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(54) Title: BIOANALYSIS OF THERAPEUTIC ANTIBODIES AND RELATED PRODUCTS USING IMMUNOPRECIPITATION AND NATIVE SCX-MS DETECTION

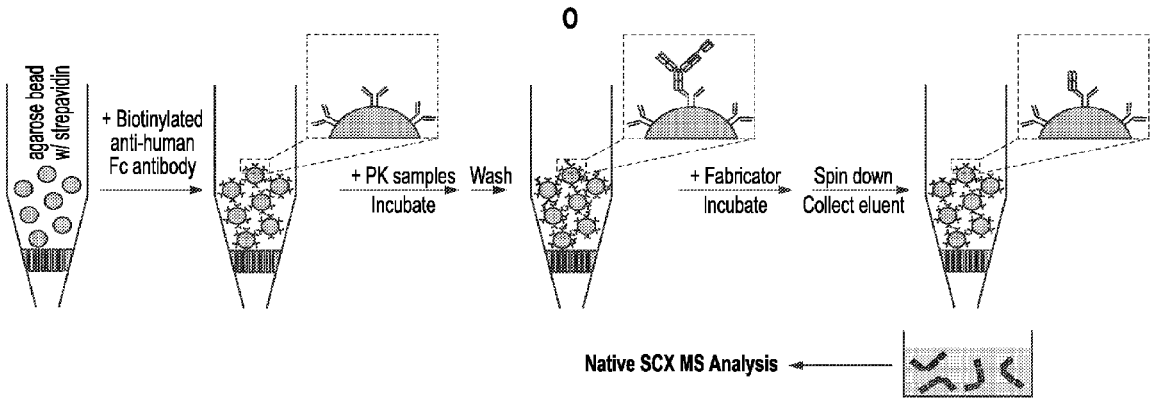

FIG. 1

(57) **Abstract:** The present invention generally pertains to methods of characterizing antibodies and related products. In particular, the present invention pertains to the use of immunoprecipitation and native strong cation exchange chromatography-mass spectrometry to specifically and sensitively detected and quantitate antibodies and related products in a sample.

[Continued on next page]

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BIOANALYSIS OF THERAPEUTIC ANTIBODIES AND RELATED PRODUCTS USING IMMUNOPRECIPITATION AND NATIVE SCX-MS DETECTION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and the benefit of U.S. Provisional Patent Application No. 63/221,439, filed July 13, 2021 which is herein incorporated by reference.

FIELD

[0002] The invention generally relates to methods for characterizing antibodies and related products.

BACKGROUND

[0003] Therapeutic peptides or proteins are expressed in cell culture suspension for production. Subsequently, the peptides or proteins are purified to remove process related impurities. The product quality attributes of the purified therapeutic peptides or proteins are extensively characterized to ensure preservation of their associated safety, efficacy, and shelf life profiles relevant to pharmacokinetics.

[0004] Alterations of therapeutic peptides or proteins may occur at any point during and after the production and/or purification process. The therapeutic peptides or proteins can become heterogeneous due to various post-translational modifications, protein degradation, enzymatic modifications, and chemical modifications. These alterations to the biophysical characteristics of biopharmaceutical products may affect associated safety, efficacy, and shelf life.

[0005] Other key features of a therapeutic peptide or protein include properties such as pharmacokinetics and pharmacodynamics that determine the abundance and timing of the therapy *in vivo*. Understanding the processing of a therapeutic *in vivo* can be essential to determining how that therapeutic is best produced and delivered, for example determining routes of administration, dosing, and therapeutic and adverse effects.

[0006] Accurately and efficiently assessing these features of a therapeutic peptide or protein, often in the context of a complex matrix such as serum that complicates detection, requires high-throughput, high-sensitivity and high-specificity techniques. It will be appreciated

that a need exists for methods and systems to achieve accurate characterization and quantitation of therapeutic peptides and proteins and their key features.

SUMMARY

[0007] A native SCX-MS method has been developed for the detection and quantitation of antibodies and related products. Immunoprecipitation with agarose beads coated in anti-human Fc antibody may be used to pull down a human antibody in a sample. The digestive enzyme IdeS or a variant thereof may be used to cleave the immobilized antibody, producing a Fab_2 fragment that may be eluted and collected. This fragment may then be subjected to native SCX-MS analysis for sensitive and robust quantitation. The method of the present invention was shown to efficiently and accurately quantitate antibodies even at low concentrations, in neat solution or in serum, as demonstrated in the Examples.

[0008] This disclosure provides a method for characterization of an antibody. In some exemplary embodiments, the method comprises: (a) immobilizing said antibody on a solid-phase substrate; (b) contacting said immobilized antibody to a digestive enzyme to produce an unbound fragment of said antibody; (c) eluting said antibody fragment; and (d) subjecting said eluate to native SCX-MS analysis to characterize said antibody.

[0009] In one aspect, said antibody is a monoclonal antibody or a bispecific antibody.

[0010] In one aspect, said immobilizing step comprises contacting a sample including said antibody to a solid-phase substrate capable of binding to said antibody. In a specific aspect, said sample is a serum sample.

[0011] In one aspect, said solid-phase substrate comprises beads. In a specific aspect, said beads are agarose beads or magnetic beads.

[0012] In a specific aspect, said binding of said solid-phase substrate is performed by an antibody adhered to said solid-phase substrate. In a further specific aspect, said antibody is an anti-Fc antibody.

[0013] In one aspect, the method further comprises a step of washing said solid-phase substrate after immobilizing said antibody.

[0014] In one aspect, said digestive enzyme is IdeS or a variant thereof. In another aspect, said antibody fragment is a Fab_2 fragment.

[0015] In one aspect, said eluting comprises a step of centrifuging said solid-phase substrate and antibody fragment.

[0016] In one aspect, said SCX system is coupled to said mass spectrometer. In another aspect, said mass spectrometer is an electrospray ionization mass spectrometer, nano-electrospray ionization mass spectrometer, or a triple quadrupole mass spectrometer.

[0017] In one aspect, said characterization of an antibody comprises quantitation of an antibody, optionally wherein said quantitation is normalized to an internal standard.

[0018] These, and other, aspects of the invention will be better appreciated and understood when considered in conjunction with the following description and accompanying drawings. The following description, while indicating various embodiments and numerous specific details thereof, is given by way of illustration and not of limitation. Many substitutions, modifications, additions, or rearrangements may be made within the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] FIG. 1 illustrates a workflow of the method of the present invention according to an exemplary embodiment.

[0020] FIG. 2 shows a comparison of the performance of different SCX columns in SCX-MS total ion chromatograms (TICs) for the separation of antibodies according to an exemplary embodiment.

[0021] FIG. 3A shows SCX-MS TICs for a range of different antibodies according to an exemplary embodiment.

[0022] FIG. 3B shows mass spectra of mAb1 at varying concentrations according to an exemplary embodiment.

[0023] FIG. 3C shows mass spectra of mAb2 at varying concentrations according to an exemplary embodiment.

[0024] FIG. 4A shows a SCX-MS TIC of mAb1 Fab_2 and internal standard mAb2 Fab_2 according to an exemplary embodiment.

[0025] FIG. 4B shows a linearity of measured mAb1 concentration between 20 pg and 20 ng in neat solution compared to an internal standard according to an exemplary embodiment.

[0026] FIG. 4C shows a linearity of measured mAb1 concentration between 20 ng and 2 µg in neat solution compared to an internal standard according to an exemplary embodiment.

[0027] FIG. 4D shows mass spectra for mAb1 at concentrations between 20 pg and 2 µg in neat solution according to an exemplary embodiment.

[0028] FIG. 5A shows a linearity of measured mAb1 concentration in serum when normalized to an internal standard according to an exemplary embodiment.

[0029] FIG. 5B shows an inset from FIG. 5A illustrating a linearity of measured mAb1 concentration at low concentrations in serum according to an exemplary embodiment.

[0030] FIG. 5C shows an inset from FIG. 5B illustrating a linearity of measured mAb1 concentration at low concentrations in serum according to an exemplary embodiment.

[0031] FIG. 6A shows a linearity of measured mAb1 concentration in serum without normalization to an internal standard according to an exemplary embodiment.

[0032] FIG. 6B shows an inset from FIG. 6A illustrating a linearity of measured mAb1 concentration at low concentrations in serum according to an exemplary embodiment.

[0033] FIG. 6C shows an inset from FIG. 6B illustrating a linearity of measured mAb1 concentration at low concentrations in serum according to an exemplary embodiment.

[0034] FIG. 7A shows a limit of detection (LOD) of mAb1 in serum in a mass spectrum according to an exemplary embodiment.

[0035] FIG. 7B shows a limit of quantitation (LOQ) of mAb1 in serum in a mass spectrum according to an exemplary embodiment.

DETAILED DESCRIPTION

[0036] Therapeutic peptides or proteins can become heterogeneous due to various post-translational modifications (PTMs), protein degradation, enzymatic modifications, and chemical modifications, which can be introduced at any point during and after the production and purification of peptides or proteins. Identification and characterization of the heterogeneous variants are critical to controlling the quality attributes of the biophysical characteristics of biopharmaceutical products. There are needs in the biopharmaceutical industry for rapid sensitive high-throughput analytical methods to control and monitor the production and

purification of therapeutic peptides or proteins, such as the production of monoclonal antibodies or antibody-drug conjugates.

[0037] Processing of a therapeutic peptide or protein *in vivo* after administration further determines features such as the efficacy and safety of the therapeutic. Properties such as the pharmacokinetics (PK) and pharmacodynamics (PD) of a peptide or protein may only become apparent after administration. Additionally, modifications to a therapeutic peptide or protein may continue to be made *in vivo*, resulting in biotransformation products that may not be predictable during manufacturing. Thus, in order to fully understand important attributes of a therapeutic, biological samples may be analyzed, which present increased complexity and challenges to sensitive and specific characterization and quantification of a protein or peptide of interest.

[0038] Electrospray ionization mass spectrometry (ESI MS)-based intact protein analysis has become an essential tool for the characterization of therapeutic proteins during development. Most commonly, MS is coupled with reversed phase liquid chromatography (RPLC) under denaturing conditions. However, the sensitivity of this method, and the signal-to-noise ratio produced by the resulting complex sample with a wide range of analyte charge states, has limits which may make it unreliable for accurate quantitation of low-abundance antibodies.

[0039] Recently, LC-MS systems comprising native ion exchange chromatography coupled online to ESI MS have been described (Yan *et al.*, 2020, *J Am Soc Mass Spectrom*, 31:2171-2179). The use of native strong cation exchange chromatography (SCX)-MS provides a number of advantages for analysis of therapeutic antibodies compared to conventional denaturing RPLC-MS. Native SCX-MS may demonstrate high sensitivity and a wide dynamic range compared to RPLC, and a superior ability to separate a target analyte from matrix, such as for example serum proteins in a serum sample. A native SCX-MS profile may also feature superior MS spatial resolution, making it easier to detect protein variants or biotransformation products.

[0040] As described above, there exists a need for sensitive methods to characterize and quantitate therapeutic proteins and peptides, such as therapeutic antibodies, in a sample. This disclosure sets forth a novel native SCX-MS method for characterizing an antibody, suitable for development of therapeutic antibodies.

[0041] Unless described otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing, particular methods and materials are now described.

[0042] The term "a" should be understood to mean "at least one" and the terms "about" and "approximately" should be understood to permit standard variation as would be understood by those of ordinary skill in the art and where ranges are provided, endpoints are included. As used herein, the terms "include," "includes," and "including" are meant to be non-limiting and are understood to mean "comprise," "comprises," and "comprising" respectively.

[0043] As used herein, the term "protein" or "protein of interest" can include any amino acid polymer having covalently linked amide bonds. Proteins comprise one or more amino acid polymer chains, generally known in the art as "polypeptides." "Polypeptide" refers to a polymer composed of amino acid residues, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof linked via peptide bonds. "Synthetic peptide or polypeptide" refers to a non-naturally occurring peptide or polypeptide. Synthetic peptides or polypeptides can be synthesized, for example, using an automated polypeptide synthesizer. Various solid phase peptide synthesis methods are known to those of skill in the art. A protein may comprise one or multiple polypeptides to form a single functioning biomolecule. In another exemplary aspect, a protein can include antibody fragments, nanobodies, recombinant antibody chimeras, cytokines, chemokines, peptide hormones, and the like. Proteins of interest can include any of bio-therapeutic proteins, recombinant proteins used in research or therapy, trap proteins and other chimeric receptor Fc-fusion proteins, chimeric proteins, antibodies, monoclonal antibodies, polyclonal antibodies, human antibodies, and bispecific antibodies. Proteins may be produced using recombinant cell-based production systems, such as the insect bacculovirus system, yeast systems (*e.g.*, Pichia sp.), and mammalian systems (*e.g.*, CHO cells and CHO derivatives like CHO-K1 cells). For a recent review discussing biotherapeutic proteins and their production, see Ghaderi et al., "Production platforms for biotherapeutic glycoproteins. Occurrence, impact, and challenges of non-human sialylation" (Darius Ghaderi et al., Production platforms for biotherapeutic glycoproteins. Occurrence, impact, and challenges of non-human sialylation, 28 BIOTECHNOLOGY AND GENETIC ENGINEERING REVIEWS 147–176 (2012), the entire teachings of which are herein incorporated). In some exemplary embodiments,

proteins comprise modifications, adducts, and other covalently linked moieties. These modifications, adducts and moieties include, for example, avidin, streptavidin, biotin, glycans (*e.g.*, N-acetylgalactosamine, galactose, neuraminic acid, N-acetylglucosamine, fucose, mannose, and other monosaccharides), PEG, polyhistidine, FLAGtag, maltose binding protein (MBP), chitin binding protein (CBP), glutathione-S-transferase (GST) myc-epitope, fluorescent labels and other dyes, and the like. Proteins can be classified on the basis of compositions and solubility and can thus include simple proteins, such as globular proteins and fibrous proteins; conjugated proteins, such as nucleoproteins, glycoproteins, mucoproteins, chromoproteins, phosphoproteins, metalloproteins, and lipoproteins; and derived proteins, such as primary derived proteins and secondary derived proteins.

[0044] In some exemplary embodiments, the protein of interest can be a recombinant protein, an antibody, a bispecific antibody, a multispecific antibody, antibody fragment, monoclonal antibody, fusion protein, scFv and combinations thereof.

[0045] As used herein, the term "recombinant protein" refers to a protein produced as the result of the transcription and translation of a gene carried on a recombinant expression vector that has been introduced into a suitable host cell. In certain exemplary embodiments, the recombinant protein can be an antibody, for example, a chimeric, humanized, or fully human antibody. In certain exemplary embodiments, the recombinant protein can be an antibody of an isotype selected from group consisting of: IgG, IgM, IgA1, IgA2, IgD, or IgE. In certain exemplary embodiments the antibody molecule is a full-length antibody (*e.g.*, an IgG1) or alternatively the antibody can be a fragment (*e.g.*, an Fc fragment or a Fab fragment).

[0046] The term "antibody," as used herein includes immunoglobulin molecules comprising four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, as well as multimers thereof (*e.g.*, IgM). Each heavy chain comprises a heavy chain variable region (abbreviated herein as HCVR or VH) and a heavy chain constant region. The heavy chain constant region comprises three domains, CH1, CH2 and CH3. Each light chain comprises a light chain variable region (abbreviated herein as LCVR or VL) and a light chain constant region. The light chain constant region comprises one domain (CL1). The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDRs), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and

four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. In different embodiments of the invention, the FRs of the anti-big-ET-1 antibody (or antigen-binding portion thereof) may be identical to the human germline sequences or may be naturally or artificially modified. An amino acid consensus sequence may be defined based on a side-by-side analysis of two or more CDRs. The term "antibody," as used herein, also includes antigen-binding fragments of full antibody molecules. The terms "antigen-binding portion" of an antibody, "antigen-binding fragment" of an antibody, and the like, as used herein, include any naturally occurring, enzymatically obtainable, synthetic, or genetically engineered polypeptide or glycoprotein that specifically binds an antigen to form a complex. Antigen-binding fragments of an antibody may be derived, for example, from full antibody molecules using any suitable standard techniques such as proteolytic digestion or recombinant genetic engineering techniques involving the manipulation and expression of DNA encoding antibody variable and optionally constant domains. Such DNA is known and/or is readily available from, for example, commercial sources, DNA libraries (including, *e.g.*, phage-antibody libraries), or can be synthesized. The DNA may be sequenced and manipulated chemically or by using molecular biology techniques, for example, to arrange one or more variable and/or constant domains into a suitable configuration, or to introduce codons, create cysteine residues, modify, add or delete amino acids, etc.

[0047] As used herein, an "antibody fragment" includes a portion of an intact antibody, such as, for example, the antigen-binding or variable region of an antibody. Examples of antibody fragments include, but are not limited to, a Fab fragment, a Fab' fragment, a F(ab')2 (or "Fab_2") fragment, a scFv fragment, a Fv fragment, a dsFv diabody, a dAb fragment, a Fd' fragment, a Fd fragment, and an isolated complementarity determining region (CDR) region, as well as triabodies, tetrabodies, linear antibodies, single-chain antibody molecules, and multi specific antibodies formed from antibody fragments. Fv fragments are the combination of the variable regions of the immunoglobulin heavy and light chains, and ScFv proteins are recombinant single chain polypeptide molecules in which immunoglobulin light and heavy chain variable regions are connected by a peptide linker. In some exemplary embodiments, an antibody fragment comprises a sufficient amino acid sequence of the parent antibody of which it is a fragment that it binds to the same antigen as does the parent antibody; in some exemplary embodiments, a fragment binds to the antigen with a comparable affinity to that of the parent

antibody and/or competes with the parent antibody for binding to the antigen. An antibody fragment may be produced by any means. For example, an antibody fragment may be enzymatically or chemically produced by fragmentation of an intact antibody and/or it may be recombinantly produced from a gene encoding the partial antibody sequence. In some exemplary embodiments, an antibody fragment may be produced by digestion with the digestive enzyme IdeS or a variant thereof. Alternatively, or additionally, an antibody fragment may be wholly or partially synthetically produced. An antibody fragment may optionally comprise a single chain antibody fragment. Alternatively, or additionally, an antibody fragment may comprise multiple chains that are linked together, for example, by disulfide linkages. An antibody fragment may optionally comprise a multi-molecular complex. A functional antibody fragment typically comprises at least about 50 amino acids and more typically comprises at least about 200 amino acids.

[0048] The term "bispecific antibody" includes an antibody capable of selectively binding two or more epitopes. Bispecific antibodies generally comprise two different heavy chains with each heavy chain specifically binding a different epitope—either on two different molecules (*e.g.*, antigens) or on the same molecule (*e.g.*, on the same antigen). If a bispecific antibody is capable of selectively binding two different epitopes (a first epitope and a second epitope), the affinity of the first heavy chain for the first epitope will generally be at least one to two or three or four orders of magnitude lower than the affinity of the first heavy chain for the second epitope, and vice versa. The epitopes recognized by the bispecific antibody can be on the same or a different target (*e.g.*, on the same or a different protein). Bispecific antibodies can be made, for example, by combining heavy chains that recognize different epitopes of the same antigen. For example, nucleic acid sequences encoding heavy chain variable sequences that recognize different epitopes of the same antigen can be fused to nucleic acid sequences encoding different heavy chain constant regions and such sequences can be expressed in a cell that expresses an immunoglobulin light chain.

[0049] A typical bispecific antibody has two heavy chains each having three heavy chain CDRs, followed by a CH1 domain, a hinge, a CH2 domain, and a CH3 domain, and an immunoglobulin light chain that either does not confer antigen-binding specificity but that can associate with each heavy chain, or that can associate with each heavy chain and that can bind one or more of the epitopes bound by the heavy chain antigen-binding regions, or that can

associate with each heavy chain and enable binding of one or both of the heavy chains to one or both epitopes. BsAbs can be divided into two major classes, those bearing an Fc region (IgG-like) and those lacking an Fc region, the latter normally being smaller than the IgG and IgG-like bispecific molecules comprising an Fc. The IgG-like bsAbs can have different formats such as, but not limited to, triomab, knobs into holes IgG (kih IgG), crossMab, orth-Fab IgG, Dual-variable domains Ig (DVD-Ig), two-in-one or dual action Fab (DAF), IgG-single-chain Fv (IgG-scFv), or κλ-bodies. The non-IgG-like different formats include tandem scFvs, diabody format, single-chain diabody, tandem diabodies (TandAbs), Dual-affinity retargeting molecule (DART), DART-Fc, nanobodies, or antibodies produced by the dock-and-lock (DNL) method (Gaowei Fan, Zujian Wang & Mingju Hao, Bispecific antibodies and their applications, 8 JOURNAL OF HEMATOLOGY & ONCOLOGY 130; Dafne Müller & Roland E. Kontermann, Bispecific Antibodies, HANDBOOK OF THERAPEUTIC ANTIBODIES 265–310 (2014), the entire teachings of which are herein incorporated). The methods of producing bsAbs are not limited to quadroma technology based on the somatic fusion of two different hybridoma cell lines, chemical conjugation, which involves chemical cross-linkers, and genetic approaches utilizing recombinant DNA technology. Examples of bsAbs include those disclosed in the following patent applications, which are hereby incorporated by reference: U.S. Ser. No. 12/823838, filed June 25, 2010; U.S. Ser. No. 13/ 488628, filed June 5, 2012; U.S. Ser. No. 14/031075, filed September 19, 2013; U.S. Ser. No. 14/808171, filed July 24, 2015; U.S. Ser. No. 15/713574, filed September 22, 2017; U.S. Ser. No. 15/713569, field September 22, 2017; U.S. Ser. No. 15/386453, filed December 21, 2016; U.S. Ser. No. 15/386443, filed December 21, 2016; U.S. Ser. No. 15/22343 filed July 29, 2016; and U.S. Ser. No. 15814095, filed November 15, 2017.

[0050] As used herein "multispecific antibody" refers to an antibody with binding specificities for at least two different antigens. While such molecules normally will only bind two antigens (*i.e.*, bispecific antibodies, bsAbs), antibodies with additional specificities such as trispecific antibody and KIH Trispecific can also be addressed by the system and method disclosed herein.

[0051] The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. A monoclonal antibody can be derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, by any means available or known in the art. Monoclonal antibodies useful with the present disclosure can be prepared using a

wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof.

[0052] In some exemplary embodiments, the protein of interest can be produced from mammalian cells. The mammalian cells can be of human origin or non-human origin can include primary epithelial cells (*e.g.*, keratinocytes, cervical epithelial cells, bronchial epithelial cells, tracheal epithelial cells, kidney epithelial cells and retinal epithelial cells), established cell lines and their strains (*e.g.*, 293 embryonic kidney cells, BHK cells, HeLa cervical epithelial cells and PER-C6 retinal cells, MDBK (NBL-1) cells, 911 cells, CRFK cells, MDCK cells, CHO cells, BeWo cells, Chang cells, Detroit 562 cells, HeLa 229 cells, HeLa S3 cells, Hep-2 cells, KB cells, LSI80 cells, LS174T cells, NCI-H-548 cells, RPMI2650 cells, SW-13 cells, T24 cells, WI-28 VA13, 2RA cells, WISH cells, BS-C-I cells, LLC-MK2 cells, Clone M-3 cells, 1-10 cells, RAG cells, TCMK-1 cells, Y-l cells, LLC-PKi cells, PK(15) cells, GHi cells, GH3 cells, L2 cells, LLC-RC 256 cells, MHiCi cells, XC cells, MDOK cells, VSW cells, and TH-I, B1 cells, BSC-1 cells, RAf cells, RK-cells, PK-15 cells or derivatives thereof), fibroblast cells from any tissue or organ (including but not limited to heart, liver, kidney, colon, intestines, esophagus, stomach, neural tissue (brain, spinal cord), lung, vascular tissue (artery, vein, capillary), lymphoid tissue (lymph gland, adenoid, tonsil, bone marrow, and blood), spleen, and fibroblast and fibroblast-like cell lines (*e.g.*, CHO cells, TRG-2 cells, IMR-33 cells, Don cells, GHK-21 cells, citrullinemia cells, Dempsey cells, Detroit 551 cells, Detroit 510 cells, Detroit 525 cells, Detroit 529 cells, Detroit 532 cells, Detroit 539 cells, Detroit 548 cells, Detroit 573 cells, HEL 299 cells, IMR-90 cells, MRC-5 cells, WI-38 cells, WI-26 cells, Midi cells, CHO cells, CV-1 cells, COS-1 cells, COS-3 cells, COS-7 cells, Vero cells, DBS-FrhL-2 cells, BALB/3T3 cells, F9 cells, SV-T2 cells, M-MSV-BALB/3T3 cells, K-BALB cells, BLO-11 cells, NOR-10 cells, C3H/IOTI/2 cells, HSDMiC3 cells, KLN205 cells, McCoy cells, Mouse L cells, Strain 2071 (Mouse L) cells, L-M strain (Mouse L) cells, L-MTK' (Mouse L) cells, NCTC clones 2472 and 2555, SCC-PSA1 cells, Swiss/3T3 cells, Indian muntjac cells, SIRC cells, Cn cells, and Jensen cells, Sp2/0, NS0, NS1 cells or derivatives thereof).

[0053] As used herein, "sample" can be obtained from any step of the bioprocess, such as cell culture fluid (CCF), harvested cell culture fluid (HCCF), any step in the downstream processing, drug substance (DS), or a drug product (DP) comprising the final formulated product. In some other specific exemplary embodiments, the sample can be selected from any

step of the downstream process of clarification, chromatographic production, viral inactivation, or filtration. In some specific exemplary embodiments, the drug product can be selected from manufactured drug product in the clinic, shipping, storage, or handling.

[0054] A sample may also be taken from a subject prior to and/or after administration of a therapeutic peptide or protein, in which case it may be a "biological sample" or "PK sample." A biological sample may be, for example, a tissue sample, a blood sample, a serum sample, a saliva sample, or a urinary sample. In an exemplary embodiment, a serum sample is taken from a subject in order to characterize and/or quantify a protein of interest after administration. In some exemplary embodiments, a biological sample is taken from a mouse.

[0055] As used herein, the term "impurity" can include any undesirable protein present in the protein biopharmaceutical product. Impurity can include process and product-related impurities. The impurity can further be of known structure, partially characterized, or unidentified. Process-related impurities can be derived from the manufacturing process and can include the three major categories: cell substrate-derived, cell culture-derived and downstream derived. Cell substrate-derived impurities include, but are not limited to, proteins derived from the host organism and nucleic acid (host cell genomic, vector, or total DNA). Cell culture-derived impurities include, but are not limited to, inducers, antibiotics, serum, and other media components. Downstream-derived impurities include, but are not limited to, enzymes, chemical and biochemical processing reagents (*e.g.*, cyanogen bromide, guanidine, oxidizing and reducing agents), inorganic salts (*e.g.*, heavy metals, arsenic, nonmetallic ion), solvents, carriers, ligands (*e.g.*, monoclonal antibodies), and other leachables. Product-related impurities (*e.g.*, precursors, certain degradation products) can be molecular variants arising during manufacture and/or storage that do not have properties comparable to those of the desired product with respect to activity, efficacy, and safety. Such variants may need considerable effort in isolation and characterization in order to identify the type of modification(s). Product-related impurities can include truncated forms, modified forms, and aggregates. Truncated forms are formed by hydrolytic enzymes or chemicals which catalyze the cleavage of peptide bonds. Modified forms include, but are not limited to, deamidated, isomerized, mismatched S-S linked, oxidized, or altered conjugated forms (*e.g.*, glycosylation, phosphorylation). Modified forms can also include any post-translational modification form. Aggregates include dimers and higher multiples of the desired product. (Q6B Specifications: Test Procedures and Acceptance Criteria for

Biotechnological/Biological Products, ICH August 1999, U.S. Dept. of Health and Humans Services).

[0056] As used herein, the general term "post-translational modifications" or "PTMs" refer to covalent modifications that polypeptides undergo, either during (co-translational modification) or after (post-translational modification) their ribosomal synthesis. PTMs are generally introduced by specific enzymes or enzyme pathways. Many occur at the site of a specific characteristic protein sequence (signature sequence) within the protein backbone. Several hundred PTMs have been recorded, and these modifications invariably influence some aspect of a protein's structure or function (Walsh, G. "Proteins" (2014) second edition, published by Wiley and Sons, Ltd., ISBN: 9780470669853). The various post-translational modifications include, but are not limited to, cleavage, N-terminal extensions, protein degradation, acylation of the N-terminus, biotinylation (acylation of lysine residues with a biotin), amidation of the C-terminal, glycosylation, iodination, covalent attachment of prosthetic groups, acetylation (the addition of an acetyl group, usually at the N-terminus of the protein), alkylation (the addition of an alkyl group (*e.g.* methyl, ethyl, propyl) usually at lysine or arginine residues), methylation, adenylation, ADP-ribosylation, covalent cross links within, or between, polypeptide chains, sulfonation, prenylation, Vitamin C dependent modifications (proline and lysine hydroxylations and carboxy terminal amidation), Vitamin K dependent modification wherein Vitamin K is a cofactor in the carboxylation of glutamic acid residues resulting in the formation of a γ-carboxyglutamate (a glu residue), glutamylation (covalent linkage of glutamic acid residues), glycylation (covalent linkage glycine residues), glycosylation (addition of a glycosyl group to either asparagine, hydroxylysine, serine, or threonine, resulting in a glycoprotein), isoprenylation (addition of an isoprenoid group such as farnesol and geranylgeraniol), lipoylation (attachment of a lipoate functionality), phosphopantetheinylation (addition of a 4′-phosphopantetheinyl moiety from coenzyme A, as in fatty acid, polyketide, non-ribosomal peptide and leucine biosynthesis), phosphorylation (addition of a phosphate group, usually to serine, tyrosine, threonine or histidine), and sulfation (addition of a sulfate group, usually to a tyrosine residue). The post-translational modifications that change the chemical nature of amino acids include, but are not limited to, citrullination (the conversion of arginine to citrulline by deimination), and deamidation (the conversion of glutamine to glutamic acid or asparagine to aspartic acid). The post-translational modifications that involve structural changes include, but are not limited

to, formation of disulfide bridges (covalent linkage of two cysteine amino acids) and proteolytic cleavage (cleavage of a protein at a peptide bond). Certain post-translational modifications involve the addition of other proteins or peptides, such as ISGylation (covalent linkage to the ISG15 protein (Interferon-Stimulated Gene)), SUMOylation (covalent linkage to the SUMO protein (Small Ubiquitin-related MOdifier)) and ubiquitination (covalent linkage to the protein ubiquitin). See European Bioinformatics Institute Protein Information ResourceSIB Swiss Institute of Bioinformatics, European Bioinformatics Institute Drs - Drosomycin precursor - Drosophila melanogaster (Fruit fly) - Drs gene & protein, http://www.uniprot.org/docs/ptmlist (last visited Jan 15, 2019) for a more detailed controlled vocabulary of PTMs curated by UniProt.

[0057] Post-translational modifications, charge variants, or size variants of a therapeutic peptide or protein may arise at any point during the production, manufacture, storage, delivery, or administration of a therapeutic peptide or protein. Additional modifications to a peptide or protein may occur *in vivo* after administration to a subject, in a process referred to as "biotransformation." Biotransformation products may have modified properties compared to a pre-administration therapeutic. Biotransformation often leads to a reduction in size of a therapeutic, such that detection methods with higher sensitivity for smaller analytes may be preferred. In some exemplary embodiments, the method of the present invention features high sensitivity for biotransformation products of a protein of interest.

[0058] In some exemplary embodiments, the method for characterizing and/or quantifying a protein of interest can optionally comprise enriching a protein of interest in the sample matrix using immunoprecipitation (IP). As used herein, the term "immunoprecipitation" can include a process of precipitating a protein antigen out of solution using an antibody that specifically binds to that particular protein. Immunoprecipitation may be direct, in which antibodies for the target protein are immobilized on a solid-phase substrate, or indirect, in which free antibodies are added to the protein mixture and later captured with, for example, protein A/G beads.

[0059] In some exemplary embodiments, the solid-phase substrate may be beads, for example agarose beads or magnetic beads. Beads may be coated in streptavidin in order to facilitate adherence to an antibody. A biotinylated "capture" antibody may then be contacted to the streptavidin-coated beads, adhering to the beads and forming "immunoprecipitation beads" capable of binding to the antigen of the adhered antibody. In some exemplary embodiments, the

adhered capture antibody may be an anti-Fc antibody, and may specifically be an anti-human Fc antibody.

[0060] An anti-human Fc antibody will preferentially bind to the Fc domain of any human antibody, such as for example a therapeutic antibody, and thus may be used to immunoprecipitate or "pull down" a therapeutic antibody from a sample, allowing it to be enriched for analysis. After immunoprecipitation of a therapeutic antibody, a digestive enzyme may be contacted to the immunoprecipitation mixture to cleave the therapeutic antibody and release antibody fragments that may then be eluted for further analysis. In an exemplary embodiment, IdeS or variants thereof are used as a digestive enzyme. IdeS cleavage produces two antibody fragments: an Fc fragment and a Fab_2 fragment. When the Fc domain of a therapeutic antibody is bound to an anti-human Fc capture antibody, cleavage with IdeS will result in the release of an unbound Fab_2 fragment, which can then be eluted for further analysis. In an exemplary embodiment, eluted Fab_2 fragments are subjected to liquid chromatography-mass spectrometry analysis, in particular native SCX-MS.

[0061] As used herein, the term "digestion" refers to hydrolysis of one or more peptide bonds of a protein. There are several approaches to carrying out digestion of a protein in a sample using an appropriate hydrolyzing agent, for example, enzymatic digestion or non-enzymatic digestion.

[0062] As used herein, the term "digestive enzyme" refers to any of a large number of different agents that can perform digestion of a protein. Non-limiting examples of hydrolyzing agents that can carry out enzymatic digestion include protease from Aspergillus Saitoi, elastase, subtilisin, protease XIII, pepsin, trypsin, Tryp-N, chymotrypsin, aspergillopepsin I, LysN protease (Lys-N), LysC endoproteinase (Lys-C), endoproteinase Asp-N (Asp-N), endoproteinase Arg-C (Arg-C), endoproteinase Glu-C (Glu-C) or outer membrane protein T (OmpT), immunoglobulin-degrading enzyme of Streptococcus pyogenes (IdeS), thermolysin, papain, pronase, V8 protease or biologically active fragments or homologs thereof or combinations thereof. For a recent review discussing the available techniques for protein digestion see Switazar et al., "Protein Digestion: An Overview of the Available Techniques and Recent Developments" (Linda Switzar, Martin Giera & Wilfried M. A. Niessen, Protein Digestion: An Overview of the Available Techniques and Recent Developments, 12 JOURNAL OF PROTEOME RESEARCH 1067–1077 (2013)).

[0063] In some exemplary embodiments, IdeS or a variant thereof is used to cleave an antibody below the hinge region, producing an Fc fragment and a Fab_2 fragment. Digestion of an analyte may be advantageous because size reduction may increase the sensitivity and specificity of characterization and detection of the analyte using LC-MS. When used for this purpose, digestion that separates out an Fc fragment and keeps a Fab_2 fragment for analysis may be preferred. This is because variable regions of interest, such as the complementarity-determining region (CDR) of an antibody, are contained in the Fab_2 fragment, while the Fc fragment may be relatively uniform between antibodies and thus provide less relevant information. Additionally, IdeS digestion has a high efficiency, allowing for high recovery of an analyte. The digestion and elution process may be performed under native conditions, allowing for simple coupling to a native LC-MS system.

[0064] IdeS or variants thereof are commercially available and may be marketed as, for example, FabRICATOR® or FabRICATOR Z®.

[0065] As used herein, the term "liquid chromatography" refers to a process in which a biological/chemical mixture carried by a liquid can be separated into components as a result of differential distribution of the components as they flow through (or into) a stationary liquid or solid phase. Non-limiting examples of liquid chromatography include reverse phase liquid chromatography, ion-exchange chromatography, size exclusion chromatography, affinity chromatography, hydrophobic interaction chromatography, hydrophilic interaction chromatography, or mixed-mode chromatography.

[0066] In some exemplary embodiments, the method for characterizing and/or quantifying a protein of interest can include the use of strong cation exchange (SCX) chromatography. Cation exchange chromatography is a subset of ion exchange chromatography that uses a stationary phase presenting a negatively charged functional group in order to capture positively charged analytes. The pH of the chromatography buffer can be gradually adjusted in order to release and elute the analytes in order of pI.

[0067] Cation exchange chromatography uses a "cation exchange chromatography material." Cation exchange chromatography can be further subdivided into, for example, strong cation exchange (SCX) or weak cation exchange, depending on the cation exchange chromatography material employed. Cation exchange chromatography materials with a sulfonic

acid group (S) may be used in strong cation exchangers, while cation exchange chromatography materials with a carboxymethyl group (CM) may be used in weak cation exchangers. Strong cation exchangers include, for example SOURCE S, which uses a functional group of methyl sulfate, and SP Sepharose, which uses a functional group of sulfopropyl. Weak cation exchangers include, for example, CM- Cellulose, which uses a functional group of carboxymethyl. SCX may be preferred because a wider range of pH buffers may be used without losing the charge of the strong cation exchanger, allowing for effective separation of analytes with a wide pI range.

[0068] Cation exchange chromatography materials are available under different names from a multitude of companies such as, for example, Bio-Rex, Macro-Prep CM (available from BioRad Laboratories, Hercules, Calif., USA), weak cation exchanger WCX 2 (available from Ciphergen, Fremont, Calif., USA), Dowex MAC-3 (available from Dow chemical company, Midland, Mich., USA), Mustang C (available from Pall Corporation, East Hills, N.Y., USA), Cellulose CM-23, CM-32, CM-52, hyper-D, and partisphere (available from Whatman plc, Brentford, UK), Amberlite IRC 76, IRC 747, IRC 748, GT 73 (available from Tosoh Bioscience GmbH, Stuttgart, Germany), CM 1500, CM 3000 (available from BioChrom Labs, Terre Haute, Ind., USA), and CM-Sepharose Fast Flow (available from GE Healthcare, Life Sciences, Germany). In addition, commercially available cation exchange resins further include carboxymethyl-cellulose, Bakerbond ABX, sulphopropyl (SP) immobilized on agarose (*e.g.* SP-Sepharose Fast Flow or SP-Sepharose High Performance, available from GE Healthcare—Amersham Biosciences Europe GmbH, Freiburg, Germany) and sulphonyl immobilized on agarose (*e.g.* S-Sepharose Fast Flow available from GE Healthcare, Life Sciences, Germany).

[0069] Cation exchange chromatography materials include mixed-mode chromatography materials performing a combination of ion exchange and hydrophobic interaction technologies (*e.g.*, Capto adhere, Capto MMC, MEP HyperCell, Eshmuno HCX, etc.), mixed-mode chromatography materials performing a combination of anion exchange and cation exchange technologies (*e.g.*, hydroxyapatite, ceramic hydroxyapatite, etc.), and the like. Cation exchange chromatography materials that may be used in cation exchange chromatography in the present invention may include, but are not limited to, all the commercially available cation exchange chromatography materials as described above.

[0070] While denaturing RPLC-MS is a conventional technique in the characterization of therapeutic proteins, native SCX-MS may provide analytical advantages as described herein. For example, native SCX-MS may provide improved sensitivity and specificity of detection. In cases where the detection limits of RPLC and SCX are comparable, SCX may provide superior data quality and a higher signal-to-noise ratio. SCX may have an improved ability to separate a target analyte from matrix proteins, for example serum proteins in a serum sample, and additionally may have an improved ability to separate biotransformation products of a protein of interest. Thus, the preferred chromatography for the method of the present invention is native SCX, and disclosed herein is a novel method of characterizing and/or quantifying a protein of interest using native SCX.

[0071] As used herein, the term "mass spectrometer" includes a device capable of identifying specific molecular species and measuring their accurate masses. The term is meant to include any molecular detector into which a polypeptide or peptide may be characterized. A mass spectrometer can include three major parts: the ion source, the mass analyzer, and the detector. The role of the ion source is to create gas phase ions. Analyte atoms, molecules, or clusters can be transferred into gas phase and ionized either concurrently (as in electrospray ionization) or through separate processes. The choice of ion source depends on the application. In some exemplary embodiments, the mass spectrometer can be a tandem mass spectrometer. As used herein, the term "tandem mass spectrometry" includes a technique where structural information on sample molecules is obtained by using multiple stages of mass selection and mass separation. A prerequisite is that the sample molecules be transformed into a gas phase and ionized so that fragments are formed in a predictable and controllable fashion after the first mass selection step. Multistage MS/MS, or MS^n, can be performed by first selecting and isolating a precursor ion (MS^2), fragmenting it, isolating a primary fragment ion (MS^3), fragmenting it, isolating a secondary fragment (MS^4), and so on, as long as one can obtain meaningful information, or the fragment ion signal is detectable. Tandem MS has been successfully performed with a wide variety of analyzer combinations. Which analyzers to combine for a certain application can be determined by many different factors, such as sensitivity, selectivity, and speed, but also size, cost, and availability. The two major categories of tandem MS methods are tandem-in-space and tandem-in-time, but there are also hybrids where tandem-in-time analyzers are coupled in space or with tandem-in-space analyzers. A tandem-in-space mass

spectrometer comprises an ion source, a precursor ion activation device, and at least two non-trapping mass analyzers. Specific m/z separation functions can be designed so that in one section of the instrument ions are selected, dissociated in an intermediate region, and the product ions are then transmitted to another analyzer for m/z separation and data acquisition. In tandem-in-time, mass spectrometer ions produced in the ion source can be trapped, isolated, fragmented, and m/z separated in the same physical device. The peptides identified by the mass spectrometer can be used as surrogate representatives of the intact protein and their post translational modifications. They can be used for protein characterization by correlating experimental and theoretical MS/MS data, the latter generated from possible peptides in a protein sequence database. The characterization includes, but is not limited, to sequencing amino acids of the protein fragments, determining protein sequencing, determining protein *de novo* sequencing, locating post-translational modifications, or identifying post translational modifications, or comparability analysis, or combinations thereof.

[0072] In some exemplary aspects, the mass spectrometer can work using nanoelectrospray or nanospray.

[0073] The term "nanoelectrospray" or "nanospray" as used herein refers to electrospray ionization at a very low solvent flow rate, typically hundreds of nanoliters per minute of sample solution or lower, often without the use of an external solvent delivery. The electrospray infusion setup forming a nanoelectrospray can use a static nanoelectrospray emitter or a dynamic nanoelectrospray emitter. A static nanoelectrospray emitter performs a continuous analysis of small sample (analyte) solution volumes over an extended period of time. A dynamic nanoelectrospray emitter uses a capillary column and a solvent delivery system to perform chromatographic separations on mixtures prior to analysis by the mass spectrometer.

[0074] In some exemplary embodiments, SCX-MS can be performed under native conditions.

[0075] As used herein, the term "native conditions" can include performing mass spectrometry under conditions that preserve non-covalent interactions in an analyte. Native mass spectrometry is an approach to study intact biomolecular structure in the native or near-native state. The term "native" refers to the biological status of the analyte in solution prior to subjecting to the ionization. Several parameters, such as pH and ionic strength, of the solution containing the biological analytes can be controlled to maintain the native folded state of the

biological analytes in solution. Commonly, native mass spectrometry is based on electrospray ionization, wherein the biological analytes are sprayed from a nondenaturing solvent. Other terms, such as noncovalent, native spray, electrospray ionization, nondenaturing, macromolecular, or supramolecular mass spectrometry can also be describing native mass spectrometry. In exemplary embodiments, native MS allows for better spatial resolution compared to non-native MS, improving detection of biotransformation products of a therapeutic protein. For detailed review on native MS, refer to the review: Elisabetta Boeri Erba & Carlo Pe-tosa, The emerging role of native mass spectrometry in characterizing the structure and dynamics of macromolecular complexes, 24 PROTEIN SCIENCE1176–1192 (2015).

[0076] In some exemplary embodiments, SCX-MS can be performed under non-native conditions. A peptide or protein of interest may be prepared by, for example, alkylation, reduction, denaturation, and/or digestion.

[0077] As used herein, the term "protein alkylating agent" refers to an agent used for alkylating certain free amino acid residues in a protein. Non-limiting examples of protein alkylating agents are iodoacetamide (IOA), chloroacetamide (CAA), acrylamide (AA), N-ethylmaleimide (NEM), methyl methanethiosulfonate (MMTS), and 4-vinylpyridine or combinations thereof.

[0078] As used herein, "protein denaturing" can refer to a process in which the three-dimensional shape of a molecule is changed from its native state. Protein denaturation can be carried out using a protein denaturing agent. Non-limiting examples of a protein denaturing agent include heat, high or low pH, reducing agents like DTT (see below) or exposure to chaotropic agents. Several chaotropic agents can be used as protein denaturing agents. Chaotropic solutes increase the entropy of the system by interfering with intramolecular interactions mediated by non-covalent forces such as hydrogen bonds, van der Waals forces, and hydrophobic effects. Non-limiting examples for chaotropic agents include butanol, ethanol, guanidinium chloride, lithium perchlorate, lithium acetate, magnesium chloride, phenol, propanol, sodium dodecyl sulfate, thiourea, N-lauroylsarcosine, urea, and salts thereof.

[0079] As used herein, the term "protein reducing agent" refers to the agent used for reduction of disulfide bridges in a protein. Non-limiting examples of protein reducing agents used to reduce a protein are dithiothreitol (DTT), ß-mercaptoethanol, Ellman's reagent,

hydroxylamine hydrochloride, sodium cyanoborohydride, tris(2-carboxyethyl)phosphine hydrochloride (TCEP-HCl), or combinations thereof.

[0080] In some exemplary aspects, the mass spectrometer can be a tandem mass spectrometer.

[0081] As used herein, the term "tandem mass spectrometry" includes a technique where structural information on sample molecules is obtained by using multiple stages of mass selection and mass separation. A prerequisite is that the sample molecules can be transferred into gas phase and ionized intact and that they can be induced to fall apart in some predictable and controllable fashion after the first mass selection step. Multistage MS/MS, or MS^n, can be performed by first selecting and isolating a precursor ion (MS^2), fragmenting it, isolating a primary fragment ion (MS^3), fragmenting it, isolating a secondary fragment (MS^4), and so on as long as one can obtain meaningful information, or the fragment ion signal is detectable. Tandem MS has been successfully performed with a wide variety of analyzer combinations. What analyzers to combine for a certain application can be determined by many different factors, such as sensitivity, selectivity, and speed, but also size, cost, and availability. The two major categories of tandem MS methods are tandem-in-space and tandem-in-time, but there are also hybrids where tandem-in-time analyzers are coupled in space or with tandem-in-space analyzers. A tandem-in-space mass spectrometer comprises an ion source, a precursor ion activation device, and at least two non-trapping mass analyzers. Specific *m/z* separation functions can be designed so that in one section of the instrument ions are selected, dissociated in an intermediate region, and the product ions are then transmitted to another analyzer for *m/z* separation and data acquisition. In tandem-in-time, mass spectrometer ions produced in the ion source can be trapped, isolated, fragmented, and *m/z* separated in the same physical device.

[0082] The peptides identified by the mass spectrometer can be used as surrogate representatives of the intact protein and their post-translational modifications. They can be used for protein characterization by correlating experimental and theoretical MS/MS data, the latter generated from possible peptides in a protein sequence database. The characterization includes, but is not limited, to sequencing amino acids of the protein fragments, determining protein sequencing, determining protein de novo sequencing, locating post-translational modifications, or identifying post-translational modifications, or comparability analysis, or combinations thereof.

[0083] As used herein, the term "database" refers to a compiled collection of protein sequences that may possibly exist in a sample, for example in the form of a file in a FASTA format. Relevant protein sequences may be derived from cDNA sequences of a species being studied. Public databases that may be used to search for relevant protein sequences included databases hosted by, for example, Uniprot or Swiss-prot. Databases may be searched using what are herein referred to as "bioinformatics tools". Bioinformatics tools provide the capacity to search uninterpreted MS/MS spectra against all possible sequences in the database(s), and provide interpreted (annotated) MS/MS spectra as an output. Non-limiting examples of such tools are Mascot (www.matrixscience.com), Spectrum Mill (www.chem.agilent.com), PLGS (www.waters.com), PEAKS (www.bioinformaticssolutions.com), Proteinpilot (download.appliedbiosystems.com//proteinpilot), Phenyx (www.phenyx-ms.com), Sorcerer (www.sagenresearch.com), OMSSA (www.pubchem.ncbi.nlm.nih.gov/omssa/), X!Tandem (www.thegpm.org/TANDEM/), Protein Prospector (prospector.ucsf.edu/prospector/mshome.htm), Byonic (www.proteinmetrics.com/products/byonic) or Sequest (fields.scripps.edu/sequest).

[0084] In some exemplary embodiments, the mass spectrometer is coupled to the chromatography system, for example, SCX.

[0085] In some exemplary embodiments, the mass spectrometer can be coupled to a liquid chromatography-multiple reaction monitoring system. More generally, a mass spectrometer may be capable of analysis by selected reaction monitoring (SRM), including consecutive reaction monitoring (CRM) and parallel reaction monitoring (PRM).

[0086] As used herein, "multiple reaction monitoring" or "MRM" refers to a mass spectrometry-based technique that can precisely quantify small molecules, peptides, and proteins within complex matrices with high sensitivity, specificity and a wide dynamic range (Paola Picotti & Ruedi Aebersold, Selected reaction monitoring–based proteomics: workflows, potential, pitfalls and future directions, 9 NATURE METHODS 555–566 (2012)). MRM can be typically performed with triple quadrupole mass spectrometers wherein a precursor ion corresponding to the selected small molecules/ peptides is selected in the first quadrupole and a fragment ion of the precursor ion was selected for monitoring in the third quadrupole (Yong Seok Choi et al., Targeted human cerebrospinal fluid proteomics for the validation of multiple

Alzheimers disease biomarker candidates, 930 JOURNAL OF CHROMATOGRAPHY B 129–135 (2013)).

[0087] In some aspects, the mass spectrometer in the method or system of the present application can be an electrospray ionization mass spectrometer, nano-electrospray ionization mass spectrometer, or a triple quadrupole mass spectrometer, wherein the mass spectrometer can be coupled to a liquid chromatography system, wherein the mass spectrometer is capable of performing LC-MS (liquid chromatography-mass spectrometry) or LC-MRM-MS (liquid chromatography-multiple reaction monitoring-mass spectrometry) analyses.

[0088] As used herein, the term "mass analyzer" includes a device that can separate species, that is, atoms, molecules, or clusters, according to their mass. Non-limiting examples of mass analyzers that could be employed are time-of-flight (TOF), magnetic electric sector, quadrupole mass filter (Q), quadrupole ion trap (QIT), orbitrap, Fourier transform ion cyclotron resonance (FTICR), and also the technique of accelerator mass spectrometry (AMS).

[0089] It is understood that the present invention is not limited to any of the aforesaid protein(s) of interest, antibody(s), antibody fragment(s), sample(s), impurity(s), PTM(s), immunoprecipitation method(s), liquid chromatography method(s) or system(s), mass spectrometer(s), alkylating agent(s), reducing agent(s), digestive enzyme(s), database(s), or bioinformatics tool(s), and any protein(s) of interest, antibody(s), antibody fragment(s), sample(s), impurity(s), PTM(s), immunoprecipitation method(s), liquid chromatography method(s) or system(s), mass spectrometer(s), alkylating agent(s), reducing agent(s), digestive enzyme(s), database(s), or bioinformatics tool(s) can be selected by any suitable means.

[0090] The present invention will be more fully understood by reference to the following Examples. They should not, however, be construed as limiting the scope of the invention.

EXAMPLES

[0091] An exemplary embodiment of the method of the present invention is illustrated in FIG. 1. The first component shown is a cartridge containing agarose beads conjugated with streptavidin moieties. Biotinylated anti-human Fc antibody is then added to the cartridge and bound to the streptavidin beads to produce immunoprecipitation beads. Biotinylated anti-human Fc may be produced or commercially purchased. An exemplary biotin-streptavidin reaction comprises incubation at about room temperature for about 15 minutes. Samples including the

analyte are then added to the cartridge and incubated to immunoprecipitate or "pull down" the analyte. An exemplary immunoprecipitation process comprises incubation at about room temperature for about 1 hour. The example illustrated is a sample from a pharmacokinetic study comprising a trispecific antibody as the protein of interest and analyte, but the method of the present invention is not limited to this example and may be applied to any appropriate sample comprising any antibody or antibody-related protein.

[0092] The sample is then washed to remove non-specifically bound components. An exemplary washing step comprises washing the cartridge with 6 cartridge volumes of HBS-EP buffer (Cytiva), followed by 6 cartridge volumes of Tris-HCl (10 mM, pH 7.5). A digestive enzyme, for example IdeS or a variant thereof, is then added to the cartridge and incubated, which leads to cleavage of the bound analyte, for example separating the Fc fragment from the Fab_2 fragment of an antibody. An exemplary digestion step comprises adding 40 units of the IdeS protein FabRICATOR® (Genovis), or 1 unit of digestive enzyme per µg of analyte, and incubating at about 37° C for about 30 minutes to about 1 hour. The cartridge is centrifuged ("spun down") to elute freed Fab_2 fragments, and the eluate is collected for subsequent native SCX-MS analysis.

[0093] Exemplary methods for native SCX-MS analysis are described in Yan *et al.*, 2020, *J Am Soc Mass Spectrom*, 31:2171-2179, which is hereby incorporated by reference. In an exemplary embodiment, SCX-MS conditions are as follows. The SCX column is YMC BioPro IEX SF 4.6 x 50 mm, 5 µm. The column temperature is 45° C. Mobile phase A (MPA) comprises 10 mM ammonium acetate, and mobile phase B (MPB) comprises 300 mM ammonium acetate. The flow rate is 0.4 mL/minute. The gradient is: 0-1 minutes: 100% MPA; 1-9 minutes: 100% MPA to 100% MPB; 9-10.5 minutes: 100% MPB; 10.5-10.6 minutes: 100% MPB to 100% MPA; and 10.6-15 minutes: 100% MPA.

[0094] The MS resolution is set at 12,500 (UHMR). The capillary spray voltage is set at 3.0 kV. The capillary temperature is set at 350° C. The S-lens RF level is set at 200. The in-source fragmentation energy is set at 100. The HCD trapping gas pressure is set at 3. Mass spectra are acquired with an m/z range window between 2000 and 15,000.

Example 1. Selection of SCX column

[0095] The performance of multiple SCX columns was compared to optimize the method of the present invention. Fab_2 fragments were prepared as described above and subjected to native SCX-MS analysis. Bioresolve SCX 2.1 x 50 mm was compared to YMC SCX 4.6 x 50 mm. SCX-MS total ion chromatograms (TICs) for each column are shown in FIG. 2, with corresponding flow rates and temperature for each experiment shown. Based on the demonstrated sensitivity of the method, YMC SCX 4.6 x 50 mm was used for further experiments, using an 8 minute gradient of 10 to 300 mM ammonium acetate buffer.

Example 2. Establishing Limit of Detection and Limit of Quantitation in Neat Solution

[0096] The native SCX-MS method of the present invention was tested on Fab_2 fragments in neat solution to establish a limit of detection (LOD). Neat solution comprised an antibody analyte and an internal standard antibody (300 pg/μL, or 600 pg on the column) in 10 mM Tris-HCl buffer (pH 7.5). A range of antibodies was tested as the analyte, with pI ranging from high to low, as shown in FIG. 3A. pI ranges of tested antibodies were between 6.28 and 8.15. Sample amounts tested ranged from 20 pg to 2 μg on the column, with concentration ranges between 10 pg/μL and 1 μg/μL.

[0097] A 15 minute SCX run was performed for each sample, each with a 0.2 mL/minute flow rate, except for Ab9. Antibodies tested included IgG1 and IgG4 antibodies, and mAbs and bsAbs, representing a diverse variety of therapeutic antibodies. The method of the present invention was capable of effectively separating and analyzing each antibody with high sensitivity. Mass spectra from two exemplary antibodies at a range of concentrations between 20 pg and 20 ng are shown in FIG. 3B and 3C. The absolute LOD of Fab_2 fragments using the method of the present invention under these conditions was determined to be 20 pg.

[0098] The LOD and limit of quantitation (LOQ) in neat solution were further assessed as shown in FIG. 4. The Fab_2 fragment of mAb1 was analyzed using native SCX-MS, with the Fab_2 fragment of mAb2 used as an internal standard, as shown in a TIC in FIG. 4A. FIG. 4B shows a comparison of the actual concentration of mAb1 compared to the intensity normalized to the internal standard as measured by the method of the present invention, at a range of concentrations between 20 pg and 20 ng. The actual versus measured concentrations show a linear relationship with a weighted R^2 of 0.9954, demonstrating the ability of the method of the present invention to accurately and sensitively quantitate an analyte at low concentrations. FIG.

4C shows the same comparison made with a range of concentrations between 20 ng and 2 µg, with a strong linear relationship demonstrated again at this higher concentration range. Exemplary mass spectra between 20 pg and 2 µg are shown in FIG. 4D, further illustrating the sensitivity and specificity of the method of the presnt invention.

Example 3. Establishing Limit of Detection and Limit of Quantitation in Serum

[0099] The robustness of the method of the present invention was further demonstrated using analytes from a mouse serum sample. Analysis of a protein of interest in serum presents numerous additional challenges, including heterogeneity of the protein of interest due to biotransformation, and interference due to a complex matrix, such as high concentration serum proteins.

[0100] Fab_2 fragments of mAb1 were prepared as previously described, and subjected to native SCX-MS analysis. The linearity of the response ratio (the measured analyte intensity normalized to an internal standard) to actual concentration of the antibody is shown in FIG. 5A. FIG. 5B and FIG. 5C show further insets, demonstrating the linearity of the response even at low concentrations. These results demonstrate the sensitivity and effectiveness of the method of the present invention in quantifying antibodies even at low concentrations in serum.

[0101] The stability of the method of the present invention was further demonstrated by plotting the linearity of the measured intensity, without normalization to an internal standard, compared to antibody concentration, as shown in FIG. 6A. FIG. 6B and 6C show further insets demonstrating the linearity of measured intensity at low concentrations in serum, even without normalization to an internal standard.

[0102] Mass spectra illustrating the LOD and LOQ of mAb1 Fab_2 in serum and in neat solution are shown in FIG. 7. The LOD was determined to be as low as 0.025 µg/mL in serum, which is equivalent to 50 pg on the SCX column, as shown in FIG. 7A. The LOQ was determined to be as low as 0.05 µg/mL in serum, which is equivalent to 100 pg on the SCX column, as shown in FIG. 7B. A signal-to-noise (S/N) ratio of 5 is indicated as a reasonable standard for establishing the LOQ. The absolute intensities of mAb1 Fab_2 detected from serum samples were higher than those detected in neat solution, suggesting that the limit of sensitivity of serum samples is due to noise from co-IPed serum protein.

[0103] In addition to the examples disclosed herein, even lower LOD and LOQ are possible using the method of the present invention in more favorable conditions that would be known to a person of skill in the art, for example using an antibody with a later elution time, or using greater washing volume during IP.

What is claimed is:

1. A method for characterizing an antibody, comprising:

(a) immobilizing said antibody on a solid-phase substrate;

(b) contacting said immobilized antibody to a digestive enzyme to produce an unbound fragment of said antibody;

(c) eluting said antibody fragment; and

(d) subjecting said eluate to native SCX-MS analysis to characterize said antibody.

2. The method of claim 1, wherein said antibody is a monoclonal antibody or a bispecific antibody.

3. The method of claim 1, wherein said immobilizing step comprises contacting a sample including said antibody to a solid-phase substrate capable of binding to said antibody.

4. The method of claim 3, wherein said solid-phase substrate comprises beads.

5. The method of claim 4, wherein said beads are agarose beads or magnetic beads.

6. The method of claim 3, wherein said binding is performed by an antibody adhered to said solid-phase substrate.

7. The method of claim 6, wherein said antibody is an anti-Fc antibody.

8. The method of claim 1, further comprising a step of washing said solid-phase substrate after immobilizing said antibody.

9. The method of claim 1, wherein said digestive enzyme is IdeS or a variant thereof.

10. The method of claim 1, wherein said antibody fragment is a Fab_2 fragment.

11. The method of claim 1, wherein said eluting comprises a step of centrifuging said solid-phase substrate and said antibody fragment.

12. The method of claim 1, wherein said SCX system is coupled to said mass spectrometer.

13. The method of claim 1, wherein said mass spectrometer is an electrospray ionization mass spectrometer, nano-electrospray ionization mass spectrometer, or a triple quadrupole mass spectrometer.

14. The method of claim 1, wherein said characterization of an antibody comprises quantitation of an antibody, optionally wherein said quantitation is normalized to an internal standard.

15. The method of claim 3, wherein said sample is a serum sample.

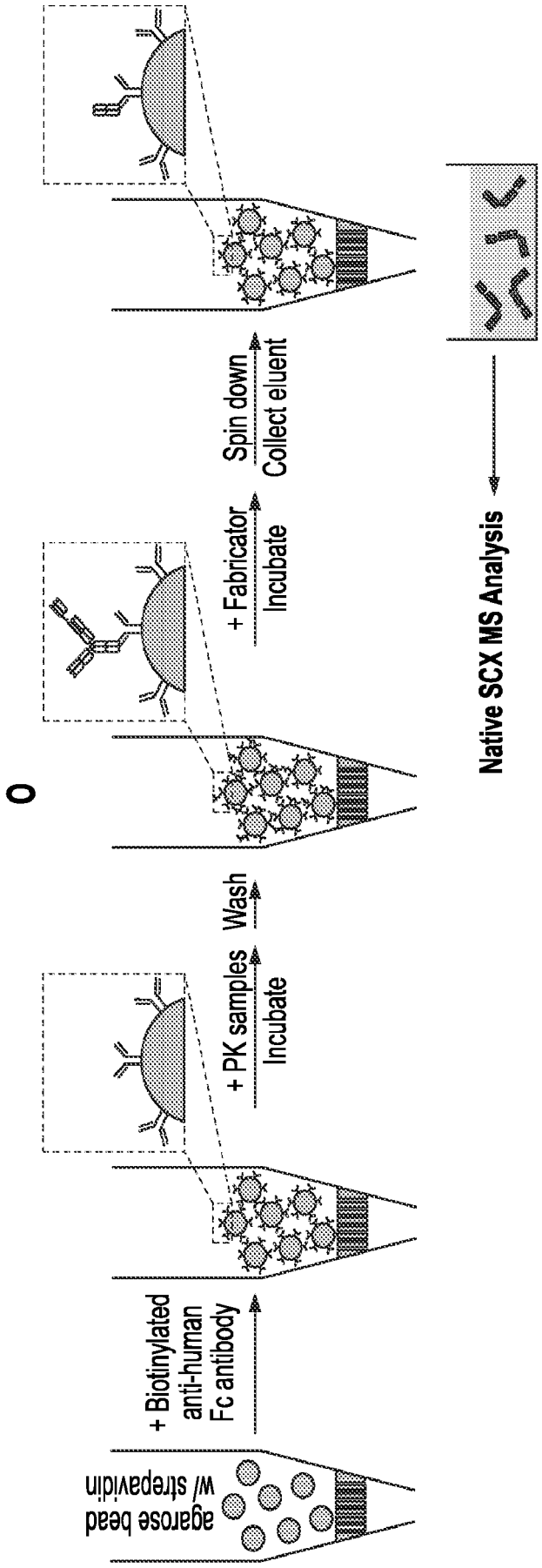

agarose bead w/ streptavidin
+ Biotinylated anti-human Fc antibody
+ PK samples
Incubate
Wash
+ Fabricator
Incubate
Spin down
Collect eluent
Native SCX MS Analysis

FIG. 1

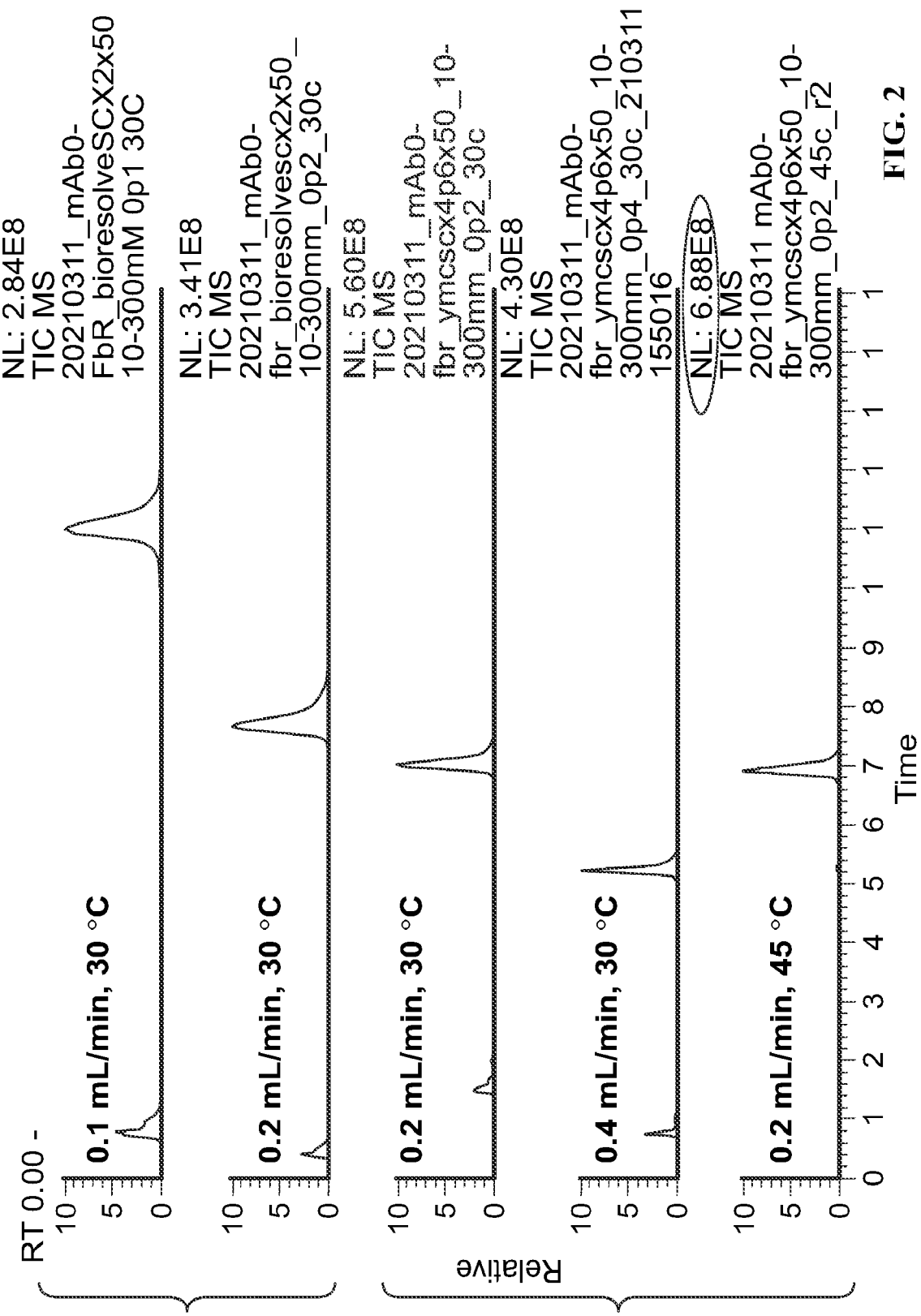

Bioresolve SCX
2.1 x 50 mm
YMC SCX
4.6 x 50 mm
RT 0.00 -
0.1 mL/min, 30 °C
NL: 2.84E8
TIC MS
20210311_mAb0-
FbR_bioresolveSCX2x50
10-300mM 0p1 30C
0.2 mL/min, 30 °C
NL: 3.41E8
TIC MS
20210311_mAb0-
fbr_bioresolvescx2x50_
10-300mm_0p2_30c
0.2 mL/min, 30 °C
NL: 5.60E8
TIC MS
20210311_mAb0-
fbr_ymcscx4p6x50_10-
300mm_0p2_30c
0.4 mL/min, 30 °C
NL: 4.30E8
TIC MS
20210311_mAb0-
fbr_ymcscx4p6x50_10-
300mm_0p4_30c_210311
155016
0.2 mL/min, 45 °C
NL: 6.88E8
TIC MS
20210311 mAb0-
fbr_ymcscx4p6x50_10-
300mm_0p2_45c_r2
Relative
Time

FIG. 2

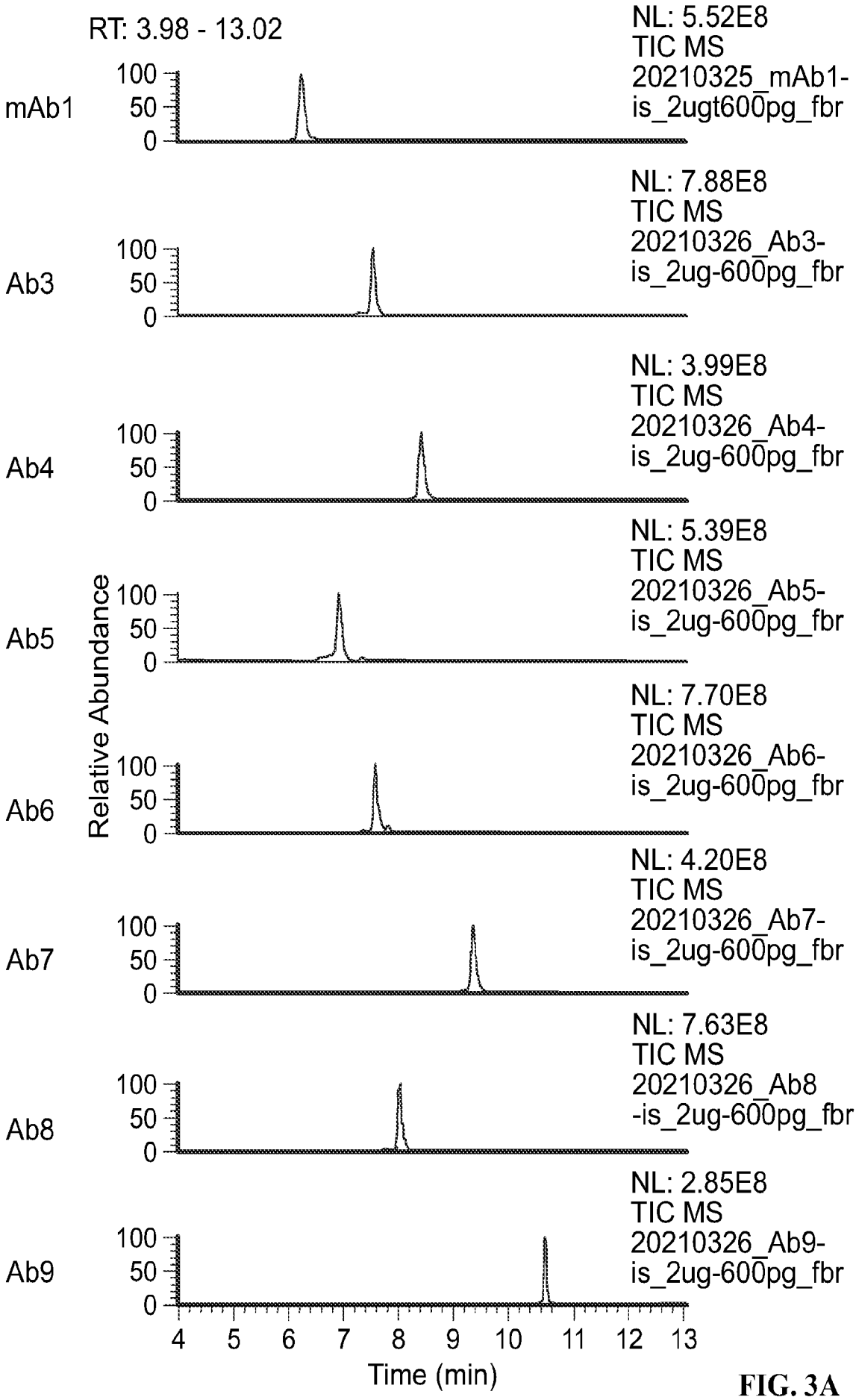

RT: 3.98 - 13.02
mAb1
NL: 5.52E8
TIC MS
20210325_mAb1-
is_2ugt600pg_fbr
Ab3
NL: 7.88E8
TIC MS
20210326_Ab3-
is_2ug-600pg_fbr
Ab4
NL: 3.99E8
TIC MS
20210326_Ab4-
is_2ug-600pg_fbr
Ab5
NL: 5.39E8
TIC MS
20210326_Ab5-
is_2ug-600pg_fbr
Ab6
NL: 7.70E8
TIC MS
20210326_Ab6-
is_2ug-600pg_fbr
Ab7
NL: 4.20E8
TIC MS
20210326_Ab7-
is_2ug-600pg_fbr
Ab8
NL: 7.63E8
TIC MS
20210326_Ab8
-is_2ug-600pg_fbr
Ab9
NL: 2.85E8
TIC MS
20210326_Ab9-
is_2ug-600pg_fbr
Relative Abundance
100
50
0
4
5
6
7
8
9
10
11
12
13
Time (min)

FIG. 3A

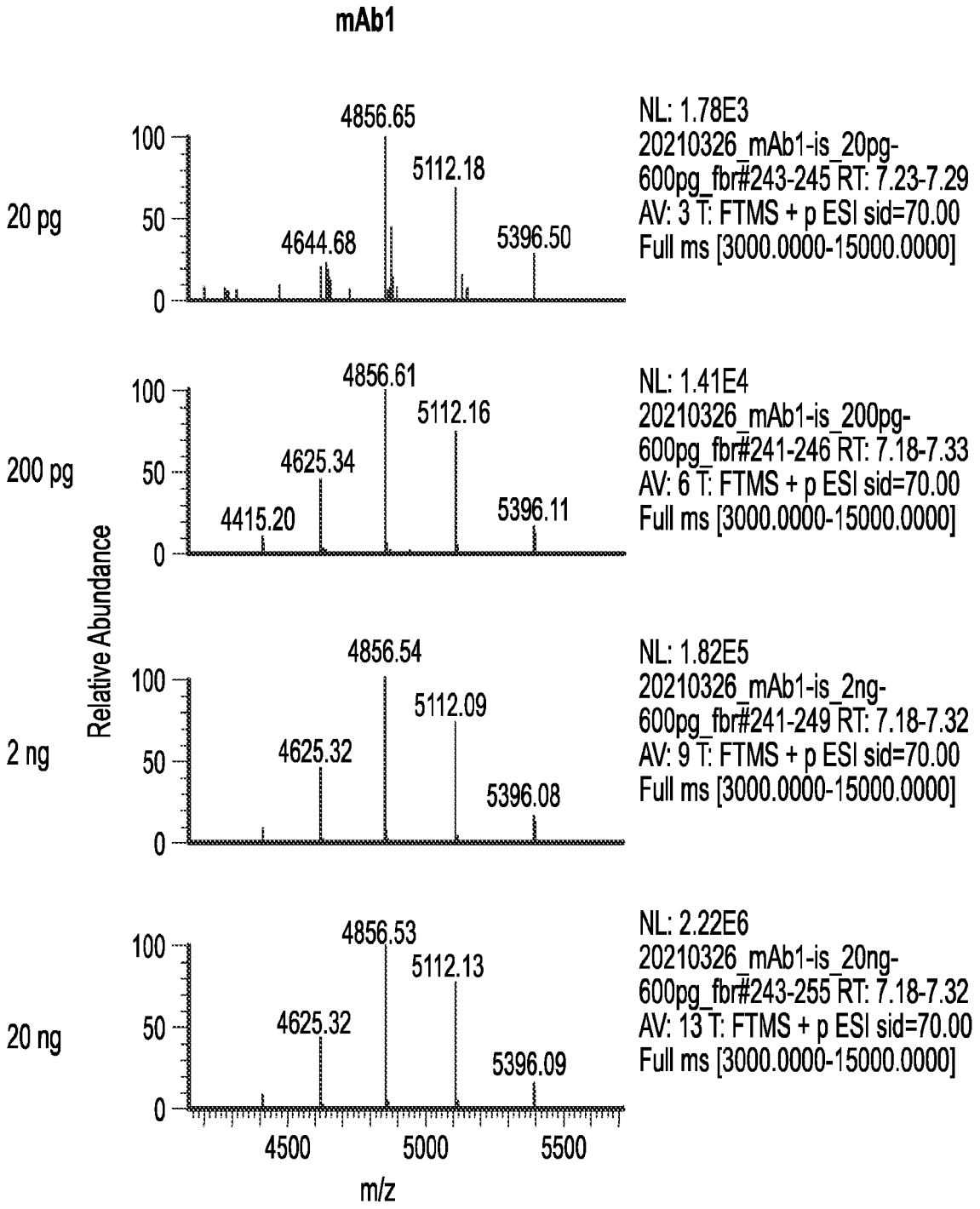

mAb1
20 pg
200 pg
2 ng
20 ng
Relative Abundance
m/z
4856.65
5112.18
4644.68
5396.50
NL: 1.78E3
20210326_mAb1-is_20pg-600pg_fbr#243-245 RT: 7.23-7.29
AV: 3 T: FTMS + p ESI sid=70.00
Full ms [3000.0000-15000.0000]
4856.61
5112.16
4625.34
4415.20
5396.11
NL: 1.41E4
20210326_mAb1-is_200pg-600pg_fbr#241-246 RT: 7.18-7.33
AV: 6 T: FTMS + p ESI sid=70.00
Full ms [3000.0000-15000.0000]
4856.54
5112.09
4625.32
5396.08
NL: 1.82E5
20210326_mAb1-is_2ng-600pg_fbr#241-249 RT: 7.18-7.32
AV: 9 T: FTMS + p ESI sid=70.00
Full ms [3000.0000-15000.0000]
4856.53
5112.13
4625.32
5396.09
NL: 2.22E6
20210326_mAb1-is_20ng-600pg_fbr#243-255 RT: 7.18-7.32
AV: 13 T: FTMS + p ESI sid=70.00
Full ms [3000.0000-15000.0000]
100
50
0
4500
5000
5500

FIG. 3B

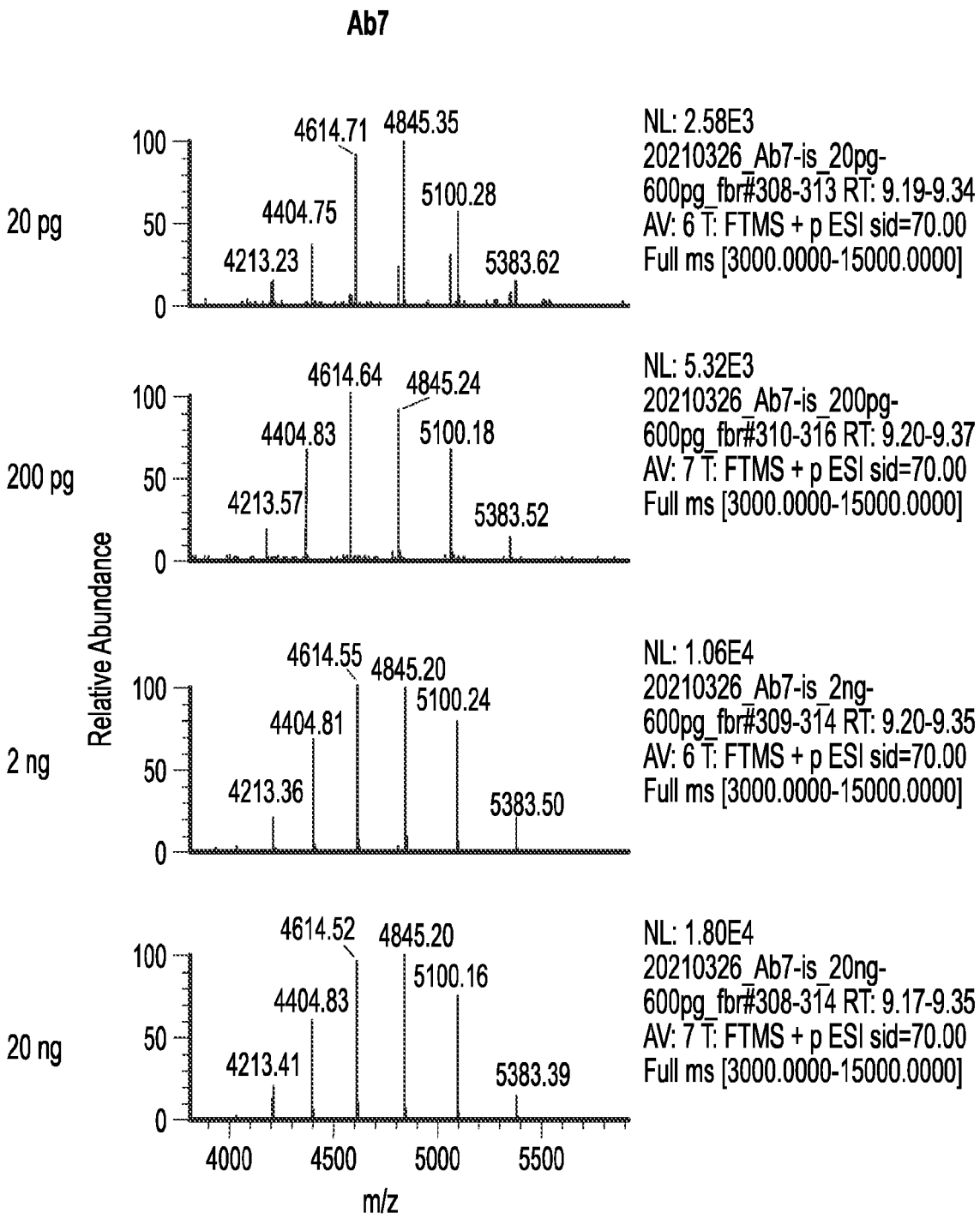

Ab7
20 pg
200 pg
2 ng
20 ng
Relative Abundance
m/z
4614.71
4845.35
4404.75
5100.28
4213.23
5383.62
NL: 2.58E3
20210326_Ab7-is_20pg-600pg_fbr#308-313 RT: 9.19-9.34
AV: 6 T: FTMS + p ESI sid=70.00
Full ms [3000.0000-15000.0000]
4614.64
4845.24
4404.83
5100.18
4213.57
5383.52
NL: 5.32E3
20210326_Ab7-is_200pg-600pg_fbr#310-316 RT: 9.20-9.37
AV: 7 T: FTMS + p ESI sid=70.00
Full ms [3000.0000-15000.0000]
4614.55
4845.20
5100.24
4404.81
4213.36
5383.50
NL: 1.06E4
20210326_Ab7-is_2ng-600pg_fbr#309-314 RT: 9.20-9.35
AV: 6 T: FTMS + p ESI sid=70.00
Full ms [3000.0000-15000.0000]
4614.52
4845.20
5100.16
4404.83
4213.41
5383.39
NL: 1.80E4
20210326_Ab7-is_20ng-600pg_fbr#308-314 RT: 9.17-9.35
AV: 7 T: FTMS + p ESI sid=70.00
Full ms [3000.0000-15000.0000]
100
50
0
4000
4500
5000
5500

FIG. 3C

FIG. 4A

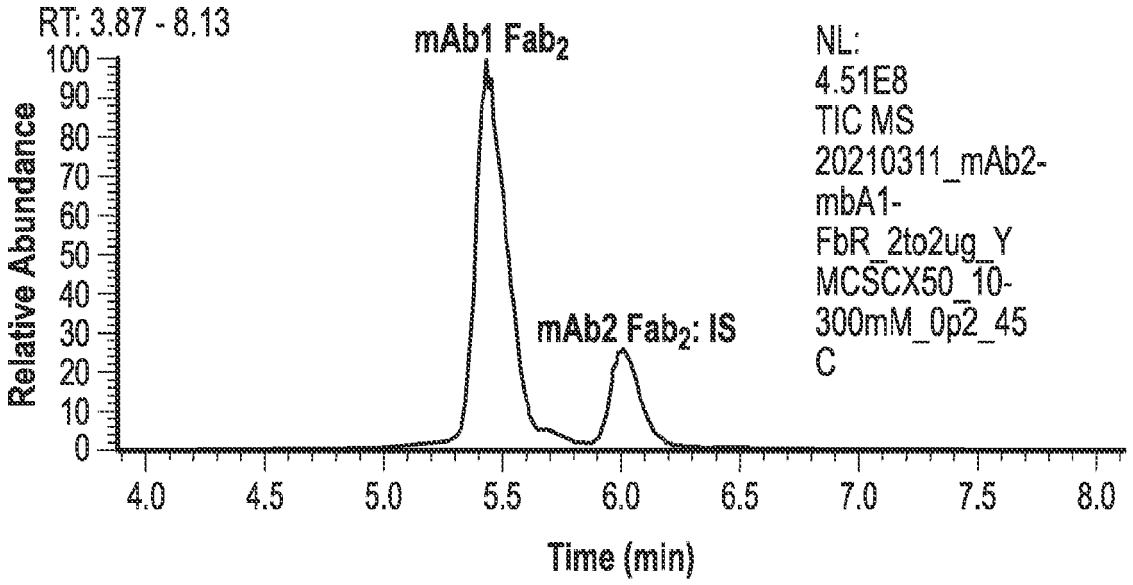
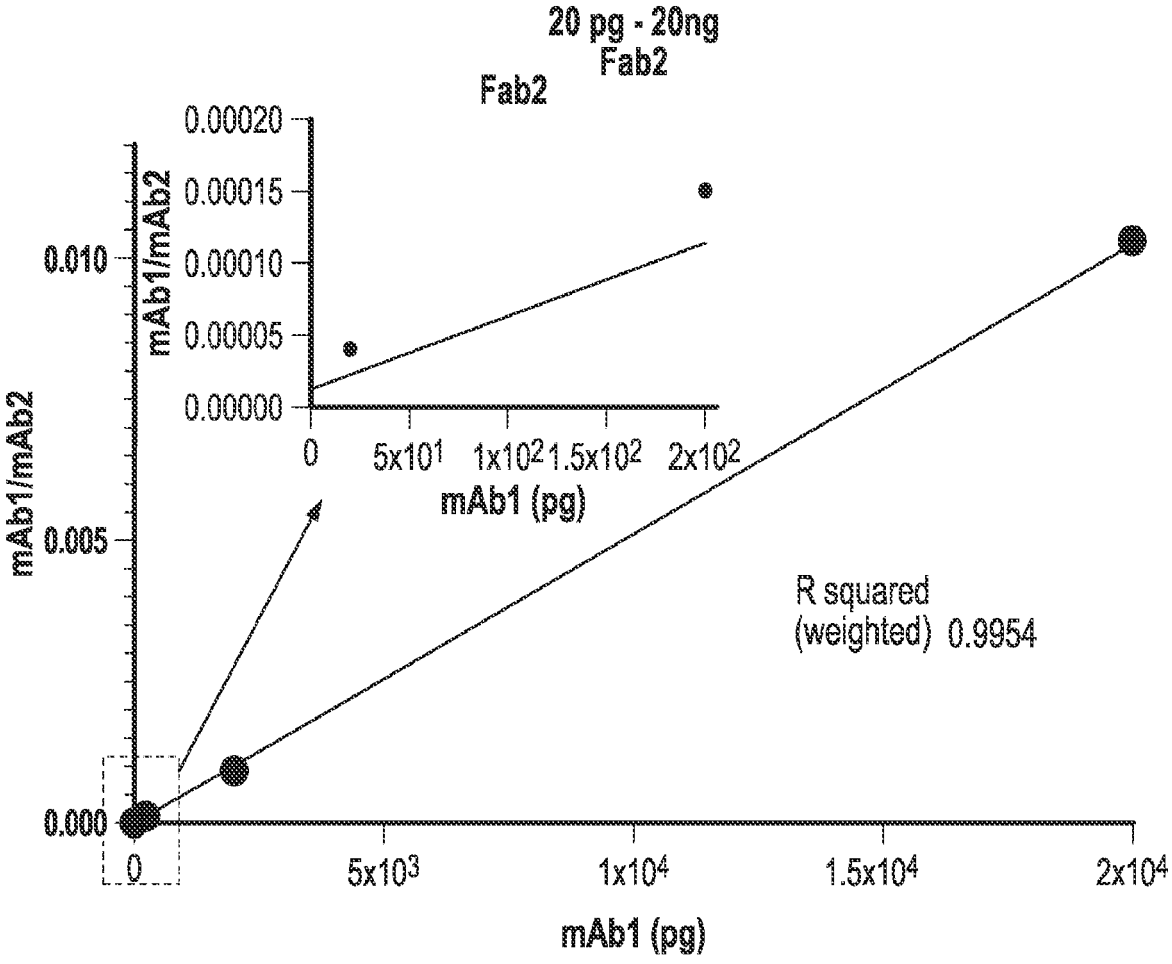

RT: 3.87 - 8.13
mAb1 Fab2
mAb2 Fab2: IS
NL:
4.51E8
TIC MS
20210311_mAb2-
mbA1-
FbR_2to2ug_Y
MCSCX50_10-
300mM_0p2_45
C
Relative Abundance
Time (min)

FIG. 4B


20 pg - 20ng
Fab2
Fab2
mAb1/mAb2
mAb1 (pg)
R squared
(weighted) 0.9954
mAb1/mAb2
mAb1 (pg)

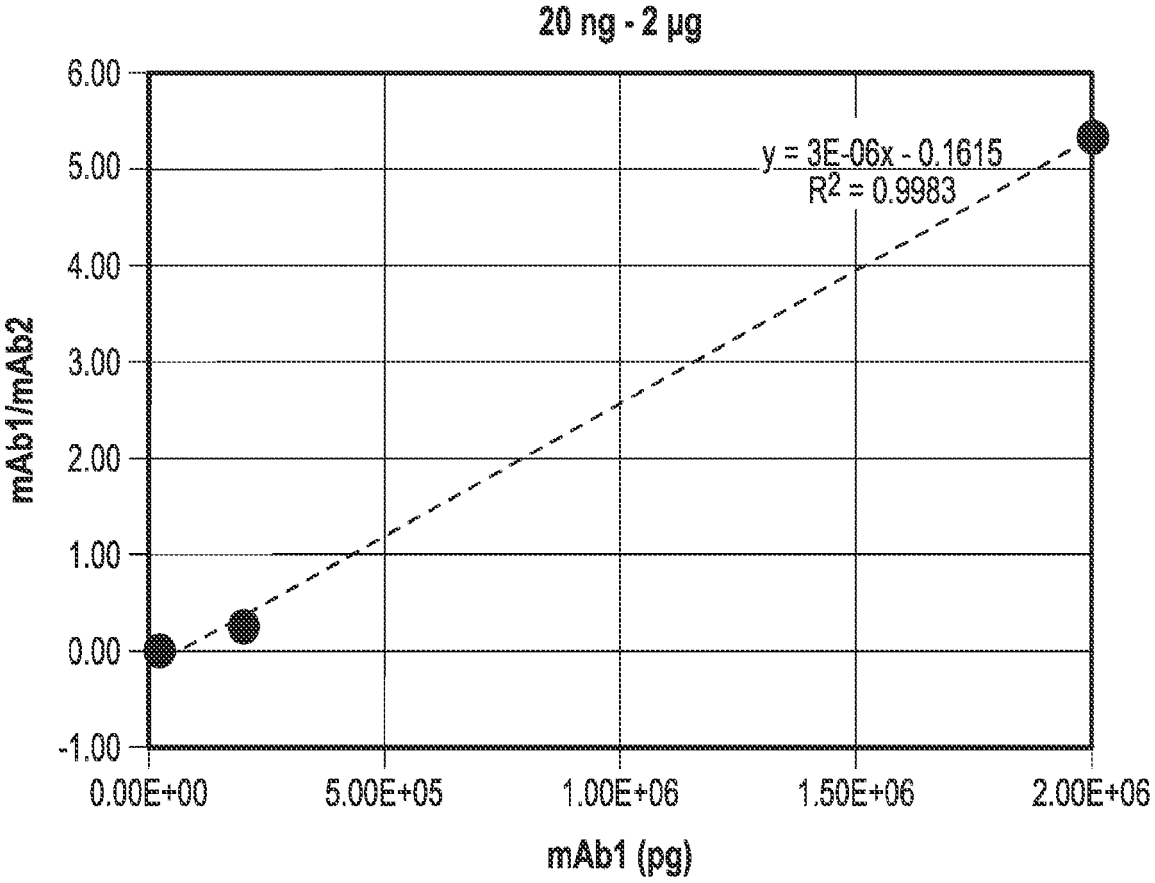

20 ng - 2 µg
6.00
5.00
4.00
3.00
2.00
1.00
0.00
-1.00
mAb1/mAb2
y = 3E-06x - 0.1615
R2 = 0.9983
0.00E+00
5.00E+05
1.00E+06
1.50E+06
2.00E+06
mAb1 (pg)

FIG. 4C

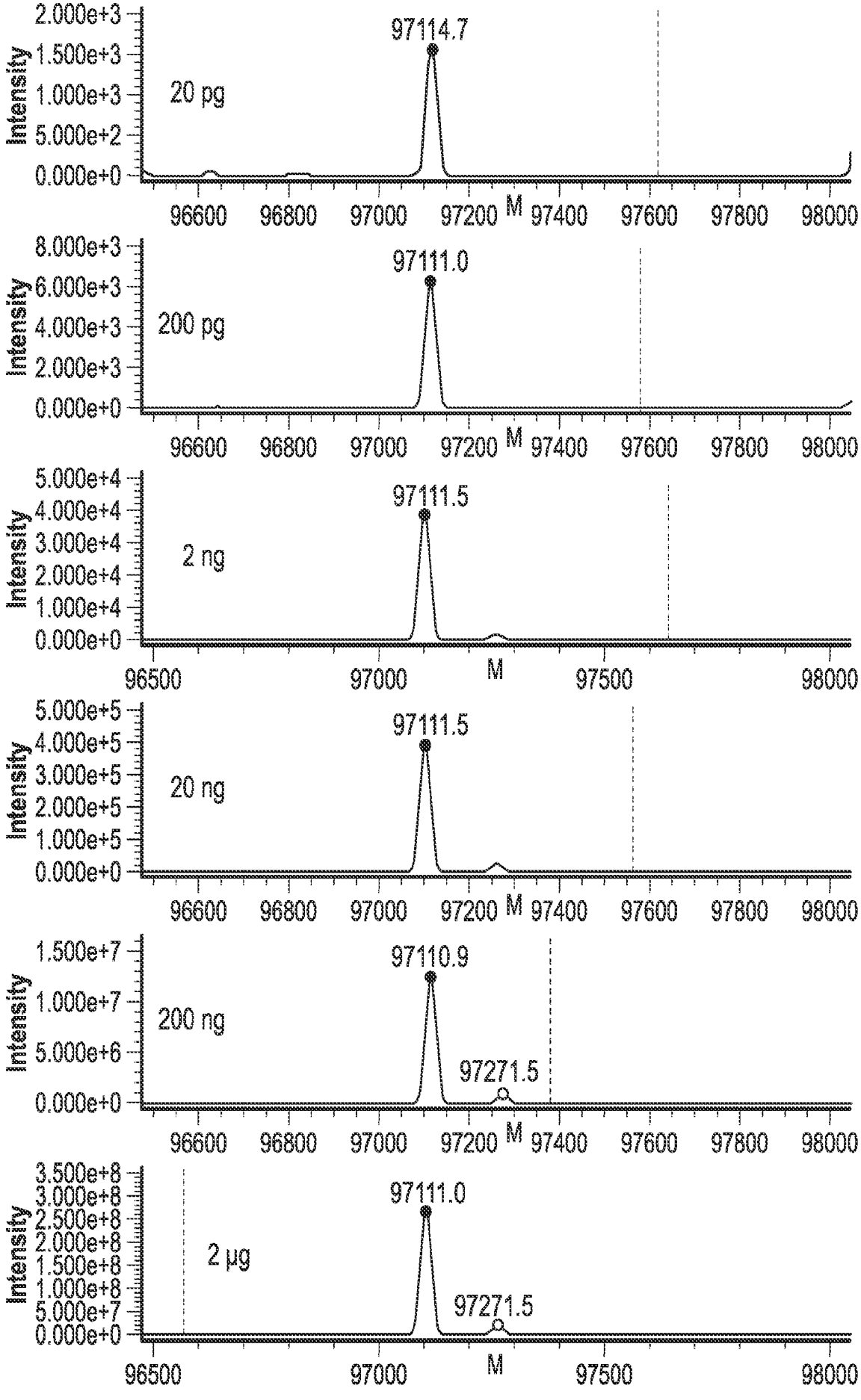
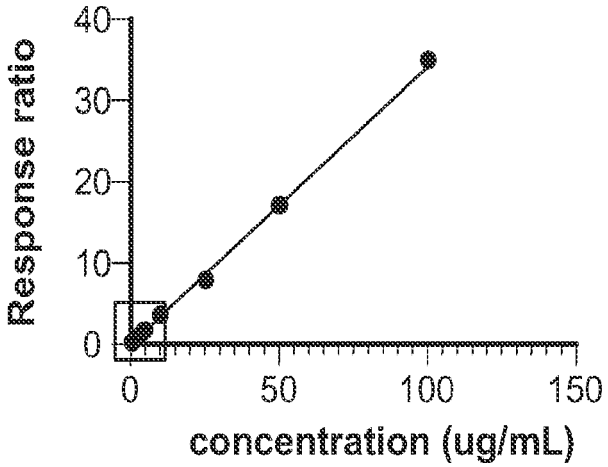

Intensity
2.000e+3
1.500e+3
1.000e+3
5.000e+2
0.000e+0
97114.7
20 pg
96600
96800
97000
97200
M
97400
97600
97800
98000
Intensity
8.000e+3
6.000e+3
4.000e+3
2.000e+3
0.000e+0
97111.0
200 pg
96600
96800
97000
97200
M
97400
97600
97800
98000
Intensity
5.000e+4
4.000e+4
3.000e+4
2.000e+4
1.000e+4
0.000e+0
97111.5
2 ng
96500
97000
M
97500
98000
Intensity
5.000e+5
4.000e+5
3.000e+5
2.000e+5
1.000e+5
0.000e+0
97111.5
20 ng
96600
96800
97000
97200
M
97400
97600
97800
98000
Intensity
1.500e+7
1.000e+7
5.000e+6
0.000e+0
97110.9
200 ng
97271.5
96600
96800
97000
97200
M
97400
97600
97800
98000
Intensity
3.500e+8
3.000e+8
2.500e+8
2.000e+8
1.500e+8
1.000e+8
5.000e+7
0.000e+0
97111.0
2 µg
97271.5
96500
97000
M
97500
98000

FIG. 4D

FIG. 5A



$1/x^2$ weighted Linear Fit: Y = 0.3405X - 0.006524
R^2 = 0.9896

FIG. 5B

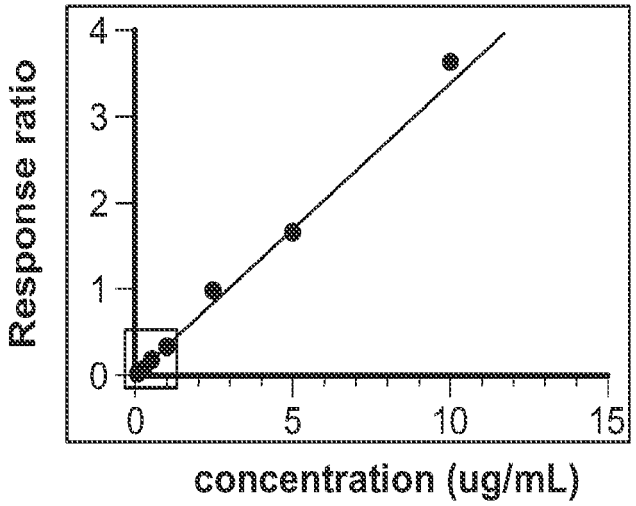


FIG. 5C

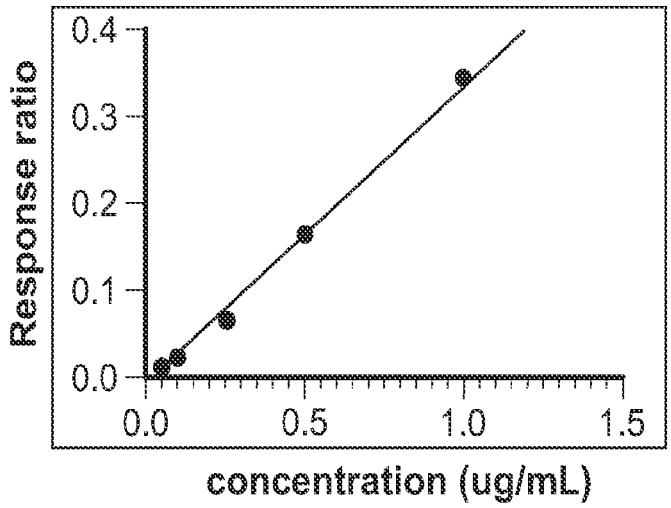
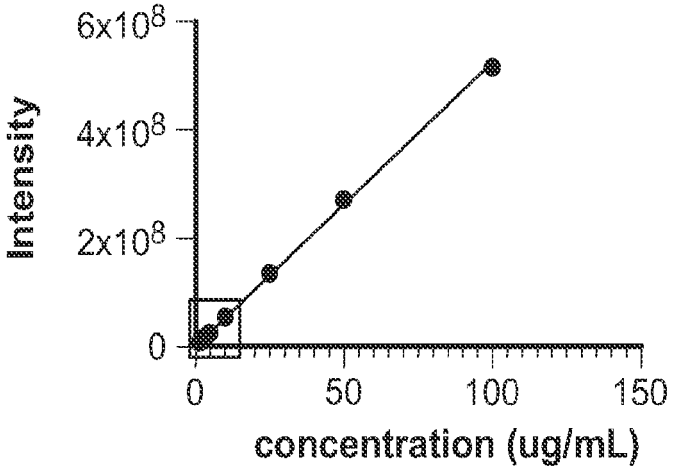


FIG. 6A



$1/x^2$ weighted Linear Fit: Y = 5267133X - 100778
R^2 = 0.9940

FIG. 6B

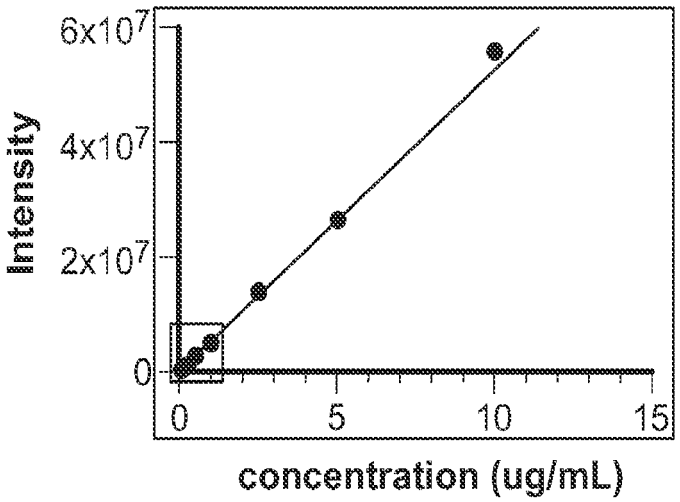
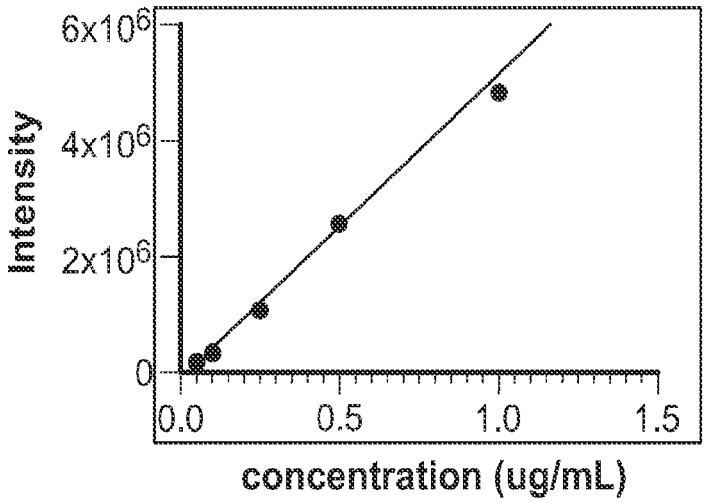


FIG. 6C



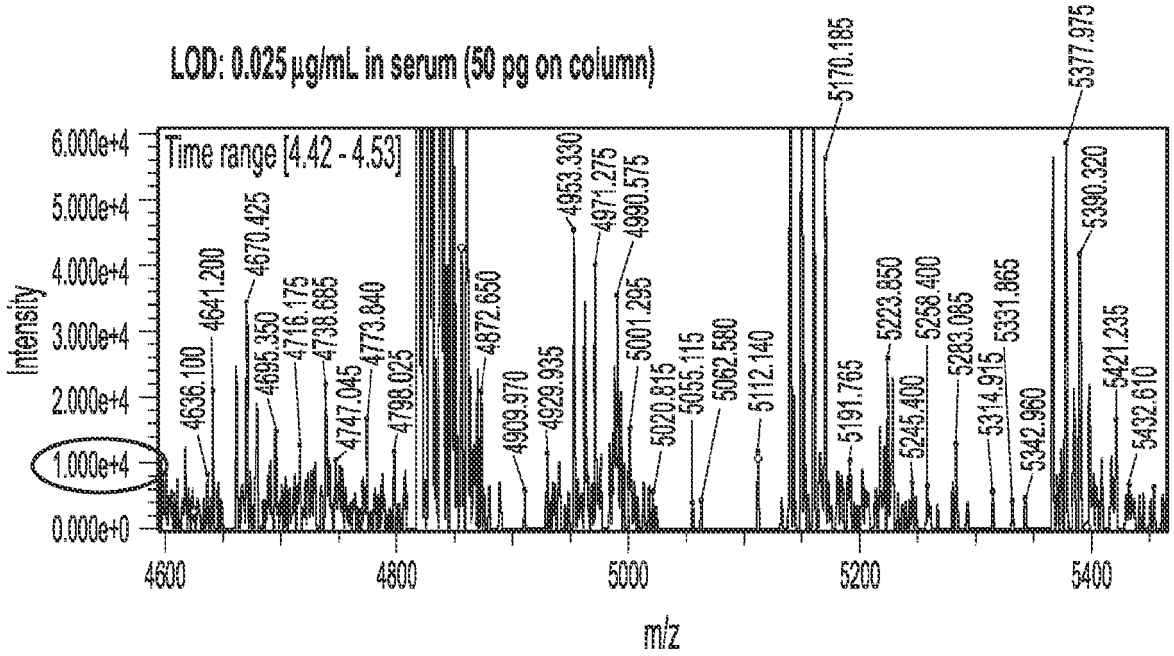

LOD: 0.025 µg/mL in serum (50 pg on column)
Time range [4.42 - 4.53]
Intensity
m/z
6.000e+4
5.000e+4
4.000e+4
3.000e+4
2.000e+4
1.000e+4
0.000e+0
4600
4800
5000
5200
5400
4636.100
4641.200
4670.425
4695.350
4716.175
4738.685
4747.045
4773.840
4798.025
4872.650
4909.970
4929.935
4953.330
4971.275
4990.575
5001.295
5020.815
5055.115
5062.580
5112.140
5170.185
5191.765
5223.850
5245.400
5258.400
5283.065
5314.915
5331.865
5342.960
5377.975
5390.320
5421.235
5432.610

FIG. 7A

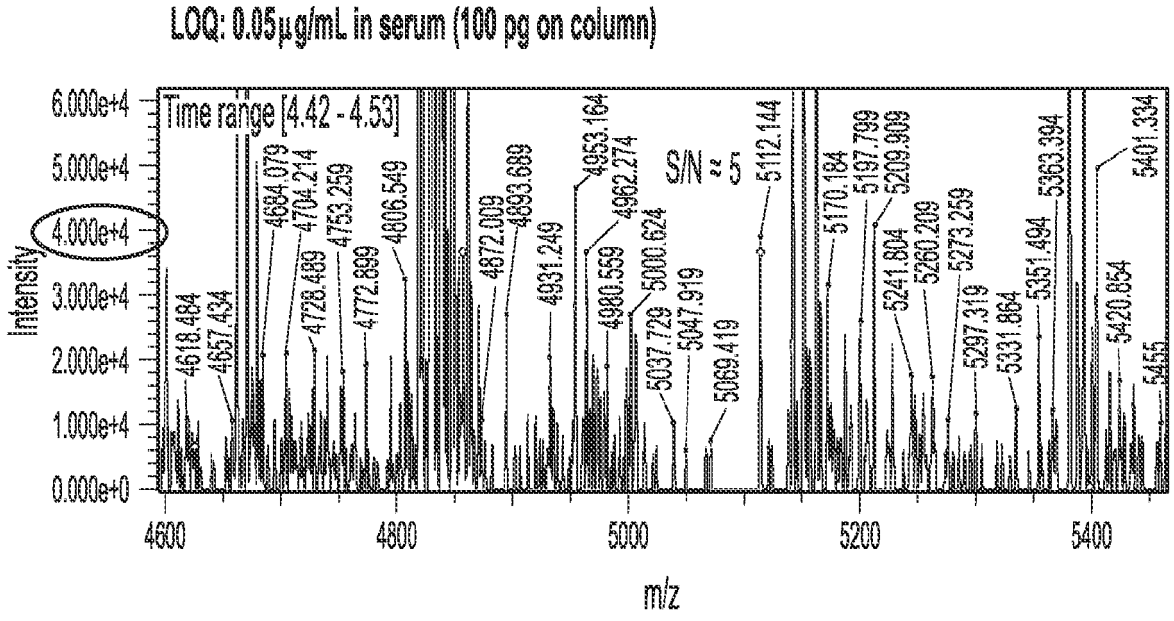

LOQ: 0.05 µg/mL in serum (100 pg on column)
Time range [4.42 - 4.53]
Intensity
m/z
S/N = 5
6.000e+4
5.000e+4
4.000e+4
3.000e+4
2.000e+4
1.000e+4
0.000e+0
4600
4800
5000
5200
5400
4618.484
4657.434
4684.079
4704.214
4728.489
4753.259
4772.899
4806.549
4872.009
4893.689
4931.249
4953.164
4962.274
4980.559
5000.624
5037.729
5047.919
5069.419
5112.144
5170.184
5197.799
5209.909
5241.804
5260.209
5273.259
5297.319
5331.864
5351.494
5363.394
5401.334
5420.854
5455

FIG. 7B