



Oligosaccharide structures and the degree of sialylation can cause variability among detection cells when using high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) technology for oligosaccharide characterization. That variability can affect quantitative results used to determine batch-to-batch consistency.

# Sialylation Levels Influence Oligosaccharide Quantitation

## Analyzing Response Variability Using High-pH Anion-Exchange Chromatography and Pulsed Amperometric Detection

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**A** protein's carbohydrate side chains affect its solubility, how it folds, its *in vivo* biological functions, and its pharmacokinetic profiles (1–4). Because of the complex nature of glycoproteins and the various recombinant DNA technology processes used to produce them, it remains a challenge to assess batch-to-batch consistency by evaluating glycosylation patterns.

Glycans can be characterized by releasing the oligosaccharides from the protein using either chemicals (5–6) or enzymes (7). The released oligosaccharides are then characterized using HPLC, fluorophore-assisted carbohydrate electrophoresis (8,9), capillary electrophoresis (10–12), mass spectrometry (13,14), or high-pH anion-exchange chromatography (15).

High-pH anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) resolves neutral and charged oligosaccharide species that differ in branch, linkage, and positional isomerism. One advantage of this method is that the oligosaccharide structures can be detected without prior derivatization. PAD cells, however, have finite lifetimes because the working electrode is subject to surface recession and fouling. That means the electrodes must be polished or replaced when baseline noises increase or when oligosaccharide peak responses begin to change.

Our investigation indicates that the variability between detector cells may limit the usefulness of HPAEC-PAD as a rugged method for quantifying glycosylation patterns.

### Materials and Methods

Recombinant human alpha-galactosidase (r-haGAL), recombinant human thyroid-stimulating hormone (rhTSH), and peptide-N-glycosidase F (PNGaseF) were obtained from Genzyme (Framingham, MA). Oligosaccharide standards for asialo biantennary (NA2), asialo biantennary with core fucosylation (NA2F), bisialylated biantennary (A2), and bisialylated biantennary with core fucosylation (A2F) were obtained from Glyko (Novato, CA). The oligosaccharide standards were reconstituted in water to a final concentration of 1 mM. The 50% (w/w) sodium hydroxide solution and sodium acetate (anhydrous) were from J.T. Baker Chemicals. The 2-mercaptoethanol came from Sigma Chemicals (St. Louis). The biodialyzer and the 1 kD molecular weight cut-off membranes were purchased from the Nest Group (Southboro, MA).

**HPAEC-PAD analysis.** N-linked oligosaccharides were released from 500  $\mu$ g of r-haGAL or 300  $\mu$ g of rhTSH with 10  $\mu$ L (6 U/mg) of PNGaseF and 150  $\mu$ M of 2-mercaptoethanol in sodium phosphate at pH 7 and 37°C for 18–20 h. Released oligosaccharides were recovered by centrifuging the digests at 14,000 rpm for 30 minutes using 10 kD molecular weight of cut-off, regenerated, cellulose membrane spin-filters.

The filtrates were vacuum dried (Savant Instruments, Farmingdale, NY) and reconstituted in 500  $\mu$ L HPLC-grade water. After dialysis using 1 kD molecular weight of cut-off, regenerated, cellulose membranes against sodium phosphate at pH 7.0 and

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**Table 1.** Structures of the oligosaccharides derived from r-h $\alpha$ Gal and rhTSH for various peaks from the chromatography profiles produced using high-pH anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD)

r-h $\alpha$ Gal Peak <sup>a</sup>	Identity	rhTSH Peak <sup>b</sup>	Identity
R <sub>G</sub> 1	Monosialylated biantennary core fucosylated	R <sub>T</sub> 1	Monosialylated biantennary
R <sub>G</sub> 2	Monophosphorylated oligomannose 6 and monophosphorylated oligomannose 7	R <sub>T</sub> 2	Bisialylated biantennary core fucosylated
R <sub>G</sub> 3	Trisialylated triantennary core fucosylated	R <sub>T</sub> 3	Bisialylated triantennary
R <sub>G</sub> 4	Hybrid monosialylated monophosphorylated	R <sub>T</sub> 4	Bisialylated biantennary
R <sub>G</sub> 5	Biphosphorylated oligomannose 7	R <sub>T</sub> 5	Trisialylated triantennary

<sup>a</sup>r-h $\alpha$ Gal-derived oligosaccharide peak in HPAEC-PAD chromatography profile  
<sup>b</sup>rhTSH-derived oligosaccharide peak in HPAEC-PAD chromatography profile

25°C, the oligosaccharides were vacuum dried, reconstituted in 300  $\mu$ L of HPLC-grade water, and filtered through 0.45  $\mu$ M regenerated, cellulose membrane spin-

filters. Several preparations of released oligosaccharides were pooled to provide a sufficient quantity of each recombinant protein from the homogenous preparation.

**Chromatography analysis.** We analyzed the oligosaccharides (50 mL of sample per injection) using a CarboPac PA100 column (4.0  $\times$  250 mm) using the DX-500 system (Dionex Corporation, Sunnyvale, CA). The column was equilibrated in 100 mM sodium hydroxide, and the oligosaccharides were eluted at 35°C using a gradient consisting of 0–60% of 500 mM sodium acetate in 100 mM sodium hydroxide for 60 minutes at a flow rate of 1 mL/min. The separated oligosaccharides were detected by electrochemical detection on an ED40 detector (PAD) equipped with a gold working electrode and an Ag–AgCl reference electrode. A four-stage potential waveform was used with the following conditions:

$$E1 = +0.1 \text{ V (0–0.4 s, integration 0.2–0.4 s),}$$

$$E2 = -1.5 \text{ V (0.41–0.42 s),}$$

$$E3 = +0.6 \text{ V (0.43 s), and}$$

$$E4 = -0.01 \text{ V (0.44–0.50 s).}$$

The resulting chromatographic data were integrated and processed using the PeakNet

data reduction software (Dionex Corp.). To assess whether the assay response for various PAD detector cells was reproducible, we analyzed three assays of derived oligosaccharides from r-h $\alpha$ GAL and rhTSH on each of six different PAD detector cells. For each PAD cell, the average value

of the triplicate analyses was used in the calculation.

**MALDI-TOF analysis.** Peaks 1–5 derived from the r-h $\alpha$ GAL and rhTSH oligosaccharide were collected, neutralized with 1 N HCl, and dialyzed against water at 25°C for 18–20 h. The samples were then

**Table 2.** Absolute peak area (nC\*sec) values from the chromatography profiles produced using high-pH anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) for specified oligosaccharide derived from r-h $\alpha$ GAL

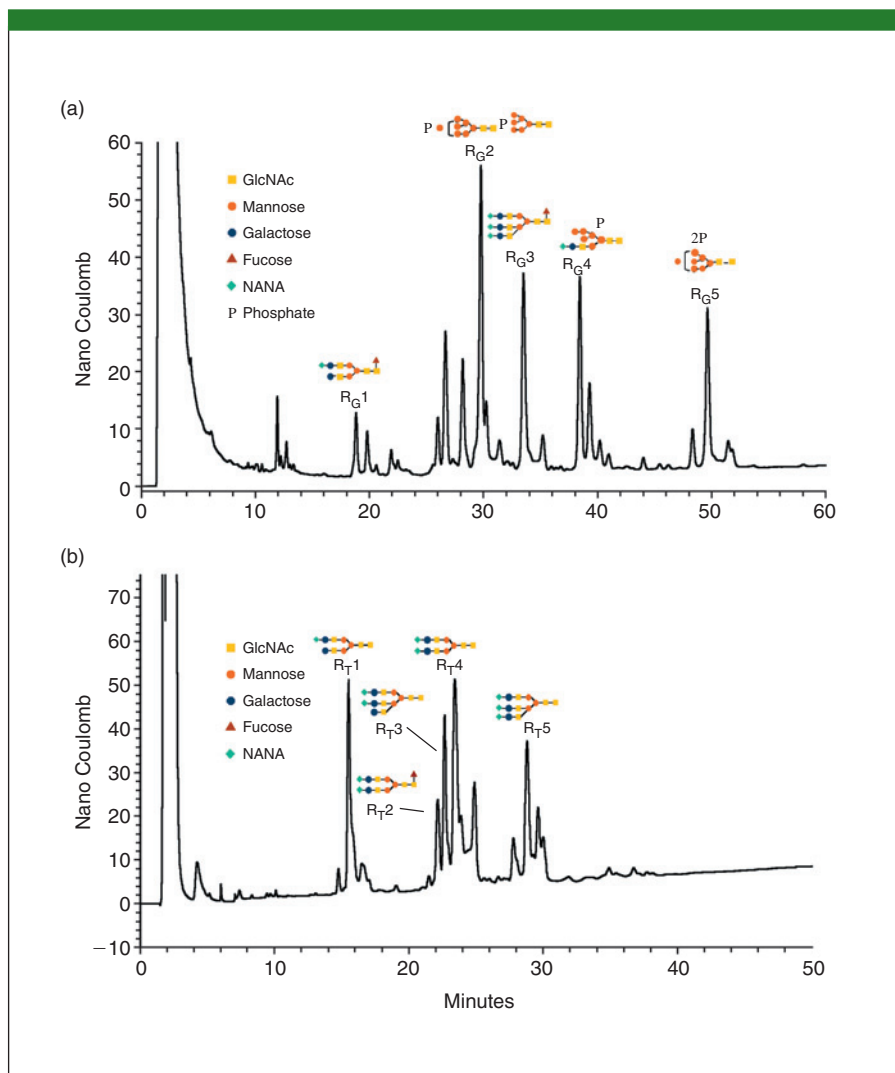
Variable	R <sub>G1</sub>	R <sub>G2</sub>	R <sub>G3</sub>	R <sub>G4</sub>	R <sub>G5</sub>
<b>Sample 1</b>					
Average <sup>a</sup>	1993617	9273046	4273536	4788080	4065661
SD <sup>b</sup>	417244	1391911	1830856	997478	516611
% CV <sup>c</sup>	20.93	15.01	42.84	20.83	12.71
<b>Sample 2</b>					
Average <sup>a</sup>	2064335	8800501	4109725	5217042	3821966
SD <sup>b</sup>	379853	1325938	1648735	1009840	633117
% CV <sup>c</sup>	18.40	15.07	40.12	19.36	16.57
<sup>a</sup> n = 6	<sup>b</sup> standard deviation		<sup>c</sup> percentage of the coefficient of variation		

**Table 3.** Relative peak area percentages from the chromatography profiles produced using high-pH anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) for specified oligosaccharide peaks in r-h $\alpha$ GAL

Variable	R <sub>G1</sub>	R <sub>G2</sub>	R <sub>G3</sub>	R <sub>G4</sub>	R <sub>G5</sub>
<b>Sample 1</b>					
Average <sup>a</sup>	8.22	38.42	16.82	19.60	16.95
SD <sup>b</sup>	0.88	2.11	3.76	0.34	1.89
% CV <sup>c</sup>	10.72	5.50	22.35	1.75	11.15
<b>Sample 2</b>					
Average <sup>a</sup>	8.66	36.98	16.55	21.71	16.11
SD <sup>b</sup>	0.93	2.10	3.76	0.24	1.95
% CV <sup>c</sup>	10.74	5.67	22.70	1.11	12.12
<sup>a</sup> n = 6	<sup>b</sup> standard deviation		<sup>c</sup> percentage of the coefficient of variation		

**Table 4.** Absolute peak area (nC\*sec) values from the chromatography profiles produced using high-pH anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) for specified oligosaccharide derived from rhTSH

Variable	R <sub>T1</sub>	R <sub>T2</sub>	R <sub>T3</sub>	R <sub>T4</sub>	R <sub>T5</sub>
<b>Sample 1</b>					
Average <sup>a</sup>	7773737	2941201	5794456	17023223	7422647
SD <sup>b</sup>	747839	411736	301594	3610299	1706852
% CV <sup>c</sup>	9.62	14.00	5.20	21.21	23.00
<b>Sample 2</b>					
Average <sup>a</sup>	8292315	2714263	5587838	16368244	7252383
SD <sup>b</sup>	696741	372358	351,729	3633608	1830326
% CV <sup>c</sup>	8.40	13.72	6.29	22.20	25.24
<sup>a</sup> n = 6	<sup>b</sup> standard deviation		<sup>c</sup> percentage of the coefficient of variation		



**Figure 1 (left).** Chromatography profiles from high-pH anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) of oligosaccharides derived from (a) r-hαGAL and (b) rhTSH; we chose the oligosaccharide peaks R<sub>G</sub>1–R<sub>G</sub>5 and R<sub>T</sub>1–R<sub>T</sub>5 for numerical analysis.

**Table 5.** Relative peak area percentages from the chromatography profiles produced using high-pH anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) for specified oligosaccharide peaks in rhTSH

Variable	R <sub>T</sub> 1	R <sub>T</sub> 2	R <sub>T</sub> 3	R <sub>T</sub> 4	R <sub>T</sub> 5
<b>Sample 1</b>					
Average <sup>a</sup>	19.42	7.18	14.38	41.14	17.87
SD <sup>b</sup>	3.83	0.34	1.93	3.78	2.05
% CV <sup>c</sup>	19.70	4.77	13.41	9.20	11.46
<b>Sample 2</b>					
Average <sup>a</sup>	21.14	6.76	14.12	40.24	17.74
SD <sup>b</sup>	4.20	0.24	1.82	3.86	2.30
% CV <sup>c</sup>	19.87	3.56	12.89	9.60	12.98

<sup>a</sup>n = 6      <sup>b</sup>standard deviation      <sup>c</sup>percentage of the coefficient of variation

analyzed using a sDHB matrix (90% 2,5-dihydroxybenzoic acid, 10% 2-hydroxy-5-methoxybenzoic acid) by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), on Bruker biflex equipment (Billerica, MA) operated in the reflectron mode with a nitrogen laser at 337 nm.

**Variability Results**

The HPAEC-PAD chromatographic profiles for r-hαGAL- and rhTSH-derived oligosaccharides (Figure 1) are resolved according to charge (related to the degree of sialylation), size, monosaccharide composition, and glycosidic linkages. We analyzed the data generated both qualitatively and quantitatively. The chromatographic profiles of the two preparations (samples 1 and 2) of each recombinant protein were compared (pattern-matching) to assess qualitative differences in the glycan structures.

**Oligosaccharide peaks.** We selected five oligosaccharide peaks (R<sub>G</sub>1–R<sub>G</sub>5 in r-hαGAL and R<sub>T</sub>1–R<sub>T</sub>5 in rhTSH) from the chromatographic profiles (peaks that spanned the entire chromatographic profile) for numerical analysis. For quantitative analyses, the results were reported either as peak areas or as relative peak area percentages (the peak area of a specified oligosaccharide peak divided by the sum of peak areas of the five specified oligosaccharide peaks).

The proposed structures of the r-hαGAL- and rhTSH-derived oligosaccharides (as identified by MALDI-TOF analysis) are tabulated in Table 1.

**PAD cell variability.** Using r-hαGAL-derived oligosaccharides as a model, significant response variability between the six PAD cells was observed. Tables 2 and 3 report the absolute peak area (in nC\*sec) and the relative peak area percentage for each specified oligosaccharide peak in the r-hαGAL samples.

The R<sub>G</sub>3 species showed significant variability (approximately 43% in peak areas and 23% in relative peak areas) between individual detector cells. These data suggest that oligosaccharide species containing more than one terminal sialic acid residue exhibited greater variability in overall PAD cell response. However,

**Table 6.** Peak area (nC\*sec) and peak area ratios for the purified oligosaccharide standards<sup>a</sup>

Variable	A2F	A2	NA2F	NA2	A2F ÷ A2	NA2F ÷ NA2	NA2F ÷ A2F	NA2 ÷ A2
Average <sup>b</sup>	5657574	3955130	3484526	3661215	1.43	0.95	0.65	0.97
SD <sup>c</sup>	1729714	1162364	597965	611652	0.03	0.02	0.16	0.20
% CV <sup>d</sup>	30.57	29.39	17.16	16.71	2.13	2.10	24.68	21.21

<sup>a</sup>The oligosaccharide standards were not evaluated on PAD cells 1 or 2 or on the first two assay occasions of PAD cell 3. On the remaining PAD cells, they were evaluated in a similar manner to the oligosaccharides derived from each recombinant protein for a total of 11 data points.  
<sup>b</sup>n = 11      <sup>c</sup>standard deviation      <sup>d</sup>percentage of the coefficient of variation

**Table 7.** Normalized peak areas from the chromatography profiles produced using high-pH anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) for specified oligosaccharide peaks in r-hαGAL test material

Variable	R <sub>G1</sub>	R <sub>G2</sub>	R <sub>G3</sub>	R <sub>G4</sub>	R <sub>G5</sub>
Average <sup>a</sup>	95.14	86.90	88.73	100.00	85.71
SD <sup>b</sup>	1.53	1.48	1.19	0.00	1.56
% CV <sup>c</sup>	1.61	1.70	1.34	0.00	1.82

<sup>a</sup>n = 6  
<sup>b</sup>standard deviation  
<sup>c</sup>percentage of the coefficient of variation

oligosaccharide species containing no or one terminal sialic acid residue(s) or phosphate residue(s) (such as mannose 6-phosphate) exhibited less variability.

To examine whether similar variability is found in oligosaccharides derived from a different recombinant protein, rhTSH-derived oligosaccharides were analyzed using the same approach (see Tables 4 and 5).

The rhTSH-derived oligosaccharides showed significant variability for the R<sub>T2</sub>, R<sub>T4</sub>, and R<sub>T5</sub> species (approximate peak variability of 14%, 22%, and 25%, respectively) as a function of the individual PAD detector cell. Peak variability was less for the oligosaccharide species R<sub>T1</sub> (about 10%) and R<sub>T3</sub> (about 6%), but the R<sub>T1</sub> variability in relative peak area was approximately 20%. Species R<sub>T2</sub>, R<sub>T4</sub>, and R<sub>T5</sub> have more than two sialic acids and are fully sialylated, whereas the R<sub>T1</sub> has only one sialic acid. Although R<sub>T3</sub> contains two terminal sialic acid residues, we detected minimal cell-related variability in that species. In R<sub>T3</sub>, however, only two of the three antennae are sialylated.

**Minimizing Variability in Analyses**

The variability found in the data from our

experiments suggests that oligosaccharide species containing more than one terminal sialic acid residue exhibit greater response variability among PAD cells. The number of occupied oligosaccharide chains with terminal sialic acid may also contribute to elevated PAD cell variability (as shown in the R<sub>T3</sub> species). Oligosaccharide species containing no or one terminal sialic acid(s) or phosphate residue(s) (such as mannose 6-phosphate) exhibit less variability.

To further examine PAD cell variability as a function of sialylation or fucosylation, we also analyzed four commercially available, purified oligosaccharide standards (A2, a bisialylated biantennary; A2F, a bisialylated biantennary with a fucosylated core; NA2, an asialo biantennary; and NA2F, an asialo biantennary with a fucosylated core). Table 6 shows the peak area and peak area ratios for these standards.

Significant variability was again apparent in the commercial standards for the sialylated species — A2F and A2 (approximately 30% in peak areas) — as a function of the individual PAD detector cell. However, the nonsialylated species, NA2F and NA2, had approximately 17% variability. The peak ratio results confirmed

that the variability in the nonsialylated species was associated with the degree of sialylation in the complex oligosaccharide species. Core fucosylation does not appear to contribute significantly to the cell–cell variability. Experiments performed with older, used, and newer PAD cells demonstrated similar variability in assay response (data not shown).

PAD cell variability can have significant implications for quantitative assays. That variability must be considered when setting numerical specifications for specific oligosaccharide structures.

**Using a multiplier.** An alternative approach (similar to that used for analyzing peptide maps) for minimizing assay variability related to the PAD detector cell is to divide the peak areas (in nC\*sec) of a specified peak by the peak area for the same peak from another sample. That quotient is the multiplier value. For example, R<sub>G4</sub> in sample 1 (designated as the reference material for this purpose) can be divided by the peak area for the same peak in sample 2 (designated as the test material). The peak areas of the other specified peaks (R<sub>G1</sub>, R<sub>G2</sub>, R<sub>G3</sub>, and R<sub>G5</sub>) in the test material are then multiplied by the multiplier value. The normalized percent of each specified peak in the test material, relative to the reference material, is then reported. Tables 7 and 8 show the results of recalculating the data for either the r-hαGAL or the rhTSH oligosaccharides using this strategy.

The advantage to using a multiplier is that the variability from the PAD detection cells decreases significantly. The disadvantage is that one of the specified

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**Sialylation** continued from page 50

oligosaccharide peaks is removed from the numerical analyses.

If the assay that profiles oligosaccharides uses HPAEC-PAD technology to produce quantitative data, we recommend that the variability in cell responses be considered. We will evaluate assay variability using disposable PAD cells (when commercially available from Dionex) and other oligosaccharide profiling methods (such as anthranilic acid labeling) as potential alternatives to the HPAEC-PAD technology (16).

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**Table 8.** Normalized peak areas from the chromatography profiles produced using high-pH anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) for specified oligosaccharide peaks in rhTSH test material

Variable	R <sub>T1</sub>	R <sub>T2</sub>	R <sub>T3</sub>	R <sub>T4</sub>	R <sub>T5</sub>
Average <sup>a</sup>	111.26	96.25	100.45	100.00	101.32
SD <sup>b</sup>	2.23	2.98	2.00	0.00	2.48
% CV <sup>c</sup>	2.01	3.09	1.99	0.00	2.45

<sup>a</sup> n = 6  
<sup>b</sup> standard deviation  
<sup>c</sup> percentage of the coefficient of variation

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