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Congruent Strategies for Carbohydrate Sequencing. 2. FragLib: An MS^n Spectral Library

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Abstract

A bottom-up approach to achieve full oligosaccharide and glycan characterization has been described that is based on an MS^n fragment spectral library and associated tools. The library, identified as FragLib, was initiated with known standards and commercially available oligomers prepared as methylated derivatives. As a component of this effort a set of software tools has been written for storing, organizing, and comparing spectral files, including the identification of isobaric mixtures. These tools provide a facile and objective evaluation of structural details including interresidue linkage, monomer identification, anomeric configuration, and branching. The tools are components of a web-based data sharing interface for sample tracking, spectral searching, and structural confirmation. Applications have been detailed with unknown samples and previously characterized glycoconjugates.

The preceding report in this series presented data indicating that spectra from various stages of ion trap disassembly (MS^n) could supply structural details for the characterization of linkage, monomer ID, and branching.[1] With complete analysis as a practical consideration, there is a fundamental need for high-throughput tools to complement proteome studies. In that regard, little could be more important than searchable spectral library files for structural confirmation. The general reproducibility of ion trap (IT) spectra, and energy independence from the modes of ionization and collisional activation (CA), make compiling an MS^n library an important research consideration.

A number of oligosaccharide libraries have been reported each having specific characteristics applicable to the needs of independent research groups. GlycosidIQ[2] was developed for computerized interpretation of oligosaccharide spectra based on matching experimental data with theoretical fragments generated from GlycoSuiteDB,[3,4] a database of glycan structures reported in the literature. Similarly, the GlycoFragment/GlycoSearchMS package compares each peak of an observed mass spectrum with the calculated fragments of all structures contained in the SweetDB database to support the interpretation of mass spectra of complex carbohydrates.[5] Both GlycosidIQ and GlycoFragment/GlycoSearchMS utilize tandem MS (MS^2) of native samples and theoretically generated spectra as reference. Tseng et al. applied a “catalog library” approach for elucidating the structures of minor components in a mixture of oligosaccharides.[6] The catalog data consist of the characteristic fragmentation patterns belonging to a set of specific substructures compiled from a library of known compounds present in the same sample that have also been characterized by other techniques. Collision-induced disassociation (CID) was used to determine the presence of the cataloged motifs. Recently, a standard trisaccharide MS^2 library has been reported that uses underivatized synthetic samples.[7]

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In this report, we summarize initial progress and introduce simple applications to demonstrate the feasibility of building and utilizing a standard MS^n carbohydrate fragment library. The goal will be to accumulate spectra of methylated standards from any stage of disassembly and couple these spectra with tools to store, search, compare, and export such data. These tools are components of a web-based data sharing interface for sample tracking, spectral searching, and structural confirmation.

EXPERIMENTAL SECTION

Sample Preparation and MS^n Experiments. MS^n spectral library building was initiated using the commercial oligosaccharides presented in the preceding report in this series,[1] and human milk oligosaccharides purchased from Calbiochem (EMD Biosciences, Inc., La Jolla, CA), (Table 1.) Porcine stomach mucin (PSM) was from Sigma (Sigma-Aldrich, St. Louis, MO) (type II, M-2387). The oligosaccharides were permethylated as described by Ciucanu and Kerek[8] and dissolved in 1:1 methanol/water containing 1 mM sodium acetate for MS^n analysis of sodium adducts. All reagents used were of the highest available purity (Sigma). Reductive base release of O-linked glycans was carried out as described by Carlson.[9] MS^n experiments were performed in positive ion mode using a linear ion trap mass spectrometer (LTQ, ThermoFinnigan, San Jose, CA) equipped with a nanospray source.

Mass Spectral Data Handling. Mass spectral data were in Xcalibur raw data format, (ThermoFinnigan). To facilitate and automate large-volume spectra handling, a utility package was developed in Python using the Xcalibur Development Kit (XDK), a suite of programmable COM objects that allows display and manipulation of Xcalibur data and access to Xcalibur files.[10]

Mass Spectral Database (FragLib) and Web Interface. A relational database was built as a central data management system. CASE tools were used during the database design and implementation phases. The database and web interface were developed on a LAMP (Linux, Apache,[11] MySQL[12] and PHP[13]) platform, which was built using freely distributable open source software. WebDot[14] was used for MS^n pathway tree layout visualization on the FragLib web interface.

Mass Spectral Comparison Tools. Library searching and spectral comparisons were performed using the NIST MS search tool.[15] For independent pairwise spectral comparisons, an in-house similarity measure was also developed for interpreting spectral matches. This measure was implemented in Python and is calculated as an "*R* score":

$$R\,\text{score} = \sum_{i=1}^{N}\left(1 - \frac{|\,\text{IntensityUnknown}_i - \text{IntensityStd}_i\,|}{\text{IntensityMax}}\right) \times \frac{1}{N} \times 1000$$

where IntensityUnknown is the relative intensity of the ion peak in the unknown (query) spectrum; IntensityStd is the relative intensity of the ion peak in the standard (reference) spectrum; IntensityMax is the maximum relative intensity of any ion peak in the standard or unknown spectra; and *N* is the number of ion peaks.

Using this similarity measure, an *R* score of zero represents completely dissimilar spectra, while an *R* score of 1000 represents identical spectra.

Data Flow and Entry Format. The major components of the library and data flow are diagrammed in Figure 1. MS^n standard spectra are from methylated glycans obtained from pure oligosaccharide standards or from previously well-characterized samples. The spectra from MS^n pathways are obtained as provisional library records. Curation is the process of "housekeeping" efforts related to the library collection, including structural annotation of the

ion fragment spectra, relevant information documentation, data cleanup, data preparation, and loading. During curation, structural assignment of the fragment spectra is confirmed. All FragLib data are stored in one centralized relational database, which provides efficient data management and flexibility for further data mining. FragLib records can be exported in various data formats including NIST-MSP and XML enabling data exchange with third party tools. For instance, the library records can be exported as a batch to the NIST MS search tool, which provides an MS spectral search engine with proven sensitivity and specificity.[15] Driven by scripts, the FragLib web interface displays data stored in the central database and allows users to query, explore, and retrieve library records from multiple entry points. One typical FragLib record page is illustrated in Figure 2. An MS^n disassembly tree is provided to visualize the hierarchical relationship among all the spectra, allowing the user to explore the data set. All related data including raw spectrum files, structural assignment (linear code and graphical representation), sample identification number, sample source, provider, and literature reference are accessible from the page.

Oligosaccharide Substructure Confirmation. Isobaric oligosaccharide substructures may generate distinct fragments in CID spectra or may generate isobaric fragments differing only in the ion intensity patterns, indicating underlying structural or stereochemical differences.[1] Therefore, spectral matching can be used for oligosaccharide substructure confirmation. For example, the CID spectra obtained from the nonreducing terminal disaccharide moiety Fuc-α (1-2)-Gal from the two human milk oligosaccharides lacto-*N*-difucosylhexaose I and lacto-*N*-fucopentaose I demonstrated high spectral similarity although they were obtained from different parent oligosaccharides along differing MS^n pathways through repeated rounds of isolation and CID (Figure 3). The MS^5 spectrum of the terminal FucHex disaccharide Fuc-α (1-2)-Gal(1-OH) (*m/z* 433), obtained from lacto-*N*-difucosylhexaose I along the pathway *m/z* 1274→834→660→433, was submitted to the FragLib spectral searching system, and the NIST search tool was invoked. The strong match against the same moiety obtained in MS^4 from lacto-*N*-fucopentaose I along the pathway *m/z* 1100→660→433, indicates likelihood of identity with the probability 95.4% (Figure 3).

RESULTS AND DISCUSSION

Application Example 1: Oligosaccharide Substructure Spectral Matching. An unknown *O*-glycan from PSM corresponding to the composition $HexNAc_2Hex2$ (*m/z* 983.2) was subjected to MS^n, giving the ion *m/z* 486.2, corresponding to a terminal HexHexNAc disaccharide. The disaccharide was isolated along the pathway *m/z* 983→708→486 giving the spectrum in Figure 4A. The MS^n pathway for this linear core-1 glycan indicates loss of the reducing end GalNAc in MS^2, followed by loss of an internal 3-linked Gal in MS^3, giving the terminal disaccharide ion *m/z* 486.2. The *m/z* 486.2 spectrum was submitted for spectral matching against two *m/z* 486.2 disaccharide ions, Gal-β(1-4)-GlcNAc-(1-ene) (Figure 4B) and Gal-β(1-3)-GlcNAc-(1-ene)(Figure 4C), isolated in MS^n from the human milk glycans lacto-*N*-neotetraose (Gal-β(1-4)-GlcNAc-β(1-3)-Gal-β(1-4)-Glc) and lacto-*N*-tetraose (Gal-β(1-3)-GlcNAc-β(1-3)-Gal-β(1-4)-Glc), re-spectively. The high spectral match for the terminal disaccharide moiety Gal-β(1-4)-GlcNAc indicated that a positive identification of the nonreducing terminal disaccharide of a putatively unknown glycan could be reliably obtained (Figure 4).

Application Example 2: Full Structure Characterization. Full details of structure are evident only in small oligomers, making MS^n disassembly a requirement.[1] The tools provided here assist glycan analysis via library matching of the substructure spectra from unknown oligosaccharides isolated along MS^n pathways, against library-stored spectra of isobaric moieties to assess underlying structural similarity. The following example demonstrates how substructure analysis is included within the framework of total glycan analysis using FragLib.

Permethylated O-linked glycans from PSM were mass profiled, and the ion corresponding to $HexNAc_2Hex_2Fuc$ (*m/z* 1157) in the MS spectrum was submitted to MS^n. The MS^2 spectrum (not shown) indicated at least two structural isomers for *m/z* 1157 based on the major peaks at *m/z* 921 and 882. The MS^2 peak at *m/z* 921 corresponds to elimination of an unsubstituted 3-linked hexose (-236), indicating a possible branched core-2 glycan. The ion at *m/z* 882 indicated a linear core-1 glycan, based on the neutral loss of an unsubstituted reducing end HexNAc (-275). The MS^2 peak *m/z* 921, of the core-2 glycan (Figure 5A), generated the trisaccharide fragment *m/z* 660 as a major peak in MS^3 (Figure 5B). MS^4 of *m/z* 660 generated the major fragment ion *m/z* 472, indicating fucose at the nonreducing terminus of the 6-linked FucGalGlcNAc— branch, as well as other ions confirming the core-2 structure (Figure 5C). The FucHex-disaccharide fragment ions *m/z* 415 (B-ion) and 433 (C-ion) were then isolated from *m/z* 660 and the MS^5 spectra were submitted for substructure spectral matching. The CID spectra of *m/z* 415 and 433 were consistent with the library-stored CID spectra of the terminal disaccharide Fuc-α(1-2)-Gal moiety obtained from the human milk glycan lacto-*N*-fucopentaose I (Figure 6A and B).

The 6-linked FucGalGlcNAc— branch (*m/z* 660) was also submitted for spectral matching. Comparison of the MS^4 spectrum with the CID spectrum of Fuc-α(1-2)-Gal-β(1-3)-GlcNAc obtained from the human milk glycan lacto-*N*-fucopentaose I indicated a high likelihood of nonidentity (the identity probability less than 2%). Consistent with the literature reports for core-2 porcine gastric mucins,[16,17] this suggested galactose is β(1-4)- linked, and not β(1-3)-linked, to GlcNAc in this glycan, a feature further indicated by the cross-ring cleavage ion *m/z* 503 (Figure 5C). Combining these attributes, we proposed the full structure of the core-2 glycan shown in Figure 5A. This glycan has been previously characterized,[16] and the CID spectrum of the Fuc-α-(1-2)-Gal-β(1-4)-GlcNAc (*m/z* 660) trisaccharide moiety was stored in the reference library.

Application Example 3: Isobaric Mixture Determination. The isomeric linear core-1 glycan indicated in the above example by the peak at *m/z* 882 in the MS^2 spectrum of $HexNAc_2Hex_2Fuc$ (*m/z* 1157) was also pursued using FragLib tools. In this example, the MS^4 spectrum of *m/z* 660 (isolated along the disassociation pathway *m/z* 1157→882→660) was submitted for spectral matching with the current FragLib reference spectra, including the recently added Fuc-α(1-2)-Gal-β(1-4)-GlcNAc spectrum (*m/z*660). However, the two top hits for *m/z* 660 reference spectra only showed moderate mass/intensity similarities to the unknown spectrum (with *R* scores as 838 and 623, respectively). The only difference between these two structures is in the Gal-GlcNAc interresidue linkage: Fuc-α(1-2)-Gal-β(1-4)-GlcNAc and Fuc-α-(1-2)-Gal-β(1-3)-GlcNAc. Comparisons between the unknown and the two reference spectra indicated significant intensity differences, but also considerable correlations in the fragment ions present among the spectra (Figure 7). Closer examination suggested the unknown could be a mixture of the two library entries. To test this hypothesis and to determine percentages in the mixture, spectra from the two standards were compiled with incremental changes in each component. These results were compared to the sample spectrum using the *R* score similarity metric. A plot of *R* score versus the mixture composition of Fuc-α(1-2)-Gal-β(1-3)-GlcNAc— indicated a spectral match in the ratio of 13% Fuc-α(1-2)-Gal-β(1-3)-GlcNAc— to 87% Fuc-α(1-2)-Gal-β(1-4)-GlcNAc— (Figure 8). This comparison plot of the mixture (13%/87%) and the unknown spectrum is shown in Figure 9 and corresponds with an earlier report indicating both isobars were observed in PSM.[17]

Uniqueness of the FragLib Approach. A comparison of FragLib to other library-based efforts is summarized in Table 2. FragLib is unique in the following aspects: (1) FragLib uses permethylated glycan samples with metal ion adduct. This combination ensures generation of reproducible structure-specific fragmentation patterns,[1] which are critical criteria for de novo oligosaccharide sequencing. In contrast, many of the structure-specific fragments defining

glycan branching, linkage, and ster-eochemistry details are absent in spectra obtained from native samples; (2) FragLib references are empirical spectra in contrast to those theoretically generated.[2-5] Additionally, the fragment patterns are specified by ion mass and relative intensities. Very often, the comparison of two isomers mass spectra reveals that the differences are only on ion relative intensities.[1] (3) FragLib spectra are obtained using MS^n. In many cases, isobars cannot be revealed in MS/MS spectra.[1] Higher order disassembly enables substructure characterization common in different glycan structures. FragLib reference structures are substructures that form “the building blocks” of glycan structures. This feature provides the opportunity to assemble fragments to determine a complete glycan structure; thus, FragLib could serve as a tool for novel structure discovery. This is different from GlycosidIQ and GlycoFragment/ GlycoSearchMS, which requires unknown structures to be in the library. (4) FragLib is a computerized system. The complexity of tracing MS^n disassembly pathways, spectra/structure searching, and huge volume spectral/structural data management requirements make manual processing a very difficult, if not impossible work.

CONCLUSIONS

A carbohydrate mass spectral library has been described that includes associated tools for library building, spectral searching, comparing, and retrieving. Library entries are methylated oligosaccharides and their MS^n fragmented products. We judge this combination of tools to be the most effective for detailing structure for both routine and high-throughput applications.[1] Spectral matches and scoring software have introduced an objective evaluation of structural details. Instrumental variables versus structural subtleties will be appreciated with growing applications and additional library entries. The library and associated tools have been applied successfully for the identification of substructure details within various glycomer samples obtained from human milk and porcine stomach mucin, even when challenged with isobaric mixtures. With a growing number of spectral records and integration with de novo carbohydrate sequencing software tools,[18] we anticipate FragLib may provide a basis for a fully integrated high-throughput sequencing platform. We further propose these strategies as a public repository for oligosaccharide spectra and disassembly products and welcome the carbohydrate community to contribute standard samples for a more comprehensive and effective library.

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(18). Lapadula AJ, Hatcher PJ, Hanneman AJ, Ashline DJ, Zhang H, Reinhold VN. Anal. Chem 2005;77:xxxx–xxxx.

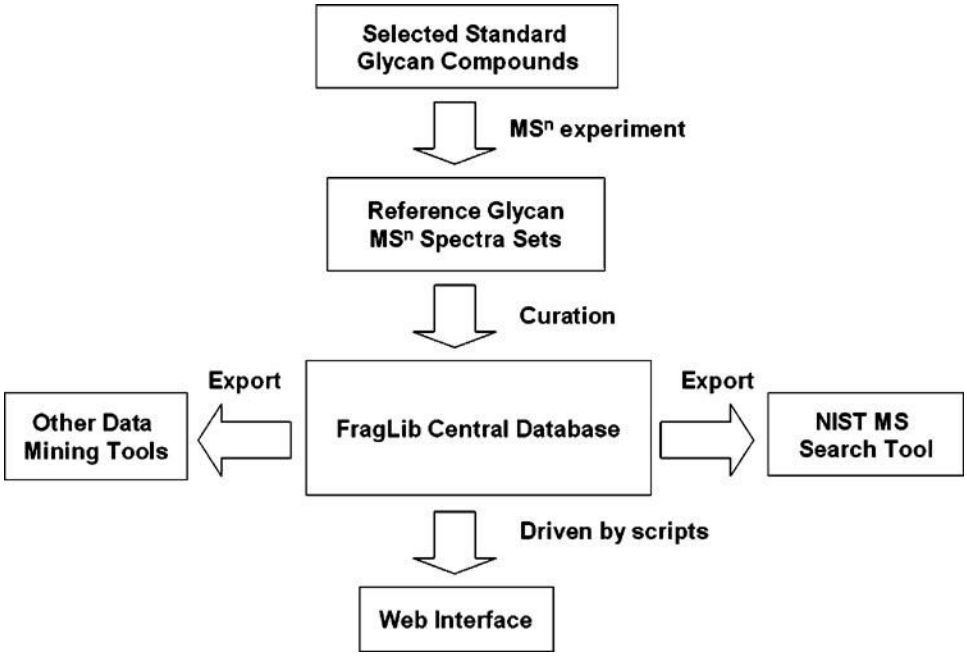


Figure 1.
FragLib system; Major components and data flow.

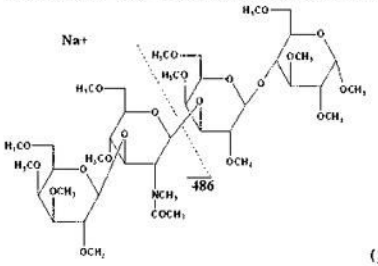
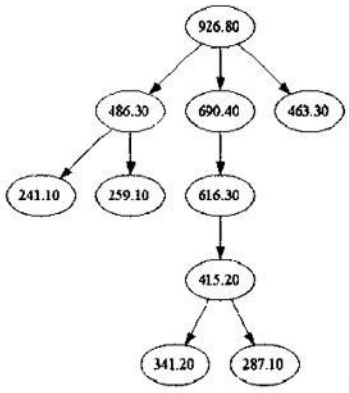
Spectrum Entry ID:	345245_N_15.1.70.1
Raw Spectrum File:	345245_N_15.raw (Download)
Spectrum Provided by:	Suddham Singh (Email)
Fragment Linear Code:	Gal-b3-GlcNAc(1,2ene)
	This motif has 4 reference spectra (Details)
Graphical Structure:	 (Zoom in)
MS^n Pathway:	926.80 -> 486.30 ->
m/z value:	486.30
Charge state:	1
Num of monomers:	2
Num of cleavages:	1
10 Largest Peaks:	412 999 | 259 959 | 444 481 | 252 134 | 250 79 208 57 | 486 48 | 241 47 | 384 33 | 268 22
Sample ID:	345245_N (Sample Tracking Details)
Sample Source:	Human Milk Glycan (From Calbiochem | Catalog No. 345245)
Literature Reference:	Feizi, T., et al. 1994. Biochemistry 33, 6342. (PubMed Link)
Sample Permethylated:	Yes
Terminal Reduced:	No
Adduct:	Na+
Sample MS^n Pathway Tree:	 (Zoom in)

Figure 2.
Example of FragLib record entry as displayed by web interface.

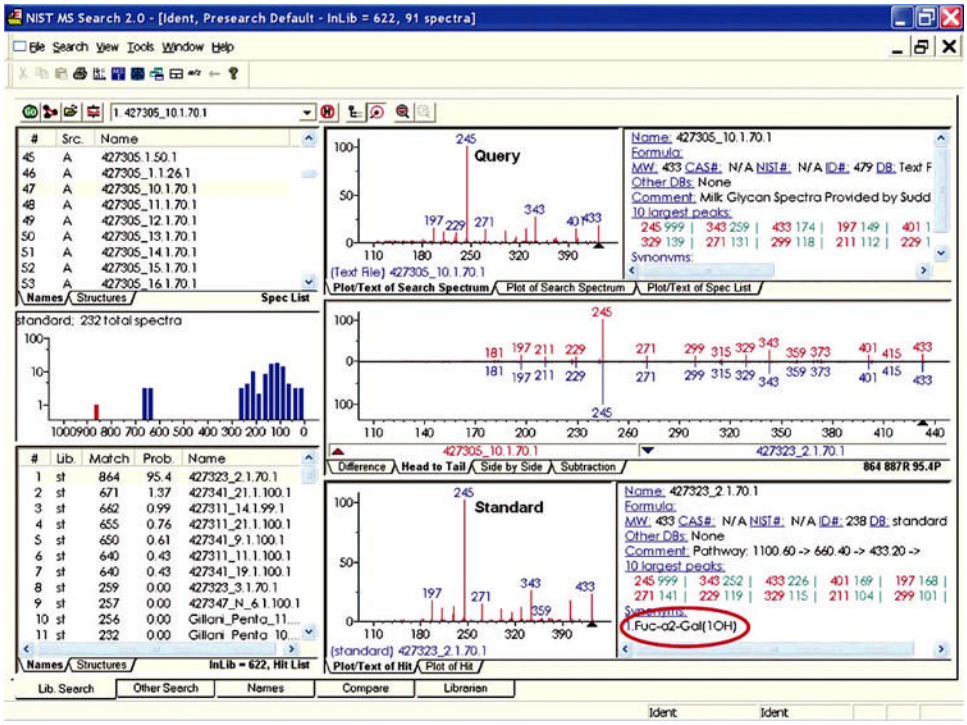


Figure 3.

Screen capture of NIST MS tool for FragLib spectral library searching; MS^5 spectrum for Fuc-α-(1-2)-Gal-(1-OH) from the human milk glycan lacto-*N*-difucosylhexaose I (isolated as *m/z* 1274→834→760→433)(top), compared with the standard spectrum for Fuc-α-(1-2)-Gal-(1-OH) stored in FragLib (MS^4 spectrum from lacto-*N*-fucopentaose I, isolated as *m/z* 1100→660→433)(bottom).

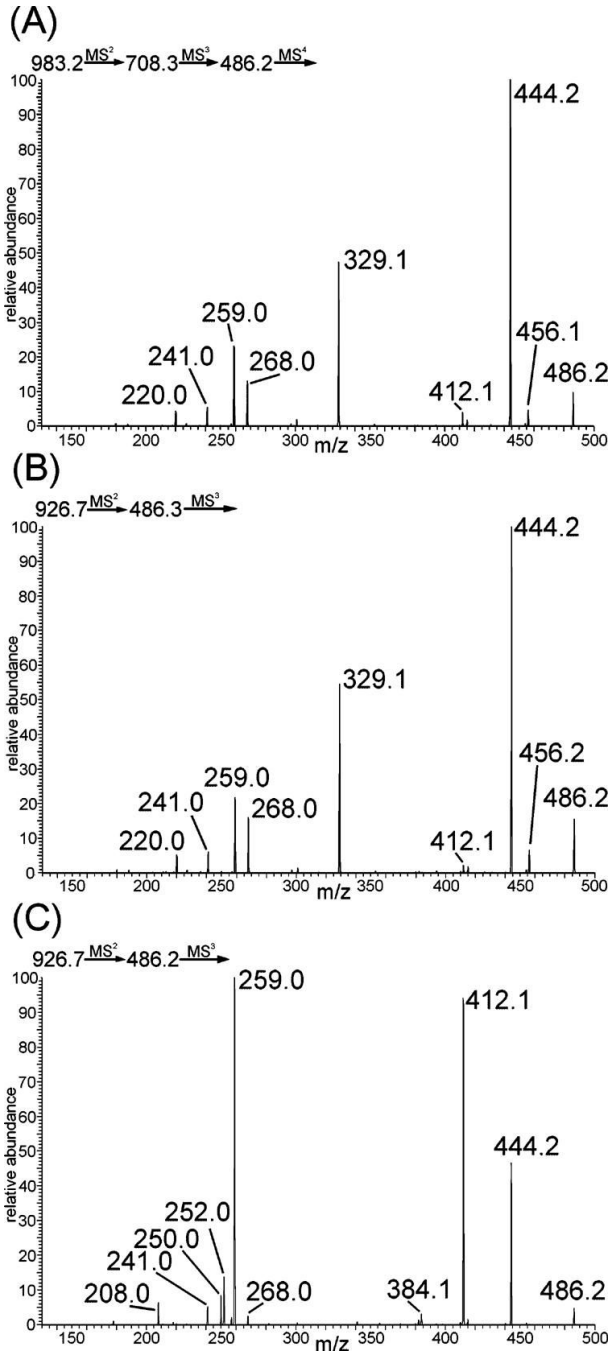


Figure 4.

Comparison of (A) an unknown PSM glycan MS^4 spectrum for the ion Hex-HexNAc (*m/z* 983→708→486) with the standard spectra of MS^n fragments,(B)Gal-β(1-4)-GlcNAc-(1-ene) (*R*score, 953), and (C) Gal-β(1-3)-GlcNAc-(1-ene)(*R*score, 325) from human milk glycans.

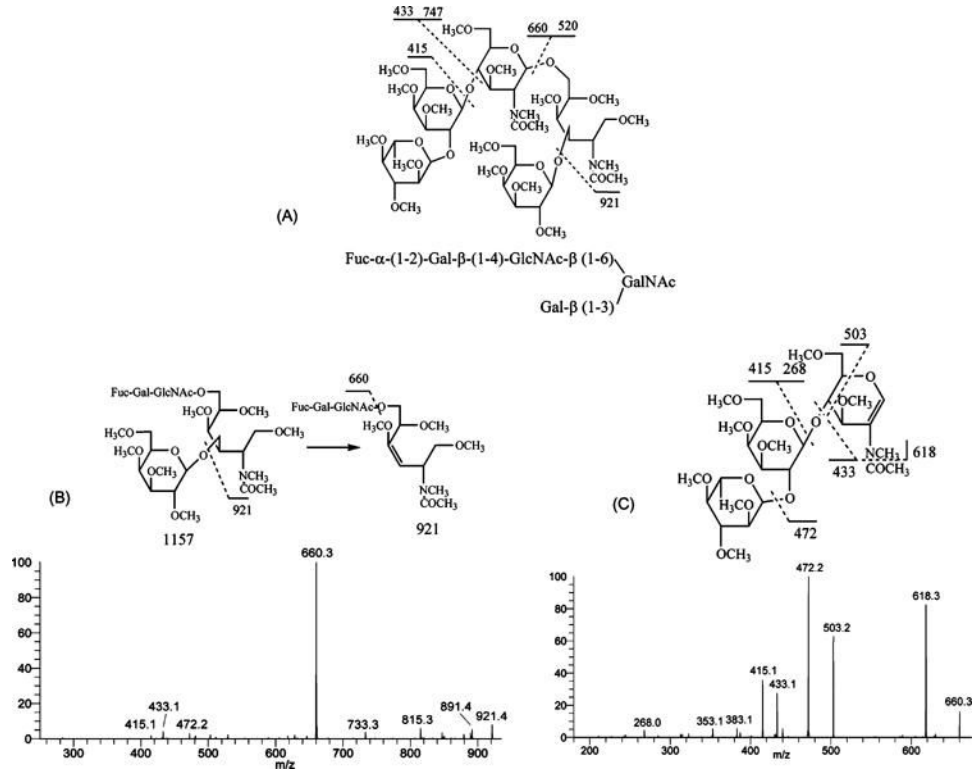


Figure 5.

Confirmation of a core-2 glycan structure from PSM. (A) MS^n results and spectral comparison giving the proposed structure for the ion *m/z* 1157→921. (B) The MS^3 of ion (*m/z* 1157→921) generated the major ion for fragment Fuc-Gal-GlcNAc (*m/z* 660). (C) Structure of the PSM glycan MS^4 ion Fuc-Gal-GlcNAc (*m/z* 1157→921→660) proposed as Fuc-α-(1-2)-Gal-β-(1-4)-GlcNAc.

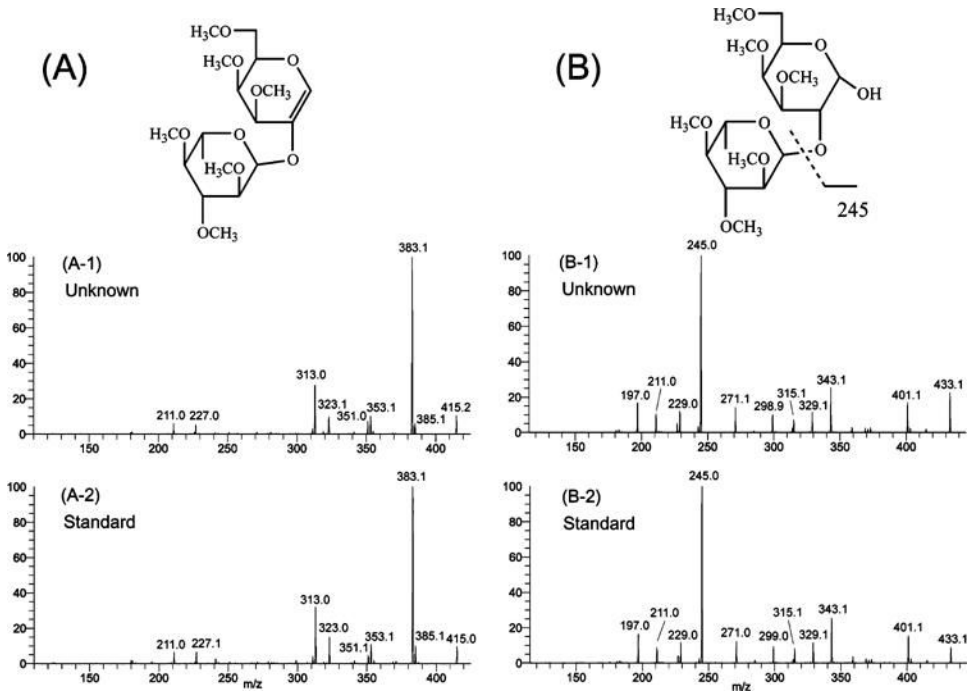


Figure 6.

Confirmation of substructures from PSM glycan. (A) Comparison of the MS^5 for the ion Fuc-Gal (*m/z* 1157→921→660→415) from PSM glycan (A-1) with the standard spectrum for Fuc-α-(1-2)-Gal-(1-ene) stored in FragLib (A-2) (*R* score, 932). (B) Comparison of the MS^5 for the ion Fuc-Gal (*m/z*1157→921→660→433) from PSM glycan (B-1) with the standard spectrum for Fuc-α(1-2)-Gal-(1-OH) stored in the FragLib (B-2) (*R* score, 917).

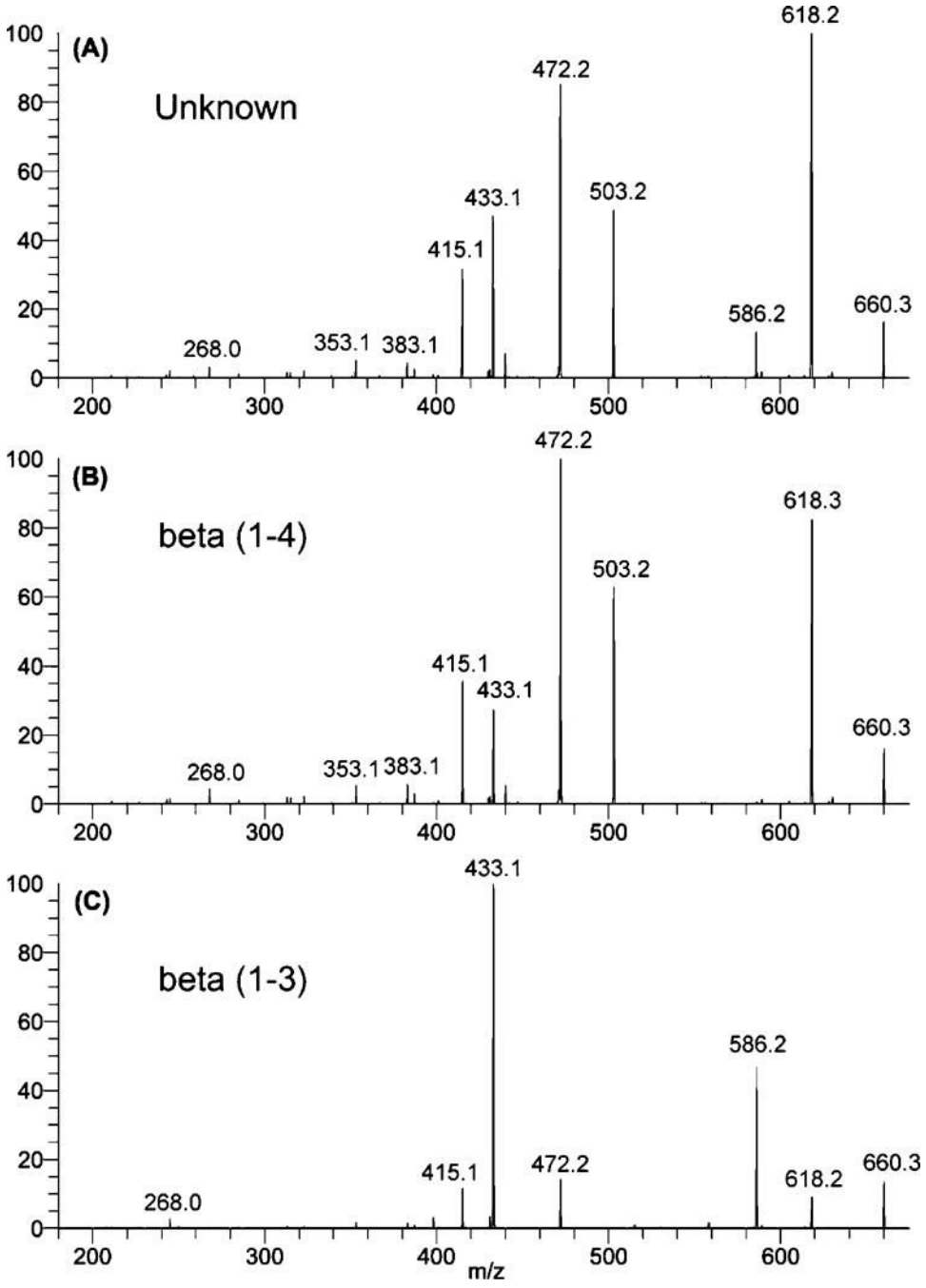


Figure 7.

Comparison of (A) an unknown PSM glycan MS^4 spectrum for ion Fuc-Hex-HexNAc (*m/z*1157→882→660) with the standard spectra for (B) Fuc-α(1-2)-Gal-β(1-4)-GlcNAc-(1-ene) from PSM glycan (*R* score, 838) and (C) Fuc-α(1-2)-Gal-β(1-3)- GlcNAc-(1-ene) from human milk glycan (*R* score, 623) stored in FragLib.

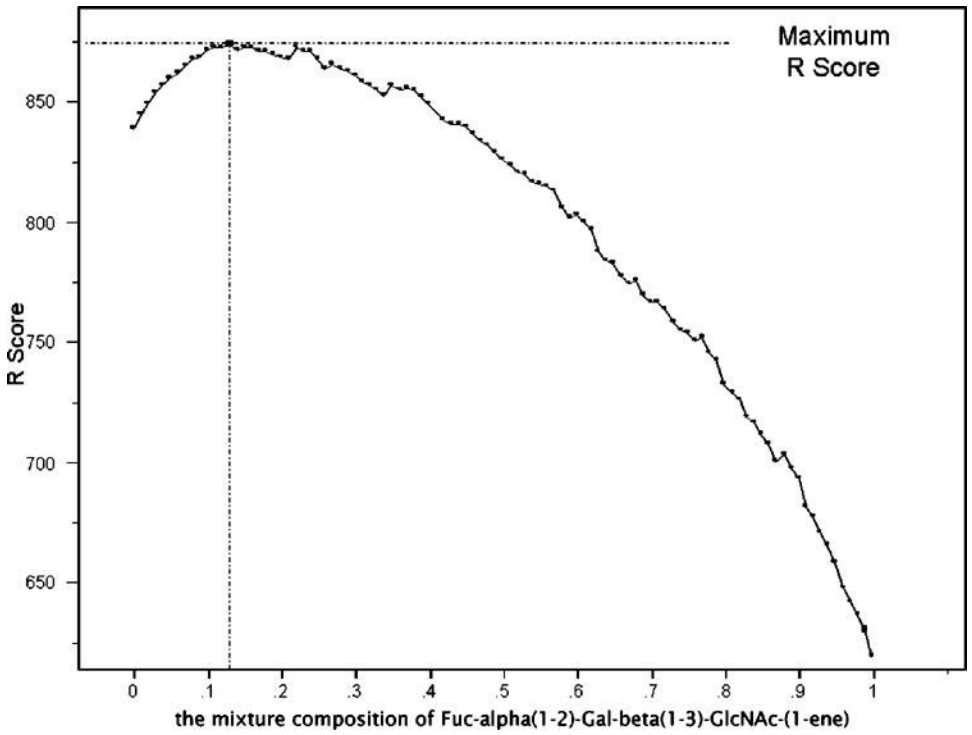


Figure 8.

Scoring for spectral comparisons. The optimal mixture composition percentages, roughly 13% Fuc-α(1-2)-Gal-β(1-3)-GlcNAc-(1-ene) and 87% Fuc-α(1-2)-Gal-β(1-4)-GlcNAc-(1-ene), were obtained by finding the mixture composition of Fuc-α(1-2)-Gal-β(1-3)-GlcNAc-(1-ene) maximizing *R* scores.

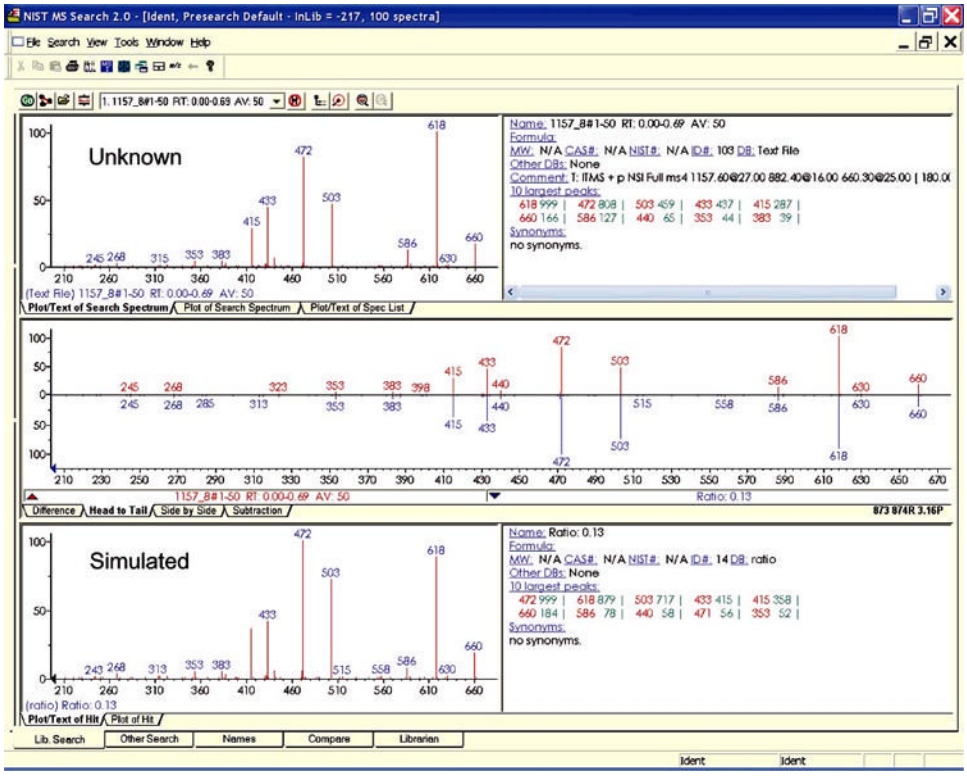


Figure 9.

Comparison of the unknown PSM glycan MS^4 spectrum of *m/z* 1157→882→660 (top) with the simulated standard mixture spectrum (bottom) at the composition percentage of 13% Fuc-α(1-2)-Gal-β(1-3)-GlcNAc-(1-ene) and 87% Fuc-α(1-2)-Gal-β(1-4)-GlcNAc-(1-ene)(*R* score, 874).

Table 1.

Human Milk Oligosaccharides Used for FragLib Building

structure	name	Calbiochem product no.
Gal-β(1-3)-GlcNAc-β(1-3)-Gal-β(1-4)-Glc	lacto-N-tetraose	345245
Fuc-α(1-2)-Gal-β(1-3)[Fuc-α(1-4)]-GlcNAc-β(1-3)-Gal-β(1-4)-Glc	lacto-N-difucosylhexaose I	427305
Gal-β(1-3)[Fuc-α(1-4)]-GlcNAc-β(1-3)-Gal-β(1-4)[Fuc-α(1-3)]-Glc	lacto-N-difucosylhexaose II	427311
Fuc-α(1-2)-Gal-β (1-3)-GlcNAc-β(1-3)-Gal-β(1-4)-Glc	lacto-N-fucopentaose I	427323
Gal-β(1-3)[Fuc-α(1-4)]-GlcNAc-β(1-3)-Gal-β(1-4)-Glc	lacto-N-fucopentaose II	427329
Gal-β(1-4)[Fuc-α(1-3)]-GlcNAc-β(1-3)-Gal-β(1-4)-Glc	lacto-N-fucopentaose III	427335
Gal-β(1-3)-GlcNAc-β(1-3)-Gal-β(1-4)[Fuc-α(1-3)]-Glc	lacto-N-fucopentaose V	427341
Gal-β(1-4)-GlcNAc-β(1-3)-Gal-β(1-4)-Glc	lacto-N-neotetraose	427347

Table 2.

Comparison of FragLib to the Prior Library-based Work in Carbohydrate Sequencing

approach/tool	glycan sample preparation	reference spectra generation method	MS^n or MS/MS	reference structure entity	reference spectral library management
FragLib	permethylated with metal ion adduct	empirical	MS^n	glycan substructure	computer-based
catalog library	native glycan	empirical	MS^n	glycan substructure	manual
GlycoFragment/ GlycoSearchMS	native glycan	theoretical	MS/MS	intact glycan	computer-based
Glycosid IQ	native glycan	theoretical	MS/MS	intact glycan	computer-based
trisaccharide MS^2 library	native glycan	empirical	MS/MS	glycan substructure	manual