N-Glycan Analysis by Orthogonal Methods: UHPLC and Multi-Capillary CE

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ABSTRACT

Sample preparation for glycoprofiling of glycoproteins has been a cumbersome, multi-day process including overnight labeling, and multiple lengthy centrifugation and vacuum drying steps. Most sample preparation protocols also use toxic reagents during the dye labeling step of glycans. Traditionally single capillary electrophoresis (CE) and liquid chromatography (LC) have been two of the most common methods used to profile N-glycans, with an average analytical time of 15-35 min for CE, and 60 min for high resolution LC. We have improved the glycoprofiling process at several levels, enabling short analysis time by highly sensitive multi-capillary CE. In addition, we have reduced overall workflow duration, hands on time, and eliminated centrifugation and vacuum drying steps. Experiments presented utilize 8-aminopyrene-1,3,6-trisulfonic acid (APTS); a well recognized dye in regulated environments. Here we present the comparability of N-Glycan profiles of human IgG prepared with GlycanAssure™ assay kit reagents on two high performance systems—the Applied Biosystems™ CE3500xL protein quality analyzer, capable of analyzing 24 samples simultaneously, and ultra-high performance liquid chromatography (UHPLC). Utilizing this workflow, we demonstrate strong similarity of N-glycan profiles between the two platforms with low %RSD of ~5% for the entire sample preparation and analysis process.

INTRODUCTION

Glycosylation of therapeutic proteins are complex traits with macro and micro-heterogeneity that affect efficacy, immunogenic responses and circulation half-life, which consequently changes PK/PD profiles. Glycosylation must be evaluated at all stages in development of drug substance from early stage cell line screening, through process optimization, formulation and eventually with every lot release. Therefore, glycosylation is considered a critical quality attribute (CQA). Changes in patients' glycome from total plasma and IgG have been found to be indicative of steps in the inflammatory cascade as well as providing biomarkers for many cancers[1].

CE and LC are two of the most common methods used for glycoprofiling. Traditionally, CE instruments used for glycoprofiling carry one capillary in which labeled glycan species are separated based on the combination of charge and size and detected using laser induced florescent (LIF). On the other hand, in LC, the glycan species are eluted based on the order of decreasing hydrophobicity and increasing size (using HILIC columns). As the nature of the separation is different between the two system, it is typical to anticipate different level of resolution among glycan species that can also result in different relative areas as well. Traditional sample preparation workflows are excessively long and cumbersome taking as long as 1-3 days. Instrument sensitivity, analytical time and system malfunctioning are some of the other major issues in traditional glycoprofiling. In addition, analytical time itself takes about 60 min on LC and 15-35 min per sample on CE.

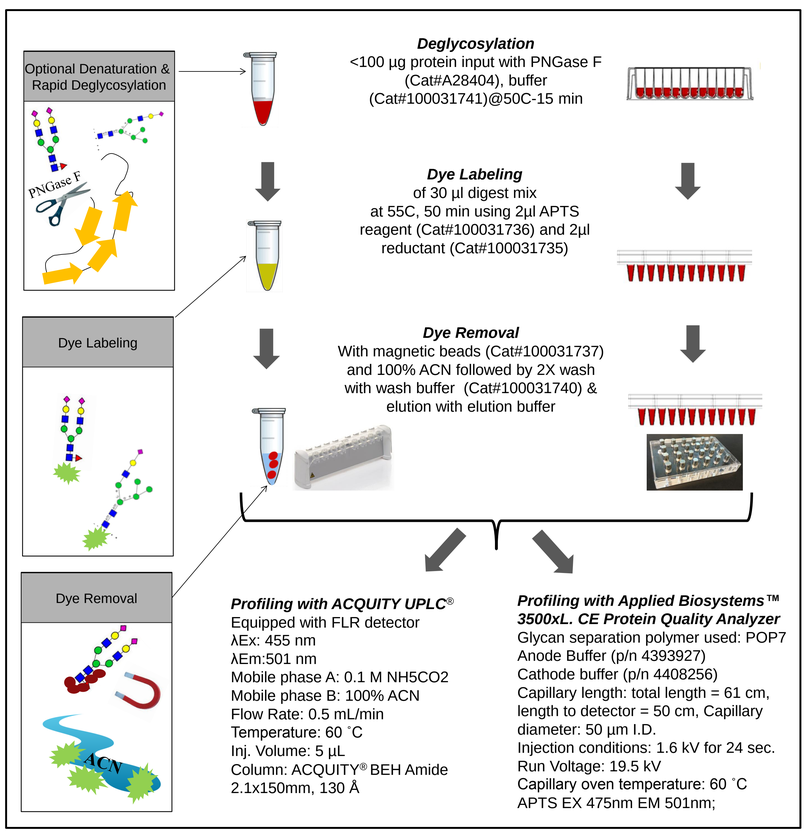
With the significant advancement in sensitivity, analytical time, and instrument robustness in the Applied Biosystems™ CE3500xL protein quality analyzer system, glycoprofiling time has been shortened to as low as <2 min per sample without any compromise in resolution. This is achieved by analysis of 24 samples simultaneously using 24 capillaries. In current work we present comparison of our sample preparation workflow using GlycanAssure™ reagents kit on both CE3500xL protein quality analyzer and UHPLC.

MATERIALS AND METHODS

Purified human serum IgG was obtained from Invitrogen (P/N 27102). Samples were prepared using the reagents provided with the GlycanAssure™ assay kit (Thermo Fisher Cat# A28676) (https://www.thermofisher.com/order/catalog/product/A28676).

10 replicates of hIgG (a well characterized reference) were prepared according to the workflow presented in scheme 1, and analyzed on both UPLC® and Applied Biosystems™ CE3500xL protein quality analyzer systems. Results are presented in 3 parts including comparison of overall glycan profile on CE 3500xL protein quality analyzer and UPLC®; comparison of relative areas between the two systems and the variation of sample prep workflow analyzed on both systems.

Scheme 1- Sample Preparation Workflow for Glycan Preparation

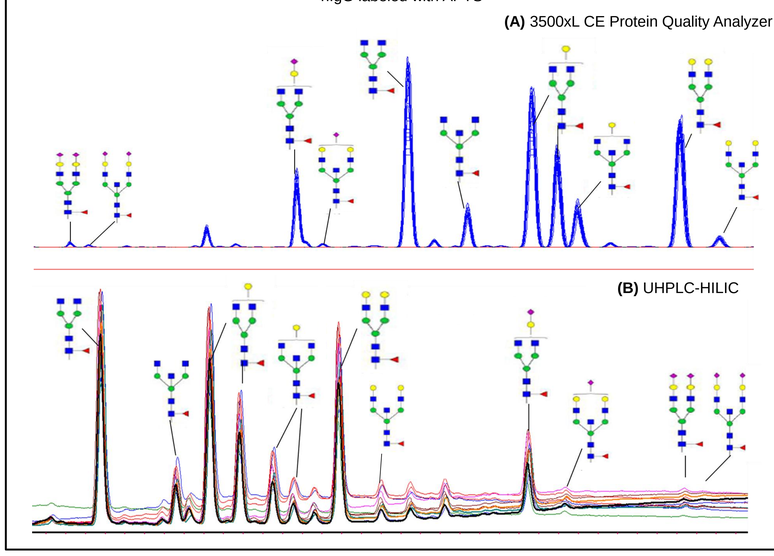


RESULTS

Comparison of N-glycoprofile and Relative Area of Major Glycan Peaks on Applied Biosystems™ CE3500xL and UHPLC

APTS labeled N-glycans from hIgG were prepared per the workflow described in scheme 1 in 10 replicates. 2µl of each sample was placed with 8 µl of loading mix (landmark red™ , Liz™ and loading buffer) in each 96-well plate for CE analysis. Another 15 µl of the samples were placed in LC vials with 45 µl ACN for LC analysis. Tentative peak assignments were performed using co-elution time with labeled standards, spiking and use of GU values. hIgG samples were shown to include a mixture of fucosylated neutral, mono and di-sialylated oligosaccharides, with some bisecting GlcNAc. 21 peaks were integrated in samples run on both systems. The normalized overlaid electropherogram of 10 replicates from CE/LIF and chromatograms from LC with N-glycan notations and structures are shown in Fig. 1 a,b. The overall profile of peak shapes and numbers were very similar on both systems including the relative area of many major glycans (Fig. 2a), indicating a good comparability between the two systems using APTS .

Figure 1. Comparison of CE electropherogram (A), with UPLC (HILIC) chromatogram (B) from replicates of hIgG labeled with APTS

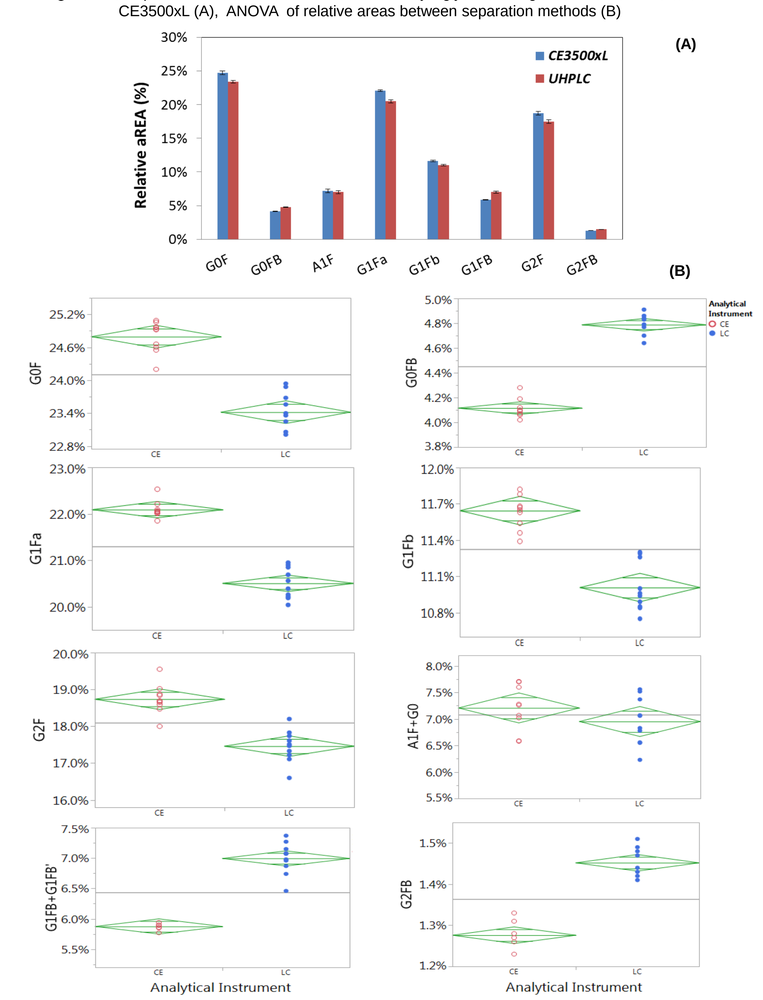


ANOVA for selected N-glycan peaks between two analytical instruments showed small, but significant differences in the relative areas obtained from these two platforms as is anticipated due to two different separation mechanism used on these two systems impacting the resolution and co-elution.

It is widely reported that similarly sized/-charged glycans migrate closely on CE which can impair the ability to resolve some peaks. For instance even upon the use of a high resolving method, G1F and G2 may not separate from each other on CE while LC can resolve G2 from G1FB. Similarly, co-elution of A1F and G0 is commonly observed on CE while G0 is well separated on the LC platform.

Among the differences, it is noteworthy that the high resolution analysis of glycan species on UHPLC takes ~60 min per sample while <2 minutes is required on the CE3500xL. With the two methods being complimentary and orthogonal, use of both methods is advisable in order to gain the best coverage. Additionally, CE would likely be a good choice for high throughput analysis of upstream samples. While LC can provide a good tool for lower throughput characterization of glycans.

Figure 2. Comparison of relative area from selected major glycans of hIgG between UHPLC and CE3500xL (A), ANOVA of relative areas between separation methods (B)

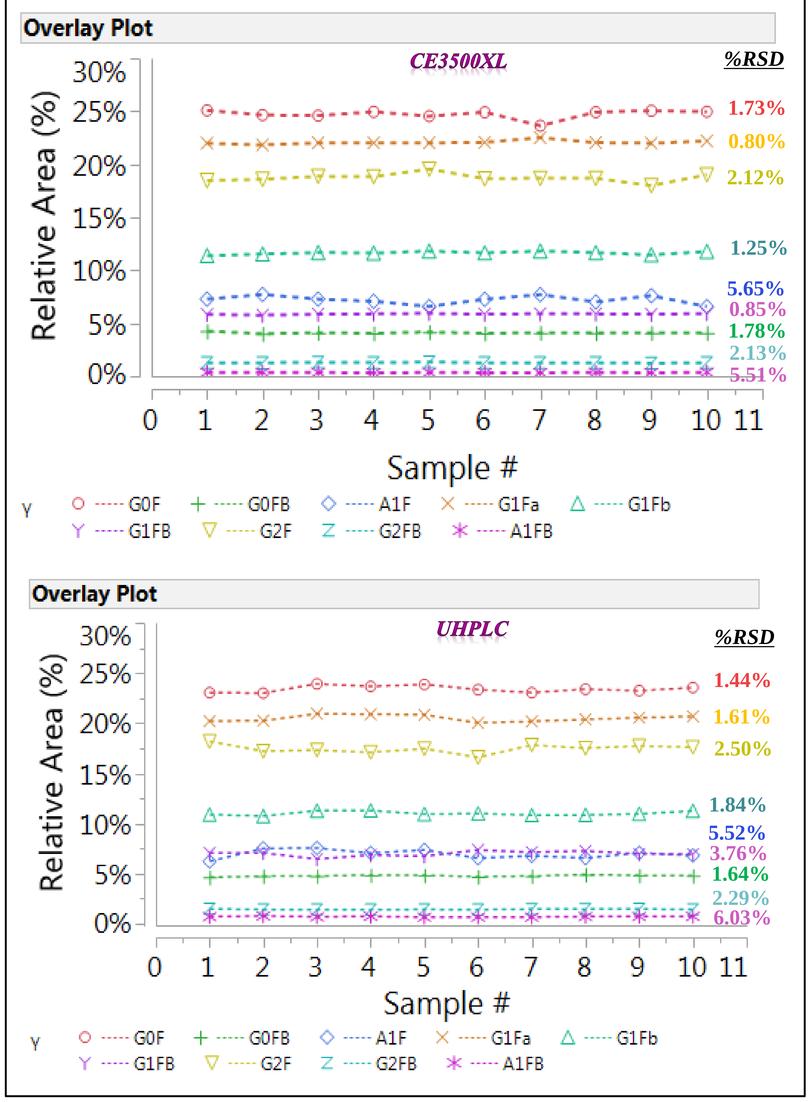


Workflow Variability Evaluation between CE3500xL & UHPLC

Sample preparation for glycan analysis typically includes many hands on steps over a period of 1-3 days. This can have a significant impact on the consistency and quality of the final results regardless of the analytical platform used. Bias from workflow can originate from several factors such as incomplete and inconsistent deglycosylation, site-specific heterogeneity, poor labeling efficiency, biased and inconsistent loss of glycans during clean up steps, and loss of sialic acids due to conditions used during labeling step.

To test the consistency of the current workflow, we evaluated the variation among 10 replicates of hIgG prepared with GlyanAssure™ assay kit reagents. A %RSD of <5% was observed for glycan peaks with relative area >1% between different sample preparation replicates when analyzed on both the CE3500xL and UHPLC. All peaks with relative area of <1% also demonstrated %RSD of <10% on both systems. This low variation demonstrates the robustness of the workflow and the high precision of analysis between different capillaries of the CE3500xL.

Figure 3. Comparison of variation between sample prep. replicates of GlycanAssure™ assay kit on CE3500xL protein quality analyzer and UHPLC



CONCLUSIONS

- UPLC/UHPLC and the CE 3500xL provide similar N-glycan profiles using the GlycanAssure™ sample preparation workflow and APTS dye.
- Comparable relative areas for the majority of major N-glycan peaks were observed on both UPLC/UHPLC and CE platforms with %RSD of <5% between sample replicates, indicating good repeatability of sample preparation and consistency between capillaries on the CE3500xL system.

REFERENCES

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TRADEMARKS/LICENSING