

# Direct Determination of Native N-linked Glycans by UHPLC with Charged Aerosol Detection

*David Thomas, Ian Acworth, Bruce Bailey, Marc Plante, and Qi Zhang  
Thermo Fisher Scientific, Chelmsford, MA*



## Overview

**Purpose:** To develop fast and sensitive HPLC methods suitable to directly measure the glycan content and profile of glycoproteins.

**Methods:** N-linked glycans were released from proteins by PNGase F. The native glycans were separated by ultra high performance liquid chromatography (UHPLC) on a column that employs both weak anion exchange and hydrophilic interaction liquid chromatography (HILIC) separation mechanisms. The native glycans were detected directly without derivatization by using charged aerosol detection.

**Results:** Precision, detection limits, and dynamic range of quantitative measurements are presented. Figures of merit include low-nanogram (low-pmol) on-column sensitivity, over two orders of magnitude of dynamic range, and peak area precision of about three percent RSD.

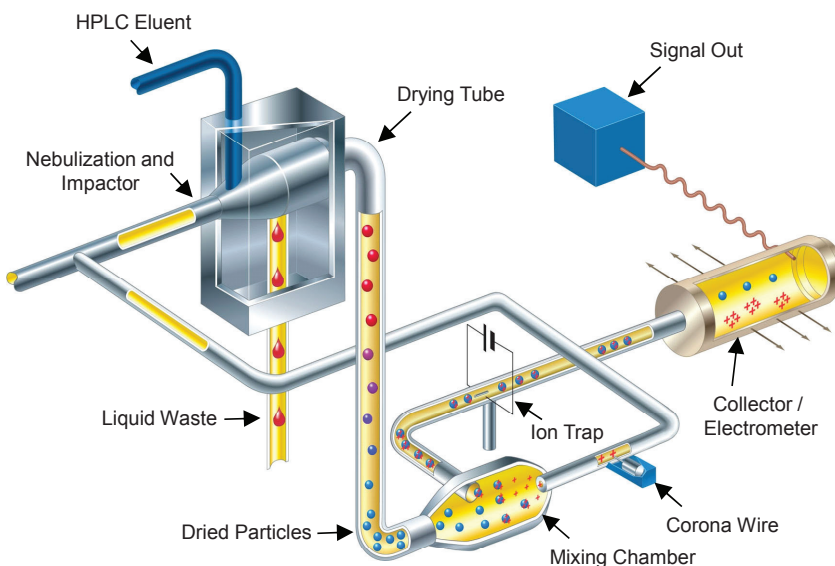
## Introduction

Glycoproteins of biological, diagnostic, or therapeutic interest owe key aspects of their normal function to the oligosaccharides attached to the protein backbone. Changes in the number, type, composition, or linkage pattern of these glycans may serve as a biomarker of disease or influence the efficacy of a biotherapeutic product.<sup>1</sup> For this reason, the ability to correctly identify and measure these glycans is of scientific interest, and to do so reliably, quickly, and inexpensively is of practical benefit. This work explores direct detection of native glycans as an alternative to the common techniques for glycan analysis that rely on derivatization reactions to render glycans detectable. The lack of a detectable chromophore in native glycans is overcome by using HPLC with charged aerosol detection, a detector that can quantitatively measure any non-volatile compound.

Figure 1 depicts the operation of the charged aerosol detector. At the top left, the liquid mobile phase from the LC column enters the detector, where it is nebulized by combining with a concentric stream of nitrogen gas or air. The fine droplets are carried by bulk gas flow to the evaporation sector where desolvation occurs to form dry particles from any nonvolatile or semivolatile species. Any remaining large droplets drain away to waste. The dry analyte particles exit from evaporation and combine with another gas stream that has been charged by a high voltage charged aerosol detection charger set near 2 kilovolts. The charged gas mixes with the dry particles and transfers positive charge to the analyte particle's surface. After passing through an ion trap that removes any high mobility species, the remaining charged particles pass to a collector and are measured by a sensitive electrometer.

Bottom line: The signal produced is directly proportional to the quantity of analyte.

**FIGURE 1. Charged aerosol detector and principle of operation**



## Methods

### Liquid Chromatography

Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system with:

- Thermo Scientific™ Dionex™ UltiMate™ 3000 Rapid Separation Diode Array Detector, DAD-3000RS
- Thermo Scientific™ Dionex™ Corona™ Veo™ Charged Aerosol Detector:
  - Evaporation Temp.: 50 °C
  - Power Function: 1.00
  - Data Collection Rate: 2 Hz

Reagents: HPLC- or LCMS-grade or better

### Data Analysis

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) 7.2

### Fast Charge Analysis

Column: Thermo Scientific™ GlycanPac™ AXH-1 column,  
1.9 µm, 2.1 × 100 mm

Column Temp.: 30 °C

Flow Rate: 0.4 mL/min

Injection Vol.: 2 µL

Mobile Phase A: 80:20 (v/v) acetonitrile:water

Mobile Phase B: 80 mM ammonium formate pH 4.4

Gradient: Time, %B: Isocratic, 25%B

### High Resolution Separation

Column: GlycanPac AXH-1 column,  
1.9 µm, 2.1 × 100 mm

Column Temp.: 30 °C

Flow Rate: 0.4 mL/min

Injection Vol.: 2 µL

Mobile Phase A: 80:20 (v/v) acetonitrile:water

Mobile Phase B: 80 mM ammonium formate pH 4.4

Gradient: Time, %B: -5, 2.5; 1, 2.5; 40, 25

### Sample Prep

OligoStandard:

Add 250 µL of HPLC grade water to one vial of Thermo Scientific™ Dionex™ OligoStandard™ Sialylated Fetuin N-linked alditols (Thermo Fisher Scientific P/N 043604). Vortex to dissolve and transfer to a plastic glass HPLC autosampler vial.

Dextran Ladder:

Reconstitute one vial (200 µg) Dextran Ladder Glycan Standard for HPLC (Sigma D3818) with 1 mL HPLC grade water per manufacturer's instructions.

Fetuin from fetal bovine serum (Sigma-Aldrich 2379) was prepared by dissolving 4 mg ±1 mg in 1 mL HPLC grade water.

IgG from human serum (Sigma-Aldrich I2511, 5.6 mg/mL, ≥ 95%) was used as received.

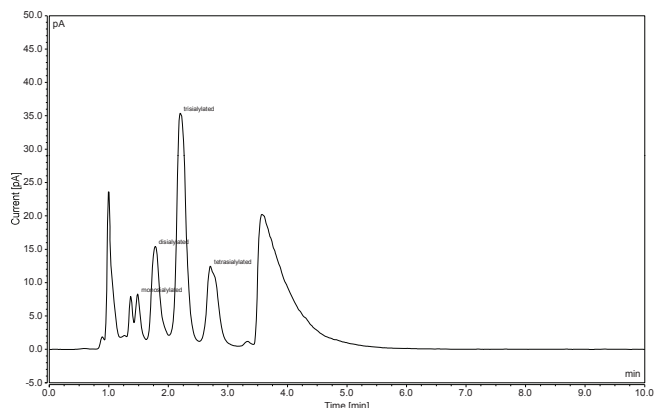
Protein PNGase F digestions were performed by using QA-Bio PNGase F Deglycosylation kit (QA-Bio P/N E-PNG01) per the manufacturer's instructions. Briefly, add 35 µL of protein solution to a plastic centrifuge tube. Add 10 µL 5x Reaction Buffer 7.5 and 2.5 µL of Denaturation Solution. Heat at 100 °C for 5 minutes. Cool. Add 2.5 µL of Triton X-100 and mix. Add 2.0 µL of PNGase F to the reaction. Incubate 18 hours at 37 °C. Centrifuge at 12,000 rpm for 10 min and inject the supernatant.

# Results

## Fast Separation by Charge

Quality control methods may aim to quickly quantify a protein's glycan pool according to charge. In the fast separation shown in Figure 2, glycans are sorted into groups consisting of mono-, di-, tri-, and tetrasialylated species. In this isocratic separation, all species elute within 10 min from the GlycanPac AXH-1 column with good resolution. Because of the uniform response of the charged aerosol detector, the relative peak areas accurately reflect the amount (pmol) within each charge group.

**FIGURE 2. Fast quantification of glycans by charge state on the GlycanPac AXH-1 column with charged aerosol detection**

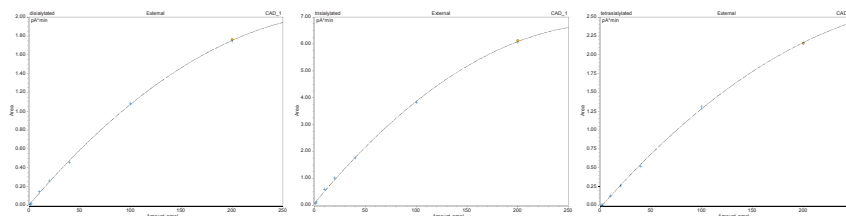


## Performance

Calibration curves for the three major charge groups of the bovine fetuin N-linked alditol standard (analyzed in duplicate) are presented in Figure 3. Standards were prepared at concentrations ranging from 0.5 to 100 pmol/μL total glycans; using 2 μL injections, the mass on column ranged from 1 to 200 pmol. The data were fit to a quadratic equation, yielding coefficients of determination,  $R^2$ , greater than 0.999 for all three analytes.

Table 1 presents a summary of the method's performance, including precision of retention time and peak area for the 200 pmol calibration standard, the coefficient of determination, and the limits of detection for the three major charge groups of the bovine fetuin N-linked alditol standard.

**FIGURE 3. Calibration data for direct detection of N-linked glycans by HPLC-charged aerosol detection**



**TABLE 1. Method performance for direct detection of glycans by HPLC-charged aerosol detection**

Component	Amount (pmol)	Ret. Time <sup>1</sup> (%RSD)	Peak Area <sup>1</sup> (%RSD)	LOD <sup>2</sup> (pmol)	R <sup>2*</sup>
Disialylated	200	0.24	3.32	8.4	0.9995
Trisialylated	200	0.32	2.41	8.5	0.9994
Tetrasialylated	200	0.13	3.80	8.7	0.9995

<sup>1</sup> for n = 10 replicates

<sup>2</sup> Hubaux-Vos method, relative to nominal total pmol on column

\* 7 levels, in duplicate, quadratic fit with no offset

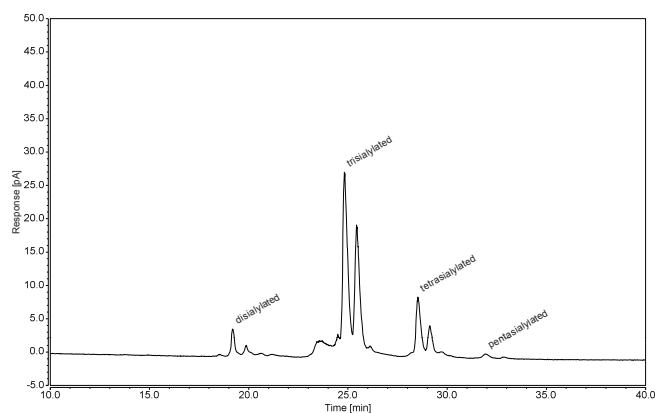
### High Resolution Separation by Charge, Size and Polarity

Often, it is advantageous to accurately profile a protein's glycan pool to assess lot to lot variability, degradation, or impurities. In the high resolution separation shown in Figure 4, glycans are selected according to charge, size, and polarity. These sialylated N-linked alditols were released from bovine fetuin by PNGase F, reduced, and purified. Here we simply reconstituted a standard in water and injected 550 pmol. The native glycans are separated by HPLC using a binary gradient consisting of acetonitrile and a volatile ammonium formate buffer and measured directly using a Dionex Corona Veo RS charged aerosol detector. Figure 5 shows the same separation for a real glycoprotein digest. In this case, although the N-linked glycans were not reduced to alditols, a similar elution profile is observed.

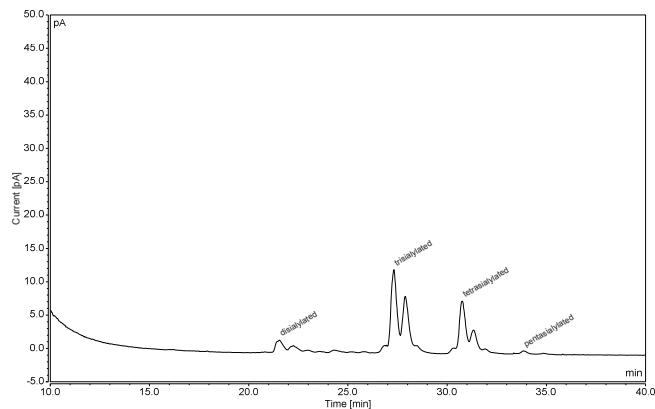
Note that there is no need to use fluorescent labeling when using charged aerosol detection, as may be necessary with other means of detecting these compounds.

As seen before, glycans of the same charge elute as a group due to the ion-exchange functionality, while the HILIC mode further separates glycans of the same charge based on their size and polarity.

**FIGURE 4. Direct charged aerosol detection of bovine fetuin N-linked alditols in a standard mixture separated with high resolution on the GlycanPac AXH-1 column**



**FIGURE 5. Direct charged aerosol detection of bovine fetuin N-linked glycans released by PNGase F digestion separated with high resolution on the GlycanPac AXH-1 column**

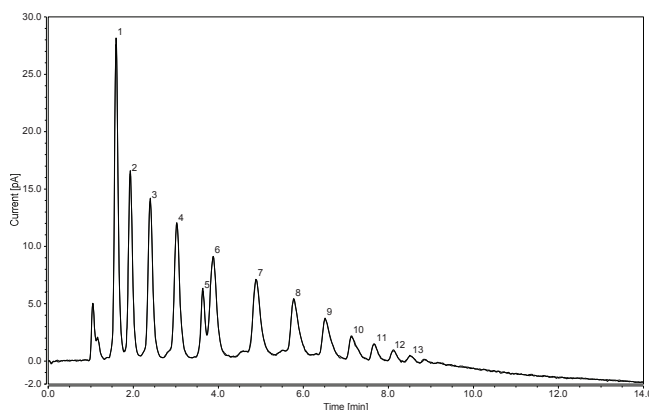


## Dextran Ladder

Many labs performing glycan analysis like to run a standard sample to help account for normal day-to-day and instrument-to-instrument variations in glycan retention time and chromatographic performance.<sup>2</sup> For example, a dextran ladder gives a characteristic profile from monomeric glucose to approximately 20-mers of glucose oligosaccharide. The elution position of each peak in this ladder is expressed as a glucose unit (GU), and is used to assign GU values to peaks in the released glycan pool. The dextran ladder may also be used to predict glycan size by comparing the retention time of the unknown glycan to that of adjacent peaks in the glycan ladder.

Here the native glycan ladder (Sigma-Aldrich D3818) is separated within about 10 minutes and shows good resolution of glucose homopolymers up to about n=13. Again, no need to spend time or money on 2-AB labeling and a clean chromatogram with no concern for reaction side products. For comparison, the product information bulletin shows 19 glucose oligos separated in 140 minutes.

**FIGURE 6. Direct detection of dextran ladder oligosaccharides separated on the GlycanPac AXH-1 column and detected with charged aerosol detection**



## Conclusion

- The HPLC method developed to measure native glycans is precise, with retention time precision better than 0.3% RSD and peak area precision of approximately 3% RSD for the major sialylated N-glycans of bovine fetuin.
- Charged aerosol detection enables sensitive, direct measurement of glycans with no need to perform labeling reactions. Detection limits for native glycans are in the low pmol (ng on-column) range.
- By responding directly to any non-volatile compound, charged aerosol detection is able to measure both native and fluorescently labeled glycans, yielding simple, accurate, and precise estimates of relative concentration even in the absence of pure primary standards.

## References

1. Varki, A. Biological Roles of Oligosaccharides: All of the Theories Are Correct. *Glycobiology*, **1993**, *3*, 97–130.
2. Guile, G. R., et al. *Anal. Biochem.*, **1996**, *240*, 210–226.

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