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(54) **MICROCHIP CAPILLARY ELECTROPHORESIS ASSAYS AND REAGENTS**

(57) MCE assays and reagents to assess purity and to identify impurities in protein drug product samples are provided. Methods for analyzing analytes in a protein drug sample are provided.

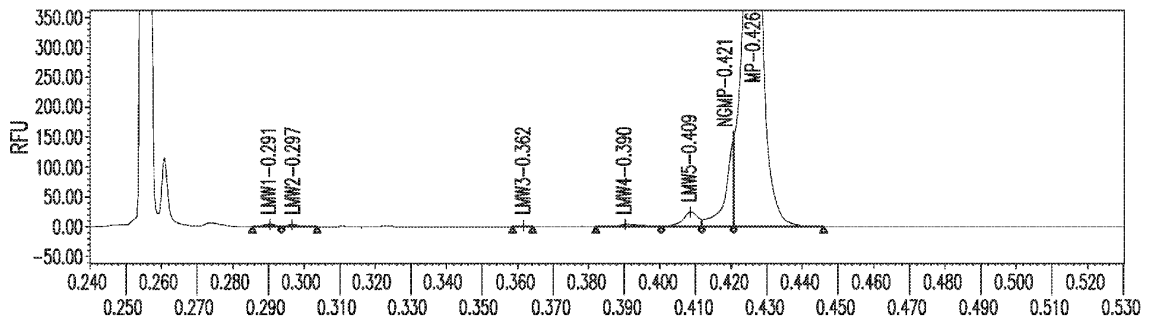


FIG. 1A

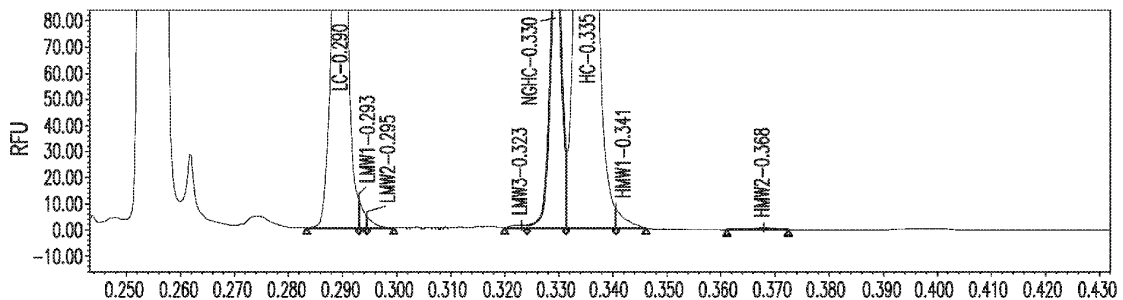


FIG. 1B

EP 4 317 959 A2

Description**TECHNICAL FIELD OF THE INVENTION**

5 [0001] Aspects of the invention are generally directed to the field of capillary electrophoresis, in particular to microchip capillary electrophoresis.

BACKGROUND OF THE INVENTION

10 [0002] Implementation of robust, reproducible, user-friendly technology is critical to meet the testing demands for biological products placed on today's Quality Control (QC) laboratories. Upgrades in technology are necessary to facilitate increased output, while continuing to generate quality analytical data and attempting to minimize the number of invalid test results and instrument-related investigations. While electrophoresis has historically been used in QC for product purity and fragmentation analysis, the methodology has transitioned from gel-based, to capillary-based, and more recently, to the microchip. Microchip Capillary Electrophoresis (MCE) allows for dramatically reduced sample analysis times, while maintaining the performance and reproducibility standards required for QC analysis (Ouimet, C., et al., Expert Opin Drug Discov., 12(2): 213-224 (2017)).

[0003] Although MCE has emerged as a promising technique with growing use in the pharmaceutical industry for characterizing biopharmaceuticals, quality control, and for drug discovery, it can be prone to assay interferences.

20 [0004] Thus, it is an object of the invention to provide improved MCE assays and compositions that reduce assay interferences.

[0005] It is another object of the invention to provide MCE assays and compositions for improving detection of impurities in a protein drug product.

SUMMARY OF THE INVENTION

25 [0006] MCE assays and reagents to assess purity and to identify impurities in protein drug product samples are provided. Methods for analyzing analytes in a protein drug sample are provided. Preferred protein drugs include, but are not limited to antibodies and antigen binding fragments thereof, fusion proteins, and recombinant proteins. The assays employ MCE techniques to separate, identify, and quantify protein product and impurities in the protein product. Impurities include, but are not limited to protein aggregates, protein fragments, protein multimers, and assay contaminants. Reducing and non-reducing buffers are also provided.

30 [0007] One embodiment provides a non-reducing aqueous electrophoresis sample buffer containing an alkylating agent, for example 155 to 175 mM 2-iodoacetamide; 0.50 to 1.5% lithium dodecyl sulfate; and 65 to 95 mM sodium phosphate, wherein the aqueous electrophoresis sample buffer has a pH of less than 7. In a preferred embodiment, the pH of the buffer is 6. In another embodiment, the aqueous buffer contains 166 mM 2-iodoacetamide, 0.81% lithium dodecyl sulfate, and 81 mM sodium phosphate.

35 [0008] A reducing buffer is also provided. In one embodiment, the reducing buffer is an aqueous electrophoresis sample buffer containing 0.5 to 1.5% lithium dodecyl sulfate, 55 to 85 mM sodium phosphate, and a reducing agent, wherein the aqueous electrophoresis sample buffer has a pH greater than 8. In a preferred embodiment, the pH of the buffer is 9. In one embodiment, the reducing buffer contains 135 to 155 mM dithiothreitol. Still another embodiment provides a reducing buffer containing 0.69 % lithium dodecyl sulfate, 69 mM sodium phosphate, and 142 mM dithiothreitol.

40 [0009] HEPES based buffers can also be used with the disclosed methods. One embodiment provides a non-reducing HEPES based aqueous electrophoresis sample buffer containing an alkylating agent, for example 55 to 75 mM 2-iodoacetamide; 0.1 to 1.0 % lithium dodecyl sulfate; 5 to 85 mM HEPES, and 5 to 115 mM sodium chloride, wherein the aqueous electrophoresis sample buffer has a pH of less than 9. In another embodiment, the pH of the buffer is 8. In still another embodiment, the aqueous buffer contains 66.4 mM 2-iodoacetamide, 0.32% lithium dodecyl sulfate, 16.2 mM HEPES, and 48.6 mM sodium chloride.

45 [0010] Another embodiment provides a reducing HEPES based aqueous electrophoresis sample buffer containing 0.05 to 0.75 % lithium dodecyl sulfate, 5 mM to 115 mM sodium chloride, 5 mM to 115 mM HEPES, and a reducing agent, wherein the aqueous electrophoresis sample buffer has a pH greater than 7. In a preferred embodiment, the pH of the buffer is 8. In one embodiment, the reducing buffer contains 35 to 50 mM dithiothreitol. Still another embodiment provides a reducing buffer containing 0.28 % lithium dodecyl sulfate, 41.5 mM sodium chloride, 13.8 mM HEPES, and 42.5 mM dithiothreitol.

50 [0011] One embodiment provides a non-reducing MCE method for identifying contaminants or impurities in a protein drug sample including the steps of adding the protein sample to a non-reducing buffer discussed above to form a buffered protein drug sample. The buffered protein drug sample is heated to between 65 to 85 °C for 5 to 15 minutes to form a denatured buffered protein drug sample. In a preferred embodiment, the buffered protein drug sample is heated to 70

°C for 10 min.

[0012] The protein drug sample is mixed with a detectable label and heated at 30 to 40 °C for 20 to 40 minutes to form a denatured labeled protein drug sample. A preferred detectable label includes, but is not limited to Dyomics DY-631 NHS Ester. Other detectable labels can be used include other dyes, fluorophores, chromophores, mass tags, quantum dots and the like and those disclosed in US Patent No. 6,924,372 which is incorporated by reference in its entirety. In a preferred embodiment, the protein drug sample with added label is heated to 35 °C for 30 minutes. Excess label is optionally removed from the sample, for example by using a spin filter.

[0013] The denatured labeled protein drug product is diluted and subjected to MCE to separate the diluted protein drug sample on a microchip capillary electrophoresis system to produce an electropherogram. In one embodiment the final concentration of a sample starting at 0.5 mg/ml that is then injected over the microchip is 9 µg/ml to MCE. The electropherogram contains peaks corresponding to the protein drug product and impurities. The method concludes by identifying peaks in the electropherogram corresponding to contaminants or impurities.

[0014] Another embodiment provides a reducing MCE method for identifying contaminants or impurities in a protein drug sample. The method begins by adding the protein sample to any one of the reducing buffers described above to form a buffered protein drug sample. The buffered protein drug sample is denatured by heating the buffered protein drug sample to 65 to 85 °C, preferably to 70 °C for 10 minutes to form a denatured protein drug sample. The protein drug sample is mixed with a detectable label and heated at 30 to 40 °C for 20 to 40 minutes to form a denatured labeled protein drug sample. In a preferred embodiment, the protein drug sample with added label is heated to 35 °C for 30 minutes. Excess label is optionally removed from the sample, for example by using a spin filter. A preferred detectable label includes, but is not limited to Dyomics D Y -631 NHS Ester. Other detectable labels that can be used include other dyes, fluorophores, chromophores, mass tags, quantum dots and the like and those disclosed in US Patent No. 6,924,372 which is incorporated by reference in its entirety.

[0015] In one embodiment, the established assay range for sample concentration is from 0.4 mg/ml to 0.6 mg/ml, corresponding to a final concentration being analyzed of about 7 µg/ml to 11 µg/ml which is subjected to MCE analysis on a microchip capillary electrophoresis system to produce an electropherogram. The method concludes by identifying peaks in the electropherogram corresponding to contaminants or impurities.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] Figure 1A shows an electropherogram of a typical non-reduced sample analysis. Figure 1B shows an electropherogram of a typical reduced sample analysis. The X-axis represents time in minutes, and the Y-axis represents the relative fluorescence units (RFU). Increased migration time corresponds to increased protein size.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

[0017] The use of the terms "a," "an," "the," and similar referents in the context of describing the presently claimed invention (especially in the context of the claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context.

[0018] Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein.

[0019] Use of the term "about" is intended to describe values either above or below the stated value in a range of approx. +/- 10%; in other embodiments the values may range in value either above or below the stated value in a range of approx. +/- 5%; in other embodiments the values may range in value either above or below the stated value in a range of approx. +/- 2%; in other embodiments the values may range in value either above or below the stated value in a range of approx. +/- 1%. The preceding ranges are intended to be made clear by context, and no further limitation is implied. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[0020] "Protein" refers to a molecule comprising two or more amino acid residues joined to each other by a peptide bond. Protein includes polypeptides and peptides and may also include modifications such as glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, alkylation, hydroxylation and ADP-ribosylation. Proteins can be of scientific or commercial interest, including protein-based drugs, and proteins include, among other things, enzymes, ligands, receptors, antibodies and chimeric or fusion proteins. Proteins are produced by various types of

recombinant cells using well-known cell culture methods, and are generally introduced into the cell by genetic engineering techniques (e.g., such as a sequence encoding a chimeric protein, or a codon-optimized sequence, an intronless sequence, etc.) where it may reside as an episome or be integrated into the genome of the cell.

[0021] "Antibody" refers to an immunoglobulin molecule consisting of four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain has a heavy chain variable region (HCVR or VH) and a heavy chain constant region. The heavy chain constant region contains three domains, CH1, CH2 and CH3. Each light chain has a light chain variable region and a light chain constant region. The light chain constant region consists of one domain (CL). The VH and VL regions can be further subdivided into regions of hyper variability, termed complementarity determining regions (CDR), 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, FR4. The term "antibody" includes reference to both glycosylated and non-glycosylated immunoglobulins of any isotype or subclass. The term "antibody" includes antibody molecules prepared, expressed, created or isolated by recombinant means, such as antibodies isolated from a host cell transfected to express the antibody. The term antibody also includes bispecific antibody, which includes a heterotetrameric immunoglobulin that can bind to more than one different epitope. Bispecific antibodies are generally described in US Patent No. 8,586,713, which is incorporated by reference into this application.

[0022] "Fc fusion proteins" comprise part or all of two or more proteins, one of which is an Fc portion of an immunoglobulin molecule, which are not otherwise found together in nature. 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); Bym 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 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 comprises two or more distinct receptor chains that bind to a one or more ligand(s). For example, an Fc-fusion protein is a trap, such as for example an IL-1 trap or VEGF trap.

[0023] The term "MCE" or "Microchip Capillary Electrophoresis" refers to microchip-based capillary electrophoresis (CE) separation of analytes.

II. MCE Assays and Buffers

[0024] Methods for analyzing analytes in a protein drug sample are provided. Preferred protein drugs include, but are not limited to antibodies and antigen binding fragments thereof, fusion proteins, and recombinant proteins. The assays employ MCE techniques to separate, identify, and quantify protein product and impurities in the protein product. Impurities include, but are not limited to protein aggregates, protein fragments, protein multimers, and assay contaminants. Reducing and non-reducing buffers are also provided.

A. Buffers

1. Non-reducing Buffers

[0025] One embodiment provides a non-reducing aqueous electrophoresis sample buffer containing 155 to 175 mM of an alkylating agent for example 2-iodoacetamide or NEM; 0.50 to 1.5% lithium dodecyl sulfate; and 75 to 95 mM sodium phosphate, wherein the aqueous electrophoresis sample buffer has a pH of less than 7. In a preferred embodiment, the pH of the buffer is 6. In another embodiment, the aqueous buffer contains 166 mM 2-iodoacetamide, 0.81% lithium dodecyl sulfate, and 81 mM sodium phosphate.

2. Reducing Buffers

[0026] A reducing buffer is also provided. In one embodiment, the reducing buffer is an aqueous electrophoresis sample buffer containing 0.5 to 1.5% lithium dodecyl sulfate, 65 to 95 mM sodium phosphate, and a reducing agent, wherein the aqueous electrophoresis sample buffer has a pH greater than 8. In a preferred embodiment, the pH of the buffer is 9.

[0027] Reducing agents are known in the art. Exemplary reducing agents include but are not limited to dithiothreitol (DTT, CAS 3483-12-3), beta-mercaptoethanol (BME, 2BME, 2-ME, b-mer, CAS 60-24-2), 2-aminoethanethiol (2-MEA-HCl, also called cysteamine-HCl, CAS 156-57-0), Tris (2-carboxyethyl) phosphine hydrochloride, (TCEP, CAS 5961-85-3), cysteine hydrochloride (Cys-HCl, CAS 52-89-1), or 2-mercaptoethanesulfonic acid sodium salt (MESNA). Other methods for reducing protein bonds are known in the art, such as an immobilized reductant column which contains resin to which a thiol-based reducing agent has been immobilized to enable the solid-phase reduction of peptide and

protein disulfide bonds. Reducing agents, including oxidizing agents, are suitable for reducing chemical interaction between polypeptides are also envisioned.

[0028] In one embodiment, reducing buffer contains 135 to 155 mM dithiothreitol.

[0029] Still another embodiment provides a reducing buffer containing 0.69 % lithium dodecyl sulfate, 69 mM sodium phosphate, and 142 mM dithiothreitol.

3. HEPES Based Non-Reducing Buffers

[0030] HEPES based buffers can also be used with the disclosed methods. One embodiment provides a non-reducing HEPES based aqueous electrophoresis sample buffer containing an alkylating agent, for example 55 to 75 mM 2-iodoacetamide; 0.1 to 1.0 % lithium dodecyl sulfate; 5 to 85 mM HEPES, and 5 to 115 mM sodium chloride, wherein the aqueous electrophoresis sample buffer has a pH of less than 9. In a preferred embodiment, the pH of the buffer is 8. In another embodiment, the aqueous buffer contains 66.4. mM 2-iodoacetamide, 0.32% lithium dodecyl sulfate, 16.2 mM HEPES, and 48.6 mM sodium chloride.

4. HEPES Based Reducing Buffers

[0031] Another embodiment provides a reducing HEPES based aqueous electrophoresis sample buffer containing 0.05 to 0.75 % lithium dodecyl sulfate, 5 mM to 115 mM sodium chloride, 5 mM to 115 mM HEPES, and a reducing agent, wherein the aqueous electrophoresis sample buffer has a pH greater than 7. In a preferred embodiment, the pH of the buffer is 8. In one embodiment, the reducing buffer contains 35 to 50 mM dithiothreitol. Still another embodiment provides a reducing buffer containing 0.28 % lithium dodecyl sulfate, 41.5 mM sodium chloride, 13.8 mM HEPES, and 42.5 mM dithiothreitol.

B. Assays

1. Non-Reducing Assays

[0032] One embodiment provides a non-reducing MCE method for identifying contaminants or impurities in a protein drug sample, the method including the steps of adding the protein sample to a non-reducing buffer discussed above to form a buffered protein drug sample. The buffered protein drug sample is heated to between 65 to 85 °C for 5 to 15 minutes to form a denatured buffered protein drug sample. In a preferred embodiment, the buffered protein drug sample is heated to 70 °C for 10 min. A detectable label is then added to the denatured buffered protein drug sample and heated at 30 to 40 °C for 20 to 40 minutes to form a denatured labeled protein drug sample. In a preferred embodiment, the denatured protein drug sample with added label is heated to 35 °C for 30 minutes. Excess label is optionally removed from the sample, for example by using a spin filter.

[0033] A preferred detectable label includes, but is not limited to Dyomics DY-631 NHS Ester. Other detectable labels can be used include other dyes, fluorophores, chromophores, mass tags, quantum dots and the like and those disclosed in US Patent No. 6,924,372 which is incorporated by reference in its entirety.

[0034] The denatured labeled protein drug product is diluted and subjected to MCE to separate the diluted protein drug sample on a microchip capillary electrophoresis system to produce an electropherogram. In one embodiment the final concentration of a sample starting at 0.5 mg/ml that is then injected over the microchip is 9 µg/ml to MCE. In another embodiment, the sample starting concentration is 0.2 mg/ml. The electropherogram contains peaks corresponding to the protein drug product and impurities. The method concludes by identifying peaks in the electropherogram corresponding to contaminants or impurities.

2. Reducing Assays

[0035] Another embodiment provides a reducing MCE method for identifying contaminants or impurities in a protein drug sample. The method begins by adding the protein drug sample to any one of the reducing buffers described above to form a buffered protein drug sample. The buffered protein drug sample is denatured by heating the buffered protein drug sample to 65 to 85 °C, preferably to 70 °C for 10 minutes to form a denatured protein drug sample. The protein drug sample with added label is then heated at 30 to 40 °C for 20 to 40 minutes form a denatured labeled protein drug sample. In a preferred embodiment, the protein drug product sample with added label is heated to 35 °C for 30 minutes. Excess label is optionally removed from the sample, for example by using a spin filter. A preferred detectable label includes, but is not limited to Dyomics DY-631 NHS Ester. Other detectable labels that can be used include other dyes, fluorophores, chromophores, mass tags, quantum dots and the like and those disclosed in US Patent No. 6,924,372 which is incorporated by reference in its entirety.

[0036] In one embodiment, the established assay range for sample concentration is from 0.4 mg/ml to 0.6 mg/ml, corresponding to a final concentration being analyzed of about 7 μg/ml to 11 μg/ml which is subjected to MCE analysis on a microchip capillary electrophoresis system to produce an electropherogram. The method concludes by identifying peaks in the electropherogram corresponding to contaminants or impurities.

C. Instrumentation

[0037] Instrumentation for conducting the disclosed MCE assays is commercially available. In a preferred embodiment, the disclosed MCE assays are performed using LabChip GXII or LabChip GXII Touch HT and LabChip® HT Protein Express Chip.

III. Proteins of Interest

[0038] The protein of interest, for example a protein drug product, assayed with the disclosed MCE assays and reagents can be any protein of interest suitable for expression in prokaryotic or eukaryotic cells and can be used in the engineered host cell systems provided. For example, the protein of interest includes, but is not limited to, an antibody or antigen-binding fragment thereof, a chimeric antibody or antigen-binding fragment thereof, an ScFv or fragment thereof, an Fc-fusion protein or fragment thereof, a growth factor or a fragment thereof, a cytokine or a fragment thereof, or an extracellular domain of a cell surface receptor or a fragment thereof. Proteins of interest may be simple polypeptides consisting of a single subunit, or complex multisubunit proteins comprising two or more subunits. The protein of interest may be a biopharmaceutical product, food additive or preservative, or any protein product subject to purification and quality standards.

[0039] In some embodiments, the protein drug product (protein of interest) is an antibody, a human antibody, a humanized antibody, a chimeric antibody, a monoclonal antibody, a multispecific antibody, a bispecific antibody, an antigen binding antibody fragment, a single chain antibody, a diabody, triabody or tetrabody, a Fab fragment or a F(ab')₂ fragment, an IgD antibody, an IgE antibody, an IgM antibody, an IgG antibody, an IgG1 antibody, an IgG2 antibody, an IgG3 antibody, or an IgG4 antibody. In one embodiment, the antibody is an IgG1 antibody. In one embodiment, the antibody is an IgG2 antibody. In one embodiment, the antibody is an IgG4 antibody. In one embodiment, the antibody is a chimeric TgG2/IgG4 antibody. In one embodiment, the antibody is a chimeric IgG2/IgG1 antibody. In one embodiment, the antibody is a chimeric IgG2/IgG1/IgG4 antibody.

[0040] In some embodiments, the antibody is selected from the group consisting of an anti-Programmed Cell Death 1 antibody (e.g. an anti-PD1 antibody as described in U.S. Pat. Appln. Pub. No. US2015/0203579A1), an anti-Programmed Cell Death Ligand-1 (e.g., an anti-PD-L1 antibody as described in U.S. Pat. Appln. Pub. No. US2015/0203580A1), an anti-D114 antibody, an anti-Angiopoietin-2 antibody (e.g., an anti-ANG2 antibody as described in U.S. Pat. No. 9,402,898), an anti-Angiopoietin-Like 3 antibody (e.g., an anti-AngPtI3 antibody as described in U.S. Pat. No. 9,018,356), an anti-platelet derived growth factor receptor antibody (e.g., an anti-PDGFR antibody as described in U.S. Pat. No. 9,265,827), an anti-Erb3 antibody, an anti-Prolactin Receptor antibody (e.g., anti-PRLR antibody as described in U.S. Pat. No. 9,302,015), an anti-Complement 5 antibody (e.g., an anti-C5 antibody as described in U.S. Pat. Appln. Pub. No. US2015/0313194A1), an anti-TNF antibody, an anti-epidermal growth factor receptor antibody (e.g., an anti-EGFR antibody as described in U.S. Pat. No. 9,132,192 or an anti-EGFRvIII antibody as described in U.S. Pat. Appln. Pub. No. US2015/0259423A1), an anti-Protein Convertase Subtilisin Kexin-9 antibody (e.g., an anti-PCSK9 antibody as described in U.S. Pat. No. 8,062,640 or U.S. Pat. No. 9,540,449), an Anti-Growth and Differentiation Factor-8 antibody (e.g. an anti-GDF8 antibody, also known as anti-myostatin antibody, as described in U.S. Pat Nos. 8,871,209 or 9,260,515), an anti-Glucagon Receptor (e.g. anti-GCGR antibody as described in U.S. Pat. Appln. Pub. Nos. US2015/0337045A1 or US2016/0075778A1), an anti-VEGF antibody, an anti-IL1R antibody, an interleukin 4 receptor antibody (e.g., an anti-IL4R antibody as described in U.S. Pat. Appln. Pub. No. US2014/0271681A1 or U.S. Pat Nos. 8,735,095 or 8,945,559), an anti-interleukin 6 receptor antibody (e.g., an anti-IL6R antibody as described in U.S. Pat. Nos. 7,582,298, 8,043,617 or 9,173,880), an anti-IL1 antibody, an anti-IL2 antibody, an anti-IL3 antibody, an anti-IL4 antibody, an anti-IL5 antibody, an anti-IL6 antibody, an anti-IL7 antibody, an anti-interleukin 33 (e.g., anti-IL33 antibody as described in U.S. Pat. Nos. 9,453,072 or 9,637,535), an anti-Respiratory syncytial virus antibody (e.g., anti-RSV antibody as described in U.S. Pat. Appln. Pub. No. 9,447,173), an anti-Cluster of differentiation 3 (e.g., an anti-CD3 antibody, as described in U.S. Pat. Nos. 9,447,173 and 9,447,173, and in U.S. Application No. 62/222,605), an anti-Cluster of differentiation 20 (e.g., an anti-CD20 antibody as described in U.S. Pat. Nos. 9,657,102 and US20150266966A1, and in U.S. Pat. No. 7,879,984), an anti-CD19 antibody, an anti-CD28 antibody, an anti-Cluster of Differentiation-48 (e.g. anti-CD48 antibody as described in U.S. Pat. No. 9,228,014), an anti-Fel d1 antibody (e.g. as described in U.S. Pat. No. 9,079,948), an anti-Middle East Respiratory Syndrome virus (e.g. an anti-MERS antibody as described in U.S. Pat. Appln. Pub. No. US2015/0337029A1), an anti-Ebola virus antibody (e.g. as described in U.S. Pat. Appln. Pub. No. US2016/0215040), an anti-Zika virus antibody, an anti-Lymphocyte Activation Gene 3 antibody (e.g.

an anti-LAG3 antibody, or an anti-CD223 antibody), an anti-Nerve Growth Factor antibody (e.g. an anti-NGF antibody as described in U.S. Pat. Appln. Pub. No. US2016/0017029 and U.S. Pat. Nos. 8,309,088 and 9,353,176) and an anti-Protein Y antibody. In some embodiments, the bispecific antibody is selected from the group consisting of an anti-CD3 x anti-CD20 bispecific antibody (as described in U.S. Pat. Appln. Pub. Nos. US2014/0088295A1 and US20150266966A1), an anti-CD3 x anti-Mucin 16 bispecific antibody (e.g., an anti-CD3 x anti-Muc16 bispecific antibody), and an anti-CD3 x anti-Prostate-specific membrane antigen bispecific antibody (e.g., an anti-CD3 x anti-PSMA bispecific antibody). In some embodiments, the protein of interest is selected from the group consisting of abciximab, adalimumab, adalimumab-atto, ado-trastuzumab, alemtuzumab, alirocumab, atezolizumab, avelumab, basiliximab, belinumab, benralizumab, bevacizumab, bezlotoxumab, blinatitmoniab, brentuximab vedotin, brodalumab, canakinumab, capromab pendetide, certolizumab pegol, cemiplimab, cetuximab, denosumab, dinutuximab, dupilumab, durvalumab, eculizumab, elotuzumab, emicizumab-kxwh, emtansinealirocumab, evinacumab, evolocumab, fasinumab, golimumab, guselkumab, ibritumomab tiuxetan, idirticizun-tab., infliximab, infliximab-abda, infliximab-dyyb, ipilimumab, ixekizumab, mepolizumab, necitumimab, nesvacumab, nivolumab, obiltoxaximab, obinutuzumab, ocrelizumab, ofatumumab, olaratumab, omalizumab, panitumumab, pembrolizumab, pertuzumab, ramucirumab, ranibizumab, raxibacumab, reslizumab, rinucumab, rituximab, sarilumab, secukinumab, siituximab, tocilizumab, tocilizumab, trastuzumab, trevogrumab, ustekinumab, and vedolizumab.

[0041] In some embodiments, the protein of interest is a recombinant protein that contains an Fc moiety and another domain, (e.g., an Fc-fusion protein). In some embodiments, an Fc-fusion protein is a receptor Fc-fusion protein, which contains one or more extracellular domain(s) of a receptor coupled to an Fc moiety. In some embodiments, the Fc moiety comprises a hinge region followed by a CH2 and CH3 domain of an IgG. In some embodiments, the receptor Fc-fusion protein contains two or more distinct receptor chains that bind to either a single ligand or multiple ligands. For example, an Fc-fusion protein is a TRAP protein, such as for example an IL-1 trap (e.g., riloncept, which contains the IL-IRAcP ligand binding region fused to the I1-1R1 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 or ziv-aflibercept, which comprises 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; see U.S. Pat. Nos. 7,087,411 and 7,279,159). In other embodiments, an Fc-fusion protein is a ScFv-Fc-fusion protein, which contains one or more of one or more antigen-binding domain(s), such as a variable heavy chain fragment and a variable light chain fragment, of an antibody coupled to an Fc moiety.

IV. Cell Culture

[0042] The protein drug product assayed with the disclosed MCE assays and reagents are produced cell cultures. The cell cultures can be a "fed-batch cell culture" or "fed-batch culture" which refers to a batch culture wherein the cells and culture medium are supplied to the culturing vessel initially and additional culture nutrients are slowly fed, in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. Fed-batch culture includes "semi-continuous fed-batch culture" wherein periodically whole culture (which may include cells and medium) is removed and replaced by fresh medium. Fed-batch culture is distinguished from simple "batch culture" whereas all components for cell culturing (including the animal cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process in batch culture. Fed-batch culture may be different from "perfusion culture" insofar as the supernatant is not removed from the culturing vessel during a standard fed-batch process, whereas in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, and the culture medium is continuously or intermittently introduced and removed from the culturing vessel. However, removal of samples for testing purposes during fed-batch cell culture is contemplated. The fed-batch process continues until it is determined that maximum working volume and/or protein production is reached, and protein is subsequently harvested.

[0043] The cell culture can be a "continuous cell culture" which is a technique used to grow cells continually, usually in a particular growth phase. For example, if a constant supply of cells is required, or the production of a particular protein of interest is required, the cell culture may require maintenance in a particular phase of growth. Thus, the conditions must be continually monitored and adjusted accordingly in order to maintain the cells in that particular phase.

[0044] The cells are cultured in cell culture medium. The terms "cell culture medium" and "culture medium" refer to a nutrient solution used for growing mammalian cells that typically provides the necessary nutrients to enhance growth of the cells, such as a carbohydrate energy source, essential (e.g., phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine) and nonessential (e.g., alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline, serine, and tyrosine) amino acids, trace elements, energy sources, lipids, vitamins, etc. Cell culture medium may contain extracts, e.g., serum or peptones (hydrolysates), which supply raw materials that support cell growth. Media may contain yeast-derived or soy extracts, instead of animal-derived extracts. Chemically defined medium refers to a cell culture medium in which all of the chemical components are known (i.e., have a known chemical structure). Chemically defined medium is entirely free of animal-derived components, such as serum- or animal-derived peptones. In one embodiment, the medium is a chemically defined medium.

[0045] The solution may also contain components that enhance growth and/or survival above the minimal rate, including hormones and growth factors. The solution may be formulated to a pH and salt concentration optimal for survival and proliferation of the particular cell being cultured.

[0046] A "cell line" refers to a cell or cells that are derived from a particular lineage through serial passaging or subculturing of cells. The term "cells" is used interchangeably with "cell population".

[0047] The term "cell" includes any cell that is suitable for expressing a recombinant nucleic acid sequence. Cells include those of prokaryotes and eukaryotes, such as bacterial cells, mammalian cells, human cells, non-human animal cells, avian cells, insect cells, yeast cells, or cell fusions such as, for example, hybridomas or quadromas. In certain embodiments, the cell is a human, monkey, ape, hamster, rat or mouse cell. In other embodiments, the cell is selected from the following cells: Chinese Hamster Ovary (CHO) (e.g., CHO K1, DXB-11 CHO, Veggie-CHO), COS (e.g., COS-7), retinal cell, Vero, CV1, kidney (e.g., HEK293, 293 EBNA, MSR 293, MDCK, HaK, BHK21), HeLa, HepG2, WI38, MRC 5, Colo25, HB 8065, HL-60, lymphocyte, e.g., Jurkat (T lymphocyte) or Daudi (B lymphocyte), A431 (epidermal), U937, 3T3, L cell, C127 cell, SP2/0, NS-0, MMT cell, stem cell, tumor cell, and a cell line derived from an aforementioned cell. In some embodiments, the cell comprises one or more viral genes, e.g., a retinal cell that expresses a viral gene (e.g., a PER.C6[®] cell). In some embodiments, the cell is a CHO cell. In other embodiments, the cell is a CHO KI cell.

V. Kits

[0048] One embodiment provides a kit including the one or more of the disclosed buffers or ingredients to make the disclosed buffers. The kit can include a container for the buffers or ingredients. The buffers can be in solution or in lyophilized form. The kit optionally also includes a second container containing a diluent or reconstituting solution for the lyophilized formulation; and optionally, instructions for the use of the solution or the reconstitution and/or use of the lyophilized buffers or powdered ingredients.

[0049] The kit may further include additional reagents needed to perform the disclosed MCE assays including one or more of a buffer, a diluent, and a filter. The buffer and reagents can be in a bottle, a vial, or test tube.

EXAMPLE

Example 1: MCE assay for purity and impurity analysis of therapeutic proteins.

Methods and Materials:

Materials:

[0050] LabChip GXII or LabChip GXII Touch HT and LabChip[®] HT Protein Express Chip were used for capillary electrophoretic separation and data collection (Perkin Elmer). Non-reducing and reducing denaturing buffers disclosed above were used for the MCE assay.

Methods:

[0051] Table 1 shows the workflow procedure for preparing a sample for an MCE assay. Briefly, protein samples were diluted to 0.5 mg/ml. 1 μ l of either non-reducing (NR) or reducing (R) denaturing buffer and 4 μ l of the diluted sample were added to a 96-well plate. The sample was mixed, centrifuged, and heated for 10 minutes at the temperature specified for the product, typically 75°C. The samples were then labeled with 5 μ M commercially available dye (for example Dyomics DY-631 NHS Ester). The samples were mixed, centrifuged, and then heated at 35°C for 30 minutes. The labeled sample was then diluted with 105 μ l of dilute stop solution. The samples were separated using LabChip GXII or LabChip GXII Touch HT.

Buffers

[0052] Stock solutions of 200 mM Sodium Phosphate Monobasic Monohydrate, 200 mM Sodium Phosphate Dibasic Hepathydrate, and 10% Lithium Dodecyl Sulfate (LDS) were prepared. Using the stock solutions and Milli-Q[®] water, solutions of 100 mM Sodium Phosphate 1% LDS pH 6 and 100 mM Sodium Phosphate 1% LDS pH 9 were prepared.

[0053] A non-reducing buffer was prepared by adding 34 μ L 1M Iodoacetamide (IAM) (prepared fresh in Milli-Q[®] water) + 166 μ L 100 mM Sodium Phosphate 1% LDS pH 6 + 5 μ L Milli-Q[®] water. The final concentrations were 166 mM 2-iodoacetamide, 0.81 % lithium dodecyl sulfate, and 81 mM Sodium phosphate.

[0054] A reducing buffer was prepared by adding 68 μ L 10x Reducing agent (500 mM dithiothreitol (DTT) + 166 μ L 100 mM Sodium Phosphate 1% LDS pH 9 + 6 μ L Milli-Q[®] water. The final concentrations were 0.69% lithium dodecyl

EP 4 317 959 A2

sulfate: 69 mM sodium phosphate, and 142 mM dithiothreitol.

Table 1. Sample preparation method for MCE assay.

Sample Preparation	
NR	R
4 μ l 0.5 mg/mL Sample 1 μ L NR Buffer	4 μ l 0.5 mg/mL Sample 1 μ L R Buffer
Mix, centrifuge, heat at Specified Temperature for 10 minutes	
Sample Labeling	
5 μ L Denatured Sample	
5 μ L 5 μ M PICO dye	
Mix, centrifuge, heat at 35°C for 30 minutes	
Final Dilution	
5 μ L Labeled Sample	
105 μ L Dilute Stop Sample	
Separation Method	
HT PICO Protein Express 200	

Results:

[0055] Microchip Capillary Electrophoresis (MCE) allows for dramatically reduced sample analysis times, while maintaining the performance and reproducibility standards required for QC analysis. An MCE assay was developed using the non-reduced and reduced denaturing buffers disclosed herein. Figures 1A-1B show a representative electropherogram showing analysis of protein in non-reduced samples and reduced samples.

[0056] While in the foregoing specification this invention has been described in relation to certain embodiments thereof, and many details have been put forth for the purpose of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein can be varied considerably without departing from the basic principles of the invention.

[0057] All references cited herein are incorporated by reference in their entirety. The present invention may be embodied in other specific forms without departing from the spirit or essential attributes thereof and, accordingly, reference should be made to the appended claims, rather than to the foregoing specification, as indicating the scope of the invention.

FURTHER NUMBERED EMBODIMENTS

[0058]

1. An aqueous electrophoresis sample buffer, comprising:

155 to 175 mM 2-iodoacetamide;
0.50 to 1.5% lithium dodecyl sulfate; and
75 to 95 mM sodium phosphate,
wherein the aqueous electrophoresis sample buffer has a pH of less than 7.

2. The aqueous buffer of [1], wherein the pH is 6.

3. The aqueous buffer of [1] or [2], comprising 166 mM 2-iodoacetamide, 0.81% lithium dodecyl sulfate, and 81 mM sodium phosphate.

4. An aqueous electrophoresis sample buffer, consisting of:

166 mM 2-iodoacetamide,

EP 4 317 959 A2

0.81 % lithium dodecyl sulfate, and
81 mM Sodium phosphate,
wherein the aqueous electrophoresis sample buffer has a pH of 6.0.

- 5 5. An aqueous electrophoresis sample buffer, comprising:
- 0.50 to 1.5% lithium dodecyl sulfate;
45 to 75 mM sodium phosphate, and
10 a reducing agent,
wherein the aqueous electrophoresis sample buffer has a pH greater than 8.
6. The aqueous buffer of [5], wherein the pH is 9.
7. The aqueous buffer of [5] or [6], comprising 135 to 155 mM dithiothreitol.
- 15 8. The aqueous buffer of any one of [5]-[7], comprising 0.69 % lithium dodecyl sulfate, 69 mM sodium phosphate, and 142 mM dithiothreitol.
9. An aqueous electrophoresis sample buffer, consisting of:
- 20 0.69% lithium dodecyl sulfate;
69 mM sodium phosphate, and
142 mM dithiothreitol,
wherein the aqueous electrophoresis sample buffer has a pH of 9.0.
- 25 10. A method for identifying contaminants or impurities in a protein drug sample, the method comprising the steps of
- adding the protein drug sample to the buffer of any one of [1] - [4] to form a buffered protein drug sample;
heating the buffered protein drug sample to 65 to 85 °C for 5 to 15 minutes to form a denatured buffered protein
30 drug sample;
adding a detectable label to the denatured buffered protein drug sample and heating it at 30 to 40 °C for 20 to
40 minutes to form a denatured labeled protein drug sample;
diluting the denatured labeled protein drug sample and subjecting it to MCE to separate the diluted protein drug
sample on a microchip capillary electrophoresis system to produce an electropherogram; and
35 identifying peaks in the electropherogram corresponding to contaminants or impurities.
11. The method of [10], wherein the buffered protein drug sample is heated at 70 °C for 10 min.
12. The method of [10] or [11], wherein the labeled protein drug sample is heated at 35 °C for 30 minutes.
- 40 13. The method of any one of [10]-[12], wherein the diluted protein drug sample is 9 µg/ml.
14. A method for identifying contaminants or impurities in a protein drug sample, the method comprising the steps of
- 45 adding a protein sample to the buffer of any one of [5]-[9] to form a buffered protein drug sample;
heating the buffered protein drug sample to 65 to 85 °C for 5 to 15 minutes to form a denatured protein drug
sample;
adding a detectable label to the denatured protein drug sample and heating it at 30 to 40°C for 20 to 40 minutes
to form a denatured labeled protein drug sample;
50 diluting the denatured labeled protein drug sample and subjecting it to MCE analysis on a microchip capillary
electrophoresis system to produce an electropherogram; and
identifying peaks in the electropherogram corresponding to contaminants or impurities.
- 55 15. The method of [14], wherein the buffered protein drug sample is heated at 70 °C for 10 min.
16. The method of [14] or [15], wherein the sample is heated at 35°C for 30 minutes.
17. The method of any one of [14]-[16], wherein the diluted protein drug sample is 9 µg/ml.

18. The method of any one of [10]-[17], wherein the detectable label is DY-631 N-hydroxysuccinimidyl ester.

19. A kit comprising the buffer according to any one of [1]-[9], and written instructions for preparing a sample for electrophoresis in the buffer.

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20. An aqueous electrophoresis sample buffer, comprising:

55 to 75 mM 2-iodoacetamide;
0.1 to 1.0 % lithium dodecyl sulfate;
5 to 115 mM sodium chloride, and
5 to 85 mM HEPES,
wherein the aqueous electrophoresis sample buffer has a pH of less than 9.

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21. An aqueous electrophoresis sample buffer, comprising:

66.4 mM 2-iodoacetamide;
0.32% lithium dodecyl sulfate;
48.6 mM NaCl, and
16.2 mM HEPES,
wherein the aqueous electrophoresis sample buffer has a pH of less than 9.

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22. The aqueous buffer of [20] or [21], wherein the pH is 8.

23. An aqueous electrophoresis sample buffer, comprising:

0.05 to 0.75 % lithium dodecyl sulfate;
5 mM to 115mM NaCl
5 mM to 85 mM HEPES, and
35 to 50 mM dithiothreitol,
wherein the aqueous electrophoresis sample buffer has a pH greater than 7.

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24. An aqueous electrophoresis sample buffer, comprising:

0.28% lithium dodecyl sulfate;
41.5 mM NaCl
13.8 mM HEPES, and
42.5 mM dithiothreitol,
wherein the aqueous electrophoresis sample buffer has a pH greater than 7.

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25. The aqueous buffer of [23] or [24], wherein the pH is 8.

Claims

45 1. An aqueous electrophoresis sample buffer, comprising:

0.50 to 1.5% lithium dodecyl sulfate;
65 to 95 mM sodium phosphate; and
a reducing agent selected from the group consisting of dithiothreitol, beta-mercaptoethanol, aminoethanethiol,
Tris (2-carboxyethyl) phosphine hydrochloride, cysteine hydrochloride and 2-mercaptoethanesulfonic acid so-
dium salt,
wherein the aqueous electrophoresis sample buffer has a pH of greater than 8.

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2. The aqueous electrophoresis sample buffer of claim 1, wherein the pH is 9.

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3. The aqueous electrophoresis sample buffer of claim 1, wherein the buffer comprises 135 to 155 mM dithiothreitol.

4. An aqueous electrophoresis sample buffer, consisting of:

EP 4 317 959 A2

0.69% lithium dodecyl sulfate;
69 mM sodium phosphate; and
142 mM dithiothreitol.

5 **5.** A method for identifying contaminants or impurities in a protein drug sample, the method comprising the steps of:

adding the protein drug sample to an aqueous electrophoresis sample buffer comprising 0.50 to 1.5% lithium dodecyl sulfate; 65 to 95 mM sodium phosphate; and a reducing agent selected from the group consisting of dithiothreitol, beta-mercaptoethanol, aminoethanethiol, Tris (2-carboxyethyl) phosphine hydrochloride, cysteine hydrochloride and 2-mercaptoethanesulfonic acid sodium salt to form a buffered protein drug sample;
10 heating the buffered protein drug sample to 65 to 85°C for 10 minutes to form a denatured buffered protein drug sample;
adding a detectable label to the denatured buffered protein drug sample and heating it at 30 to 40°C for 20 to 40 minutes to form a denatured labeled protein drug sample;
15 subjecting the denatured labeled protein drug to microchip capillary electrophoresis to produce an electropherogram; and
identifying peaks in the electropherogram corresponding to contaminants or impurities.

20 **6.** The method of claim 5, wherein the buffered protein drug sample is heated at 70°C for 10 min.

7. The method of claim 5, wherein the denatured buffered protein drug sample is heated at 35°C for 30 minutes.

8. The method of claim 5, wherein the detectable label is DY-631 N-hydroxysuccinimidyl ester.

25 **9.** An aqueous electrophoresis sample buffer, comprising:

0.05 to 0.75% lithium dodecyl sulfate;
5 to 115 mM sodium chloride;
5 to 115 mM HEPES, and
30 a reducing agent selected from the group consisting of dithiothreitol, beta-mercaptoethanol, aminoethanethiol, Tris (2-carboxyethyl) phosphine hydrochloride, cysteine hydrochloride and 2-mercaptoethanesulfonic acid sodium salt,
wherein the aqueous electrophoresis sample buffer has a pH of greater than 7.

35 **10.** The aqueous electrophoresis sample buffer of claim 9, wherein the pH is 8.

11. The aqueous electrophoresis sample buffer of claim 9, wherein the buffer comprises 35 to 50 mM dithiothreitol.

40 **12.** An aqueous electrophoresis sample buffer, consisting of:

0.28% lithium dodecyl sulfate;
41.5 mM sodium chloride;
13.8 mM HEPES, and
42.5 mM dithiothreitol.

45 **13.** A method for identifying contaminants or impurities in a protein drug sample, the method comprising the steps of:

adding the protein drug sample to an aqueous electrophoresis sample buffer comprising 0.05 to 0.75% lithium dodecyl sulfate; 5 to 115 mM sodium chloride; 5 to 115 mM HEPES and a reducing agent selected from the group consisting of dithiothreitol, beta-mercaptoethanol, aminoethanethiol, Tris (2-carboxyethyl) phosphine hydrochloride, cysteine hydrochloride and 2-mercaptoethanesulfonic acid sodium salt to form a buffered protein drug sample;
50 heating the buffered protein drug sample to 65 to 85°C for 10 minutes to form a denatured buffered protein drug sample;
adding a detectable label to the denatured buffered protein drug sample and heating it at 30 to 40°C for 20 to 40 minutes to form a denatured labeled protein drug sample;
55 subjecting the denatured labeled protein drug to microchip capillary electrophoresis to produce an electropherogram; and

EP 4 317 959 A2

identifying peaks in the electropherogram corresponding to contaminants or impurities.

14. The method of claim 13, wherein the buffered protein drug sample is heated at 70°C for 10 min.

5 **15.** The method of claim 13, wherein the denatured buffered protein drug sample is heated at 35°C for 30 minutes.

16. The method of claim 13, wherein the detectable label is DY-631 N-hydroxysuccinimidyl ester.

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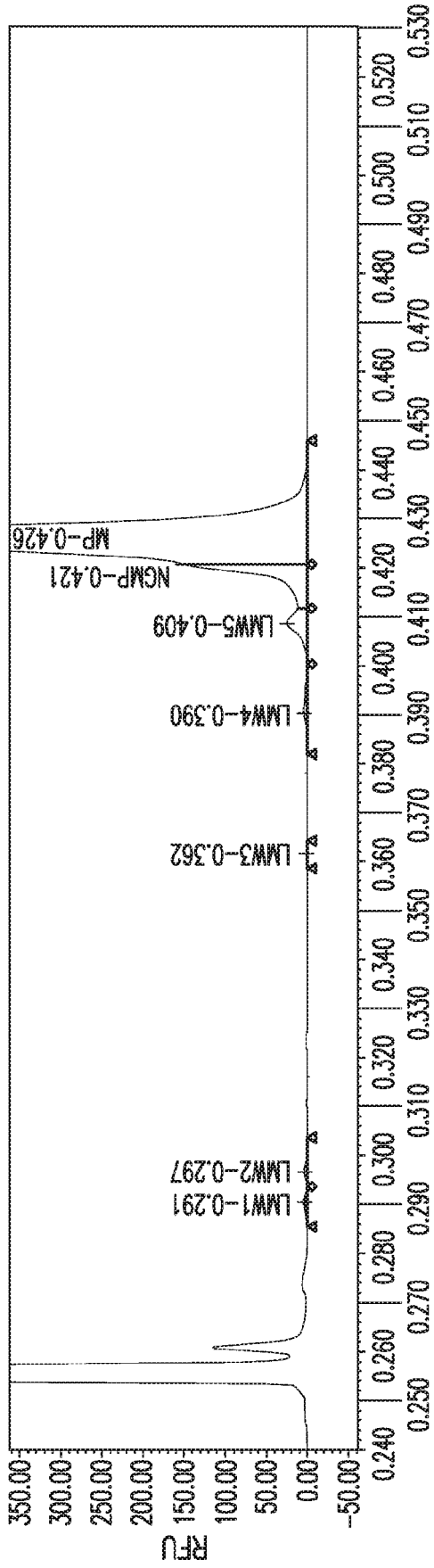


FIG. 1A

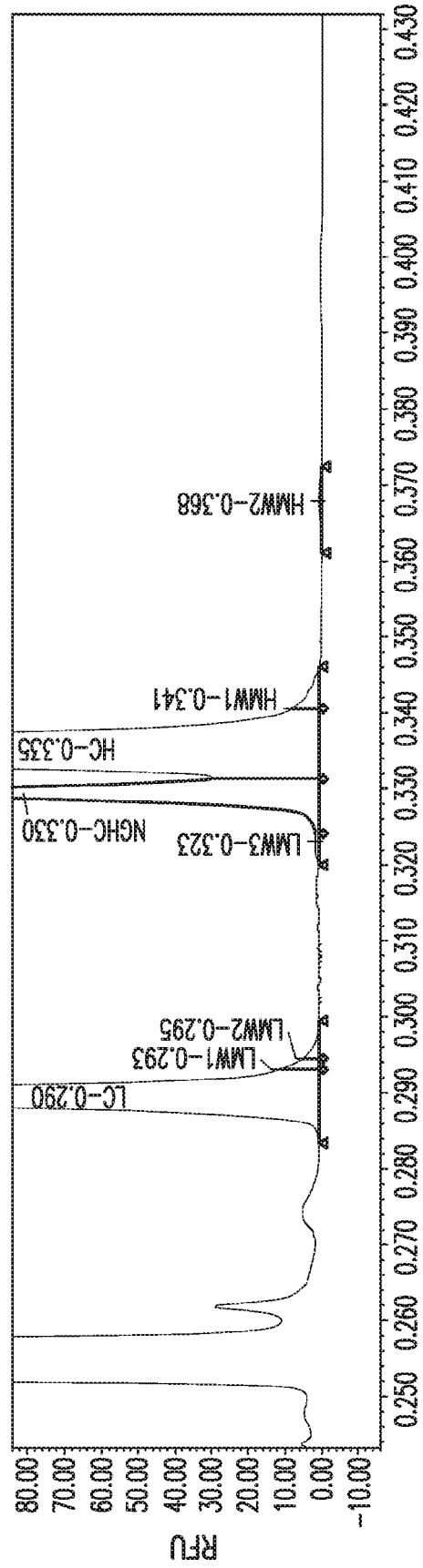


FIG. 1B

REFERENCES CITED IN THE DESCRIPTION

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