGSGSGTDFTLK	1303.62	2.74			Unavailable
SSSGFTCQVTHE	1339.56	1.27			P01846
TAPSVYPLAPCGR	1388.71	7.85			IgG1a-b,IgG4b,IgG5b,IgG6b,L8B180,K7 ZLA7,L8B0Z4
LLGASVLGVGDIHR	1406.81	1			Unavailable
Unknown	1415.62				Unavailable
Unknown	**1467.28**		2911.26 3073.31	G0F G1F	Unavailable
Unknown	1495.73				Unavailable
LVESGGGLVQPGGSLR	1525.85	11			L8B0S2,L8B180,L8B0S7
QSNNKYAASSYLAL	1529.76	0.39			P01846
AGGTTVTQVETTKPSK	1604.85	0.6			P01846
YAASSYLALSASDWK	1632.79	0.24			P01846
VVSVLPIQHQDWLK	1661.92	−10.4			IgG1a-b,IgG3,IgG6b,L8B180,K7ZLA7,L8B0Z4
QEYREDFVLTVTGK	1667.83	12.7			Unavailable
APASYFVQSVLTVSAK	1667.9	0			K7ZJP7

Peptide sequence	m/z (M + H)+	Error (ppm)	m/z glycoform	Glycoform	Identification source[*]
Unknown	1718.91				Unavailable
TVIYSTNSRPTGVPSR	1734.91	1.73			P01846
QLIYSTNNRPTGVPSR	1802.95	3.88			Unavailable
QLIYQTNSRPTGVPSR	1816.97	1.98			IPR 007110 (Ig-like)
SSSGFTCQVTHEGTIVEK	1966.92	1.32			P01846
AAPTVNLFPPSSEELGTNK	1972	1.06			P01846
ASGVPDRFSGSGSGTDFTLK	1985.96	1.86			Unavailable
FTDETLVSDLQPSLDRAR	2063.04	0.15			Unavailable
ATLVCLISDFYPGAVTVWK	2083.1	3.26			P01846
AGPLGWFERRPEPPPGPPSK	2172.14	2.39			Unavailable
QSNNKYAASSYLALSASDWK	2204.06	0.82			P01846
Unknown	2717.07				Unavailable
Unknown	2832.04				Unavailable

*L8B180,L8B0Z4,L8B0S2,L8B0S7,K7ZLA7 porcine IgG heavy chain entries (Fc) [22]; P01846, porcine IgG lambda constant domain (Fab); K7ZJP7, IgM heavy chain; P01786, mouse IgG heavy chain; P01857, human IgG heavy chain; IgGs. from [3]; IPR, GPM entries.

Table 1.
*Masses and sequences of peptides found in a glycopeptide-enriched fraction from porcine IgG (wild-type) tryptic digestion products. Red: Labeled manually; black: Sequenced and/or matched by GPM [24]. Glycopeptides, **m/z** values in bold not observed, only glycoforms.*
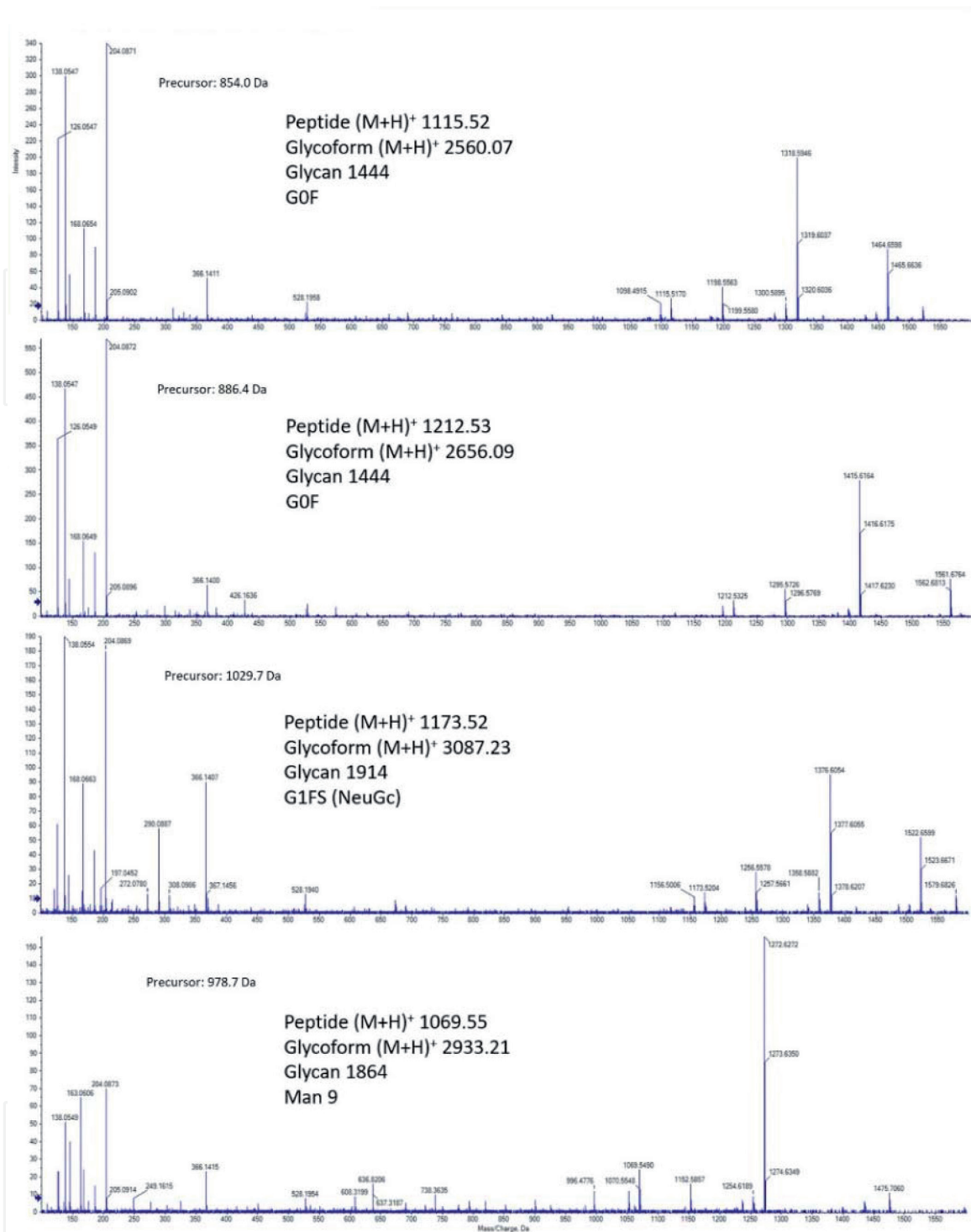
Figure 2.
Tandem mass spectra of doubly or triply charged ions of glycopeptides enriched from the tryptic products of porcine wild-type IgG. (a) GoF glycoform of EAQFNSTYR, (b) GoF of undetermined peptide, (c) G1FS of EEQFNSTYR, (d) Man-9 of undetermined peptide.

3.2 Fragmentation of porcine wild-type IgG with papain and fabulous™ followed by SEC

Antibody samples were first fragmented on immobilized papain, and thus it is expected that only IgG-related products will be present in the mixture. As shown by **Figure 3a**, all antibody was fragmented (intact antibody would have appeared at ca. 4.5 min). In general, when IgG is incubated with papain with a reducing agent, one or more peptide bonds in the hinge region are split, producing three fragments of similar size: two Fab fragments and one Fc fragment. The Fc may remain intact

based on conditions and enzyme used [9, 10]. The cleavages occur at cysteines around position 271 (in **Figure A1**), about 10 amino acids from the IdeS cleavage site [29]. In human IgG subtypes (IgG1-4), there are on average of three cysteines in the range of hinge region positions 265–275 to make the numbers correspond with those of **Figure A1**, where papain cleavages can be initiated [29]. Porcine IgG has similar cysteine motifs in these positions; however, **Figure A1** shows different lengths of amino acid chains in the hinge region, which did not seem to prevent fragmentation. Interestingly in this papain-fragmentation experiment, the Fc did not remain intact, but the Fab did. **Figure 3a** shows significant separation although not at the baseline, but which still allowed the collection of Fab and Fc fractions.

In order to further isolate Fab and Fc segments, collected fractions were analyzed in turn by SDS-PAGE. This allowed identification by mass, with confirmation by linear mode MALDI-TOF-MS (not shown) and in-gel tryptic digestion of the bands (analyzed by reflector mode MALDI-TOF-MS and HPLC-MS, next section). The first major peak in **Figure 3a** was identified as originating from the Fab, and the second peak was identified as the Fc. The intact Fab fragment is larger and thus elutes prior to the split Fc, while the opposite is usually true in the case of human IgG [15].

Fabulous™ is a recombinant cysteine protease which under reducing conditions digests in the hinge region of antibodies from many species and subclasses, including human, mouse, rat, and rhesus monkey, yielding Fab and Fc fragments [11]. Some specific fragmentation site information is available for human IgG1, mouse IgG1, and rabbit IgG, but no information at all pertains to the fragmentation of porcine
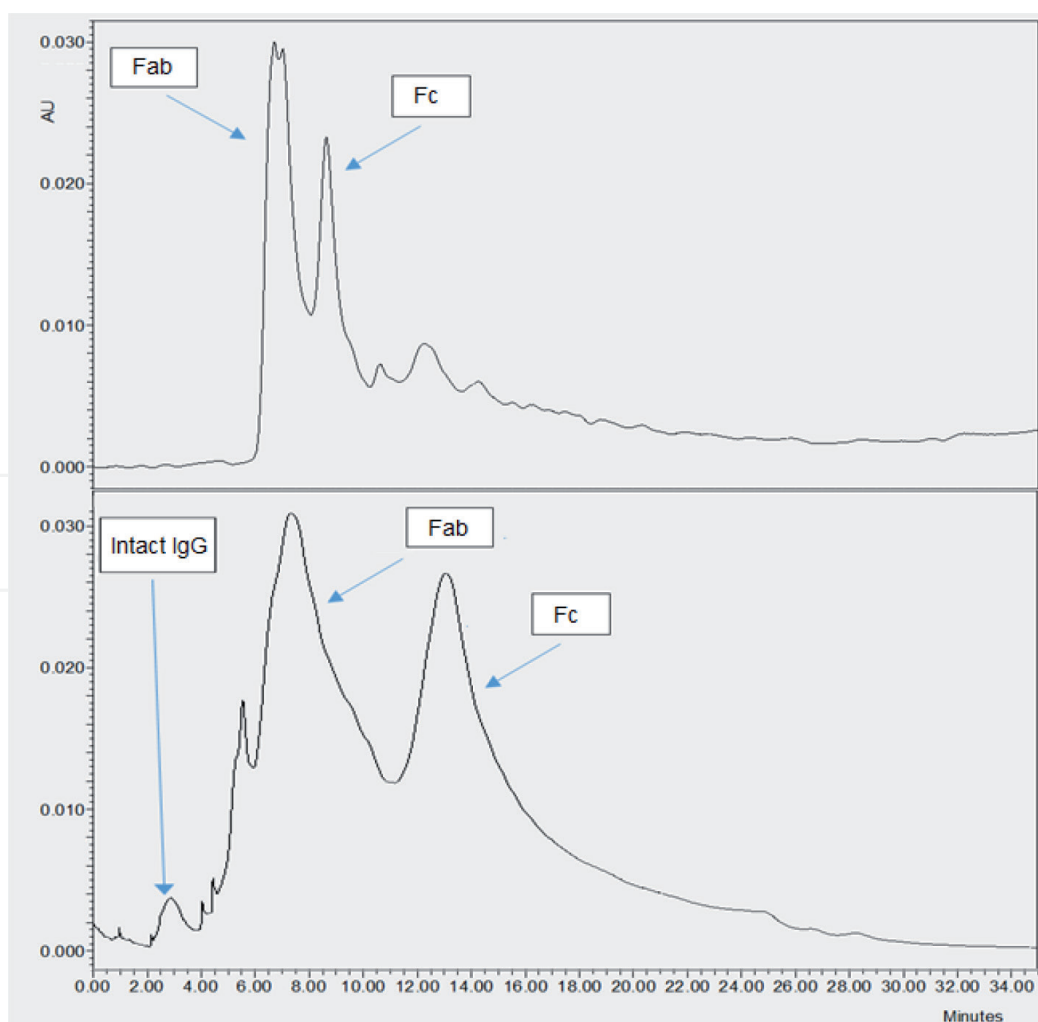

Figure 3.
Size exclusion chromatograms obtained for 200 μg of wild-type porcine IgG fragmented by (a) immobilized papain and (b) Fabulous™.

IgG. Looking at all porcine IgG subtype sequences (see alignment in **Figure A1**), they have a motif similar to that of rabbit IgG (KP^{270}I/CPP) [11], that is, with a potential fragmentation site between isoleucine I and cysteine C: CP^{270}I/CPG or CP^{270}I/CPA.

In general, when IgG molecules are incubated with Fabulous™ in the presence of a reducing agent, one or more peptide bonds in the hinge region are split next to a cysteine, producing two Fab fragments and one Fc. As the reducing agent is present during the digestion reaction, it is likely that all interchain thiols will be reduced.

In this experiment with Fabulous™ using 100 μg of pig IgG, a small portion of the antibody was not fragmented; otherwise the Fc was split into two halves and the Fab fragments remained intact. The HPLC-SEC chromatogram is shown in **Figure 3b**. The first major peak was indicative of the Fab fragment, and the second peak eluting after identified as the Fc, as verified later by SDS-PAGE and further tryptic digestion. As noticed in the chromatograms of **Figure 3**, elution times are different, that is, longer in portion b. This is due to gradual deterioration of the column, as both experiments depicted in a and b were performed several months apart.

The fact that Fabulous™ (28,724 Da [11]) was free in solution and not immobilized as in the case of papain involves that it would elute in the SEC chromatogram (**Figure 3b**), most probably in the second peak with the Fc. If this proprietary enzyme has a sequence similar to that of papain, subsequent proteolysis by trypsin is likely to occur extensively, as many lysine and arginine residues are present in papain's sequence [31].

3.3 SDS-PAGE separation of porcine IgG fragments

For papain-produced fragments, a nonreducing gel experiment was performed on the previously collected SEC fractions (**Figure 4a**). This experiment allowed confirming the identity of the fragments. A reducing SDS-PAGE experiment was also performed on intact pig IgG to serve as a control (**Figure 4b**). Bands were then excised from the gel, followed by in-gel tryptic digestion. This helped to single out heavy and light chains for differentiating between Fab and Fc according to their respective known glycopeptides and peptides [2, 3]. In **Figure 4a**, lanes 6–9 correspond to a fraction containing both Fab and Fc collected at the junction of SEC peaks in **Figure 3a**. A single 50 kDa Fab band and two 25 kDa bands were obtained. The Fab band is positioned just below the 50 kDa marker band and clearly below the Fc band in **Figure 4b**, and the Fab should have a lower molecular weight than intact Fc [32].
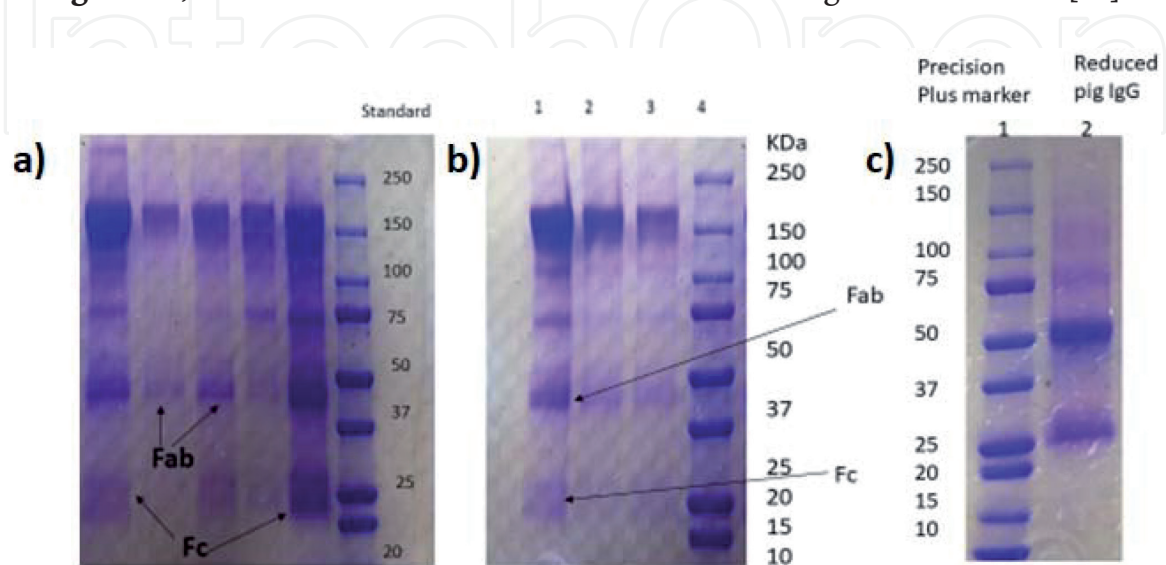

Figure 4.
(a) Fab and Fc SEC fractions from papain-fragmented porcine IgG on nonreducing SDS-PAGE and (b) from Fabulous™-fragmented porcine IgG, (c) whole porcine IgG runs under reducing conditions.

Both bands appearing in the Fc region were digested independently with trypsin as denoted by "upper Fc band" and "lower Fc band." It appears from further results that they belong to different IgG subtypes. For Fabulous™ fragments, the same experiment was conducted and results are shown in **Figure 4b**. According to gel separations, papain and Fabulous™ had very similar fragmentation effects on porcine IgG.

3.4 MALDI-MS of in-gel tryptic-digested Fc bands

Figure 5a shows the tryptic products for the upper Fc band (papain generated) in **Figure 4a**. The main glycopeptide observed has three main glycoforms at *m/z* 2472, 2618, and 2780 as [M + H]$^+$ ions. In **Figure 5b** (lower Fc band), two series of glycopeptides are observed, that is, the same as above and another one at *m/z* 2414, 2560, and 2722. The amino acid sequences of these glycopeptides were verified by MALDI-MS/MS as EEQFNSTYR and EAQFNSTYR, respectively [2, 3], with glycoforms as indicated in the figure. PNGase de-glycosylation of these two samples led to the spectrum of **Figure 5c**, where peptides at *m/z* 1117 (EAQFDSTYR) and 1175 (EEQFDSTYR) were detected, while the analysis of released glycans was not successful. Similar MALDI-MS and MS/MS results were obtained for the Fabulous™-generated porcine IgG that combined upper and lower Fc fragments digested with trypsin. Besides glycopeptides from the Fc region, other Fc γ-domain peptides were observed, as identified from the database [22] in **Table 2**.
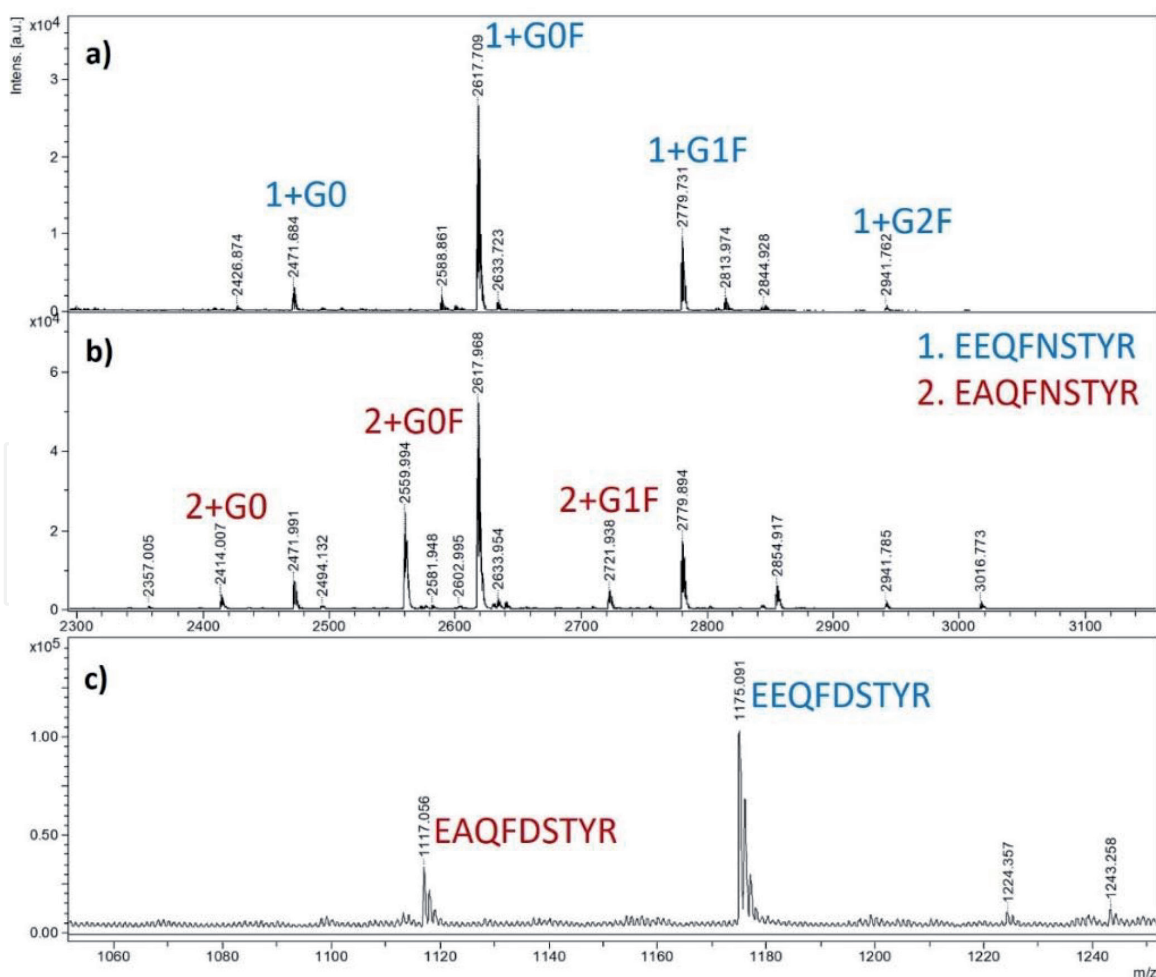

Figure 5.
*Reflector positive mode MALDI-TOF-MS spectra of (a) tryptic digestion products from pig IgG Fc fragment (higher 25 kDa band) and (b) lower Fc fragment. Both bands were excised from the gel run under non-reducing conditions shown in **Figure 4a**. (c) De-glycosylated peptides after removal of glycans to give peptides at m/z 1117 and 1175.*

Peptide sequence	*m/z* (M + H)+	Error ppm	*m/z* glyco-form	Glyco-form	Source*
Unknown	807.28				Unavailable
Unknown	842.38				Unavailable
Unknown	870.41				Unavailable
DLPAPITR	882.5	−5.78			IgG2ba-b,IgG4a-b,IgG6a-b, L8B180,L8B0S7,L8B0Z4
Unknown	905.33				Unavailable
Unknown	951.33				Unavailable
Unknown	993.36				Unavailable
Unknown	1033.4				Unavailable
Unknown	1107.4				Unavailable
EAQFNSTYR	**1115.5**	−22.51	2560.39	G0F	IgG6a,L8B180,L8B0Z4
Unknown	1165.4				Unavailable
EEQFNSTYR	**1173.50**	−14.6	2471.60 2779.62 2779.63	G0 G0F G1F	IgG1a-b,IgG2a-b,IgG4a-b,IgG5a-b, IgG6b,L8B0S7,L8B0S2,K7ZLA7
Unknown	1209.5				Unavailable
Unknown	1261.4				Unavailable
VNNVDLPAPITR	1308.6	−66.51			IgG1a-b,K7ZLA7
Unknown	1330.4				Unavailable
SNGQPEPEGNYR	1347.6	−1.48			IgG1a,IgG6a-b,L8B180,L8B0S2, K7ZLA7,L8B0Z4
Unknown	1374.4				Unavailable
Unknown	1392.2				Unavailable
Unknown	1427.6				Unavailable
Unknown	1475.6				Unavailable
Unknown	1503.4				Unavailable
LVESGGGLVQPGGSLR	1525.7	−74.23			L8B180,L8B0S7

Peptide sequence	*m/z* (M + H)+	Error ppm	*m/z* glyco-form	Glyco-form	Source*
VSSQNIQDFPSVLR	1589.8	42.9			K7ZJP7 (IgM HC const. region)
YAASSYLALSASDWK	1632.8	−24.5			P01846 UniprotKB (Ig λ const. region)
VVSVLPIQHQDWLK	1662	13.7			IgG1a-b,IgG3,IgG6b,L8B180,K7ZLA7, L8B0Z4
Unknown	1677.5				Unavailable
Unknown	1693.7				Unavailable
Unknown	1725.4				Unavailable
Unknown	1740.6				Unavailable
Unknown	1754.4				Unavailable
STGKPTLYNVSLVLSDT	1794.8	−60.4			K7ZJP7 (IgM HC const. region)
EPQVYTLSPSAEELSR	1805.8	−34.1			IgG6a-b,L8B180,L8B0Z4
EPQVYTLPPPAEELSR	1825.9	2.9			IgG1a-b,K7ZLA7
EPQVYTLPPPTEELSR	1855.9	−2.32			IgG4b
TTPPQQDVDGTFFLYSK	1943.8	−50.7			IgG1a-b,K7ZLA7
TTPPQQDVDGTYFLYSK	1959.9	−1.07			IgG2a-b,IgG3,IgG4a-b,IgG6a,L8B180, L8B0S7,L8B0Z4
AAPTVNLFPPSSEELGTNK	1971.9	−36.6			P01846 UniprotKB (Ig λ const. region)
Unknown	2135.8				Unavailable
Unknown	2162.9				Unavailable
Unknown	2211				Unavailable
GLEGLAYIGYTGVITDYADSVK	2305.4	86.8			L8B180

*L8B180,L8B0Z4,L8B0S2,L8B0S7,K7ZLA7, porcine IgG heavy chain entries (Fc) [22]; P01846, porcine IgG lambda constant domain (Fab); K7ZJP7, IgM heavy chain; P01786, mouse IgG heavy chain; P01857, human IgG heavy chain; IgGs, from [3]; IPR, GPM entries.

Table 2.
Masses and sequences of peptides found by MALDI-MS in porcine IgG (wild-type) tryptic digestion products of combined Fc bands obtained by papain and Fabulous™ fragmentation. Peptides and glycopeptides in red were sequenced manually after MS/MS and/or tentatively identified by mass fingerprinting.

3.5 In-gel tryptic-digested fab bands analyzed by MALDI-MS

The bands just below 50 kDa from both papain and Fabulous™ digestion processes were excised from the gel and digested with trypsin. MALDI-MS spectra of the products were obtained, and display no obvious glycopeptides as found in the Fc. Some peaks could be identified as light and heavy chain Fab peptides. Fab peptide masses and sequences available are listed in **Table 3**.

Sequence	*m/z* ESI	*m/z* MALDI	Error ppm	Identification source
FSGAISGNK	880.45	880.39	−70.8	P01846 (Ig λ const. region)
Unknown	951.33	951.21		Unavailable
Unknown	993.36	993.56		Unavailable
Unknown		1052.22		Unavailable
Unknown		1119.29		Unavailable
Unknown		1141.26		Unavailable
Unknown	1209.48	1209.28		Unavailable
Unknown		1242.51		Unavailable
FSGSGSGTDFTLK	1303.62	1303.61	−4.91	Unavailable
Unknown		1339.55		Unavailable
Unknown		1374.27		Unavailable
Unknown		1383.95		Unavailable
Unknown		1419.30		Unavailable
Unknown		1476.02		Unavailable
Unknown	1503.39	1504.12		Unavailable
Unknown		1526.39		Unavailable
Unknown		1584.35		Unavailable
Unknown		1622.45		Unavailable
YAASSYLALSASDWK	1632.79	1632.85	36.7	P01846 (Ig λ const. region)
Unknown		1660.16		Unavailable
Unknown		1735.19		Unavailable
Unknown		1762.23		Unavailable
QLIYSTNNRPTGVPSR	1802.95	1803.00	27.73	Unavailable
Unknown		1826.41		Unavailable
Unknown		1866.38		Unavailable
Unknown		1910.24		Unavailable
Unknown		1942.26		Unavailable
AAPTVNLFPPSSEELGTNK	1972.00	1972.18	90.2	P01846 (Ig λ const. region)
Unknown		2034.37		Unavailable
FTDETLVSDLQPSLDRAR	2063.04	2063.17	62.9	Unavailable
Unknown	2135.81	2136.40		Unavailable
Unknown	2210.95	2211.46		Unavailable
Unknown		2338.64		Unavailable
Unknown		2377.63		Unavailable
Unknown		2408.38		Unavailable
VTLTCLVTGFYPPDIDVEWQR	2509.24	2509.39	58.5	IgG4a

Sequence	*m/z* ESI	*m/z* MALDI	Error ppm	Identification source
Unknown		2691.50		Unavailable
Unknown		2807.49		Unavailable

Table 3.
*Fab tryptic peptides from porcine IgG fragmented with papain and fabulous™. Second column (m/z ESI): Already identified in **Table 1**. Error: Measured using the MALDI m/z values against calculated values. Sequences in red are assigned from mass only based on **Table 1**.*

Overall, MALDI-TOF-MS experiments allowed suggesting that efficient separation of Fab and Fc fragments from each other occurred, using the SEC/SDS-PAGE procedure. There was a minimal number of overlapping peptides between Fab and Fc MALDI spectra (**Tables 2** and **3**). In **Table 3**, peptides in red had already been sequenced and appeared in **Table 1**; peptides whose *m/z* values appear in the second column were also observed in **Table 1**, although not sequenced. Overlapping peptides between **Tables 2** and **3** are at *m/z* 2509 (unknown), 2884 (glycoform of *m/z* 1115), and *m/z* 1677 (IgM). The presence of IgM in the sample had been noticed in an earlier report [3].

3.6 Tryptic products of Fabulous™-fragmented porcine IgG analyzed by HPLC/MS

Each sample of digestion products from Fc and Fab bands were analyzed twice by HPLC/MS, and results are summarized in **Table 4**. In **Figure A2**, total ion chromatograms (TIC) of the pig Fab and Fc tryptic products showed differences in the retention times of some peaks as expected. Peaks observed at the same retention times (e.g., 4.41–4.46, 10.35, and 23.10 min) and common to all injections corresponded to singly charged ions of small compounds of *m/z* < 700 and were not considered in the analysis, as they probably were non-peptide contaminants. Peaks at 5.96 min also present in both chromatograms corresponded to a mass 1660 peptide from the Fab, AGGTTVTQVETTKPSK. Non-glycosylated peptides in **Table 4** were identified through MS/MS and database search, while glycopeptides were assigned by mass only in reference to entries in **Table 1**.

As Fabulous™ was possibly still present in the mixture at the time of tryptic digestion of the Fc gel band, analogous papain tryptic digestion products were sought for by *m/z*, but not found. Only one papain peptide with sequence YIDETNK (*m/z* 882.4203) could be present according to its nominal mass; however, the observed *m/z* 882.4998 peptide ions are closer to the calculated mass of IgG DLPAPITR, 882.4939. As seen in **Table 4**, many peptides remain un-sequenced/unidentified, and others are identified as either Fab or Fc peptides of porcine IgG. Interestingly, there were some very good matches with human and mouse IgG, which is not surprising given relatively high levels of homology between mammal species in general. However even with all the heavy chain sequences available in the literature for porcine IgG (see footnote of **Table 4** and **Figure A1**), none of them contained the human- and murine-assigned peptides. It is also interesting to observe that most peptides are predominant in either the Fab or Fc injections and that overlapping peptides are clearly more present in one sample than in the other. Accordingly, all Fab P01846 peptides (*m/z* 880, 1175, 1274, 1375, 1533, 1590, and 1661) were more abundant in the Fab than the Fc sample, although all these peptides showed some level of overlap. The same reasoning is applicable to Fc peptides, including the *N*-glycoforms of EAQFNSTYR and EEQFNSTYR. It is also possible to know the origin of most unidentified peptides in **Table 4** by comparing their normalized abundances. Other *N*-glycopeptides reported in **Table 1** were not detected in these Fab and Fc RPLC/MS experiments, possibly due to the lack of glycopeptide enrichment and to sample loss during the SEC and SDS-PAGE procedures.

Mass (calc.)	m/z (M + H) + (exp.)	m/z error (ppm)	Normalized abundance		Sequence	Sources of identification[*]
			Pig Fab wt (avg)	Pig Fc wt (avg)		
516.301	517.309	0.00	0.0	23.9	VDKR	IgG1a-b,IgG2a-b,IgG3,IgG4a-b
585.322	586.330	−1.97	76.1	1972.6	PGGSLR	P01786
712.372	713.380	−4.25	8.0	891.4	LVESGGGL	P01786
768.412	769.420		0.0	9.4		
806.345	807.353		652.0	22.4		
811.439	812.447	−5.63	6.1	424.4	LVESGGGLV	P01786
816.416	817.424		0.0	78.4		
824.372	825.380		0.0	9.1		
826.434	827.442		749.4	1176.6		
827.511	828.519		478.3	166.0		
834.421	835.429		0.0	65.1		
837.492	838.500	−5.12	14.2	0.0	ALPAPIEK	P01857
841.502	842.510		977.9	1260.9		
844.291	845.299		264.0	0.0		
850.418	851.426		0.0	42.4		
861.432	862.440		220.3	52.3		
872.373	873.381		0.0	74.7		
872.397	873.405		0.0	15.5		
879.445	880.453	−0.45	1559.7	479.8	FSGAISGNK	P01846
881.492	882.500	−5.78	0.0	410.6	DLPAPITR	IgG2ba-b,IgG4a-b, IgG6a-b,L8B180,L8B0S7,L8B0Z4

Mass (calc.)	*m/z* (M + H) + (exp.)	*m/z* error (ppm)	Normalized abundance		Sequence	Sources of identification[*]
			Pig Fab wt (avg)	Pig Fc wt (avg)		
888.366	889.374		0.0	44.9		
917.391	918.399		254.9	54.6		
919.450	920.458		17.9	135.1		
935.856	936.864		2502.6	2690.0		
939.510	940.518	7.61	86.4	407.8	LVESGGGLVQ	P01786
990.341	991.349		264.4	5.6		
994.506	995.513		13.2	518.7		
994.512	995.520		7.6	311.7		
1043.899	1044.907		1589.5	1690.0		
1044.554	1045.562	−2.30	5106.9	6511.7	LSSPATLNSR	Unavailable
1057.831	1058.839		1179.7	1190.5		
1066.539	1067.547	−1.47	0.0	8.5	VDGVEVHNAK	P01857
1103.601	1104.609		0.0	17.0		
1164.577	1165.585		66.4	199.4		
1174.613	1175.621	−5.01	468.4	169.5	TQGVETTKPSK	P01846
1208.640	1209.648		0.0	790.8		
1238.648	1239.656		1.6	343.5		
1259.553	1260.561		3.7	480.5		
1260.536	1261.544		15.5	1386.4		
1273.685	1274.693	−1.89	324.0	104.5	VTQGVETTKPSK	P01846
1296.678	1297.686		9.3	727.0		
1298.485	1299.493		30.8	263.2		
1302.609	1303.617	0.00	2446.2	908.9	FSGSGSGTDFTLK	Unavailable

Mass (calc.)	*m/z* (M + H) + (exp.)	*m/z* error (ppm)	Normalized abundance		Sequence	Sources of identification[*]
			Pig Fab wt (avg)	Pig Fc wt (avg)		
1307.718	1308.726	−1.30	139.6	13772.8	VNNVDLPAPITR	IgG1a-b,K7ZLA7
1308.702	1309.710		13.2	3997.2		
1312.672	1313.680	−1.22	2.4	306.7	ESGGGLVQPGGSLR	P01786
1316.625	1317.633		896.9	223.7		
1329.687	1330.695		20.3	434.9		
1330.670	1331.678		87.9	1324.0		
1334.648	1335.656		23.1	374.1		
1340.548	1341.556		131.8	45.0		
1345.666	1346.674		5.8	2149.3		
1346.587	1347.595	1.41	47.2	658.2	SNGQPEPEGNYR	IgG1a,IgG5b,IgG6b,L8B180K7ZLA7,L8 B0Z4
1347.568	1348.576		50.8	1702.0		
1350.620	1351.628		7.9	512.8		
1368.616	1369.624		16.4	484.9		
1374.732	1375.740	−2.30	742.1	219.7	TVTQGVETTKPSK	P01846
1376.558	1377.566		457.8	61.0		
1390.608	1391.616		3.4	308.0		
1391.593	1392.601		6.6	898.0		
1399.808	1400.816		1434.9	1503.7		
1433.682	1434.690		8.5	226.8		
1449.688	1450.696		17.8	958.3		

Mass (calc.)	m/z (M + H) + (exp.)	m/z error (ppm)	Normalized abundance		Sequence	Sources of identification[*]
			Pig Fab wt (avg)	Pig Fc wt (avg)		
1514.667	1515.675		100.8	1436.0		
1524.822	1525.830	−2.62	1005.8	20216.6	LVESGGGLVQPGGSLR	L8B180, L8B0S7
1530.664	1531.672		0.3	599.0		
1532.803	1533.811	−0.91	646.1	171.1	GTTVTQGVETTKPSK	P01846
1546.798	1547.806		64.1	2405.4		
1562.772	1563.780		192.8	4750.7		
1578.744	1579.752	47.70	4.7	439.3	EEQFNSTYR +2GlcNAc	IgG1a-b,IgG2a-b,IgG4a-b,IgG5a-b,IgG6b,L8B0S7,L8B0S2,K7ZLA7
1589.823	1590.831	−1.93	647.8	181.7	GGTTVTQGVETTKPSK	P01846
1624.841	1625.849		321.8	72.8		
1642.851	1643.859	−0.92	1827.2	614.7	AGGTTVTQGVETTKPSK	[C-term] neutral loss
1660.864	1661.872	0.26	11688.6	4028.5	AGGTTVTQGVETTKPSK	P01846
1682.829	1683.837		346.8	77.9		
1698.811	1699.819		1146.9	308.5		
1719.703	1720.711		10.5	471.9		
1735.702	1736.710		0.0	480.4		
1753.805	1754.813		2.7	493.2		
1769.797	1770.805		4.8	723.1		

Mass (calc.)	m/z (M + H) + (exp.)	m/z error (ppm)	Normalized abundance		Sequence	Sources of identification[*]
			Pig Fab wt (avg)	Pig Fc wt (avg)		
1801.950	1802.958	3.60	191.0	99.2	QLIYSTNNRPTGVPSR	Unavailable
1807.752	1808.760		4.8	287.3		
1824.931	1825.939	2.90	4.0	670.0	EPQVYTLPPPAEELSR	IgG1a-b,K7ZLA7
1854.932	1855.940	−2.32	0.0	1499.6	EPQVYTLPPPTEELSR	IgG4b
1862.867	1863.875		0.0	695.8		
1919.930	1920.938		1.8	0.0		
1958.924	1959.932	−1.07	3.4	2082.2	TTPPQQDVDGTYFLYSK	IgG4a-b, IgG6a, L8B180, L8B0S7, IgG2a, IgG2b, IgG3
2092.889	2093.897		649.7	14.8		
2251.915	2252.923		0.0	750.1		
2267.910	2268.918		0.0	356.8		
2289.908	2290.916		0.0	72.8		
2355.931	2356.939		0.0	62.0		
2413.960	2414.968	−11.42	0.0	2793.9	G0 of EAQFNSTYR	IgG6a,L8B180,L8B0Z4
2435.939	2436.947		0.0	86.5		
2451.911	2452.919		0.0	90.0		
2558.988	2559.995	−19.69	0.0	25.6	G0F EAQFNSTYR	IgG6a,L8B180,L8B0Z4
2576.008	2577.016		0.0	439.0		
2617.036	2618.044	−2.66	3.5	1257.9	G0F EEQFNSTYR	IgG1a-b,IgG2a-b,IgG4a-b,IgG5a-b,IgG6b,L8B0S7,L8B0S2,K7ZLA7

Mass (calc.)	m/z (M + H) + (exp.)	m/z error (ppm)	Normalized abundance		Sequence	Sources of identification[*]
			Pig Fab wt (avg)	Pig Fc wt (avg)		
2639.006	2640.014		0.0	706.3		
2654.984	2655.992		2.5	1048.1		
2670.955	2671.963		0.0	200.5		
2692.958	2693.966		0.0	49.2		
2779.092	2780.100	−1.36	0.0	1327.0	G1F EEQFNSTYR	IgG1a-b,IgG2a-b,IgG4a-b,IgG5a-b,IgG6b,L8B0S7,L8B0S2,K7ZLA7
2801.070	2802.078		2.8	244.1		
2817.033	2818.041		0.0	380.8		
2941.166	2942.174	5.90	0.0	266.4	G2F EEQFNSTYR	IgG1a-b,IgG2a-b,IgG4a-b,IgG5a-b,IgG6b,L8B0S7,L8B0S2,K7ZLA7
3004.216	3005.224		0.0	68.7		
3086.182	3087.190	−1.33	0.0	159.4	G1FS EEQFNSTYR	IgG1a-b,IgG2a-b,IgG4a-b,IgG5a-b,IgG6b,L8B0S7,L8B0S2,K7ZLA7

[*]*L8B180,L8B0Z4,L8B0S2,L8B0S7,K7ZLA7, porcine IgG heavy chain entries (Fc) [22]; P01846, porcine IgG lambda constant domain (Fab); K7ZJP7, IgM heavy chain; P01786, mouse IgG heavy chain; P01857, human IgG heavy chain; IgGs, from [3]; IPR, GPM entries.*

Table 4.
Tryptic peptides from pig IgG Fab and Fc fragment analyzed by HPLC-MS/MS.

3.7 Comparative aspects of both workflows

The initial RPLC-MS/MS experiment on the enriched whole IgG digest produced the most useful information in terms of glycopeptides. Besides the latter, many peptides were identified as originating from the Fc or Fab portion, while for other peptides origin remained unknown. This first workflow allowed detecting *N*-glycopeptides from the Fab portion of the antibody, although their sequences could not be completely assigned. The second workflow necessitated more intensive work than the first one. Size-exclusion chromatography or SDS-PAGE was not sufficient on its own to properly separate Fab and Fc, and a combination of both was preferable. Even then, RPLC/MS results show partial overlaps for many peptides, although most peptides were much more abundant in one sample (Fc or Fab) than in the other. In terms of ionization modes used in the second workflow, ESI yielded more details on sialylated glycopeptides, but did not allow the observation of Fab *N*-glycopeptides. As analyses by MALDI were the result of depositing all tryptic products of a gel band onto one target spot, it is probable that the signals of sialylated glycopeptides or low concentration Fab glycopeptides were overtaken competitively by peptides that are more abundant.

4. Conclusions

Porcine immunoglobulins constitute a complex ensemble of biomolecules, with several subtypes whose amino acid sequences are not clearly assigned and described in the literature (see **Figure A1**). In this study, three state-of-the-art mass spectrometers were used to characterize tryptic peptides, offering a considerable amount of complementary information owing to the great sensitivities of these instruments, given the small amounts of IgGs used. Indeed, fragmentation of porcine IgG into its Fc and Fab portions was achieved for the first time using papain and Fabulous™ on 200 µg or less of antibody. Fragments needed separation by both SEC and SDS-PAGE before analysis by MALDI-TOF-MS and HPLC/MS. These separation procedures did not eliminate the overlapping of Fab and Fc tryptic peptides entirely; however, there was a significant level of discrimination. This workflow resulted in better knowledge about the origin of tryptic peptides from IgG.

During the course of this work, the several sources of sequence information on porcine IgG found in **Figure A1** were used to verify HPLC-MS/MS, MALDI-TOF-MS, and HPLC-MS data. Most Fc peptides were from the gamma constant region, where some Fab peptides were identified as belonging to the constant portion of the lambda chain. The HPLC-MS/MS method of tryptic peptide without previous Fab-Fc fragmentation was the most efficient in terms of useful data generated per amount of sample used, although many peptides could not be related to either Fc or Fab. This study highlights the need for detailed pertinent sequence information for porcine IgG, which is not a commonly studied set of biomolecules. Future work will involve the quantification of IgG subtypes according to unique peptide sequences that are already known in each subtype.

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Conflict of interest

None of the authors has a conflict of interest.

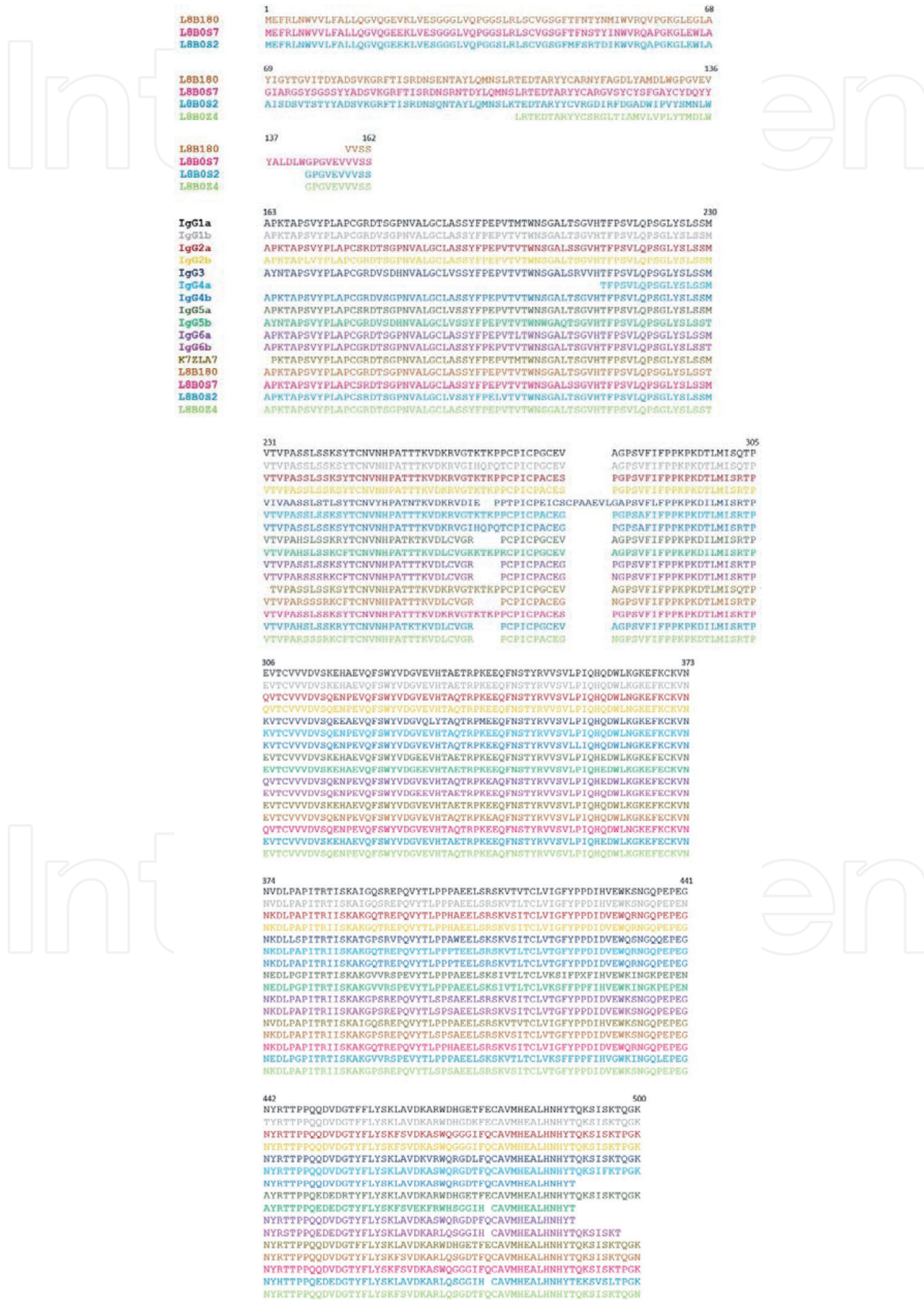## A. Appendix: supplementary materials

Figure A1.
Alignment of sequences available in the literature for the porcine IgG heavy chains. IgGn: from Ref. [3]. Others: UniprotKB [22].
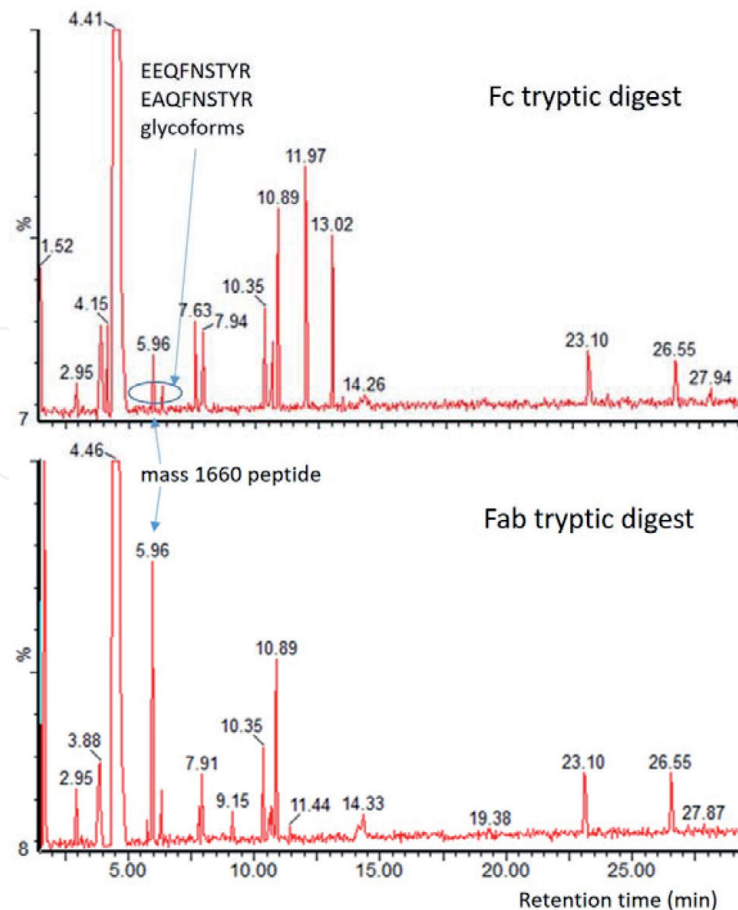
Figure A2.
HPLC/MS total ion chromatograms obtained for the tryptic products of wild-type porcine IgG Fabulous™ fragments, (a) Fc and (b) Fab.

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