HPLC enrichment/isolation of proteins for post-translational modification studies from complex mixtures

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ABSTRACT

The paper describes a macroporous RP-HPLC method for separation and isolation/enrichment of proteins from complex mixtures. The method is robust and efficient; using 2.1 or 4.6 mm diameter columns provides sufficient material for subsequent proteomic analysis. The main advantage of the method is that most protein variants are isolated in the same fraction, as separation is not based on differences in isoelectric point. This is highly advantageous for studying complex mixtures and post-translational modifications. Examples related to glycosylation analysis are discussed in detail.

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HIGHLIGHTS:

- Macroporous RP-HPLC for enrichment/isolation of proteins from complex mixtures
- Non-biased protein enrichment for analyzing glycosylation
- Sample preparation method for glycosylation analysis

KEYWORDS:

Mass spectrometry; HPLC; sample preparation; proteins; glycosylation

1 Introduction

The structural and functional diversity of proteins is, in a large part, due to post-translational modifications (PTM, like glycosylation, phosphorylation, ubiquitination). One of the most common PTM of proteins is glycosylation, which is implicated in various biological functions like transport of proteins, immune response and cell-cell communication. Relation between glycosylation of human plasma proteins and various diseases has also been shown [1, 2].

Blood plasma and other biological samples are very complex mixtures; often containing a wide range of proteins. Due to this complexity, characterization of the glycosylation pattern of plasma proteins typically requires the use of a range of sample preparation and/or isolation methods, like immunoaffinity isolation, plasma fractionation, glycoprotein enrichment etc.

Glycoprotein/glycopeptide enrichment is often performed using various lectins. This is an excellent choice for enriching glycoproteins. However, selective binding of diverse glycoforms influences enrichment, and may cause significant bias in the glycosylation pattern determined [3].

Probably the most efficient way to isolate *a single protein component* of a complex mixture (e.g. in order to determine its glycosylation pattern) is based on immunoaffinity binding. However, antibodies are often not available, they may have non-specific binding properties, and are variable among different producers [4, 5]. When analysis of glycosylation pattern (or other PTMs) of several glycoproteins is required, immunoaffinity may not be a feasible choice for protein isolation.

Enrichment and/or fractionation of the complex protein mixture is also a common alternative. For analysis of *large number of diverse proteins* the best choice is often reducing the complexity of the biological mixture by fractionation. There are many possibilities, each with advantages and disadvantages. Multidimensional separation techniques (like 2D gel electrophoresis) are more efficient, but are time consuming, and often not feasible for high throughput analysis [6, 7]. Fractionation can be performed either on the intact protein mixture, or following the digestion of the proteins [8]. The latter case typically involves proteolytic enzymes (like trypsin). Fractionation at the peptide level has the advantage that there are a wide variety of well-established analytical methodologies for separation of small and medium size molecules. The disadvantages are, that the protein digest is even more complex than the original mixture; and different components of the same protein are collected in different fractions.

Fractionation at the protein level is also feasible [7, 9], although the analytical methodologies are not so well defined. Most frequently ion exchange, size exclusion, hydrophobic interaction, partition, affinity chromatography, dye-ligand chromatography and electrophoretic methods are applied [10, 11]. These techniques are efficient, and often separate not only the proteins, but also various protein variants: sequence variants (e.g. genetic variants); diverse PTMs; and among the latter various glycoforms.

When the intention is the study of different PTMs of the same protein (like the glycosylation pattern) collecting all variants of a given protein in one fraction is often considered highly advantageous. This typically precludes the use of ion-exchange chromatography or electrophoretic methods; as these separate proteins based on the number of charges (and therefore glyco- or phosphoproteins with the different number of charged groups) [12]. On the other hand, the use of reversed-phase HPLC is most likely advantageous from this point of view,

as it separates proteins with high efficiency, but the different protein variants are expected to elute together. In the present paper we focus on this approach, and apply it to the study of site-specific glycosylation patterns and genetic variants.

In order to enhance performance of HPLC, first we deplete human plasma of the most abundant proteins. In the present case only human serum albumin (HSA) and immunoglobulin G (IgG) are depleted, because we would like to study the glycosylation of some high abundance proteins, as well. Note that, depletion of other high abundance proteins further increases the ability to analyze glycosylation of low abundance components. Note also that while immunoaffinity binding has its limitations, for the purpose of plasma depletion this is an excellent choice; as the objective is only to remove the bulk of the most abundant plasma proteins. Reversed phase HPLC of intact proteins was made feasible by the introduction of large pore size (macroporous) columns. While this is not novel technology, nevertheless requires extensive optimization to obtain good performance [13-15]. After optimizing HPLC performance, fractions were collected, containing a few, highly enriched proteins. Site-specific glycosylation patterns were determined for the individual components, using tryptic digestion and nano-HPLC-MS(/MS) analysis. Due to the high specificity of MS/MS, determination of the site-specific glycosylation pattern of even *several plasma proteins* is feasible.

2 Material and methods

2.1 Samples and chemicals

Human serotransferrin standard and human alpha-1-acid glycoprotein standard were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Haptoglobin standard was purchased from Fluka Chemie GmbH (Sigma-Aldrich®, Zwijndrecht, Netherlands). Human blood plasma sample was obtained from a healthy volunteer, it was divided into aliquots and stored at -20 °C.

1,4-dithio-L,D-threitol (DTT) and 2-iodoacetamide (IAA) were purchased from Fluka Chemie GmbH (Sigma-Aldrich®, Zwijndrecht, Netherlands). RapiGest SF (lyophilized sodium-3-[(2-methyl-2-undecyl-1,3-dioxolan-4-yl)-methoxyl]-1-propane-sulfonate) was obtained from Waters (Milford, MA, USA). Mass spectrometry grade trypsin (Promega Corporation, Madison, WI, USA) and proteomics grade PNGase F from Elizabethkingia meningoseptica (Sigma-Aldrich®, St. Louis, MO, USA) were used.

All other reagents were purchased from Sigma-Aldrich® (St. Louis, MO, USA).

2.2 Removal of albumin and IgG

To reduce sample complexity, two high abundance proteins: albumin and IgG were removed from human plasma with Agilent Multiple Affinity Removal Spin Cartridge HSA/IgG (Agilent Technologies, Santa Clara, CA, USA).

According the manufacturer's standard protocol 50 μL plasma sample was loaded onto the cartridge and instructions were followed. In addition, at the end of the depletion steps K_2HPO_4

and citric acid were added to the diluted samples to prevent aggregation (30-30 mM concentration in the samples), and samples were concentrated with 10 kDa centrifuge filters. After filtration, volume of the depleted plasma sample was 25 μL (i.e. half of the original plasma sample volume). We have checked by MS based standard proteomics experiments that (1) 66% of the total protein amount was removed from the sample; (2) 80% of the total albumin amount is removed from the sample.

2.3 RP-HPLC fractionation of depleted plasma samples

Proteins were isolated from depleted plasma samples using HPLC: Acquity UPLC® System (Waters, Milford, MA, USA) with Binary Solvent Manager, Sample Manager, Column Heater and TUV-detector.

The following macroporous HPLC columns were tested: Agilent mRP-C18 High-Recovery Protein Column (4.6 × 50 mm, Agilent Technologies, Santa Clara, CA, USA); Poros R2 (Poly(Styrene-Divinylbenzene), 10 μm, 2.1 × 100 mm, Applied Biosystems, Foster City, CA, USA); BioSuite pC18 Column 500 (7 μm, 2.0 × 150 mm, Waters, Milford, MA, USA), Acquity UPLC BEH300 (C18, 1.7 μm, 2.1 × 100 mm, Waters, Milford, MA, USA) and Aeris™ WidePore XB-C18 (3.6 μm, 2.1 × 150 mm, Phenomenex, Torrance, CA, USA). We have selected the Poros R2 column for detailed optimization based on the preliminary tests.

Tests were performed using standard proteins (alpha-1-acid glycoprotein, serotransferrin, haptoglobin), deglycosylated standard proteins and depleted plasma sample. Deglycosylated alpha-1-acid glycoprotein and deglycosylated serotransferrin were prepared the following way:

to 11 µL sample, containing 300 pmol protein standard, 2 µL NH_4HCO_3 (200 mM) and 10 µL PNGase F (500 U/mL) were added and digested at 37 °C overnight.

Standard and deglycosylated standard proteins were injected in the 30 pmol – 5 nmol range, depleted plasma samples were injected in 0.5-15 µL volume.

Optimized gradient for depleted plasma samples (Poros R2 column): The column temperature was 65 °C, the flow rate was 1 mL/min. The gradient started with 20% B, kept here for 0.7 min, after this followed a 15 min long gradient from 20% to 70% solvent B. The next step was increasing to 95% solvent B in 0.1 min, kept there for 1.5 min (washing). Finally returning to 20% B in 0.1 min, and kept there for 6 min (equilibration). Solvent A was water containing 0.07 v/v% trifluoroacetic acid and solvent B was acetonitrile containing 0.07% v/v trifluoroacetic acid.

Samples of 500 µL fractions were collected manually using the timer of the MassLynx software and UV-detection (280 nm). To each fraction 1.3 µL NH_3 solution (25 w/w%) to neutralize TFA, and 1.8 µL K_2HPO_4 (500 mM) to prevent aggregation was added and fractions were concentrated to 30 µL with SpeedVac (miVac Duo Concentrator, Genevac Ltd., Ipswich, Suffolk, UK).

2.4 In-solution digestion

Samples of 30 µL volume (collected/concentrated as described above) were digested by the protocol optimized for small sample volume and described before [16]. The main steps were the following: 5 µL NH_4HCO_3 (200 mM) was added to the sample, proteins were unfolded and reduced with 3 µL RapiGest SF (0.5 w/v%) and 1.5 µL DTT (100 mM) for 30 min at 60 °C.

Alkylation was performed adding 4 μL NH_4HCO_3 (200 mM) and 2 μL IAA (200 mM) for 30 min at room temperature in dark. Fractions were digested with 0.5–1.5 μL trypsin (40 μM) for 180 min at 37 °C. The digestion was stopped by adding 1.5 μL formic acid, followed by 30 min incubation time at 37 °C. The samples were centrifuged at 13500 rpm (corresponding to 17000 g) for 10 min.

2.5 nano LC-MS(/MS) measurements

The samples prepared as described above were subjected to several nano-HPLC-MS(/MS) experiments. The first objective is to determine the protein composition of the plasma fraction collected; the second to identify glycoforms of the major glycoproteins in the given fraction; the third is to determine the glycosylation pattern, i.e. the relative proportion of various glycoforms of the same protein.

The digested fractions were analyzed using nanoAcquity UPLC (Waters, Milford, MA, USA) coupled to a high resolution QTOF Premier mass spectrometer (Waters, Milford, MA, USA).

The chromatographic conditions were during all MS(/MS) measurements the following: the peptides were separated on a reversed-phase analytical column (C18, 1.7 μm BEH particles, 75 μm i.d. × 200 mm, Waters, Milford, MA, USA). The column temperature was 55 °C. Before the analytical column a Symmetry C18 trap column (180 μm i.d. × 20 mm, Waters Milford, MA, USA) was used. Solvent A was water containing 0.1 v/v% formic acid and solvent B was acetonitrile containing 0.1 v/v% formic acid. The gradient was the following: using 250 nL/min flow rate starting with a 4 min gradient from 3% to 8% B, then a 65 min long gradient going to

40% solvent B. Washing was done using 450 nL/min flow rate and a 2 min long gradient from 40 to 75% solvent B, and kept there for 18 min. After that returning to 3% B in 2 min, and equilibration was done for 18 min.

To determine the protein composition of the collected macroporous HPLC fractions, the fractions were digested as described above and the resulted peptides were identified by data dependent analysis with the most intense 3 peaks selected. The mass spectrometer operated in positive electrospray ionization mode. The capillary voltage was 2.3 kV, nanoflow 1 bar, source temperature 90 °C, cone voltage 35 V. The parent ion was selected in the 400–1800 m/z range, MS/MS spectra were acquired in the 50-2000 m/z range. Collision gas was argon, at 4.05×10^{-3} mbar. Collision energy was varied in the 7-70 eV range. Genetic variants of alpha-1-acid glycoprotein were identified using these conditions, as well.

Glycosylation sites and major site-specific glycoforms were identified with tandem mass spectrometry. The parent ion was selected in the 780-2000 m/z range, MS/MS spectra were acquired in the 150-3000 m/z range. Collision energy was varied in the 5-55 eV range. Minor glycoforms and relative quantitation were measured with single stage mass spectrometry in extended dynamic range mode. Scans were acquired in the 500-2000 m/z range. Other instrumental parameters were same as described above.

Site-specific glycosylation pattern of components of the fractions has been analyzed and characterized by the method described earlier in detail [17].

2.6 Data evaluation

Proteins in each fraction and genetic variants of alpha-1-acid glycoprotein were identified from MS/MS measurements using ProteinLynx Global Server v.2.3 (Waters, Milford, MA, USA) and searched against v.2011_10 of SwissProt sequence database with human taxonomy using Mascot Server v.2.2 (Matrix Science, London, UK). One missed cleavage was allowed, carbamidomethyl cysteine was set as fixed modification.

MS/MS spectra corresponding to major glycopeptides were automatically evaluated by our computer software GlycoMiner v.1.13 Beta [18]. For each glycosylation site, the tryptic peptides with possible glycan structures were identified. Minor glycopeptide identification and relative glycopeptide quantitation were performed from MS measurements using the in-house developed computer software GlycoPattern v.2.0 [17]. The software calculates the retention time windows of minor glycoforms based on the known retention time of the major glycoforms. Minor glycoforms are identified based on retention time and exact mass. Internally, GlycoPattern uses an isotope model to increase the confidence of the identification. For each glycoforms the extracted ion chromatograms are generated and the chromatographic peaks are integrated.

3 Results and discussion

In the current work we have optimized a workflow for RP-HPLC fractionation of complex protein mixtures, in particular human blood plasma. The primary aim was to collect the different variants of a given protein in one fraction. Fractionation significantly simplifies sample complexity, and is particularly advantageous for analyzing protein variants, like various PTMs.

Still, even after fractionation, each collected fraction is a complex mixture, containing more than one co-eluting protein and their variants. When all variants of a given protein are collected in one fraction, this makes subsequent identification, qualitative and quantitative analysis of PTMs more reliable, more robust and more sensitive. Chromatographic behavior of proteins under RP conditions are expected to depend mainly on their hydrophobicity; and in most cases on their peptide backbone; single point mutations or changes in the PTMs should only have a minor, often negligible effect. This expectation will be studied in detail below. Note that RP-HPLC fractionation is only the first step, PTM characterization typically requires several further stages for sample preparation and analysis. This may be based on procedures which separate various PTMs from each other (like in 2D gel electrophoresis); or on procedures (like mass spectrometry), which are capable of dealing with a mixture of various PTMs.

Important parameters and properties of the fractionation method were tested and optimized. The main steps of fractionation and analysis are the following: affinity depletion of high abundance proteins; RP-HPLC fractionation of the remaining proteins; proteolysis (tryptic digestion) of the collected fractions; nano-HPLC-MS(/MS) measurements; and complex data analysis to determine site-specific glycosylation pattern. The complete workflow is presented in Fig. A.

The first issue to consider is the amount of proteins needed to be isolated/enriched. This, in turn, depends on the objective and methodology of further analysis or utilization. In the present case the objective is analytical characterization based on mass spectrometry. This methodology is sufficiently sensitive, that using 4.6 or 2.1 mm diameter analytical HPLC (or UHPLC) columns for sample preparation is adequate – although the loading capacity should be tested and optimized. When larger sample amounts are needed for subsequent analysis (e.g. for most 2D gel

electrophoresis studies), multiple HPLC runs or the use of larger diameter column may be required.

The whole analytical procedure starts with depletion of the blood plasma sample (removal of the most abundant protein components). This is a commonly used method to simplify subsequent analysis. Typically the most abundant 2, 7, 14 or 20 proteins may be removed by affinity binding, using various kits. In the present study we use the simplest variant, removing albumin and IgG. It is often recommended that blood plasma should be reduced and alkylated before depletion and HPLC analysis. This somewhat improves chromatographic behavior (resolution and shape of peaks). However, we found that following reduction and alkylation new peaks appear in the chromatogram (most likely due to changes of the chromatographic contact area); and a significant amount of the reduced protein precipitates from the solution. (This may partly be avoided by the addition of K_2HPO_4 and citric acid to the solution; and depends significantly on the individual plasma sample). To avoid these problems, we have used (and recommend) depletion and subsequent RP-HPLC fractionation on the native plasma, i.e. *without* reduction and alkylation.

The range of macroporous columns is relatively limited, nevertheless there are several types available. We have made a tentative comparison of five different columns, mainly with respect to semi-preparative applications (Agilent mRP-C18, BioSuite pC18, Poros R2, Acquity UPLC BEH300, Aeris^{TM} WidePore XB-C18). Our primary concern was their loading capacity and avoiding cross-contamination of samples (i.e. a blank injected after a plasma sample should not contain any proteins). Based on preliminary tests we have selected the Poros R2 column for detailed optimization – but detailed comparison of these columns is outside the scope of the present paper.

263 Length and slope of the gradient, trifluoroacetic acid content of solvents and flow rate have been
264 optimized for the best achievable resolution, while keeping the peak/fraction volume manageable
265 [19]. Trifluoroacetic acid content of solvents should be low; best results were obtained in the
266 0.05-0.1% range; the optimum depends on column type. Without trifluoroacetic acid the proteins
267 remain bound to the column, but higher concentration of trifluoroacetic acid decreases the pH
268 extremely, which results the precipitation of proteins and hydrolization of sugar bonds. In order
269 to avoid unnecessary dilution of the sample the flow rate was limited to 1 mL/min, and elution
270 time was 20 min. Using these parameters reasonable selectivity was obtained, even injecting as
271 much as 15 μL sample (Fig. B).

272 Using the conditions described above proteins were collected in 20, 0.5 min wide, 500 μL
273 volume fractions; from 4 to 14 min retention time based on the chromatogram shown in Fig. B.
274 Prior to subsequent sample analysis NH_3 solution and K_2HPO_4 solution were added to neutralize
275 trifluoroacetic acid and to prevent further precipitation. Then, the 500 μL fractions were
276 concentrated to 30 μL volume using vacuum centrifugation. Protein recovery of the complete
277 procedure has been tested with standards and optimized for the minimal sample loss. It was
278 found that special tubing: ethylene tetrafluoroethylene (ETFE), needle: fluorinated ethylene
279 propylene (FEP), and low protein affinity eppendorf tubes were needed to obtain over 90%
280 protein recovery for major plasma proteins.

281 Reproducibility of the chromatographic method has been tested with various protein standards
282 and plasma samples using UV-detection. Reproducibility (standard deviation) of retention times
283 is 0.15%; that of the peak areas is 1.4%. Reproducibility of the whole procedure (which includes
284 plasma depletion as well); with respect to retention times is the same (0.15%); but that of peak
285 areas is significantly worse, 9.3%. This shows that plasma depletion is less reproducible than

chromatographic analysis. The standard deviations shown are the averages measured for three protein standards (and the same protein components of plasma), and were calculated using 3 replicates.

As described above, using macroporous RP-HPLC one can reasonably expect that different variants of a given protein should elute in one chromatographic peak, i.e. the PTMs have only minor influence on the retention time. As this is of major importance in the present study, we have performed various tests on genetic variants and on glycoproteins to confirm this assumption. In case of glycosylation, glycosylated and deglycosylated proteins; and glycoforms containing acidic and neutral sugars were studied.

In the case of standard (commercial) serotransferrin sample, the UV chromatogram in Fig. C shows a single peak (although with a well-defined shoulder). It is the well-known behavior of commercial serotransferrin standard, might be attributed to natural charge or oxidized variants [11]. We have fractionated this peak into 5 narrow (0.1 min, 100 μL) fractions; digested these with trypsin, and studied them in conventional nano-HPLC-MS and MS/MS experiments (details are described in the Material and methods part, 2.5). The results show, that all fractions contain serotransferrin; only one genetic variant was observed. We have also determined that the glycosylation pattern of serotransferrin does not show changes among the various fractions. Deglycosylated serotransferrin standard was injected onto the RP-HPLC column as well, and found to behave very similar to the glycosylated standard: peak shape and retention time were identical. We have also studied the effect of deglycosylation in the case of alpha-1-acid glycoprotein, in which case ~40% of the molecular mass is due to the sugar chains. In this case the retention time did change, but only by 0.5 min. This suggest, that even when most of the protein surface is covered by sugar units, the retention time is predominantly determined by the

peptide backbone. Note also that changes in the glycosylation pattern (in contrast to complete deglycosylation) of the protein has a negligible effect on retention, as described below with respect to glycoform analysis.

We have performed an analogous study in the case of blood plasma. We have tried to follow elution of serotransferrin in the plasma sample, but due to the complexity of the electrospray signals the results were equivocal. To confirm the assumption that various glycoforms of serotransferrin in the plasma sample indeed elute in a single chromatographic peak the following test was performed. The RP-HPLC fractions were collected as described above. The fractions were digested using trypsin, and the intensity of the molecular ions of the three most abundant peptide fragments of serotransferrin were determined using a conventional nano-HPLC-MS experiment. The relative amount of serotransferrin in each fraction was determined using label-free quantitation; using the average abundance of the three most abundant unique peptide signals. The result is shown in Fig. C (dots marked, scale at the right side). This clearly indicates that serotransferrin is collected predominantly in one fraction only; a minor amount (ca. 2%) in a neighboring fraction, while other fractions contain only traces of serotransferrin (either due to peak tailing or cross-contamination).

Chromatographic behavior of proteins containing multiple amino acid modifications were studied in the case of alpha-1-acid glycoprotein. This protein has two different genetic variants (ORM1 and ORM2), and has 21 variable amino acid sites (out of 200 amino acids). After fractionation we have digested the protein mixtures, and identified peptide fragments characteristic for the two genetic variants. Two peptide pairs were selected for analysis, ^{171}SDVVYTDWK^{179} and ^{154}EQLGEFYEALDCLR^{167} for ORM1; and ^{171}SDVMYTDWK^{179} and ^{154}EQLGEFYEALDCLCIPR^{170} for ORM2. The peptides were identified in a conventional

proteomics experiment using nanoHPLC-MS/MS, as described in the Material and methods part. All of these peptides were found in the same protein fraction, indicating that the protein variants co-elute under the studied conditions.

Reproducibility of fraction collection was also studied. This is illustrated the case of beta-2-glycoprotein_1; shown in Fig. D. Fractions were collected in three separate experiments; and the relative amount of beta-2-glycoprotein_1 was determined in each fraction using label-free quantitation; as described above by serotransferrin. The results show that beta-2-glycoprotein_1 is collected predominantly in one fraction only. Standard deviation of relative intensities in the various fractions is 0.8% SD, using 3 replicates.

Combining the presently described fractionation method with glycoprotein analysis [17], we have successfully characterized the detailed, site-specific glycosylation pattern of several plasma proteins. The reproducibility of glycosylation patterns (relative abundance of various glycoforms) so determined in three replicates is, on average, 15% SD. This is identical to that described before for standard protein samples [17].

Most studied proteins gave results similar to those discussed above (one chromatographic peak). One notable difference is haptoglobin; which showed a single peak having a very (8 min) long tail (probably due to strong column binding; observed in the standard, using UV detection, Fig. E.1). We have collected the various fractions from plasma; after digestion and nano-HPLC-MS(/MS) analysis we have determined the glycosylation pattern of haptoglobin in the various fractions; shown in Fig. E.2. This illustrates that, even if proteins show large tailing, this is predominantly not due to separation of glycoforms. This confirms that proteins collected in one fraction are suitable for determining representative glycosylation patterns.

We have performed a further experiment to show that RP-HPLC fractionation does not compromise determination of glycosylation patterns. In other words, the glycosylation pattern determined after fractionation is the same as that determined on the original sample. Due to limited sensitivity, this was checked on a standard protein (alpha-1-acid glycoprotein). The glycosylation pattern at site ^{56}N (corresponding to the ^{52}NEEYNK^{57} glycopeptide) was determined for the commercial standard protein without fractionation, and after fractionation using the methodology discussed above. The results are shown in Fig. F, and show that fractionation does not change the glycosylation pattern (e.g. due to selective binding or some other effects). Between the two samples 13% SD was calculated from all glycopeptides of alpha-1-acid glycoprotein, using 3 replicates.

As described above, loading capacity was a major issue optimizing the present methodology. This determines the amount of proteins isolated/fractionated by RP-HPLC for further studies. Determination of PTMs is often limited by sensitivity, even if high sensitivity mass spectrometry is used for analysis. In most proteomics experiments it is sufficient to identify a few (the most sensitive) peptide fragments of protein to give positive identification and relative (label-free) quantitation. Analyzing PTMs requires a far, 100-1000-10 000 fold larger sample amount than that required for protein identification for the two main reasons: (1) Peptides containing the PTM unit have often limited sensitivity. This is a particularly serious issue for glycopeptides. (2) Many PTMs are minor components, e.g. phosphorylation may be present in a few %; minor (but possibly biologically important) glycoforms have 1% or less relative abundance.

Using the Poros R2 2.1 mm diameter column allowed us to inject ca. 15 μL depleted plasma onto the column, without significantly deteriorating column performance. The total protein amount in this is approximately 800 μg (~13 nmol). This calculation is based on the following: Total

protein content of plasma is ~80 g/L; from which ~66% is removed by depletion, so ~27 g/L remains in the sample. In the experiments 50 µL plasma is used, this means ~1300 µg protein in the 25 µL depleted fraction. Onto the column 15 µL of 25 µL is injected, which is ~800 µg. Assuming a 60 kDa average protein molecular mass, this converts to ~13 nmol total protein amount injected. This means that major plasma proteins can be collected in one fraction in 20–1000 pmol amount. This is typically sufficient to obtain (after proteolytic digestion) high quality glycosylation patterns using nano-HPLC-MS(/MS) methodology. In order to perform 2D gel electrophoresis experiments using the common Coomassie Staining typically larger protein amounts are needed. This may require repeated sample fractionations, and pooling several fractions for subsequent analysis.

4 Conclusions

The paper demonstrates the usefulness of RP-HPLC methodology for sample preparation, enrichment and (in certain cases) isolation of proteins from complex mixtures. The use of macroporous stationary phases is the key to utilize HPLC for separation of macromolecules. This methodology is not new [9, 20], but the availability of efficient, commercially available columns is a relatively recent development. Most other protein separation methods (like capillary electrophoresis or 2D gel electrophoresis) rely on isoelectric focusing, which is a very efficient way for separating not only proteins, but protein variants as well. RP-HPLC separates protein variants in much less degree: on the one hand this restricts the separation power (or peak capacity); on the other hand this does not separate most protein variants. This was shown in case of genetic variants and glycoforms, and other PTMs (like phosphoproteins) are expected to

behave similarly. As protein variants are not resolved, this facilitates unbiased determination of the PTM distribution, which is very important in PTM studies.

In the present paper we have shown that various glycoforms are collected in the same fraction and protein isolation in this way does not introduce bias. This was established comparing glycosylation patterns with and without fractionation, and these were (within experimental error) identical. Mass spectrometry (arguably the most common method for protein analysis) requires only small sample amounts. Therefore, one injection onto a 2.1 or 4.6 mm diameter (analytical, high efficiency) HPLC column usually provides a sufficient amount of sample for subsequent analysis. The suggested macroporous RP-HPLC method is robust; which facilitates widespread applications.

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7 Appendices

Fig. A: Workflow for characterization of site-specific glycosylation pattern of plasma proteins.

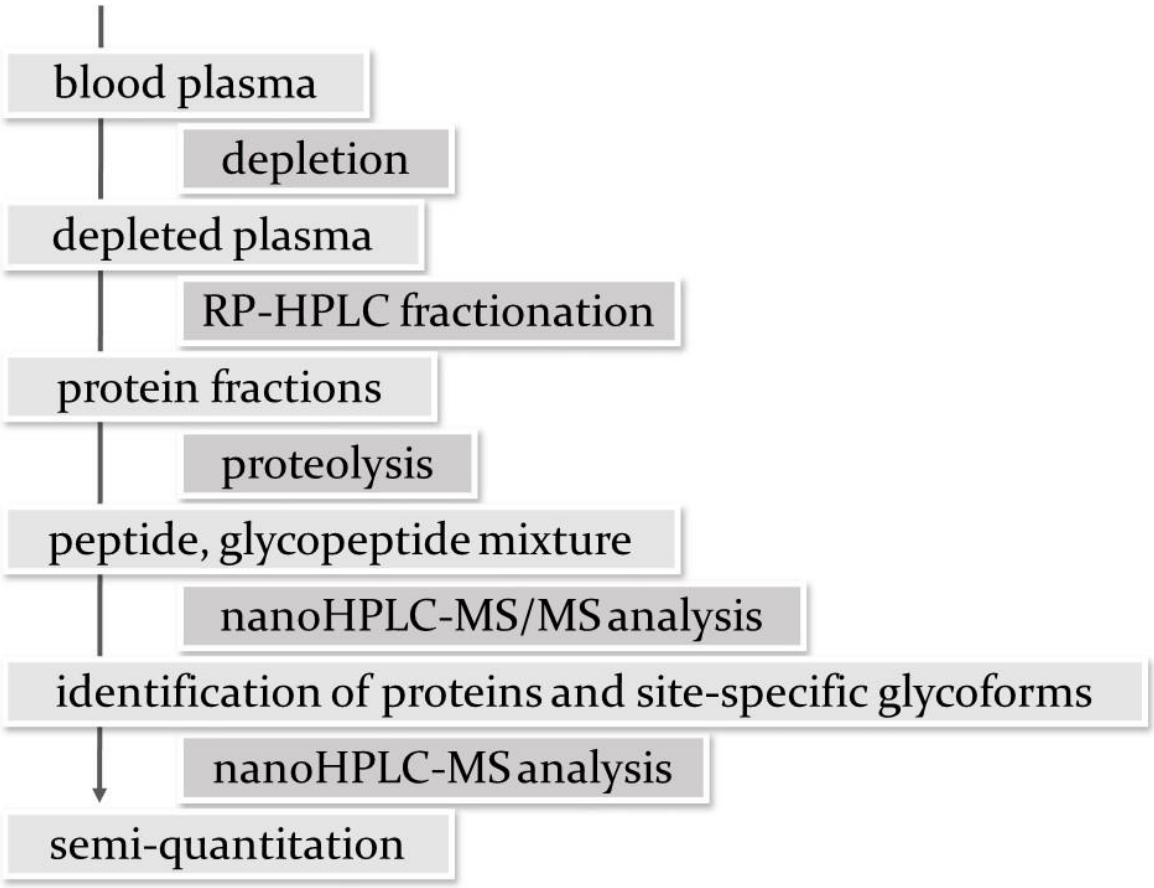
Fig. B: Depleted plasma chromatogram obtained on Poros R2 column. As the chromatogram shows reasonable selectivity was obtained after the injection of 15 µL depleted plasma sample.

Fig. C: UV chromatogram of standard serotransferrin and distribution of plasma serotransferrin among fractions collected at different retention times. In the latter case human blood plasma was fractionated; the individual fractions were digested and studied in separate nano-HPLC-MS experiments. Label-free quantitation (based on the sum of the three most abundant peptide signals of serotransferrin) was used to determine the relative amount of serotransferrin in the various fractions, indicated by diamond shapes in the Figure. The results show that serotransferrin was indeed successfully isolated in a single chromatographic fraction from blood plasma.

Fig. D: Reproducibility of fraction collection. Protein distribution in the collected fractions is presented in case of beta-2-glycoprotein_1. 3 replicates are presented with different marker types.

Fig. E.1 and Fig. E.2: 1) UV chromatogram of standard haptoglobin and 2) glycosylation pattern of plasma haptoglobin characterized from fractions collected at different retention time. The normalized distribution of glycoforms in case of ^{184}N glycosylation site (^{179}MVSHHNLTTGATLINEQWLLTTAK^{202} glycopeptide) is presented here.

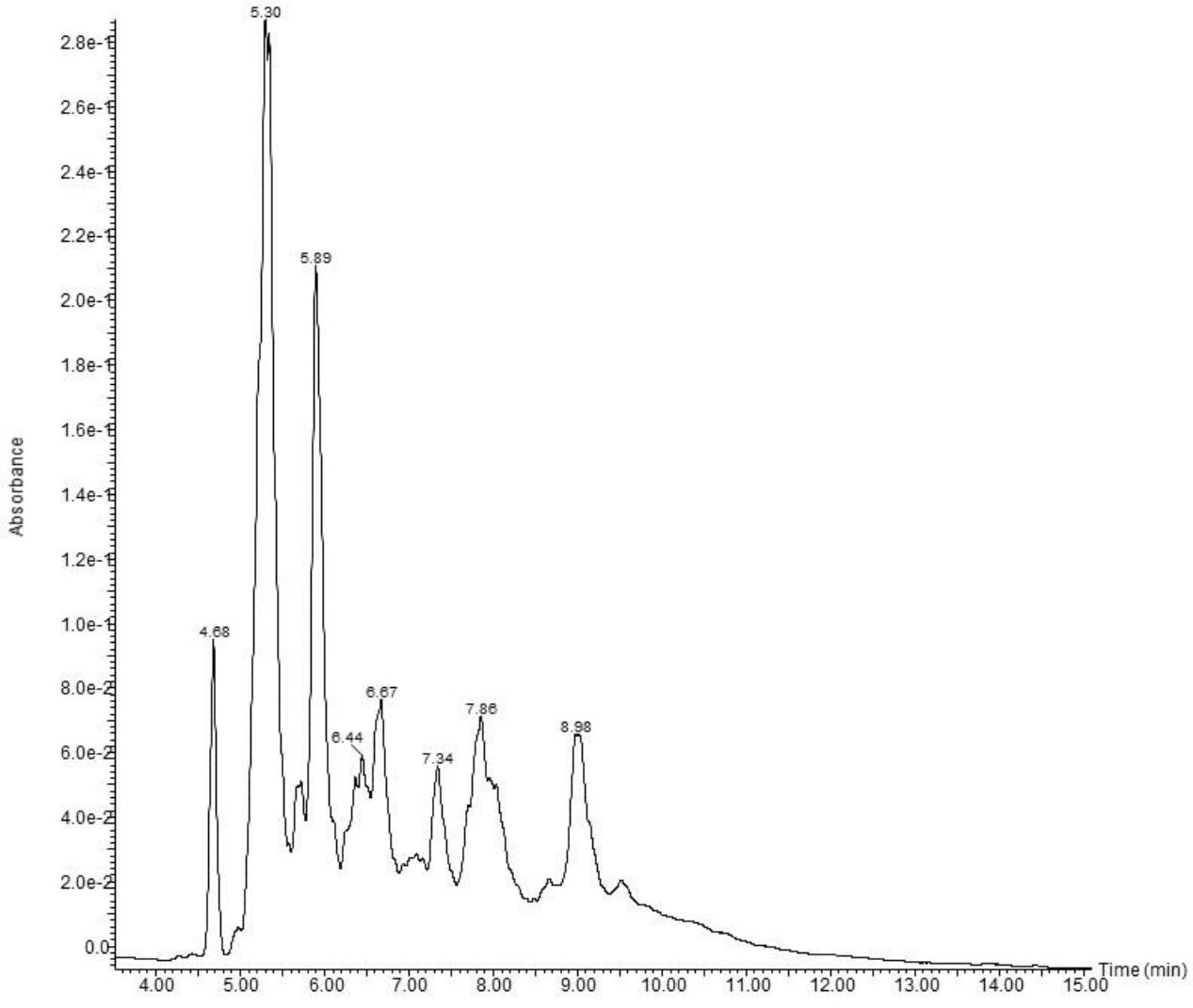
Fig. F: Glycosylation pattern at the ^{56}N site (^{52}NEEYNK^{57} glycopeptide) of alpha-1-acid glycoprotein standard without fractionation and after fractionation. The same pattern was determined after the glycoprotein standard was "fractionated" by RP-HPLC; and the fraction collected was analyzed in the same way.


blood plasma
depletion
depleted plasma
RP-HPLC fractionation
protein fractions
proteolysis
peptide, glycopeptide mixture
nanoHPLC-MS/MS analysis
identification of proteins and site-specific glycoforms
nanoHPLC-MS analysis
semi-quantitation

495

496 Fig. A

497



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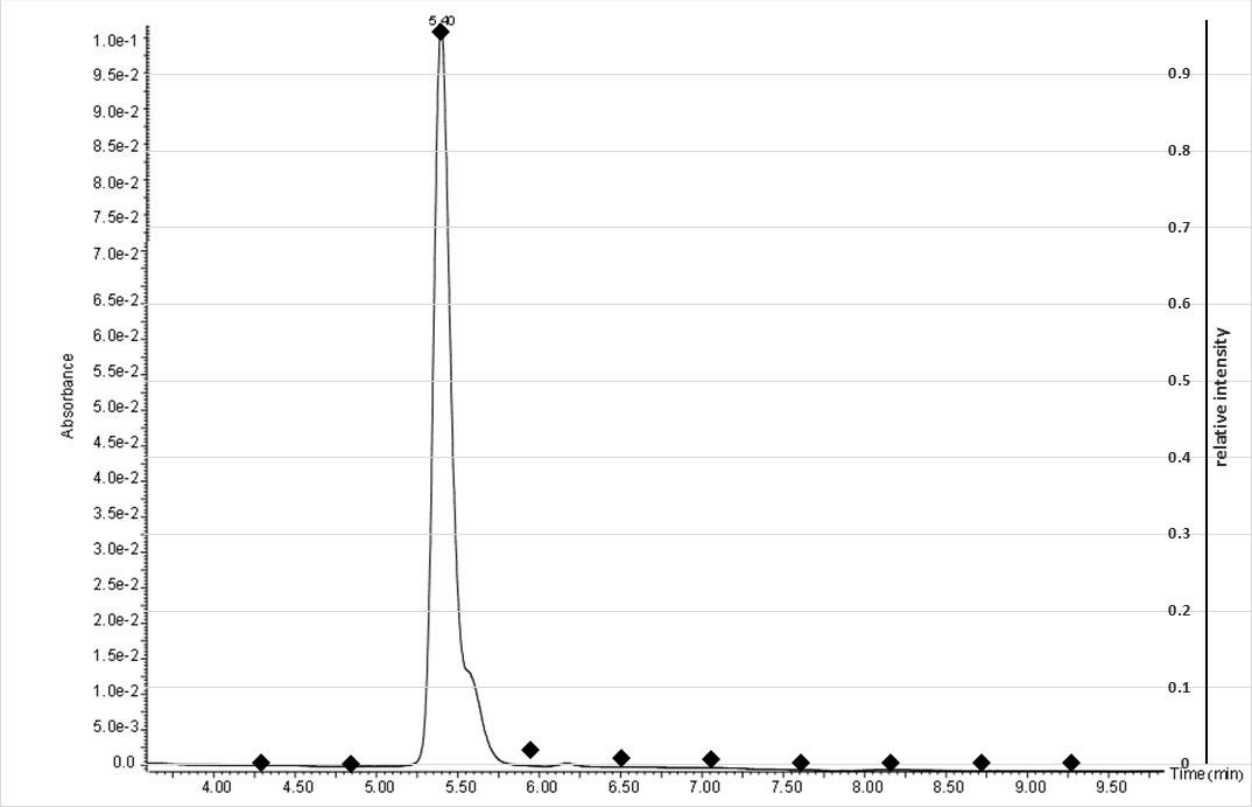
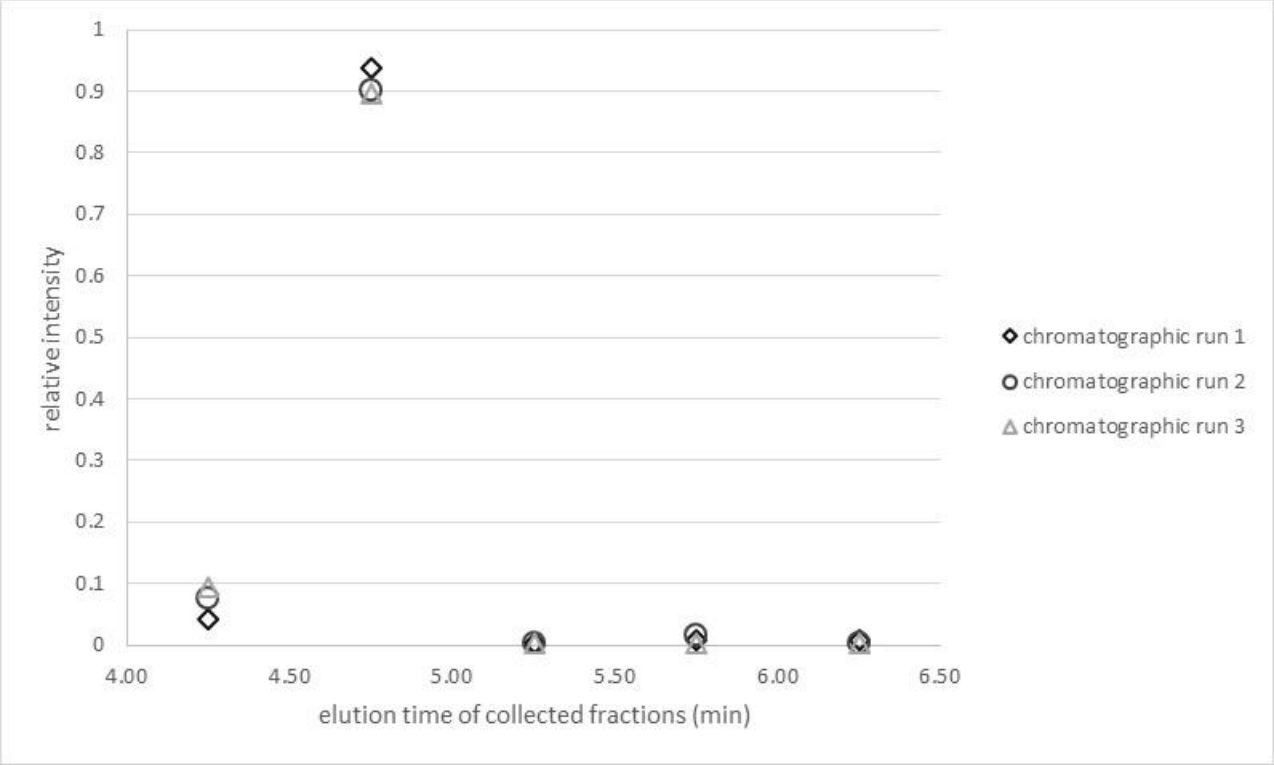
499 Fig. B

500 

501 Fig. C

502



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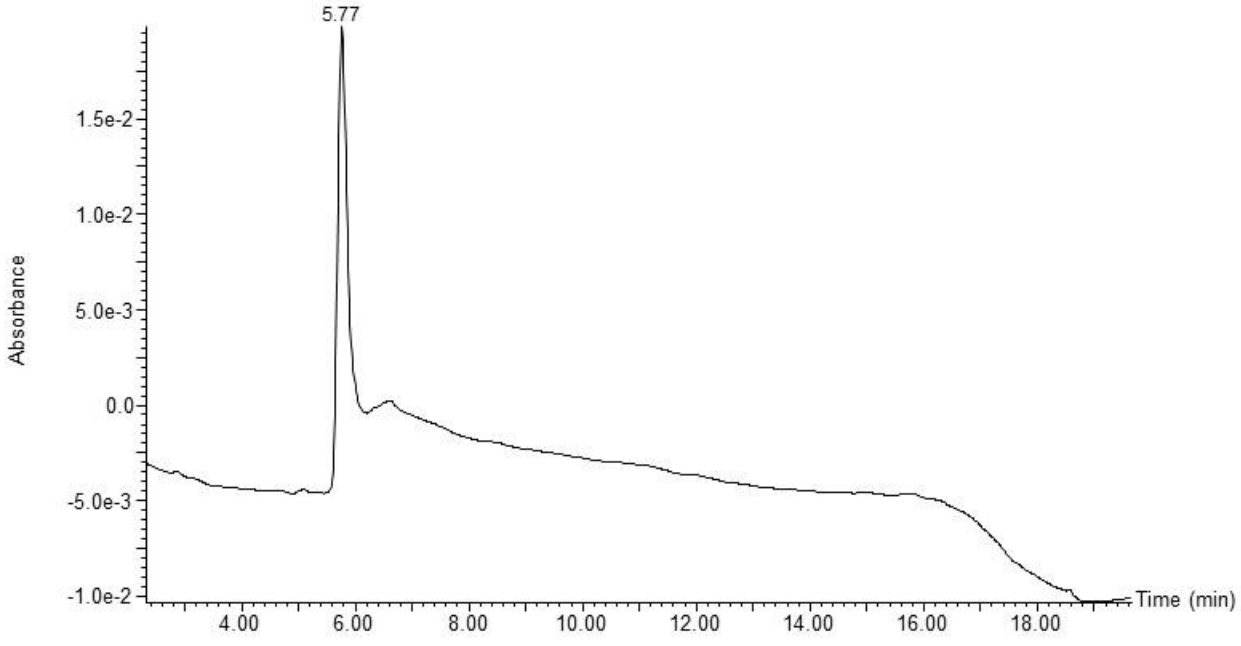
504 Fig. D



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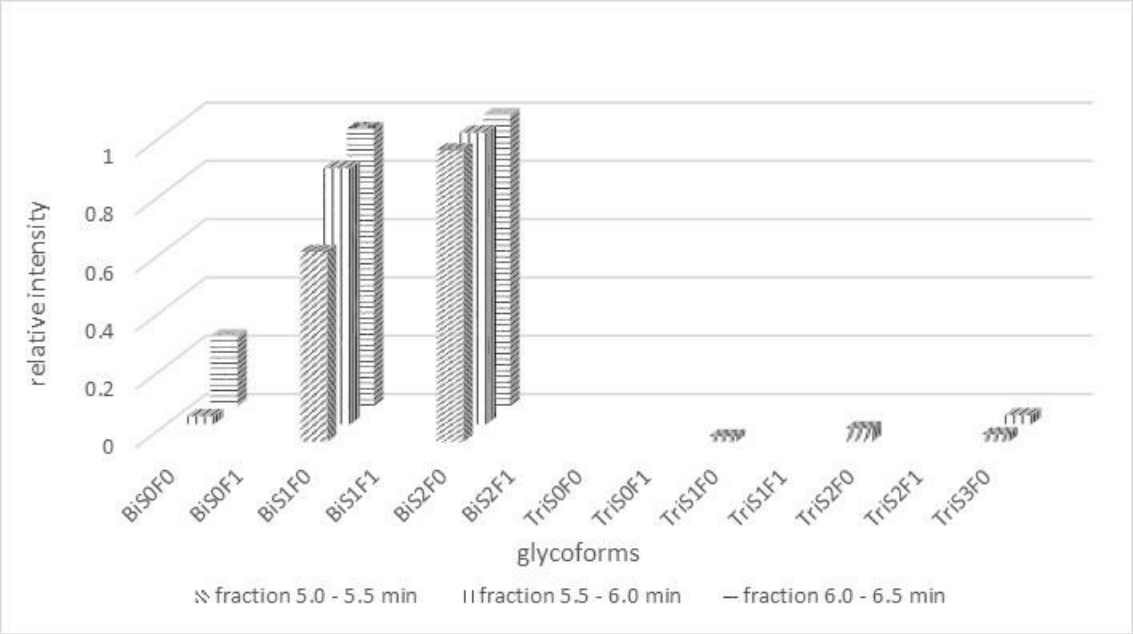
506 Fig. E.1



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508 Fig. E.2

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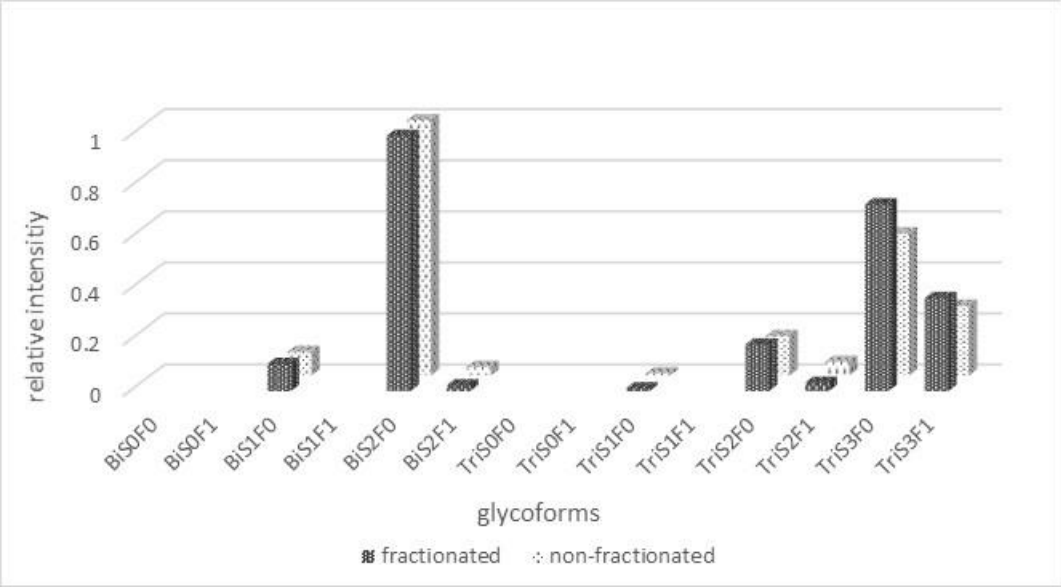

510

511 Fig. F