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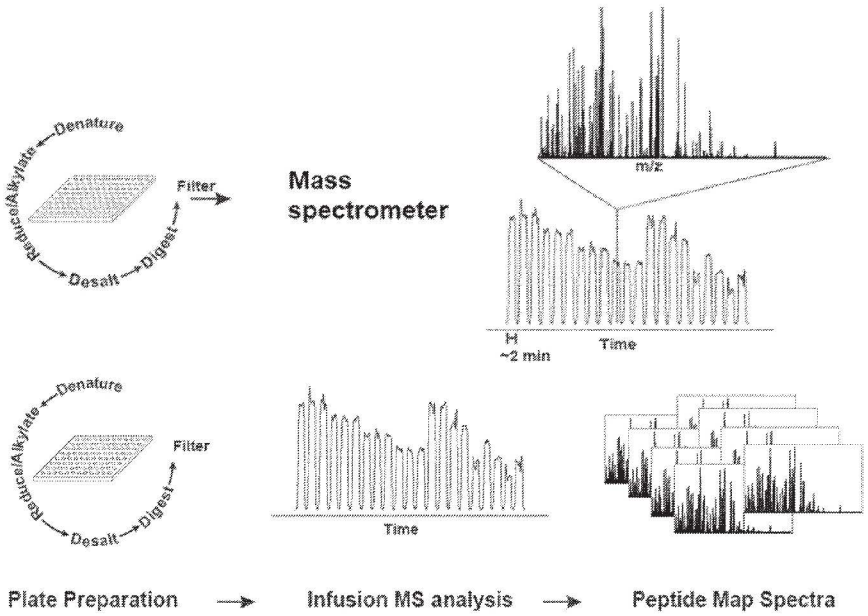

Fig. 2

(57) Abstract: A high throughput peptide mapping platform combining a plate-based sample preparation method with direct infusion MS analysis. Protein samples are cleaned, reduced, alkylated, digested, and acid quenched in a filter plate. Samples are then each infused sequentially directly to a mass spectrometer device without additional conventional chromatography or purification steps. The result is an effective assay for identifying site specific modifications in therapeutic antibodies, with their initial results showing high fidelity to liquid chromatography based methods (>90% sequence coverage).

[Continued on next page]

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HIGH THROUGHPUT INFUSION PEPTIDE MAPPING

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority from United States Provisional Patent Application No. 63/487,739, filed March 1, 2023, which is incorporated by reference herein to the extent that there is no inconsistency with the present disclosure.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with government support under GM108538 awarded by the National Institutes of Health. The government has certain rights in the invention.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0003] Incorporated by reference in its entirety herein is a computer-readable nucleotide/amino acid sequence listing identified as follows: One 7.39 KB XML file named "339885_74-22_WO.xml" created on February 28, 2024.

BACKGROUND OF THE INVENTION

[0004] Biotherapeutic proteins are vital medicines for a wide range of diseases with extracellular targets. However, these protein-based drugs acquire deleterious modifications during production and storage that can impact safety and efficacy. Production, formulation, and storage conditions must be optimized for stability of these drugs to ensure molecular fidelity. Numerous assays are implemented to monitor their quality, such as capillary isoelectric focusing for charge variants, top-down or middle-down mass spectrometry (MS) for oxidation analysis, glycan analysis to check for humanization, and peptide mapping for site specific modification levels. Of these, only peptide mapping generates site specific modification levels used to identify critical quality attributes (CQAs). Understanding the specific residues that are prone to modifications that affect efficacy is a powerful tool for both development of robust production and formulations as well as demonstrating control over the production process. Peptide mapping is especially powerful for the analysis of monoclonal antibodies (mAbs) because their sequences typically only differ in specific regions. Modification sites that do not have deleterious effects in one mAb can be de-prioritized in other mAbs.

[0005] Peptide mapping entails liquid chromatography (LC) coupled with MS (LC-MS) analysis of enzymatically produced peptides from the target protein. Typically, peptides composing the complete sequence and common chemical and biological modifications (e.g., oxidation, deamidation, isomerization, glycosylation, clipping, etc.) are observed. The signals for the modified and non-modified (native) peptides are compared to generate and estimate of the modification stoichiometry. These measurements can be correlated with bioactivity/binding analyses to inform strategies for production, formulation, and storage of biotherapeutic proteins to specifically protect the critical amino acids.

[0006] Demand for peptide mapping analysis is growing substantially due to both the power of the technique as well as an increase in the number of biotherapeutic proteins being developed commercially. However, the collection of peptide mapping data is both cumbersome and complicated, requiring long data collection times and complicated data analyses. The throughput of the method is inherently limited by the requirement to use chromatography. Short chromatographic methods can increase throughput, but at the cost of decreased resolution that can prevent the detection of deamidated peptides, which are often difficult to separate from their native forms. Additionally, chromatographic columns must be equilibrated for extended times prior to separation and may require blank runs after every analysis to limit carryover. Although extensively developed, chromatographic peak selection algorithms are not flawless and often require at least a manual check to confirm the bounds were set appropriately and/or modified manually to the correct positions.

[0007] Accordingly, what is needed is an improved high throughput system for sample preparation and analysis comparable with conventional peptide mapping methods. Preferably, the system is able to analyze polypeptides and other molecules to generate MS data without the need for a chromatography step prior to injection of the analytes into the mass spectrometer while retaining the accuracy required by end users, such as pharmaceutical companies and those monitoring the quality and efficacy of biotherapeutics.

SUMMARY OF THE INVENTION

[0008] The present invention provides an alternative to LC-MS based protein mapping assays. The alternative methods and systems described herein have the potential to significantly increase peptide analysis throughput while providing accuracy and sequence coverage comparable to conventional protein mapping methods.

[0009] In one aspect, the present invention provides a method for preparing and analyzing multiple polypeptides via direct infusion mass spectrometry (MS). “Direct infusion”, also

known as “direct injection”, refers to the direct analysis of a sample by mass spectrometry with little to no separation or purification steps being performed on analytes within the sample prior to the sample being inserted into the MS device. Most often this is accomplished by use of electrospray ionization where a liquid sample is infused into an electrospray emitter directly. The sample is then ionized by electrospray ionization and the ions are then subsequently measured by mass spectrometry.

[0010] An attribute of the direct infusion approach is that there are essentially no online separations of the sample. Instead, the entirety of sample’s chemical diversity is at once subjected to analysis by electrospray ionization mass spectrometry (ESI-MS). In an embodiment, the sample is a complex mixture of polypeptides resulting from a proteolytic digest of a single protein or simple protein mixture, including but not limited to a monoclonal antibody (mAb) or antibody drug conjugate. This mixture may contain several hundred polypeptides and, following direct infusion, m/z peaks in the resulting mass spectrum are detected for many, if not the majority, of the polypeptides in the mixture. The removal of conventional chromatography and other separation steps can significantly improve the time and costs requirements of such analyses.

[0011] One embodiment of the invention provides a method for analyzing a plurality of polypeptides in a sample comprising the steps of: a) simultaneously injecting the plurality of polypeptides into an ion source of a mass spectrometer (MS) device and generating a plurality of precursor ions from the polypeptides; and b) performing one or more MS scans on each precursor ion generated from the plurality of polypeptides, thereby generating spectral data for each precursor ion. Preferably, multiple MS scans of each precursor ion are performed. The MS scans may be a full mass range scan (which provides a full mass spectrum of each analyte), a narrow mass range scan (which provides a mass spectrum over a specified m/z range), a segmented mass range scan, or combinations thereof. With a segmented mass range scan, the mass range of interest is broken into two or more segments, each segment is scanned in succession, and the resulting scans are combined together. This increases the spectral dynamic range allowing smaller ion intensities to be seen. In an embodiment, the sample is a liquid sample that is directly injected into an electrospray emitter of an MS device, including but not limited to an Orbitrap MS device. Preferably, the MS device provides a resolving power equal to or greater than 100,000, has a scan rate of at least 0.5 scans per second (0.5 Hz), and the multiple scans are performed over a time period of at least 10 seconds. Additionally, the method preferably does not comprise an additional liquid or gas chromatography step and/or salt removal step.

[0012] Injecting the plurality of polypeptides into the ion source of the MS device optionally includes preparing the polypeptides comprising the steps of: a) denaturing the plurality of polypeptides thereby generating a plurality of denatured polypeptides; b) treating the plurality of denatured polypeptides with a reducing agent, thereby generating a plurality of reduced polypeptides; c) treating the plurality of reduced polypeptide with a first solution causing the plurality of reduced polypeptide to precipitate out of the first solution, thereby generating a polypeptide precipitate; and e) dissolving the polypeptide precipitate thereby generating a plurality of dissolved polypeptides, followed by injecting the plurality of dissolved polypeptides into the ion source to generate precursor ions.

[0013] In a further embodiment, mass spectrometry data is obtained on each of the plurality of dissolved or digested polypeptides. The mass spectrometry data may include mass-to-charge ratios obtained from MS1 data, MS2, or both. Optionally, the spectral data is generated by averaging spectral data obtained from multiple scans for each precursor ion. Site specific mutations, post translational modifications, or combinations thereof, are detected and/or measured in the polypeptides. Optionally, the biological activity of each of the polypeptides is measured and a link between the biological activity and the site specific mutations, post translational modifications, or combinations thereof, are recorded for each polypeptide.

[0014] Optionally, the polypeptides are generated from a protein of interest that is chemically or enzymatically digested. In an embodiment, the plurality of polypeptides are biotherapeutics (or portions thereof), including but not limited to antibodies and/or therapeutic proteins. Preferably, a specific sequence or region of a control polypeptide or polypeptide standard is known, such as a region of an antibody that affects activity or stability of the antibody, or a region that is susceptible to degradation or modification. Accordingly, a further embodiment of the invention comprises comparing one or more specific regions of each of the plurality of polypeptides with a corresponding region of a control polypeptide and determining if a chemical modification is present in one or more of the plurality of polypeptides.

[0015] In an embodiment, the spectral data generated for each precursor ion is compared to calculated mass-to-charge values for predicted polypeptides and modified polypeptides able to be generated from the protein. It is then determined whether the precursor ions correspond to one or more of the predicted polypeptides or modified polypeptides of the protein. “Sequence coverage” is used herein to refer to the amount of a protein amino acid sequence that corresponds to precursor ions detected and analyzed by the MS device.

Preferably, the present method provides a sequence coverage of at least 90%, at least 92%, at least 95%, or 100% of the of the protein's amino acid sequence.

[0016] In a further embodiment, at least a portion of the plurality of precursor ions are fragmented, thereby generating product ions (also referred to as fragment ions). The mass-to-charge ratios of the product ions are measured to generate product ion mass spectrometry data, and the generated product ion mass spectrometry data is compared with predicted fragment ion data of the protein. Comparing the generated product ion mass spectrometry data to the predicted fragment ion data of the protein can confirm the spectral data for the precursor ions and can confirm the sequence order of the polypeptides that generate the precursor ions, which can provide sequence confirmation.

[0017] The direct infusion method of the present invention also enables relative quantification of the polypeptides in the sample. The peak intensity of two or more detected precursor ions in MS1 events or their product ions in MS/MS events are compared to each other and the relative abundance in the sample of each polypeptide generating the precursor ions is then calculated from the compared peak intensities. Preferably, the polypeptides generating the two or more detected precursor ions comprise a first polypeptide and a variant of the first polypeptide having a modified amino acid sequence, a modified structure, or a modified chemical formula. As a result, the relative abundance of the first polypeptide to the modified polypeptide in the sample is able to be calculated. Such modifications to the polypeptide include, but are not limited to, oxidation, deamidation, isomerization, glycosylation, substitutions of amino acids, and clipping. For example, specific regions a manufactured antibody or therapeutic protein may be compared to a control protein or antibody to determine if the manufactured antibody or therapeutic protein has undergone a modification that would affect the stability or activity of the molecule.

[0018] Optionally, the MS device provides a resolving power equal to or greater than 100,000, or equal to or greater than 200,000, or equal to or greater than 480,000, or equal to or greater than 960,000. Additionally, the MS device preferably has a scan rate of at least one scan per second (1 Hz), at least two scans per second (2 Hz), at least five scans per second (5 Hz), or at least ten scans per second (10 Hz). In many conventional MS methods using liquid chromatography, the MS device has a time period of approximately 6-10 seconds to scan the analytes eluting from the chromatography column before the analytes are moved to make room for the next batch of analytes. However, using the direct infusion method of the present invention allows the MS device to perform multiple scans over a time period of at least 5 seconds, over a time period of at least 10 seconds, over a time period of

at least 20 seconds, over a time period of at least 30 seconds, over a time period of at least 45 seconds, over a time period of at least 60 seconds, over a time period of at least 90 seconds, or over a time period of at least 120 seconds.

[0019] An additional embodiment of the invention provides methods for preparing a polypeptide or a plurality of polypeptides using a method that enables rapid MS analysis compatible with direct infusion and without requiring additional purification or separation steps. This method is also beneficial in that it does not remove small polypeptides that are often removed during chromatography or other separation steps, thereby allowing MS detection and analysis of these polypeptides.

[0020] In an embodiment, the present invention provides a method for analyzing a polypeptide comprising the steps of: a) denaturing the polypeptide thereby generating a denatured polypeptide; b) treating the denatured polypeptide with a reducing agent, thereby generating a reduced polypeptide; c) treating the reduced polypeptide with a first solution causing the reduced polypeptide to precipitate out of the first solution, thereby generating a polypeptide precipitate; d) optionally, washing the polypeptide precipitate with a second solution; and e) dissolving the polypeptide precipitate with a third solution thereby generating a dissolved polypeptide. In a further embodiment, the dissolved polypeptide is then preferably digested, thereby generating a digested polypeptide; and the dissolved polypeptide or digested polypeptide is directly injected into an ion source of a mass spectrometer device. Preferably, the method further comprises obtaining mass spectrometry data on the digested polypeptide and measuring site specific mutations, post translational modifications, or combinations thereof, in the polypeptide. The mass spectrometry data may include MS1 data, MS2, or both. Preferably, the method does not comprise an additional salt removal step (other than what is achieved by step d), an additional liquid chromatography step, an additional gas chromatography step, and/or other additional separation steps.

[0021] In an embodiment, the present invention provides a method for analyzing one or more polypeptides comprising the steps of: a) depositing the one or more polypeptides in a container or carousel having one or more separated wells or chambers, wherein the one or more polypeptides are deposited in separate wells or chambers in the container; b) denaturing the one or more polypeptides thereby generating one or more denatured polypeptides; c) treating the one or more denatured polypeptides with a reducing agent, thereby generating one or more reduced polypeptides; and d) treating the one or more reduced polypeptides with a first solution causing the one or more reduced polypeptide to

precipitate out of solution, thereby generating one or more polypeptide precipitates. Preferably the container or carousel is a multi-well plate, including but not limited to filter plates, and the one or more polypeptides are deposited in separate wells in the multi-well plate. In an embodiment, the one or more polypeptides are a plurality of polypeptides.

[0022] The one or more polypeptide precipitates are then optionally washed with a second solution one or more times. Preferably, the method further comprises dissolving the one or more polypeptide precipitates, either washed or unwashed, with a third solution thereby generating one or more dissolved polypeptides, and, optionally, digesting the one or more dissolved polypeptides or polypeptide precipitates, thereby generating one or more digested polypeptides. Each of the one or more dissolved polypeptides or digested polypeptides is injected from the container into an ion source of a mass spectrometer device able to generate mass spectrometry data on the plurality of polypeptides. Preferably, the contents of each well or container is sequentially injected into the ion source. In an embodiment, the one or more polypeptides are a plurality of polypeptides.

[0023] The denaturing step comprises treating the one or more polypeptides with a chemical agent able to denature proteins and polypeptides as is known in the art. In an embodiment, the denaturing step comprises contacting each of the one or more polypeptides with one or more of guanidinium chloride, urea, a detergent, or solutions thereof. Preferably, the one or more polypeptides are contacted with a solution containing of guanidinium chloride or urea.

[0024] The reducing agent may be any chemical agent known in the art able to reduce the denatured polypeptides, including but not limited to dithiolthreitol (DTT) and solutions thereof. Optionally, the one or more denatured polypeptides or reduced polypeptides are additionally treated with an alkylating agent. The alkylating agent may be any alkylating agent known in the art suitable for use with polypeptides including, but not limited to, iodoacetate, iodoacetic acid, iodoacetamide (all referenced herein as IAA) and solutions thereof.

[0025] As used herein, the first solution may be any chemical agent able to cause polypeptides, particularly the reduced polypeptides, to precipitate out of solution, including acids, organic solvents, salts, and metals as is known in the art. Preferably, the first solution comprises an organic solvent, such as methanol (MeOH).

[0026] As used herein, the second solution is optionally used to wash the one or more polypeptide precipitates in order to reduce or remove salts and other unwanted

contaminants. In an embodiment, the second solution comprises water, MeOH, and combinations thereof. Preferably, the second solution comprises 70%-95% MeOH, such as a 20/80 or 10/90 water to MeOH mixture.

[0027] As used herein, the third solution is used to dissolve the one or more polypeptide precipitates back into solution, and may be any solution able to dissolve the polypeptide precipitates, including but not limited to ammonium acetate, ammonium bicarbonate, and other buffer solutions.

[0028] The digesting step comprises treating the one or more dissolved polypeptides with an enzyme or a chemical agent able to digest proteins and polypeptides as is known in the art. In an embodiment, the denaturing step comprises contacting each of the dissolved polypeptides with a proteolytic enzyme, including but not limited to trypsin, pepsin, chymotrypsin, papain, calpain, serrapeptase, thermolysin, carboxypeptidase, and combinations thereof. Optionally, an acid quenching step is performed on the resulting digested polypeptides by adding an acidic solution, including but not limited to a formic acid solution, to the digested polypeptides.

[0029] Methods described herein are advantageous in that they can prepare the one or more polypeptides for MS analysis in a sufficiently purified form so as to allow the dissolved polypeptides or digested polypeptides to be injected into a MS device without requiring additional salt removal steps and/or chromatography steps. For example, in an embodiment, the method does not comprise an additional salt removal step other than what is achieved by washing the polypeptide precipitates with the second solution. In a similar embodiment , the method does not comprise a liquid chromatography step and/or or other additional separation step.

[0030] In an embodiment, the biological activity of each of the analyzed polypeptides is compared with the biological activity of a control peptide. Where the polypeptides are antibodies or other biotherapeutics, the biological activity of the analyzed polypeptides are compared with the desired or expected activity of the corresponding normal active antibody or biotherapeutic to determine if the analyze polypeptides have altered activity and to determine if the altered activity corresponds to a chemical or structural modification. For example, therapeutic or commercially produced antibodies can be compared to the normal active antibody to determine if chemical modifications affecting activity have arisen during production or storage. Examples of chemical modifications that may alter the biological

activity of the polypeptides include, but are not limited to, oxidation, deamidation, isomerization, glycosylation, substitutions of amino acids, and clipping.

[0031] In an embodiment, at least 50 samples, each generating a plurality of polypeptides, are analyzed within 24 hours using the above methods on a single mass spectrometry device, preferably at least 100 samples within 24 hours, preferably at least 150 samples within 24 hours, preferably at least 200 samples within 24 hours, preferably at least 250 samples within 24 hours, preferably at least 300 samples within 24 hours, preferably at least 400 samples within 24 hours, preferably at least 500 samples within 24 hours, or more preferably at least 600 samples within 24 hours. In an embodiment, each sample is analyzed at an average time of ≤ 5.0 min per sample, preferably ≤ 4.5 min per sample, preferably ≤ 4.0 min per sample, preferably or ≤ 3.0 min per sample. Optionally, each sample is an antibody or therapeutic protein that is digested to generate a plurality of polypeptides.

[0032] In an embodiment, the sample being analyzed comprises a mixture of at least 50 different polypeptides, at least 100 different polypeptides, at least 150 different polypeptides, at least 200 different polypeptides, or at least 300 different polypeptides. In conventional LC-MS methods, small polypeptides are often removed from the sample and are not analyzed. In an embodiment of the present invention, the generated spectral data preferably includes spectral data from precursor or product ions generated from polypeptides having ten amino acids or less, eight amino acids or less, six amino acids or less, or four amino acids or less. In an embodiment, mass spectrometry data is obtained for over 50% of the amino acids of the analyzed polypeptides, preferably over 60% of the amino acids of the analyzed polypeptides, preferably over 70% of the amino acids of the analyzed polypeptides, preferably over 80% of the amino acids of the analyzed polypeptides, preferably over 85% of the amino acids of the analyzed polypeptides, preferably over 90% of the amino acids of the analyzed polypeptides, or preferably over 95% of the amino acids of the analyzed polypeptides. Preferably, obtaining mass spectrometry data includes detecting and/or measuring specific mutations, post translational modifications, or combinations thereof, of the amino acids.

[0033] In an embodiment, the one or more polypeptides are treated so as to induce a chemical modification in the polypeptides. For example, the one or more polypeptides are treated with a chemical reagent, including but not limited to H_2O_2, or heat, time, light, etc. to generate susceptibility to chemical modifications in the polypeptides. In a further embodiment, the one or more peptides are a plurality of polypeptides deposited in a multi-

well plate, wherein the plurality of polypeptides are deposited in separate wells, and varying concentrations of the chemical reagent (e.g., H_2O_2) or varying temperatures are added to different wells.

[0034] In an embodiment, the present invention provides a system for analyzing a plurality of polypeptides comprising:

a) a series of containers or a multi-well plate able to contain a plurality of polypeptides in separate containers or wells;

b) a reagent kit comprising, separately from one another, a denaturing solution, a reducing agent solution, a first solution able to cause reduced polypeptides to precipitate, a second solution wherein the second solution is a wash solution, a third solution able to dissolve polypeptide precipitates, and optionally, a solution comprising an enzyme able to digest the plurality of polypeptides; and

c) an automated system for injecting the plurality of peptides in each of the separate containers or wells into a mass spectrometer or other molecular analyzer. Preferably, the automated system is able to inject the plurality of peptides directly into the mass spectrometer or other molecular analyzer without going through a separation system, such as a chromatography columns or system. The series of containers may be one or more, preferably two or more, five or more, ten or more, or twenty or more, containers, including but not limited to test tubes.

[0035] Optionally, the reagent kit additionally comprises an alkylating agent solution and an acid quench solution. In an embodiment, the reagent kit comprises guanidinium chloride, urea, or a detergent as part of a denaturing solution, dithiolthreitol (DTT) as part of the reducing agent solution, iodoacetate, iodoacetic acid, and/or iodoacetamide (IAA) as part of the alkylating agent solution, methanol (MeOH) as the first solution, a MeOH/water mixture as the second solution, ammonium acetate as the third solution, and trypsin as part of the digesting enzyme solution.

[0036] In a further embodiment, the multi-well plate is a filter plater and the system optionally further comprises an automated fluid delivery system for adding each solution and/or reagent to each well and draining each well of any liquid after each step. In an embodiment, the reagent kit comprises an additional chemical reagent solution able to induce a chemical modification in the polypeptides, including but not limited to oxidation. For example, the reagent kit may comprise one or more H_2O_2 solutions. Optionally, the reagent kit comprises solutions of the chemical reagent in varying concentrations, wherein the solutions of varying concentrations may be added to different wells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0037] FIG. 1 shows different regions and modifications of an antibody that may be analyzed using peptide mapping with a mass spectrometer.

[0038] FIG. 2 illustrates a sample preparation step performed in a filter plate in an embodiment of the present invention.

[0039] FIG. 3 shows a mass spectrum collected from an antibody sample analyzed using a method in an embodiment of the present invention.

[0040] FIG. 4A shows a representative mass spectrum where oxidation of a peptide results in stereoisomers and split peaks in conventional LC-MS peptide mapping.

[0041] FIG. 4B shows a representative mass spectrum using the direct infusion method of the present invention where all isomers have the same mass and coalesce into a single peak that additionally has a distinguishable mass shift.

[0042] FIGs. 4C and 4D show peptide mapping results of a digested NIST mAb for both LC-MS analysis and direct infusion MS (inf-MS) analysis of the present invention (DTLMISR in Fig. 4C - SEQ ID NO:1; FNWYVDGVEVHNAK in Fig. 4D - SEQ ID NO:2).

[0043] FIG. 4E provides a graph showing untreated and H_2O_2 treated NIST mAb, Sigma mAb, infliximab, rituximab, and cetuximab samples prepared and analyzed by both conventional LC-MS peptide mapping and inf-MS peptide mapping of an embodiment of the present invention.

[0044] FIG. 4F shows that the methods are able to provide consistent results across the different antibodies for untreated and H_2O_2 treated samples.

[0045] FIG. 5A shows a zoomed-in m/z region from an MS/MS spectrum of a peptide (GFYPSDIAVEWESNGQPENNYK – SEQ ID NO:3) from a mAb sample analyzed by an inf-MS method of an embodiment of the present invention.

[0046] FIGs. 5B-5C provide graphs showing that the inf-MS method of an embodiment of the present invention generates comparable results to convention peptide mapping for calculating the ratios of deamidated peptides to native peptides in mAb samples.

[0047] FIGs. 6A-6B shows MS data comparing the ability of an inf-MS method of an embodiment of the present invention to generate accurate and comparable glycan occupation data for mAb samples.

[0048] FIG. 7 shows a mass spectrum resulting from direct injection of a tryptic digest of the therapeutic antibody adalimumab according to an embodiment of the present invention.

[0049] FIG. 8 shows the sequence of the adalimumab heavy chain (SEQ ID NO: 4) and light chain (SEQ ID NO:5) annotated with detected polypeptides.

[0050] FIG. 9 shows MS/MS analysis and sequence confirmation of polypeptide #94 from the adalimumab digestion (SEQ ID NO: 6).

[0051] FIG. 10 is a plot showing the percentage of theoretically calculated m/z peaks that could occur from a direct injection of the tryptic peptides of adalimumab that can be resolved from one another at various mass analyzer resolving powers. The x-axis is resolving power calculated at m/z 200. At a resolving power of about 100,000, approximately 80 percent of possible m/z peaks were resolved.

[0052] FIG. 11 is a graphic showing whether various glycosylated and modified variants of the peptide EEQYNSTYR (SEQ ID NO:7) can be detected (lighter colored boxes) in the MS1 signal by accurate mass. Note for the peptides that exist at higher abundances (> 5%) the signals are easily observed in the MS1 scan. For those with relative abundances lower than ~ 3.5%, reduction of the m/z range down as low as 2 m/z can allow for detection.

[0053] FIG. 12 shows a MS/MS scan confirming the amino acid sequence of MS1 identified m/z peaks for (GFYPSDIAVEWESNGQPENNYK – SEQ ID NO:3) and allowing for detection and quantification of deamidation. A key to detecting deamidation in peptides is the use of very high resolving powers to distinguish the deamidated peptide product ions from those generated by the ^{13}C isotope containing non-modified version.

DETAILED DESCRIPTION OF THE INVENTION

[0054] Definitions

[0055] In general the terms and phrases used herein have their art-recognized meaning, which can be found by reference to standard texts, journal references and contexts known to those skilled in the art. The following definitions are provided to clarify their specific use in the context of the invention.

[0056] As used herein, the term "precursor ion" is used herein to refer to an ion which is produced during ionization stage of mass spectrometry analysis, including the MS1 ionization stage of MS/MS analysis.

[0057] As used herein, the term "product ion" is used to refer to an ion which is produced during a fragmentation process of a precursor ion, including the MS2 ionization stage of MS/MS analysis.

[0058] As used herein, the term "mass spectrometry" (MS) refers to an analytical technique for the determination of the elemental composition of an analyte, including but not limited to polypeptides. The mass spectrometry principle consists of ionizing analytes to generate charged species (i.e., precursor ions) or species fragments (i.e., product ions) and measurement of their mass-to-charge ratios. Conducting a mass spectrometric analysis of an analyte results in the generation of mass spectrometry data relating to the mass-to-charge ratios of the analyte and analyte product ions. Mass spectrometry data corresponding to analyte ion and analyte ion fragments is presented in mass-to-charge (m/z) units representing the mass-to-charge ratios of the analyte precursor ions and/or analyte product ions. In tandem mass spectrometry (MS/MS or MS2), multiple rounds of mass spectrometry analysis are performed. For example, samples containing a mixture of polypeptides can be ionized and the resulting precursor ions separated according to their mass-to-charge ratio. Selected precursor ions can then be fragmented and further analyzed according to the mass-to-charge ratio of the product ions.

[0059] As used herein, the term "mass spectrometer" refers to a device which creates ions from a sample, separates the ions according to mass to charge ratios, and measures the abundance of each detected m/z peak. Because charge (z) can be calculated from the spectra, mass can be determined. Mass spectrometers include multistage mass spectrometers which fragment the mass-separated precursor ions and separate the product ions by mass-to-charge ratio one or more times. Multistage mass spectrometers include tandem mass spectrometers which

fragment the mass-separated precursor ions and separate the product ions by mass-to-charge ratio once.

[0060] As used herein, the term "ion source" refers to a component of a mass spectrometer which produces ions from a sample. Examples of ion sources include, but are not limited to, electrospray ionization (ESI) sources, matrix assisted laser desorption/ionization (MALDI) sources, atmospheric pressure chemical Ionization (APCI) sources.

[0061] As used herein, the term "signal-to-noise ratio" refers to the level of a desired signal to the level of background noise.

[0062] As used herein, the term "mass-to-charge ratio" refers to the ratio of the mass of a species (i.e., a precursor ion or product ion) or to the charge state of a species. The term "m/z unit" refers to a measure of the mass to charge ratio. The Thomson unit (abbreviated as Th) is an example of an m/z unit and is defined as the absolute value of the ratio of the mass of an ion (in Daltons) to the charge of the ion (with respect to the elemental charge).

[0063] "Mass spectrometer resolving power", often termed resolution, is a quantitative measure of how well m/z peaks in a mass spectrum are separated (i.e., resolved). There are a variety of conventions to calculate resolving power. The IUPAC definition is: Resolving power (R) : $R = m/\Delta m$. This equation illustrates how to calculate resolving power (R) where m is the mass corresponding to the peak and Δm is the spacing between that peak and the nearest neighbor peak. Another, definition for resolving power is: Resolving power (R) : $R = m/(m\ 1/2)$, where m is the mass corresponding to the peak (m) and m½ is a variable referring to the full width at half maximum of the peak (m½ = FWHM). With the second definition, two peaks at m/z 500 and 501 are just barely discernible if the resolving power is 500. This method of calculating resolution is particularly useful as it provides a metric to assess peak width regardless of whether there is a nearby neighbor to compare it to.

[0064] "Scan rate" refers to the number of scans per second performed by the mass spectrometer device. For example a scan rate of 10 Hz means the acquisition of 10 spectra per second.

[0065] "Mass spectrometer dynamic range" can be defined as the ratio between the minimal and the maximal concentration of compounds that can be detected simultaneously in a given sample. For example, mass spectrometers suitable for the present invention typically have a dynamic range of at least 3 orders of magnitude, at least 4 orders of magnitude, at least 5 orders of magnitude, or at least 6 orders of magnitude.

[0066] The term "protein" refers to a class of compounds comprising one or more polypeptide chains and/or modified polypeptide chains. Proteins can be modified by naturally occurring processes such as post-translational modifications or co-translational modifications. Exemplary post-translational modifications or co-translational modifications include, but are not limited to, phosphorylation, glycosylation, deamidation, lipidation, prenylation, sulfonation, hydroxylation, acetylation, methylation, methionine oxidation, the addition of cofactors, proteolysis, and assembly of proteins into macromolecular complexes. Modification of proteins can also include non-naturally occurring derivatives, analogues and functional mimetics generated by chemical synthesis. Exemplary derivatives include chemical modifications such as alkylation, acylation, carbamylation, iodination or any modification that derivatizes the protein.

[0067] The terms "peptide" and "polypeptide" are used synonymously in the present description, and refer to a class of compounds composed of amino acid residues chemically bonded together by amide bonds (or peptide bonds). Peptides and polypeptides are polymeric compounds comprising at least two amino acid residues or modified amino acid residues. Modifications can be naturally occurring or non-naturally occurring, such as modifications generated by chemical synthesis. Modifications to amino acids in peptides include, but are not limited to, phosphorylation, glycosylation, deamidation, lipidation, prenylation, sulfonation, hydroxylation, acetylation, methylation, methionine oxidation, alkylation, acylation, carbamylation, iodination and the addition of cofactors. Peptides and polypeptides can be generated by substantially complete digestion or by partial digestion of proteins.

[0068] Overview

[0069] Peptide mapping is a vital tool for biotherapeutic drug development. Demand for this technique is increasing due to demand within pharmaceutical companies to use site-specific information to inform therapeutic strategies, as well

as from groups, such as the FDA, to ensure that these therapies are safely produced and administered. A typical pharmaceutical company is likely to conduct thousands of peptide mapping assays each year. However, the costs associated with existing techniques is substantial given the time required (~60 minutes per sample), the requisite equipment (advanced LC-MS systems), and the need for highly skilled technicians.

[0070] In particular, biotherapeutic proteins, such as antibodies, acquire deleterious modifications during production and storage (**Figure 1**). Peptide mapping assays, such as liquid chromatography-mass spectrometry (LC-MS) analyses of tryptic digests of these molecules, can identify and measure site-specific changes that ultimately reduce bioactivity. The results from these assays can also be used to make improvements and changes to production and formulation strategies with the goal of protecting critical amino acids.

[0071] The growing number of biotherapeutic proteins is additionally driving an increase in demand for these assays. However, the inherent requirement to use liquid chromatography and other purification techniques severely limits the throughput (~60 minutes per sample) of existing mapping techniques. As a result, alternatives to conventional methods of sample preparation and protein mapping are of interest, particularly solutions that could increase throughput while retaining specificity.

[0072] In an embodiment, the present invention provides a high throughput peptide mapping platform by combining a plate-based sample preparation method with direct infusion MS analysis. Protein samples are reduced, alkylated, digested, and acid quenched in a filter plate (a type of multi-well plate). Samples are then each infused sequentially using a NanoMate® or other automated infusion system directly to a mass spectrometer (i.e., a high resolution Orbitrap). The result is an effective assay for identifying site specific modifications in therapeutic antibodies, with their initial results showing high fidelity to LC based methods (>90% sequence coverage) for methionine and tryptophan oxidation, asparagine glycosylation, protein n-terminal pyrolysis, clipping, amino acid sequence variants, and succinimide intermediates of asparagine deamidation and aspartic acid isomerization. Adding targeted ultrahigh resolution MS2 scans (500,000 resolving power at 200 m/z) into the scan sequence further enabled the ability to measure site-specific deamidation.

[0073] Earlier alternative conventional methods have attempted to directly inject “digested” anitbodies into a MS device. However, such methods still require a time

consuming or resource intensive separation step (such as a gas phase separation or purification step) and may not provide sufficient sequence coverage.

[0074] The high-throughput direct infusion method in an embodiment of the present invention can provide similar information to conventional LC-MS methods but taking only approximately 0.1 to 3 minutes, preferably approximately 0.1 to 2 minutes, or approximately 0.1 to 1 minute per sample, which could translate to a 20X increase in throughput.

[0075] As a result, the sequence coverage provided by the present invention compares favorably to conventional methods despite faster analysis time and simpler preparation. In an embodiment, the infusion method of the present invention performs similarly to conventional LC-MS peptide mapping in comparing methionine and tryptophan oxidation levels of NIST monoclonal antibody treated with increasing amounts of hydrogen peroxide (>90% sequence coverage). Additional comparisons for antibody deamidation and glycosylation have also been completed with similar results.

[0076] EXAMPLES

[0077] Example 1 – This example describes a peptide mapping method for high throughput sample preparation and analysis by performing direct infusion mass spectrometry analyses on tryptic peptide mixes without chromatography. A plate-based sample preparation has been developed that is compatible with direct injection MS analysis. This method is capable of rapidly preparing and analyzing 96 samples completely within 8 hours (4 hours of sample preparation, and 4 hours of MS analysis). Protein samples are cleaned, reduced, alkylated, digested and acid quenched in a filter plate. Samples are then filtered and infused sequentially using a NanoMate® system directly to the mass spectrometer. With the unbiased sample preparation method and high resolution Orbitrap survey scans, >95% sequence coverage are observed through exact mass comparison to theoretical peptide masses. This alone is useful in the rapid confirmation of the biotherapeutic identity. Additionally, methionine oxidation, tryptophan oxidation, and asparagine glycosylation levels can be measured from these scans. Adding targeted ultrahigh resolution MS2 scans (500,000 resolving power at 200 m/z) into the scan sequence enables measurement of site-specific deamidation

[0078] Materials and Methods. For filter well plate preparation, combine 50 µg of antibody (<20 µL) with 100 µL 8 M guanidinium chloride (GnHCl) in a filter well. To reduce cysteines, add 2.5 µL 1M DTT to each well and incubate at 37C for 15 minutes. To alkylate cysteines, add 6 µL 1 M IAA to each well and incubate at room temperature in the dark for 15 minutes,

then quench with 3.5 µL 1 M DTT. To precipitate the protein add 200 µL MeOH to each well and incubate at room temperature for 5 minutes. Centrifuge at 3,000 RCF for 10 minutes and discard filtrate. Wash each well with 200 µL 90% MeOH, centrifuge at 3,000 RCF for 10 minutes, discard filtrate, repeat washing three more times.

[0079] To each well add 100 µL 50 mM ammonium acetate, and 2 µg of trypsin. Incubate the digestion at 37C for 2 hours, then quench by adding 5 µL 10% formic acid. Centrifuge the plate for 10 minutes at 3,000 RCF, collect the filtrate in a clean 96 well plate. Transfer the plate to NanoMate® stage for analysis.

[0080] Plate Method. Developing a plate-based sample preparation that is compatible to infusion MS was key to matching the throughput of the sample preparation with that of the data collection. Previously, studies have reported plate-based peptide mapping methods. However, these have been coupled to chromatographic separation which removes the salt from the rest of sample during the loading step. These methods are unlikely to be compatible with direct infusion without additional desalting steps. These examples describe a method (see generally **Figure 2**) where all sample preparation steps are performed in a filter plate. In this example, the samples are first added to 8 M guanidine chloride in an individual filter well, followed by reduction and alkylation with dithiolthreitol (DTT) and iodoacetate (IAA), respectively. The protein is then precipitated with methanol and washed repeatedly to desalt the sample, all within the same well. The protein is then resuspended in ammonium acetate and digested with trypsin (still in the well). The digestion is then quenched with formic acid and the peptides are eluted from the filter to a second 96-well plate and are ready for analysis.

[0081] Any preparation method must be easily compatible with varied formulations. This plate method is compatible with antibody samples with concentrations greater than 2 mg/mL; less concentrated samples may need to be pre-concentrated prior to analysis. Sample matrices will commonly have salts and detergents, which are not compatible with infusion-MS and must be removed prior to analysis. In this method, the precipitation step removes detergents better than gel permeation chromatography or molecular weight cutoff filters.

[0082] Once the samples have been prepared they can be infused into a mass spectrometer using electrospray ionization. An Advion Nanomate® system is typically used which directly accepts the 96 well plate of samples and automates the infusion and

ionization step. The resultant ionized tryptic peptides can be analyzed with any interfaced mass spectrometer – such as Orbitrap hybrid systems capable of high-resolution MS and MS^n experiments. Shown in **Figure 3** is a partial mass spectrum that has been collected from an antibody sample using the methods described herein. All annotated m/z peaks are identified peptides resulting from the mAb. Sequence coverage is generally above 95%. From these data, the presence of modifications, truncations, or any sequence variations contained within the mAb sample can be quickly determined. Specific examples are provided below of how these data can be analyzed and leveraged to determine sequence variations that are of high importance to the biopharmaceutical industry.

[0083] Oxidation analysis. Protein therapeutics are susceptible to oxidative damage prior to being administered to the patient. Primarily, methionine and tryptophan residues acquire deleterious oxidation modifications. When these modifications occur in the complementarity determining region (CDR) or fragment crystallizable (FC) region, this chemical modification can decrease bioactivity. Conventional peptide mapping experiments separate out the oxidized and native versions of a given peptide using chromatography and mass spectrometry. The oxidation level is then estimated by dividing the signal from the oxidized peptide by the total signal from the peptide of interest. Oxidation is a chemical process and thus leads to stereoisomers in methionine and positional isomers in tryptophan. These stereoisomers partially separate by chromatography and can lead to split peaks (**Figure 4A**) which complicate data analysis – typically requiring an expert analyst to manually inspect each data file. Direct infusion MS analysis forgoes this complication since all isomers have the exact same mass and coalesce into a single peak in the mass spectrum. Further, oxidation itself imparts a mass shift of 16 Da and is easily distinguished by MS from the corresponding native version without need for physical separation using chromatography (**Figure 4B**).

[0084] Since peptide mapping is typically performed with chromatographic separation prior to MS analysis, oxidation results were compared for both LC-MS and inf-MS (**Figures 4C** and **4D**). Peptides (DTLMISR in Fig. 4C - SEQ ID NO:1; and FNWYVDGVEVHNAK in Fig. 4D - SEQ ID NO:2) from digested NIST mAb treated with H_2O_2 were titrated into untreated NIST mAb digests to setup response curves to compare conventional peptide mapping to inf-MS. These results demonstrate the measurements from these two methods strongly correlate (r^2 <0.98). Untreated and H_2O_2 treated NIST mAb, Sigma mAb, infliximab, rituximab, and cetuximab samples were prepared and analyzed by both conventional peptide mapping and inf-MS. Results (**Figure 4E**) show that the oxidation changes from untreated to H_2O_2 treated samples are similar with both methods. The methods provided

consistent results across the different antibodies for untreated and H_2O_2 treated samples (**Figure 4E**).

[0085] Deamidation analysis. Deamidation converts asparagine residues into aspartic acid and iso-aspartic acid. This modification can alter both the charge state and structure. Using conventional peptide mapping methods, deamidated peptides are difficult to separate from the unmodified versions by reversed-phase chromatography and the extra modified species further complicates data analysis. Deamidation adds 1 Da mass shift to the peptide; however, this mass shift is 19 mDa different from the first ^{13}C isotope of the native peptide. These species are indistinguishable under most mass spectrometry conditions. However, high resolving power (e.g., ~500,000 resolution at 200 m/z) can often separate deamidated species from their native counterparts, however, larger peptides with higher charge and m/z are more difficult to resolve. MS/MS fragmentation produces product ions with lower charge states and thus are more easily resolved.

[0086] **Figure 5A** presents a zoom-in of this m/z region from an MS/MS spectrum of the GFYPSDIAVEWESNGQPENNYK (SEQ ID NO:1) (commonly referred to as "PENNYK") peptide from a mAb sample analyzed by inf-MS. Note the y9 product ion isotope distribution indicates resolution of the deamidated and native PENNYK peptides (shown by the arrow). **Figures 5B** and **5C** provide evidence that inf-MS can generate comparable results for calculating the ratios of deamidated to native peptides in mAb samples.

[0087] Glycan analysis. Glycosylation occurs on mAbs and determining the extent, location, and type of glycosylation is similarly of high importance to the biopharmaceutical field. **Figures 6A** and **6B** provide data comparing the ability of inf-MS to generate accurate and comparable glycan occupation data for mAb samples. These data can be obtained from either the MS m/z signals (as used here) of the various peptides or through MS/MS spectra.

[0088] Other types of modifications and variations that are detectable using this approach include aspartic acid isomerization, glutamine and glutamic acid protein N-terminal cyclization, des-lysine, sequence clipping, and finally sequence variants.

[0089] Example 2 – This example describes an additional general filter plate preparation method suitable for the present invention. Approximately 50 µg of a polypeptide, such as an antibody, is added to each desired well of a filter plate and denatured using guanidinium chloride (GnHCl). Different amounts of the polypeptide may be used depending on the

polypeptide to be analyzed. The polypeptide is reduced and/or alkylated, precipitated out of solution using MeOH, and then optionally washed. The precipitate is dissolved in approximately 25 mM ammonium acetate and digested using trypsin or other known digestive enzyme or chemical agent. An acid quenching step is optionally performed and then the polypeptide fragments are injected into a mass spectrometer or other analyzing device, without the need for a chromatography step or salt removing step.

[0090] Example 3 - **Figure 7** presents a spectrum resulting from direct infusion of polypeptides that result from the trypsin digestion of a therapeutic antibody (adalimumab, i.e., Humira®), prepared as described herein, into an Orbitrap hybrid mass spectrometer. The spectrum contains hundreds of discrete m/z peaks (~ 500 isotopic clusters) that result from the various tryptic peptides of the digested mAb. To identify which polypeptides and potential modifications are present, *in silico* peptide masses are generated from trypsin digestion of the known protein sequence, and each peptide's theoretical m/z values are calculated in the expected charge states. Those calculated values are then matched to the measured m/z values using a m/z tolerance. This tolerance is typically ranges from 1 to 20 parts per million (5 to 10 ppm being common, and 5 ppm being the typical choice in this experiment) and results in accurate mapping of the measured m/z values to theoretical m/z values calculated from the expected peptide sequences.

[0091] This process, i.e., direct injection, is particularly effective because the mixture is not very complex relative to other biological mixtures (such as cell lysates), with the vast majority of the detected m/z peaks resulting from the various tryptic peptides of the antibody. In **Figure 7**, the most abundant m/z peaks are annotated for each peptide sequence. Polypeptides are detected spanning the entire light and heavy chain (**Figure 8**) allowing confirmation of the expected sequence of the mAb. To determine the relative abundance of the various peptides and their modifications, the peak intensities were used. For example, the intensity of the modified and unmodified versions of a given peptide m/z signal can be compared to calculate the relative amounts of each. Occasionally a protease cleavage site is missed and the resulting longer peptide, contains two or more of the expected tryptic peptides (see peptide 55, for example, in **Figure 8**). Such missed cleavage peptides may be used to confirm the sequence and to also inform on the relative abundances of any modifications/variants.

[0092] A further level of confidence can be added by performing tandem MS experiments (MS/MS). These MS/MS experiments can be collected in the traditional data dependent acquisition mode (DDA), in a targeted fashion, or in a data independent mode (DIA). No

matter how they are triggered, the product ions in these MS/MS spectra can be used to confirm the accurate mass MS1 peak assignments. With just the MS1 scan, only the intact mass of the polypeptide is known. That mass indicates if the polypeptide's mass is consistent with the expected amino acids but does not indicate if the amino acids are in the correct sequence order. Also, the mass accuracy of the instrument is not always high enough to guarantee that the detected polypeptide is not composed of a different combination of amino acids (and possibly modifications) which happen to sum to a similar m/z. By fragmenting each m/z in the MS1 scan, the resulting MS/MS spectrum allows the order of the amino acids and site-specific location of any modifications to be determined. A modification or sequence variant can then be localized, or the stoichiometry of a particular modification can be determined (i.e., deamidation). **Figure 9** presents an example of MS/MS analysis of peptide #94 from adalimumab.

[0093] Example 4 – This example describes the sequence coverage and sequence confirmation of an analyzed protein. Sequence coverage is generally defined here as the amount of the protein sequence being analyzed that has been detected. For example, if the protein analyte in question contains 100 amino acids and peptides containing 90 of those amino acids are observed, then it is reported that 90% of the sequence has been covered.

[0094] In the case of mAbs, the sequence of both heavy and light chain are included in this calculation. In this example (adalimumab, **Figure 7**), all the amino acids present in both the heavy and light chains were detected in at least one tryptic peptide. Therefore, this analysis resulted in 100% sequence coverage. For all antibodies tested with the direct infusion method of the present invention, all have resulted in sequence coverage of $\geq$92%.

[0095] Detection of a polypeptide is done in two ways. First, and as described above, the exact mass of the observed m/z signals are compared with the predicted m/z values for all possible polypeptides. If a match is made within a specific mass tolerance window (typically 1 to 10 ppm), this peptide is reported and part of the sequence is indicated as being detected. A further level of confirmation can be obtained by collecting MS/MS (**Figure 9**). In this way, predicted product ions of the matched sequence are compared to those observed in the experimental MS/MS spectrum. Established algorithms and tools for this comparison are known in the art and are used to calculate a confidence score and ultimately to confirm the detection of the matched peptide sequence. In **Figure 9**, at least one of the two typical fragments (b/y, c/z, a/x, see Chu et al., Int. J Mass Spec, 2015, 390: 24-27) was observed for each backbone site along the sequence, which indicates 100% sequence

confirmation. As a result, it is not only known which amino acids, and potentially modifications, are present, it is also known what order the amino acids are in.

[0096] Example 5 – There are several key figures of merit that are required in the MS system to achieve the results described in the examples above. First, given there are hundreds of polypeptides directly injected into the MS system, the mass analyzer used must have the capability to resolve these, often closely spaced, peptide m/z signals. Theoretical analysis shows that resolving powers in excess of approximately 100,000 (@ m/z 200) provide the best performance (**Figure 10**). That is, at this resolving power approximately 80% of the possible m/z peaks can be resolved.

[0097] Second, because the sample is being directly injected and, is complex with some polypeptides having much lower abundances than others, the MS analyzer should have the largest dynamic range possible. Ideally, the system should allow the detection of a peptide that is up to 1,000 times, preferably up to 10,000 times, more abundant than another polypeptide, i.e., up to four orders of magnitude. Such capability would allow for the detection of modified and variant peptides as low as 0.1% relative abundance. In an experiment, as many ions were injected into the Orbitrap analyzer as possible (approximately one million) and polypeptides with as low as 0.8 % relative abundance were detected. Signal to noise ratios, and detection limits, can also be increased by narrowing the m/z range of ions that are allowed to be injected and polypeptides have been observed with as low as 0.02 % relative abundance with this approach. **Figure 11** shows how detection sensitivity is improved for low level modified peptides as the injection m/z range is narrowed.

[0098] Third, another key figure of merit for the MS system and mass analyzer in the above experiments is the ability to provide accurate mass measurement. For the above results, the system was able to provide at least 50 ppm mass accuracy, at least 25 ppm mass, and at least 10 ppm mass accuracy. Mass accuracy of 5 ppm or even 1 ppm allows more ions to be positively identified.

[0099] Fourth, the MS system ideally can perform tandem MS experiments. While not necessary, this capability allows for the confirmation of sequences as described above. In the case of detecting deamidation, for example, the MS/MS analysis requires high resolution analysis so that the deamidation modification can be distinguished from the isotopic cluster of the non-modified peptides (see **Figure 12**).

[00100] Having now fully described the present invention in some detail by way of illustration and examples for purposes of clarity of understanding, it will be obvious to one of ordinary skill in the art that the same can be performed by modifying or changing the invention within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any specific embodiment thereof, and that such modifications or changes are intended to be encompassed within the scope of the appended claims. For example, it should be understood that the reagents, solution concentrations, and their amounts used in the above examples may be modified or depending on the specific polypeptides to be analyzed.

[00101] When a group of materials, compositions, components or compounds is disclosed herein, it is understood that all individual members of those groups and all subgroups thereof are disclosed separately. Every formulation or combination of components described or exemplified herein can be used to practice the invention, unless otherwise stated. Whenever a range is given in the specification, for example, a temperature range, a time range, or a composition range, all intermediate ranges and subranges, as well as all individual values included in the ranges given are intended to be included in the disclosure. Additionally, the end points in a given range are to be included within the range. In the disclosure and the claims, “and/or” means additionally or alternatively. Moreover, any use of a term in the singular also encompasses plural forms.

[00102] As used herein, “comprising” is synonymous with "including," "containing," or "characterized by," and is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. As used herein, "consisting of" excludes any element, step, or ingredient not specified in the claim element. As used herein, "consisting essentially of" does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claim. Any recitation herein of the term “comprising”, particularly in a description of components of a composition or in a description of elements of a device, is understood to encompass those compositions and methods consisting essentially of and consisting of the recited components or elements.

[00103] One of ordinary skill in the art will appreciate that starting materials, device elements, analytical methods, mixtures and combinations of components other than those specifically exemplified can be employed in the practice of the invention without resort to undue experimentation. All art-known functional equivalents, of any such materials and

methods are intended to be included in this invention. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Headings are used herein for convenience only.

[00104] All publications referred to herein are incorporated herein to the extent not inconsistent herewith. Some references provided herein are incorporated by reference to provide details of additional uses of the invention. All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. References cited herein are incorporated by reference herein in their entirety to indicate the state of the art as of their filing date and it is intended that this information can be employed herein, if needed, to exclude specific embodiments that are in the prior art.

Claims

1. A method for analyzing one or more polypeptides comprising the steps of:

a) depositing the one or more polypeptides in a container or carousel having a plurality of separated wells or chambers, wherein the one or more polypeptides are deposited in separate wells or chambers in the container;

b) denaturing the one or more polypeptides thereby generating one or more denatured polypeptides;

c) treating the one or more denatured polypeptides with a reducing agent, thereby generating one or more reduced polypeptides;

d) treating the one or more reduced polypeptides with a first solution causing the one or more polypeptides to precipitate out of the first solution, thereby generating one or more polypeptide precipitates;

e) washing the one or more polypeptide precipitates with a second solution;

f) dissolving the one or more polypeptide precipitates with a third solution thereby generating one or more dissolved polypeptides;

g) optionally, digesting the one or more dissolved polypeptides thereby generating one or more digested polypeptides;

h) injecting each of the one or more dissolved polypeptides or digested polypeptides from each well or chamber of the container or carousel into an ion source of a mass spectrometer device.

2. The method of claim 1 further comprising obtaining mass spectrometry data on each of the one or more digested polypeptides and detecting and/or measuring site specific mutations, post translational modifications, or combinations thereof, in each of the polypeptides.

3. The method of claim 2 further comprising measuring biological activity of each of the one or more polypeptides, and recording a link between the biological activity and the site specific mutations, post translational modifications, or combinations thereof, for each polypeptide.

4. The method of claim 3 further comprising comparing the biological activity of each of the one or more polypeptides with a control polypeptide.

5. The method of claims 1-4 comprising comparing one or more specific regions of each of the one or more polypeptides with a corresponding region of a control polypeptide and determining if a chemical modification is present at the one or specific regions in one or more of the one or more polypeptides.

6. The method of claims 1-5 wherein sample are analyzed at an average time of ≤ 4.0 min per sample.

7. The method of claims 1-5 wherein at least 100 samples are analyzed on a same mass spectrometry device within 24 hours.

8. The method of claims 1-7 wherein the one or more polypeptides are each an antibody.

9. The method of claims 1-8 wherein the denaturing step comprises contacting each of the one or more polypeptides with guanidinium chloride, urea, or a detergent.

10. The method of claims 1-9 further comprising treating the one or more denatured polypeptides or reduced polypeptides with an alkylating agent.

11. The method of claims 1-10 wherein the first solution comprises MeOH.

12. The method of claims 1-11 wherein the second solution comprises a mixture of water and MeOH.

13. The method of claims 1-12 wherein the third solution comprises ammonium acetate or ammonium bicarbonate.

14. The method of claims 1-12 wherein the digesting step comprises contacting the one or more dissolved polypeptides with a proteolytic enzyme.

15. The method of claims 1-14 wherein the digesting step comprises contacting the one or more dissolved polypeptides with trypsin.

16. The method of claims 1-15 further comprising performing an acid quenching step on the one or more digested polypeptides.

17. The method of claims 1-16 wherein the method does not comprise an additional salt removal step.

18. The method of claims 1-17 wherein the method does not comprise a liquid chromatography step.

19. The method of claims 1-18 wherein the container or carousel is a multi-well plate and the one or more polypeptides are a plurality of polypeptides deposited in separate wells in the multi-well plate.

20. The method of claims 1-19 wherein mass spectrometry data is obtained for over 90% of the amino acids of the analyzed one or more polypeptides.

21. The method of claims 2-20 wherein the obtained mass spectrometry data comprises detection and/or measurements of specific mutations, post translational modifications, or combinations thereof, of amino acids in the one or more polypeptides.

22. The method of claims 1-21 wherein the one or more polypeptides are treated before the denaturing step so as to induce a chemical modification in the polypeptides.

23. The method of claim 22 wherein the one or more peptides are treated with a chemical reagent, heat, time, and/or light, to generate susceptibility to chemical modification in the polypeptides.

24. The method of claims 22-23 wherein the one or more polypeptides are deposited in a multi-well plate, and varying concentrations of the chemical reagent or varying temperatures are added to different wells.

25. A method for analyzing a polypeptide comprising the steps of:

a) denaturing the polypeptide thereby generating a denatured polypeptide;

b) treating the denatured polypeptide with a reducing agent, thereby generating a reduced polypeptide;

c) treating the reduced polypeptide with a first solution causing the reduced polypeptide to precipitate out of the first solution, thereby generating a polypeptide precipitate;

d) washing the polypeptide precipitate with a second solution;

e) dissolving the polypeptide precipitate with a third solution thereby generating a dissolved polypeptide;

f) optionally digesting the dissolved polypeptide thereby generating a digested polypeptide;

g) directly injecting the dissolved polypeptide or digested polypeptide into an ion source of a mass spectrometer device.

26 The method of claim 25 further comprising obtaining mass spectrometry data on the digested polypeptide and measuring site specific mutations, post translational modifications, or combinations thereof, in the polypeptide.

27. The method of claims 25-26 wherein the polypeptide is an antibody.

28. The method of claims 25-27 wherein the denaturing step comprises contacting the polypeptide with guanidinium chloride, urea, or a detergent.

29. The method of claims 25-28 further comprising treating the denatured polypeptide or reduced polypeptide with an alkylating agent.

30. The method of claims 25-29 wherein the first solution comprises MeOH.

31. The method of claims 25-30 wherein the second solution comprises water, MeOH, or combinations thereof.

32. The method of claims 25-31 wherein the third solution comprises ammonium acetate or ammonium bicarbonate.

33. The method of claims 25-32 wherein the digesting step comprises contacting the dissolved polypeptide with a proteolytic enzyme.

34. The method of claims 25-33 wherein the digesting step comprises contacting the dissolved polypeptide with trypsin.

35. The method of claims 25-34 further comprising performing an acid quenching step on the digested polypeptide.

36. The method of claims 25-35 wherein the method does not comprise an additional salt removal step.

37. The method of claims 25-36 wherein the method does not comprise a liquid chromatography step.

38. A system for analyzing a plurality of polypeptides comprising:

a) a series of containers or a multi-well plate able to contain a plurality of polypeptides in separate containers or wells;

b) a reagent kit comprising, separately from one another, a denaturing solution, a reducing agent solution, a first solution able to cause reduced polypeptides to precipitate, a second solution wherein the second solution is a wash solution, a third solution able to dissolve polypeptide precipitates, and optionally, a solution comprising an enzyme able to digest the plurality of polypeptides; and

c) an automated system for injecting the plurality of peptides in each of the separate containers or wells into a mass spectrometer or other molecular analyzer after the plurality of peptides have been treated with the solutions of the reagent kit.

39. The system of claim 38 wherein the reagent kit additionally comprises an alkylating agent solution, an acid quench solution, or both.

40. The system of claims 38-39 wherein the first solution comprises methanol (MeOH), the second solution comprises a MeOH/water mixture, the third solution comprises ammonium acetate, the denaturing solution comprises guanidinium chloride, urea, or a detergent, the reducing agent solution comprises dithiolthreitol (DTT), the alkylating agent solution comprises iodoacetate (IAA), and the digesting enzyme solution comprises trypsin.

41. The system of claim 40 wherein the series of containers or the multi-well plate is a filter plater and the system further comprises an automated fluid delivery system for adding each solution to each well and draining each well of any liquid after each step.

42. The system of claim 41 wherein the reagent kit comprises an additional chemical reagent solution able to induce a chemical modification in the polypeptides.

43. The system of claim 42 wherein the additional chemical reagent solution comprises H_2O_2.

44. The system of claims 42-43 wherein the reagent kit comprises solutions of the chemical reagent in varying concentrations, wherein the solutions of varying concentrations are added to different wells containing the polypeptides.

45. A method for analyzing a plurality of polypeptides in a sample comprising the steps of:

simultaneously injecting the plurality of polypeptides into an ion source of a mass spectrometer (MS) device and generating a plurality of precursor ions; and

performing one or more MS scans on each precursor ion generated from the plurality of polypeptides, thereby generating spectral data for each precursor ion,

wherein the MS device provides a resolving power equal to or greater than 100,000, has a scan rate of at least 0.5 scans per second, and wherein the one or more scans are performed over a time period of at least 10 seconds.

46. The method of claim 45 further comprising generating the plurality of polypeptides by digesting a protein.

47. The method of claim 46 further comprising comparing the generated spectral data for each precursor ion to calculated mass-to-charge values for predicted polypeptides and modified polypeptides able to be generated from the protein, and determining if each precursor ion corresponds to one or more of the predicted polypeptides or modified polypeptides of the protein.

48. The method of claim 47, wherein spectra data provides a sequence coverage of at least 90% of the protein amino acid sequence.

49. The method of claim 47 further comprising fragmenting at least a portion of the plurality of precursor ions, thereby generating product ions, and measuring mass-to-charge ratios of the product ions, thereby generating product ion mass spectrometry data, and comparing the generated product ion mass spectrometry data with predicted fragment ion data of the protein and confirming sequence order of the plurality of precursor ions that correspond to one or more of the possible polypeptides or modified polypeptides of the protein.

50. The method of claims 47, wherein the protein is an antibody or therapeutic protein.

51. The method of claims 45-50, wherein generating the spectral data comprises averaging the spectral data obtained from the multiple scans for each precursor ion.

52. The method of claims 45-51 further comprising comparing peak intensity of two or more detected precursor ions and calculating a relative abundance in the sample of each polypeptide generating the two or more detected precursor ions.

53. The method of claim 52, wherein the polypeptides generating the two or more detected precursor ions comprise a first polypeptide and a variant of the first polypeptide having a modified amino acid sequence, one or more modified amino acid side chains, a modified structure, and/or a modified chemical formula.

54. The method of claims 45-53, wherein the MS device is able to detect a polypeptide in the sample that is 1,000 times less abundant than another polypeptide

55. The method of claims 45-54, wherein the MS device provides a resolving power equal to or greater than 200,000.

56. The method of claims 45-55, wherein the multiple scans are performed over a time period of at least 5 seconds.

57. The method of claims 45-55, wherein the multiple scans are performed over a time period of at least 60 seconds.

58. The method of claims 45-57, wherein the generated spectral data includes spectral data from product ions generated from polypeptides having four amino acids or less.

59. The method of claims 45-58, wherein the sample comprises a mixture of at least 50 different polypeptides.

60. The method of claims 45-59, wherein the sample is a liquid sample directly injected into an electrospray emitter of the MS device.

61. The method of claims 45-60, wherein the method does not comprise a liquid chromatography step or a salt removal step.

62. The method of claims 45-61, wherein injecting the plurality of polypeptides into the ion source of the MS device comprises the steps of:

a) denaturing the plurality of polypeptides thereby generating a plurality of denatured polypeptides;

b) treating the plurality of denatured polypeptides with a reducing agent, thereby generating a plurality of reduced polypeptides;

c) treating the plurality of reduced polypeptide with a first solution causing the plurality of reduced polypeptide to precipitate out of the first solution, thereby generating a polypeptide precipitate;

e) dissolving the polypeptide precipitate thereby generating a plurality of dissolved polypeptides; and

f) injecting the plurality of dissolved polypeptides into the ion source.

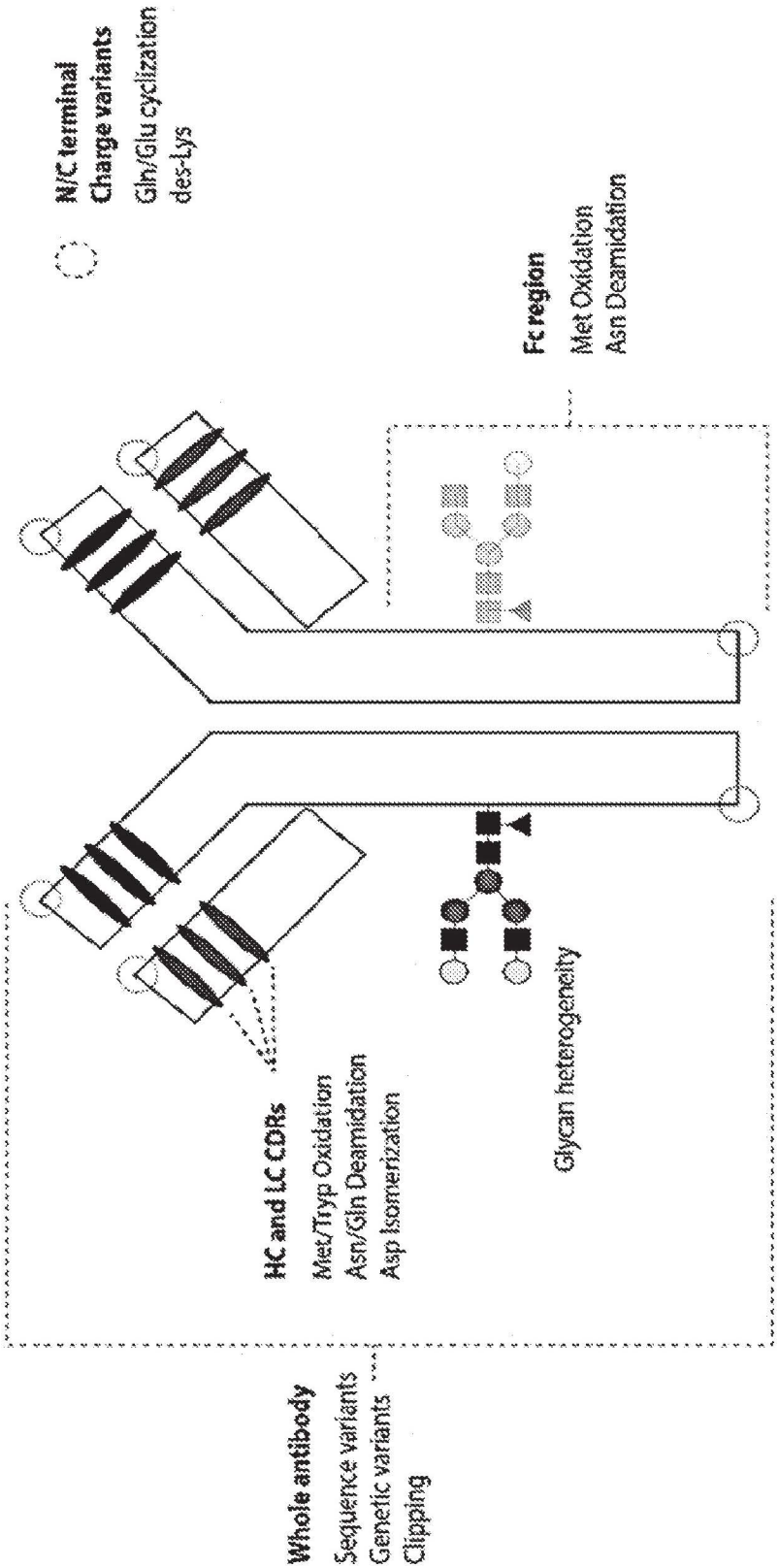
N/C terminal
Charge variants
Gln/Glu cyclization
des-Lys
Fc region
Met Oxidation
Asn Deamidation
HC and LC CDRs
Met/Tryp Oxidation
Asn/Gln Deamidation
Asp Isomerization
Glycan heterogeneity
Whole antibody
Sequence variants
Genetic variants
Clipping

Fig. 1

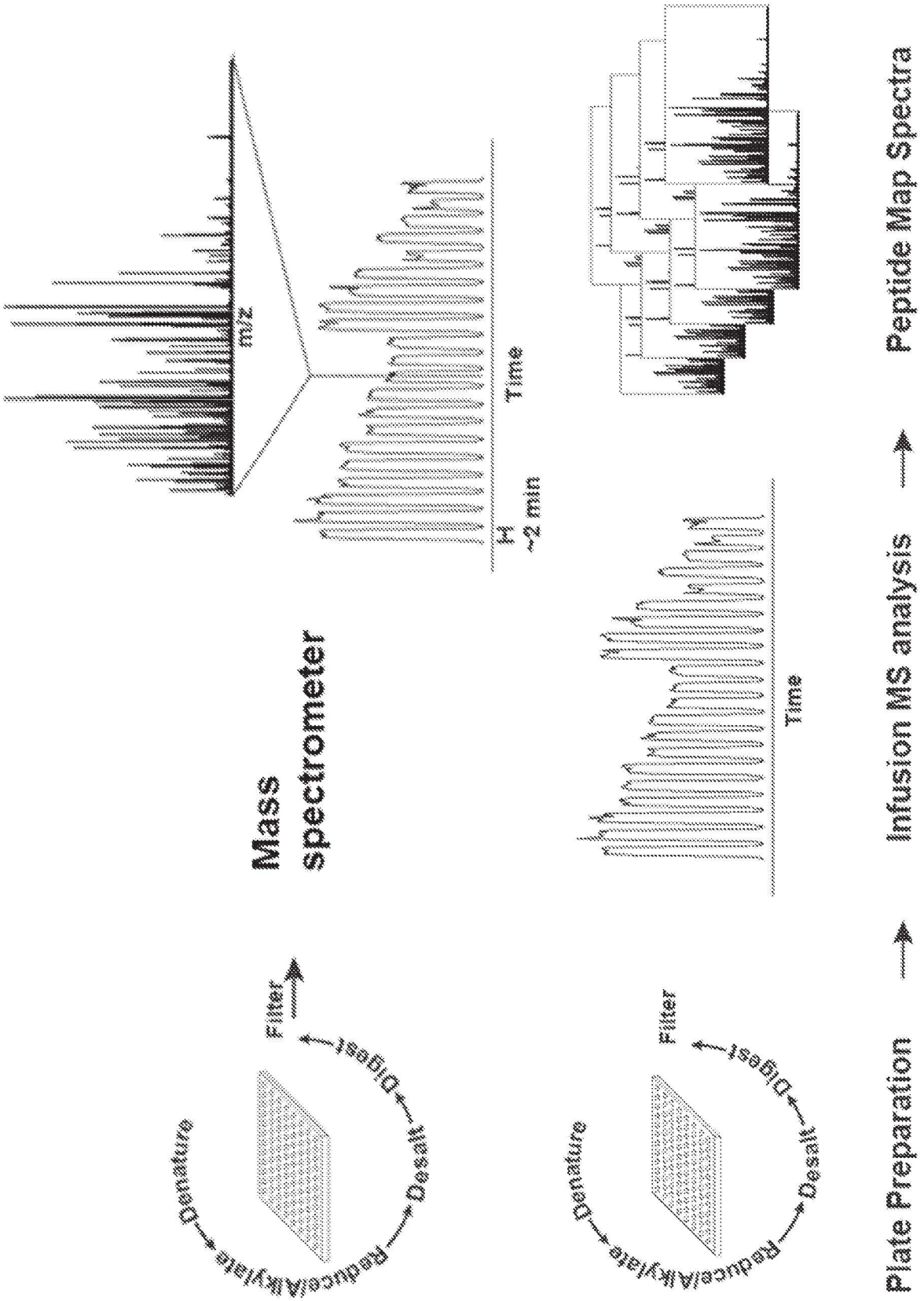
Plate Preparation
Denature
Reduce/Alkylate
Desalt
Digest
Filter
Infusion MS analysis
Time
Mass spectrometer
m/z
Time
~2 min
Peptide Map Spectra

Fig. 2

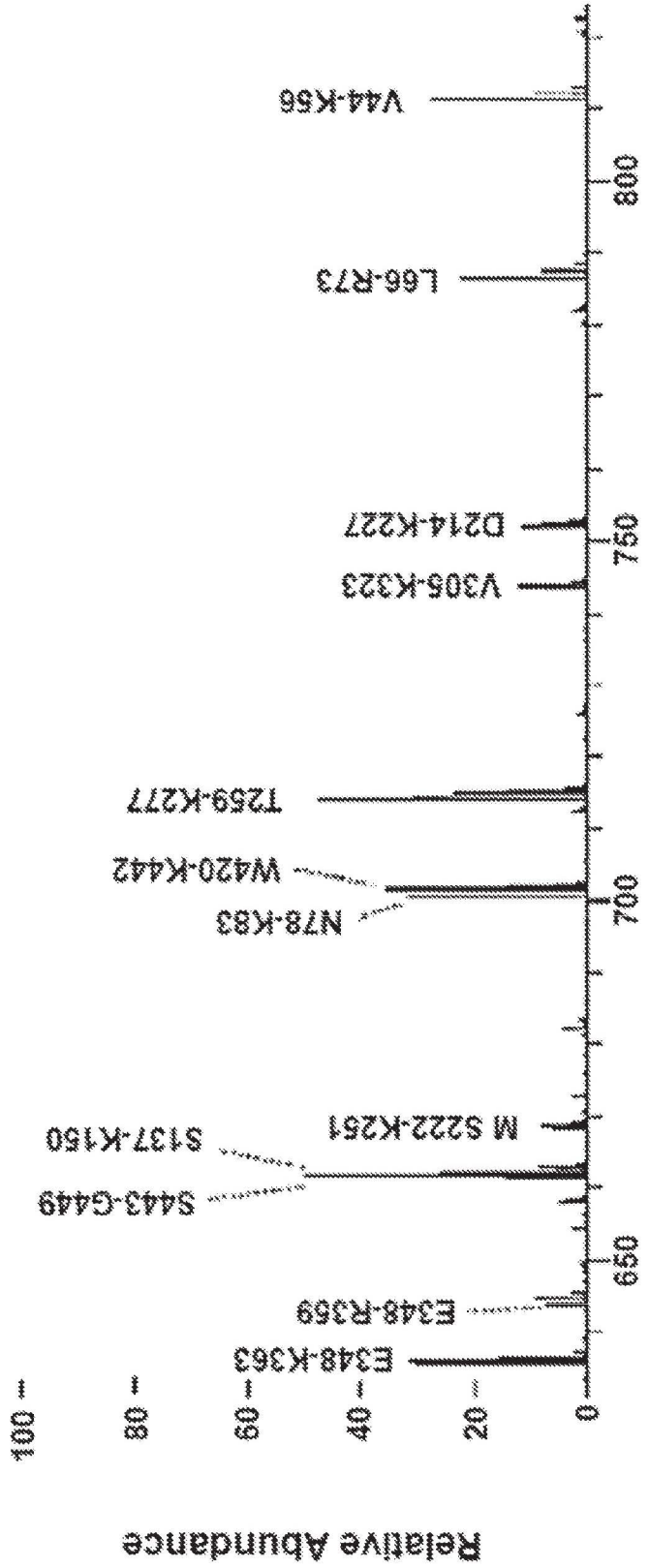
Relative Abundance
100
80
60
40
20
0
650
700
750
800
E348-K363
E348-R359
S443-G449
S137-K150
M S222-K251
N78-K83
W420-K442
T259-K277
V305-K323
D214-K227
L66-R73
V44-K56

Fig. 3

Conventional LC-MS Peptide Mapping

$$\text{Level} = \left(\frac{\text{Oxidized (area)}}{\text{Oxidized (area)} + \text{Native (area)}} \right) X\ 100$$

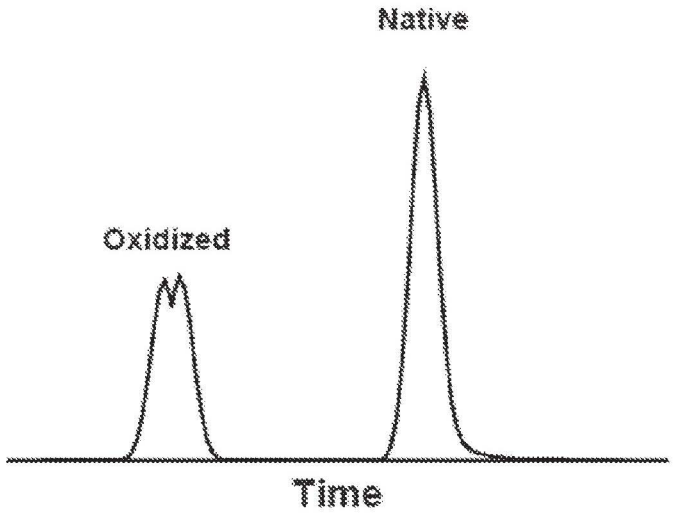

Fig. 4A

Infusion Peptide Mapping

$$\text{Level} = \left(\frac{\text{Oxidized (height)}}{\text{Oxidized (height)} + \text{Native (height)}} \right) X\ 100$$

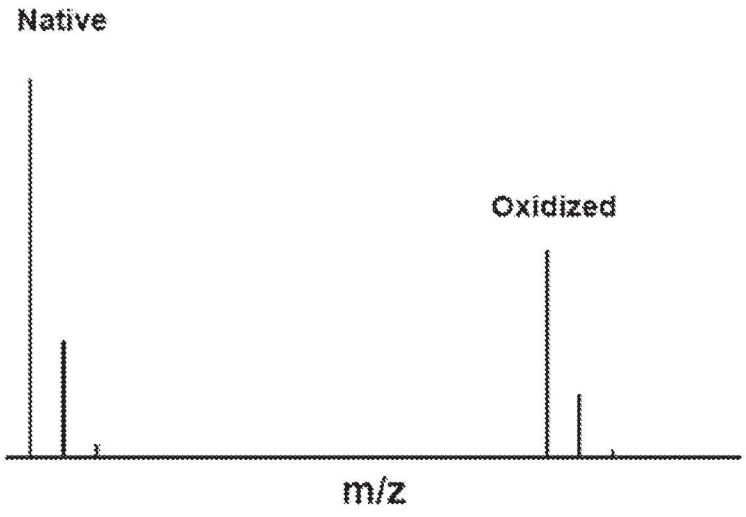

Fig. 4B

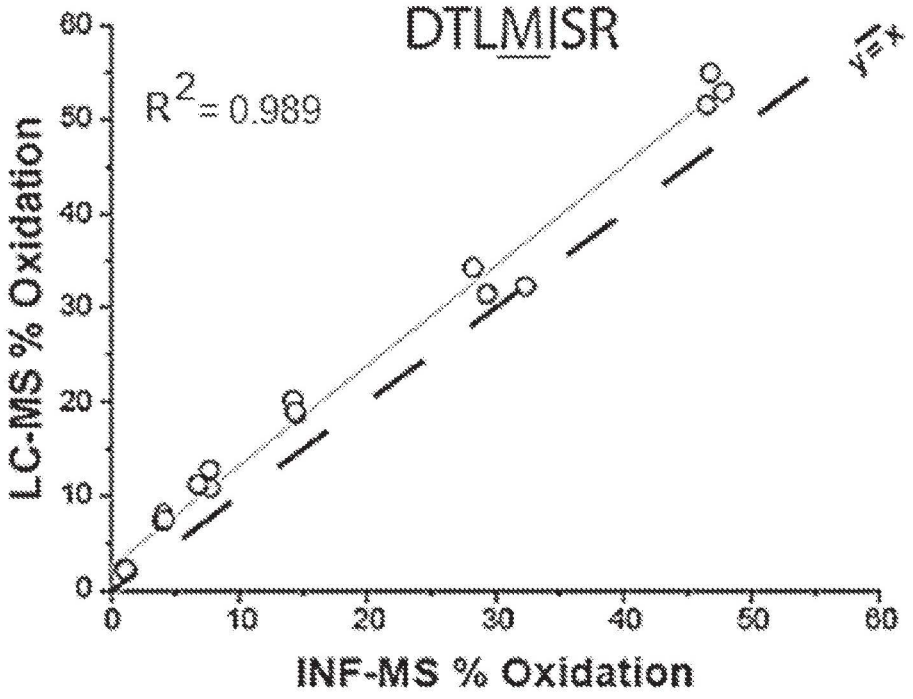
DTLMISR
R^2 = 0.989
y=x
LC-MS % Oxidation
INF-MS % Oxidation
0
10
20
30
40
50
60

Fig. 4C

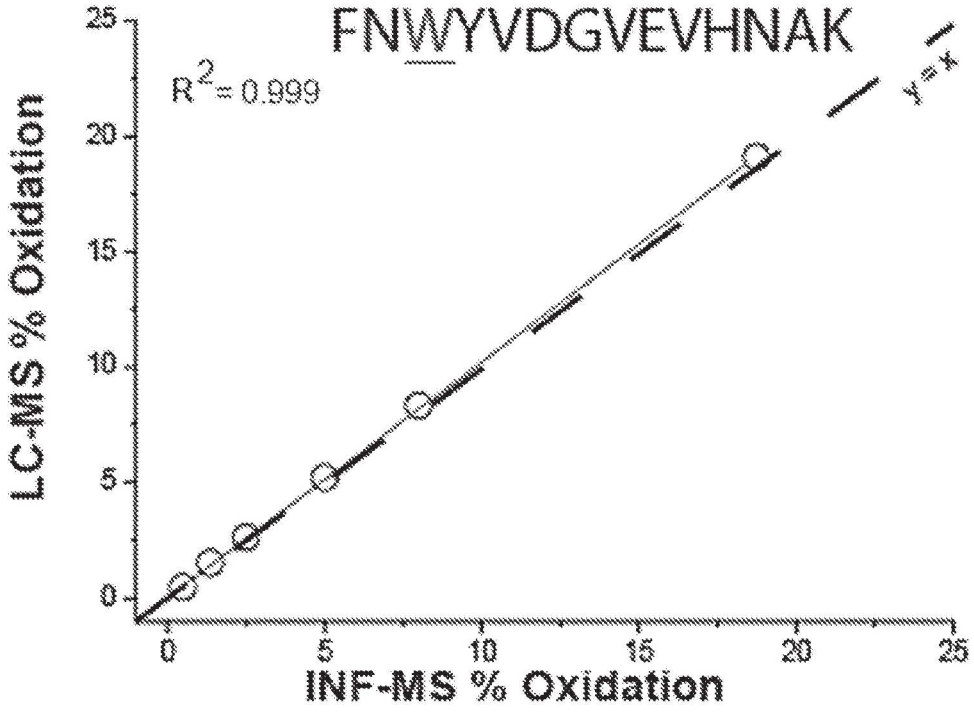
FNWYVDGVEVHNAK
R^2 = 0.999
y=x
LC-MS % Oxidation
INF-MS % Oxidation
0
5
10
15
20
25

Fig. 4D

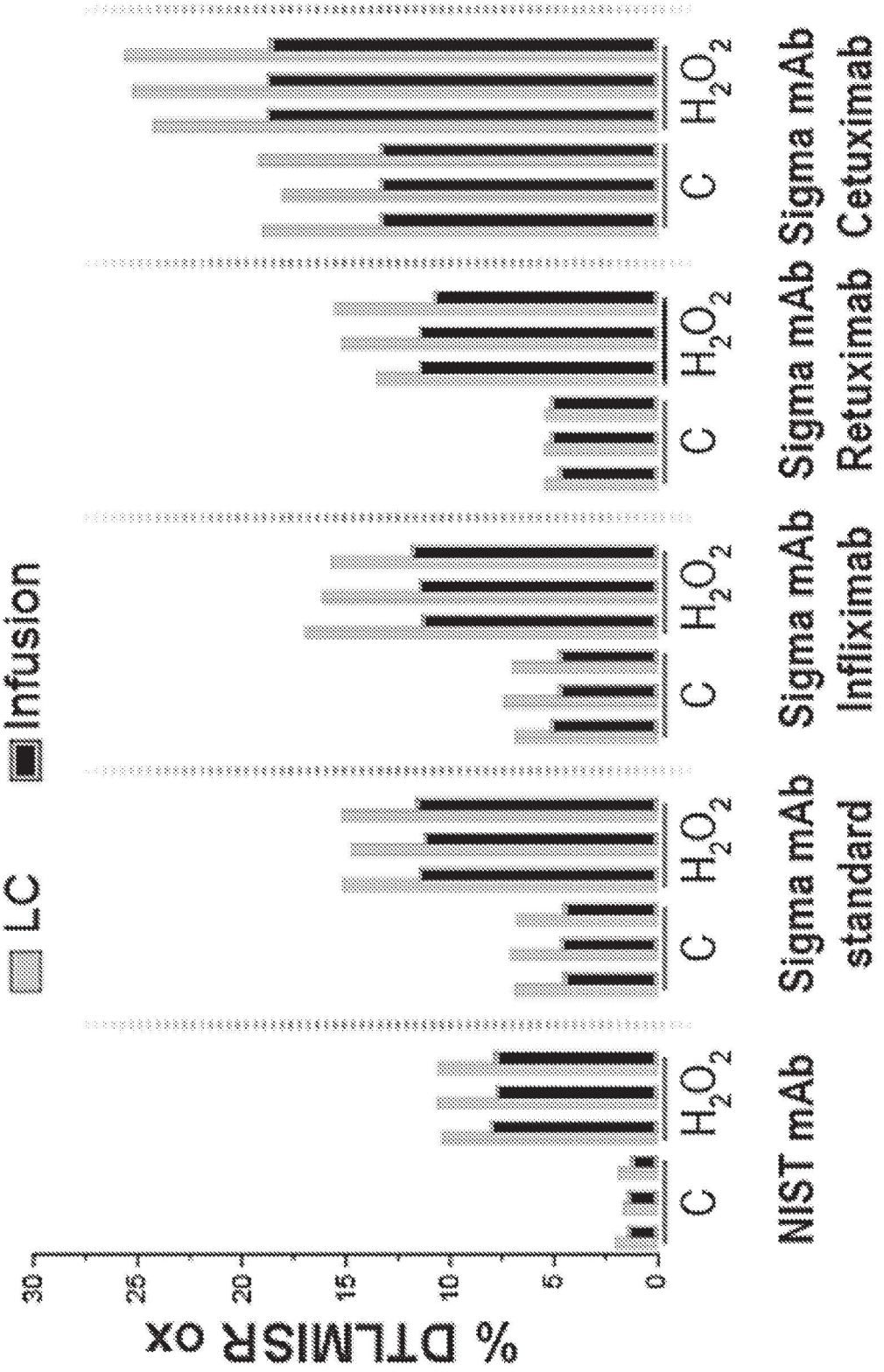
% DTLMISR ox
30
25
20
15
10
5
0
LC
Infusion
C
H_2O_2
NIST mAb
Sigma mAb standard
Sigma mAb Infliximab
Sigma mAb Retuximab
Sigma mAb Cetuximab

Fig. 4E

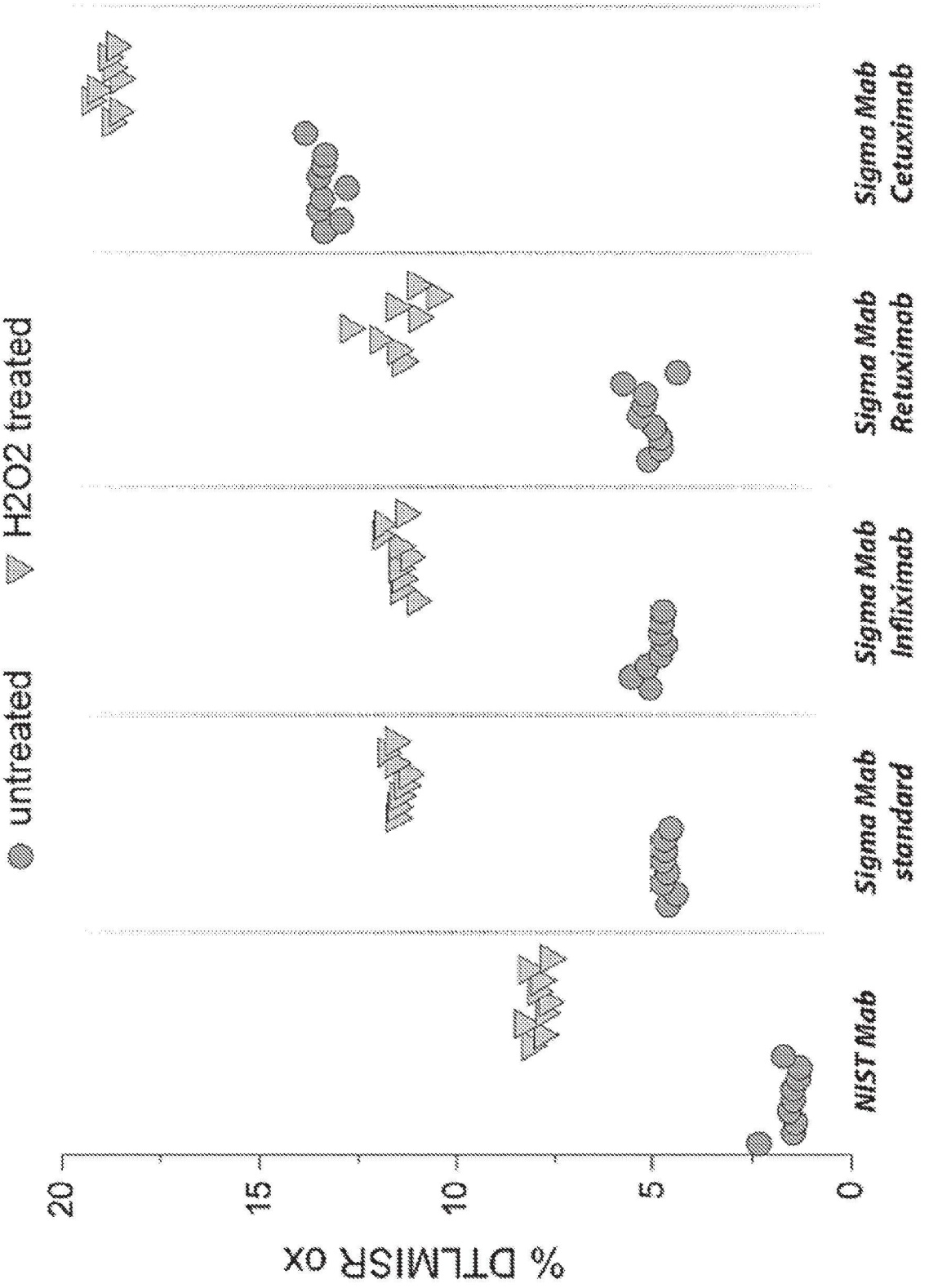
% DTLMISR ox
20
15
10
5
0
NIST Mab
Sigma Mab standard
Sigma Mab Infliximab
Sigma Mab Retuximab
Sigma Mab Cetuximab
untreated
H2O2 treated

Fig. 4F

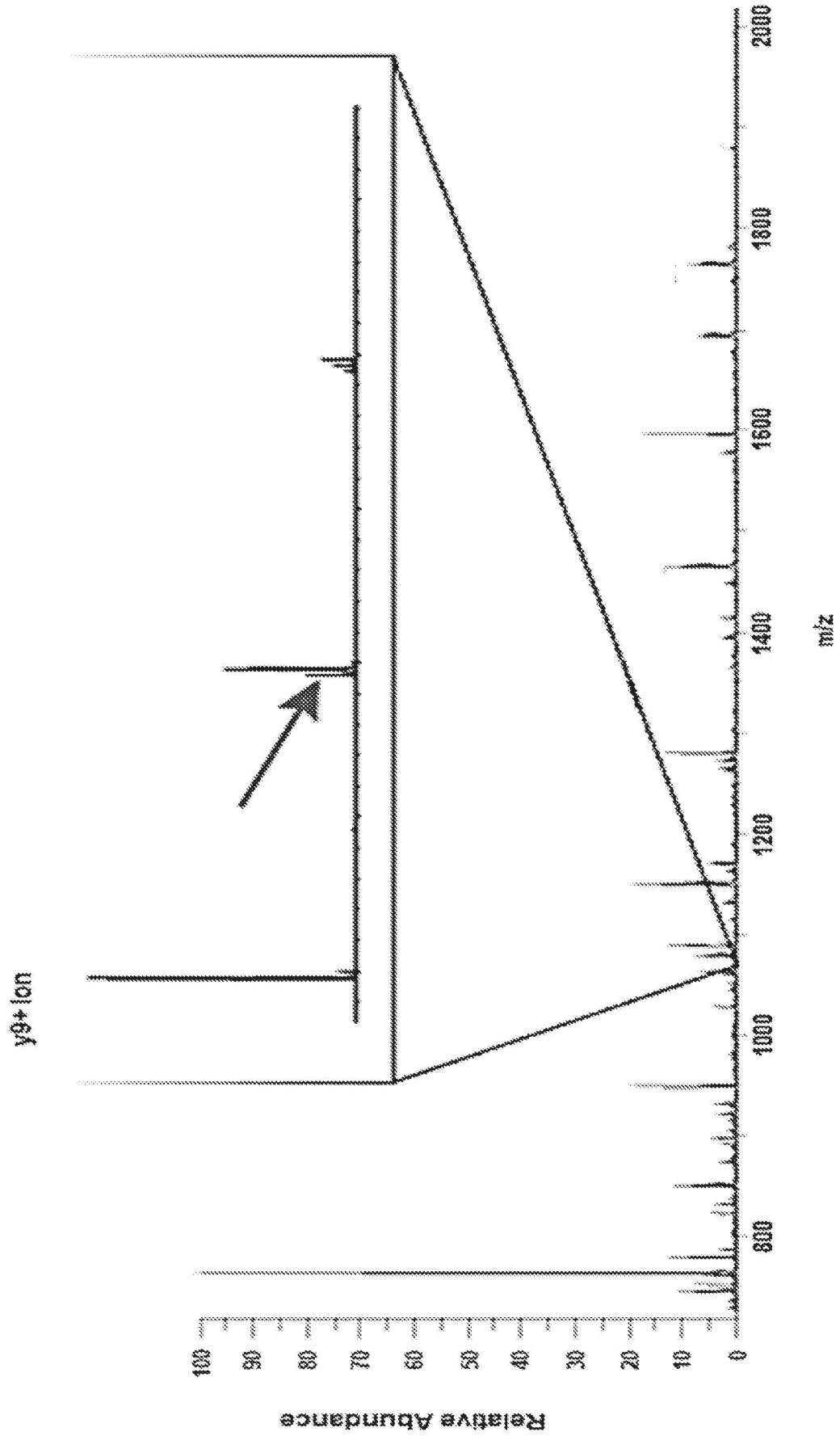
y9+ ion
Relative Abundance
m/z
100
90
80
70
60
50
40
30
20
10
0
800
1000
1200
1400
1600
1800
2000

Fig. 5A

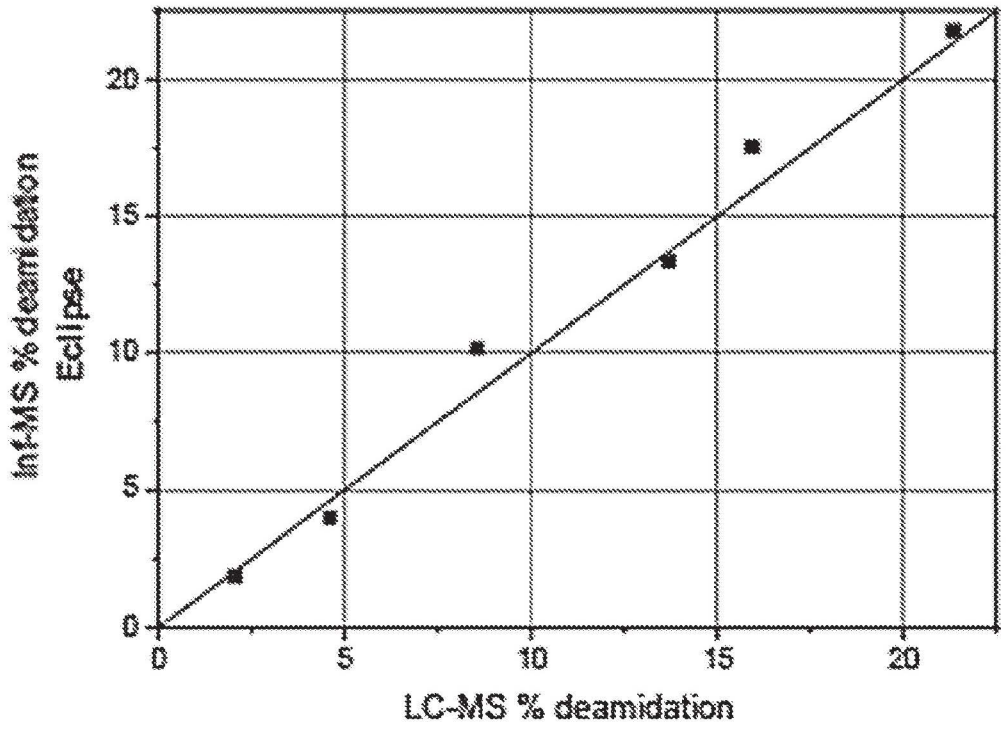
Int-MS % deamidation
Eclipse
LC-MS % deamidation
0
5
10
15
20

Fig. 5B

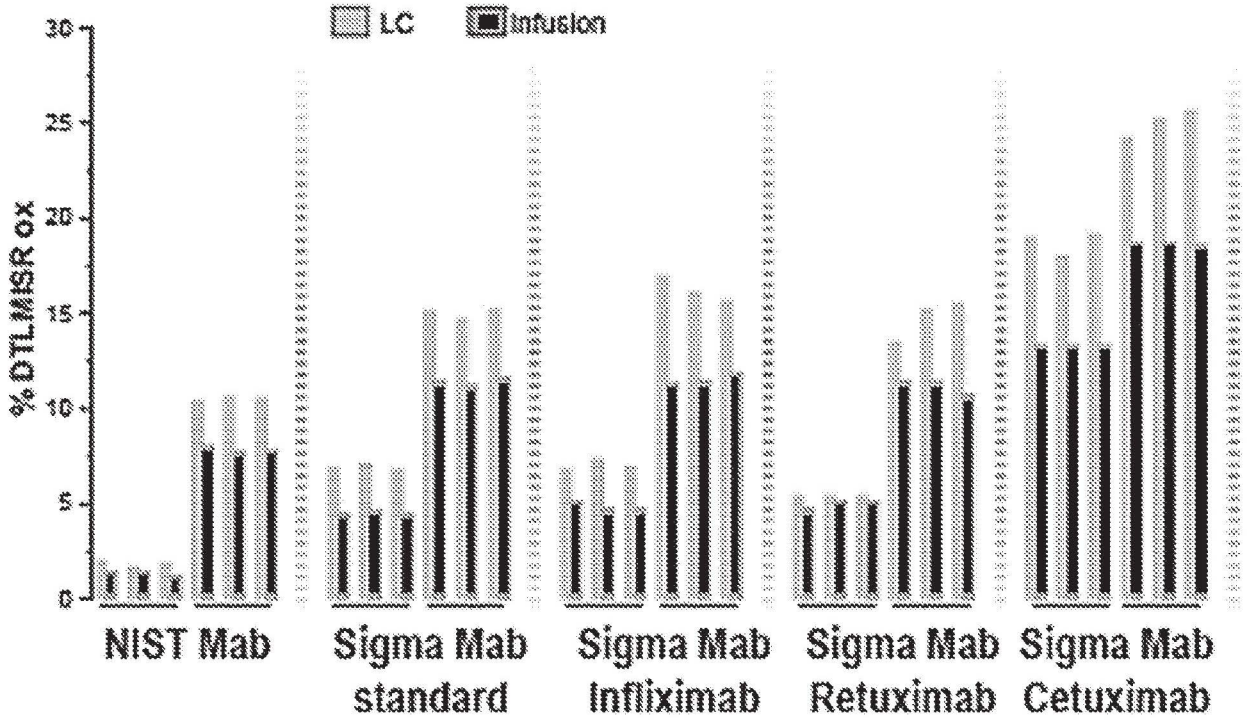
LC
Infusion
% DTLMISR ox
NIST Mab
Sigma Mab standard
Sigma Mab Infliximab
Sigma Mab Retuximab
Sigma Mab Cetuximab

Fig. 5C

Glycan	% by LC	% by INF
G0F	40.3	47.5
G1F	47.9	43.2
G2F	11.8	9.3

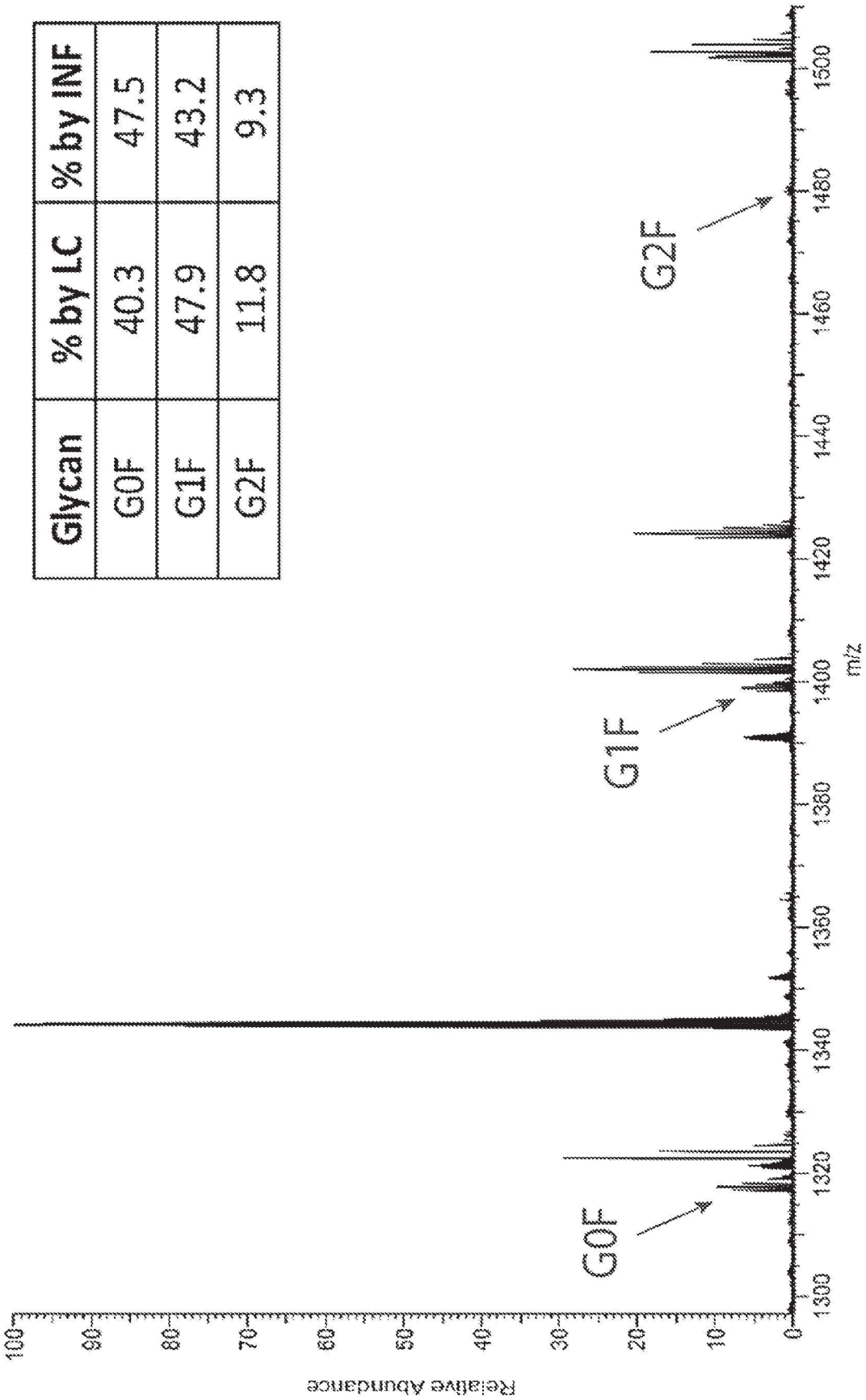

Fig. 6A

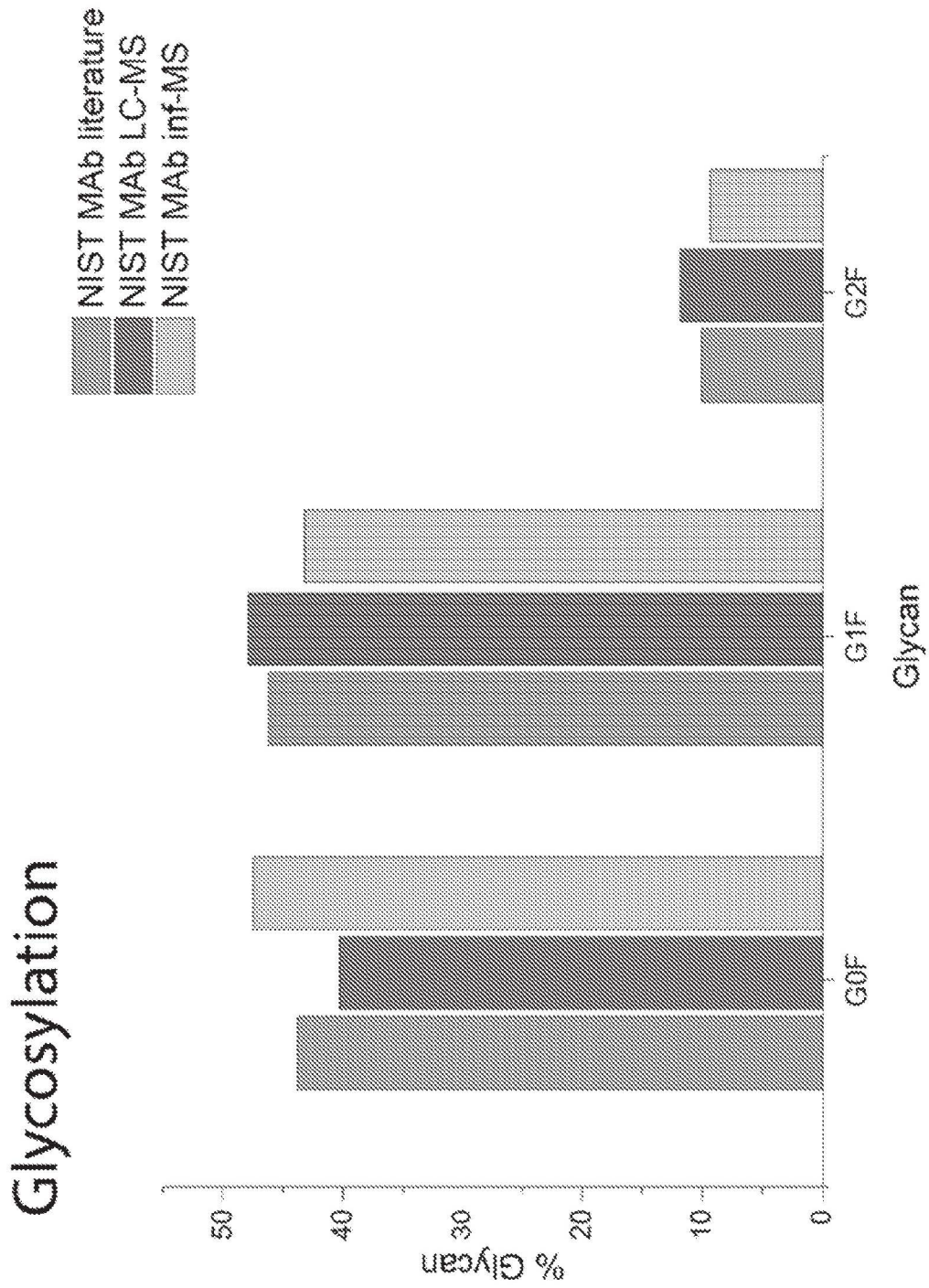
Glycosylation
NIST MAb literature
NIST MAb LC-MS
NIST MAb inf-MS
% Glycan
0
10
20
30
40
50
G0F
G1F
G2F
Glycan

Fig. 6B

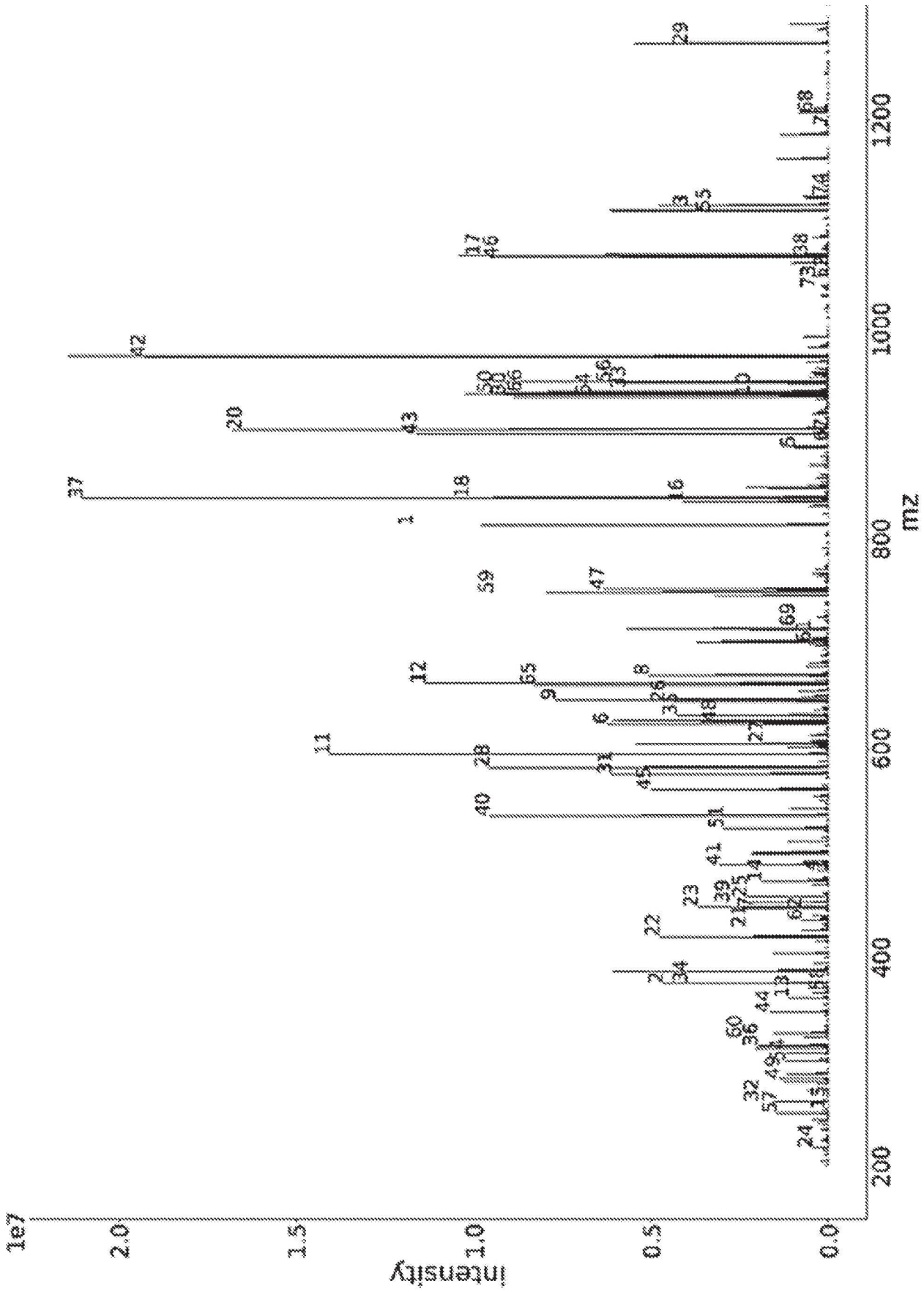

Fig. 7

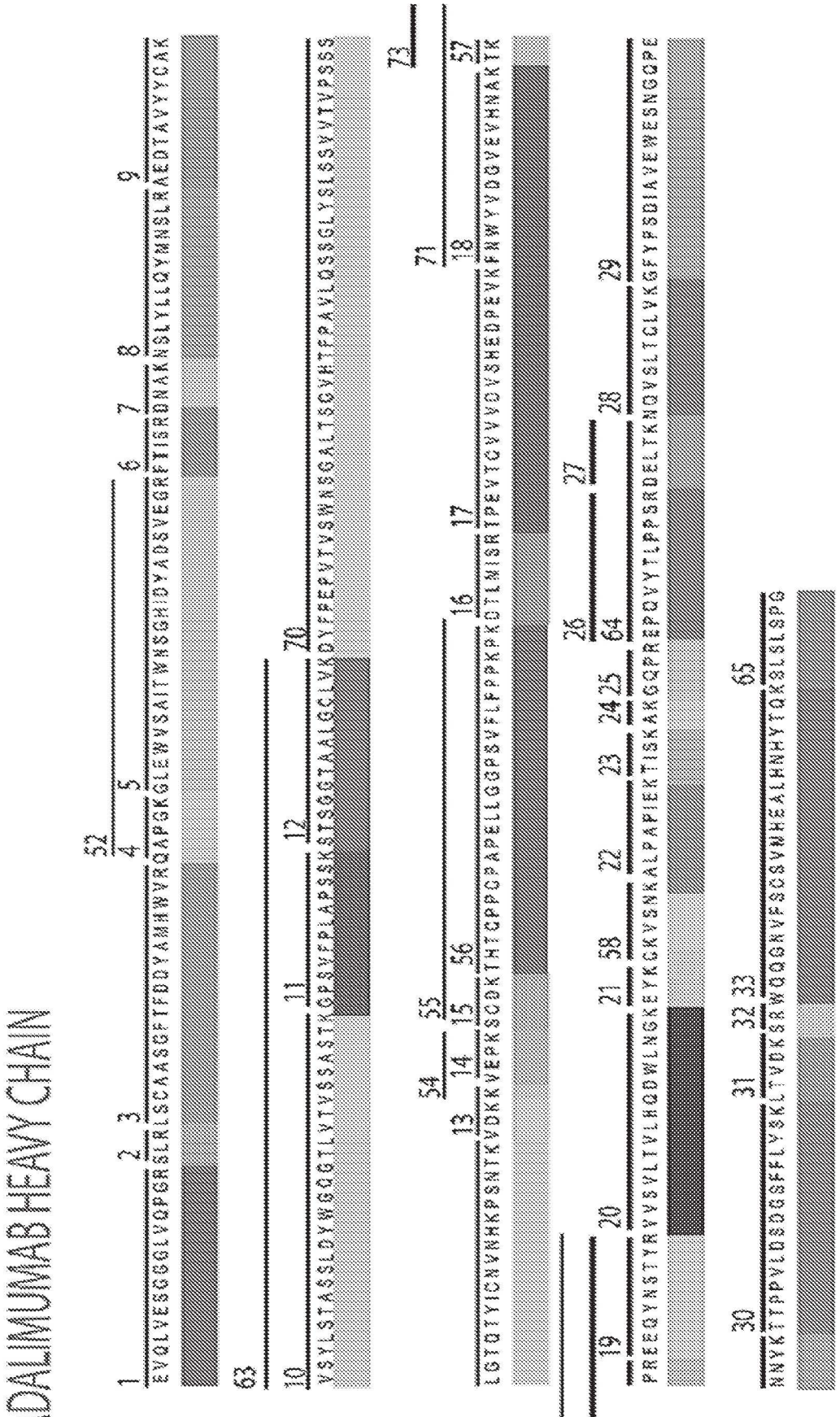

Fig. 8

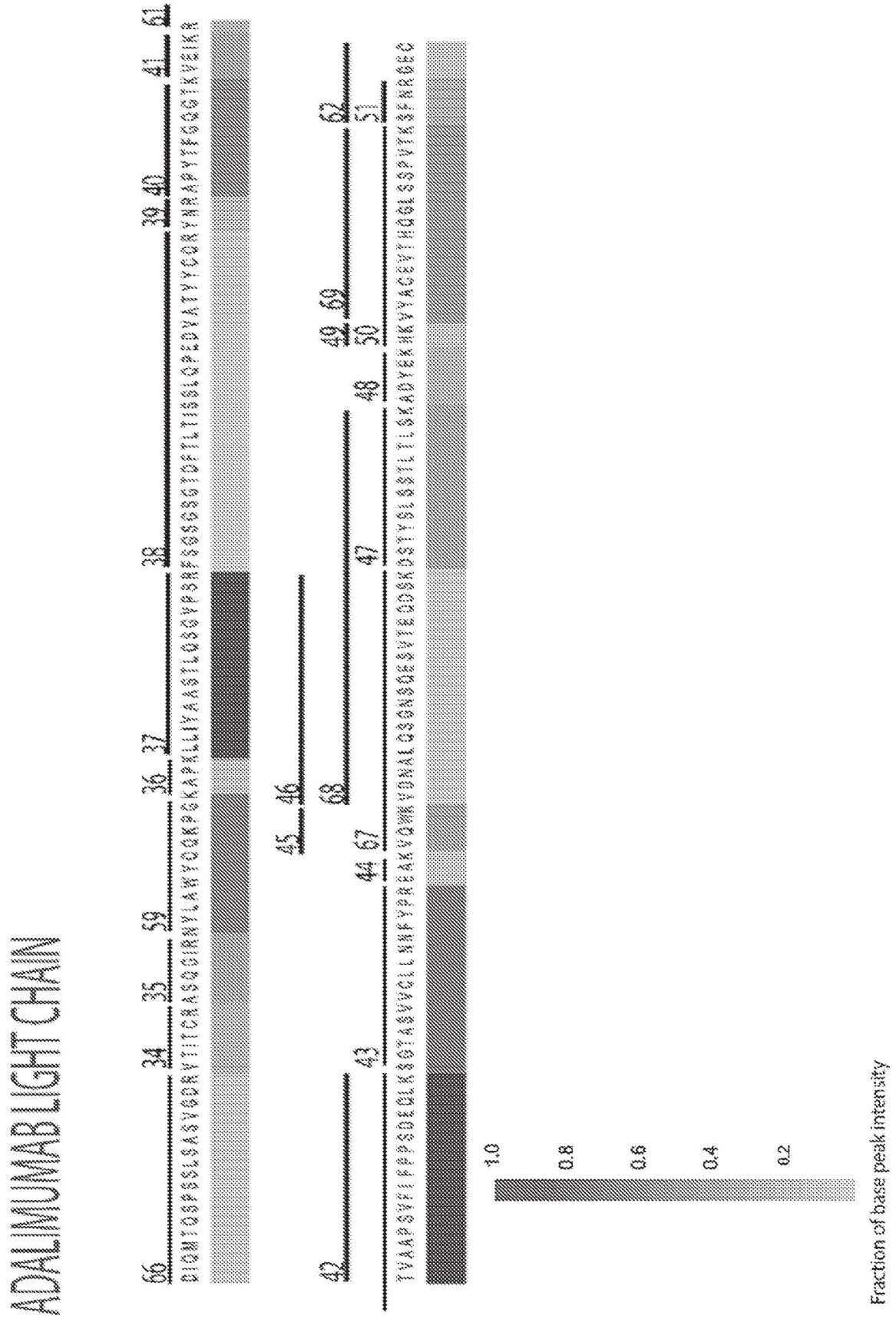

Fig. 8 continued

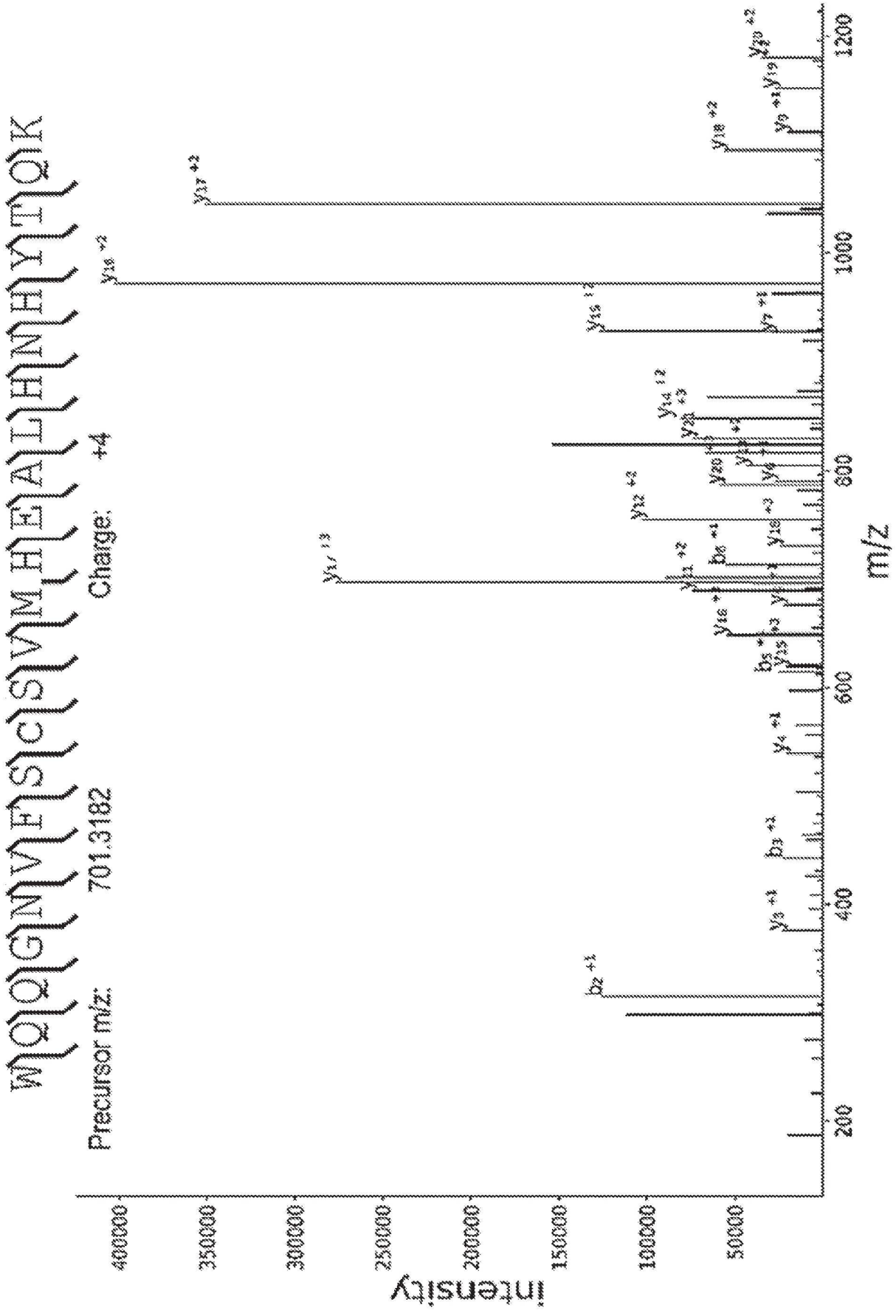
WQQGMVEFSCSVVMHEALHNHYTQK
Precursor m/z: 701.3182
Charge: +4
intensity
m/z
400000
350000
300000
250000
200000
150000
100000
50000
200
400
600
800
1000
1200

Fig. 9

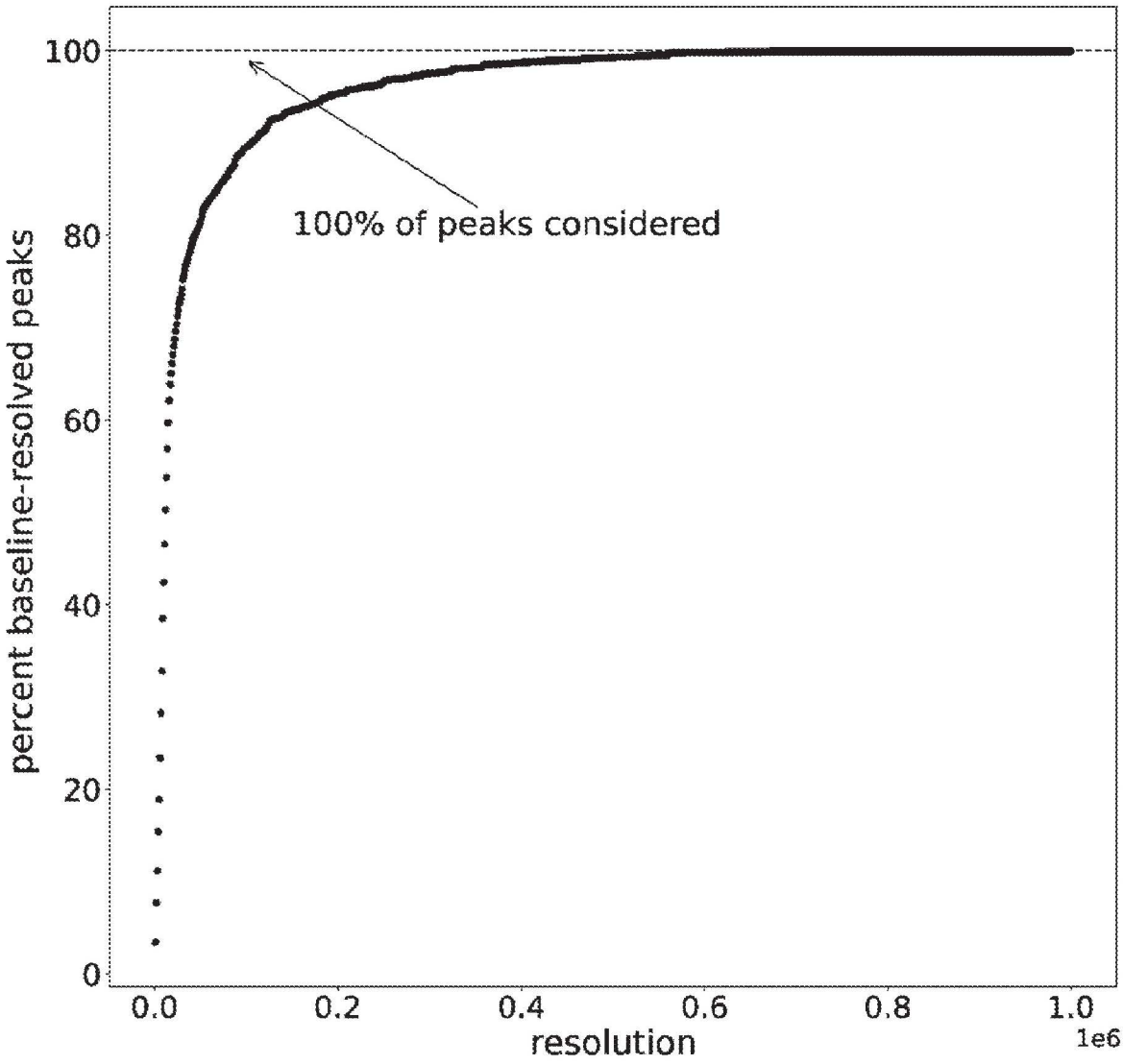
100
80
60
40
20
0
percent baseline-resolved peaks
100% of peaks considered
0.0
0.2
0.4
0.6
0.8
1.0
resolution
1e6

Fig. 10

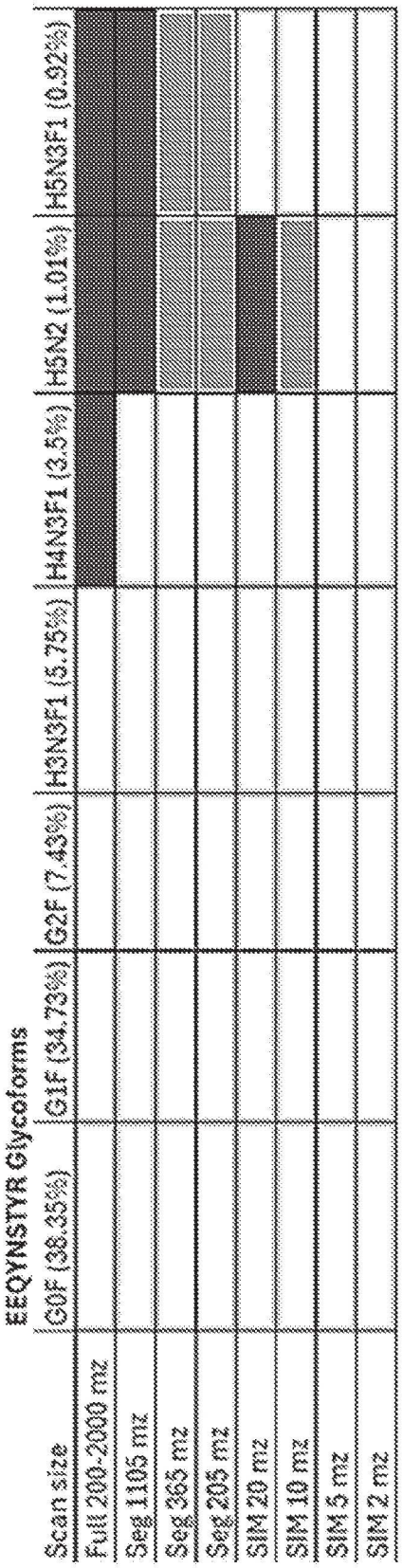
EEQYNSTYR Glycoforms
Scan size
G0F (38.35%)
G1F (34.73%)
G2F (7.43%)
H3N3F1 (5.75%)
H4N3F1 (3.5%)
H5N2 (1.01%)
H5N3F1 (0.92%)
Full 200-2000 mz
Seg 1105 mz
Seg 365 mz
Seg 205 mz
SIM 20 mz
SIM 10 mz
SIM 5 mz
SIM 2 mz

Fig. 11

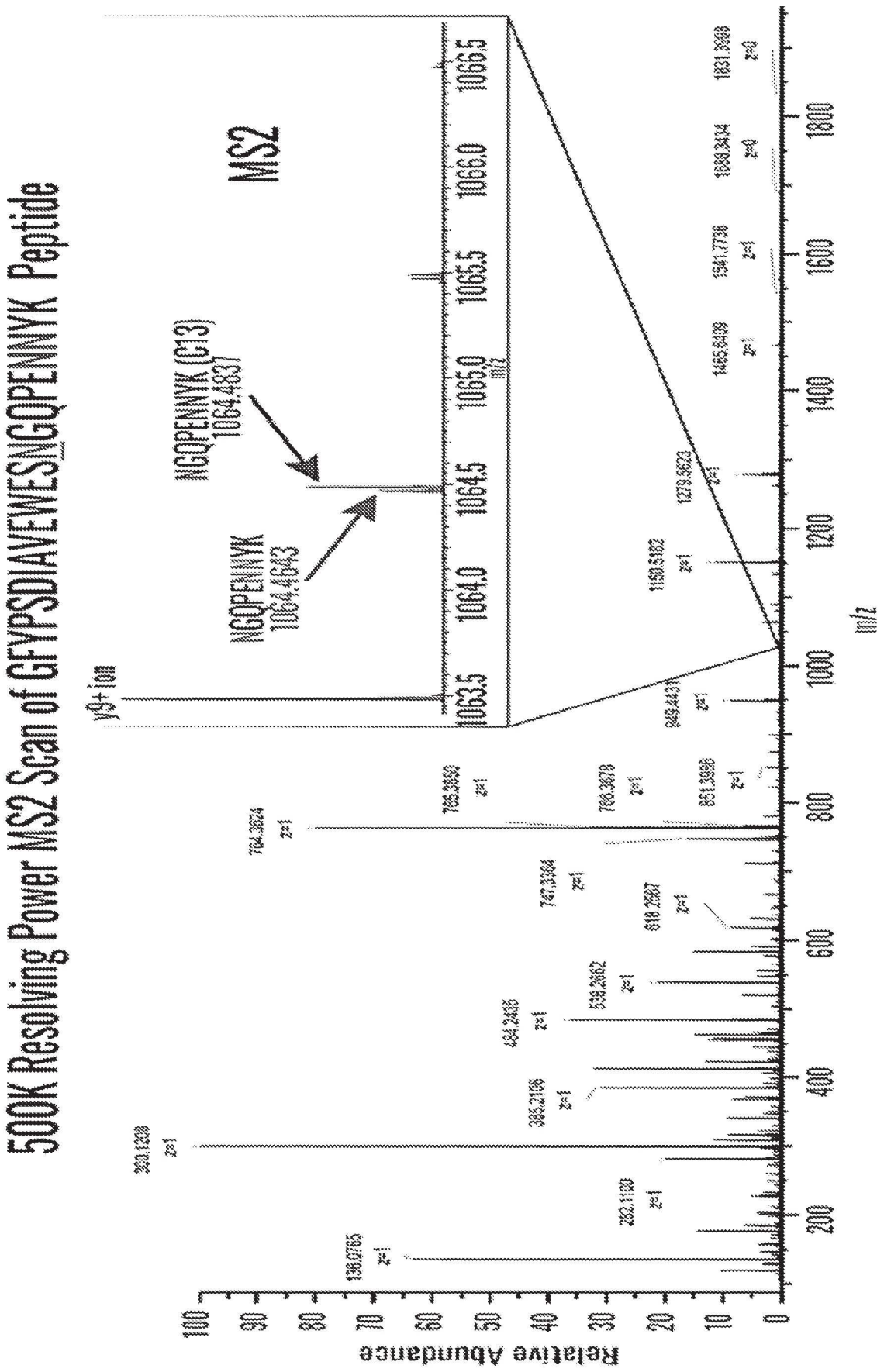
500K Resolving Power MS2 Scan of GFYPSDIAVEWESNGQPENNYK Peptide
MS2
NGQPENNYK (C13)
1064.4837
NGQPENNYK
1064.4643
y9+ ion
m/z
Relative Abundance

Fig. 12