A Comparative Study of Analytical Parameters for Proteins with Different Degrees of Glycosylation

Application Note

Authors

Nicole Engel, Victor U. Weiss, Martina Marchetti-Deschmann, and Günter Allmaier
Research Group Bio- and Polymer Analysis, Institute of Chemical Technology and Analytics, Vienna University of Technology
Vienna, Austria

Christian Wenz, Andreas Rüfer, Martin Kratzmeier, and Susanne Glück
Agilent Technologies R&D and Marketing GmbH & Co. KG
Waldbronn, Germany

Abstract

Glycosylation, either co- or post-translational, is one important form of protein modification conveying protein stability, as well as contributing to folding and recognizability. Consequently, glycoproteins are the focus of basic research, bioengineering, and biotechnology. This Application Note describes the analysis of three biological relevant glycoproteins using the Agilent 2100 Bioanalyzer system. Human transferrin, human antitrypsin, and bovine acid glycoprotein were examined with the Agilent High Sensitivity Protein 250 and Protein 230 assays in regard to molecular weight determination (sizing), dynamic range for quantitation, and sensitivity for the selected analytes. Selected glycoproteins vary in their degree of glycosylation, influencing their behavior during capillary gel electrophoresis and sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). Both methods were compared with respect to characteristic analytical parameters. In general, microchip capillary gel electrophoresis (MCGE) separated the selected glycoproteins in less than a minute with high sensitivity and high reproducibility. However, the determined molecular weights of all analytes exceeded the theoretical values. Moreover, the occurrence of broadening peaks correlated to the degree of glycosylation.

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Introduction

The attachment of sugar moieties to proteins greatly influences their stability, solubility, folding, and most importantly, their bioactivity. Moreover, glycosylation patterns have been found to play a major role in various biological processes concerning immunology, development, and cancer biology[1]. To gain a better understanding of the biological function of a glycoprotein, or to control the integrity of an engineered product, it is of utmost importance to find new bioanalytical methods with high sensitivity and reliability to characterize such analytes in a fast manner.

The Agilent 2100 Bioanalyzer system is capable of rapid on-chip protein analysis with high reproducibility and accuracy regarding quantitation and sizing. It offers two different assays, the Protein 230 (P230) and the High Sensitivity Protein 250 (HSP-250) kits, for the electrophoretic separation of proteins up to 230 and 250 kDa, respectively. Electrophoresis of denatured proteins is carried out in a sieving matrix and buffer containing SDS. Sensitivities of the assays can exceed Coomassie Blue and silver-stained SDS-PAGE analyses[2,3]. Furthermore, both assays exhibit similar levels of reproducibility and accuracy, but differ in regard to labeling procedure and sensitivity. Proteins analyzed with the P230 Kit are not directly labeled but entrapped in SDS micelles containing fluorescence dye. In contrast, in the HSP-250 assay, proteins are covalently labeled with the HSP-250 dye prior to electrophoretic separation showing overall higher sensitivity.

To assess the electrophoretic behavior of glycoproteins with the 2100 Bioanalyzer system, three biological relevant proteins with varying glycosylation moieties were examined with both assays and compared to traditional SDS-PAGE. The characteristics of the selected glycoproteins, human transferrin, human antitrypsin, and bovine acid glycoprotein, are summarized in Table 1. All samples were analyzed with respect to reproducibility and accuracy of sizing and quantitation, sensitivity, peak pattern, and peak shape.

Table 1. Summary of the characteristics of human transferrin, human antitrypsin, and bovine acid glycoprotein.

Glycoprotein	Amino acids	N-glycosylation sites	MW_{lit} (MS) (kDA)	MW_{lit} (SDS-PAGE) (kDA)	Approximate glycan content
Transferrin[4,5]	697	Asn^{413}, Asn^{611}	80	80	6 %
Antitrypsin[6,7]	394	Asn^{46}, Asn^{83}, Asn^{247}	51	52	13 %
Acid glycoprotein[8]	184	Asn^{16}, Asn^{39}, Asn^{76}, Asn^{86}, Asn^{118}	33.8	42	37 %

Experimental

Materials

Human serum transferrin (≥ 98 %), bovine acid glycoprotein (99 %), human antitrypsin (salt free, lyophilized powder), dithiothreitol (DTT, BioUltra), silver nitrate and sodium carbonate (both analytical grade), as well as formaldehyde solution (35 % wt., for molecular biology) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Sodium thiosulfate pentahydrate, ethanol, and acetic acid (all analytical grade) were obtained from Merck (Darmstadt, Germany). NuPAGE 4–12 % *Bis-Tris* gels, 4x lithium dodecyl sulfate (LDS) sample buffer (106 mM *Tris* HCl, 141 mM *Tris* Base, 2 % LDS, 10 % Glycerol, 0.51 mM EDTA, 0.22 mM Serva Blue G250, 0.175 mM Phenol Red, pH 8.5), 20x MES SDS running buffer (50 mM MES, 50 mM *Tris* Base, 0.1 % SDS, 1 mM EDTA, pH 7.3), and BenchMark Protein Ladder were purchased from Invitrogen (Darmstadt, Germany). The Agilent 2100 Bioanalyzer system, P230 and HSP-250 kits were obtained from Agilent Technologies (Waldbronn, Germany).

SDS-PAGE

Samples in 1x LDS sample buffer and 50 mM DTT were run on NuPAGE 4–12 % *Bis-Tris* gels in 1x MES SDS running buffer at 120 V (const.) and 60 mA (max.). For molecular weight determination, BenchMark Protein Ladder was applied. Protein bands were seen by silver staining[9]. The gel was washed with 50 % ethanol and 5 % acetic acid for 20 minutes, with 50 % ethanol for 10 minutes, and three times with water for 20 minutes each. Afterwards, the gel was incubated in 0.2 % sodium thiosulfate pentahydrate for 1 minute and washed twice with water for 1 minute each. Subsequently, the gel was incubated with 1 % silver nitrate at 4 °C for 20 minutes and again washed twice with water for 1 minute each. The final incubation was in 2 % sodium carbonate and 0.04 % formaldehyde solution until protein bands were visible. Staining was stopped with 5 % acetic acid.

Protein labeling for HSP-250 analysis

All proteins and the HSP-250 ladder were labeled according to the description in the Agilent High Sensitivity Protein 250 Kit Guide[10]. Briefly, 10 µL of protein solution was mixed with 1 µL HSP-250 labeling dye and incubated on ice for 30 minutes. The labeling reaction was stopped by adding 1 µL of ethanolamine and incubating on ice for 10 minutes. For reproducibility and sensitivity experiments, 700 µg/mL glycoprotein solutions were labeled and diluted to the required concentration for chip analysis.

On-chip analysis

The chip-based glycoprotein analysis was performed on the Agilent 2100 Bioanalyzer system either with the P230 or the HSP-250 assay. Each chip was prepared according to the respective kit guide[10,11]. Briefly, samples were diluted 1:200 with water prior to analysis with the HSP-250 assay. Aliquots of 4 µL were incubated with 2 µL of sample buffer in the presence of DTT at 95 °C for 5 minutes. Samples analyzed with the HSP-250 assay were directly applied

to the chip, whereas 84 µL water were added to the samples used with the P230 assay before applying 6 µL of the dilution to the chip.

Results and Discussion

All three glycoproteins were analyzed with the 2100 Bioanalyzer system using the P230 and the HSP-250 kit, as well as with SDS-PAGE and subsequent silver staining. Glycoprotein sizing on a MCGE system, as with the 2100 Bioanalyzer solution, faces the same pre-analytical problems as traditional slab gel electrophoresis. Hydrophilic and bulky carbohydrate moieties on the amino acid backbone lead to a hampered interaction with detergents in comparison to unmodified proteins. In MCGE and SDS-PAGE, it can be observed that glycoproteins migrate slower in gel electrophoresis and, therefore, are detected at higher apparent molecular weight (MW)[12].

Figure 1 displays SDS-PAGE gels and gel-like images of electrophoretic separations of transferrin, antitrypsin, and acid glycoprotein with the P230 and the HSP-250 kit, respectively. Having two N-glycosylation sites, transferrin, with a MW of 80 kDa, represents the protein with the lowest glycan content (approximately 6 % of total MW) of the three investigated analytes (Table 1). Whereas a single band with the expected MW of 80 kDa is visible by SDS-PAGE, MCGE gives an average MW (MWexp) of ~90 kDa (89.3 ± 1.3 kDa for P230 and 90.4 ± 1.4 kDa for HSP-250) for both assays equaling a MW overdetermination of about 12.5 % (Figure 1A).

The average MW was calculated for both assays from over 15 measurements. For MCGE, the relevant signals are sharp and distinct (small peak width), as seen in the exemplary electropherograms presented in Figures 2A and 2C. The peak shape can be compared to nonglycosylated proteins (for example, the protein ladder which is always applied on the same protein chip as the samples).

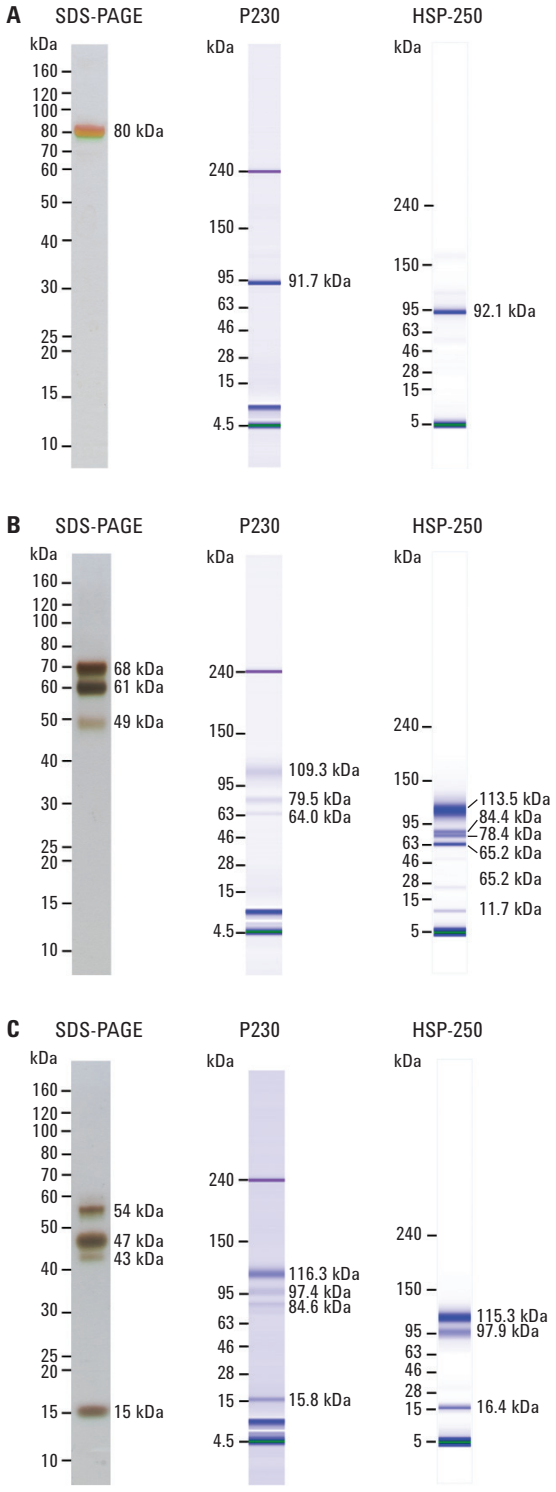

Figure 1. Comparison of the electrophoretic separation of (A) human transferrin, (B) human antitrypsin, and (C) bovine acid glycoprotein with SDS-PAGE, P230, and HSP-250 assays. For SDS-PAGE analysis, 250 ng of transferrin (A), 695 ng of antitrypsin (B), and 348 ng of acid glycoprotein (C) were applied. For the Agilent 2100 Bioanalyzer measurements, 550 µg/mL of each glycoprotein were applied.

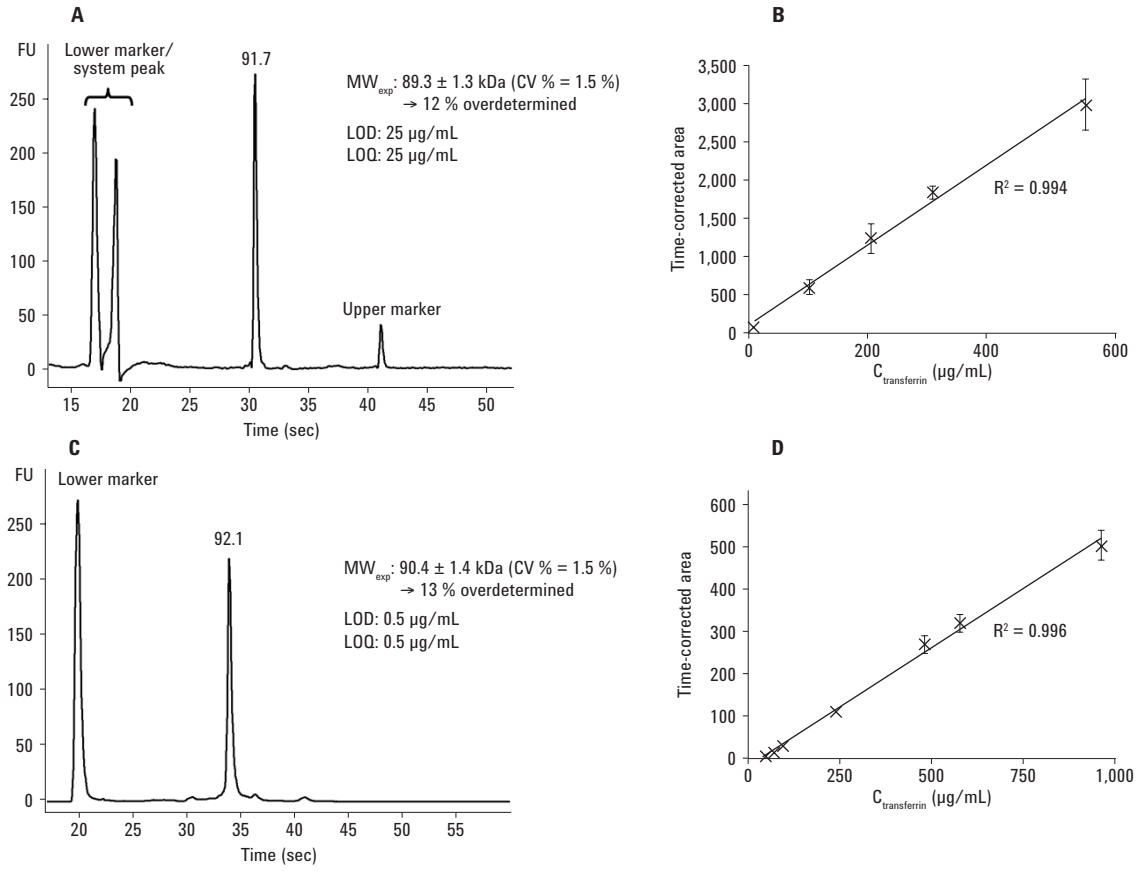

Figure 2. Gel electrophoretic analysis of transferrin on the Agilent 2100 Bioanalyzer system. 550 µg/mL transferrin was separated using (A) the P230 and (C) the HSP-250 assays (exemplary results depicted). The correlation coefficient (R^2) was determined to be (B) 0.994 for the P230 and (D) 0.996 for the HSP-250 assays.

Antitrypsin, which exhibits three N-glycosylation sites, has an expected MW of 51 kDa and an approximate glycan content of 13 % of the overall MW (Table 1). The SDS-PAGE gel shows three distinct bands with MWs of 49, 61, and 68 kDa (Figure 1B), respectively. These three bands can also be detected with the P230 assay, yet the determined average MWs of 63.4 ± 1.0, 78.8 ± 1.0, and 108.1 ± 1.4 kDa exceeded the expected values by over 100 % in the most abundant component (Figure 3A). A further increase in MW was observed for the HSP-250 run (65.0 ± 0.8, 84.3 ± 1.2, and 112.0 ± 1.2 kDa), which can be explained by the altered behavior of labeled glycoproteins compared to nonlabeled ones. The highly sensitive assay allowed for the detection of a fourth component with a MW of 78.0 ± 1.3 kDa not visible by SDS-PAGE (Figure 3C). In comparison to transferrin, the antitrypsin signals of the 2100 Bioanalyzer measurements were broader without causing greater deviations in sizing (approximately 1.1–1.6 % for each signal).

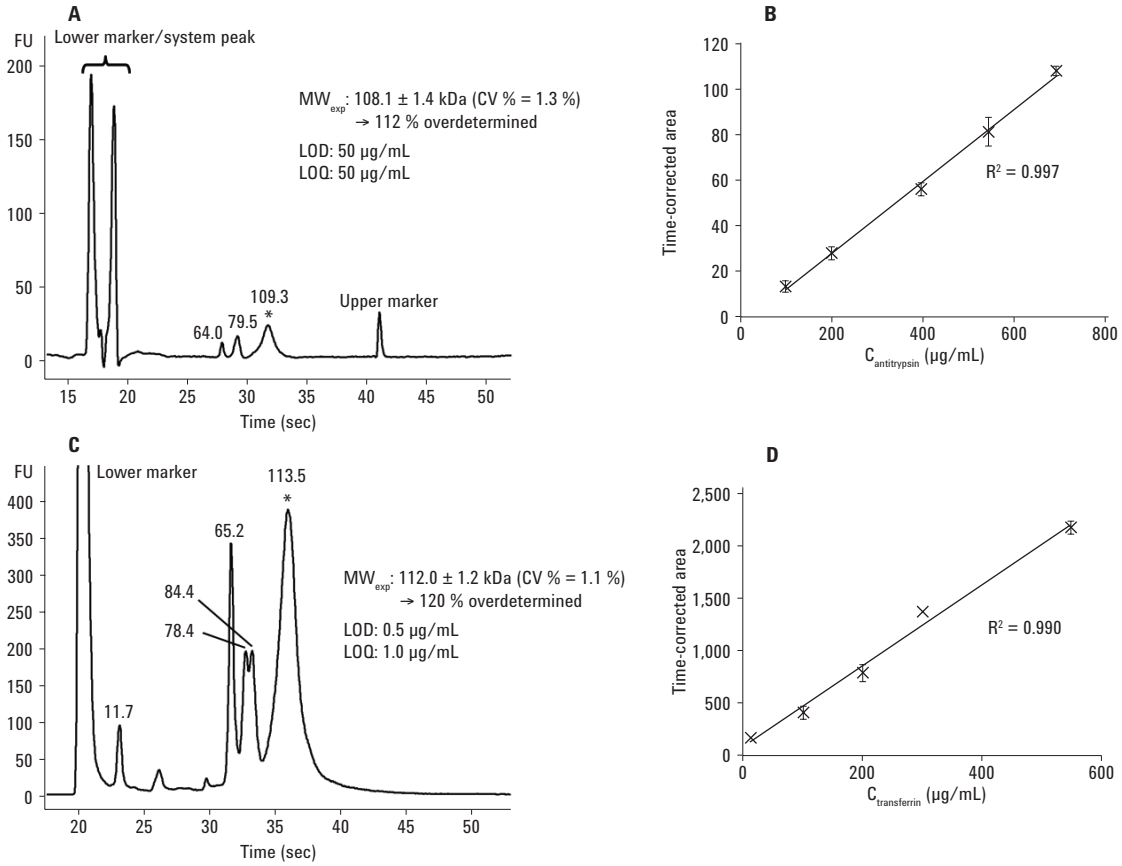

Figure 3. Gel electrophoretic analysis of antitrypsin with the Agilent 2100 Bioanalyzer system. 550 µg/mL antitrypsin was separated using (A) the P230 and (C) the HSP-250 assays (exemplary results depicted). The * marks the most abundant and evaluated signal. The correlation coefficient (R^2) was determined to be (B) 0.997 for the P230 and (D) 0.990 for the HSP-250 assays.

The third analyte, acid glycoprotein, has a MW of 33.8 kDa and exhibits five N-glycosylation sites leading to a glycan content of approximately 37 % (Table 1). Due to this high percentage of attached carbohydrates, an even higher deviation of the measured MWs from literature was expected, as well as an increase in peak heterogeneity compared to the two previously investigated glycoproteins. Corresponding SDS-PAGE and 2100 Bioanalyzer results are presented in Figure 1C, confirming these anticipations. Four protein bands with MWs of 15, 43, 47, and 54 kDa could be seen on the SDS-PAGE gel. The 47 kDa compound was the most abundant one. The 2100 Bioanalyzer investigations using the P230 assay also identified four compounds, but with much higher MWs (15.6 ± 0.2, 84.7 ± 0.7, 98.5 ± 1.2, and 116.3 ± 1.2 kDa, respectively). The largest one being the most abundant. In contrast, the HSP-250 assay only separated three compounds exhibiting similar MWs (15.9 ± 0.6, 95.8 ± 1.8, and 113.6 ± 1.3 kDa) when compared to the P230 kit (Figure 4C).

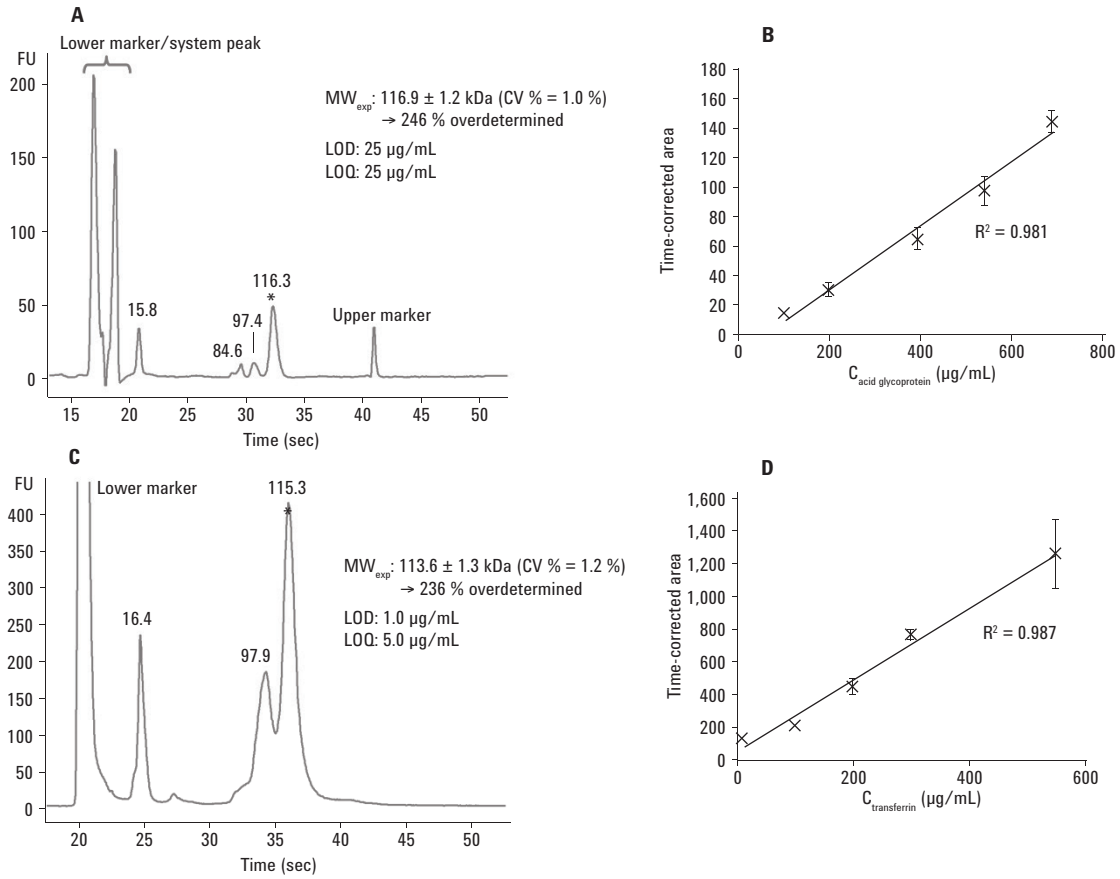

Figure 4. Gel electrophoretic analysis of acid glycoprotein with the Agilent 2100 Bioanalyzer system. 550 µg/mL acid glycoprotein was separated using (A) the P230 and (C) the HSP-250 assays. (exemplary results depicted). The * marks the most abundant and evaluated signal. The correlation coefficient (R^2) was determined to be (B) 0.981 for the P230 and (D) 0.987 for the HSP-250 assays.

To evaluate the sizing reproducibility, sample triplets were measured for each analyte either on three consecutive (inter-chip) or on one 2100 Bioanalyzer chip (intra-chip). Both assays showed low deviations of obtained MW values (less than 1.0 % for intra- and less than 1.7 % for inter-chip reproducibility). Such values lie perfectly within the specifications of both kits with a determined sizing reproducibility of 3 % for measurements with bovine serum albumin[10,11].

The quantitation accuracy and reproducibility were determined for all glycoproteins, and both assays on the 2100 Bioanalyzer system, respectively. Triplets of several different concentrations of each glycoprotein were measured and plotted against corresponding time corrected areas. Figures 2B and 2D display linear regressions of transferrin analyzed with the P230 and the HSP-250 assays. In both cases, good linearity could be determined with correlation coefficients (R^2) of over 0.99 with average deviations of approximately 10 %. The latter was slightly lower for the P230 assay. Antitrypsin also showed good correlation with linear regressions of R^2 being 0.99 or more as demonstrated (Figures 3B and 3D). Deviations of time corrected areas were, on average, approximately 8 % for the P230 and approximately 10 % for the HSP-250 assay, and were always smaller than 17 % for single measurements. In comparison to transferrin and antitrypsin, the correlation coefficients of the linear regressions of acid glycoprotein were slightly lower with values of 0.981 and 0.987 for the P230 and HSP-250 assays, respectively (Figures 4B and 4D). Furthermore, the related deviations were slightly higher for both assays, but always lower than 19 %. Altogether, all glycoproteins could be quantified within the specifications of both assays, which define the quantitation reproducibility with a CV % lower than 20 % as determined for bovine serum albumin[10,11].

Besides the quantitation accuracy and reproducibility, the sensitivity of both assays was investigated for all three analytes. The limit of detection (LOD) defines the concentration with a signal-to-noise ratio (S/N) over 2 and the limit of quantification (LOQ) has an S/N over 3. The specific values for each glycoprotein are presented in Figures 2A/C, 3A/C, and 4A/C. The LOD and LOQ rose with increasing degree of glycosylation in case of the HSP-250 assay. For the P230 assay, antitrypsin showed highest values when compared to transferrin and acid glycoprotein.

Conclusion

Three glycoproteins with varying glycosylation degrees were analyzed on the Agilent 2100 Bioanalyzer system using the P230 and the HSP-250 assays and compared to results from standard SDS-PAGE experiments (Table 2). In general, MWs determined with the 2100 Bioanalyzer system exceeded SDS-PAGE values depending on the degree of glycosylation, as N-glycans interfered with detergent attachment resulting in flawed values of seemingly higher MWs. Additionally, the width of respective peaks broadened in accordance to the number (that is, heterogeneity) of glycan moieties. Consequently, sizing of intact glycoproteins with gel based electrophoretic separation can be difficult. In contrast, quantitation experiments with glycoproteins on the 2100 Bioanalyzer system showed reproducible and accurate results with increasing deviations concomitant to increasing degrees of glycosylation. However, reproducibility of sizing results still lay within the specifications of both assays. Also, the LOD and LOQ tended to show a correlation with the amount of protein carbohydrate modifications.

Table 2. Summary of results for the glycoproteins human transferrin, human antitrypsin and bovine acid glycoprotein for SDS-PAGE, the P230, and HSP-250 assays.

Glyco-protein	Approx. glycan content	MW_{lit} (MS) in kDa	MW_{exp} (SDS-PAGE) in kDa	MW_{exp} (P230) in kDa	MW_{exp} (HSP-250) in kDa	MW_{exp} (HSP-250) in kDa	Average CV % (P230)*	Average CV % (HSP-250)*	LOD/LOQ (P230) in µg/mL	LOD/LOQ (HSP-250) in µg/mL
Transferrin	6 %	80	80	89.3 ± 1.3	90.4 ± 1.4	90.4 ± 1.4	9	10	25/25	0.5/0.5
Antitrypsin	13 %	51	49, 61, 68	63.4 ± 1.0 78.8 ± 1.0 108.1 ± 1.4	65.0 ± 0.8 78.0 ± 1.3 84.3 ± 1.2 112.0 ± 1.2	65.0 ± 0.8 78.0 ± 1.3 84.3 ± 1.2 112.0 ± 1.2	8	10	50/50	0.5/1
Acid glycol-protein[8]	37 %	33.8	15, 43, 47, 54	15.6 ± 0.2 84.7 ± 0.7 98.5 ± 1.2 116.3 ± 1.2	15.9 ± 0.6 95.8 ± 1.8 113.6 ± 1.3	15.9 ± 0.6 95.8 ± 1.8 113.6 ± 1.3	8	11	25/25	1/5

* Average CV %: average deviation of quantitation concerning the evaluated signal.

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