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***Highlights (for review)**

- Methacrylate-based monolithic reactors with immobilized PNGase F were prepared in capillary format.
- Oriented and non-oriented immobilization procedures were compared.
- The prepared PNGase F reactors were used for deglycosylation of glycoproteins followed by CE/LIF and/or MALDI/MS analysis.

ORIENTED IMMOBILIZATION OF PEPTIDE-N-GLYCOSIDASE F ON A MONOLITHIC SUPPORT FOR GLYCOSYLATION ANALYSIS

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Abstract

In this paper we report on a novel oriented peptide-N-glycosidase F (PNGase F) immobilization approach onto methacrylate based monolithic support for rapid, reproducible and efficient release of the N-linked carbohydrate moieties from glycoproteins. The glutathione-S-transferase-fusion PNGase F (PNGase F-GST) was expressed in *E. coli* using regular vector technology. The monolithic pore surface was functionalized with glutathione via a succinimidyl-6-(iodoacetyl-amino)-hexanoate linker and the specific affinity of GST towards glutathione was utilized for the oriented coupling. This novel immobilization procedure was compared with reductive amination technique commonly used for non-oriented enzyme immobilization via primary amine functionalities. Both coupling approaches were compared using enzymatic treatment of several glycoproteins, such as ribonuclease B, fetuin and immunoglobulin G followed by MALDI/MS and CE-LIF analysis of the released glycans. Orientedly immobilized PNGase F via GST-glutathione coupling showed significantly higher activity, remained stable for several months, and allowed rapid release of various types of glycans (high-mannose, core fucosylated, sialylated, etc.) from glycoproteins. Complete protein deglycosylation was obtained as fast as in several seconds when using flow-through immobilized microreactors.

Keywords: enzyme microreactor, oriented immobilization, monolith, PNGase F, deglycosylation.

Abbreviations: AIBN, 2,2´-azobisisobutyronitrile, APTS, 8-aminopyrene-1,3,6-trisulphonic acid; EDMA, ethylene dimethacrylate; GMA, glycidyl methacrylate; GSH, glutathione; GST, glutathione-S-transferase; PNGase F, peptide-N-glycosidase F.

1 Introduction

Microreactors are becoming important tools in many research and development areas ranging from organic synthesis [1] to diesel fuel production [2,3]. Immobilized enzyme reactors are under rapid recent development, especially for bioanalytical purposes related to the omics fields. Besides the most common immobilized trypsin reactors [4,5] other enzymes, including, e.g., the endoproteinase LysC have been also successfully used [6]. While many different forms of solid support can be used for enzyme immobilization, including chromatographic particles [7], self-assembled magnetic beads [8] or open fused silica capillary surfaces [9], porous monoliths represent a promising choice due to their excellent mechanical and chemical properties, which can be easily fine-tuned for a plethora of special applications [10].

Enzymatic release of glycans from glycoproteins represents a key step in analytical glycomics. Peptide-N-glycosidase F (PNGase F) is one of the most frequently used endoglycosidases utilized to release N-linked glycans. The common in-solution deglycosylation method is a relatively time-consuming process requiring several hours up to overnight for complete removal of all N-linked glycans. While it has been shown that the deglycosylation time can be reduced to minutes by microwave irradiation [11,12] or pressure-cycling [13], the simplest way to speed up the reaction is increasing the enzyme to substrate ratio by immobilization of PNGase F on a solid support. Both particulate and monolithic supports were used for PNGase F immobilization via non-specific coupling chemistries including CNBr-activated Sepharose 4B [14], glycidoxypropyltrimethoxysilane modified silica beads [15], carboxylated nanodiamonds [16], or acrylic polymer particles containing amine functionalities [17]. Solid supports with immobilized PNGase F can be suspended in the glycoprotein solution [16], packed into a column [14], capillary [17] or microfluidic chip [15] in order to create flow-through reactors. Monolithic PNGase F reactors prepared in capillary format were reported earlier [18-20] as promising alternatives to packed column reactors. Palm and Novotny immobilized PNGase F on the surface of polyacrylamide based monoliths via succinimide functionalities [18]. Immobilization of PNGase F on monolithic support via azlactone chemistry [19] or reductive amination [20] was just recently published.

Currently, all coupling methods employ non-specific reactions between the primary amines of PNGase F with reactive functionalities generated on a solid support surface. Since amino groups are also present at the active site of the enzyme molecule, the resulting non-oriented immobilization may negatively affect accessibility of the active site leading to reduced activity of the immobilized enzyme. It can be expected that enzyme immobilization

through a selected functional group instead of randomly reacting amines will result in predictable, and therefore better performing microreactors.

With the use of recombinant DNA technology a wide variety of proteins, including enzymes, can be produced with specific sequences allowing site selective binding. Such fusion systems were developed for purification of recombinant proteins from cell lysates through affinity capture. While the tags are usually removed from the protein molecules before further use, they offer a special advantage as specific moieties for oriented immobilization. In particular, a discrete modification using a highly specific tag attached to the enzyme molecule during its synthesis can be employed for direct coupling reactions away from the active center of the enzyme molecule.

In this work we report on a novel oriented PNGase F immobilization method onto a methacrylate based monolithic support prepared in a 250 μm ID fused silica capillary for rapid and efficient N-glycan removal from glycoproteins. Our approach is based on the affinity of glutathione (GSH) immobilized on the monolithic surface towards glutathione-S-transferase fusion PNGase F (PNGase F-GST). The developed oriented immobilization was compared with the non-oriented approach in enzymatic treatments of several standard glycoproteins including ribonuclease B, fetuin and immunoglobulin G, followed by off-line MALDI/MS and CE-LIF analysis of released glycans.

2 Experimental part

2.1 Materials and reagents

Glycidyl methacrylate (GMA), ethylene dimethacrylate (EDMA), 1-dodecanol, cyclohexanol, 2,2´-azobisisobutyronitrile (AIBN), 3-(trimethoxysilyl)propyl methacrylate, sodium periodate, sodium cyanoborohydride, chloroform, iodomethane, dithiotreitol, and iodoacetamide were purchased from Sigma-Aldrich (Prague, Czech Republic). GMA and EDMA were purified by passing them through a column containing basic alumina inhibitor remover (Sigma-Aldrich). Succinimidyl-6-[(iodoacetyl)amino]hexanoate was purchased from Chem-Impex International (Wood Dale, IL). 8-aminopyrene-1,3,6-trisulphonic acid (APTS) was purchased from Beckman Coulter (Brea, CA).

PNGase F-GST was expressed in *E. coli* using regular vector technology and purified by affinity chromatography [21,22]. The expression of PNGase F-GST was confirmed by SDS-PAGE of cell lysates followed by Coomassie Brilliant Blue R-250 staining.

Polyclonal human immunoglobulin G (hIgG), bovine ribonuclease B (RNase B), bovine fetuin, PNGase F, glutathione, super-dihydroxybenzoic acid (9:1 mixture of dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid) and sinapinic acid were purchased from Sigma-Aldrich.

Polyimide-coated fused-silica capillary (250 μm ID, 375 μm OD) was purchased from Polymicro Technologies (Phoenix, AZ).

2.2 Instrumentation

MALDI/MS measurements were carried out using an AB SCIEX TOF/TOF 5800 system (Framingham, MA, USA). CE-LIF separations were performed on a P/ACE MDQ instrument (Beckman Coulter) equipped with an Ar-ion laser (488 nm excitation wavelength) for fluorescent detection.

The elemental composition of monolithic columns was determined by scanning electron microscopy with energy dispersive X-ray analysis (SEM/EDAX) on a JEOL JSM-5500 LV instrument equipped with an analyzer IXRF Systems and a detector Gresham Sirius 10.

2.3 Preparation of monolithic poly(glycidyl methacrylate-co-ethylene dimethacrylate) support

The inner surface of the fused silica capillary was first activated with 3-(trimethoxysilyl)propyl methacrylate [23]. The polymerization mixture consisting of 25.5% GMA, 17.5% EDMA, 40% 1-dodecanol, 17% cyclohexanol, and 1% AIBN (with respect to monomers) (all percentages w/w) was purged with nitrogen for 10 min. The vinylized capillary was filled with this mixture and thermally initiated polymerization was carried out in a water bath at 60 °C for 24 h. After polymerization, the porogenic solvents were removed from the monolith by pumping acetonitrile through the column at a flow rate of 60 μL/h for 1 h.

2.4 Oriented immobilization of PNGase F-GST

The poly(GMA-co-EDMA) monolithic column was filled with a 30% (v/v) solution of ammonium hydroxide, sealed with silicone septa and heated in a column oven to 70 °C for 3 h. The modified column was washed with water (150 μL/h, 1 h) and dimethyl sulfoxide (DMSO) (150 μL/h, 15 min). The resulting primary amine functionalized monolithic column

was flushed with a solution of succinimidyl-6-[(iodoacetyl)amino]hexanoate in DMSO (10 mg/mL) at a flow rate of 150 µL/h for 3 h in dark at room temperature. Next, a solution of glutathione (10 mg/mL) in 20 mmol/L Tris-HCl buffer, pH 8.3 was pumped through the iodoacetate functionalized column at a flow rate of 150 µL/h for 3 h in dark at room temperature. The scheme of the activation procedure is in Figure 1. The glutathione modified column was washed with the 20 mmol/L Tris-HCl buffer, pH 8.3. PNGase F-GST (1 mg/mL) was dissolved in 10 mmol/L phosphate buffered saline (PBS). The enzyme solution was pumped through the glutathione modified monolithic column at room temperature for 2.5 h with a flow rate of 100 µL/h. The PNGase F-GST conjugated monolith was then washed with 10 mmol/L PBS (100 µL/h, 1 h) and stored at 4 °C before further use.

2.5 Non-oriented immobilization of PNGase F-GST

The poly(GMA-co-EDMA) monolithic column was filled with 0.5 mol/L sulfuric acid, sealed with silicone septa and heated in a column oven to 70 °C for 3 h. The monolithic column was washed with water (150 µL/h, 1 h) followed by rinsing with a freshly prepared solution of 0.1 mol/L sodium periodate (150 µL/h, 1.5 h). PNGase F-GST (1 mg/mL) was dissolved in 10 mmol/L PBS, containing 3 mg/mL sodium cyanoborohydride. The enzyme solution was pumped through the aldehyde functionality modified monolith at room temperature for 2.5 h with a flow rate of 100 µL/h. The monolith conjugated with PNGase F-GST was then washed with 10 mmol/L PBS (100 µL/h, 1 h) and stored at 4 °C before further use.

2.6 Sample preparation

Stock solutions (5 mg/mL) of ribonuclease B, fetuin and hIgG were prepared in 5 mmol/L sodium bicarbonate, pH 8.0 containing 10 % acetonitrile. Ribonuclease B and fetuin were denaturated before deglycosylation by the addition of dithiotreitol to the glycoprotein solution in a final concentration of 5 mmol/L and incubation at 65 °C for 15 min. Next, iodoacetamide was added in a final concentration of 5 mmol/L and the solution was incubated in dark at room temperature for 15 min. Before deglycosylation, the glycoprotein solutions were diluted by 5 mmol/L sodium bicarbonate, pH 8.0, containing 10 % acetonitrile.

2.7 Release of N-glycans using immobilized PNGase F-GST

Deglycosylation using immobilized PNGase F-GST was carried out at room temperature for 1-6 min. The glycoprotein solutions (1.0-2.5 mg/mL) were pumped through the monolithic reactors (2.5-10 cm) at different flow rates by means of a syringe pump. The released glycans were collected in microvials and stored at 4 °C before further analysis.

2.8 Release of glycans using soluble PNGase F and PNGase F-GST

Deglycosylation of denaturated ribonuclease B (50 μg) was performed using soluble PNGase F-GST and commercially available PNGase F (Sigma Aldrich). The enzyme was added to the protein solution in a concentration of 0.1 nmol/L and deglycosylation was carried out at 37 °C for 2 h. The released glycans were collected in microvials and stored at 4 °C before further analysis.

2.9 Labeling and purification of the released glycans

The deglycosylated proteins were removed from the collected fractions (50 μL) using 10 kDa MWCO centrifugal filter units (Millipore, Billerica, MA) before the glycan labeling step. The glycan-containing supernatants were dried in a SpeedVac concentrator and labeled via reductive amination by the addition of 2 μL of 50 mmol/L APTS in 15% acetic acid and 4 μL of 1 mol/L sodium cyanoborohydride in tetrahydrofuran. The labeling reaction was performed at 37 °C overnight. Labeled glycans were purified using cleanup cartridges (Prozyme, Hayward, CA) following the protocol suggested by the manufacturer. The APTS labeled glycan solutions were collected in microvials, dried in a SpeedVac concentrator and redissolved in water (50 μL) before CE-LIF analysis.

2.10 CE-LIF analysis of the labeled glycans

CE-LIF separations of APTS labeled glycans were performed on a Beckman P/ACE MDQ instrument using a neutral coated N-CHO (PVA) capillary (Beckman Coulter, 50 μm ID/365 μm OD, 60-cm total length, 50-cm effective length) and a commercially available carbohydrate separation gel buffer (Beckman Coulter) as a background electrolyte. The instrument was equipped with a 488 nm laser module and a 520 nm cut-off filter for LIF detection. Samples were injected by pressure at 6.9 kPa (1 psi) for 5 s. The capillary was rinsed with the separation buffer between injections for 5 min at 206.8 kPa (30 psi) in order to avoid any sample carryover. All separations were performed at 25 °C by applying −30 kV separation voltage (E = 500 V/cm).

2.11 Permethylation

Permethylation of N-linked glycans released from fetuin was performed according to the published procedure in [24]. Briefly, spin columns were packed with sodium hydroxide beads (20-40 mesh) washed with DMSO and spun down at 1000 rpm for 2 min. Before permenthylation, proteins were removed from the collected fractions (50 µL) using 10 kDa MWCO centrifugal filters, dried in a SpeedVac concentrator and redissolved in a solution containing 140 µL DMSO, 54 µL iodomethane and 6 µL water. Next, the sample was passed through the spin column at 1000 rpm for 2 min. The process was repeated 10 times. The permethylated glycans were extracted with chloroform and washed repeatedly with water. The extracts were dried in a SpeedVac concentrator and redissolved in a solution (50 µL) containing 50 % methanol and 50 % 5 mmol/L sodium bicarbonate.

2.12 MALDI/MS analysis of proteins and released glycans

Before MALDI/MS analysis of proteins, the collected fractions were 10 times diluted by water and mixed with a sinapinic acid solution (10 mg/mL, prepared in 50% acetonitrile containing 0.1% trifluoroacetic acid) in a 1:1 (v/v) ratio. Proteins were analyzed in the positive linear ion mode. The released glycans (native or permethylated) were analyzed by MALDI/MS without any dilution/pre-concentration steps. The samples were mixed with super-dihydroxybenzoic acid solution (20 mg/mL, prepared in water) in 1:1 (v/v) ratio and analyzed in the positive reflectron ion mode.

3 Results and discussion

In this work we describe a novel oriented immobilization technique of PNGase F on monolithic support. Prior to immobilization the activity of the expressed and purified PNGase F-GST was tested by in-solution deglycosylation of bovine ribonuclease B (RNase B). RNase B is a relatively small glycoprotein containing four disulfide bonds and a single glycosylation site at Asn34, where five to nine mannose residues can be attached to the chitobiose core. The released glycans were APTS labeled and analyzed by CE-LIF. Figure 1–2 shows the electrophoretic profile of the APTS-labeled glycans released from RNase B, confirming efficient expression and good isolation of the active PNGase F-GST. Table 1 delineates the glycan structures and GU_{CE} values for all significant peaks corresponding to APTS-labeled RNase B glycans detected by CE-LIF.

We have compared the in solution digestion activity of the PNGase F-GST conjugate to that of the commercially available PNGase F (Sigma Aldrich) under the same conditions. The obtained electropherograms of APTS-labeled glycans released from ribonuclease B did not show any difference between the enzyme treatments. Furthermore, the samples treated using soluble enzymes were also analyzed by SDS-PAGE. The complete deglycosylation of ribonuclease B was observed after treatment by both enzymes, confirming that PNGase F-GST has similar activity as the commercially available counterpart.

3.1 Immobilization of PNGase F-GST

The oriented approach required modification of the monolithic pore surface by immobilized GSH. Therefore, the poly(GMA-co-EDMA) monolith prepared by thermally initiated radical polymerization in a 250 µm ID fused silica capillary was activated via a multistep procedure ~~shown in Figure 2~~ (Figure 1). The monolith was first treated with an aqueous ammonia solution and then functionalized with succinyl-6-[(iodoacetyl)amino]hexanoate to assure the oriented coupling of GSH to the monolith. Iodoacetyl groups react specifically and efficiently with sulfhydryl functionalities forming covalent thioether bonds, permanently attaching GSH to the monolithic column. Each step of the monolith modification was characterized by fluorescent microscopy as well as SEM/EDAX analysis. The specific reaction of fluorescamine with primary amines forming a highly fluorescent product was employed for qualitative characterization of the efficient amination of the monolith as well as the immobilization of GSH via sulfhydryl functionalities. Furthermore, the SEM/EDAX analysis confirmed the presence of iodine (1.54 wt%) and sulfur (0.15 wt%) in the iodoacetyl- and GSH-modified monolithic columns. In the last step, a solution of PNGase F-GST was flushed through the GSH-modified column resulting in the required oriented immobilization of PNGase F via the GST tag.

3.2 Optimization of the PNGase F-GST reactor performance

RNase B was used as a model glycoprotein for digestion condition optimization. It has been shown that globular glycoproteins with several disulfide bridges are often resistant to PNGase F cleavage requiring a denaturation step to increase deglycosylation efficiency [18,19]. Therefore, the effect of RNase B denaturation was studied in our initial experiments. Since MALDI/MS was chosen for the analysis of the native glycans released from RNase B, enabling rapid generation of the desired information, no detergents were used for protein denaturation. RNase B was only reduced by dithiotreitol before endoglycosidase treatment

using a monolithic reactor containing PNGase F-GST prepared by oriented immobilization. Solutions of native or denatured RNase B (1 mg/mL) were flushed through the 10 cm long reactor at room temperature setting the respective residence times for 1 min. The eluents were collected and analyzed by MALDI/MS. The resulting neutral glycans exhibited low ionization efficiency; therefore, another deglycosylation step was performed in 5 mmol/L sodium bicarbonate solution, pH 8.0 containing 10% (v/v) acetonitrile and the released glycans were detected as sodium cationized molecular species. The glycan structures and mass values for peaks corresponding to the RNase B glycans are summarized in Table 1. The initial experiments attempting deglycosylation of native RNase B using the immobilized PNGase F-GST reactor were unsuccessful and only low signal peaks were found in the MALDI/MS spectrum that corresponded to the released glycans (Figure 3a). It was also confirmed by MALDI/MS analysis in the linear mode, where the peaks corresponding to glycosylated protein (m/z~15.3 kDa) dominated the mass spectrum (Figure 3b). In contrast, denaturation of RNase B prior to enzymatic treatment significantly improved deglycosylation efficiency. The peaks corresponding to the intact or partially deglycosylated RNase B disappeared and only peaks corresponding to the completely deglycosylated protein were found in the mass spectrum (Figure 3d). The signal intensities of peaks corresponding to released glycans increased as shown in Figure 3c.

3.3 Effect of the immobilization technique on reactor efficiency

The oriented immobilization via the GST tag was compared with the non-oriented reductive amination technique introduced in the monolithic area by Petro et al. [25], which was based on the reaction of the primary amines of PNGase F-GST with aldehyde functionalities generated on the monolithic surface.

In order to see the difference in the efficiency of both reactors, very short deglycosylation times of 30 seconds were used for the RNase B treatment. A solution of denaturated RNase B (2.5 mg/mL) was flushed through 2.5 cm long monolithic reactors at room temperature and the collected fractions were analyzed by MALDI/MS in linear mode as shown in Figure 4. Only partial deglycosylation of RNase B was obtained by the PNGase F-GST reactor prepared by non-oriented immobilization as shown in the MALDI spectrum containing the peaks of original glycosylated protein (m/z ~6.7 kDa and ~15.3 kDa) (Figure 4a). In contrast to non-oriented PNGase F-GST immobilization, the oriented immobilization technique provided an efficient monolithic reactor as demonstrated by complete RNase B deglycosylation (Figure 4b).

It has been shown that the immobilization chemistry can affect the density of immobilized enzyme and consequently the performance of the enzyme reactor. For example, trypsin immobilization via azlactone functionalities can improve the reactor performance in comparison to the widely used coupling via epoxy groups [6]. However, both procedures lead to non-oriented immobilization of enzyme, which can negatively affect accessibility of the active site and reduce activity of the immobilized enzyme.

In the next experiments, bovine fetuin was used to examine the performance of the immobilized PNGase F-GST monolithic reactors. Bovine fetuin is an acidic glycoprotein containing four O-linked (Ser253, Thr262, Ser264, Ser323) and three N-linked (Asn81, Asn138, Asn158) glycosylation sites, with heavily sialylated glycans attached. A solution of denatured bovine fetuin (1 mg/mL) was flushed through 10 cm long monolithic reactors at a flow rate to maintain the deglycosylation time of 1 min. The direct comparison of deglycosylation performance of both types of reactors i.e., with oriented and non-oriented immobilization is shown in Figure 5. With non-oriented enzyme immobilization the main product peaks of ~20.8 kDa and ~41.5 kDa represent the molecular masses of the doubly and singly charged partially deglycosylated protein (Figure 5b). On the other hand, the MALDI/MS analysis of fetuin treated by the reactor prepared by oriented immobilization of PNGase F-GST provided the mass spectrum containing the main peaks of ~19.6 kDa and ~39 kDa corresponding to the molecular masses of the doubly and singly charged bovine fetuin after complete release of N-linked glycans (Figure 5c). The fetuin glycans released by immobilized PNGase F-GST (oriented immobilization) were also detected by MALDI/MS (Figure 6); however, due to the low stability of sialic acid residues in acidic glycans the permethylation step was performed prior to mass spectrometry analysis. The glycan structures and mass values for all significant peaks corresponding to the permethylated sodium cationized fetuin glycans are summarized in Table 2.

The MS spectra shown in Figures 4 and 5 clearly indicate the significant efficiency difference between the monolithic PNGase F-GST reactors prepared by non-oriented (Figures 4a and 5b) and oriented (Figures 4b and 5c) immobilization techniques. Since the same monolith and the same enzyme was used for preparation of both reactors, the main reason for this difference can be attributed to the immobilization techniques where the oriented PNGase F molecules provided higher density with more accessible active sites.

It should be emphasized that both types of reactors, i.e., with oriented and non-oriented immobilization were very stable. The reactors were used repeatedly within a period of five months, washed with buffer and stored in refrigerator between experiments. No

apparent decrease in activity of immobilized PNGase F-GST was observed in comparison to the initial experiments as demonstrated by complete deglycosylation of RNase B (2.5 mg/mL) in 15 seconds using the repeatedly used monolithic reactor.

3.4 Deglycosylation of human immunoglobulin G

In order to demonstrate the deglycosylation performance of the immobilized PNGase F-GST reactor with larger proteins, analysis of polyclonal human immunoglobulin G (hIgG) was performed. IgG contains a single conserved N-glycosylation site at Asn297 on both heavy chains in the C_H2 domain of the Fc region. Since the denaturation process resulted in precipitation of hIgG, deglycosylation was carried out without denaturation. A solution of native hIgG was pumped through the monolithic PNGase F-GST reactor prepared by oriented immobilization at a flow rate maintaining 3 min residence time. Figure 7 shows the capillary electrophoretic profile of the APTS-labeled glycans released from hIgG. The structures and GU_{CE} values for all significant peaks corresponding to APTS-labeled glycans detected by CE-LIF are summarized in Table 3. The native glycans released from hIgG were also detected by MALDI/MS, but the losses of sialic acid residues from released glycans were often observed. However, this can be minimized by glycan permethylation prior to the MS analysis as demonstrated above during the analysis of acidic fetuin glycans. The obtained data clearly demonstrate the efficient release of all respective glycans from hIgG using the immobilized PNGase F-GST monolithic reactor.

4 Conclusions

In summary, the described oriented immobilization of PNGase F on porous monolithic support significantly increased the efficiency of the flow through microreactor. Complete protein deglycosylation could be obtained in seconds with oriented or minutes with non-oriented immobilized reactors, compared to several hours when in-solution digestion is used. The key component for achieving the highest processing speed was with permeable monolithic support and oriented immobilization of the enzyme allowing complete deglycosylation of RNase B (2.5 mg/mL) in 15 seconds using a 25 mm long, 250 μm id microreactor. Such a processing speed can significantly shorten the glycoprotein analysis workflow from several hours to several minutes or even less in comparison to traditional in-solution glycoprotein treatment. Furthermore, the immobilized PNGase F reactor enables the integration of glycoprotein deglycosylation into multidimensional capillary [19,20] or

microfluidic [15] based platforms incorporating glycoprotein digestion, protein removal, glycan capture and separation followed by MS analysis and open up new automated protocol without any manual interference for a broad range of glycoprotein analysis.

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Figure captions

Figure 1~~2~~. Scheme of monolith modification by glutathione. 1 - succinimidyl-6-[(iodoacetyl)amino]hexanoate, 2 – glutathione.

Figure ~~1~~2. Capillary electrophoresis profile of the APTS-labeled glycans released from RNase B using in solution PNGase F-GST digestion.

Figure 3. MALDI/MS spectra of bovine RNase B (b, d) and glycans (a, c) released by immobilized PNGase F-GST reactor: (a, b) native RNase B, (c, d) denaturated RNase B. Protein concentration: 1.0 mg/mL, deglycosylation time: 1 min. The individual detected glycans are listed in Table 1.

Figure 4. MALDI/MS spectra of bovine RNase B after treatment using the PNGase F-GST reactor prepared by non-oriented (a) and oriented (b) immobilization. Protein concentration: 2.5 mg/mL, deglycosylation time: 30 s~~ec~~.

Figure 5. MALDI/MS spectra of bovine fetuin: before (a) and after treatment using the PNGase F-GST reactor prepared by non-oriented (b) and oriented (c) immobilization. Protein concentration: 1.0 mg/mL, deglycosylation time: 1 min.

Figure 6. MALDI/MS spectrum of permethylated glycans released from bovine fetuin in the PNGase F-GST reactor prepared by oriented immobilization. Protein concentration: 1.0 mg/mL, deglycosylation time: 1 min. The individual detected glycans are listed in Table 2.

Figure 7. Capillary electrophoresis profile of the APTS-labeled glycans released from human IgG using the PNGase F-GST reactor prepared by oriented immobilization. Protein concentration: 2.5 mg/mL, deglycosylation time: 3 min. The individual detected glycans are listed in Table 3.

Figure 1

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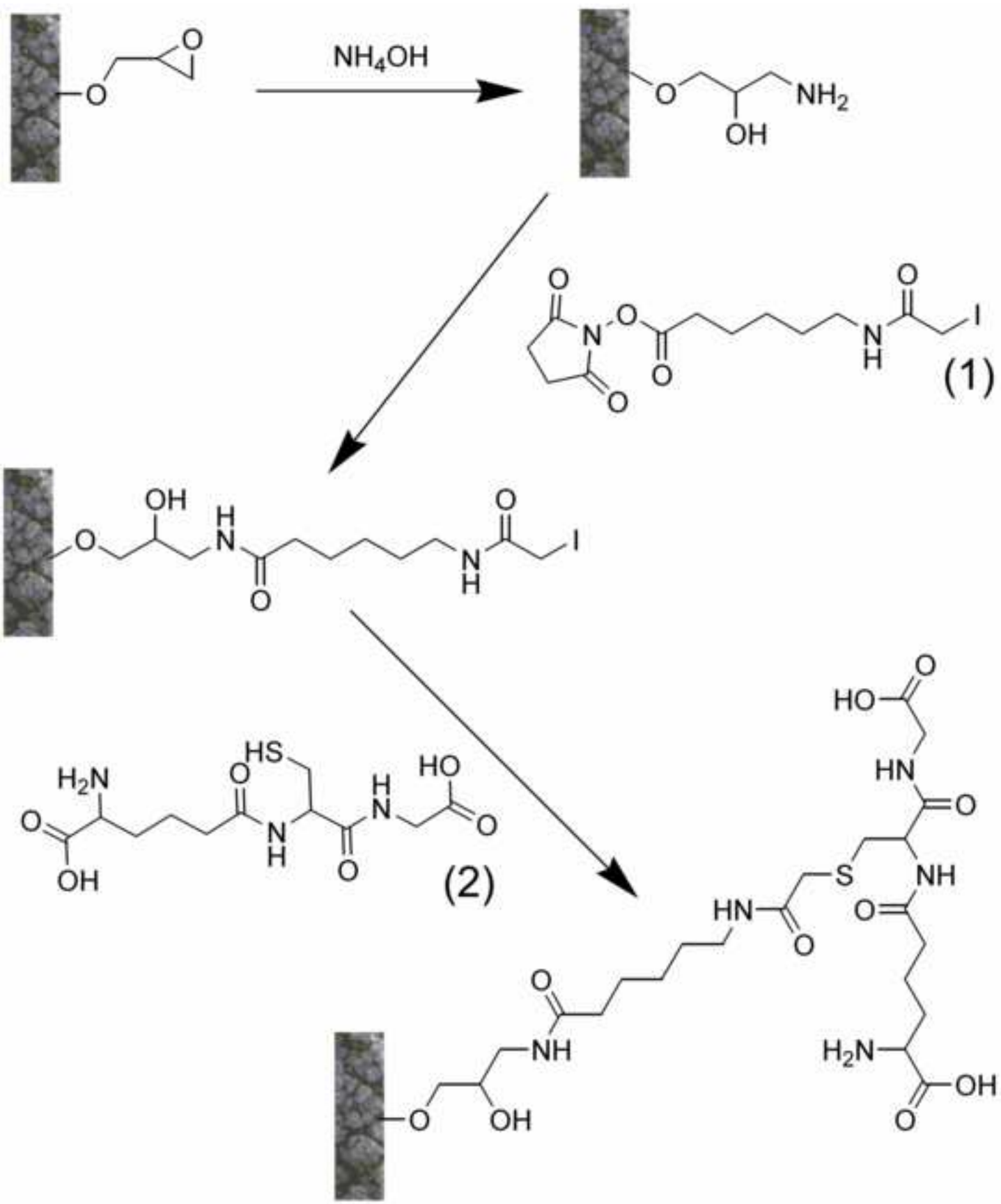

Figure 2

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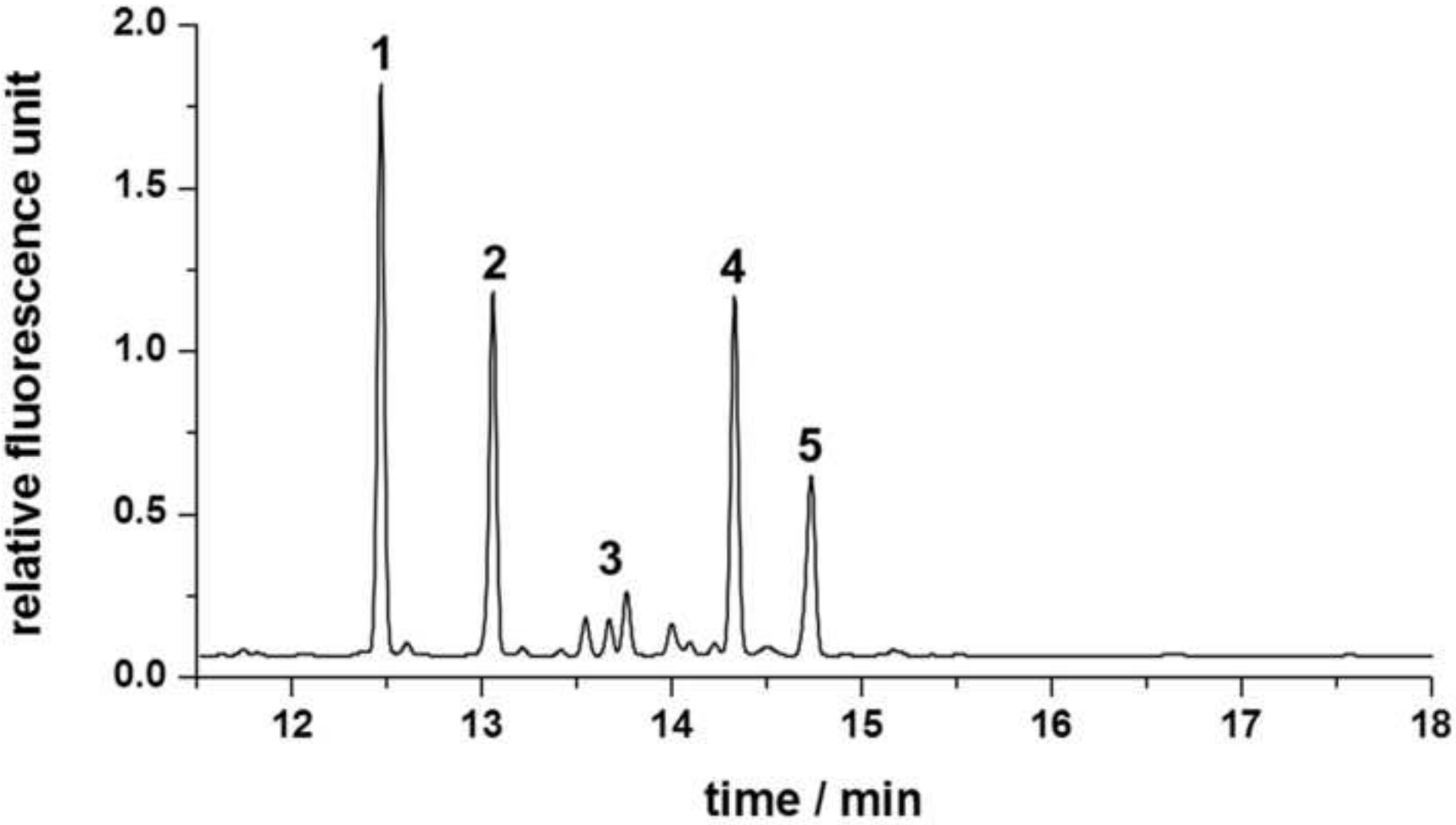

Figure 3

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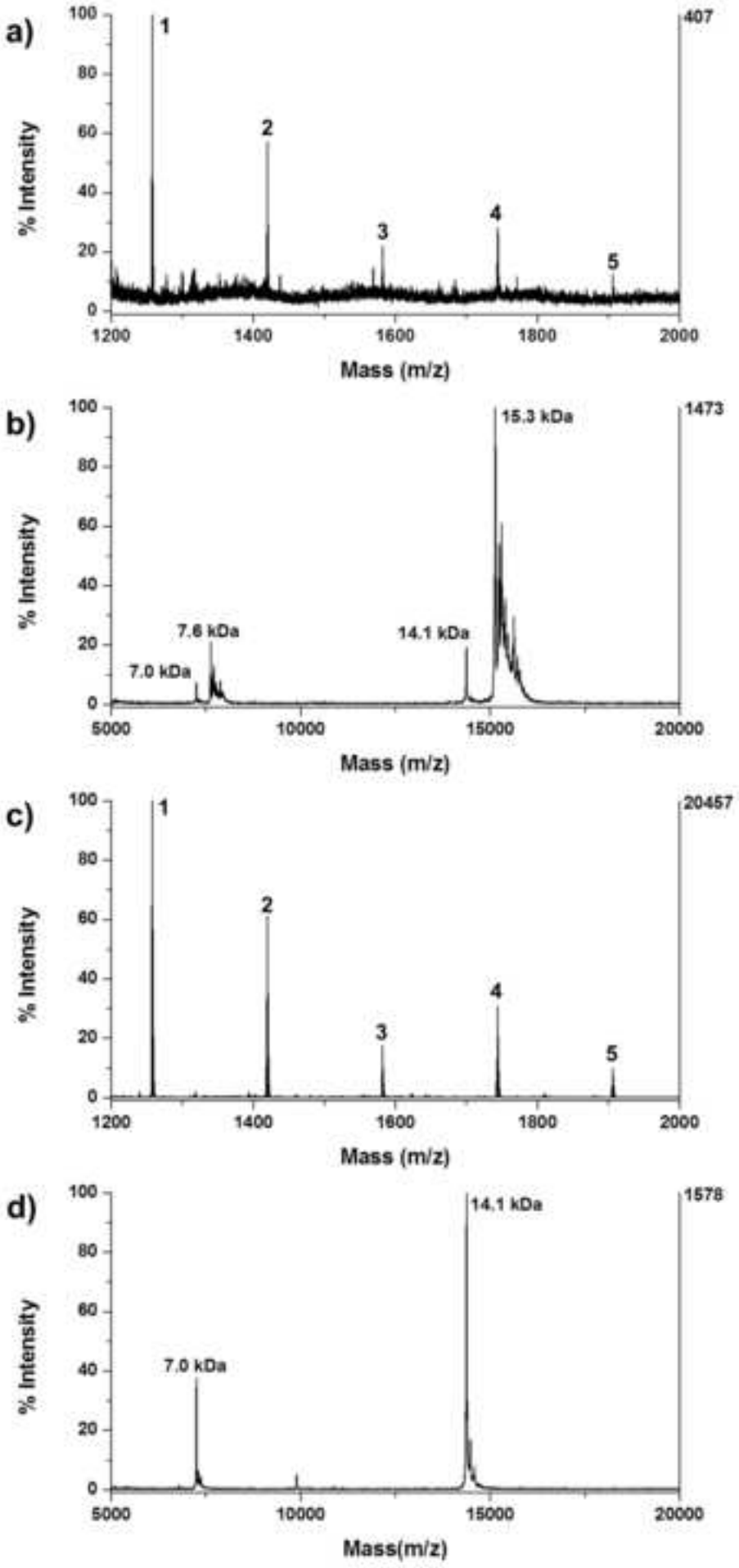

Figure 4
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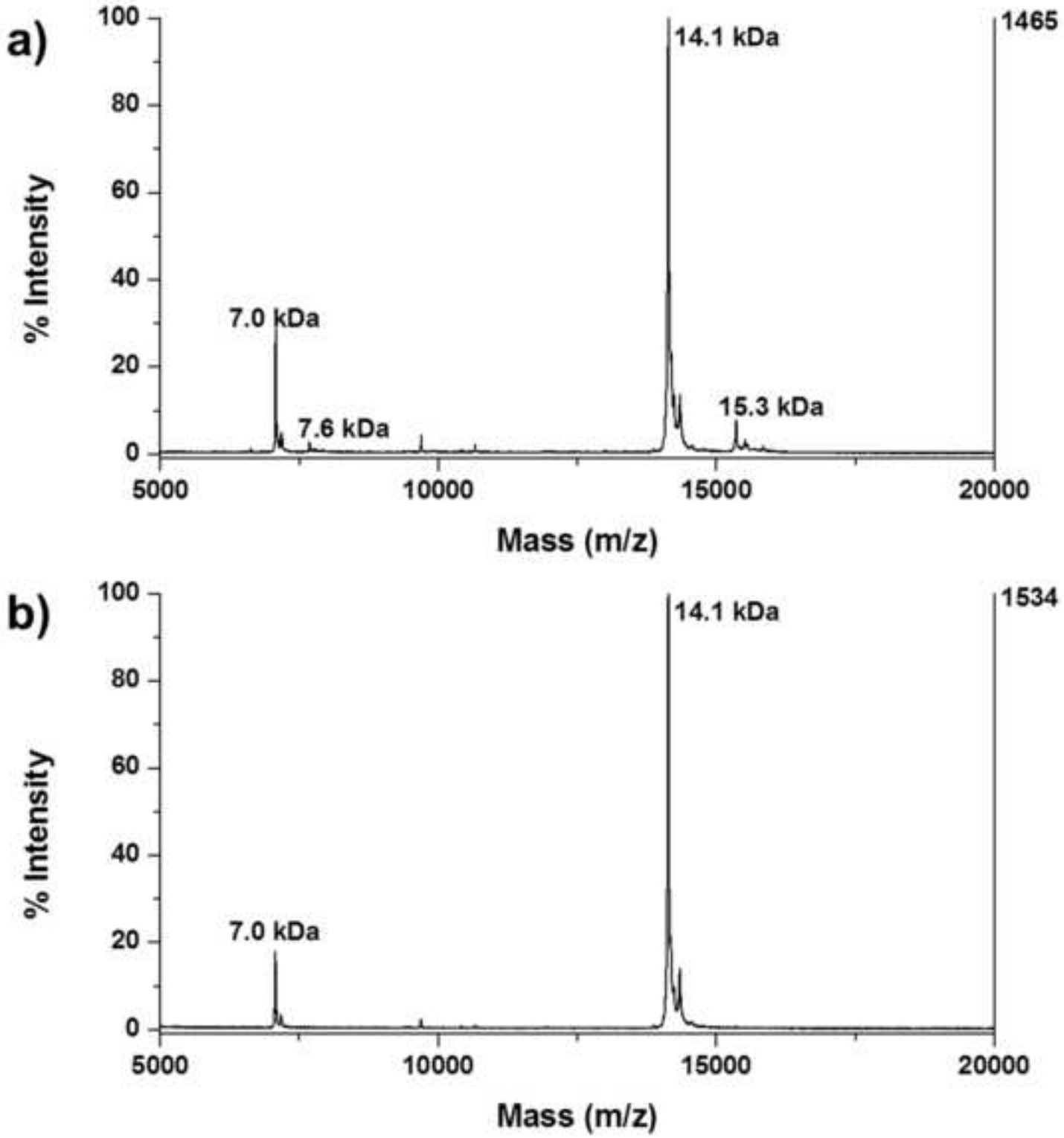

Figure 5

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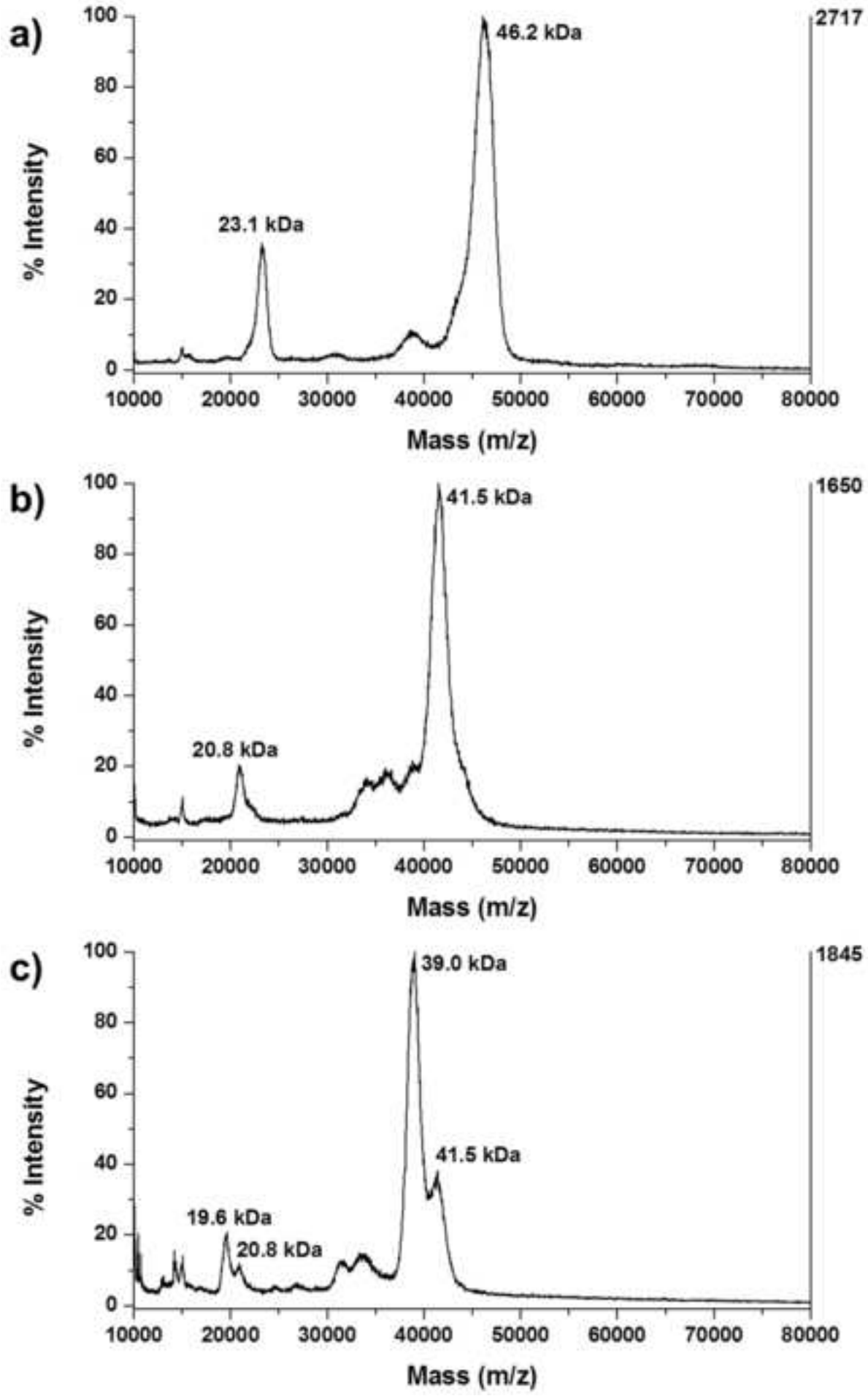

Figure 6

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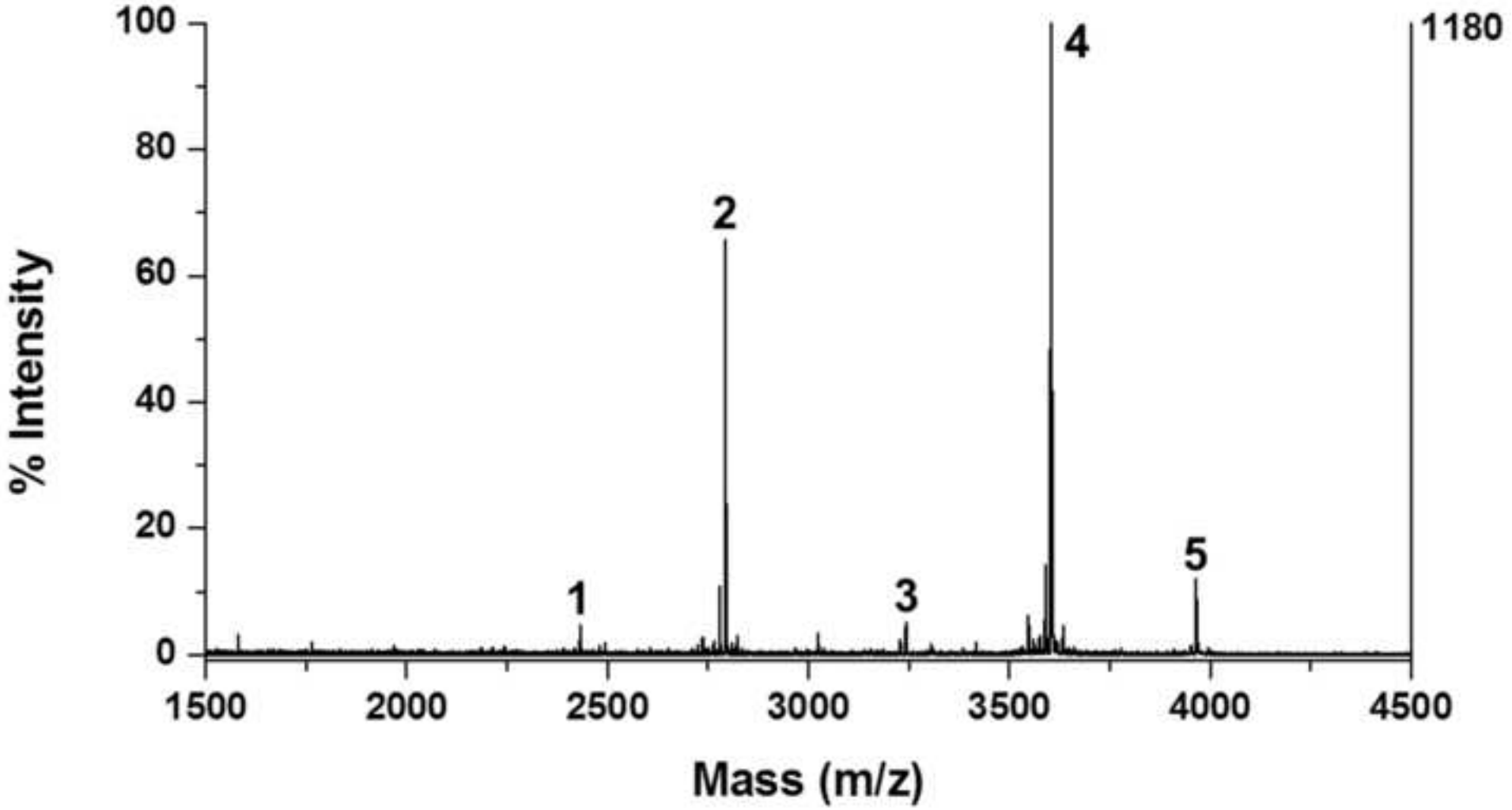

Figure 7

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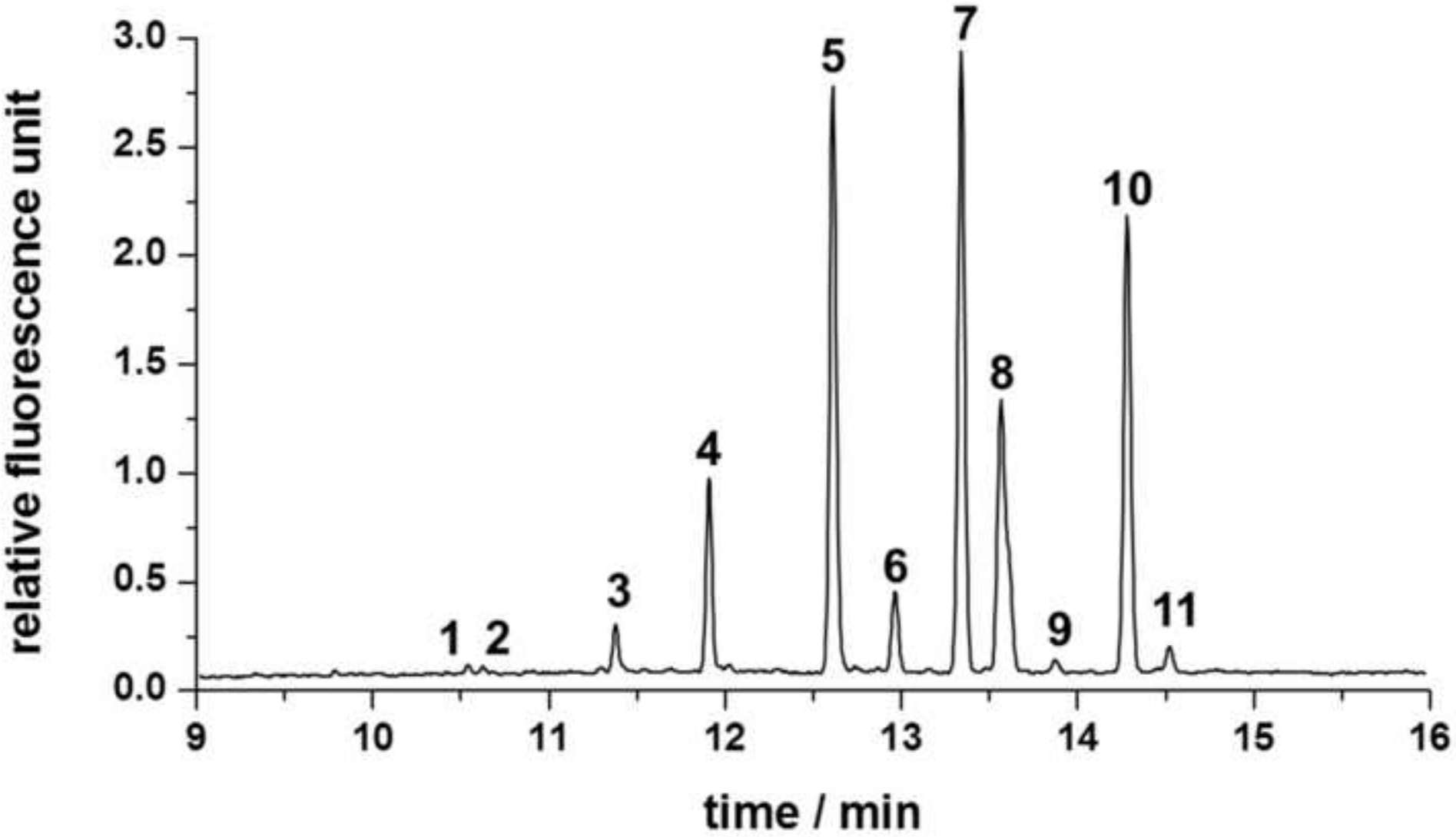
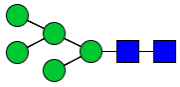
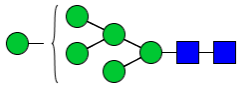
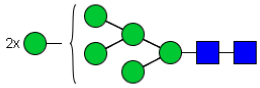
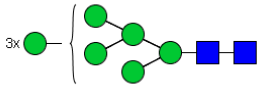
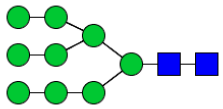


Table 1

Table 1. Glycans released from bovine RNase B.

Peak No.	Structure	GU_{CE} value	[M+Na]$^+$
1		6.76	1257.56
2		7.62	1419.57
3		8.35; 8.54; 8.67	1581.68
4		9.03; 9.18; 9.54	1743.74
5		10.13	1905.79

2

Table 2

Table 2. Glycans released from bovine fetuin.

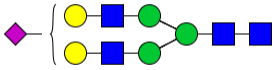
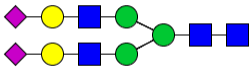
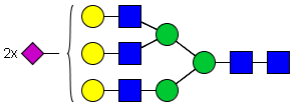
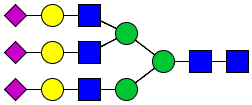
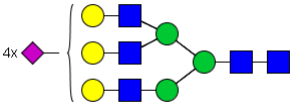
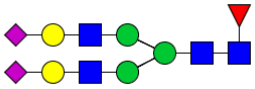
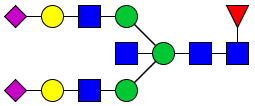
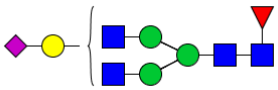
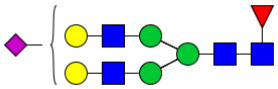
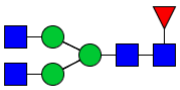
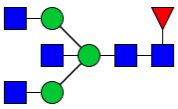
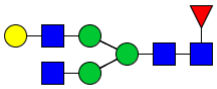
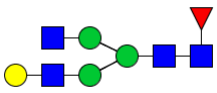
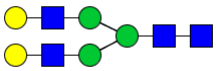
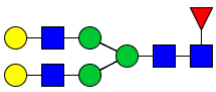
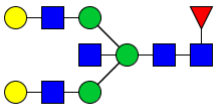
Peak No.	Structure	$[M+Na]^+$
1		2430.88
2		2791.98
3		3241.11
4		3602.22
5		3963.32

Table 3

Table 3. Glycans released from human IgG.

Peak No.	Structure	GU_{CE} value
1		4.83
2		4.92
3		5.91
4		6.11
5		7.69
6		8.20
7		8.75
8		9.08

9		9.12
10		10.12
11		10.47

2