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## INTRODUCTION

Low molecular weight heparins (LMWH) - Enoxaparin, Parnaparin, Certoparin, Deltaparin, Tinzaparin, etc. - have been reported to be the key drugs and are as safe and effective as Unfractionated Heparin (UFH) [1,4] which further necessitates in-depth study of new regulatory drugs. Despite its medical and biological importance, LMWHs are relatively uncharacterized in terms of its chemical structures due to its structural complexity. Liquid Chromatography coupled with Mass Spectrometry workflows have been increasingly used for potential high throughput identifications of the LMWH oligosaccharides [2-6]. However, lack of adequate software tools to interpret the complex data generated by LC-MS methods is the main bottleneck in achieving high throughput heparin characterization. We have developed SimGlycan® v. 5.60 to streamline the data analysis.

## METHODS

**Sample Preparation:** Digestion mixture was prepared by mixing 20µL of Enoxaparin sodium (20mg/ml in deionized water) using the method explained in Zhang et al (2013). The digested reaction mixture was filtered using 0.45mm filter and used as final test solution for RPIP-UPLC-MS analysis.

**LC Analysis:** HPLC: Shimadzu HPLC System on Agilent SB C18 (4.6 x 100 mm, and 1.8µm columns connected in serial). Mobile phases: A (Water with 15mM Hexylamine) and B (75% ACN with 15mM Hexylamine).

**Mass Spectrometry:** Data acquisition was performed with a TripleTOF® 5600 (SCIEX, Concord, ON) in negative electrospray ionization mode. LC-MS2 experiments were conducted for structural elucidation.

**Data Analysis:** SimGlycan 5.60 software (PREMIER Biosoft, USA) was used for LC-MS and MS/MS data analysis.

## DATABASE CURATION

To facilitate automatic and rapid identification of LMWH oligosaccharides, we have created a database in SimGlycan software containing 317 probable structural isomers constructed based on the following core motifs (Figure: 1) reported by Zhang et al., 2013 [4]. Each structure is modified with varying number of substituents namely SO<sub>3</sub>, Ac, or H.

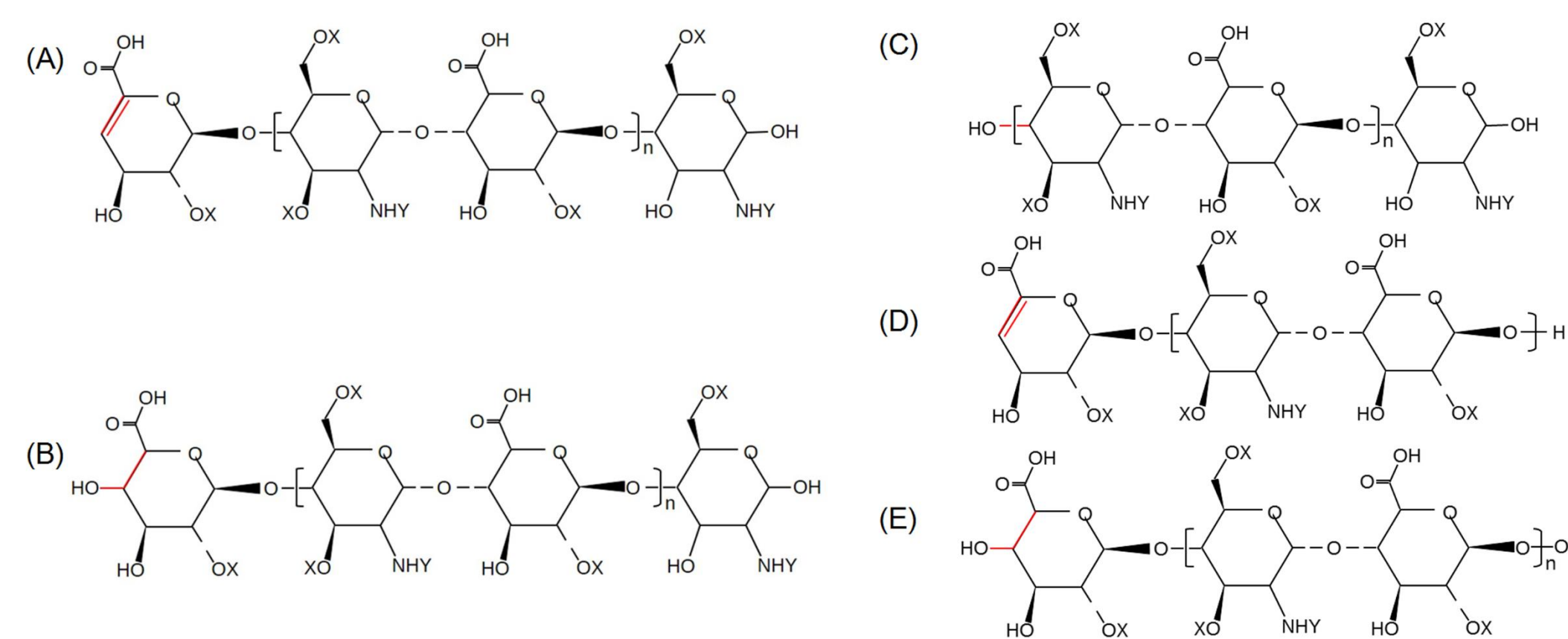


Figure 1: Structures of oligosaccharides found in enoxaparin. (a) Main oligosaccharides with even numbers of units and unsaturated uronic acid at the nonreducing end; (b) oligosaccharides with even numbers of units and saturated uronic acid at the nonreducing end; (c, e) oligosaccharides with odd numbers of units and saturated uronic acid at the nonreducing end; (d) oligosaccharides with odd numbers of units and unsaturated uronic acid at the nonreducing end. X = SO<sub>3</sub> or H, Y = SO<sub>3</sub>, Ac, or H. (Zhang et al., 2013).

A pictorial representation of the SimGlycan software workflows for creating a custom database has been shown in Figures 2-5.

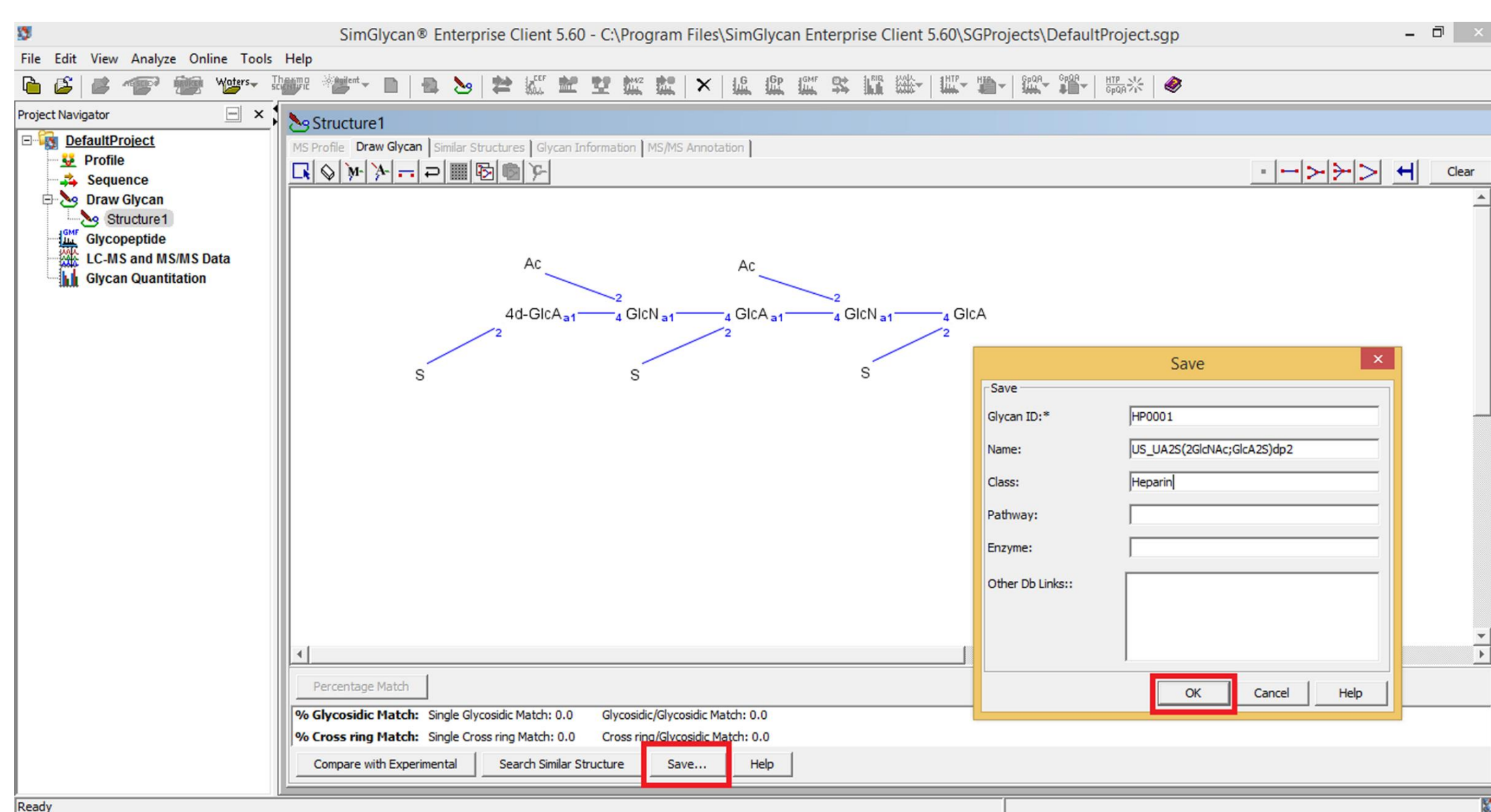


Figure 2: Typical graphical user interface (GUI) of SimGlycan software facilitating user to draw glycan structure that can be saved as KCF file format.

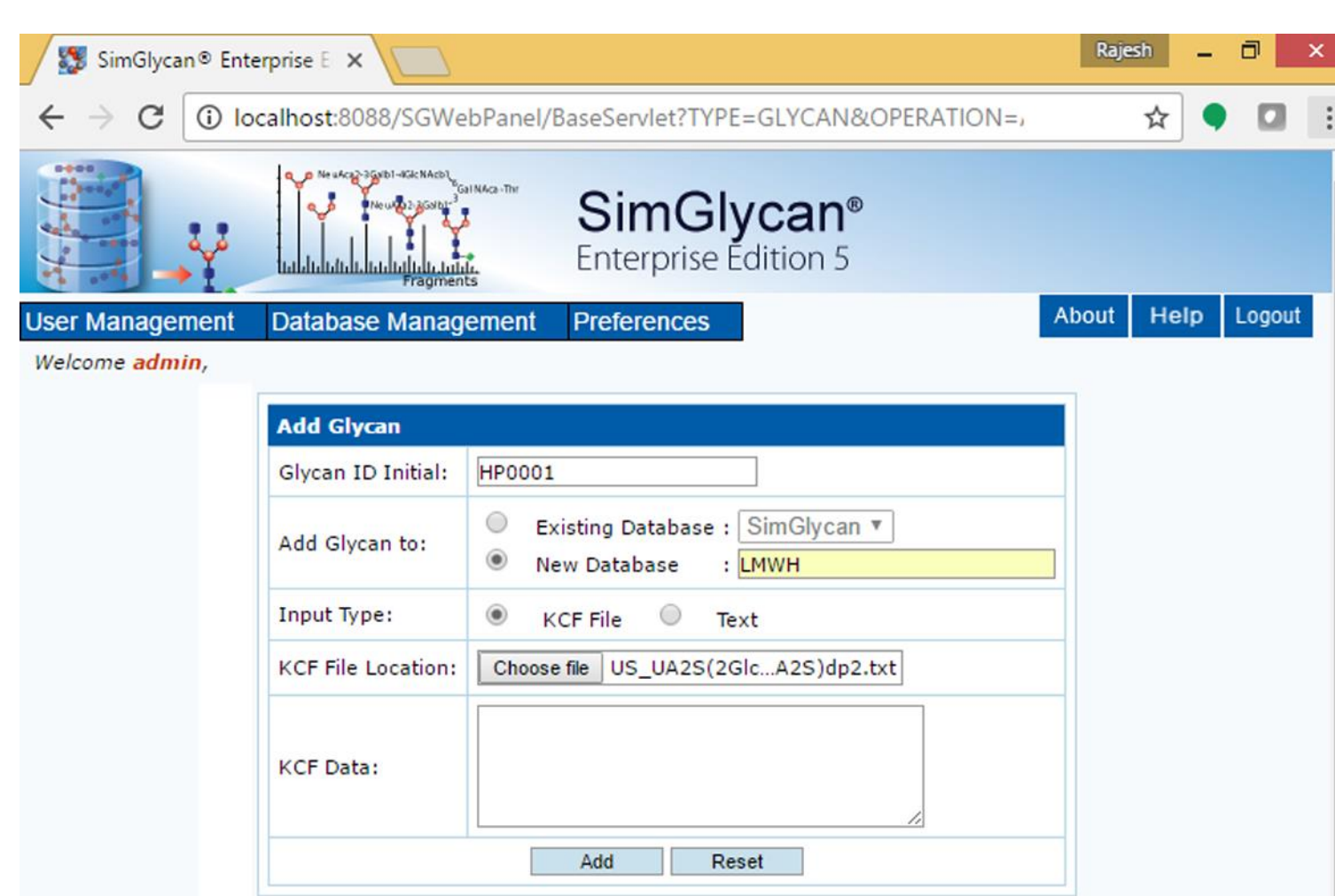


Figure 3: Typical GUI of SimGlycan software to add glycans into an existing or new database.

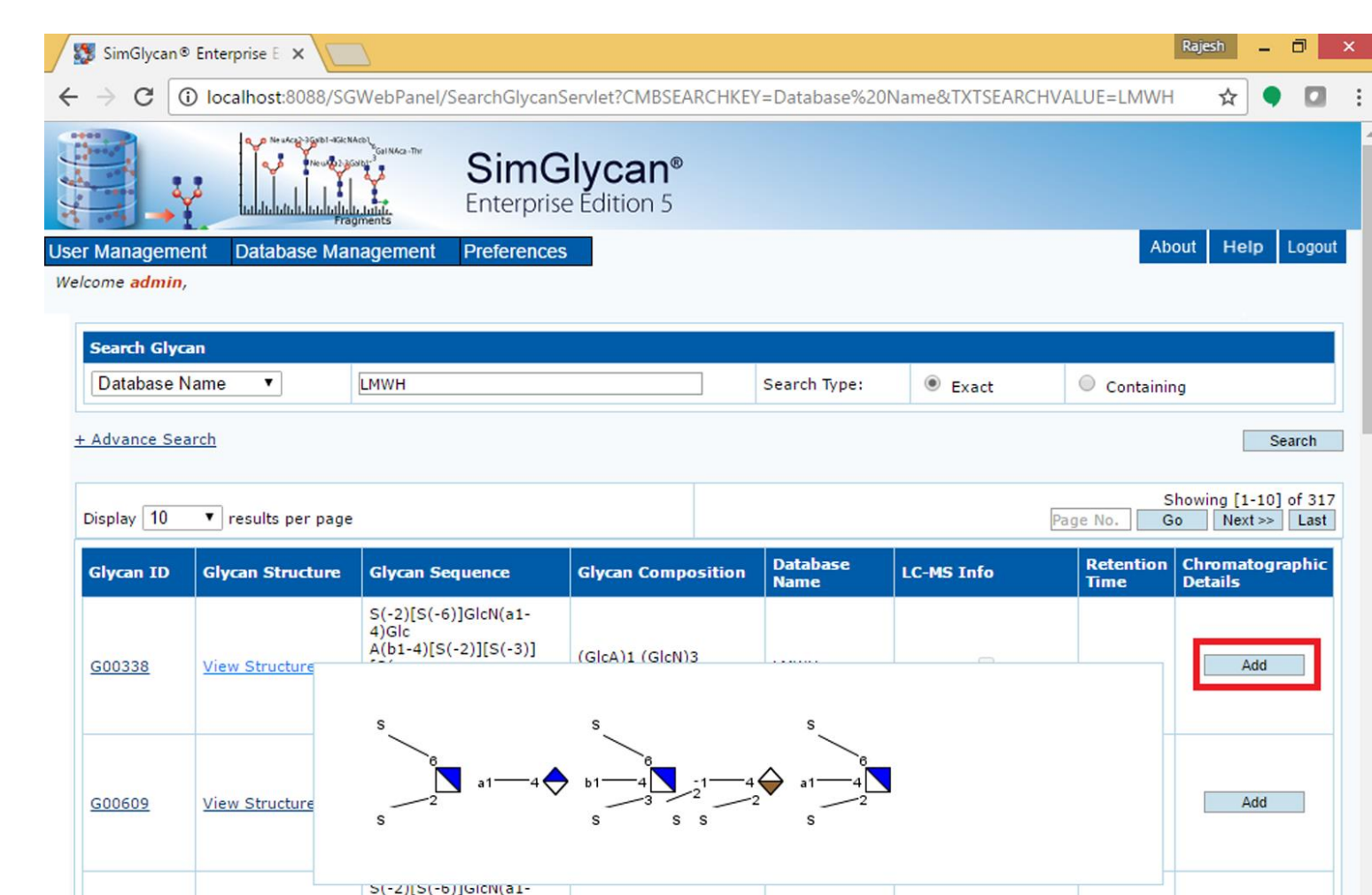


Figure 4: Typical GUI of SimGlycan software web page displaying searched glycans.

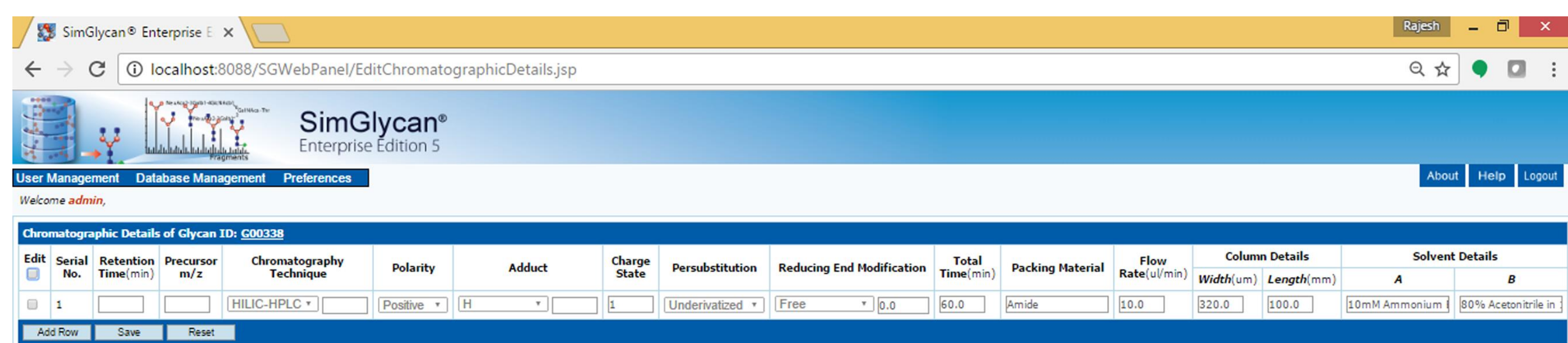


Figure 5: Typical GUI of SimGlycan software allowing user to store detailed information from LC-Experiments.

Once the database is created, user can instruct the SimGlycan software to perform the MS/MS glycan data analysis for structural identification of unknown glycans by considering the candidate's structures available in the curated database. Figure 6 shows the schematic representation illustrating the SimGlycan software workflow for identification of probable glycan structures using observed MS/MS data.

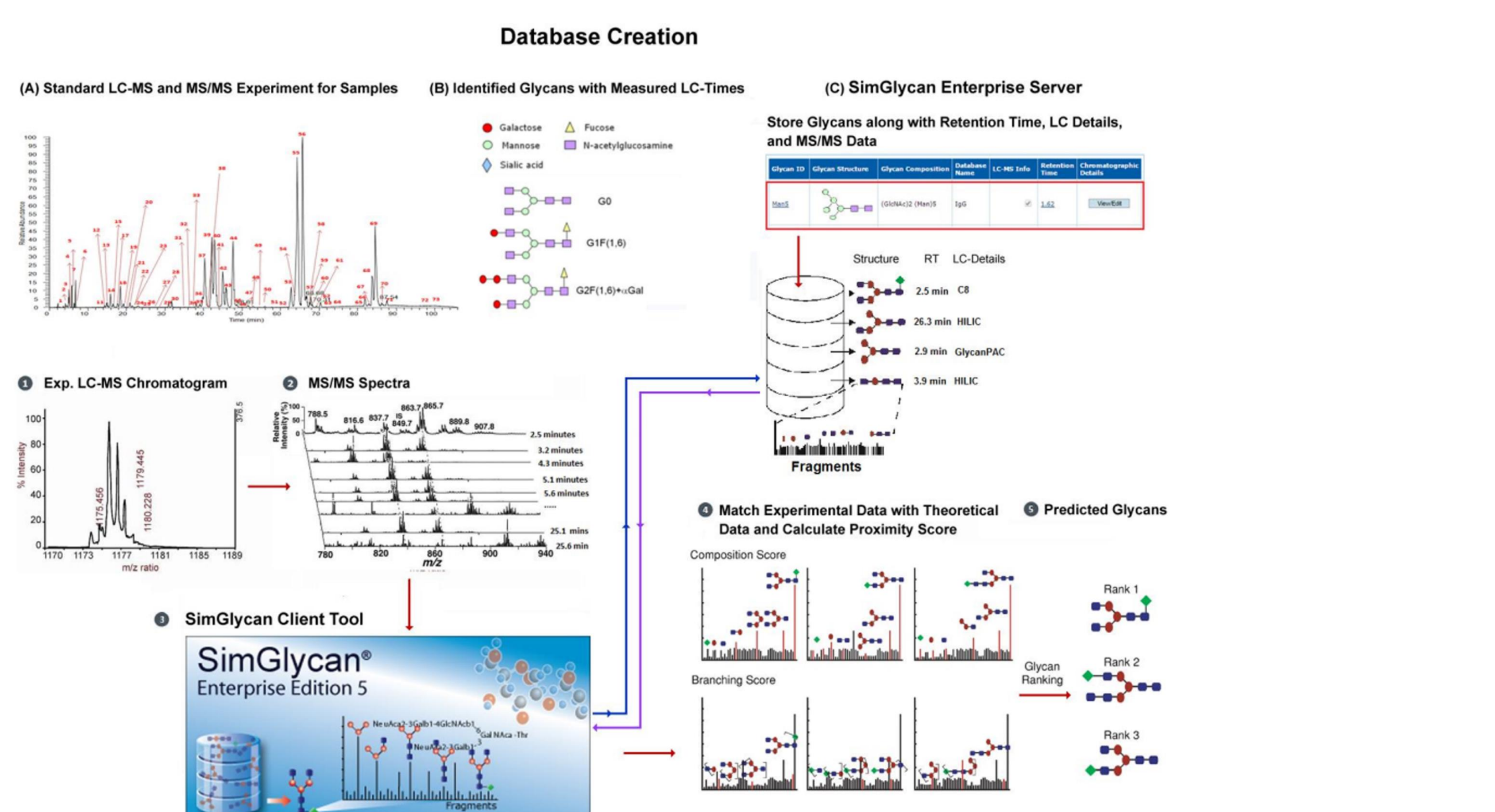


Figure 6: Schematic representation of glycan and glycopeptide data analysis workflow using SimGlycan software.

## RESULTS AND DISCUSSION

The LC-MS2 data were subjected into SimGlycan MS/MS database search using the parameter settings displayed in the figures 7(A-C). The search was performed on the custom database containing the 317 probable heparin structures. Also, only the MS/MS scans with precursor m/z having deprotonated ion species were considered for the database search even though some oligosaccharides were observed in the spectra with hexylamine adduct.

The search resulted in the identification of 84 enoxaparin oligosaccharides ranging from dp0 to dp10 with or without acetylation, variable sulphation degrees, and high mass accuracy (Table-1). The identified glycans can be reviewed in the SimGlycan software project management panel – MS/MS scans that are annotated with glycans after the database search are highlighted in blue color; red color: no result found; black color: search was not performed due to either non-availability of adequate MS/MS spectrum list, precursor m/z, or precursor m/z value lesser than theoretical m/z value of all the carbohydrate residues supported by SimGlycan software (Figure 8). Identified glycans are listed in the results pane. Structures, fragments and other information can be viewed in a single workspace (Figure 8). The MS/MS spectra will be automatically annotated with the fragment ions of the identified structures (Figure 9).

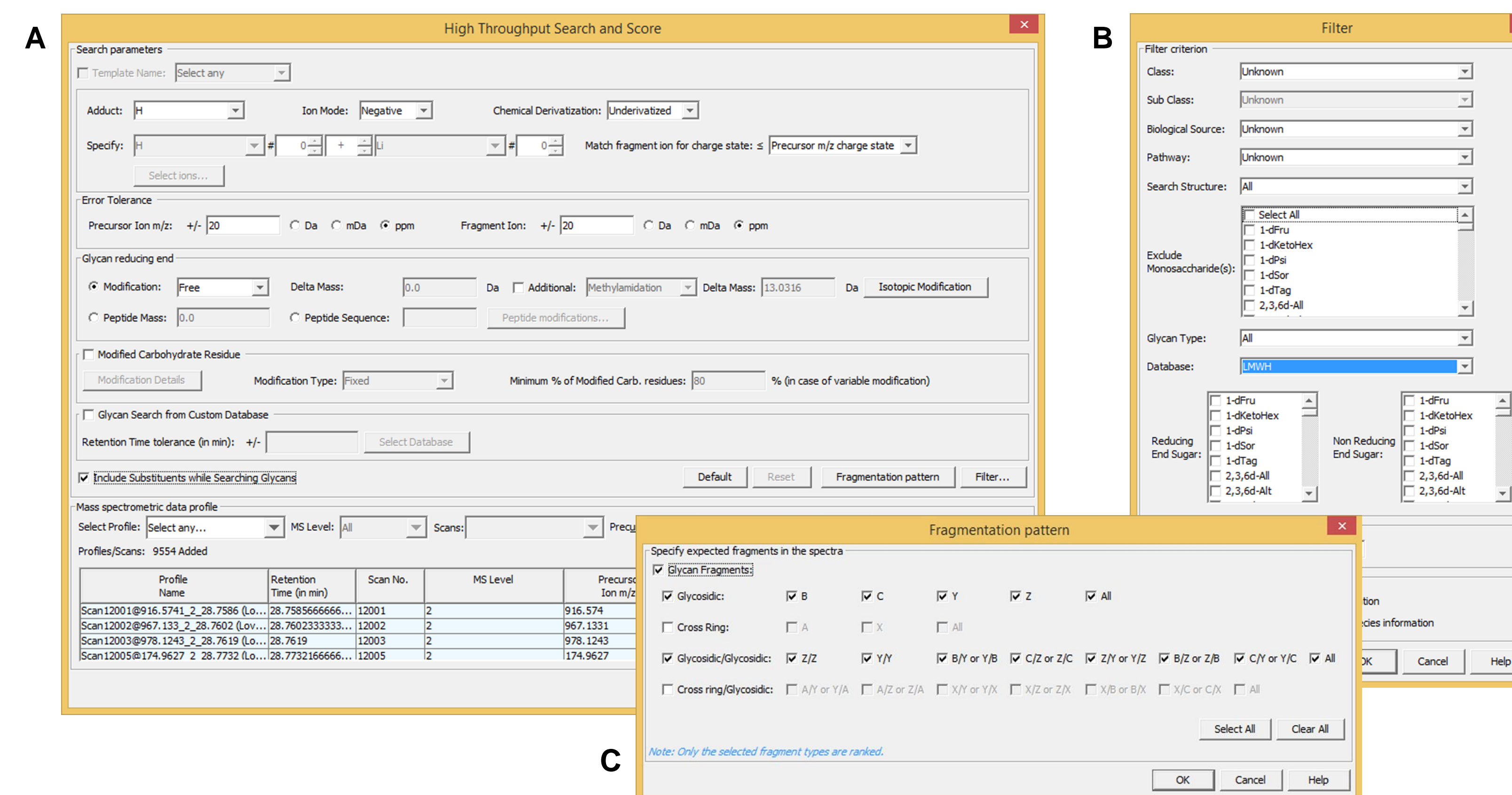


Figure 7: Typical GUIs of SimGlycan software showing (A) Search parameter dialog – user has to tick the box: Include substituents while searching glycans, (B) Filters such as Class, Sub-Class, Biological Source, etc., (C) The fragmentation patterns specific to the experimental settings.

RT	m/z	z	Composition	dp	Core motif	RT	m/z	z	Composition	dp	Core motif
0.02	225.1644	3	(44-GlcA1) (GlcA1) (GlcA2)	1	A	24.41	636.1761	1	(GlcA1) (GlcA2) (HexA1) (S7)	2	C
0.06	1242.2744	2	(GlcA1) (GlcA1) (S1)	10	B	24.77	845.6243	3	(44-GlcA1) (GlcA1) (GlcA2)	7	D
0.17	1069.5155	3	(44-GlcA1) (GlcA1) (GlcA2) (S9)	9	D	24.78	845.6243	3	(44-GlcA1) (GlcA1) (GlcA2)	7	D
0.27	170.8405	3	(44-GlcA1) (GlcA1) (GlcA1) (S3)	1	D	25.14	594.8376	2	(GlcA3) (GlcA4) (S1)	3	C
0.79	1200.7996	2	(44-GlcA1) (GlcA2) (GlcA2) (GlcA1)	6	A	25.15	913.0516	3	(44-GlcA1) (GlcA1) (GlcA2) (GlcA1)	7	A
0.79	1224.7917	3	(44-GlcA1) (GlcA1) (GlcA1) (S1)	10	A	25.24	796.0064	3	(44-GlcA1) (GlcA1) (GlcA2)	6	A
0.79	1234.756	3	(44-GlcA1) (GlcA1) (GlcA1) (S1) (S2)	10	A	25.25	796.0064	3	(44-GlcA1) (GlcA1) (GlcA2)	6	A
0.81	1019.4634	2	(GlcA1) (GlcA2)	5	B	26.23	846.0334	3	(Ac)1 (GlcA1) (GlcA2)	7	C
0.89	242.9493	3	(GlcA1) (GlcA1) (GlcA1) (S1)	1	B	26.25	846.0334	3	(Ac)1 (GlcA1) (GlcA2)	7	C
0.90	264.9294	1	(GlcA1) (HexA1) (S2)	2	B	27.91	964.0311	2	(44-GlcA1) (GlcA1) (GlcA2) (GlcA1)	11	A
0.95	514.8904	1	(GlcA1) (GlcA2) (S3)	1	C	28.70	1028.0061	1	(GlcA3) (GlcA3) (S1)	2	B
0.99	620.8496	3	(GlcA1) (GlcA2)	5	C	28.81	1024.9879	3	(44-GlcA1) (GlcA1) (GlcA2) (GlcA1)	8	A
1.46	396.1154	1	(44-GlcA1) (GlcA1) (GlcA2) (S3)	0	B	29.97	792.542	3	(Ac)1 (GlcA1) (GlcA2) (S1)	7	B
1.06	396.1169	3	(44-GlcA1) (GlcA1) (GlcA2) (S3)	3	A	29.96	904.0451	3	(GlcA1) (GlcA2)	7	B
1.41	336.185	3	(44-GlcA1) (GlcA1) (GlcA2) (S3)	2	A	29.13	1101.1222	3	(GlcA1) (GlcA1) (S1)	8	B
2.97	592.7244	2	(44-GlcA1) (GlcA1) (GlcA2) (S9)	3	D	29.26	1128.2206	3	(GlcA1) (GlcA1) (S1)	9	B
5.44	514.2859	1	(Ac)1 (GlcA1) (GlcA1) (S1)	1	C	29.97	792.542	3	(Ac)1 (GlcA1) (GlcA2) (S1)	6	B
5.79	514.3246	1	(GlcA1) (GlcA1) (GlcA1) (S1)	1	D	30.11	1499.8036	2	(GlcA1) (GlcA1) (S1)	4	A
6.72	514.0156	1	(44-GlcA1) (GlcA1) (GlcA1) (S1)	1	D	30.13	337.1366	1	(44-GlcA1) (GlcA1) (S1)	0	A
8.81	514.0145	1	(44-GlcA1) (GlcA1) (GlcA1) (S2)	1	D	30.27	1070.229	3	(Ac)1 (GlcA1) (GlcA1) (S1)	9	C
11.38	514.3223	1	(44-GlcA1) (Ac)1 (GlcA1) (GlcA1)	1	D	30.39	1070.8976	3	(GlcA1) (GlcA1) (S1)	9	C
16.44	695.0994	2	(44-GlcA1) (GlcA1) (GlcA1) (GlcA1)	3	A	30.44	692.3212	2	(GlcA1) (GlcA2)	7	A
16.50	677.0815	1	(GlcA1) (GlcA2) (HexA1) (S6)	1	B	30.58	898.1813	3	(44-GlcA1) (GlcA1) (GlcA2)	7	A
16.53	619.9238	3	(44-GlcA1) (GlcA1) (GlcA2) (GlcA1)	5	D	30.61	1031.522	2	(44-GlcA1) (GlcA1) (GlcA2) (GlcA1)	5	A
16.53	619.9214	3	(44-GlcA1) (Ac)1 (GlcA1) (GlcA2) (S1)	5	D	30.74	843.0847	2	(44-GlcA1) (GlcA1) (GlcA2)	4	A
16.62	592.9719	2	(GlcA1) (GlcA1) (GlcA1) (S9)	3	C	30.76	1460.0311	3	(44-GlcA1) (GlcA1) (GlcA2) (S9)	8	A
19.16	675.0809	1	(44-GlcA1) (GlcA1) (GlcA2) (S2)	1	A	31.00	1054.1471	1	(44-GlcA1) (GlcA1) (GlcA2) (GlcA1)	2	A
19.16	675.0809	2	(44-GlcA1) (GlcA1) (GlcA2) (S1)	3	A	32.51	1029.1907	1	(GlcA3) (GlcA3)	2	B
19.15	853.1054	1	(GlcA1) (GlcA1) (GlcA1) (S9)	2	E	32.71	1069.8196	3	(Ac)1 (GlcA1) (GlcA1) (GlcA1) (S9)	9	D
19.64	853.1043	1	(GlcA1) (GlcA1) (GlcA1) (S1)	1	D	32.71	1137.8667	3	(44-GlcA1) (GlcA1) (GlcA2) (GlcA1)	9	B
22.10	1011.6648	1	(44-GlcA1) (GlcA1) (GlcA2) (S1)	2	A	32.86	1124.1954	3	(44-GlcA1) (GlcA1) (GlcA1) (S1)	9	A
22.10	1011.6648	2	(44-GlcA1) (GlcA1) (GlcA2) (S1)	5	A	32.89	1123.5337	3	(44-GlcA1) (GlcA1) (GlcA2) (S1) (S1)	9	B
22.10	1011.6648	3	(44-GlcA1) (GlcA1) (GlcA2) (S1) (S1)	8	A	39.61	379.1579	1	(Ac)1 (GlcA1) (GlcA1) (S1)	0	A
22.96	824.1094	1	(GlcA1) (GlcA1) (HexA1) (S9)	2	C	41.00	1633.2048	1	(Ac)1 (GlcA1) (GlcA1) (S2)	0	A
22.91	395.4931	3	(GlcA2) (GlcA2)	3	C	41.03	337.1884	1	(44-GlcA1) (GlcA1)	0	A
22.90	555.9781	1	(44-GlcA1) (GlcA1) (GlcA1) (S1)	1	D	41.08	529.3048	1	(GlcA2) (GlcA1) (S2)	1	E
23.16	515.9999	1	(GlcA1) (GlcA2) (S9)	1	C	43.48	679.1462	3	(GlcA1) (GlcA1) (S1)	5	B
23.32	567.9969	2	(GlcA1) (GlcA1) (GlcA1) (S1)	1	C	44.53	173.1292	1	(GlcA2) (GlcA1) (S1)	1	B
23.37	557.9637	1	(GlcA1) (GlcA1) (GlcA1) (S2)	1	C	44.83	733.0942	1	(GlcA2) (GlcA1) (GlcA1) (S1)	1	B
24.00	716.5311	1	(44-GlcA1) (GlcA1) (GlcA1) (GlcA1) (S1)	1	A	47.01	691.1188	1	(GlcA2) (GlcA2)	1	B
24.08	716.5319	1	(44-GlcA1) (GlcA1) (GlcA1) (GlcA1) (S1)	1	A	47.19	691.117	1	(GlcA1) (GlcA1) (S1)	1	B
24.34	824.9275	1	(44-GlcA1) (Ac)1 (GlcA1) (GlcA2)	8	A	47.26	515.2989	1	(GlcA1) (GlcA1) (S1)	1	C

Table 1: List of identified Unique compositions using tandem MS data for Enoxaparin. (RT = Retention Time; m/z = mass to charge ratio; z = Charge state; dp = degree of polymerization).

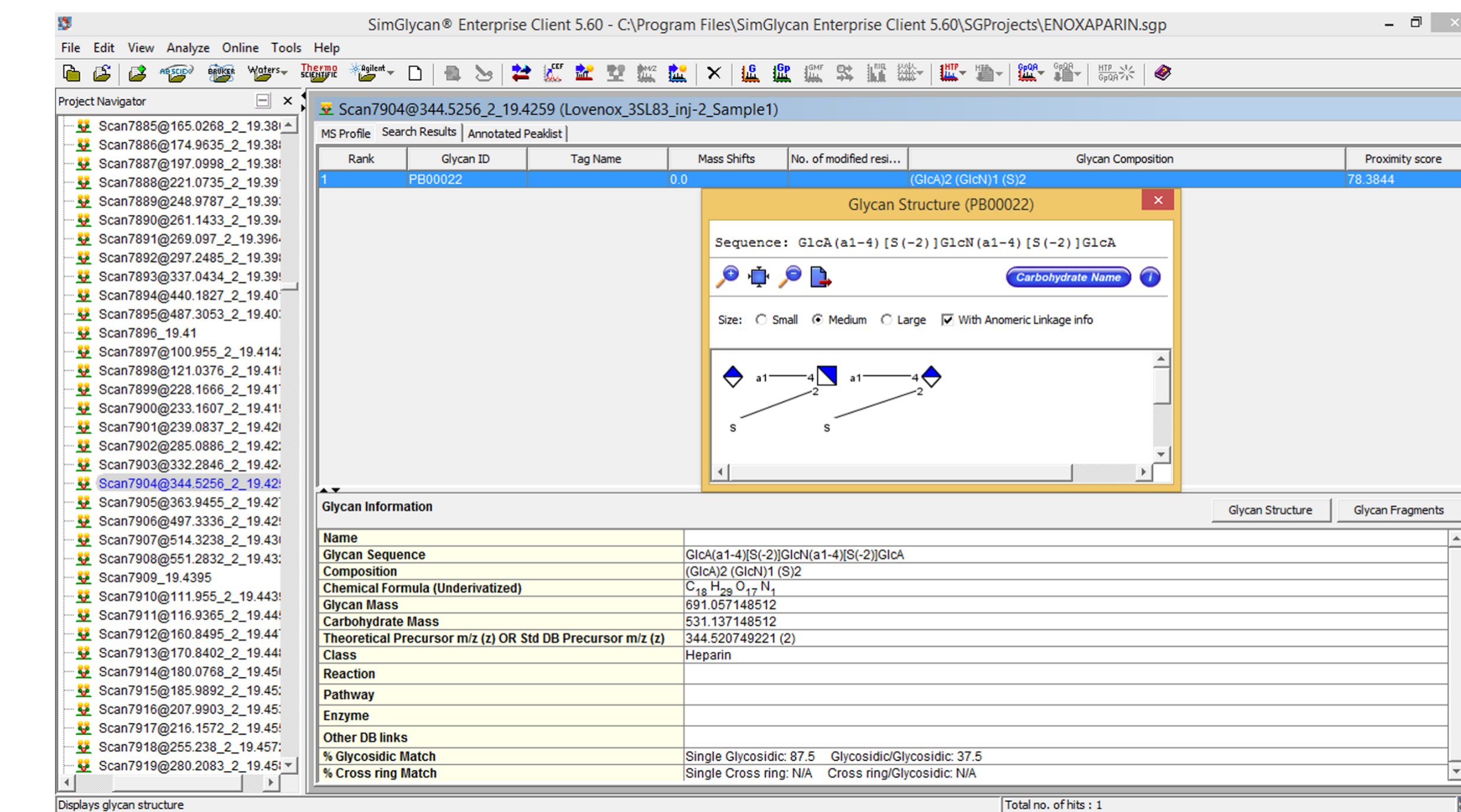


Figure 8: Typical GUI of SimGlycan software showing search results pane.

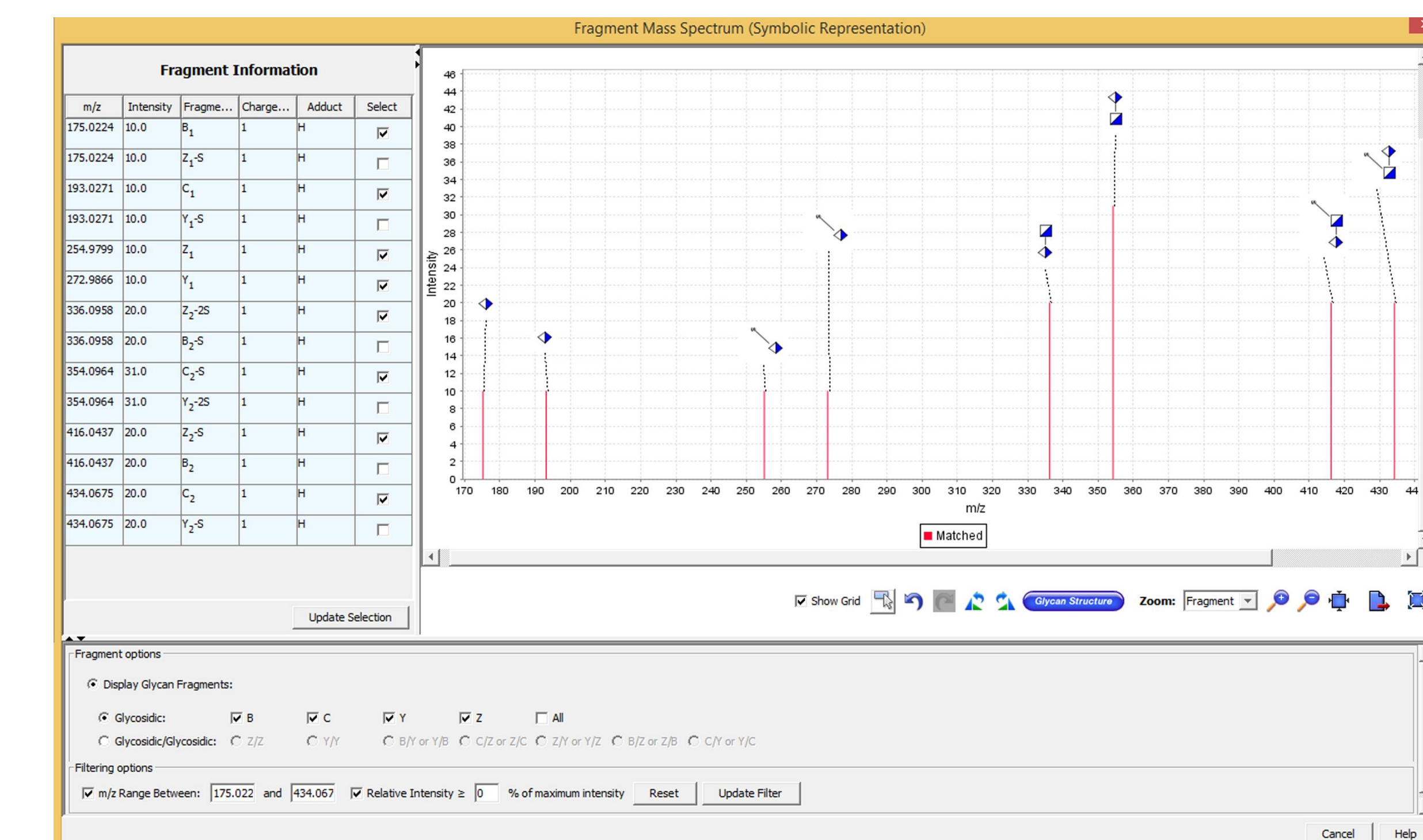


Figure 9: Typical GUI of SimGlycan software showing MS/MS spectrum annotated with fragment ions of an identified structure.

**Portable Reports:** Spreadsheet based reports facilitate easy reviewing of results for further verification, downstream analysis and dynamic information sharing. One major challenge with spreadsheet based report format is to save glycan structures into spreadsheet cells so that information such as retention time, precursor m/z, glycan ID etc. can be processed using spreadsheet operations to further organize the results. SimGlycan software generates report in MS excel file wherein glycan structures are also exported along with other structure specific information (Figure 10).

Figure 10: Typical view of MS excel file containing results outputted by SimGlycan software after performing MS/MS database search.

## CONCLUSION

1. A method for structural analysis of LMWH oligosaccharides using a high-resolution chromatography coupled with a High Performance fast scanning TripleTOF® 5600 system with SimGlycan® 5.60 software.
2. A custom database named "LMWH" containing 317 LMWH oligosaccharide structures curated from published literature was created.
3. 84 components of enoxaparin were identified—The tandem MS data was subjected to SimGlycan database search with the following parameters – precursor/product ions charge states: 1, 2, and 3; Database: LMWH, and error tolerances of precursor/product ions: 20 ppm.
4. Highly reproducible results were obtained in terms of both retention times and areas based on extracted ion chromatograms, which are important for evaluating various LMWH preparations and lot-to-lot variability.

## NOVEL ASPECT

Software solution for automated and rapid structural characterization of low molecular heparin oligosaccharides using LC-MS2 data.

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