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A separation-free quantitative MS-based profiling approach using 2-AA isotopically labeled substrates for high-throughput glycan screening

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INTRODUCTION AND PURPOSE

Fast, sensitive, robust methods for 'high-level' glycan profiling are necessary during various stages of a biotherapeutic product's lifecycle, including clone selection, process changes, and quality control for lot release testing. Traditional glycan screening involves chromatographic or electrophoretic separation, and although reproducible, these methods can be time consuming. Even ultra high performance chromatographic and microfluidic integrated LC/MS systems, which work on the tens of minute time scale, become lengthy when hundreds of samples are analyzed. Comparatively, a direct infusion MS-based glycan screening method acquires data on a millisecond time scale, exhibits exquisite sensitivity and reproducibility, and is amenable to automated peak annotation. Here, we demonstrate a quantitative high-throughput MS-based profiling approach using stable isotope 2-AA for rapid 'high-level' glycan profiling of recombinant IgG proteins.

MATERIALS AND METHODS

N-linked glycans from 50 µg of a rIgG2 were released enzymatically using a Barocycler (PBI Inc., South Easton, MA). Protein, detergents, and salts were removed by C18 and porous graphitized carbon SPE procedures using a PhyNexus MEA system (PhyNexus Inc., San Jose, CA). A 100 µL aliquot of 30 mg/mL 2-$^{12}[C_6]$AA or 2-$^{13}[C_6]$AA solution and 50 µL of 1 M sodium cyanoborohydride in tetrahydrofuran were added to the released glycans and incubated at 80°C for 1 hr. Excess reducing-end label was removed using an automated normal-phase clean-up on a PhyNexus MEA system. Mass spectrometric data was obtained on a LTQ mass spectrometer (Thermo Scientific, San Jose, CA) equipped with a TriVersa Nanomate™-automated nanospray ion source (Advion, Ithaca, NY) and an Applied Biosystems 4800 Proteomics analyzer (Applied Biosystems, Framingham, MA), equipped with 355 nm Nd:YAG laser.

Process time for 96 samples			
	UPLC-RP-MS	Agilent "Lab-on-a-chip"	Stable isotopic profiling
PNGase F release	2.0 hours	N/A (performed on chip)	30 minutes
Derivatization	1.0 hours	N/A (no derivatization)	1.0 hours
Clean up	1.0 hours	N/A (no derivatization)	1.0 hours
Data collection	31.7 hours (20 minutes / sample)	16.0 hours (10 minutes / sample)	< 1 minute (0.5 seconds / sample for MS^n)
Total time	35.7 hours	16.0 hours	3.0 hours

Figure 1. Process time for 96 samples using various glycan analysis methodologies. During the development and commercialization of biotherapeutic molecules, the detection and detailed characterization of the drug's glycan array is a regulatory requirement. However, for certain operations, such a clone selection, process changes, and quality control fast 'high-level' high-throughput glycan population screening methods are more appropriate and satisfactory. Even with the "instantaneous" sample preparations now available it is still evident that the bottleneck in glycan analysis are lengthy chromatographic separation times. separation free MS-based relative quantitation analysis, that handles up to 384 individual paired samples in approximately 3 hours.

REFERENCES

1. Prien et al. Anal Chem. 2010 Feb 15;82(4):1498-508.

RESULTS

Proof-of-Concept study (Figures 2-4)

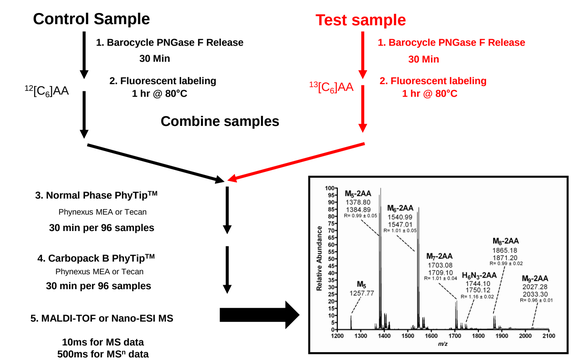


Figure 2. 2-AA stable isotope glycan mapping workflow. Figure 2 illustrates an experimental workflow for comparative glycan mapping of **upwards of 384 paired samples in approximately 3 hours.** The development of this high-throughput workflow is based on three core features: (1) the availability of 2-aminobenzoic acid as $^{12}[C_6]$ and $^{13}[C_6]$ stable isotopic variants; (2) adaptation of manual glycan purification procedures to a robotic automated purification platform; and (3) separation-free relative quantitative stable isotope MS-based analysis via direct infusion, which by-passes lengthy chromatographic separation times. Briefly, equimolar amounts of protein are independently released enzymatically using a Barocycler and differentially labeled with either the $^{12}[C_6]$AA or $^{13}[C_6]$AA fluorescent tag. Alternatively, for "absolute" quantitation exact molar amounts of $^{13}[C_6]$AA labeled glycan standards may be used. The differentially labeled samples are then blended during an automated normal phase label removal step on a PhyNexus MEA purification system. The mixed sample undergoes a subsequent desalting via an automated porous graphitized carbon step. The equimolar differentially labeled paired sample is then subjected to MS-based relative quantitative analysis (1).

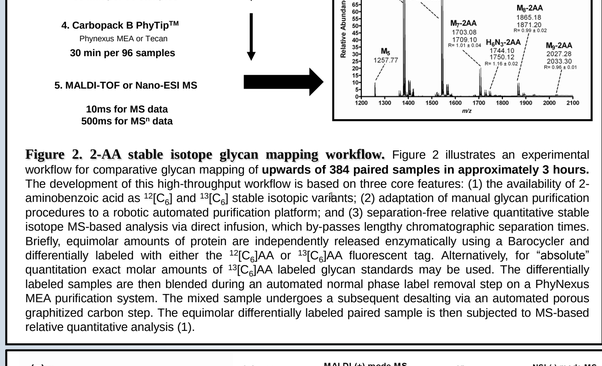


Figure 3. Relative quantitation of $^{12}[C_6]$AA and $^{13}[C_6]$ AA labeled glycan standards. Various molar ratios (1:1; 2:1; 5:1; 10:1) of $^{12}[C_6]$AA and $^{13}[C_6]$AA labeled oligomannose-5 standard (M_5) derived from porcine thyroglobulin were used to determine the feasibility and dynamic range of a separation-free relative quantitation MS-based approach. Five replicates of each individual molar ratio (1:1; 2:1; 5:1; 10:1) were analyzed by positive mode MALDI-TOF MS and negative mode ESI-MS. Figure 3 (Panels a-d) represent MALDI-TOF MS spectra of the M_5 standard ratios (1:1; 2:1; 5:1; 10:1). The parent ions, *m/z* 1379.04 and *m/z* 1385.06, represent intact sodium adducted M5-2-$^{12}[C_6]$AA and M5-2-$^{13}[C_6]$AA species, respectively. As expected, the $^{13}[C_6]$AA labeled species exhibits a mass offset of +6 Da. Importantly, the isotopic mass difference provides sufficient *m/z* value dispersion so each singly charged isotopically labeled species may be distinguished and quantified without analog interference. As the spectra (Figure 3, Panels a-d) demonstrate the ratios of M5-2-$^{12}[C_6]$AA to M5-2-$^{13}[C_6]$AA corresponds closely to the expected theoretical molar ratios (1:1; 2:1; 5:1; 10:1). The ratio of $^{13}[C_6]$/$^{12}[C_6]$ was calculated from the sum of the peak intensities of the isotopic distribution from each of the $^{13}[C_6]$AA and $^{12}[C_6]$AA labeled M_5 species (Figure 3, Panels e and f)(1).

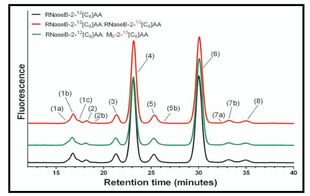


Figure 4. Rapid resolution reverse-phase separation of $^{12}[C_6]$AA and $^{13}[C_6]$AA stable isotope fluorescently derivatized glycan from bovine RNase B. Three 2-AA derivatized glycan samples: (1) 2-$^{12}[C_6]$AA labeled glycans derived from RNase B; (2) a 1:1 sample mixture of $^{13}[C_6]$AA and $^{12}[C_6]$AA labeled glycans from RNase B 'spiked' with 7.4 pmol of 2-$^{13}[C_6]$AA labeled M5 standard from porcine thyroglobulin were used to assess whether or not $^{12}[C_6]$AA and $^{13}[C_6]$AA labeled glycans coelute during chromatographic experimentation. Figure 4 depicts the reverse-phase chromatographic profiles of three 2-AA derivatized glycan samples. The profiles of the $^{12}[C_6]$-2-AA derivatized glycans from RNase B and the 1:1 mixture of $^{12}[C_6]$-2-AA and $^{13}[C_6]$-2AA derivatized glycans from RNase B are indistinguishable confirming the stable isotope variants exhibit identical chromatographic behavior to their non-isotopic reciprocal. The blended sample of $^{12}[C_6]$AA labeled RNase B glycans and $^{13}[C_6]$AA labeled M_5 standard presents a slightly different profile. Peaks 1b, 3, 4, and 5 appear suppressed, which is consistent with the addition of supplemental amounts of M_5 standard introduced into the sample mixture. Equally unsurprising, is the peak area of the peaks 1a, 5b, and 7a, which represent the elution points of the various M_5 isomers increased accordingly . These findings illustrate 2-AA stable isotope labeled glycan analogs co-elute during reverse-phase chromatography and do not exhibit chromatographic variance(1).

Case Study: Monitoring glycan distribution during manufacturing process changes (Figures 5-7)

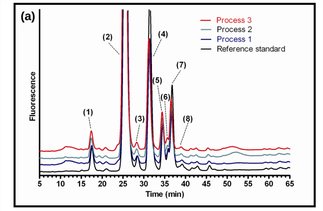
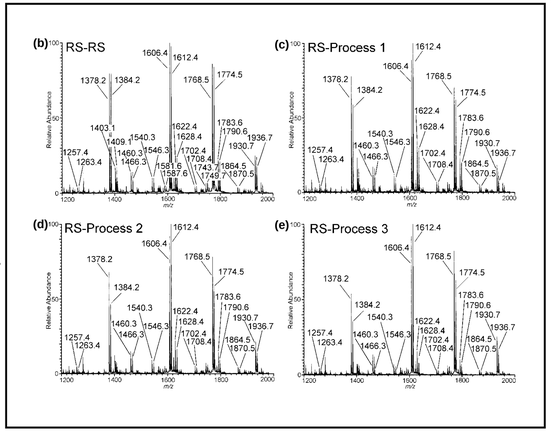


Figure 5. Traditional glycan screening involves chromatographic or electrophoretic separation, and although reproducible, these methods can be time consuming. Figure 5 (Panel a) illustrate an HPAEC-2AB glycan mapping method used for comparability testing between a rIgG reference standard and its development under three different manufacturing process changes. Peaks labeled 1 through 8 are the glycans monitored during process change for this particular rIgG. Please note that the method takes 65 minutes for a single chromatographic run. MS-based relative quantitative analysis is quick and reproducible. **Figure 5 (Panel b)** illustrates an equimolar mixture of $^{13}[C_6]$AA and $^{12}[C_6]$AA labeled glycans from rIgG reference standard. **Figure 5 (Panels c-e)**, represent equimolar mixtures of $^{12}[C_6]$AA labeled glycans from a rIgG reference standard and $^{13}[C_6]$AA labeled glycans from rIgG developed under different process conditions. MS-based data acquisition is on the millisecond time-scale. Please see **Figure 6** for glycan species elution position and corresponding 2-AA stable isotopic mass/charge (*m/z*) values.



HPAEC Peak	MS-based Peak: 2-$^{12}[C_6]$AA *m/z* Na+ Adduct	MS-based Peak: 2-$^{13}[C_6]$AA *m/z* Na+ Adduct	Theoretical Mass, Da	Identification	Glycan structure*‡
1	1403.1	1409.1	1259.5	D1H3N3-2AA **A1F-G0**	
2	1606.7	1612.4	1462.6	D1H3N4-2AA **A2F-G0**	
3	1257.4	1263.4	1113.5	H3N3-2AA A1-G0	
4	1768.5	1774.5	1624.3	D1H4N4-2AA **A2F-G1**	
5	1460.3	1466.3	1316.6	H3N4-2AA A2-G0	
6	1930.7	1936.7	1786.8	D1H5N4-2AA **A2F-G2**	
7	1378.2	1384.2	1234.5	H5N2-2AA **M5**	
8	1622.4	1628.4	1478.6	H4N4-2AA **A2-G1**	

* Glycan structural elucidation obtained through serial exoglycosidase digestion and MALDI-TOF MS analysis
‡ Glycan structures that are monitored for comparability during manufacturing process change.

Figure 6. HPAEC elution positions and corresponding 2-AA derivatized sodium adducted mass/charge (*m/z*) values. These eight glycan species highlighted in **Figure 5 (Panels a-e)** are monitored to assess the potential impact changes in the manufacturing process, particularly transfers of site and scale, might have on the product. Both traditional HPAEC mapping and 2-AA stable-isotopic comparative analysis easily track changes in glycan distribution.

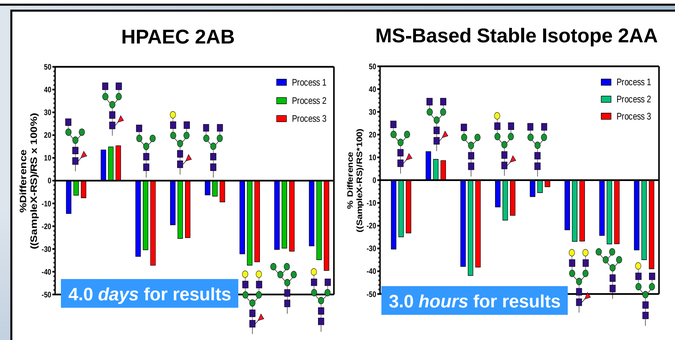


Figure 7. Comparison of the major individual glycan species based on % Difference. Both HPAEC mapping and 2-AA stable isotopic MS-based comparative methodologies derive comparability results highlighting the same relative distribution trends spurred by changes in the manufacturing process . Improvement in operational efficiency is the major advantage of MS-based 2-AA stable isotopic mapping over traditional chromatographic separation for comparability testing. Here, the 2-AA MS-based approach takes a total of 3 hours for data acquisition for the 4 sample types, while the HPAEC methodology took over 4 days to acquire comparable data. The improvements in operation efficiency are evident **(Figure 6 and 7)**.

CONCLUSION

Here, we introduced and apply a reproducible high throughput MS-based quantitative workflow, which includes the incorporation of differentially labeled stable isotope labeled tags, robotic sample purification, and separation free MS-based relative quantitation analysis, that handles up to 384 individual paired samples in approximately 3 hours. It is noteworthy that the majority of this analysis time is associated with the sample preparation, including enzymatic deglycosylation, labeling, and clean up. Improvements in sample processing throughput were discussed in this report, however further efficiencies could be gained through the utility of immobilized PNGase F, and the development of rapid labeling chemistries. In addition to the obvious time savings, reductions in consumable and disposal costs are also gained. These cost savings come from the elimination of expensive chromatographic columns, complete reduction in acetonitrile use as well as the associated removal hazardous HPLC waste. As such, signification cost avoidances and time savings can be amassed through the implementation of stable isotope MS-based quantitation methodology for all routine 'high level' high-throughput screening activities.