

HPAE-PAD Peak Area Response of Glycoprotein Oligosaccharides

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Key Words

Dionex CarboPac Column, Carbohydrate, *N*-Linked, *O*-Linked

Goal

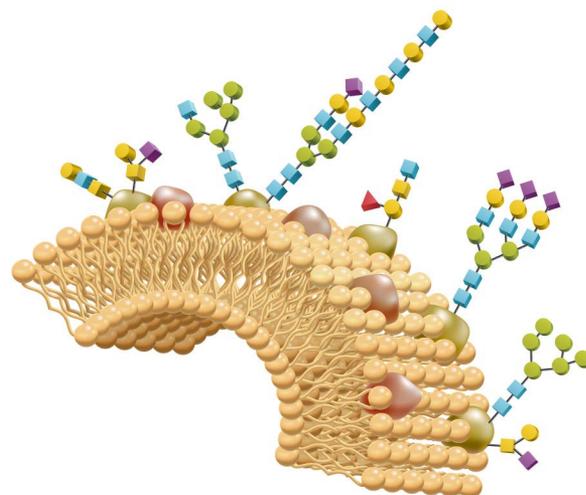
To summarize and discuss the scientific literature concerning HPAE-PAD peak area response of glycoprotein oligosaccharides

Introduction

High-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) was first described for separations of asparagine-linked (*N*-linked) oligosaccharides derived from glycoproteins in 1988.¹ Shortly thereafter HPAE-PAD was cited as a key technique for characterizing recombinant therapeutic glycosylation because it can be used for monosaccharide, sialic acid, and oligosaccharide analyses (*N*-linked and Ser/Thr-linked [*O*-linked] oligosaccharides). This oligosaccharide analysis is now commonly referred to as oligosaccharide profiling.²

In her 2010 review of carbohydrate analysis for protein therapeutics, Higgins noted that HPAE-PAD is one of the common methods for oligosaccharide profiling.³ The success of HPAE-PAD for this analysis is due to both its separation and detection capabilities. The Thermo Scientific™ Dionex™ CarboPac™ columns designed for oligosaccharide separation—the Dionex CarboPac PA1, PA100, and PA200 columns—resolve closely related oligosaccharide structures including linkage isomers, which are isobaric structures that only differ in the linkage of one monosaccharide. PAD requires only high-fmol to low-pmol amounts of oligosaccharides, no sample derivatization (i.e., it is direct detection), and no cost to purchase and dispose of organic solvents.

Higgins' review of HPAE-PAD also states, "Different oligosaccharides respond differently to this form of detection, making quantification—or even relative



quantification of different oligosaccharides—impossible.” The other issue for oligosaccharide quantification independent of the oligosaccharide profiling method is the absence of a large number of inexpensive oligosaccharide standards. Although it is true that a change in oligosaccharide structure can result in a change in electrochemical response, the degree of change has not prevented companies from successfully characterizing and testing glycoprotein therapeutics by HPAE-PAD oligosaccharide profiling. Most importantly, HPAE-PAD is sensitive to lot-to-lot variation of the glycoprotein's oligosaccharides.

This technical note reviews data from the peer-reviewed scientific literature evaluating electrochemical response of glycoprotein-derived and related oligosaccharides to show that the changes of electrochemical response with structure are not extreme. HPAE-PAD is then compared to other common methods of oligosaccharide profiling.

Literature Studies of HPAE-PAD Glycoprotein Oligosaccharide Responses

Study 1

The first published study of HPAE-PAD oligosaccharide response evaluated 35 synthetic oligosaccharides ranging from di- to nonasaccharides, including 11 phosphorylated and 5 sialylated oligosaccharides.⁴ To quantify response, the amount of each oligosaccharide was measured by HPAE-PAD monosaccharide analysis. Then, after injecting a known amount of oligosaccharide, the response per pmol was calculated and divided by the response per pmol of glucose (derived from multiple injections of a known amount of glucose). Stated another way, oligosaccharide response was normalized to the response of glucose, which equaled 1.0.

The authors noted that the compositional analysis had an error of 10–15% and the response (peak area) from repeat injections of the oligosaccharide varied as much as 5%, with 1% typical. Thus, it is reasonable to assume that the responses discussed in the following paragraphs pertaining to this study can vary by 10% or more.

The response range of the 35 synthetic oligosaccharides was 0.2–3.2. The one diphosphorylated oligosaccharide in the study (a disaccharide) had the 0.2 response factor. The 10 monophosphorylated oligosaccharides, which were either di- or trimannose oligosaccharides, had responses that ranged from 0.5 to 1.2. The two monophosphorylated oligosaccharides that can be compared to the only dimannose oligosaccharide in the study had responses of 0.5 and 0.6, compared to 1.1 for the unphosphorylated disaccharide. Thus, the addition of phosphate generally caused a loss of electrochemical response, with the addition of a second phosphate causing a 5-fold loss in response. This has also been observed for HPAE-PAD of inositol phosphates where monophosphorylated inositols had approximately 10 times more response than diphosphorylated inositols, and nearly 100 times more response than triphosphorylated inositols.⁵ Response loss is likely due in part to the loss of oxidizable hydroxyl groups.

Among the other oligosaccharides, the general trend showed greater response with increased size. The response of neutral disaccharides ranged from 1.1 to 1.3, linear tri- and tetraoligosaccharides ranged from 1.5 to 2.0, and the branched penta- to nonaoligosaccharides from 2.4 to 3.2, with the two nonaoligosaccharides having the highest responses of 3.1 and 3.2. There were also seven sialylated oligosaccharides in this study, one disialylated and six monosialylated oligosaccharides. Though negatively charged like the phosphorylated oligosaccharides, these oligosaccharides did not exhibit inhibition of response. Their response varied from 1.4 to 2.7 for the monosialylated tri- to pentaoligosaccharides, with a trend of more response as size increased. The disialylated oligosaccharide had a response of 1.4, compared to 2.1 for the monosialylated version, suggesting that increased sialylation may cause reduction in response. Free sialic acid (*N*-acetylneuraminic acid) had a response of 1.1, close to that of glucose.

Study 2

The first study of HPAE-PAD response of *N*-linked oligosaccharides from glycoproteins used bovine fetuin.⁶ Bovine fetuin, which has three *N*-linked attachment sites that have mostly di- to pentasialylated triantennary oligosaccharides, is a commonly used protein to evaluate glycoprotein methods. This study's first step involved using semipreparative HPAE-PAD to purify 10 oligosaccharides. Oligosaccharide response was determined relative to glucose with a response of 1.0 (as described in Study 1). Here, the authors assumed that 5 pmol of *N*-acetylglucosamine (GlcNAc, measured as glucosamine) equals 1 pmol of oligosaccharide. The ratio of 5 to 1 was derived from the fact that a triantennary complex-type *N*-linked oligosaccharide has five GlcNAcs (two for the chitobiose and one for each antenna).

The 10 studied oligosaccharides (Figure 1)—7 trisialylated triantennary, 2 tetrasialylated triantennary, and 1 pentasialylated triantennary—had an average response of $4.8 \pm 14\%$. These oligosaccharides represent the major oligosaccharides from bovine fetuin. They ranged in size from 14 to 16 monosaccharide units and their responses ranged from 3.8 to 6.1. There was no evidence of decreased response with increased sialylation, the possibility suggested from the single example in Study 1.

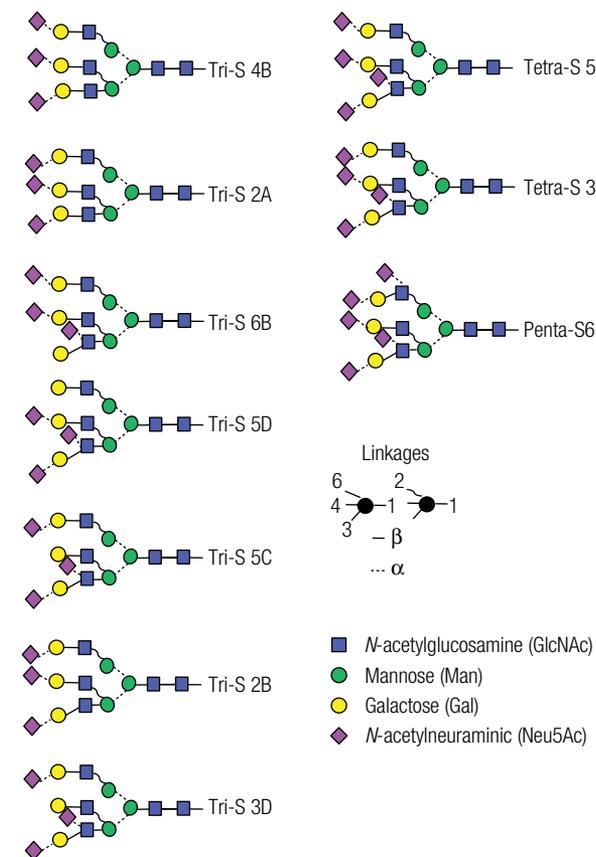


Figure 1. The 10 sialylated *N*-linked oligosaccharides from bovine fetuin evaluated in Study 2.⁶

Figure 2 shows a bar graph of the HPAE-PAD peak area responses determined in Study 2. This study showed that for a glycoprotein containing three oligosaccharide attachment sites, each containing complex *N*-linked oligosaccharides with typical oligosaccharide microheterogeneity, the range of response is less than 2-fold, with most of the oligosaccharides having similar responses.

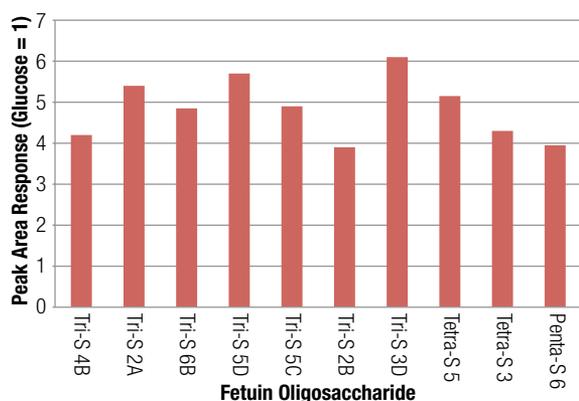


Figure 2. Bar graph of HPAE-PAD responses of the 10 sialylated *N*-linked oligosaccharides in Figure 1.

Study 3

While studying the oligosaccharide structures on a yeast glycoprotein, the Study 3 authors who used HPAE-PAD as part of the oligosaccharide characterization, studied the HPAE-PAD response of four high-mannose *N*-linked oligosaccharides found on the yeast glycoprotein.⁷ Response was measured in the same manner as in Studies 1 and 2; however, because these were high-mannose oligosaccharides, 2 pmol of GlcNAc (the chitobiose) were equal to 1 pmol oligosaccharide.

The four oligosaccharides had 10, 11, 12, and 13 monosaccharide units (i.e., 8 to 11 mannoses [Man₈, Man₉, Man₁₀, and Man₁₁]), and had responses of 2.4, 2.6, 2.3, and 2.3, respectively. In other words, their electrochemical responses were similar. Unlike the previous study, the four oligosaccharides were reduced. If reduction impacts response it is minor, because the three studies discussed thus far show that for *N*-linked oligosaccharides and synthetic oligosaccharides that resemble *N*-linked oligosaccharides, the range of responses is small (with the exception of a set of structures having a range of phosphorylation). Like Study 2, this study demonstrated that the range of response is less than 2-fold in a glycoprotein showing typical oligosaccharide microheterogeneity.

Study 4

A 1996 study of HPAE-PAD oligosaccharide response measured the responses of 28 oligosaccharides from human milk.⁸ Each of the oligosaccharides was a purchased standard of >95% purity. An equal molar amount of each standard was injected three to six times to determine response with the assumption that the labeled value of each standard was accurate.

For this study, the response of the tetrasaccharide lacto-*N*-tetraose, a precursor of many human milk oligosaccharides, was set to equal 1.0. Using this criterion, the response range of 15 other neutral (uncharged) oligosaccharides ranging in size from tri- to nonaoligosaccharides was 0.68 to 3.54. Fourteen of the fifteen contained fucose and the lowest responder was the nonfucosylated oligosaccharide. In general, response increased with size, but the highest responder was a tetrasaccharide, difucosyllactose. The next highest responder was an octasaccharide with a response of 2.69.

Eight mono- and two disialylated oligosaccharides were also part of Study 4 and their responses ranged from 0.4 to 1.48. This study also measured the response of Neu5Ac and two of the sialylated oligosaccharides measured in the Study 1.⁴ Neu5Ac had a response of 0.8. Therefore, to make a rough comparison of the results of the two studies, the values determined in Study 4 are multiplied by the ratio of 1.1 to 0.8 (or 1.375) to yield values of 1.63 and 1.00 for 6-sialyllactose and 3-sialyllactose, respectively. This compares favorably to the values of 1.9 and 1.4 determined in Study 1, especially considering the different methods of measuring the quantities of carbohydrate injected and that the two studies used different HPAE-PAD separation and detection conditions.

The authors of Study 4 noted that the position of fucose and Neu5Ac significantly influenced detector response. The responses of the four monofucosylated derivatives of lacto-*N*-tetraose ranged from 0.86 to 1.84. The authors also noted the difference in responses of the two sialylated lactoses discussed above.

Study 5

Another 1996 study also investigated the HPAE-PAD responses of human milk oligosaccharides using 14 neutral and six acidic oligosaccharides.⁹ Neutral oligosaccharide response values were calculated relative to the response of the tetrasaccharide stachyose; acidic oligosaccharide response values were calculated relative to the response of galacturonic acid. Responses were measured from 10 injections with the relative standard deviations all below 5%. The purities of the oligosaccharide studies were evaluated by HPAE-PAD and, although not stated specifically, these values were probably used to adjust the amount injected versus the 100% value from the label.

The 14 neutral oligosaccharides that ranged in size from a trisaccharide to a heptasaccharide had molar response values that ranged from 0.49 to 1.05 (stachyose = 1.0). It may be difficult to compare molar response values from this study to Study 4 because the internal standards are added to the milk prior to sample preparation; therefore, there is an assumption that the internal standards are fully recovered. Despite that caveat, there are a number of oligosaccharide response factors that can be compared. For example, both studies measure the responses of lacto-*N*-fucopentaose I, II, and III. In the study currently being discussed, the measured responses are 0.78, 0.84,

and 0.94, respectively, and the response of lacto-*N*-tetraose is 0.74. Therefore, taking the response values from Study 4 and multiplying them by 0.74 provides values that can be used to compare to Study 5. Those calculated values are 0.64, 1.13, and 1.36, respectively.

Considering the different chromatographic conditions, the different standards, and the caveat mentioned earlier, the values of the two studies are remarkably consistent and trend in the same manner. Conversely, the values for 2'-fucosyllactose and 3'-fucosyllactose in this study are 0.7 and 0.49, compared to the converted values of 0.62 and 1.61 from Study 4. Although the values for 2'-fucosyllactose are close, the 3' values are much different: one 3' value is less relative to 2'-fucosyllactose whereas one 3' value is more. Overall, both studies show there is not a large range of HPAE-PAD response values for the neutral oligosaccharides from human milk.

The six sialylated oligosaccharides (one mono- and two disialylated) had relative response factors ranging from 1.2 to 3.2 (galacturonic acid = 1.0). Two of the oligosaccharides evaluated, 6-sialyllactose and 3-sialyllactose, had response factors of 2.0 and 1.2, respectively. Using the reported response of Neu5Ac of 1.6, the values can be converted to the values of Study 1 by multiplying the response factors by 0.688 (1.1/1.6). This calculation yields 1.38 and 0.83, respectively. So for the three studies, the response factors—converted to the scale of the first study (glucose = 1.0)—are 1.9, 1.63, and 1.38 for 6-sialyllactose, and 1.4, 1.0, and 0.83 for 3-sialyllactose.

Considering the different conditions, standards, and so forth, these values are remarkably similar and consistent in that the 6-sialyllactose is more responsive than the 3-sialyllactose. This study also noted that for three tested neutral oligosaccharides and two tested acidic oligosaccharides the response factors were linear over tested ranges that spanned between one and two orders of magnitude.

In addition to 6-sialyllactose and 3-sialyllactose, the response factors of the other four sialylated oligosaccharides were studied in both milk oligosaccharide publications. The Neu5Ac response was 1.6 in Study 5 and 0.8 in Study 4, so the values for this study can be simply multiplied by 0.5 to compare to the values from the other study. That comparison and a comparison of the 6-sialyllactose and 3-sialyllactose values from the two studies using the same rationale are shown in Table 1. Considering the previously discussed differences in the two studies, the values are remarkably similar with only the response values for sialyl-lacto-*N*-tetraose b differing by more than 20%.

Table 1. Comparison of sialylated human milk oligosaccharide electrochemical response factors from Studies 4 and 5.

Study Number	Response Factor: Disialyl-Lacto- <i>N</i> -Tetraose	Response Factor: Sialyl-Lacto- <i>N</i> -Tetraose a	Response Factor: Sialyl-Lacto- <i>N</i> -Tetraose b	Response Factor: Sialyl-Lacto- <i>N</i> -Tetraose c	Response Factor: 6-Sialyllactose	Response Factor: 3-Sialyllactose
4	1.33	0.88	1.05	1.48	1.19	0.73
5	1.5*	0.85*	1.6*	1.6*	1.0*	0.6*

*The values determined in Study 5 were multiplied by 0.5, the ratio of the Neu5Ac responses in the two studies, to normalize the values to Study 4.

Study 6

There has been one published report of HPAE-PAD response factors for *O*-linked oligosaccharides.¹⁰ These oligosaccharides were reduced to model the state of *O*-linked oligosaccharides released from glycoproteins by beta elimination under reducing conditions. The responses of 10 reduced sialylated *O*-linked oligosaccharides were measured relative to Neu5Ac α 2,6GalNAc-ol set to a response of 1.0. The amount of standard injected was determined by acid hydrolysis of the standard followed by measurement of the amount of galactosaminitol (1 mole per mole of oligosaccharide). The 10 structures ranged from disaccharides to a hexasaccharide (Figure 3) and their relative PAD responses ranged from 0.68 to 1.6, with half the structures 1.0 ± 0.2 . The response variation is low among these 10 common sialylated mammalian *O*-linked oligosaccharides, as shown in a bar graph of their responses (Figure 4).

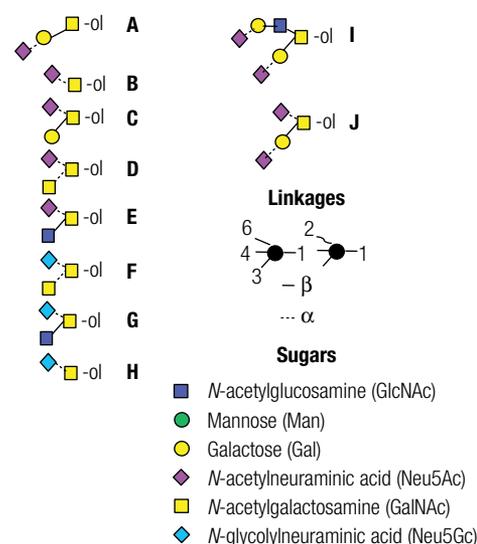


Figure 3. The 10 *O*-linked oligosaccharides studied in Reference 10.

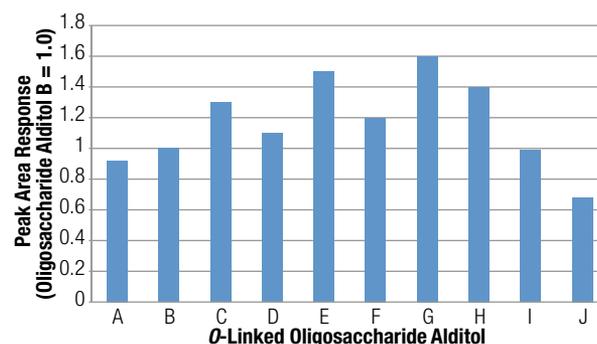


Figure 4. Bar graph of HPAE-PAD responses of the 10 *O*-linked oligosaccharides in Figure 3.

Study 7

A 2009 publication that reported an HPAE-PAD separation method for oligosaccharides from recombinant glycoproteins showed the separation of 18 *N*-linked oligosaccharides commonly found on monoclonal antibodies that ranged in size from a penta- to a dodecaoligosaccharide.¹¹ The separation was of 4.5 pmol of each oligosaccharide and the peak height of each was reported. Although this is not a sound comparison of response between oligosaccharides because their peak widths and therefore their areas vary, it does provide a rough comparison. The authors discuss response factors in the article but note that the data are not shown. In discussing response factors the authors state, “The response factors varied slightly between the different oligosaccharides.” They note that the high-mannose oligosaccharides between 3 and 9 mannose units had approximately twice the response of the neutral oligosaccharides containing fucose, and that the responses of the other neutral oligosaccharides and the acidic (sialylated) oligosaccharides had responses between those two groups. They also point out that the standards were purchased as qualitative standards and they did not know the accuracy of the reported quantity of each standard.

Table 2 summarizes the seven studies discussed. Overall, the variation in response of these oligosaccharides—which represent a broad range in terms of both size and composition—is far less than an order of magnitude. In addition, sets of similar structures have little variation of response. Therefore, despite the limitation noted regarding the unknown exact amount of oligosaccharide injected, one can still conclude that the variation in response of these oligosaccharides is small. In fact, the three studies that determined the amount of oligosaccharide by monosaccharide analysis (Studies 2, 3, and 6) had low amounts of response variation despite the fact the acid hydrolysis involved in monosaccharide analysis has its own variability.^{6,7,10} One might argue that those studies looked at more uniform sets of structures, however, those sets of structures are more representative of the typical oligosaccharide profiling assay. The small amount of variation observed in these studies makes HPAE-PAD oligosaccharide analysis a suitable technique for determining changes in a recombinant glycoprotein’s oligosaccharide composition in a comparability assay.

Table 2. Summary of the oligosaccharide electrochemical response studies.

Number			Type(s) of Oligosaccharides	Size Range of Oligosaccharides (No. of Monosaccharides)	Electrochemical Response Referenced to	Range of Response Values	General Conclusion(s)
Study	Reference	Oligosaccharides					
1	4	35	Synthetic, resembling <i>N</i> -linked and <i>O</i> -linked with 11 phosphorylated and 5 sialylated	2–9	Glucose = 1.0	All: 0.2–3.2 10 Monophosphorylated: 0.5–1.2 7 Sialylated: 1.4–2.7 Neutral disaccharides: 1.1–1.3 Neutral linear tri- and tetrasaccharides: 1.5–2.0 Neutral penta- to nonaoligosaccharides: 2.4–3.2	1. Addition of phosphate reduces response. 2. Addition of sialic acid increases response. 3. Response increases with size.
2	6	10	Sialylated complex-type <i>N</i> -linked from bovine fetuin	14–16	Glucose = 1.0	3.8–6.1	1. Average response is 4.8 +/- 14%. 2. Response range is <2-fold for typical site heterogeneity.
3	7	4	Reduced high-mannose type <i>N</i> -linked from <i>Saccharomyces SUC2</i> invertase	10–13	Glucose = 1.0	2.3–2.6	Response range is small for typical site heterogeneity.
4	8	33	Human milk: 28 Blood group: 5	Milk: 3–7 Blood: 2–7	lacto- <i>N</i> -tetraose = 1.0	All: 0.68–3.54 8 Sialylated: 0.4–1.4 8 Blood Group: 1.19–2.33	1. Response increases with size. 2. Positions of fucose and sialic acid influence detector response.
5	9	20	Human milk	3–7	For 14 neutral oligosaccharides: stachyose = 1.0 For 6 sialylated oligosaccharides: galacturonic acid = 1.0	Neutrals: 0.49–1.0 Sialylated: 1.2–3.2	1. Response ranges are small for neutral and charged milk oligosaccharides. 2. For 5 tested oligosaccharides, responses were linear in the range tested (1 to 2 orders of magnitude).
6	10	10	Reduced sialylated <i>O</i> -linked	2–6	Neu5Acα2, 6GalNAc-ol = 1	0.68–1.6	Response range is small for 10 common sialylated mammalian <i>O</i> -linked oligosaccharides.
7	11	18	<i>N</i> -linked from IgG	5–12	No reference used; nominally, 4.5 pmole of each as injected	Peak heights: not valuable to a large variation in peak widths	The authors noted only “slight variation” of response factors for the 18 oligosaccharides studied.

Comparison of HPAE-PAD to Other Glycoprotein Oligosaccharide Profiling Techniques

There are numerous methods for glycoprotein oligosaccharide profiling and this undoubtedly reflects the difficulty of the analysis. Higgins noted that due to the complexity of oligosaccharides, at least two techniques can be required to confirm the identity of some oligosaccharides.³ So it is likely that a lab will be employing at least two techniques to characterize the oligosaccharides on a glycoprotein. Therefore, this section will focus on two techniques for which there is the most data for comparison to HPAE-PAD:

- Reductive amination fluorescent labeling followed by hydrophilic interaction/normal-phase chromatography
- Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) of underivatized oligosaccharides

Two collaborative studies highlight the difficulty of quantifying oligosaccharides. In the first study, 20 labs characterized the oligosaccharides from transferrin and IgG.¹² This study represented many techniques for oligosaccharide profiling and quantitation, including:

- Reductive amination fluorescent labeling followed by reversed-phase chromatography, hydrophilic interaction/normal-phase chromatography, or capillary electrophoresis
- MALDI-TOF MS of either permethylated or underivatized oligosaccharides
- Electrospray ionization mass spectrometry (ESI-MS) of underivatized oligosaccharides, derivatized oligosaccharides, or glycopeptides; sometimes preceded by chromatography

The study concluded that MALDI-TOF MS of permethylated glycans yielded good quantification and the results correlated with the chromatographic results.

This study also reported the results of quantifying the galactosylation levels of three IgG samples by 16 of the labs. The RSDs for the three samples were 23, 20, and 19% but were higher for the subset of five labs doing chromatography after fluorescent labeling. The data from those labs had RSDs of 29, 24, and 23%, respectively. This highlights the between-lab reproducibility issue for this technique that was noted in that paper's Table V, which listed as a disadvantage of chromatography after fluorescent labeling: "various conditions for derivatization". The same table notes as an advantage: "independence of detector response on size or structure".¹² Although this last statement is commonly believed, it is likely false.

In his review of fluorescent derivatization methods for HPLC of glycoprotein carbohydrates, Anumula notes, "Fluorescence intensity and the reactivity of the tag depend on molecular structure and the reaction conditions. Although the fluorescence phenomenon is well understood at the molecular level, it is difficult to predict

the intensity differences quantitatively."¹³ It is also important that the standard and samples be labeled under conditions that are as similar as possible because the sample environment impacts the degree of reaction. For some samples it may be difficult to match the environment to get equivalent yields, which will impact quantification. It is also important that conditions be as mild as possible because sialic acid will be lost from oligosaccharides. Even under mild conditions, it is generally assumed that there is some loss of sialic acids.

In a comparison of fluorescent labeling techniques for oligosaccharides and the introduction of a simple purification method (acetone precipitation) the authors wrote in a discussion on the rise of carbon LC (i.e., use of a porous graphitized carbon column under reversed-phase conditions) for LC ESI-MS for underivatized oligosaccharides, "Labeling inevitably leads to the generation of by-products by incomplete derivatization and desialylation—even under optimized conditions."¹⁴ Conversely, mild conditions for the anthranilic acid labeling reaction were reported to yield little to no desialylation of labeled glycosphingolipid-derived oligosaccharides.¹⁵ Samples should also be analyzed promptly because labeled products can degrade. Other disadvantages associated with these derivatization techniques include the extra time for derivatization and the expense of purchasing and disposing of not only the hazardous chemicals used for derivatization, but also the organic solvents used for chromatography. Conversely, compared to native oligosaccharides, labeled oligosaccharides ionize better for MS analysis.

The belief that detector response for fluorescently labeled oligosaccharides is independent of size and structure is similar to the former dogma that carbohydrates labeled with tritium by reduction with tritiated borohydride had the same specific activity. Gabriel and Ashwell showed that this was a false assumption in a set of experiments in which a variety of small carbohydrates were tritiated and their specific activity measured.¹⁶ With tritiated talose as a reference, the specific activities ranged from 51 to 182% of talose.

In Table 6 of Anumula's review, he compares HPAE-PAD to HPLC-fluorescence. The comparison is generally sound, but it is this author's opinion that some corrections are needed. HPAE-PAD is described as "high resolution" and HPLC-fluorescence as "very high resolution". The opinion here is that the resolution for both techniques can best be described as similar. Furthermore, since Anumula's publication, the Dionex CarboPac PA200 column was introduced, a product that yields higher oligosaccharide resolution than previous Dionex CarboPac columns. There are also new higher-resolution columns for HPLC-fluorescence. So again, the two techniques are nearly equivalent in terms of resolution.

Anumula's Table 6 also states that HPAE-PAD has "nonspecific response to electrochemically active compounds (e.g., amino acids, peptides, and thiols)". It fails to mention that under the detection conditions used, these

compounds are detected at 10–100 times less sensitivity compared to carbohydrates and that these compounds will not be found in significant amounts in nearly all oligosaccharide samples analyzed. The table also lists that HPLC-fluorescence is ~200 times more sensitive than HPAE-PAD. Although it is more sensitive, it is unlikely 200 times more so.

In Figure 3 of their article, Grey et al. showed an HPAE-PAD separation of 4.5 pmol each of 18 oligosaccharide standards.¹¹ From that chromatogram it is easy to conclude that the sensitivity is no less than 1 pmol for each oligosaccharide. To be 200 times more sensitive would require HPLC-fluorescence to detect 5 fmol. If that is indeed possible, one would undoubtedly have to derivatize a larger sample to be able to inject 5 fmol, whereas 1 pmol of an underivatized sample can be directly analyzed by HPAE-PAD.

The second collaborative study concerning quantification of *N*-linked oligosaccharides involved 11 labs from the United Kingdom.¹⁷ In this second study, the organizer provided the samples with the oligosaccharides already released so that one variable could be controlled. The participating labs used essentially the same techniques as the first study and noted that none of the participants employed HPAE-PAD in the study.

There was a wide range of results for some of the questions posed in this second collaborative study, again highlighting the problem of oligosaccharide quantification. For example, the charge state determined for human IgG varied widely as did the percentage of Man-5 structure determined for ribonuclease B (29 to 62%). The authors noted that MS methods identified more oligosaccharides, especially sialylated oligosaccharides, than the chromatography methods, which in this study were those that used fluorescent labeling. While not mentioned in the paper, one can hypothesize that this may be in part due to loss of sialic acid during the labeling reaction. Poor resolution by HPLC was one of the reasons cited for the deficiency, but the lack of standards—also a problem for HPAE-PAD—is a limiting factor in identification of structures. Not surprisingly, MS, when combined with chromatography, identified the most structures.

Both collaborative studies highlight the difficulty of oligosaccharide analysis—regardless of the technique—and emphasize that more than one technique is needed for oligosaccharide identification. After identification, profiling can monitor consistency.

A few papers have compared MALDI-TOF MS to HPAE-PAD and they reach similar conclusions. In Field et al.'s 1996 paper, they monitored oligosaccharide degradation by MALDI-TOF MS and HPAE-PAD.¹⁸ The two methods showed similar rates of oligosaccharide degradation. The authors state that they had considered both methods to be more qualitative than quantitative, but the precision of both methods and the similar results suggest to them that both methods are capable of accurate quantification. They also note that not all types of oligosaccharides were evaluated and that MALDI-TOF

MS does not detect sialylated structures well. This is probably the result of using 2,5-dihydroxybenzoic acid as the matrix. Shortly after that publication, the authors found that using 2,4,6-trihydroxyacetophenone (THAP) as the matrix improved the ionization of sialylated oligosaccharides.¹⁹

In Harvey's 2005 review of MALDI-TOF MS and ESI-MS for structural determinations of *N*-linked oligosaccharides he details the difficulties of measuring sialylated oligosaccharides by MALDI-TOF MS.²⁰ Even when using THAP, there is in-source and post-source fragmentation that causes a loss of sialic acids. He recommends forming methyl esters of the sialic acids by reacting with methyl iodide and thus stabilizing the sialic acids. Zhou et al. permethylated *N*-linked oligosaccharides, including sialylated oligosaccharides, released from isoelectric focusing gel bands containing glycoproteins and used MALDI-TOF MS to confirm the results of HPAE-PAD, suggesting again that the two techniques yield similar results.²¹

Harvey also noted the importance of having a clean sample for MALDI-TOF MS of oligosaccharides and reviewed a number of sample cleanup processes. Field et al. mention that a changing electrode surface hinders consistent response by HPAE-PAD. Since their publication, this has been remedied by the development of a waveform that prevents electrode surface degradation²² and the introduction of disposable gold working electrodes, which have a more consistent surface than conventional working electrodes.

Another 1996 publication studying the oligosaccharides of soybean peroxidase also compared HPAE-PAD and MALDI-TOF MS.²³ The authors noted that the two techniques complemented each other well for the analysis of high-mannose type *N*-linked oligosaccharides, but that HPAE-PAD was able to reveal the distribution of isomers (isobaric) to which MALDI-TOF MS was blind. Outside the area of glycoprotein oligosaccharide analysis, a study compared MALDI-TOF MS to HPAE-PAD for the determination of amylopectin chain length distribution from debranched starch.²⁴ The major conclusion was that although MALDI-TOF MS was able to perform the analysis in less time per sample with better sensitivity, it was less reproducible than HPAE-PAD and overestimated the amounts of polymers with degrees of polymerization >21.

In a more recent publication, Grey et al. reported an improved HPAE-PAD separation of monoclonal antibody oligosaccharides and compared their results to MALDI-TOF MS.¹¹ Like Field et al.,¹⁸ they noted that although the techniques are based on different detection principles, the results were similar. Thus, Grey et al. concluded that the relative glycan responses were similar for the two methods. The difference between the two techniques for individual oligosaccharide abundance was often <2.5%. The authors additionally noted that although MALDI-TOF MS was faster, the precision of HPAE-PAD was significantly better; HPAE-PAD was able to identify structural isomers and it could simultaneously analyze neutral and sialylated oligosaccharides.

Conclusion

An examination of the peer-reviewed scientific literature shows that although oligosaccharide response does vary with structure in HPAE-PAD, the variation is small, especially among sets of similar oligosaccharides like those found on many glycoproteins. The literature also suggests that this variation is similar to what is observed with MALDI-TOF MS analysis of oligosaccharides. Although it is commonly believed that there is little variation of response with structure for fluorescently labeled oligosaccharides, that belief is likely false; furthermore, there are also questions of sample degradation and whether the sample has been completely labeled.

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