Title: GlyCombo enables rapid, complete glycan composition identification across diverse glycomic sample types

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Abstract

Glycans are sugar-based polymers found to modify biomolecules including lipids and proteins, as well as occur unconjugated as free polysaccharides. Due to their ubiquitous cellular presentation, glycans mediate crucial biological processes and are frequently sought after as biomarkers for a wide range of diseases. Identification of glycans present in samples acquired with mass spectrometry (MS) is a cornerstone of glycomics research, thus, the ability to rapidly identify glycans in each acquisition is integral to glycomics analysis pipelines. Here we introduce GlyCombo (https://github.com/Protea-Glycosciences/GlyCombo), an open-source, freely available software tool designed to rapidly assign monosaccharide combinations to observed and fragmented precursors in an MS experiment.

GlyCombo was evaluated across six diverse datasets, demonstrating MS vendor, derivatization, and glycan-type neutrality. Compositional assignments using GlyCombo are shown to be faster than the current, predominant approach, GlycoMod, a closed-source web application. Finally, the comprehensiveness of glycan feature identification is exhibited in Skyline, a software that requires pre-defined transitions that are derived from GlyCombo output files.

Introduction

Glycans are comprised of individual monosaccharides that are capable of modifying diverse biomolecules such as lipids and proteins. Additionally, glycans can exist as unconjugated free polysaccharides. Glycosylation processes in nature are comprised of a wide range of monosaccharides and their derivatives, numbering in at least in the hundreds[1]. There are a variety of methods that have been employed to detect and measure glycans, however mass spectrometry (MS) has become the method of choice for rapid characterisation of sample glycosylation[2]. The ability to separate glycans by mass for mass-based detection provides a relatively unbiased approach to detecting both the usual and unusual glycans in nature.

Upon completion of any glycomics MS data acquisition, the initial analysis will include the identification of the glycans detected. Unlike proteomics, which is template-derived, and therefore able to use the genome as a bioinformatic search space, the glycosylation of a given sample cannot be predicted[3], and requires bioinformatic confirmation. One such approach is combinatorial monosaccharide analysis and is most popularly used in the form of GlycoMod[4,5]. GlycoMod is a Perl program designed to find all possible combinations of a glycan from an experimentally determined mass, by searching a precomputed list of masses[6]. Despite being published in 2001, it remains frequently in use but is limited by its closed source nature, and online-only access.

MS-based glycomics analyses have changed significantly as the monosaccharides and glycan structures observed experimentally challenge existing paradigms. This is compounded by improvements in data acquisition and bioinformatics that have allowed the glycoscience field to detect, quantify and share the data, describing more glycans than ever before[7–10].Considerable advancements have been made in the glycan bioinformatic search space including GlycReSoft[11], and GlycoNote[12], but throughput is lacking, and the monosaccharide search spaces are limited to routine and expected *N*-glycan structures.

To address these challenges and complement the advantages of GlycoMod, we describe an open-source, offline program called GlyCombo which combinatorially assigns monosaccharide combinations to glycan masses acquired MS. Taking advantage of collaborative standards initiatives, we employ the mzML file format[13] to enable accessible, and complete glycan composition assignments in an automated manner while also delivering fast and efficient combinatorial analysis using dynamic programming[14].

Methods

Benchmark datasets and comparisons

Raw files were downloaded from published datasets on Glycopost[10]. Datasets were chosen to cover negative and positive MS polarities, glycan types (NG, OG and GSL), resolutions (2D and 3D ion traps, and Orbitrap), and vendors (Bruker and Thermo Fisher Scientific). All benchmarks were performed on a consumer-grade Lenovo laptop equipped with an AMD Ryzen 6 Pro 5850U CPU, 16 GB of RAM and a 500 GB SSD. Search times for GlycoMod[4] were based on the loading timeline in Firefox network developer tools.

Raw file conversion to mzML

All downloaded files were converted to mzML file format[13] by MSConvert[15]. For low-resolution ion trap data with unassigned precursor charge states, an additional filter of "Charge State Predictor" was used without overwriting existing charges, single charge % TIC = 0.9, and only 1 charge per precursor. The mzML files were then directly read by GlyCombo.

GlyCombo design and Implementation

GlyCombo was developed in C# and is available open source under the Apache 2.0 license (https://github.com/Protea-Glycosciences/GlyCombo). The first process, mass list generation, extracts precursor *m/z* values from MS2 scans along with charge state and polarity to calculate neutral precursor mass values. This list is exported in the parameters file at the end of a given search in GlyCombo and was used for GlycoMod comparisons.

Neutral precursor mass values were adjusted for combinatorial analysis based on user-specified mass error, reducing end formats, and glycan derivatisation status. High-resolution files were searched with 50 ppm mass error, and low-resolution files were searched with 0.6 Da mass error. Based on presets or user-specified monosaccharide ranges, limits were then applied to the combinatorial analysis of monosaccharide masses to identify matches for the starting neutral precursor mass list. The same monosaccharide ranges were applied to GlycoMod for direct comparisons. Dynamic programming was used to efficiently identify all possible matching monosaccharide combinations for each precursor mass.

Skyline analysis

Output skyline transition lists were directly imported into Skyline[16–18] with minimum *m/z* 50 and maximum *m/z* 10,000. High resolution raw files were analysed with the following full-scan settings: 3 peaks for centroided mass analyser at 50 ppm mass accuracy. Low resolution raw files were analysed with the following full scan settings: 3 peaks for TOF mass analyser at 5000 resolution. Glycan precursors were filtered for quality, only including those with a minimum isotopic dot product (idotp) of 0.9.

Data availability

GlyCombo output files, converted mzML files, and Skyline assays are available on Panorama Public[19] at: https://panoramaweb.org/Protea%20Glycosciences%20Pty%20Ltd/GlyCombo/project-begin.view. Search time values and compositional identification values can be found in Supplementary File 1.

Results and Discussion

Combinatorial glycan composition determination has been demonstrated to be suitable for identifying glycans in MS acquisitions of glycan-containing samples. With a diverse range of sample preparation, acquisition methods, and vendors, profiling glycans by their precursor mass is a robust initial approach with minimal assumptions. To enhance the identification of glycan precursors in acquired MS data, we developed an open-source Windows application for the rapid extraction of precursor *m/z* values from the mzML file format, a vendor neutral file type that enables cross-platform compatibility.

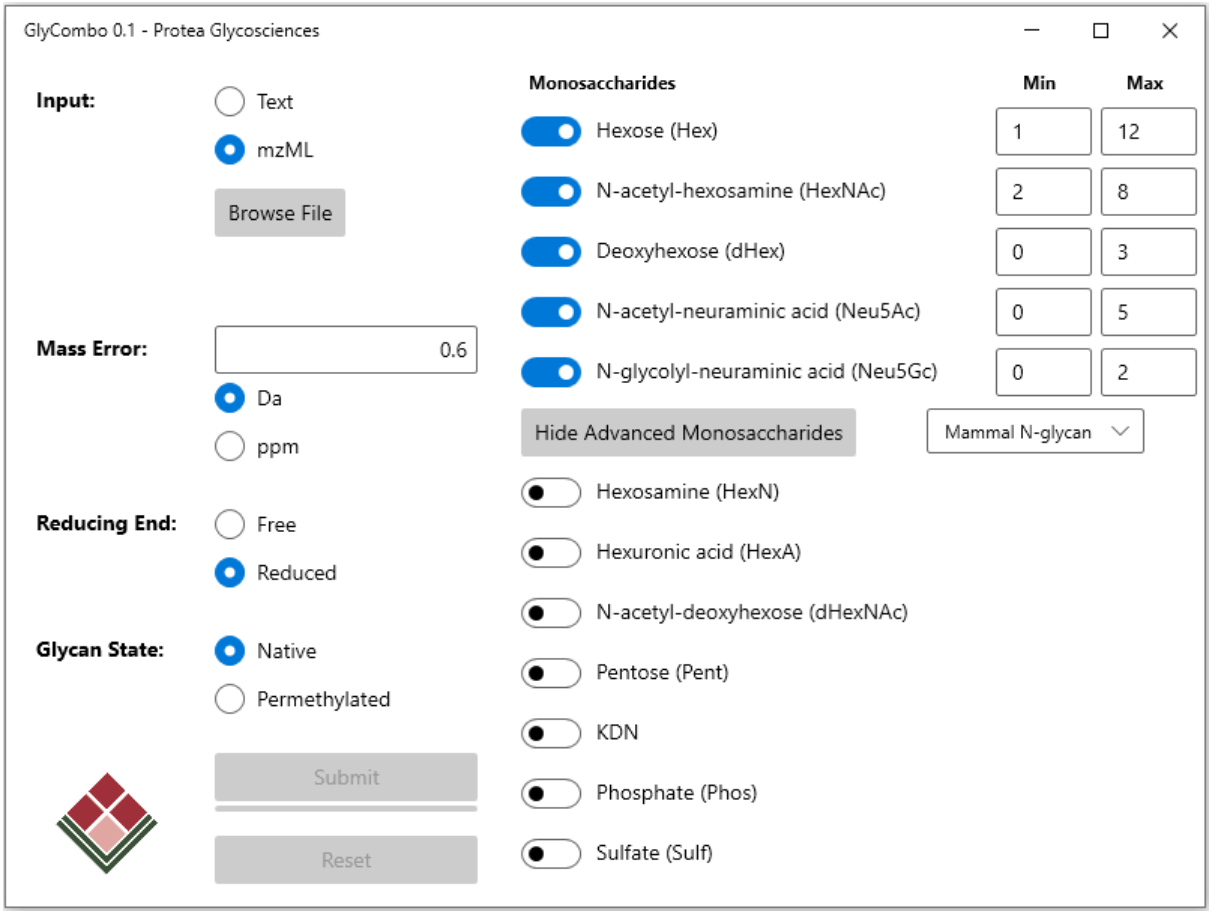

Figure 1 The input and settings page of GlyCombo found at https://github.com/Protea-Glycosciences/GlyCombo.

Many aspects of the glyco-analytical pipeline can affect the resulting *m/z* values observed in these mzML files, and as a result, GlyCombo is run through a GUI that requests user specifications regarding the mass error, reducing end format, glycan state, and expected monosaccharide ranges (**Fig 1**). Unlike GlycoMod, we also provide hexosamine (*i.e.* isomerically ambiguous glucosamine) as a new monosaccharide for combinatorial analysis, enabling the analysis of biomedically important polysaccharides such as heparan sulfate[20]. While the current selection of monosaccharides is not comprehensive, it serves as a starting point for future versions to expand where necessary.

Other aspects integral for correct data interpretation, such as MS polarity, charge state, and precursor *m/z* are automatically extracted from mzML files and used to build a neutral mass list that is searched, enabling direct comparisons to other platforms that require a glycan mass list as input (**Fig 2**). As brute-force combinatorial analysis lacks throughput for applications such as these (like the classic computer science coin change problem[21]), dynamic programming[14] was implemented as the solution to calculate monosaccharide combinations to match a given neutral mass.

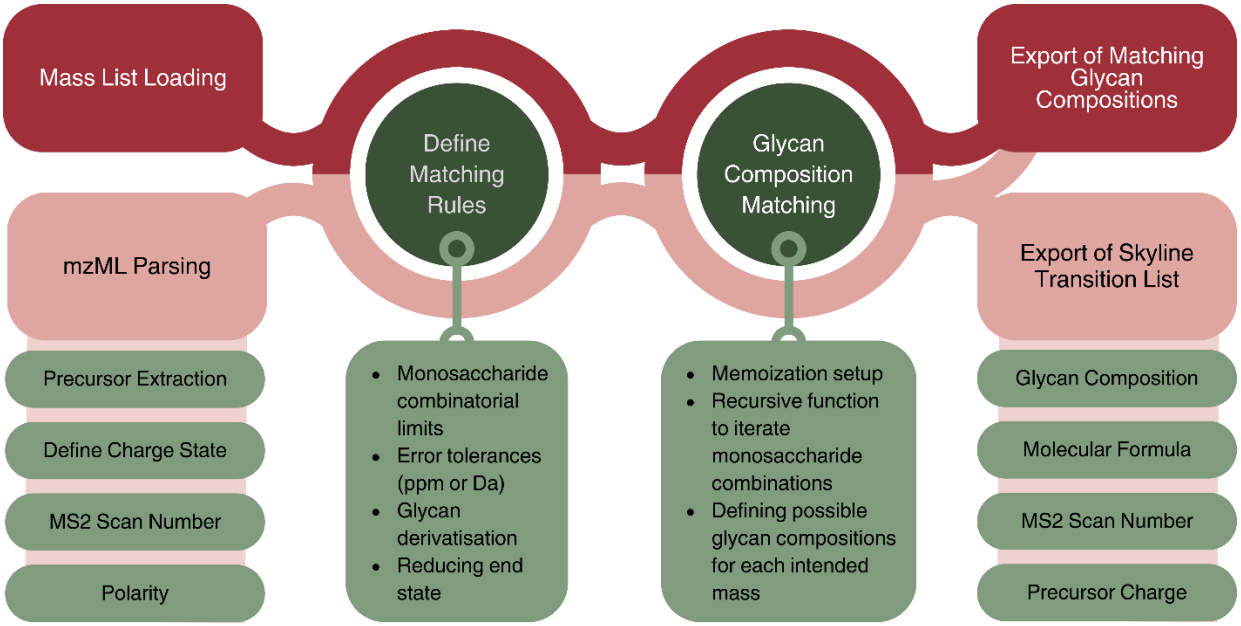

Figure 2 Process diagram describing the input, processes, and output for GlyCombo.

GlyCombo works best with mzML input as it enables the output of three files. The first is a csv file which includes glycan compositions matches, mass error of the matches, and the scan number of the respective MS2 spectrum. The second is a similar csv file which has additional columns enabling direct import into tools which generate extracted ion chromatograms (EIC) such as Skyline. The final file is a parameters file, giving the specific parameters used to generate the given output, as well as the list of precursors searched. In this work, we have used this list of precursors to benchmark our platform against one of the most popular approaches for glycan composition identification, GlycoMod. Users are also given the ability to use a text-based list of glycan masses within the GUI. In this case, the output is limited to a csv file which includes the glycan composition matches and mass error of the matches.

To benchmark our software, and ensure compatibility with existing approaches, six datasets were selected and one raw file downloaded from their respective GlycoPost accessions (**Table 1**). To demonstrate broad applicability across multi-glycomics[22], these datasets cover a range of glycan types: *N*-glycans (NG), *O*-glycans (OG) and glycans released from glycosphingolipids (GSL). For suitability of different polarities and derivatisation states, we also included permethylated and native glycans acquired in positive and negative modes. The vendor-neutrality of our approach was also assessed with dataset generated on Bruker and Thermo Fisher Scientific instruments.

Table 1 Benchmark datasets used in this study, selected to represent a wide range of glycomics approaches including glycan type, sample preparation, liquid chromatography (LC), MS polarity, MS vendor, and MS1 resolution.

Data set	LC	MS mode	MS1 resolution	Additional challenge	GlycoPost accession	Raw file name
1[23]	PGC	-	High	Native	GPST000188	20081907_Fetuin
2[24]	HILIC	+	High	Native	GPST000338	Native_CSF_03_55c_200mins_010223
3[25]	C18	+	High	Permethylated	GPST000242	Fetuin_Perm_Ng_C18_50cm
4[18]	PGC	-	Low	Thermo	GPST000029	CA_U87MG_CL_LAD_NG_090117
5[26]	PGC	-	Low	GSL, Bruker	GPST000239	54-GSL glycans - SW1116 - technical replicate 2
6[27]	PGC	-	Low	O-glycan	GPST000060	TT_190920bPRG4_2

GlyCombo was developed with three qualities in mind: broad accessibility, rapid output, and completeness of glycan detection. Benchmarking was performed by searching the same neutral mass list generated by GlyCombo between both GlycoMod and GlyCombo, using the same monosaccharide ranges, and search time was recorded in triplicate. As shown in **Fig 3A**, all datasets were compatible with our software and it generally performed faster than GlycoMod, with up to 3x faster search times. Despite this, dataset 3 was almost 2x slower with our software, causing these search speed discrepancies to be investigated.

Unlike GlyCombo, GlycoMod (and other search tools such as GlycReSoft[11] and GlycoNote[12]) utilise matching rules based on literature review and assumed biosynthetic pathways to limit the combinatorial burden. This is demonstrated by the GlycoMod *N*-glycan search function whereas the *O*-glycan search function does not have such rules beyond a mass limit of 5 kDa[5]. A comparison of the number of glycan compositions identified based on the glycan masses observed in each dataset (**Fig 3B**) confirmed that the *N*-glycan search function yielded fewer matches, and when assessed with the *O*-glycan function, similar combinations were identified at longer search times (*e.g.* dataset 3 processing time increased from 180 to 550 seconds, 50% slower than GlyCombo).

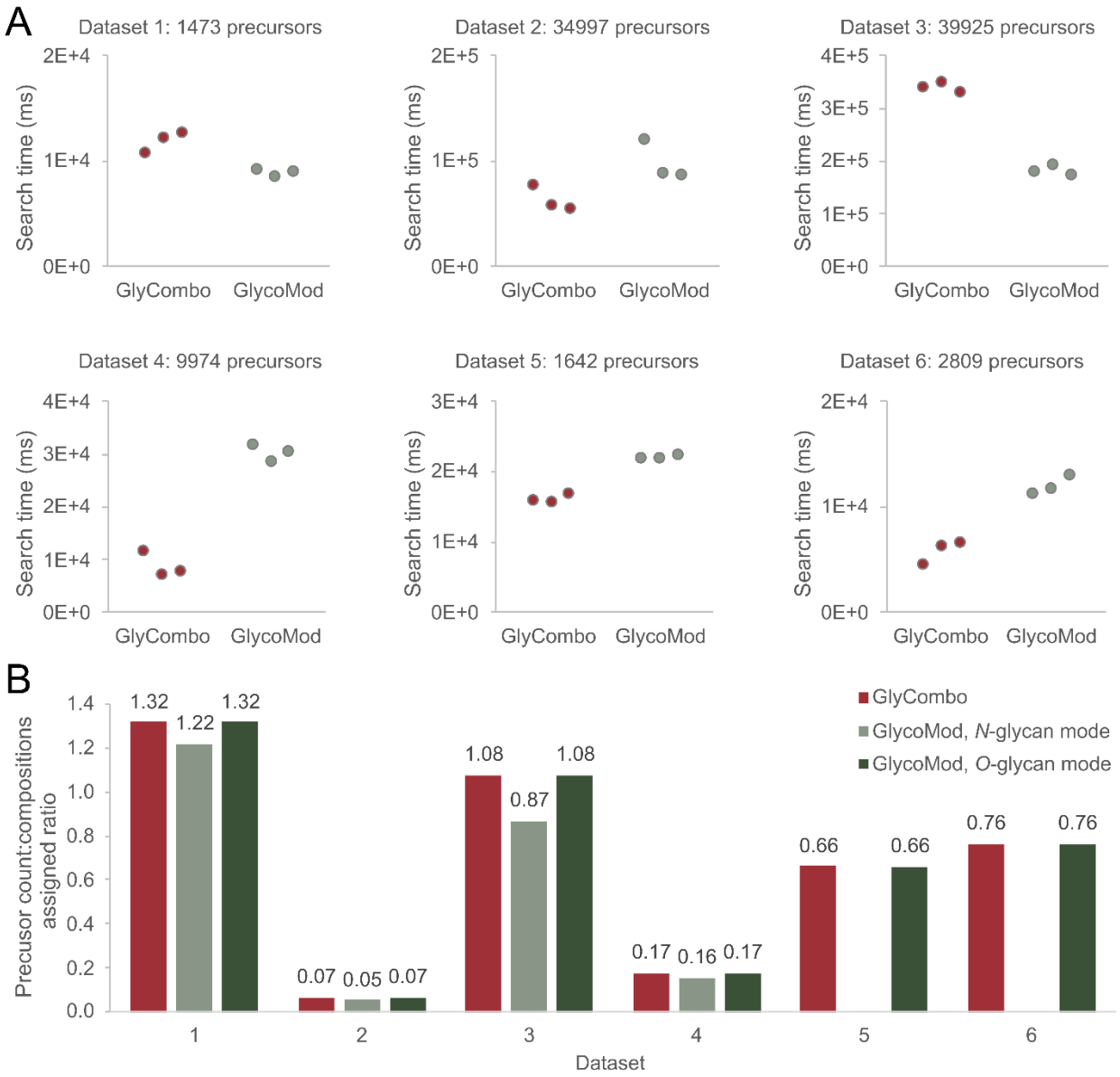

Figure 3 Benchmark comparison of compositional assignment between GlyCombo, a local application, and GlycoMod, a server-based web-app. **A** Comparison of search time for each dataset based on recommended GlycoMod settings **B** Compositional identifications made per dataset across software platforms, represented as a ratio of the number of precursors in the mzML file

The rapid analysis speed of GlyCombo could enable exciting new directions which have been realised in other MS applications such as proteomics and metabolomics. At an average search time of 7.5 milliseconds per precursor, real-time instrument acquisition methods could be devised which have been demonstrated to improve analytical depth[28] and real-time method optimisation[29]. Additionally, as downloading raw files and mzML conversion took longer than the combinatorial search itself, repository-scale reanalysis could prove a promising alternative, enabling iterative reanalyses for new insights[30].

Combinatorial analysis as performed here is often the first step of a bioinformatic pipeline, serving as a way of filtering out non-glycan molecules such as contaminants that have been detected by MS. For this reason, completeness of glycan detection is greatly desired for subsequent quality checks including retention time filtering, MS2 scoring and precursor isotopic distribution evaluation.

Here, we used Skyline[16–18], a software tool that uses pre-defined molecule transitions to generate EICs to remove poor quality combinatorial matches (idotp > 0.9 for the first three isotopes) and visualise the glycan profile of three datasets which feature *N*-glycans and glycans released from glycosphingolipids (**Fig 4**). In all three datasets, qualitatively identical plots of detected glycans were observed when these EICs were compared to base peak chromatograms (BPC) of the original raw files, demonstrating that GlyCombo is effective at glycan detection across these diverse datasets.

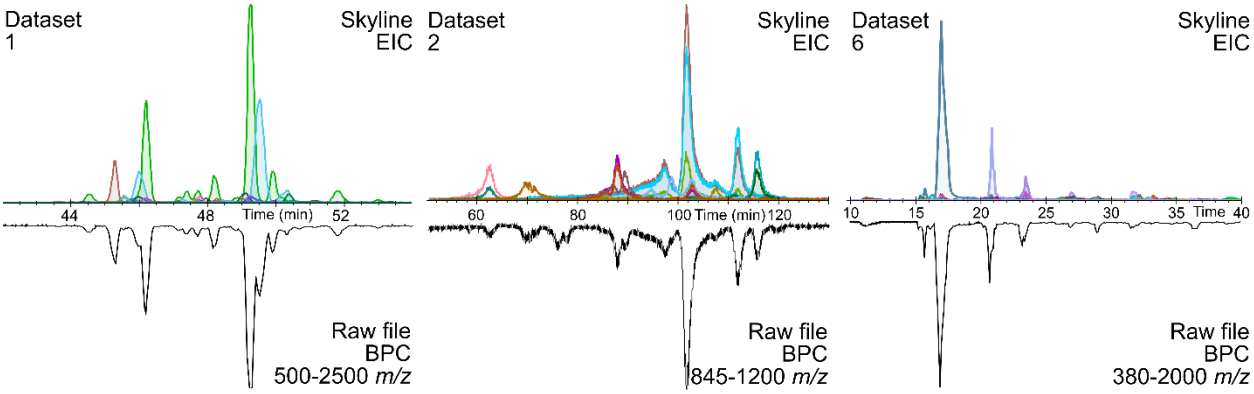

Figure 4 Comparison of glycan composition extracted ion chromatograms (EIC) generated by GlyCombo for Skyline to raw file base peak chromatograms (BPC) qualitatively recapitulate the feature profiles

Although our software extracts and rapidly searches mzML files, instrument acquisition schemes can incorrectly identify the monoisotopic peak (known as off-by-one errors[31]), leading to incorrectly calculated neutral glycan masses. This is also exacerbated by our requirement of an MS2 scan for a given glycan composition to be detected. The use of glycan precursor isotopic distribution filtering ensures that these effects are mitigated, due to scoring the abundance of the first three isotopes of each glycan and subsequent removal of glycan compositions given poor scores.

Another notable limitation of this precursor mass-based combinatorial approach is the inability to assess glycan topology or glycosidic linkages. As glycans are frequently present in multiple isomeric forms or structures, and each isomer has differing biomarker potential, additional forms of separation beyond precursor mass are needed including liquid chromatography stationary phases (such as porous graphitised carbon and hydrophilic interaction columns benchmarked here), and diagnostic product ions from MSn spectra[32]. GlyCombo output includes scan numbers to aid in subsequent downstream MS2 annotation and structure elucidation by software such as GlycoWorkBench[33] and spectral library searching[12].

Conclusions

The identification of glycans in raw files serves as the first foundational step of glycomics data analysis. We describe a new open-source computational tool, GlyCombo, that is capable of rapidly elucidating possible glycan compositions from MS analyses. In this paper we utilize GlyCombo and benchmark its performance against the current state-of-the-art combinatorial solution for glycomics, GlycoMod. The experimental results exemplify the speed and robustness of GlyCombo for use with *N*-glycans, *O*-glycans, glycans released from glycosphingolipids, in combination with multiple polarities and derivatisation states. In addition to faster search times, broader accessibility, and completeness of annotation, processing is automated, and output are specifically formatted to connect with downstream processes including GlycoWorkBench for structural annotation, and Skyline for quality control and quantitation.

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