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(54) **Titre : CARACTERISATION DE PROTEINES PAR SPECTROMETRIE DE MASSE PAR CHROMATOGRAPHIE D'ECHANGE D'ANIONS (AEX-MS)**
(54) **Title: CHARACTERIZATION OF PROTEINS BY ANION-EXCHANGE CHROMATOGRAPHY MASS SPECTROMETRY (AEX-MS)**

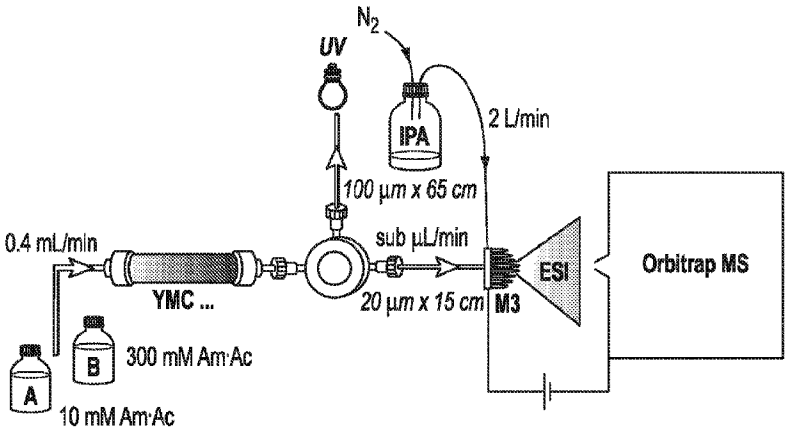

FIG. 3

(57) **Abrégé/Abstract:**
The present invention generally pertains to methods of characterizing charge variants of a protein. In particular, the present invention pertains to the use of anion exchange chromatography-mass spectrometry (AEX-MS) methods using a salt-gradient. The present invention is particularly useful for charge variant analysis of IgG4 subclasses.

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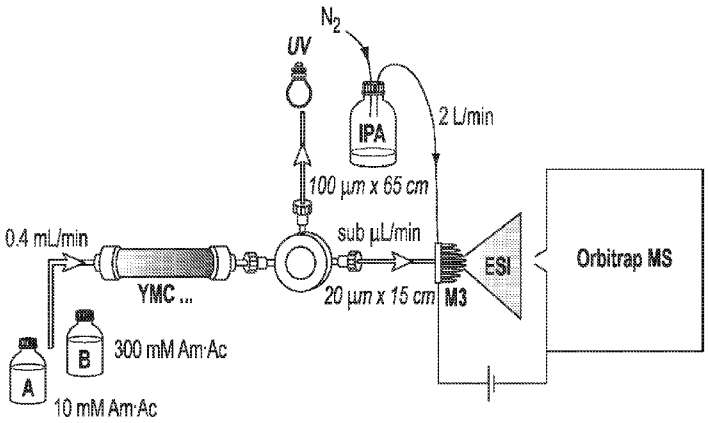

FIG. 3

(57) Abstract: The present invention generally pertains to methods of characterizing charge variants of a protein. In particular, the present invention pertains to the use of anion exchange chromatography-mass spectrometry (AEX-MS) methods using a salt-gradient. The present invention is particularly useful for charge variant analysis of IgG4 subclasses.

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CHARACTERIZATION OF PROTEINS BY ANION-EXCHANGE CHROMATOGRAPHY MASS SPECTROMETRY (AEX-MS)

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to and the benefit of U.S. Provisional Patent Application No. 63/221,447, filed July 13, 2021, which is herein incorporated by reference. This application also claims priority to and the benefit of Provisional Application No. U.S. 63/305,177, filed January 31, 2022. which also is in herein incorporated by reference.

FIELD

[0001] The present invention generally pertains to methods for characterizing charge variants of proteins using anion-exchange chromatography coupled to mass spectrometry using increasing salt gradient elution.

BACKGROUND

[0002] Therapeutic proteins are important drugs for the treatment of cancer, autoimmune disease, infection and cardiometabolic disorders, and they represent one of the fastest growing product segments of the pharmaceutical industry. They must meet very high standards of purity. Thus, it can be important to monitor impurities at different stages of drug development, production, storage and handling.

[0003] More than 600 different types of post-translational modifications are known for proteins, many of them being of extremely low abundance, causing subtle changes in physicochemical properties and posing an extreme challenge to analytical methods required for their characterization. For proteins produced using recombinant methods, post-translational modifications represent critical product quality attributes, which may impact drug potency and patient safety, as has been shown for monoclonal antibodies (mAbs). Charge-sensitive separation modes such as ion exchange chromatography (IEC), capillary electrophoresis, and capillary isoelectric focusing are routinely used for the separation and characterization of charge variants, due to their high selectivity, allowing for the separation of protein variants with minimal difference in their properties, *i.e.*, net surface charge. However, charge-based separation methods for intact proteins are generally not ideally suited for coupling to mass spectrometry (MS) due to non-

compatibility of the buffer systems. The methods developed so far by coupling IEC with MS have only been suitable for proteins with isoelectric point (pI) less than about 6.0.

[0004] Several therapeutic proteins developed based on IgG1 and IgG4 can have pI greater than 6.0. Development, purification, and characterization of these proteins require an efficient analytical method. It will be appreciated that there is a long felt need in the art for an efficient method for characterizing charge variants of proteins irrespective of their pIs.

SUMMARY

Exemplary embodiments disclosed herein satisfy the aforementioned demands by providing methods for characterizing charge variants of a protein using native AEX-MS.

[0002] This disclosure provides a method for characterization of such charge variants of a protein of interest. In one exemplary embodiment, the method comprises: (a) loading a sample having a protein of interest and at least one charge variant of said protein of interest to an anion-exchange chromatography (AEX) column; (b) applying an increasing salt concentration gradient to the loaded anion exchange column to obtain an eluate; (c) collecting at least one fraction from (b); and (d) subjecting said at least one fraction to mass spectrometry analysis to characterize said at least one charge variant of said protein of interest.

[0003] In one aspect of the embodiment, the method further comprises applying an increasing salt concentration gradient to the loaded anion exchange column, wherein the salt gradient is an elution gradient and ranges from about 10 mM to about 600 mM ammonium salt. In one specific aspect, the ammonium salt is ammonium acetate. In another specific aspect, the salt gradient is an elution gradient and ranges from about 10 mM to about 300 mM ammonium salt. In another specific aspect, the increasing salt concentration gradient applied is linear.

[0004] In one aspect of the embodiment, the method further comprises monitoring the eluate of (b) for ultraviolet absorbance and collecting said at least one fraction that is eluted prior to elution of the protein of interest. In another aspect of the embodiment, the method further comprises monitoring the eluate of (b) for ultraviolet absorbance and collecting said at least one fraction that is eluted after elution of the protein of interest.

[0005] In one aspect of the embodiment, the protein of interest has a pI value of greater than about 6.2. In a specific aspect of the embodiment, the protein of interest has a pI value of about

6.2 to about 7.0. In a specific aspect of the embodiment, the protein of interest has a pI value of about 6.2 to about 8.7.

[0006] In one aspect of the embodiment, the protein of interest is an IgG4-based monoclonal antibody. In another aspect of the embodiment, the protein of interest is an IgG1-based monoclonal antibody. In yet another aspect of the embodiment, the protein of interest is a bispecific monoclonal antibody.

[0007] In one aspect of the embodiment, the method further comprises reducing flow rate from (c) via a flow-splitter prior to subjecting said at least one fraction to a mass spectrometer.

[0008] In one aspect of the embodiment, said at least one charge variant can be either an acidic variant or a basic variant of the protein of interest. For example, the acidic variants can include deamidation, glycation, glucuronylation, and/or high molecular weight species of the protein of interest, and the basic variants can include C-terminal lysine, and glycosylated species.

[0009] In one aspect of the embodiment, the method further comprises removing Fc N-glycosylation from said protein of interest prior to loading said sample on said AEX column. This step can improve the AEX separation due to conversion of Asparagine residues to Aspartic acid. In a specific aspect of this embodiment, the Fc N-glycosylation from said protein of interest is removed using PNGase F. In another specific aspect of this embodiment, the method can further comprise treating said sample to digestion conditions.

[0010] In some exemplary embodiments, the mass spectrometer is run under native conditions.

[0011] In one aspect of the embodiment, the method further comprises treating said sample to digestion conditions prior to loading said sample on AEX column. In one specific aspect, the digestion conditions include use of IdeS or a variant thereof.

[0012] In one exemplary embodiment, the method comprises: (a) subjecting a sample having a protein of interest and at least one charge variant to deglycosylation conditions; (b) loading said sample to an anion-exchange chromatography (AEX) column; (c) applying an increasing salt concentration gradient to the loaded anion exchange column to obtain an eluate; (d) collecting at least one fraction from (c); and (e) subjecting said at least one fraction to mass spectrometry analysis to characterize said at least one charge variant of said protein of interest.

[0013] In one aspect of the embodiment, the method further comprises applying an increasing salt concentration gradient to the loaded anion exchange column, wherein the salt gradient is an elution gradient and ranges from about 10 mM to about 600 mM ammonium salt. In one specific aspect, the ammonium salt is ammonium acetate. In another specific aspect, the salt gradient is an elution gradient and ranges from about 10 mM to about 300 mM ammonium salt. In another specific aspect, the increasing salt concentration gradient applied is linear.

[0014] In one aspect of the embodiment, the method further comprises monitoring the eluate of (c) for ultraviolet absorbance and collecting said at least one fraction that is eluted prior to elution of the protein of interest. In another aspect of the embodiment, the method further comprises monitoring the eluate of (c) for ultraviolet absorbance and collecting said at least one fraction that is eluted after elution of the protein of interest.

[0015] In one aspect of the embodiment, the protein of interest has a pI value of greater than about 6.2. In a specific aspect of the embodiment, the protein of interest has a pI value of about 6.2 to about 7.0. In a specific aspect of the embodiment, the protein of interest has a pI value of about 6.2 to about 8.7.

[0016] In one aspect of the embodiment, the protein of interest is an IgG4-based monoclonal antibody. In another aspect of the embodiment, the protein of interest is an IgG1-based monoclonal antibody. In yet another aspect of the embodiment, the protein of interest is a bispecific monoclonal antibody.

[0017] In one aspect of the embodiment, the method further comprises reducing flow rate from (d) via a flow-splitter prior to subjecting said at least one fraction to a mass spectrometer.

[0018] In one aspect of the embodiment, said at least one charge variant can be either an acidic variant or a basic variant of the protein of interest. For example, the acidic variants can include deamidation, glycation, glucuronylation, and/or high molecular weight species of the protein of interest, and the basic variants can include C-terminal lysine and glycosylated species.

[0019] In one aspect of the embodiment, the method further comprises removing Fc N-glycosylation from said protein of interest prior to loading said sample on AEX column. This step can improve the AEX separation due to conversion of Asparagine residues to Aspartic acid. In a

specific aspect of this embodiment, the Fc N-glycosylation from said protein of interest is removed using PNGase F.

[0020] In some exemplary embodiments, the mass spectrometer is run under native conditions.

[0021] In one aspect of the embodiment, the method further comprises treating said sample to digestion conditions prior to loading said sample on said AEX column. In one specific aspect, the digestion conditions include use of IdeS or a variant thereof.

[0022] In one exemplary embodiment, the method comprises: (a) subjecting a sample having a protein of interest and at least one charge variant to digestion conditions and deglycosylation conditions; (b) loading said sample to an anion-exchange chromatography (AEX) column; (c) applying an increasing salt concentration gradient to the loaded anion exchange column to obtain an eluate; (d) collecting at least one fraction from (c); and (e) subjecting said at least one fraction to mass spectrometry analysis to characterize said at least one charge variant of said protein of interest.

[0023] In one aspect of the embodiment, the digestion conditions include the use of IdeS or a variant thereof.

[0024] In one aspect of the embodiment, the method further comprises applying an increasing salt concentration gradient to the loaded anion exchange column, wherein the salt gradient is an elution gradient and ranges from about 10 mM to about 600 mM ammonium salt. In one specific aspect, the ammonium salt is ammonium acetate. In another specific aspect, the salt gradient is an elution gradient and ranges from about 10 mM to about 300 mM ammonium salt. In another specific aspect, the increasing salt concentration gradient applied is linear.

[0025] In one aspect of the embodiment, the method further comprises monitoring the eluate of (c) for ultraviolet absorbance and collecting said at least one fraction that is eluted prior to elution of the protein of interest. In another aspect of the embodiment, the method further comprises monitoring the eluate of (c) for ultraviolet absorbance and collecting said at least one fraction that is eluted after elution of the protein of interest.

[0026] In one aspect of the embodiment, the protein of interest has a pI value of greater than about 6.2. In a specific aspect of the embodiment, the protein of interest has a pI value of about

6.2 to about 7.0. In a specific aspect of the embodiment, the protein of interest has a pI value of about 6.2 to about 8.7.

[0027] In one aspect of the embodiment, the protein of interest is an IgG4-based monoclonal antibody. In another aspect of the embodiment, the protein of interest is an IgG1-based monoclonal antibody. In yet another aspect of the embodiment, the protein of interest is a bispecific monoclonal antibody.

[0028] In one aspect of the embodiment, the method further comprises reducing flow rate from (d) via a flow-splitter prior to subjecting said at least one fraction to a mass spectrometer.

[0029] In one aspect of the embodiment, said at least one charge variant can be either an acidic variant or a basic variant of the protein of interest. For example, the acidic variants can include deamidation, glycation, glucuronylation, and/or high molecular weight species of the protein of interest, and the basic variants can include C-terminal lysine and glycosylated species.

[0030] In one aspect of the embodiment, the method further comprises removing Fc N-glycosylation from said protein of interest prior to loading said sample on said AEX column. This step can improve the AEX separation due to conversion of Asparagine residues to Aspartic acid. In a specific aspect of this embodiment, the Fc N-glycosylation from said protein of interest is removed using PNGase F.

[0031] In some exemplary embodiments, the mass spectrometer is run under native conditions.

BRIEF DESCRIPTION OF THE DRAWINGS

[0032] FIG. 1 illustrates the concepts of a pH gradient and a salt gradient according to an exemplary embodiment.

[0033] FIG. 2A shows a pH buffer range of salts used for pH gradients according to an exemplary embodiment.

[0034] FIG. 2B shows a pI range of antibodies according to an exemplary embodiment.

[0035] FIG. 2C shows pI values of antibodies tested with the AEX-MS method of the invention according to an exemplary embodiment.

[0036] FIG. 3 illustrates a high salt-tolerant native AEX-MS system according to an exemplary embodiment.

[0037] FIG. 4A shows a trace of a pH gradient in AEX-MS according to an exemplary embodiment.

[0038] FIG. 4B shows a trace of a salt gradient in AEX-MS according to an exemplary embodiment.

[0039] FIG. 5A shows a native AEX-MS analysis of antibodies of varying pI according to an exemplary embodiment.

[0040] FIG. 5B shows a native AEX-MS analysis of antibodies of varying pI according to an exemplary embodiment.

[0041] FIG. 5C shows a native AEX-MS analysis of antibodies of varying pI according to an exemplary embodiment.

[0042] FIG. 5D shows the pI and effectiveness of AEX separation for each of the antibodies of FIG. 5A-C.

[0043] FIG. 6A shows AEX-TICs of antibodies of varying type and pI according to an exemplary embodiment.

[0044] FIG. 6B shows a zoomed in view of the AEX-TICs of FIG. 6A according to an exemplary embodiment.

[0045] FIG. 7 shows an AEX-TIC of a deglycosylated, FabRICATOR-digested IgG4 mAb according to an exemplary embodiment.

[0046] FIG. 8A shows an SCX-TIC of an antibody according to an exemplary embodiment.

[0047] FIG. 8B shows an AEX-TIC of an antibody according to an exemplary embodiment.

[0048] FIG. 9 illustrates deglycosylation of an antibody using PNGase F according to an exemplary embodiment.

[0049] FIG. 10A shows an AEX-TIC comparison of a non-deglycosylated and a deglycosylated antibody according to an exemplary embodiment.

[0050] FIG. 10B shows an AEX-TIC comparison of a non-deglycosylated and a deglycosylated antibody according to an exemplary embodiment.

[0051] FIG. 10C shows an AEX-TIC comparison of a non-deglycosylated and a deglycosylated antibody according to an exemplary embodiment.

[0052] FIG. 11A shows an AEX-TIC comparison of a non-deglycosylated and a deglycosylated antibody according to an exemplary embodiment.

[0053] FIG. 11B shows a zoomed-in view of the AEX-TIC of FIG. 11A according to an exemplary embodiment.

[0054] FIG. 12 shows an AEX-TIC comparison of a non-deglycosylated and a deglycosylated antibody according to an exemplary embodiment.

[0055] FIG. 13 shows an AEX-TIC comparison of a non-deglycosylated and a deglycosylated antibody according to an exemplary embodiment.

[0056] FIG. 14A shows an AEX-TIC comparison of a non-deglycosylated method control of an antibody and a non-deglycosylated near acidic fraction of the antibody according to an exemplary embodiment.

[0057] FIG. 14B shows a zoomed-in view of the AEX-TICs of FIG. 14A according to an exemplary embodiment.

[0058] FIG. 14C shows an AEX-TIC comparison of a deglycosylated method control of an antibody and a deglycosylated near acidic fraction of the antibody according to an exemplary embodiment.

[0059] FIG. 14D shows a zoomed-in view of the AEX-TICs of FIG. 14C according to an exemplary embodiment.

[0060] FIG. 15A shows an AEX-TIC comparison of a non-deglycosylated antibody from drug substance and a non-deglycosylated total acidic fraction of the antibody according to an exemplary embodiment.

[0061] FIG. 15B shows an AEX-TIC comparison of a deglycosylated antibody from drug substance and a deglycosylated total acidic fraction of the antibody according to an exemplary embodiment.

[0062] FIG. 16 illustrates Fc charge variants readily separable by AEX-MS, including glycosylation, deamidation and C-terminal lysines, according to an exemplary embodiment.

[0063] FIG. 17A shows AEX-TICs of deglycosylated Fc regions of various antibodies according to an exemplary embodiment.

[0064] FIG. 17B shows a zoomed-in view of the AEX-TICs of FIG. 17A according to an exemplary embodiment.

[0065] FIG. 18A shows an AEX-TIC of a control and a stressed deglycosylated Fc sample according to an exemplary embodiment.

[0066] FIG. 18B shows a quantification of the charge variants of FIG. 18A according to an exemplary embodiment.

[0067] FIG. 19 illustrates Fc exchange of a bispecific antibody according to an exemplary embodiment.

[0068] FIG. 20 shows an AEX-TIC of a non-deglycosylated bsAb FabRICATOR digest according to an exemplary embodiment.

DETAILED DESCRIPTION

[0069] Thorough characterization of biotherapeutic proteins is an essential requirement at all stages of development and final product quality control. Each protein will have several different variant forms due to multiple post-translational modifications that can alter the charge distribution on the surface of the protein. This can be due to a modification which directly alters the charge state or by indirectly altering the accessible surface charge through a conformational change. These modifications can be chromatographically separated using ion-exchange (IEX) analysis.

[0070] Methods employing ion exchange chromatography coupled on-line to mass spectrometry have previously been developed in order to characterize charge variants in intact proteins. However, the methods published so far illustrate characterization of proteins with respect to their charge variants, wherein the protein has a pI of less than 6.0. For example, Leblanc *et al.*

characterized charge variants of human serum albumin (HSA) (pI = 4.7) using anion exchange chromatography (AEX) coupled online with a MS. See Leblanc *et al. J. Chroma. B,* 2018, 1095: 87–93.

[0071] Fussl *et al.* also reported a pH gradient-based AEX method, which was directly interfaced to a mass spectrometry for characterization of charge variants at the intact protein level under native conditions. Fussl *et al. J. Proteome Res.*, 2019, 18: 3689–3702. However, pH gradient elution on the developed method only facilitated chromatographic selectivity and generic applicability for anionic proteins with pI values <6.5, such as transferrin (pI = 5.2-5.6), ovalbumin (pI = 4.5), and Asialo α-1-AGP (pI = 2.8-3.8). *Id.* at 3690.

[0072] Very recently, Van Schaik *et al.* applied AEX-MS to monitoring multiple quality attributes of biopharmaceutical erythropoietin (EPO, pI = 4.4-5.1). All the aforementioned methods employ a pH-gradient elution to separate the proteins and their charge variants.

[0073] The present invention discloses an AEX method suitable for characterizing charge variants of proteins, which can have pI values of about 6.0 to about 7.0. Two major mechanisms for elution of proteins from AEX column were analyzed to study the separation of charge variants from proteins with a higher pI value than what was previously been published. See, for example FIG. 1. In salt gradient elution, proteins are pushed down the column from exchange site to exchange site by competing with the salt ions in the salt gradient. A focusing effect occurs as the salt concentration increases. With pH gradient elution, proteins will elute from the column when the pH reaches the point where they have little to no charge. A further decrease in pH during the gradient will make the protein more cationic and therefore repelled from the column resin. This is an important difference from salt gradients, as they will then have very little interaction with the rest of the column.

[0074] Advantages of pH gradients are also observed with an increase in the sample loading capacity, as the proteins refocus well at the pH of elution, irrespective of the position where they were originally bound on the column. This also compensates the effect of high salt in the sample, thereby reducing sample matrix effects. This routinely used and favored elution method of pH gradient elution was not useful for proteins having pI of more than about 7 as it is difficult to achieve the required pH using buffers which can be compatible with MS devices (*i.e.*, linear pH gradients are difficult to achieve in the desired range of pH over 7.0 using MS-compatible salts,

which have a buffering gap near the pI range of antibodies). For example, proteins having higher pI compared to what has been previously published would fall into a buffering gap wherein no MS-compatible salts can be used. See for example, FIG. 2A. Theoretically, this gap makes it difficult for AEX to separate antibodies, due to their relatively high pI, as shown in FIG. 2B. IgG1 antibodies tested tended to have a higher pI relative to IgG4 antibodies, as shown in FIG. 2B and 2C.

[0075] This disclosure sets forth a novel native AEX-MS method for characterizing charge variants for proteins by AEX-MS using a salt gradient.

[0076] 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. All publications mentioned are hereby incorporated by reference.

[0077] 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.

[0078] As used herein, the term "protein" includes 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, related naturally occurring structural variants, and synthetic non-naturally occurring analogs thereof. “Synthetic peptides or polypeptides’ 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. A protein may contain one or multiple polypeptides to form a single functioning biomolecule. A protein 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. In another exemplary aspect, a protein can include antibody fragments, nanobodies, recombinant antibody chimeras, cytokines, chemokines, peptide hormones, and the

like. Proteins may be produced using recombinant cell-based production systems, such as the insect baculovirus system, yeast systems (*e.g.*, Pichia sp.), mammalian systems (*e.g.*, CHO cells and CHO derivatives like CHO-K1 cells). For a review discussing biotherapeutic proteins and their production, see Ghaderi et al., "Production platforms for biotherapeutic glycoproteins. Occurrence, impact, and challenges of non-human sialylation," (BIOTECHNOL. GENET. ENG. REV. 147-175 (2012)). In some exemplary embodiments, proteins comprise modifications, adducts, and other covalently linked moieties. Those 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.

[0079] In some exemplary embodiments, the protein of interest can be an antibody, a bispecific antibody, a multispecific antibody, antibody fragment, monoclonal antibody, or an Fc fusion protein.

[0080] 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 V_H) and a heavy chain constant region. The heavy chain constant region comprises three domains, C_H1, C_{H2} and C_H3. Each light chain comprises a light chain variable region (abbreviated herein as LCVR or V_L) and a light chain constant region. The light chain constant region comprises one domain (C_{L1}). The V_H and V_L 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 V_H and V_L 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, FR4. In different exemplary

embodiments, 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, *e.g.*, 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.

[0081] 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 fragment, a Fc 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. An antibody fragment may be produced by various 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. 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.

[0082] 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.

[0083] The term "Fc fusion proteins" as used herein includes part or all of two or more proteins, one of which is an Fc portion of an immunoglobulin molecule, that are not fused in their natural state. Preparation of fusion proteins comprising certain heterologous polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, e.g., by Ashkenazi *et al.*, Proc. Natl. Acad. Sci USA 88: 10535, 1991; Byrn *et al.*, Nature 344:677, 1990; and Hollenbaugh *et al.*, "Construction of Immunoglobulin Fusion Proteins", in Current Protocols in Immunology, Suppl. 4, pages 10.19.1-10.19.11, 1992. "Receptor Fc fusion proteins" comprise one or more of one or more extracellular domain(s) of a receptor coupled to an Fc moiety, which in some embodiments comprises a hinge region followed by a CH2 and CH3 domain of an immunoglobulin. In some embodiments, the Fc-fusion protein contains two or more distinct receptor chains that bind to a single or more than one ligand(s). For example, an Fc-fusion protein is a trap, such as for example an IL-1 trap (*e.g.*, Rilonacept, which contains the IL-1 RAcP ligand binding region fused to the IL-1R1 extracellular region fused to Fc of hIgG1; see U.S. Pat. No. 6,927,004, which is herein incorporated by reference in its entirety), or a VEGF Trap (*e.g.*, Aflibercept, which contains the Ig domain 2 of the VEGF receptor Flt1 fused to the Ig domain 3 of the VEGF receptor Flk1 fused to Fc of hIgG1; *e.g.*, SEQ ID NO:1; see U.S. Pat. Nos. 7,087,411 and 7,279,159, which are herein incorporated by reference in their entirety)

[0084] 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.

[0085] As used herein, the term "charge variants", refers to the full complement of product variants including, but not limited to acidic species, and basic species (*e.g.*, Lys variants). In certain embodiments, such variants can include product aggregates and/or product fragments, to the extent that such aggregation and/or fragmentation results in a product charge variation.

[0086] As used herein, the term "acidic species" refers to the variants of a protein which are characterized by an overall acidic charge. Exemplary acidic species can include, but are not limited to, deamidation variants, afucosylation variants, oxidation variants, methylglyoxal (MGO) variants, glycation variants, and citric acid variants. Exemplary structure variants include, but are not limited to, glycosylation variants and acetonation variants. Exemplary fragmentation variants include any modified protein species from the target molecule due to dissociation of peptide chain, enzymatic and/or chemical modifications, including, but not limited to, Fc and Fab fragments, fragments missing a Fab, fragments missing a heavy chain variable domain, C-terminal truncation variants, variants with excision of N-terminal Asp in the light chain, and variants having N-terminal truncation of the light chain. Other acidic species variants include variants comprising unpaired disulfides, host cell proteins, and host nucleic acids, chromatographic materials, and media components. Commonly, acidic species elute later than the main peak during CEX or later than the main peak during AEX analysis

[0087] As used herein, the term "basic species" refers to the variants of a protein, for example, an antibody or antigen-binding portion thereof, which are characterized by an overall basic charge, relative to the primary charge variant species present within the protein. Exemplary basic species can include, but are not limited to, lysine variants, isomerization of aspartic acid, succinimide formation at asparagine, methionine oxidation, amidation, incomplete disulfide bond formation, mutation from serine to arginine, aglycosylation, fragmentation and aggregation. Commonly, basic species elute later than the main peak during CEX or earlier than the main peak during AEX analysis.

[0088] As used herein, "ion exchange chromatography" can refer to separations including any

method by which two substances are separated based on differences in their respective ionic charges, either on the molecule of interest and/or chromatographic material as a whole or locally on specific regions of the molecule of interest and/or chromatographic material, and thus can employ either cationic exchange material or anionic exchange material. Ion exchange chromatography separates molecules based on differences between the local charges of the molecules of interest and the local charges of the chromatographic material. A packed ion-exchange chromatography column or an ion-exchange membrane device can be operated in a bind-elute mode, a flowthrough mode, or a hybrid mode. After washing the column or the membrane device with an equilibration buffer or another buffer, product recovery can be achieved by increasing the ionic strength (*i.e.*, conductivity) of the elution buffer to compete with the solute for the charged sites of the ion exchange matrix. Changing the pH and thereby altering the charge of the solute can be another way to achieve elution of the solute. The change in conductivity or pH may be gradual (gradient elution) or stepwise (step elution). Anionic or cationic substituents may be attached to matrices in order to form anionic or cationic supports for chromatography. Non-limiting examples of anionic exchange substituents include diethylaminoethyl (DEAE), quaternary aminoethyl (QAE) and quaternary amine (Q) groups.

[0089] 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 eluted for detection and/or characterization. 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). The choice of ion source depends heavily on the application.

[0090] In some embodiments, the mass spectrometer can be an electrospray-mass spectrometer.

[0091] As used herein, the term "electrospray ionization" or "ESI" refers to the process of spray ionization in which either cations or anions in solution are transferred to the gas phase via formation and desolvation at atmospheric pressure of a stream of highly charged droplets that result from applying a potential difference between the tip of the electrospray needle containing the solution and a counter electrode.

[0092] In some exemplary embodiments, the electrospray ionization mass spectrometer can be a nano-electrospray ionization mass spectrometer.

[0093] 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.

[0094] 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 for fast protein sequencing 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).

[0095] In some exemplary embodiments, mass spectrometry can be performed under native conditions.

[0096] As used herein, the term "native conditions" or "native MS" or "native ESI- MS" can include a performing mass spectrometry under conditions that preserve no-covalent interactions in an analyte. For detailed review on native MS, refer to the review: Elisabetta Boeri Erba & Carlo Petosa, *The emerging role of native mass spectrometry in characterizing the structure and dynamics of macromolecular complexes*, 24 PROTEIN SCIENCE1176–1192 (2015). Some of the distinctions between native ESI and regular ESI are illustrated in table 1 and FIG. 1 (Hao Zhang *et al.*, *Native mass spectrometry of photosynthetic pigment-protein complexes*, 587 FEBS Letters 1012–1020 (2013)).

[0097] In some exemplary embodiments, the mass spectrometer can be a tandem mass spectrometer.

[0098] 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 have been successfully performed with a wide variety of analyzer combinations. What analyzers to combine for a certain application is 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.

[0099] 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 can include, 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.

[0100] As used herein, the term "database" refers to bioinformatic tools which provide the possibility of searching the uninterpreted MS-MS spectra against all possible sequences in the database(s). Non-limiting examples of such tools are Mascot (http://www.matrixscience.com), Spectrum Mill (http://www.chem.agilent.com), PLGS (http://www.waters.com), PEAKS (http://www.bioinformaticssolutions.com), Proteinpilot (http://download.appliedbiosystems.com//proteinpilot), Phenyx (http://www.phenyx-ms.com), Sorcerer (http://www.sagenresearch.com), OMSSA

(http://www.pubchem.ncbi.nlm.nih.gov/omssa/), X!Tandem (http://www.thegpm.org/TANDEM/), Protein Prospector (http://www.http://prospector.ucsf.edu/prospector/mshome.htm), Byonic (https://www.proteinmetrics.com/products/byonic) or Sequest (http://fields.scripps.edu/sequest).

[0101] 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.

[0102] As used herein, the term "hydrolyzing agent" refers to any one or combination 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 trypsin, endoproteinase Arg-C, endoproteinase Asp-N, endoproteinase Glu-C, outer membrane protease T (OmpT), immunoglobulin-degrading enzyme of Streptococcus pyogenes (IdeS), chymotrypsin, pepsin, thermolysin, papain, pronase, and protease from *Aspergillus Saitoi.* Non-limiting examples of hydrolyzing agents that can carry out non-enzymatic digestion include the use of high temperature, microwave, ultrasound, high pressure, infrared, solvents (non-limiting examples are ethanol and acetonitrile), immobilized enzyme digestion (IMER), magnetic particle immobilized enzymes, and on-chip immobilized enzymes. 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" (J. Proteome Research 2013, 12, 1067-1077). One or a combination of hydrolyzing agents can cleave peptide bonds in a protein or polypeptide, in a sequence-specific manner, generating a predictable collection of shorter peptides.

EXAMPLES

[0103] IgG1- and IgG4-based therapeutic mAb samples were either directly analyzed or treated with PNGase F and/or FabRICATOR before being injected and separated on a YMC BioPro QA-F SAX column (4.6 x 100 mm). A previously reported native LC-MS platform was applied to accommodate the analytical flowrate (0.4 mL/min) with nanoelectrospray ionization (NSI). Ammonium acetate-based mobile phases ranging from 10 to 300 mM were used to develop salt-based gradient for mAb elution. To elucidate variant peaks resulting from either site-specific deamidation or deglycosylation reactions, offline fractionation and tryptic peptide

mapping analyses were performed. Synthetic peptides were also used to differentiate deamidation products (*e.g.*, iso-Asp vs Asp) based on retention time alignment.

[0104] To develop the method of the present invention, AEX was first investigated using either pH or salt gradient modes, as illustrated in FIG. 1. The system used was a Dionex Ultimate 3000 HPLC – Q Exactive UHMR. An exemplary system and method for AEX-MS is described in Yan *et al.*, 2020, *J Am Soc Mass Spectrom*, 31:2171-2179, which is hereby incorporated by reference, and illustrated in FIG. 3.

[0105] Experiments using AEX-MS with a pH gradient or a salt gradient are shown in FIG. 4A and 4B. A linear pH gradient with the desired pH range cannot be achieved for AEX analysis of mAbs using MS-compatible ammonia-based mobile phases. However, a linear salt gradient can be achieved using MS-compatible mobile phases. The developed native LC-MS platform can tolerate salt concentrations up to 600 mM.

[0106] A wide range of antibodies were tested using the AEX-MS system to determine which mAbs would most benefit from AEX separation. AEX TICs of tested antibodies are shown in order of increasing pI in FIG. 5A, 5B, and 5C, with a summary of the results in 5D. Generally, mAbs with relatively lower pI's (pH < 7) had better AEX separation.

[0107] Another comparison of mAbs and bsAbs was conducted using the AEX-MS method of the invention. Full-scale AEX-TICs are shown in FIG. 6A and 3x zoom AEX-TICs are shown in FIG. 6B. The native AEX-MS method was shown to be suitable for most IgG4 molecules with moderate pI, allowing for the identification of various acidic and basic species, and less useful for IgG1 mAbs with high pI. Generally, AEX separation improved for molecules with lower pI. Common acidic variants observed included deamidation, glycation, and NeuAc. Common basic variants observed included partial and non-glycosylated species, and species with C-terminal lysines.

[0108] Further analysis was conducted on deglycosylated IgG4 mAbs that were treated with IdeS (FabRICATOR® from Genovis) to perform Fc attribute monitoring, as shown in FIG. 7. Specific acidic peaks were found to correlate to site-specific deamidation. Identities were confirmed by fractionating a deglycosylated and FabRICATOR®-digested thermally-stressed

IgG4 sample, performing subsequent reduced peptide mapping, and comparing the results against synthetic peptides.

[0109] A comparison was made between SCX-MS and AEX-MS for Ab21, as shown in FIG. 8A versus 8B. AEX-MS was able to identify a greater number of variants compared to SCX-MS, demonstrating that for proteins with lower pI's, AEX can be a useful alternative to SCX.

[0110] Further improvements were made to the method of the present invention. AEX separation was improved by deglycosylating the analyte antibody with PNGase F, lowering the pI of the sample, as illustrated in FIG. 9. Non-deglycosylated and deglycosylated antibodies were subjected to AEX-MS analysis and the AEX-TICs were compared. Deglycosylation lowered mAb pI, resulting in later elution and improved AEX separation and resolution, as shown in FIG. 10A-C. For mAbs with relatively high pI's (pH > 7), the deglycosylation step can help the retention of the molecule, but the improvement in resolution was not as significant as for mAbs with lower pI's.

[0111] A further comparison of non-deglycosylated and deglycosylated mAb subjected to AEX-MS is shown in FIG. 11A, with a zoom in in FIG. 11B. The method was able to detect a large number of basic and acidic species and differentiate different protein modifications. After deglycosylation, overall resolution was improved. The basic variants detected included C-terminal lysines and converted partially glycosylated species. The acidic variants detected included deamidation, glycation/glucuronylation, and high molecular weight (HMW) species.

[0112] A similar comparison was carried out for another antibody as shown in FIG. 12. Again, AEX resolution was greatly improved after deglycosylation. Basic variants detected included C-terminal lysines, non-glycosylated species, and converted partially glycosylated species. Acidic variants detected included deamidation and glycation/glucuronylation species.

[0113] AEX-MS analysis with and without deglycosylation was carried out for another antibody as shown in FIG. 13. Again, AEX resolution was greatly improved after deglycosylation. Basic variants detected included C-terminal lysine and converted partially glycosylated species. Acidic variants detected included deamidation, glycation/glucuronylation, and NeuAc species.

[0114] There are a variety of potential applications for the method of the invention. For example, AEX-MS was used for in-depth characterization of enriched charge variants as shown in

FIG. 14. Method control samples (Mtd ctrl) were compared to fractionated near acidic species (Acidic 1) in non-deglycosylated and glycosylated samples and subjected to AEX-MS analysis. Basic variants observed in the method control include succinimide and C-terminal lysine, as shown in FIG. 14A and 14B. Acidic variants observed include deamidation, glycation/glucuronylation and sialic acid (NeuAc). The method was capable of further separating charge variants into site-specific deamidation sites. After deglycosylation, additional succinimide peaks were also identified, as shown in FIG. 14C and 14D.

[0115] A different enriched charged variant analysis was carried out on another antibody as shown in FIG. 15. Antibody drug substance was compared to a total acidic species fraction. The total acidic pool consists of acidic variants such as deamidation, glycation/glucuronylation and sialic acid (NeuAc), as shown in FIG. 15A. The acidic variants could be further separated into site-specific deamidation sites. After deglycosylation, additional resolution of HMW species was observed, as shown in FIG. 15B

[0116] A second application explored was the use of AEX-MS for high-resolution Fc attribute monitoring. After deglycosylation, AEX-MS can simultaneously monitor glycosylation states, C-terminal lysines, and site-specific deamidation, as illustrated in FIG. 16. Because the Fc region of related antibodies, for example IgG4s, is shared, analysis of Fc attributes provides representative information on a wide range of antibodies. An exemplary analysis of a range of proteins is shown in FIG. 17A and 17B, with different peaks representing variants in glycosylation, C-terminal lysines and site-specific deamidation. The method was demonstrated to be broadly applicable to IgG4 antibodies.

[0117] This analysis was further applied to monitor stability samples, as shown in FIG. 18. An AEX-TIC showing a separation of modified antibody species is shown in FIG. 18A, with two overlapping traces indicating a naïve sample (in black, lower trace) and a stressed sample (in red, higher trace). The stressed sample was stored at 25° C for 6 months prior to analysis. The abundance of different charged species can be compared and quantified as shown in FIG. 18B. In this example, use of the AEX-MS method of the present invention allowed for the discovery that acidic variants of the antibody (see, for example, A1) increased after thermal stress. The site-specific deamidation site A1 likely correlates with VSNK abased on previous studies.

[0118] A further application explored is using AEX-MS to monitor Fc and Fc* exchange in a bispecific antibody. It is known that IgG4 can undergo rapid Fc exchange after removing the hinge disulfide bonds. For a bsAb, the Fc and Fc* will exchange into a 1:2:1 ratio of Fc*/Fc* : Fc*/Fc : Fc/Fc products after FabRICATOR® digestion, as illustrated in FIG. 19. The AEX-MS method of the present invention was applied to a bsAb FaBRICATOR® digest and proved sensitive enough to detect Fc exchange and separate Fc species in the predicted 1:2:1 ratio as shown in FIG. 20. In this example Fc* exhibited much higher C-terminal lysine compared to Fc.

[0119] A salt-gradient based native AEX-MS method has been developed that can be potentially applied to a range of protein analysis, including charge variant analysis of mAbs with relatively low pI's. The AEX separation profile and resolution of mAbs can be further improved by deglycosylation. AEX is very sensitive to glycosylation states, as well as C-terminal lysine and deamidation. Common basic variants observed in AEX-MS include C-terminal lysine, non-glycosylated or partially glycosylated variants, and succinimide. Common acidic variants observed in AEX-MS include NeuAc, deamidation, glycation and glucuronylation.

[0120] AEX can be used orthogonally for in-depth characterization of enriched charge variant samples at BLA stage. AEX-MS can also be used for high-resolution Fc attribute monitoring, and in particular for monitoring site-specific deamidations.

What is claimed is:

1. A method for characterizing at least one charge variant of a protein of interest, comprising:
    a. loading a sample having a protein of interest and at least one charge variant of said protein of interest to an anion-exchange chromatography (AEX) column;
    b. applying an increasing salt concentration gradient to the loaded anion exchange column to obtain an eluate;
    c. collecting at least one fraction from b); and
    d. subjecting said at least one fraction to mass spectrometry analysis to characterize said at least one charge variant of said protein of interest.
2. The method of claim 1, wherein the salt gradient is an elution gradient and ranges from about 10 mM to about 600 mM ammonium salt.
3. The method of claim 1 further, wherein the salt gradient is an elution gradient and ranges from about 10 mM to about 300 mM ammonium salt.
4. The method of claim 1, wherein the increasing salt concentration gradient applied is linear.
5. The method of claim 3, wherein the increasing salt concentration gradient applied is linear.
6. The method of claim 1 further comprising monitoring the eluate of b) for ultraviolet absorbance and collecting said at least one fraction that is eluted prior to or after elution of the protein of interest.
7. The method of claim 1, wherein the protein of interest has a pI value of greater than about 6.2.
8. The method of claim 1, wherein the protein of interest is an IgG4-based monoclonal antibody.
9. The method of claim 1, wherein the protein of interest is an bispecific monoclonal antibody.
10. The method of claim 1, wherein the at least one charge variant is deamidation, glycation, glucuronylation, high molecular weight species, C-terminal lysine, or glycosylated species of the protein of interest.

11. The method of claim 1 further comprising removing Fc N-glycosylation from said protein of interest prior to loading said sample on AEX column.

12. The method of claim 1 further comprising treating said sample to digestion conditions prior to loading said sample on AEX column.

13. The method of claim 12, wherein the digestion conditions include use of IdeS or variant thereofs.

14. The method of claim 1, wherein the mass spectrometer is run under native conditions.

15. A method for characterizing at least one charge variant of a protein of interest, comprising:

    a. subjecting a sample having a protein of interest and at least one charge variant to deglycosylation conditions;

    b. loading said sample to an anion-exchange chromatography (AEX) column;

    c. applying an increasing salt concentration gradient to the loaded anion exchange column to obtain an eluate;

    d. collecting at least one fraction from c); and

    e. subjecting said at least one fraction to mass spectrometry analysis to characterize said at least one charge variant of said protein of interest.

16. The method of claim 15, wherein the salt gradient is an elution gradient and ranges from about 10 mM to about 600 mM ammonium salt.

17. The method of claim 15, wherein the salt gradient is an elution gradient and ranges from about 10 mM to about 300 mM ammonium salt.

18. The method of claim 15, wherein the increasing salt concentration gradient applied is linear.

19. The method of claim 18, wherein the increasing salt concentration gradient applied is linear.

20. The method of claim 15 further comprising monitoring the eluate of b) for ultraviolet absorbance and collecting said at least one fraction that is eluted prior to or after elution of the protein of interest.

21. The method of claim 15, wherein the protein of interest has a pI value of greater than about 6.2.

22. The method of claim 15, wherein the at least one charge variant is deamidation, glycation, glucuronylation, high molecular weight species, C-terminal lysine, or glycosylated species of the protein of interest.

23. The method of claim 15 further comprising treating said sample to digestion conditions prior to loading said sample on AEX column.

24. The method of claim 15, wherein the digestion conditions include use of IdeS or variant thereof.

25. The method of claim 15, wherein the mass spectrometer is run under native conditions

26. A method for characterizing at least one charge variant of a protein of interest, comprising:

    a. subjecting a sample having a protein of interest and at least one charge variant to digestion condition and deglycosylation condition;

    b. loading said sample to an anion-exchange chromatography (AEX) column;

    c. applying an increasing salt concentration gradient to the loaded anion exchange column to obtain an eluate;

    d. collecting at least one fraction from c); and

    e. subjecting said at least one fraction to mass spectrometry analysis to characterize said at least one charge variant of said protein of interest.

27. The method of claim 26, wherein the mass spectrometer is run under native conditions

28. The method of claim 26, wherein the digestion conditions include use of IdeS or variant thereof.

29. The method of claim 26, wherein the salt gradient is an elution gradient and ranges from about 10 mM to about 600 mM ammonium salt.

30. The method of claim 26, wherein the salt gradient is an elution gradient and ranges from about 10 mM to about 300 mM ammonium salt.

31. The method of claim 26, wherein the increasing salt concentration gradient applied is linear.

32. The method of claim 30, wherein the increasing salt concentration gradient applied is linear.

33. The method of claim 26 further comprising monitoring the eluate of b) for ultraviolet absorbance and collecting said at least one fraction that is eluted prior to or after elution of the protein of interest.

34. The method of claim 26, wherein the protein of interest has a pI value of greater than about 6.2.

35. The method of claim 26, wherein the at least one charge variant is deamidation, glycation, glucuronylation, high molecular weight species, C-terminal lysine, or glycosylated species of the protein of interest.

36. The method of claim 26 further comprising treating said sample to digestion conditions prior to loading said sample on AEX column.

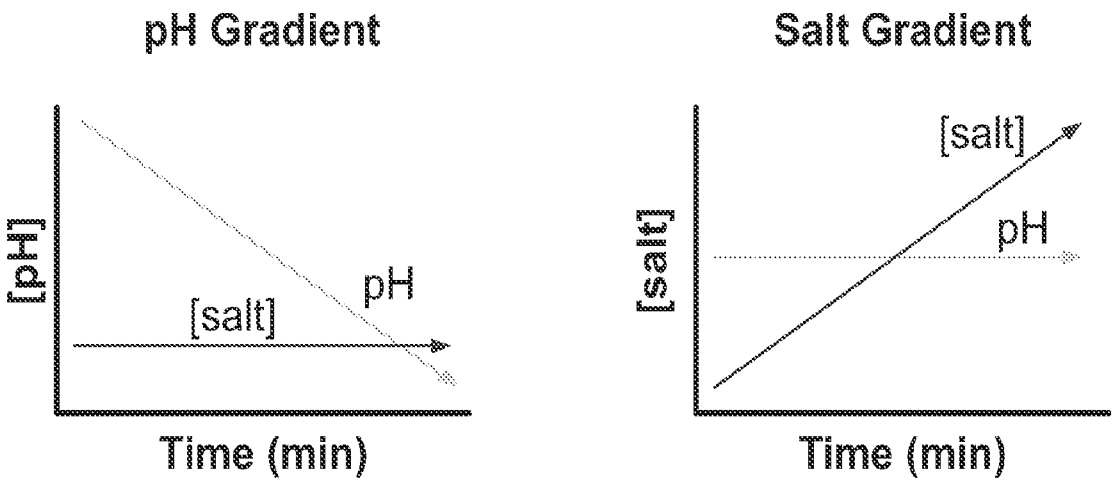

pH Gradient
[pH]
pH
[salt]
Time (min)
Salt Gradient
[salt]
[salt]
pH
Time (min)

FIG. 1

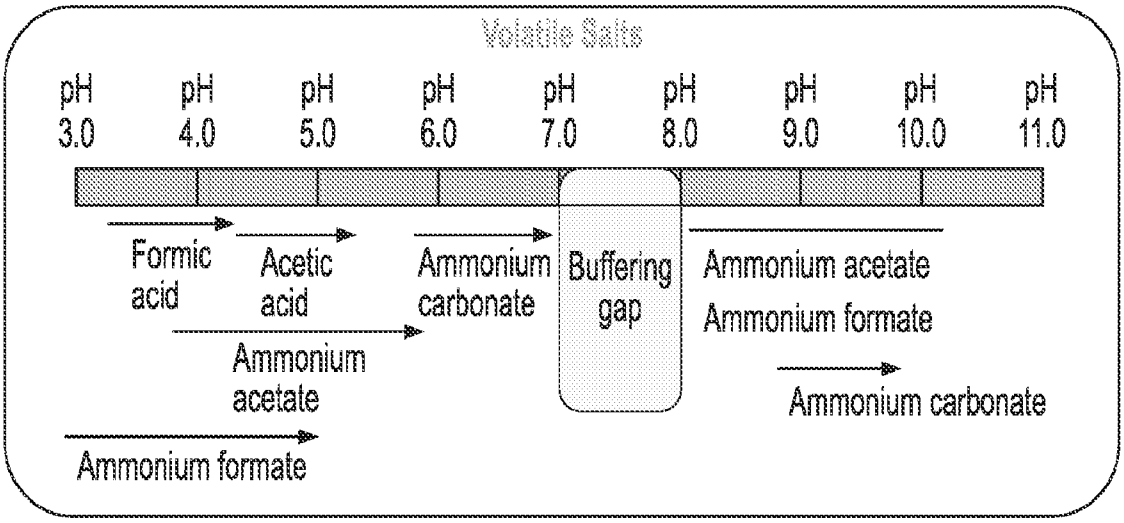


FIG. 2A

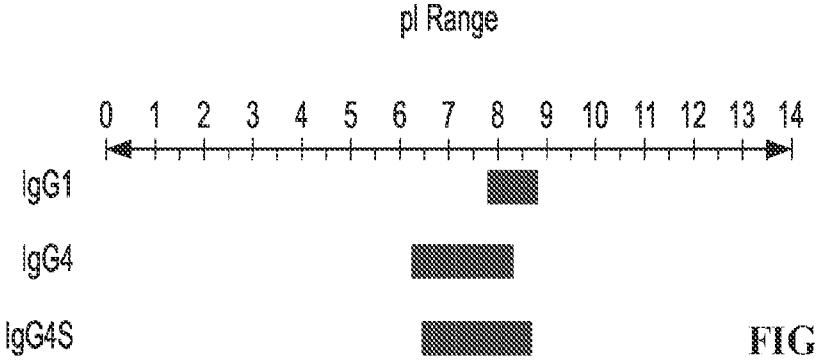


FIG. 2B

mAbs/bsAbs	Isotype	pI
Ab1	IgG1	7.82
Ab2	IgG1	7.98
Ab3	IgG1	8.30
Ab4	IgG1	8.30
Ab5	IgG1	8.32
Ab6	IgG1	8.32
Ab7	IgG1	8.61
Ab8	IgG1	8.66
Ab9	IgG1	8.79
Ab10	IgG1	6.51
Ab11	IgG4S	6.62
Ab12	IgG4S	7.37
Ab13	IgG4S	7.59
Ab14	IgG4S	7.66
Ab15	IgG4S	8.59
Ab16	IgG4S	8.67

mAbs/bsAbs	Isotype	pI
Ab17	IgG4	6.28
Ab18	IgG4	6.42
Ab19	IgG4	6.57
Ab20	IgG4	6.82
Ab21	IgG4	6.82
Ab22	IgG4	6.86
Ab23	IgG4	6.86
Ab24	IgG4	6.87
Ab25	IgG4	6.92
Ab26	IgG4	7.33
Ab27	IgG4	7.60
Ab28	IgG4	7.82
Ab29	IgG4	7.89
Ab30	IgG4	8.02

FIG. 2C

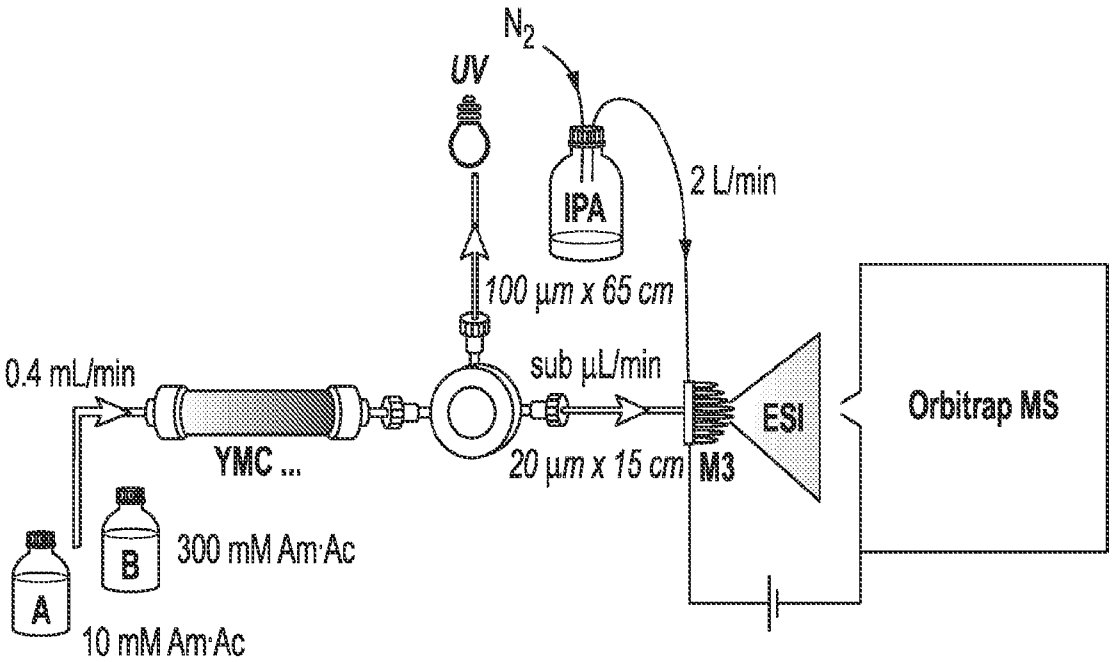
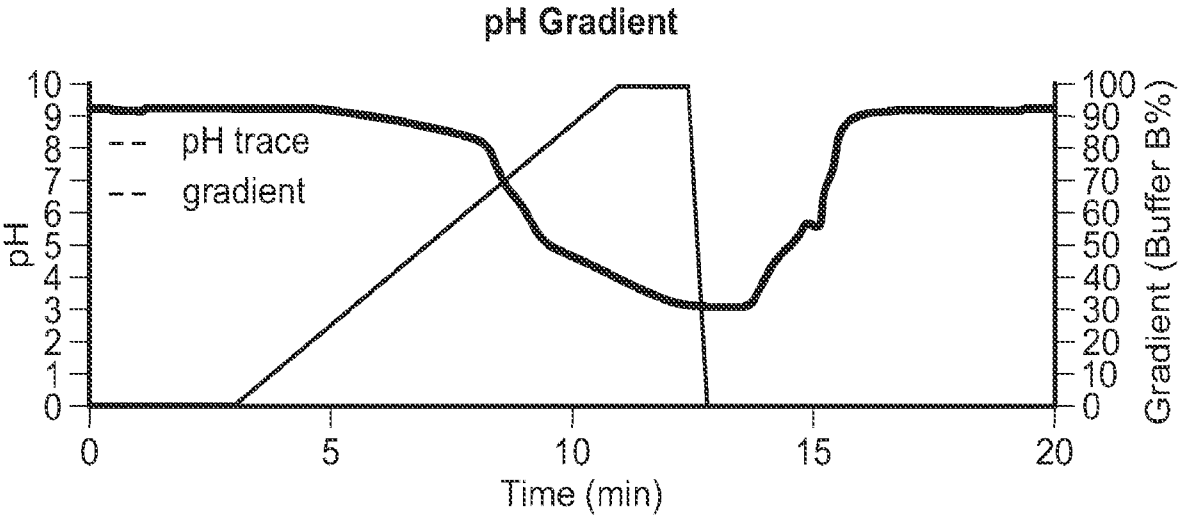
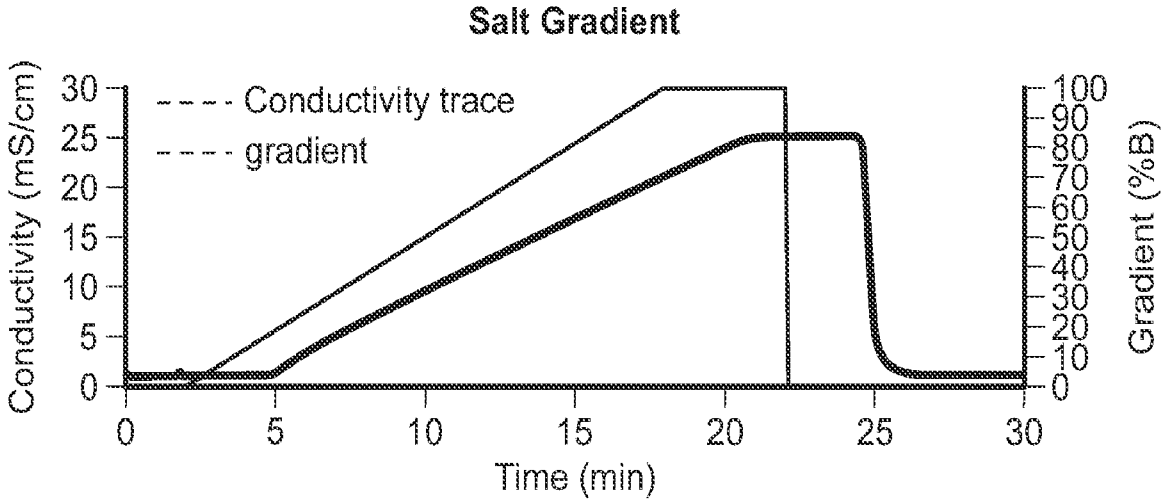

N2
UV
IPA
2 L/min
100 µm x 65 cm
0.4 mL/min
sub µL/min
ESI
Orbitrap MS
YMC ...
20 µm x 15 cm
M3
B
300 mM Am·Ac
A
10 mM Am·Ac

FIG. 3



MPA: 10 mM ammonium acetate, pH 9.0
MPB: 50 mM ammonia, pH 5.5

FIG. 4A



MPA: 10 mM ammonium acetate
MPB: 300 mM ammonium acetate

FIG. 4B

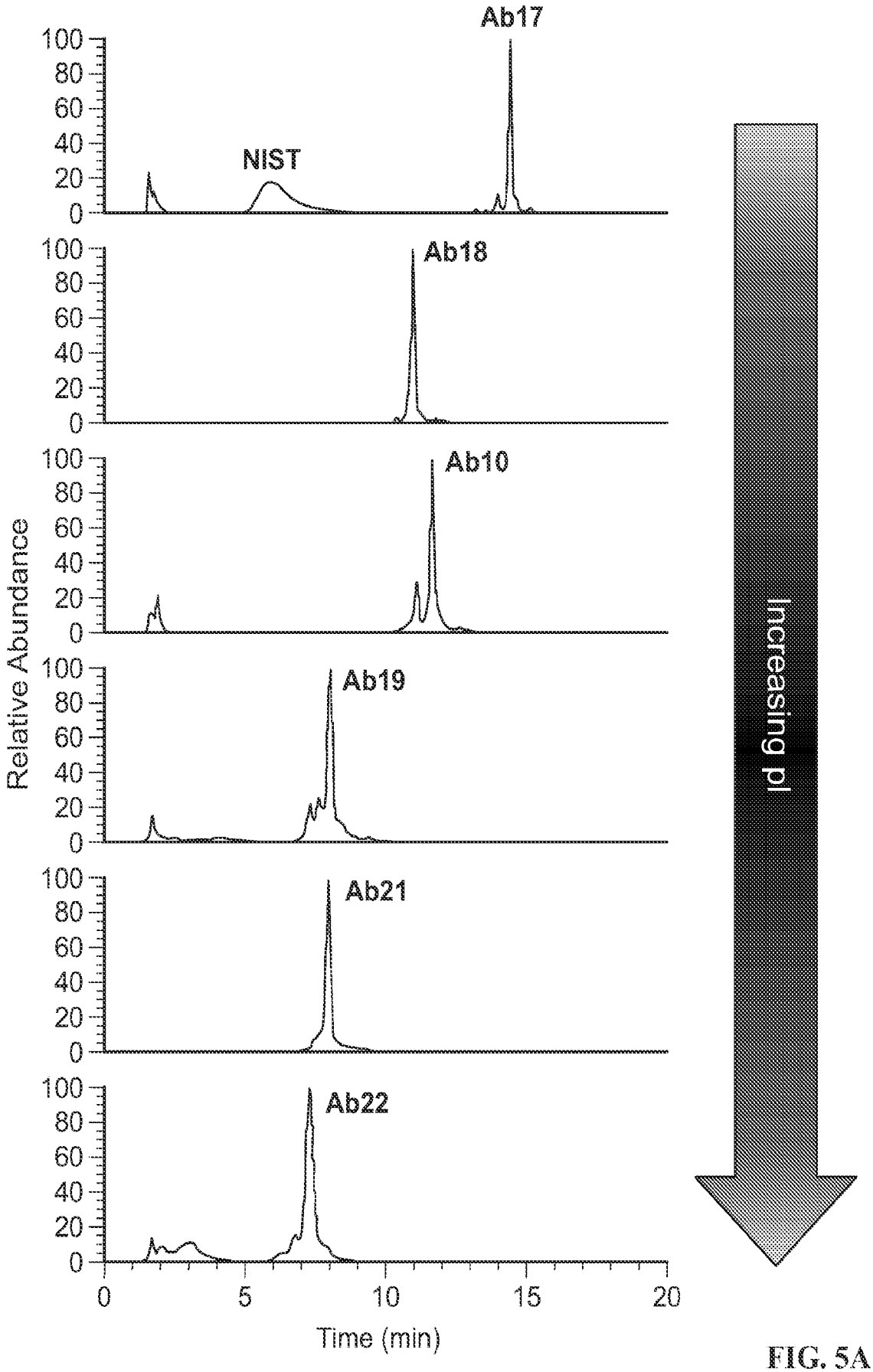

Ab17
NIST
Ab18
Ab10
Ab19
Ab21
Ab22
Relative Abundance
Time (min)
Increasing pI

FIG. 5A

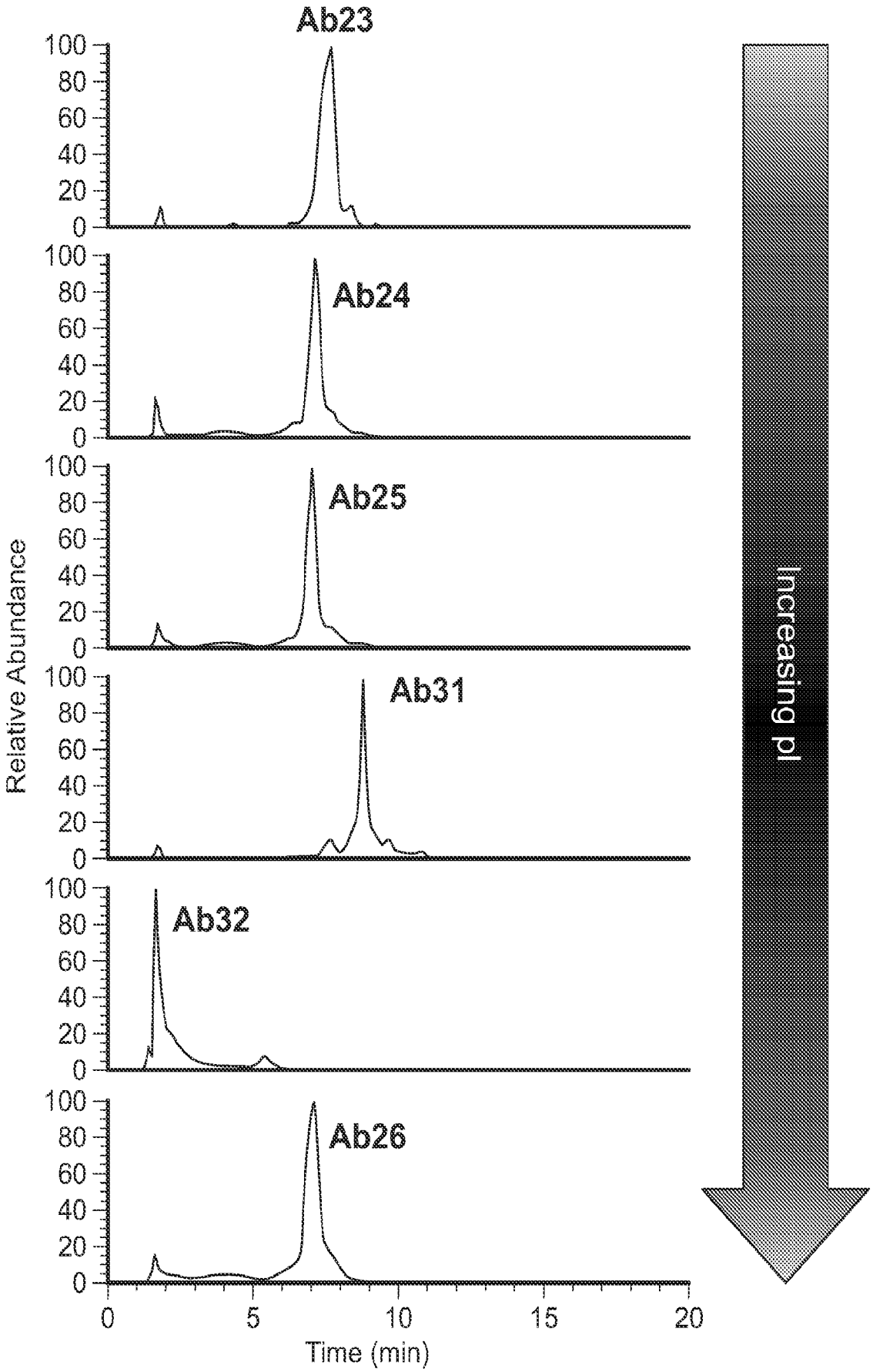

Ab23
Ab24
Ab25
Ab31
Ab32
Ab26
Relative Abundance
Time (min)
Increasing pI

FIG. 5B

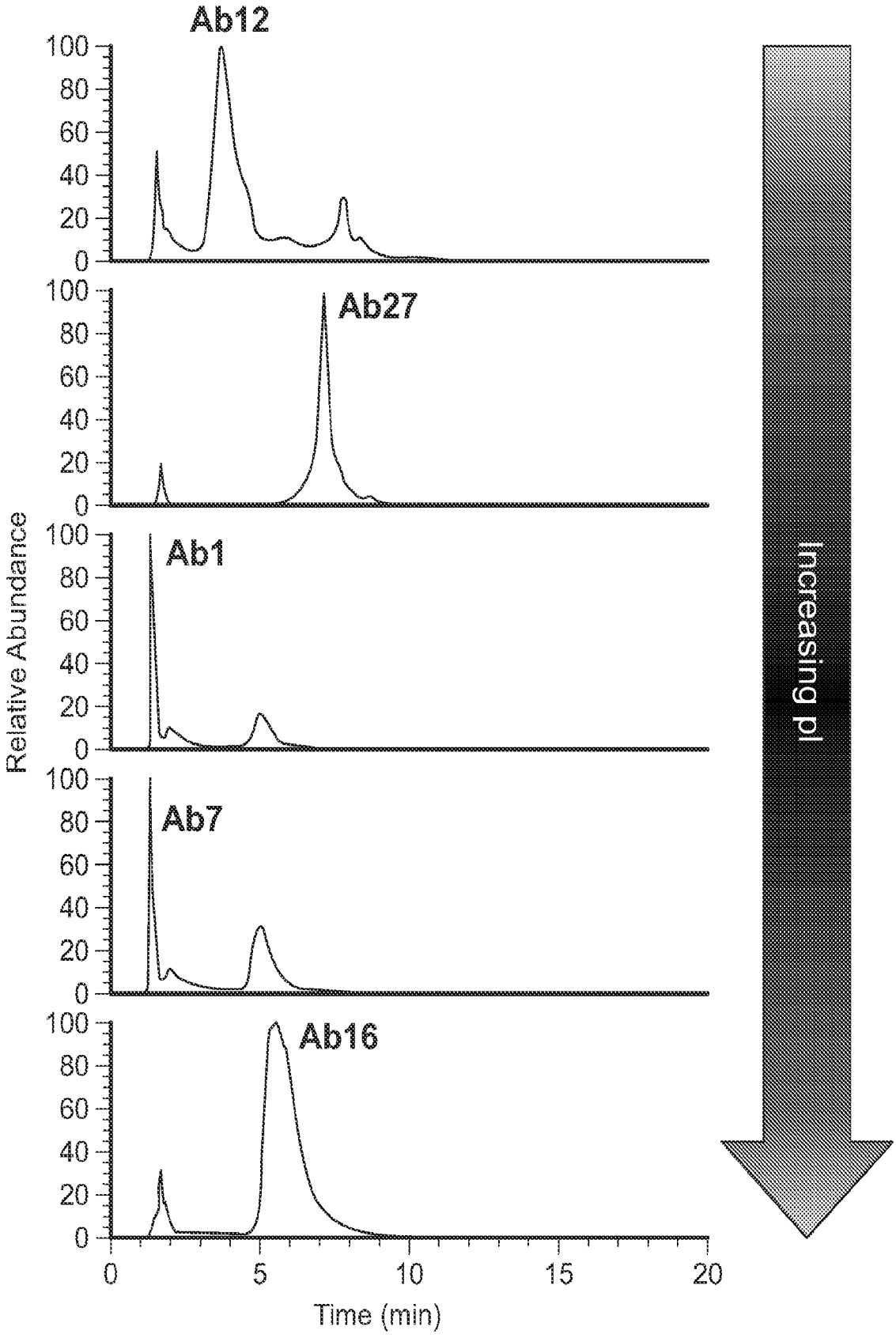

Ab12
Ab27
Ab1
Ab7
Ab16
Relative Abundance
Time (min)
Increasing pI

FIG. 5C

mAb	pI	AEX Separation
Ab17	6.28	Good
Ab18	6.42	Good
Ab10	6.51	Good
Ab19	6.57	Good
Ab21	6.82	Good
Ab22	6.86	Good
Ab23	6.86	Some
Ab24	6.87	Some
Ab25	6.92	Some
Ab31	7.02	Some
Ab32	7.28	Poor
Ab26	7.33	Some
Ab12	7.37	Poor
Ab27	7.60	Some
Ab1	7.82	Poor
Ab7	8.61	Poor
NISTmAb	8.64	Poor
Ab16	8.67	Poor

FIG. 5D

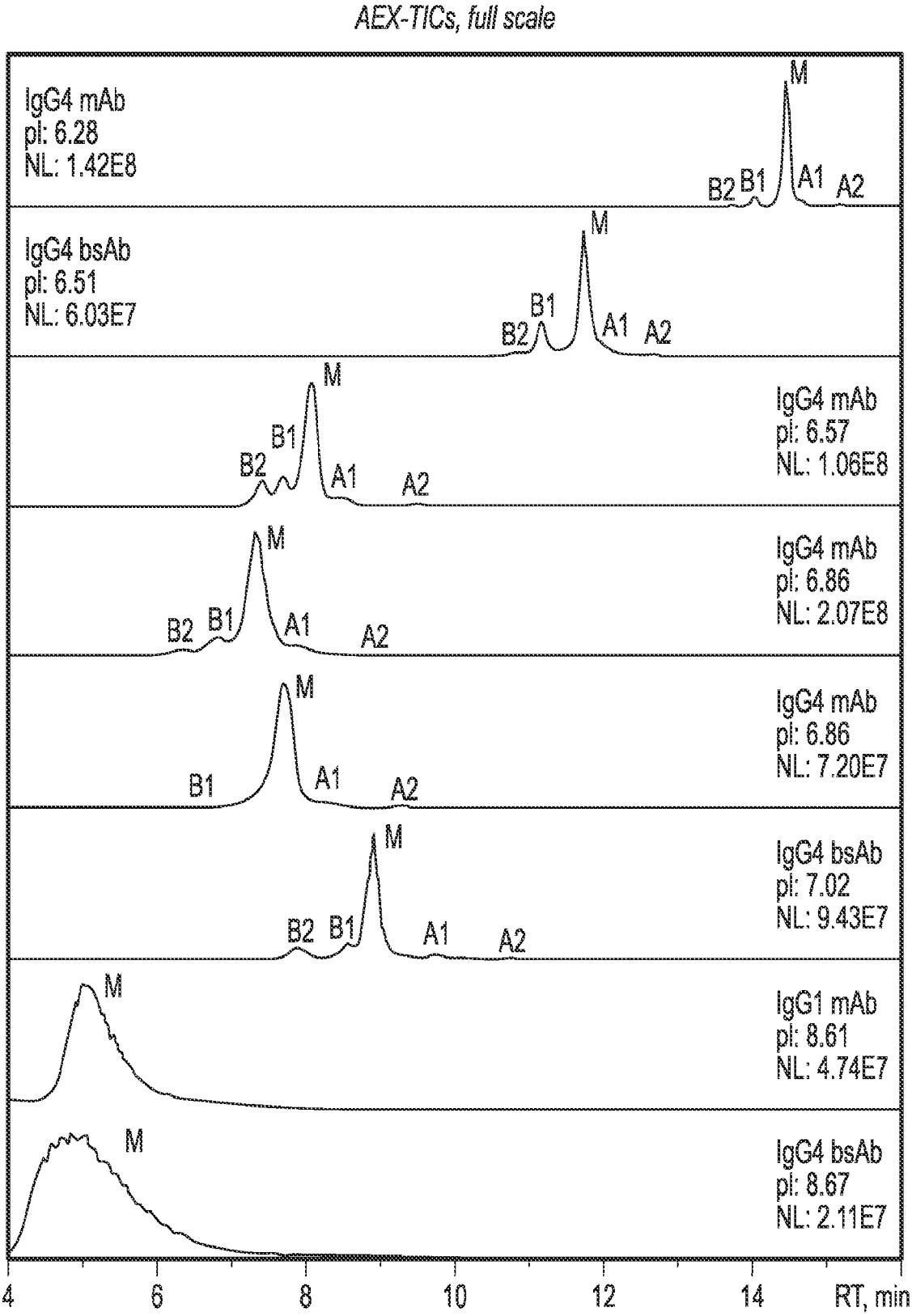

AEX-TICs, full scale
IgG4 mAb
pI: 6.28
NL: 1.42E8
IgG4 bsAb
pI: 6.51
NL: 6.03E7
IgG4 mAb
pI: 6.57
NL: 1.06E8
IgG4 mAb
pI: 6.86
NL: 2.07E8
IgG4 mAb
pI: 6.86
NL: 7.20E7
IgG4 bsAb
pI: 7.02
NL: 9.43E7
IgG1 mAb
pI: 8.61
NL: 4.74E7
IgG4 bsAb
pI: 8.67
NL: 2.11E7
M
B2
B1
A1
A2
4
6
8
10
12
14
RT, min

FIG. 6A

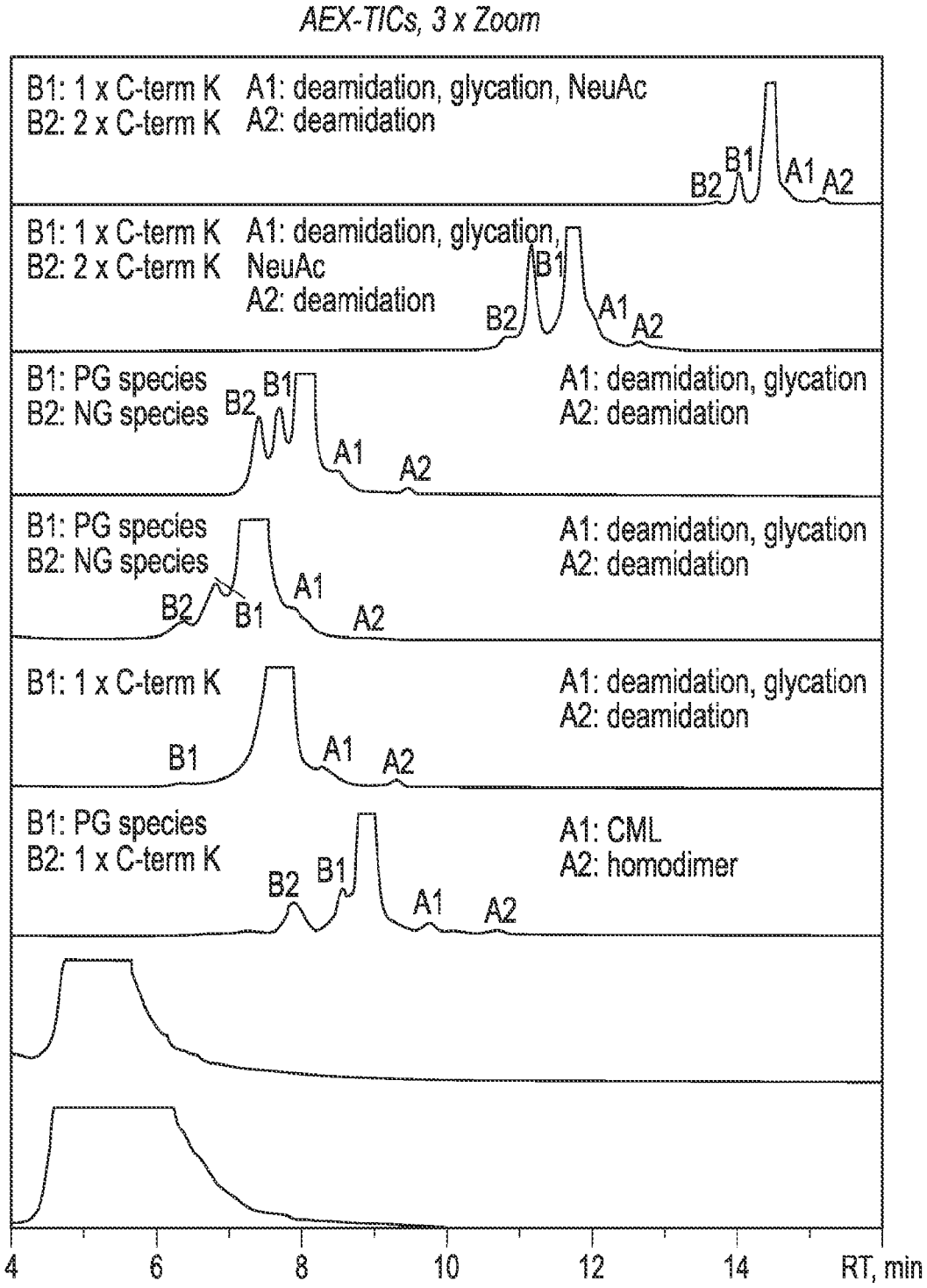

AEX-TICs, 3 x Zoom
B1: 1 x C-term K
B2: 2 x C-term K
A1: deamidation, glycation, NeuAc
A2: deamidation
B2
B1
A1
A2
B1: 1 x C-term K
B2: 2 x C-term K
A1: deamidation, glycation, NeuAc
A2: deamidation
B1: PG species
B2: NG species
A1: deamidation, glycation
A2: deamidation
B1: 1 x C-term K
B1: PG species
B2: 1 x C-term K
A1: CML
A2: homodimer
4
6
8
10
12
14
RT, min

FIG. 6B

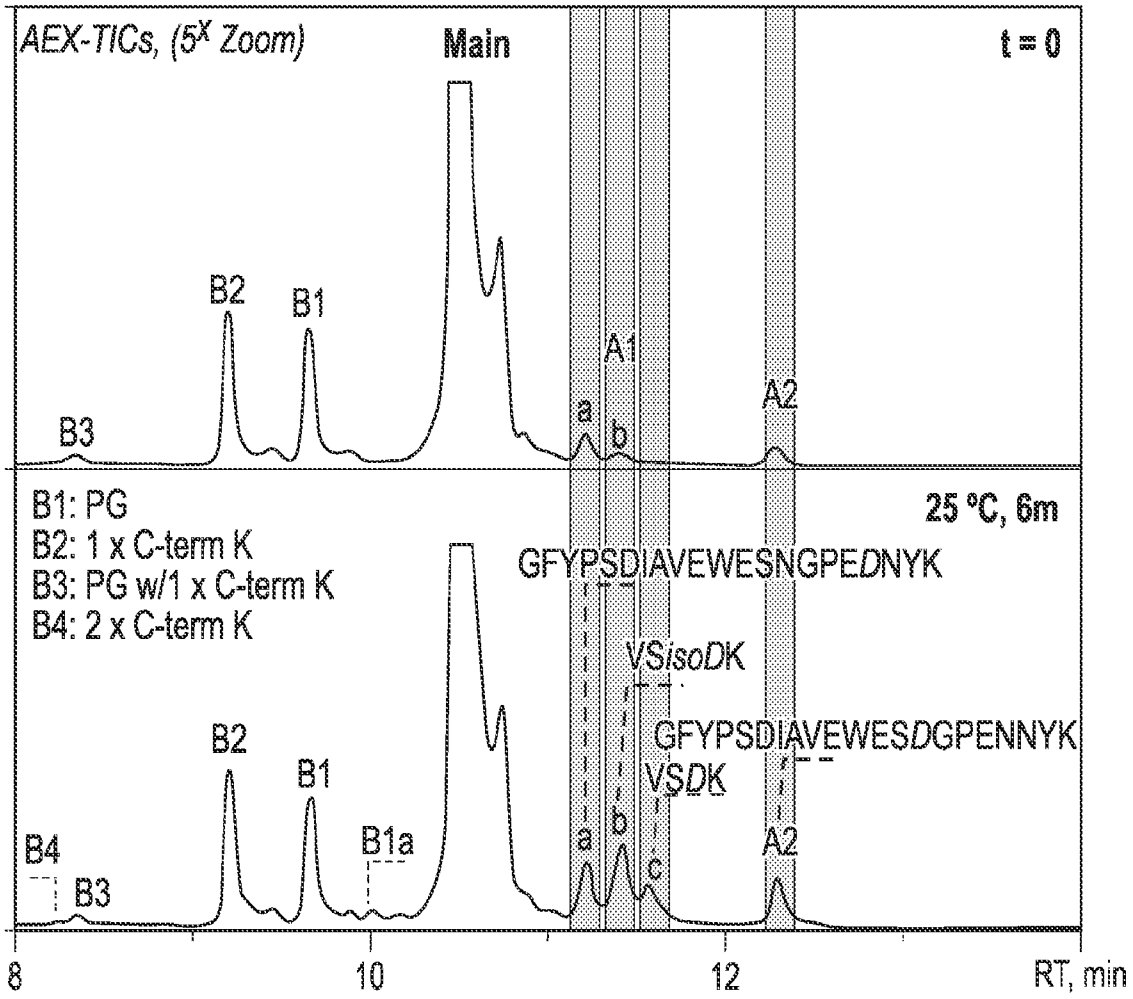

AEX-TICs, (5X Zoom)
Main
t = 0
B2
B1
A1
B3
a
b
A2
B1: PG
B2: 1 x C-term K
B3: PG w/1 x C-term K
B4: 2 x C-term K
25 °C, 6m
GFYPSDIAVEWESNGPEDNYK
VSisoDK
GFYPSDIAVEWESDGPENNYK
VSDK
B2
B1
B4
B3
B1a
a
b
c
A2
8
10
12
RT, min

FIG. 7

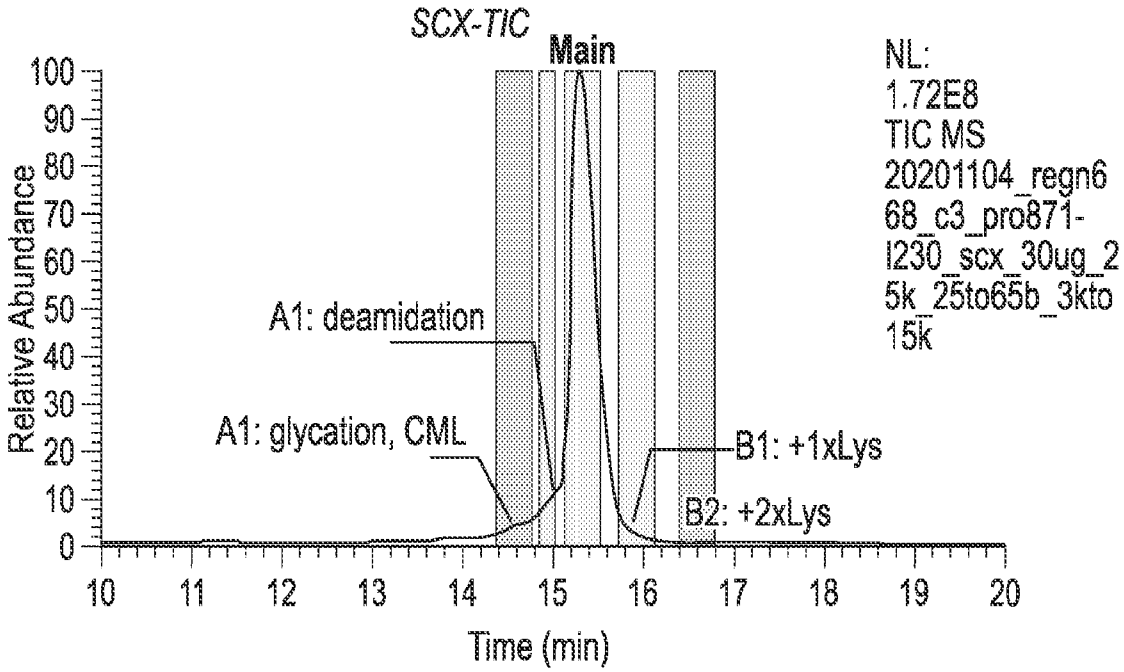

SCX-TIC
Main
NL:
1.72E8
TIC MS
20201104_regn6
68_c3_pro871-
l230_scx_30ug_2
5k_25to65b_3kto
15k
A1: deamidation
A1: glycation, CML
B1: +1xLys
B2: +2xLys
Relative Abundance
Time (min)

FIG. 8A

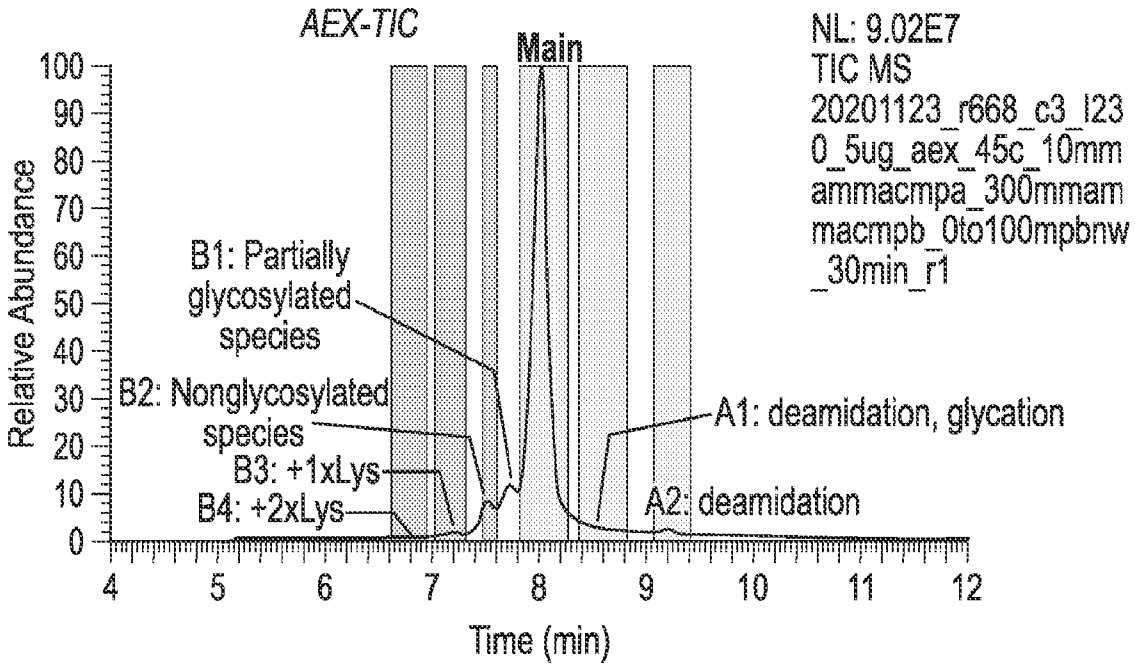

AEX-TIC
Main
NL: 9.02E7
TIC MS
20201123_r668_c3_l23
0_5ug_aex_45c_10mm
ammacmpa_300mmam
macmpb_0to100mpbnw
_30min_r1
B1: Partially
glycosylated
species
B2: Nonglycosylated
species
B3: +1xLys
B4: +2xLys
A1: deamidation, glycation
A2: deamidation
Relative Abundance
Time (min)

FIG. 8B

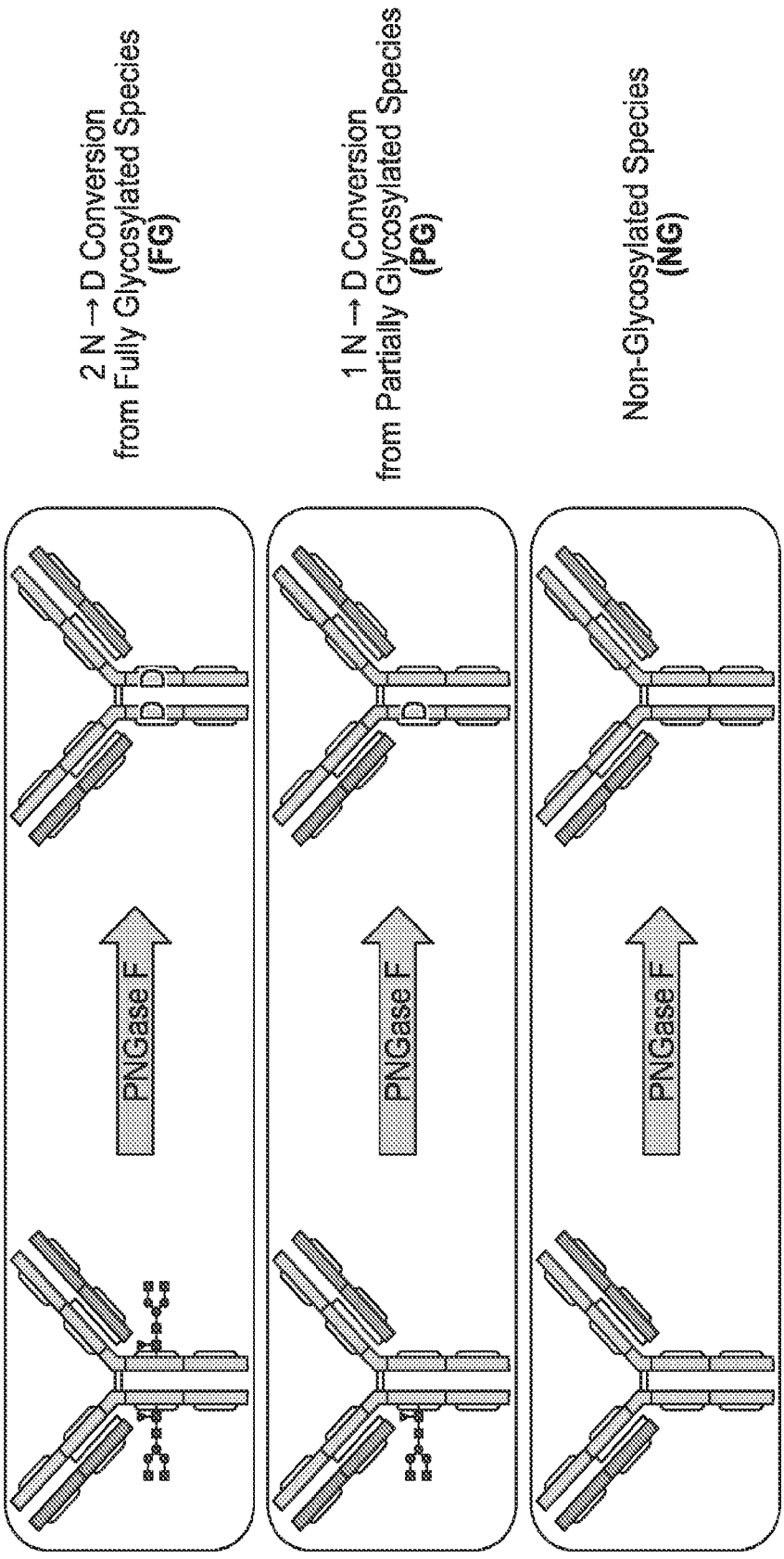

2 N → D Conversion
from Fully Glycosylated Species
(FG)
1 N → D Conversion
from Partially Glycosylated Species
(PG)
Non-Glycosylated Species
(NG)
PNGase F
PNGase F
PNGase F

FIG. 9

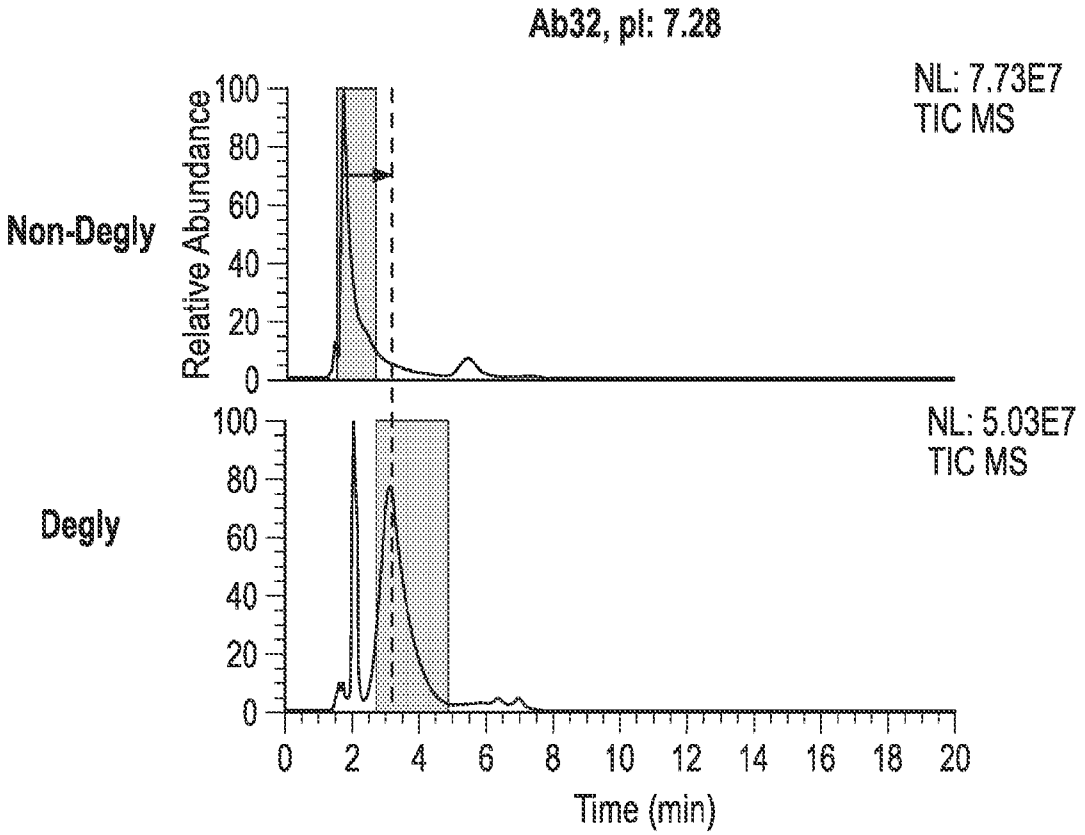


FIG. 10A

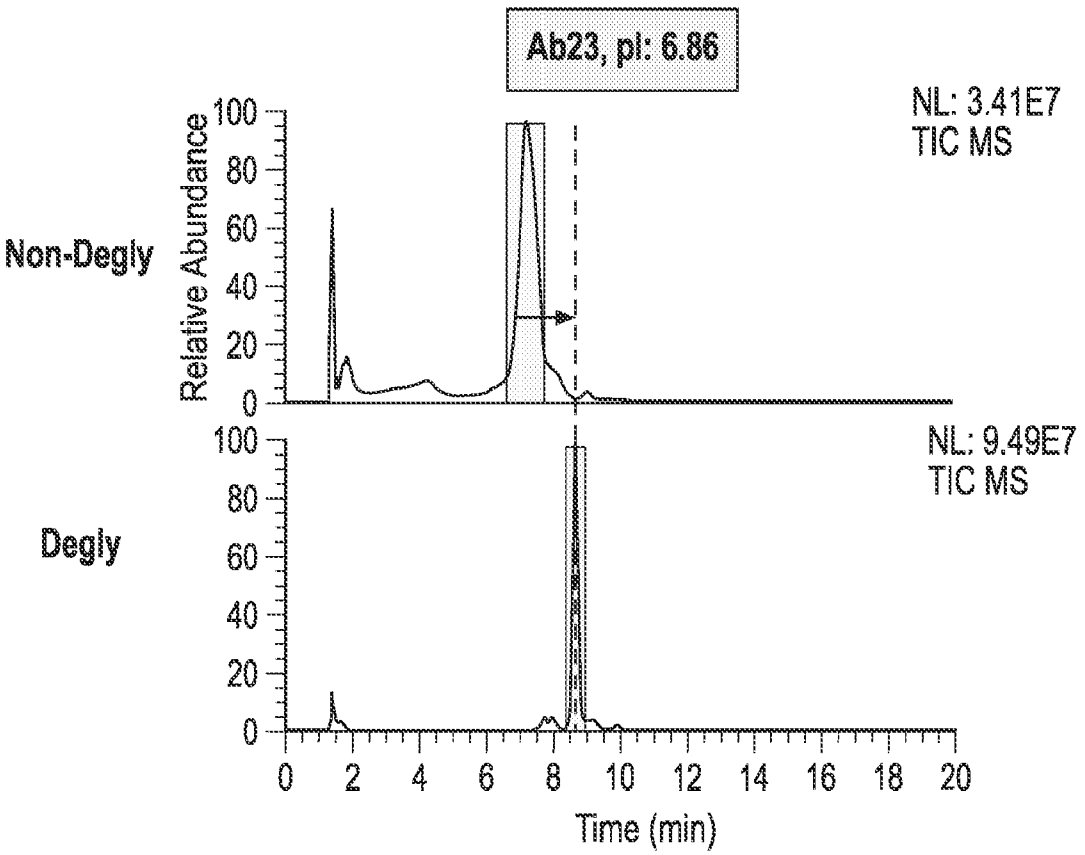


FIG. 10B

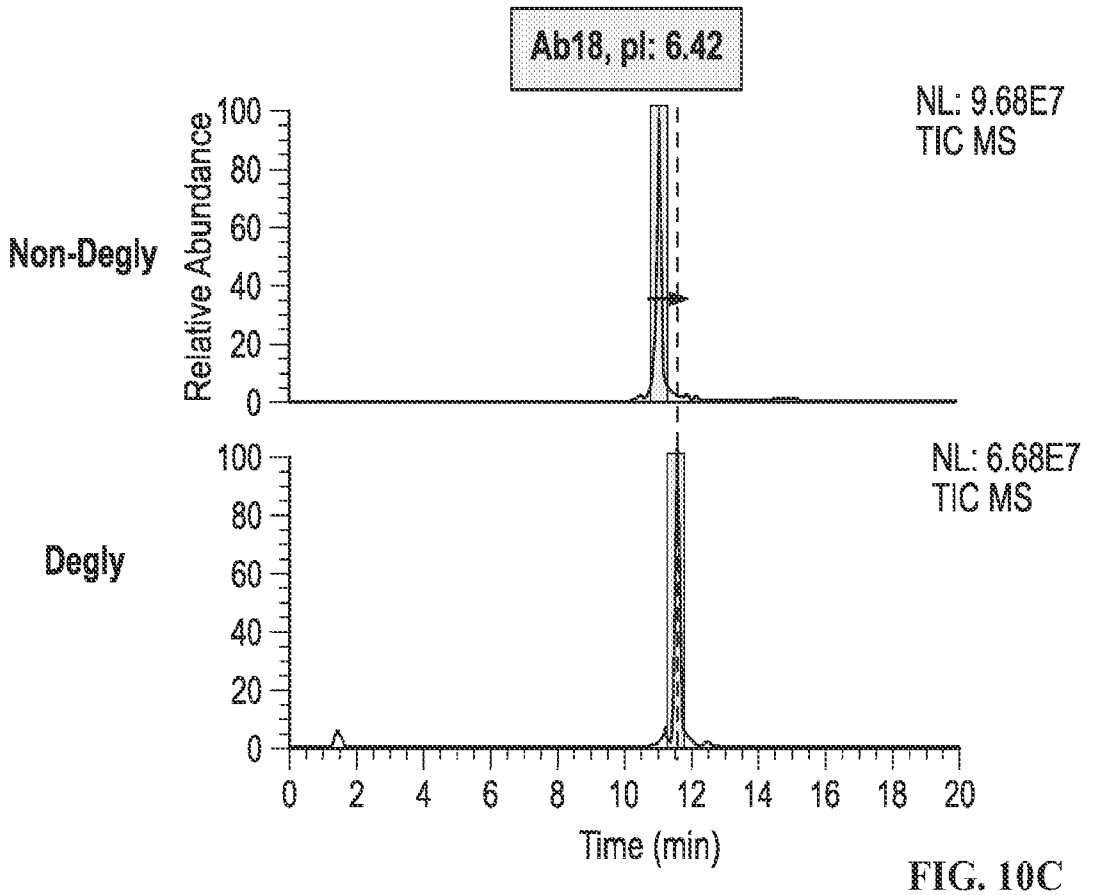


FIG. 10C

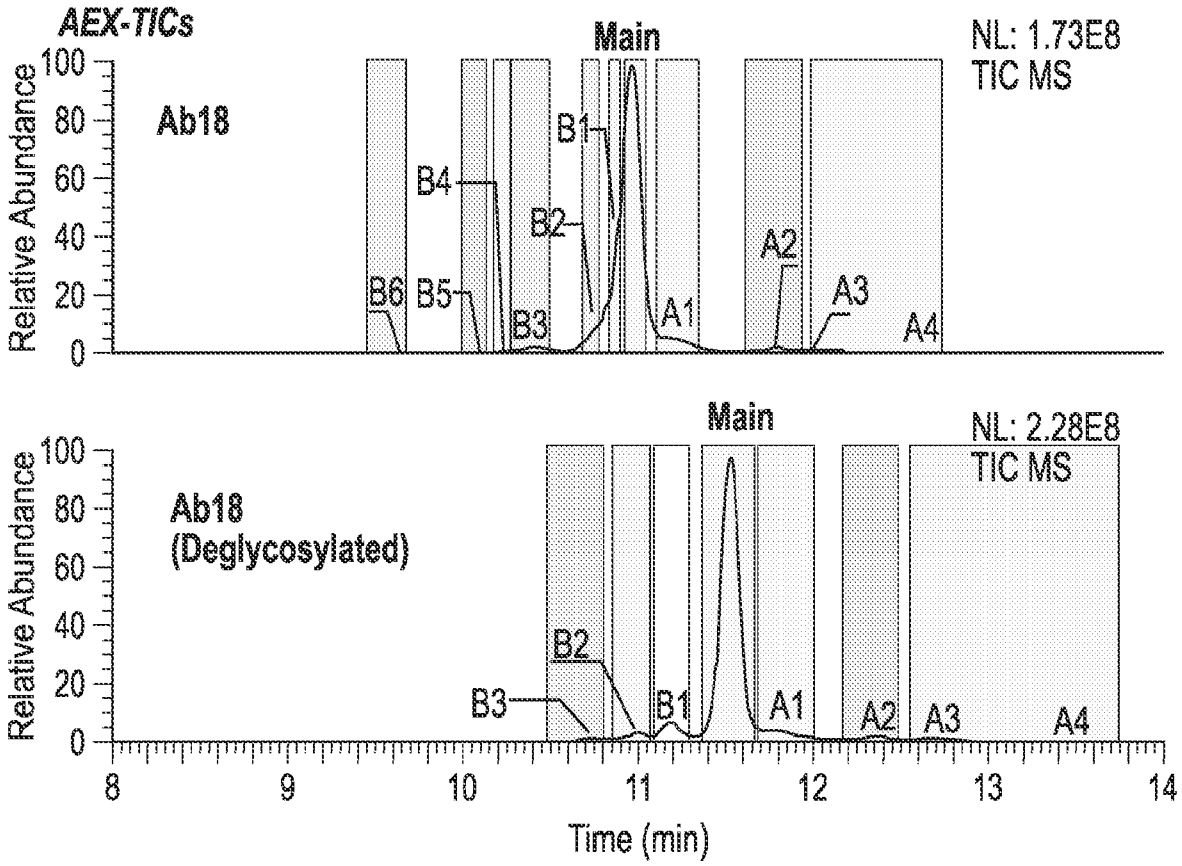


FIG. 11A

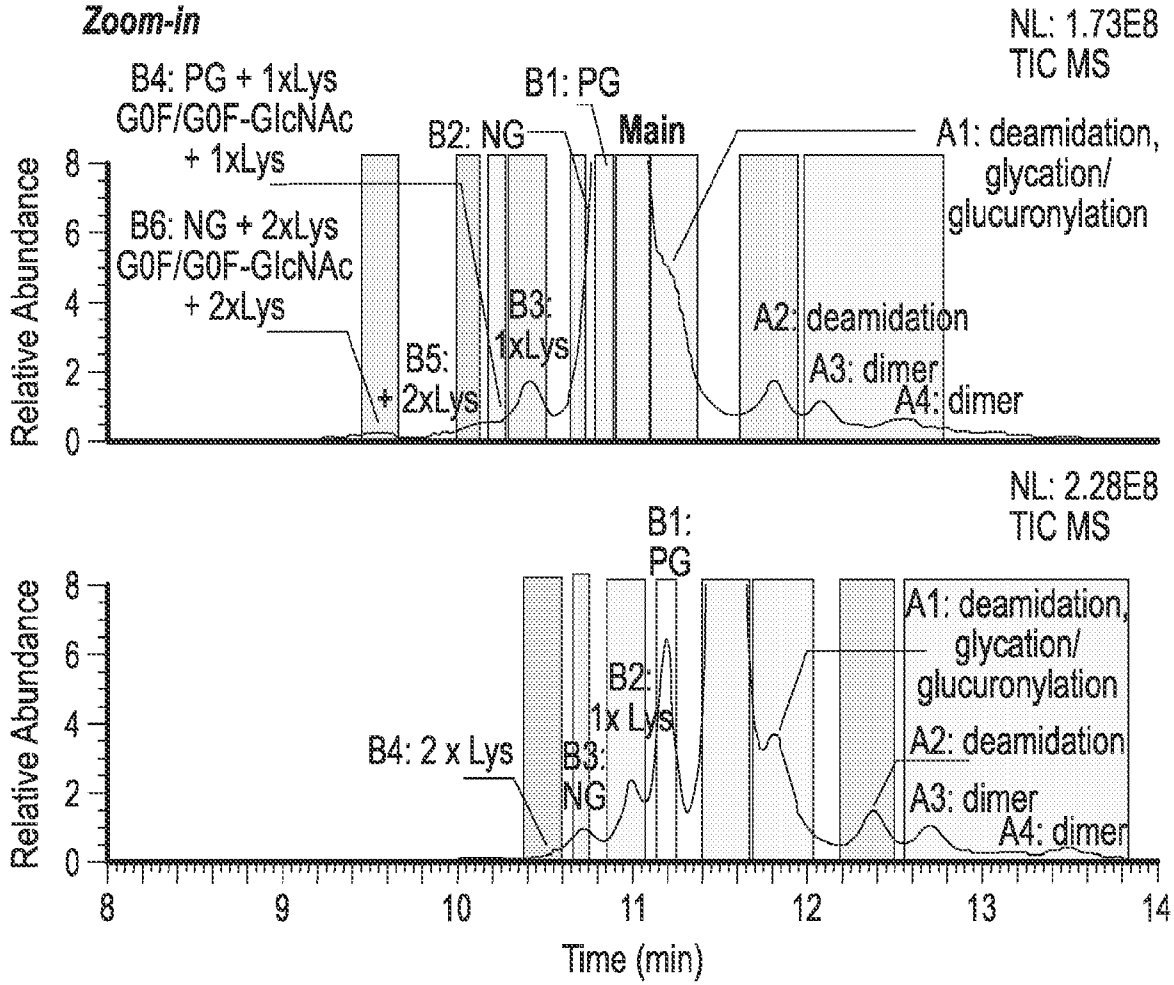

Zoom-in
NL: 1.73E8
TIC MS
B4: PG + 1xLys
G0F/G0F-GlcNAc
+ 1xLys
B6: NG + 2xLys
G0F/G0F-GlcNAc
+ 2xLys
B2: NG
B1: PG
Main
A1: deamidation,
glycation/
glucuronylation
B3:
1xLys
B5:
+ 2xLys
A2: deamidation
A3: dimer
A4: dimer
Relative Abundance
NL: 2.28E8
TIC MS
B1:
PG
B2:
1x Lys
B4: 2 x Lys
B3:
NG
A1: deamidation,
glycation/
glucuronylation
A2: deamidation
A3: dimer
A4: dimer
Relative Abundance
Time (min)

FIG. 11B

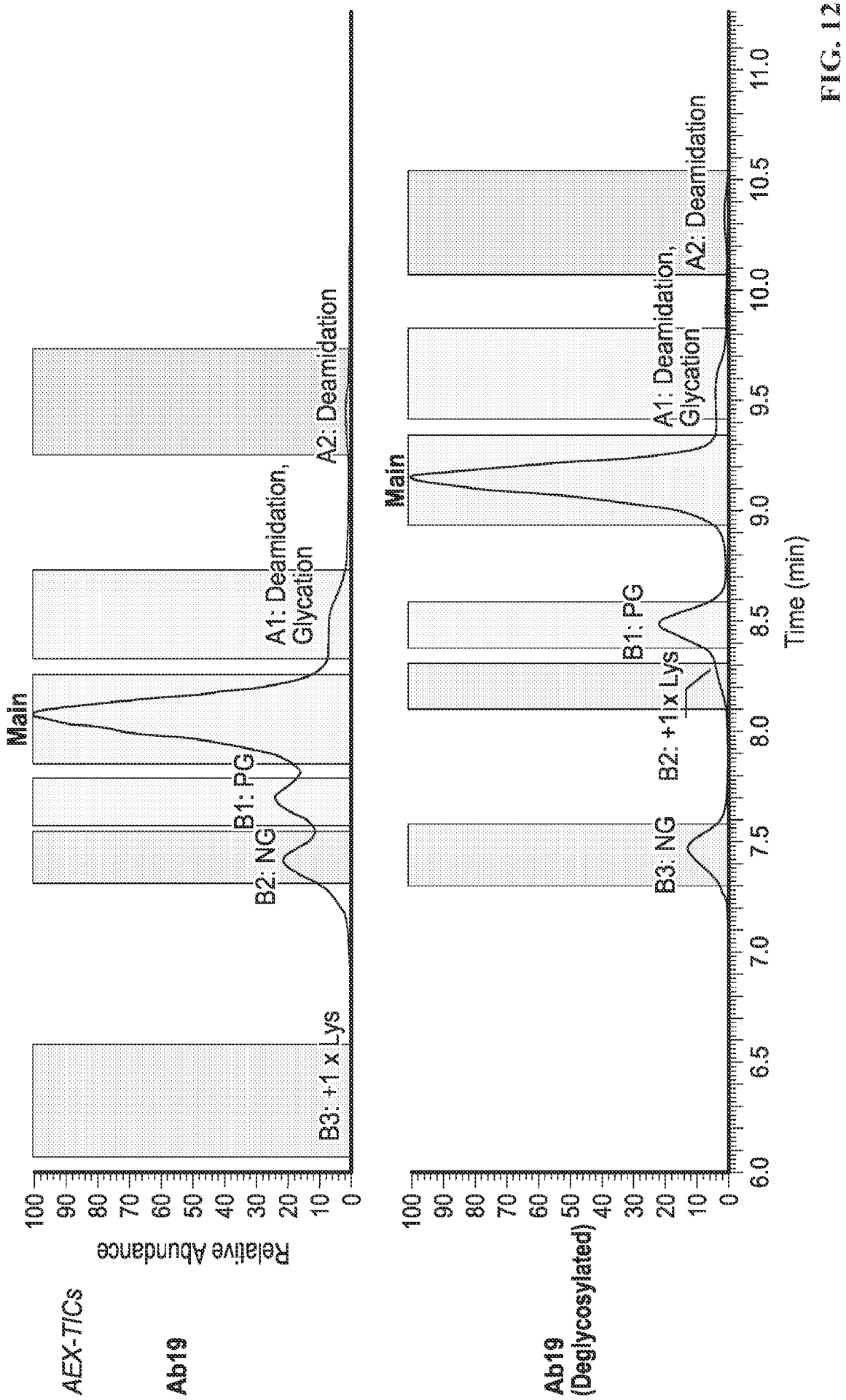

AEX-TICs
Ab19
Relative Abundance
Main
A1: Deamidation, Glycation
A2: Deamidation
B1: PG
B2: NG
B3: +1 x Lys
Ab19 (Deglycosylated)
Time (min)

FIG. 12

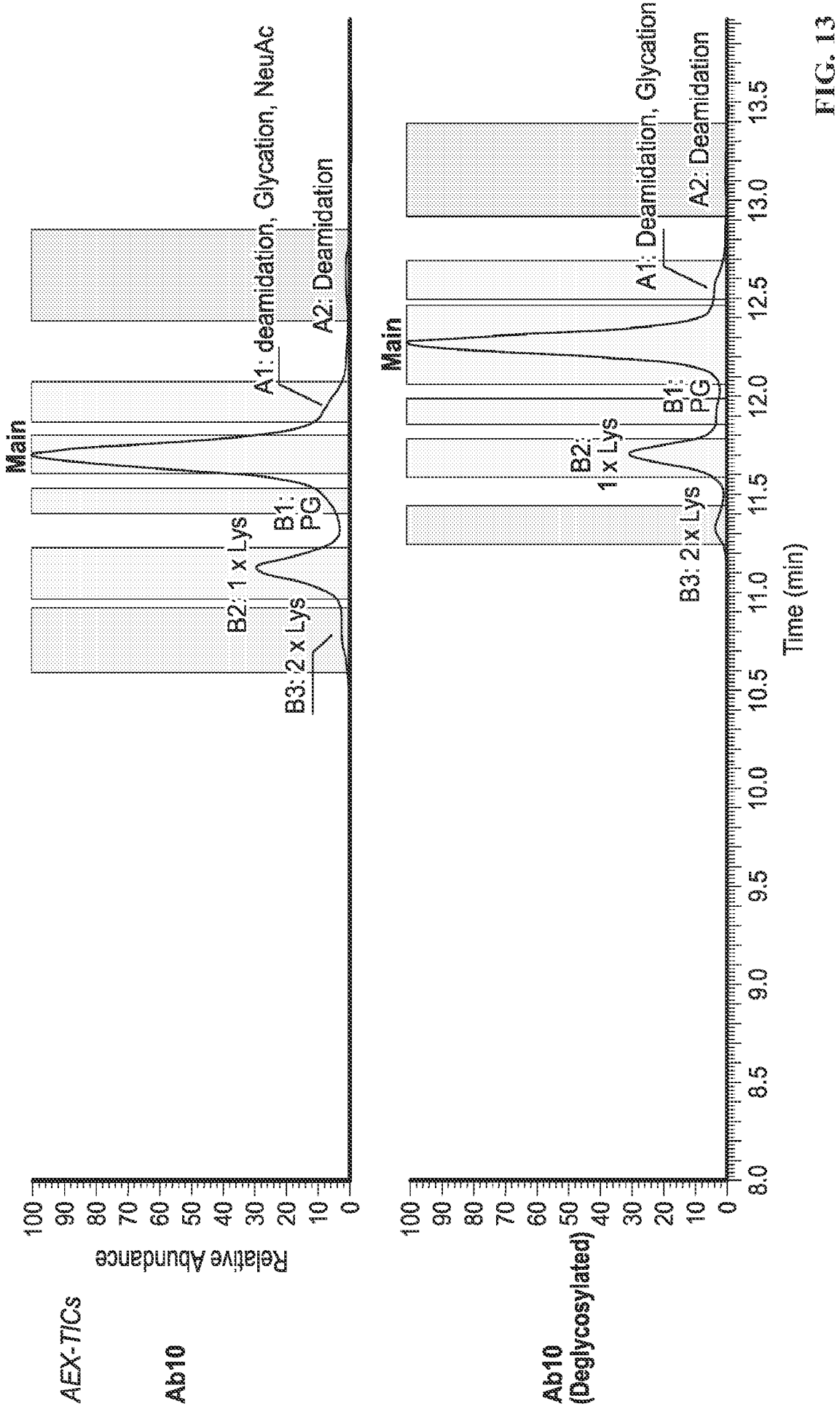

AEX-TICs
Ab10
Relative Abundance
Main
A1: deamidation, Glycation, NeuAc
A2: Deamidation
B1: PG
B2: 1 x Lys
B3: 2 x Lys
Ab10 (Deglycosylated)
Main
A1: Deamidation, Glycation
A2: Deamidation
B1: PG
B2: 1 x Lys
B3: 2 x Lys
Time (min)

FIG. 13

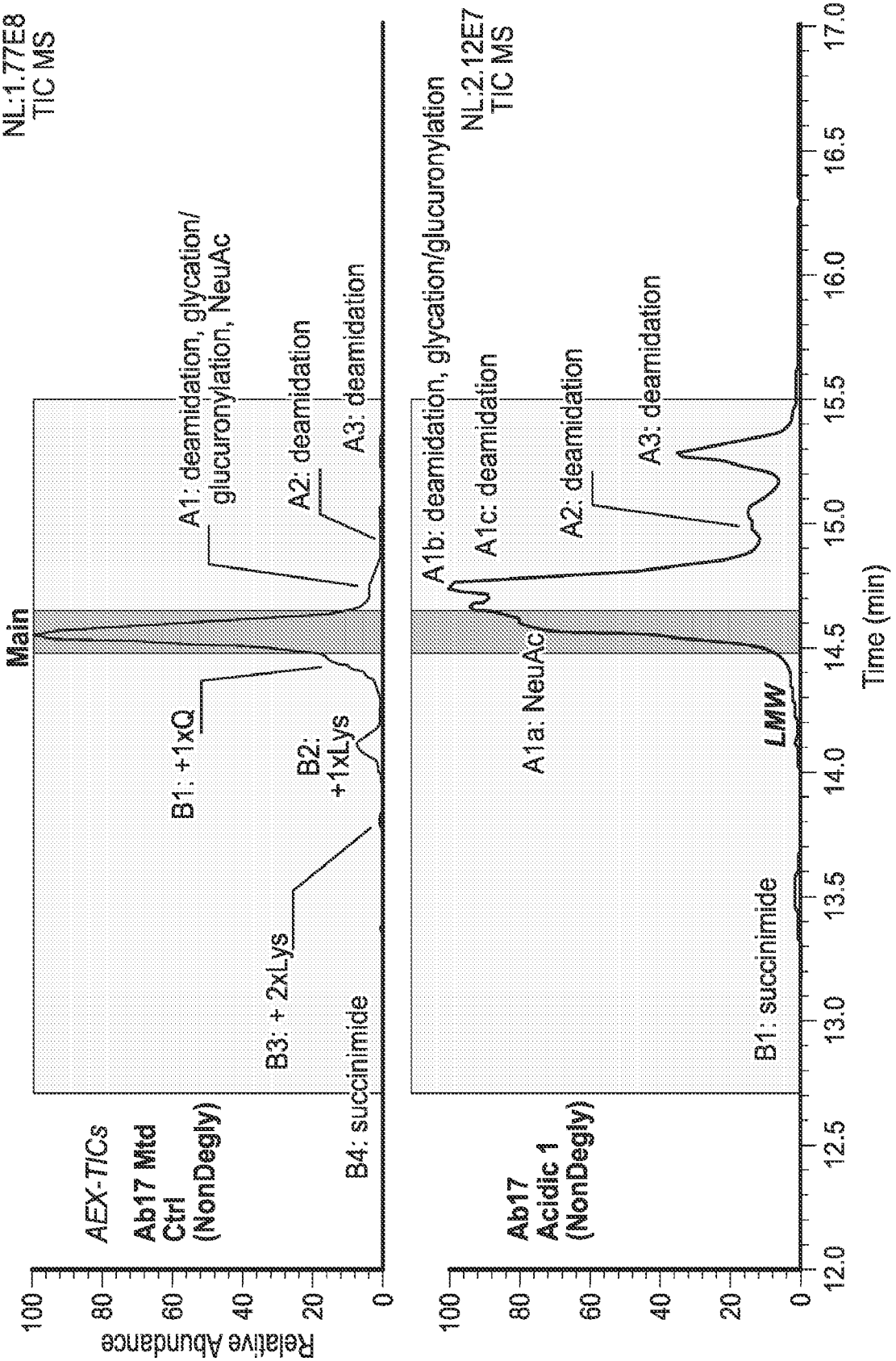

AEX-TICs
Ab17 Mtd Ctrl (NonDegly)
Ab17 Acidic 1 (NonDegly)
Relative Abundance
Time (min)
NL:1.77E8 TIC MS
NL:2.12E7 TIC MS
Main
LMW
A1: deamidation, glycation/ glucuronylation, NeuAc
A2: deamidation
A3: deamidation
B1: +1xQ
B2: +1xLys
B3: + 2xLys
B4: succinimide
A1a: NeuAc
A1b: deamidation, glycation/glucuronylation
A1c: deamidation
B1: succinimide

FIG. 14A

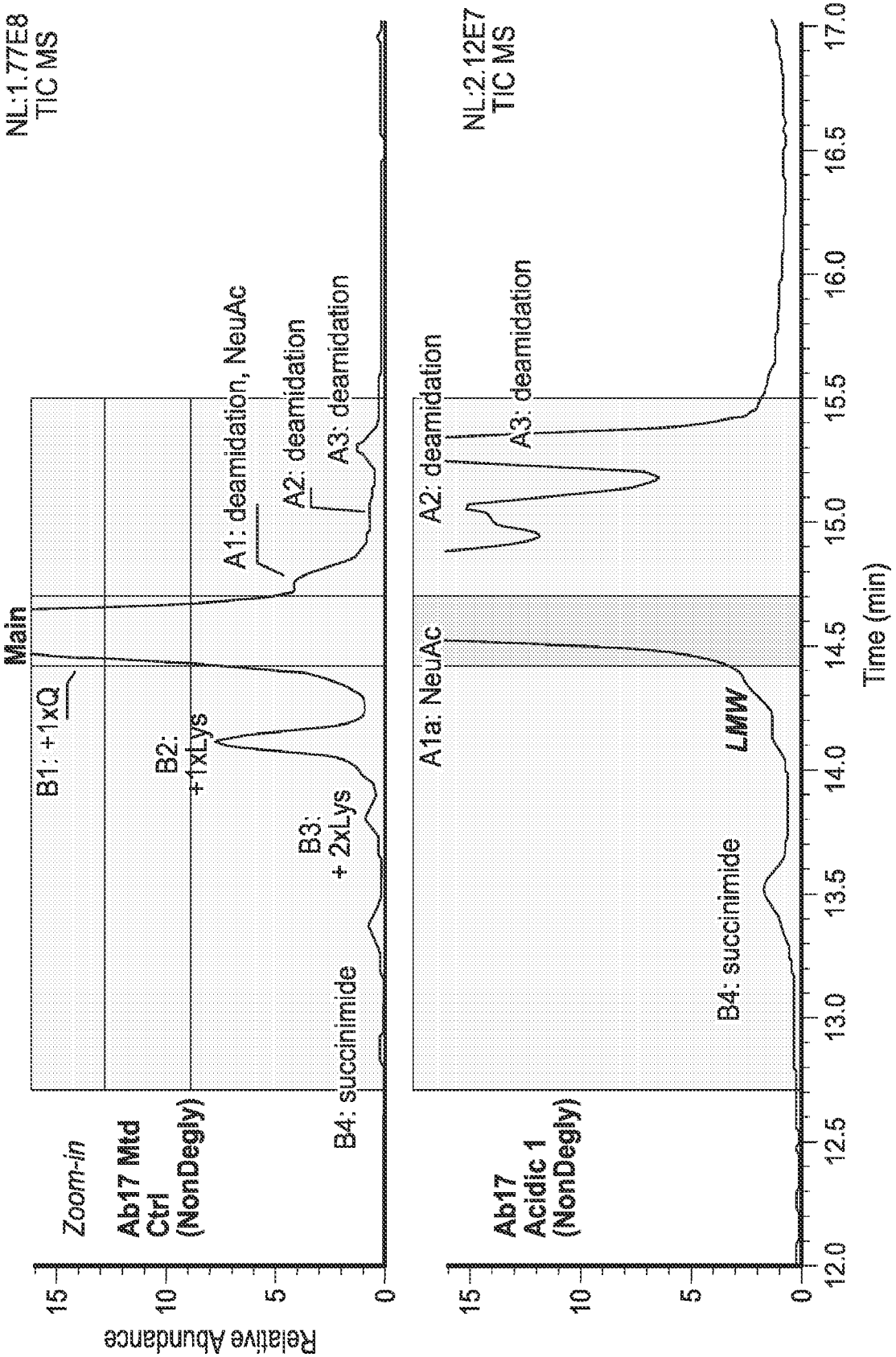

NL:1.77E8
TIC MS
Main
B1: +1xQ
B2:
+1xLys
B3:
+ 2xLys
B4: succinimide
A1: deamidation, NeuAc
A2: deamidation
A3: deamidation
Zoom-in
Ab17 Mtd
Ctrl
(NonDegly)
NL:2.12E7
TIC MS
A1a: NeuAc
A2: deamidation
A3: deamidation
LMW
B4: succinimide
Ab17
Acidic 1
(NonDegly)
Relative Abundance
Time (min)
0
5
10
15
12.0
12.5
13.0
13.5
14.0
14.5
15.0
15.5
16.0
16.5
17.0

FIG. 14B

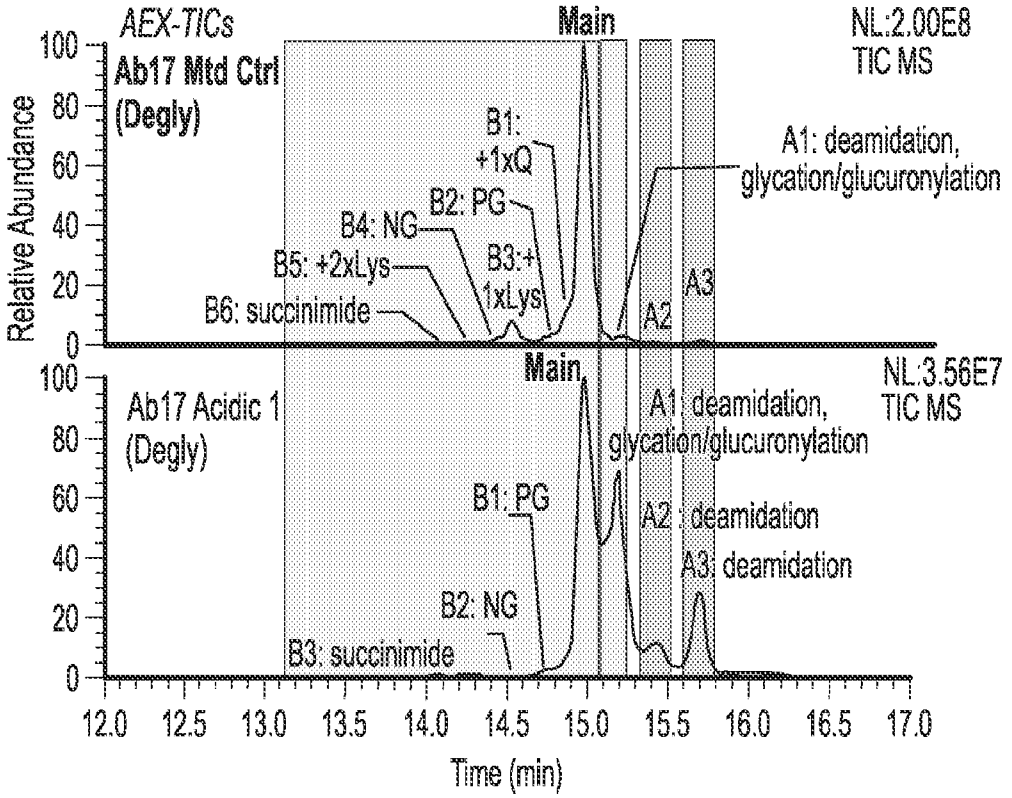


FIG. 14C

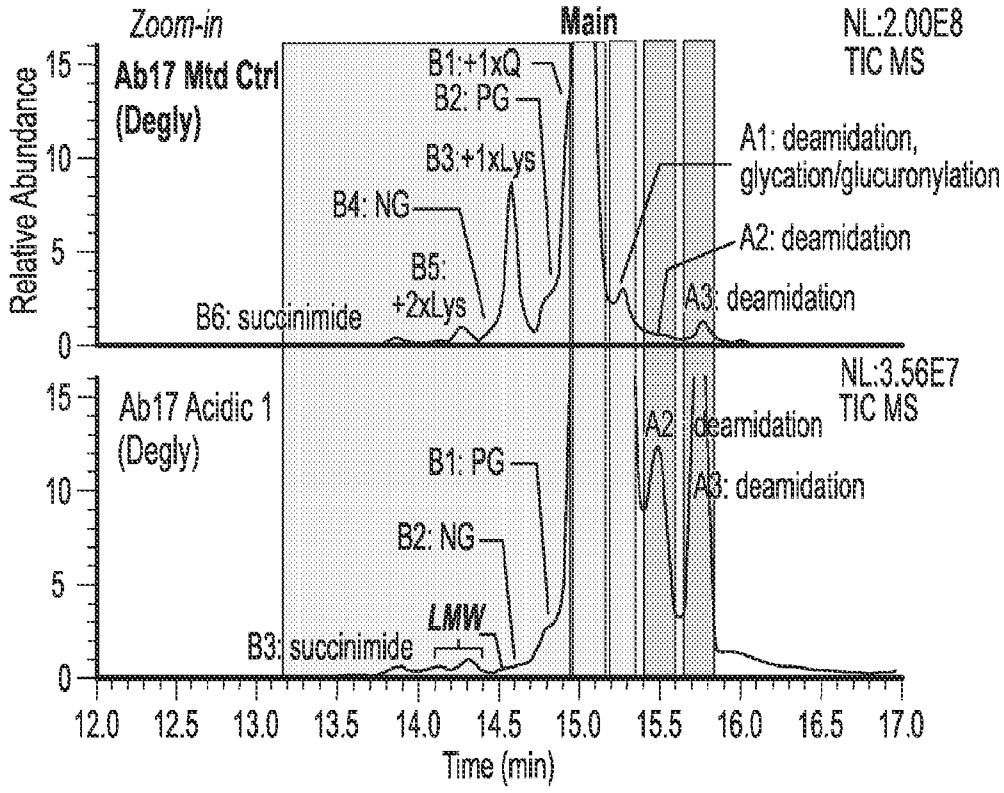


FIG. 14D

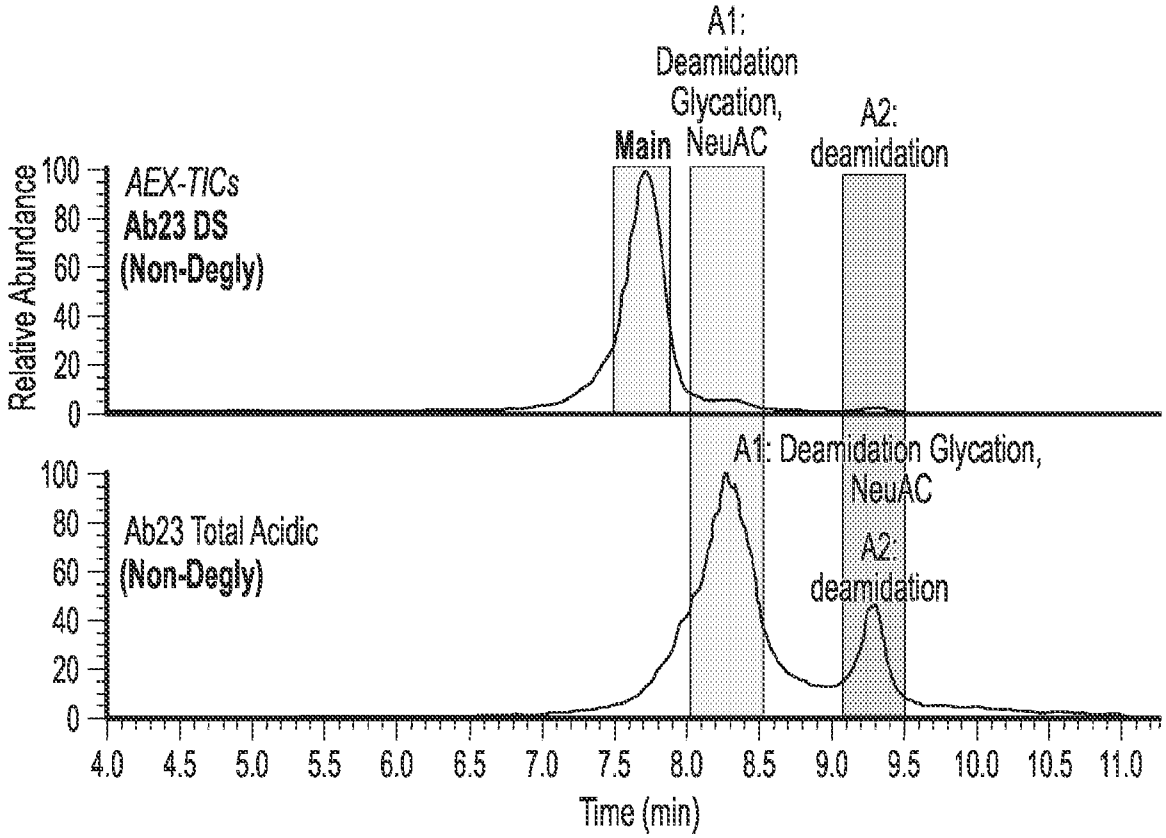


FIG. 15A

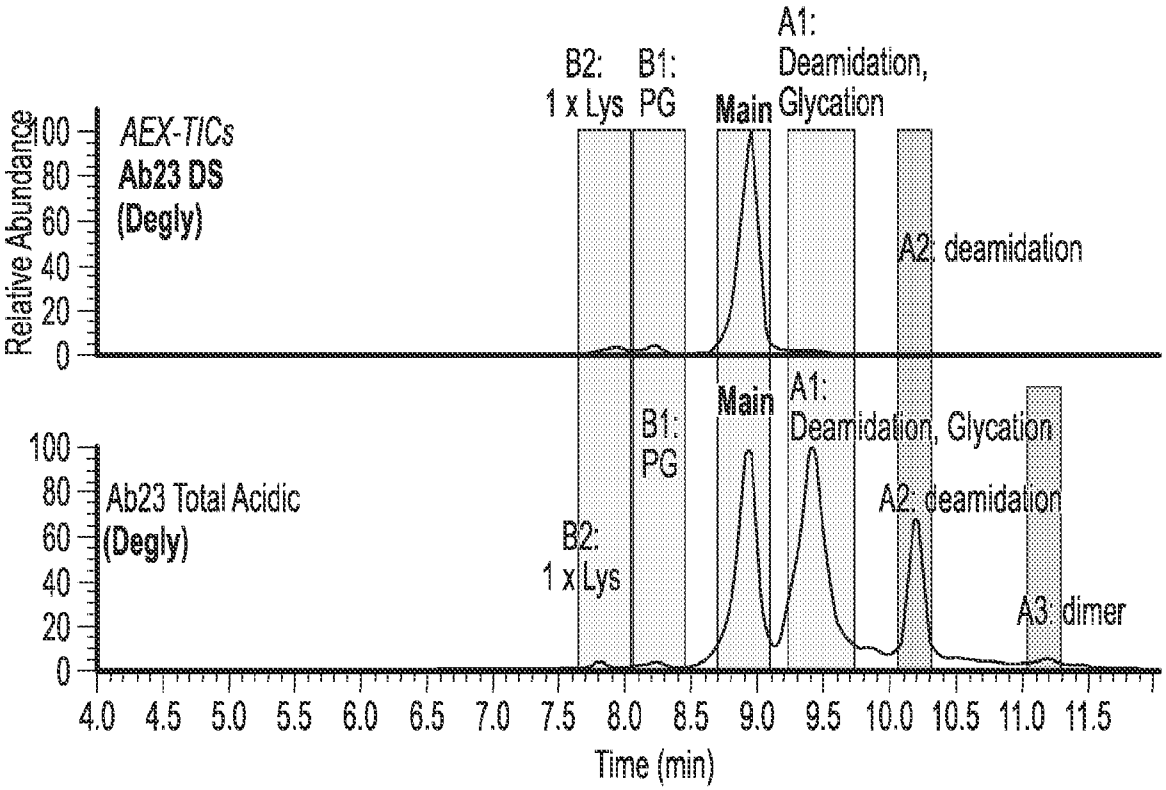


FIG. 15B

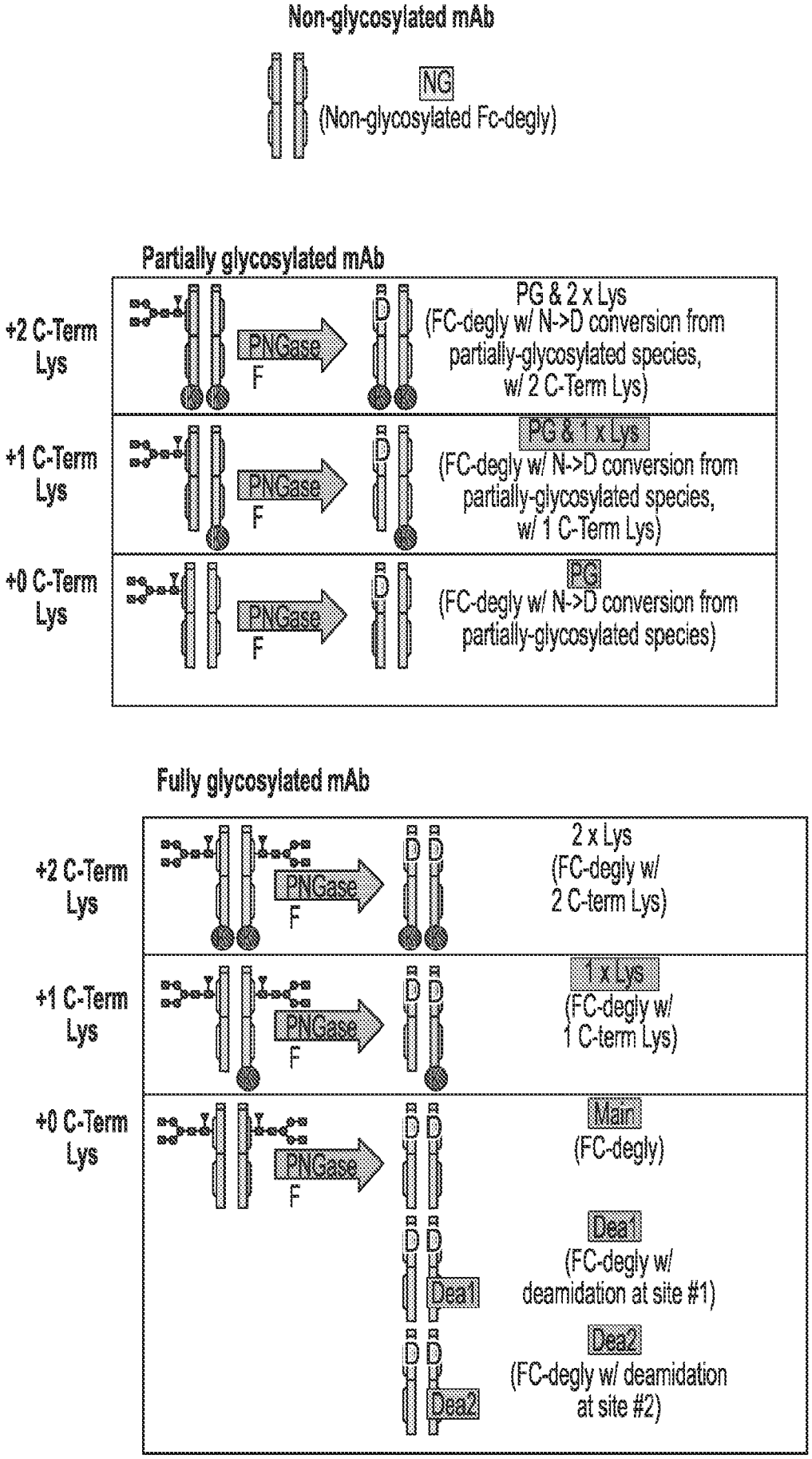

Non-glycosylated mAb
NG
(Non-glycosylated Fc-degly)
Partially glycosylated mAb
+2 C-Term Lys
PNGase F
PG & 2 x Lys
(FC-degly w/ N->D conversion from partially-glycosylated species, w/ 2 C-Term Lys)
+1 C-Term Lys
PNGase F
PG & 1 x Lys
(FC-degly w/ N->D conversion from partially-glycosylated species, w/ 1 C-Term Lys)
+0 C-Term Lys
PNGase F
PG
(FC-degly w/ N->D conversion from partially-glycosylated species)
Fully glycosylated mAb
+2 C-Term Lys
PNGase F
2 x Lys
(FC-degly w/ 2 C-term Lys)
+1 C-Term Lys
PNGase F
1 x Lys
(FC-degly w/ 1 C-term Lys)
+0 C-Term Lys
PNGase F
Main
(FC-degly)
Dea1
(FC-degly w/ deamidation at site #1)
Dea2
(FC-degly w/ deamidation at site #2)

FIG. 16

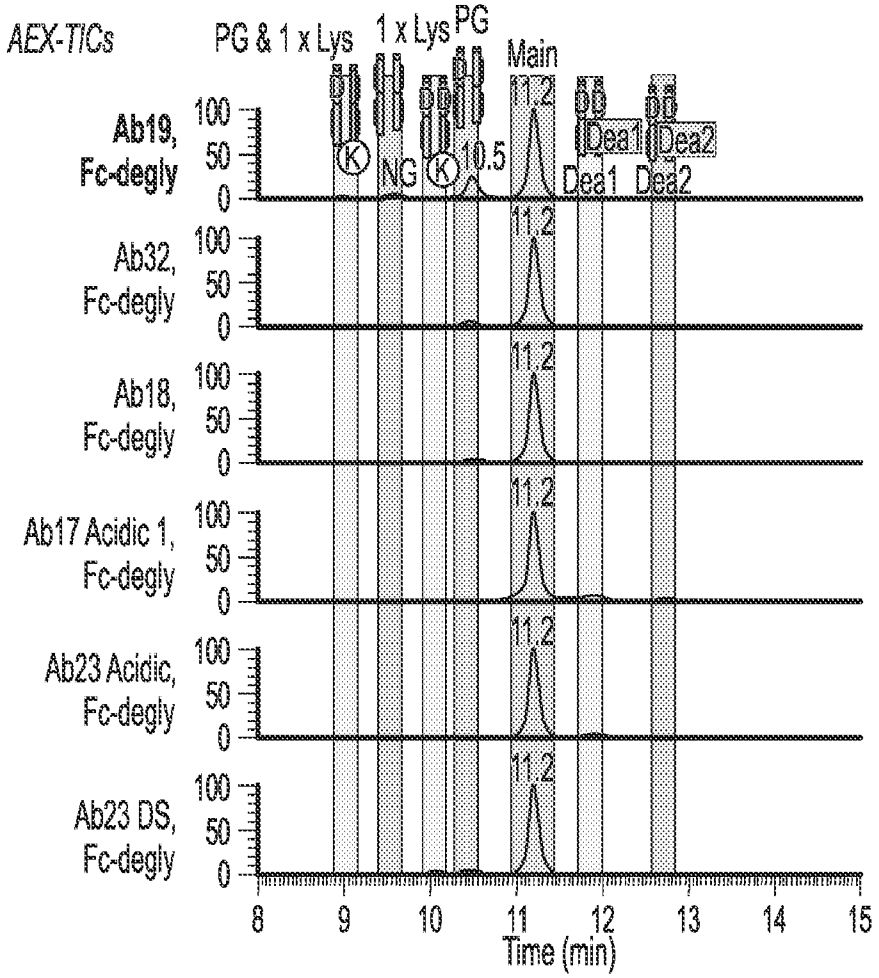


FIG. 17A

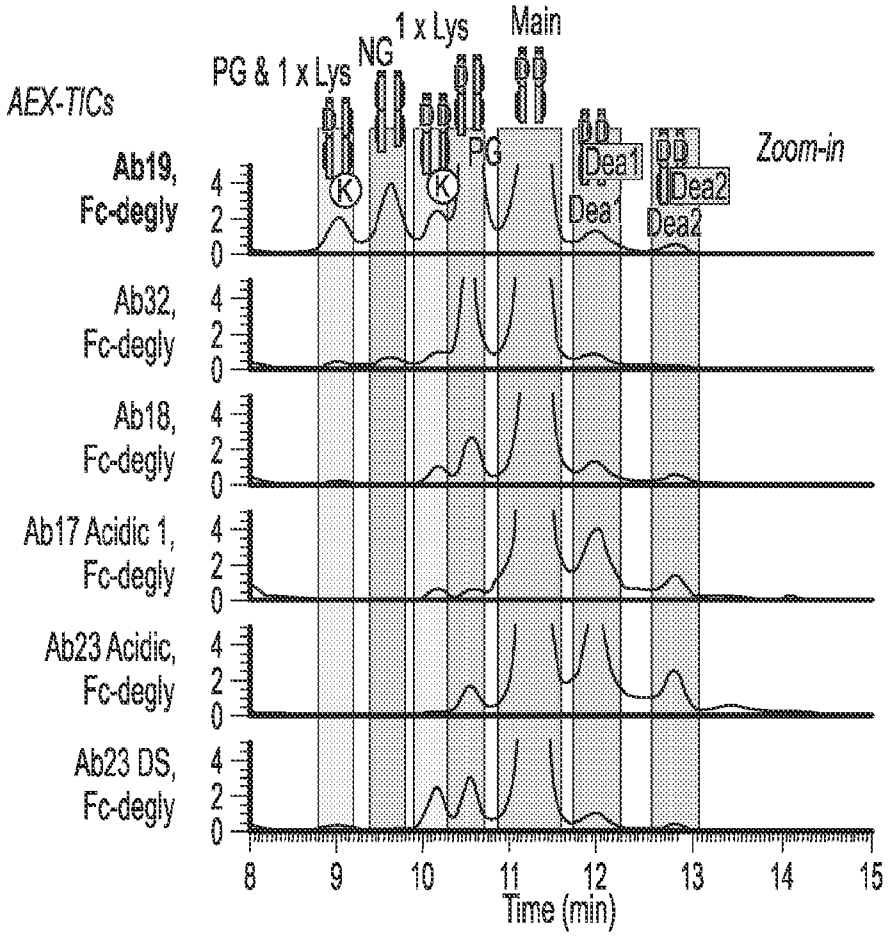


FIG. 17B

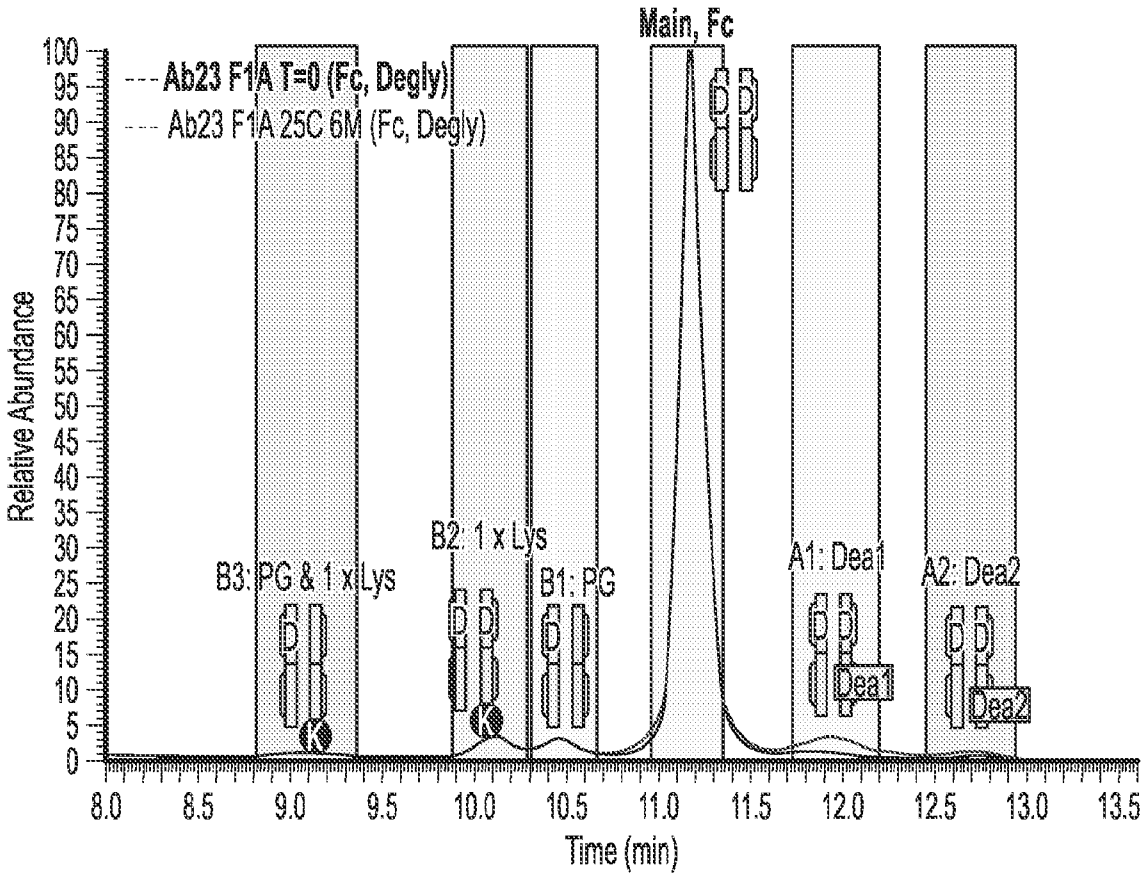


FIG. 18A

Peak	Relative Abundance (%)	
	T0	25C 6M
B3	0.9%	0.9%
B2	2.3%	2.5%
B1	1.8%	1.8%
Main	93.2%	90.9%
A1	1.2%	2.9%
A2	0.6%	1.0%

FIG. 18B

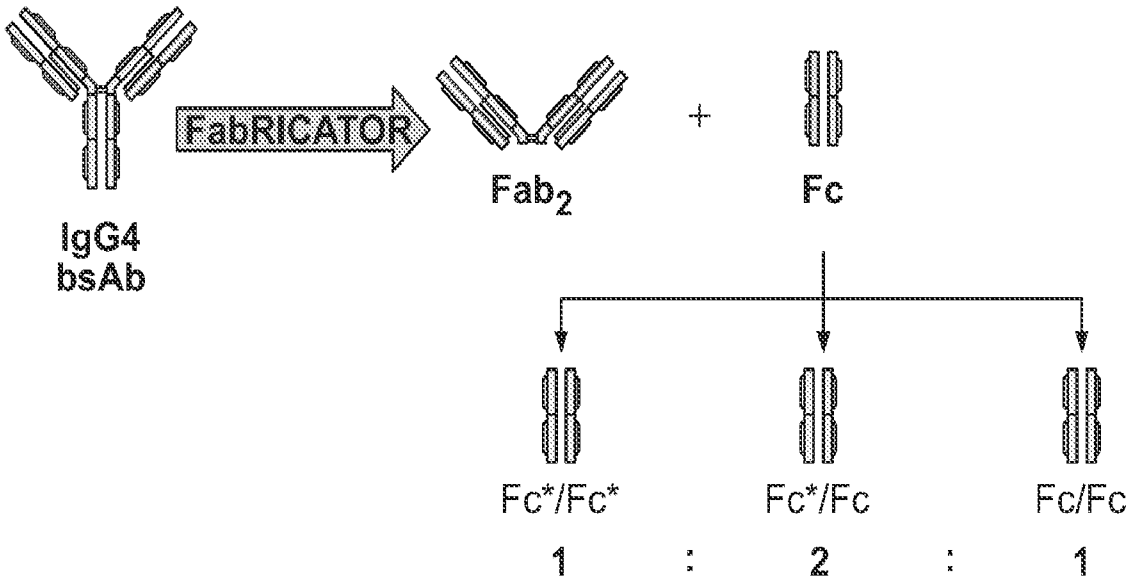


FIG. 19

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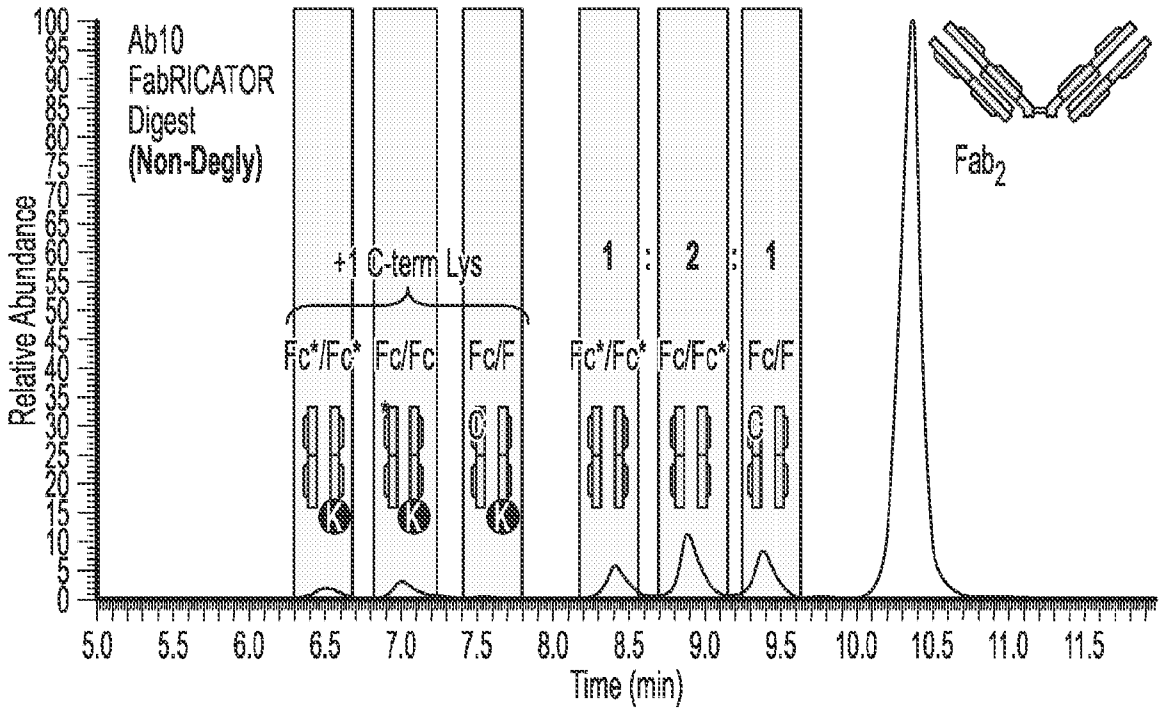
Ab10
FabRICATOR
Digest
(Non-Degly)
Relative Abundance
+1 C-term Lys
1 : 2 : 1
Fc*/Fc*
Fc/Fc
Fc/F
Fc*/Fc*
Fc/Fc*
Fc/F
Fab2
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
11.0
11.5
Time (min)

FIG. 20