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(54) NANOGL PLATFORM TECHNOLOGY FOR LONG-TERM BIOLOGICS THERAPY

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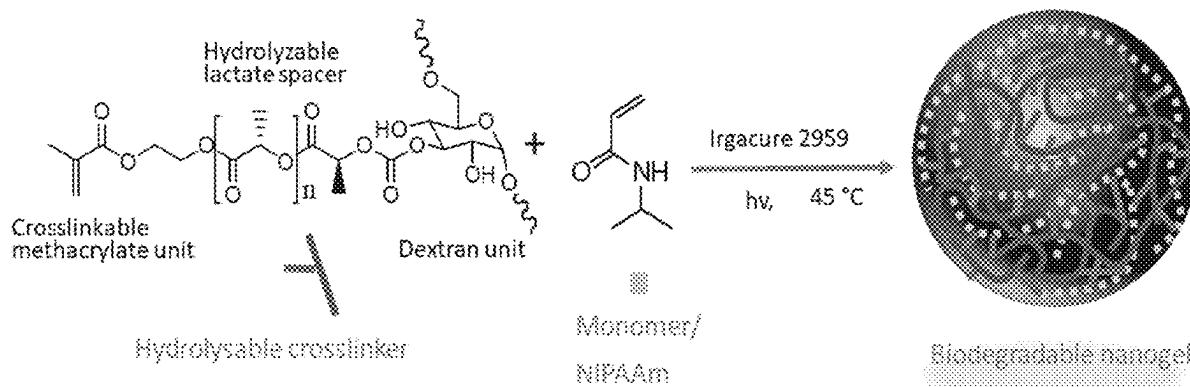
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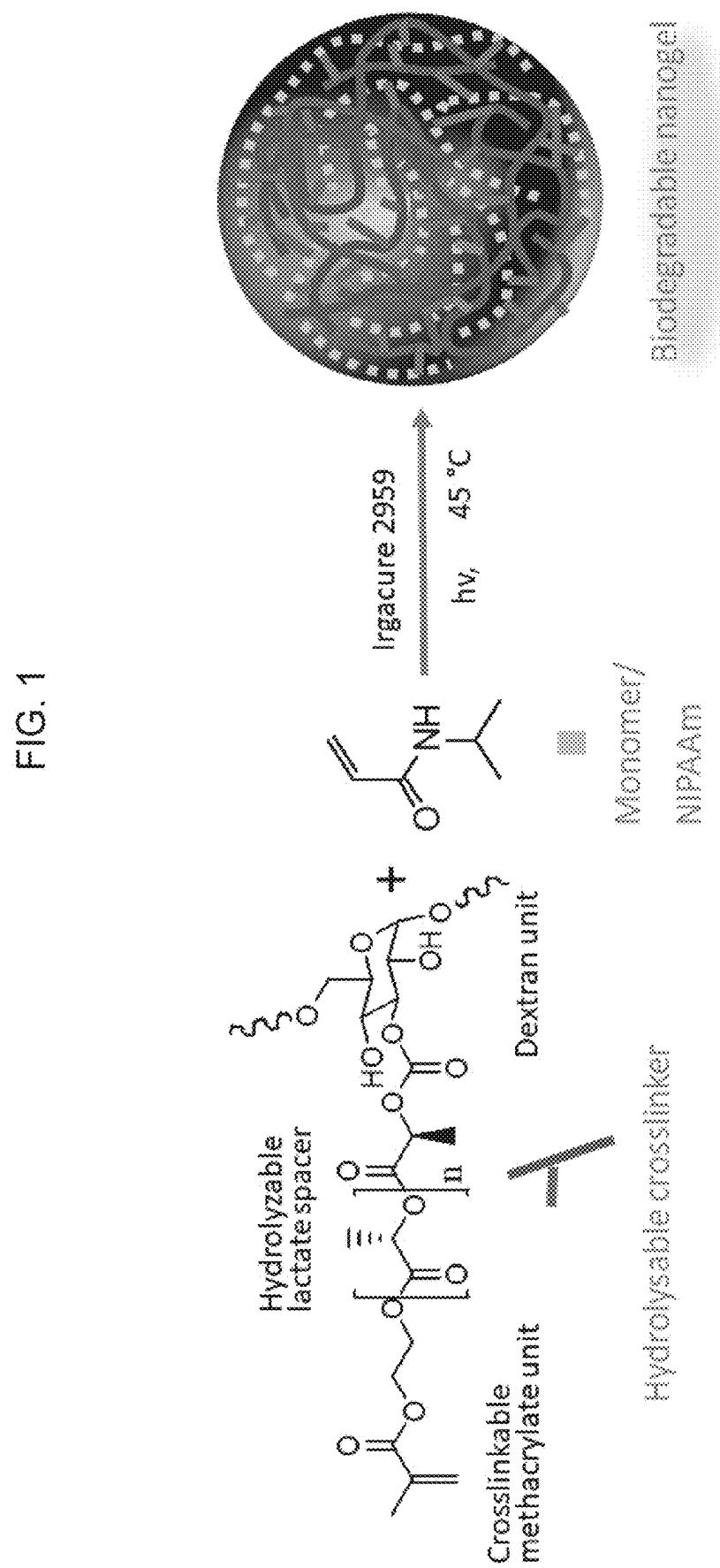
ABSTRACT

Biologics, including peptides, proteins, antibodies, nucleic acids (DNA and RNA), oligonucleotides, vaccines, or complex combinations of these substances, are important for treating various types of diseases and tissue and organ regeneration. However, biologics are not stable and have short half-lives, making effective delivery to patients difficult. Therefore, there is an unmet need in the art to increase the stability and half-lives of biologics for long-term bioavailability, therapy, treatment and repair. This invention provides a nanogel platform technology that can load biologics in aqueous solution with high loading efficiency without using any organic solvent and also sustain the release of active biologics in the body for more than 2 months.

Related U.S. Application Data

(60) Provisional application No. 63/334,938, filed on Apr. 26, 2022, provisional application No. 63/334,896, filed on Apr. 26, 2022.





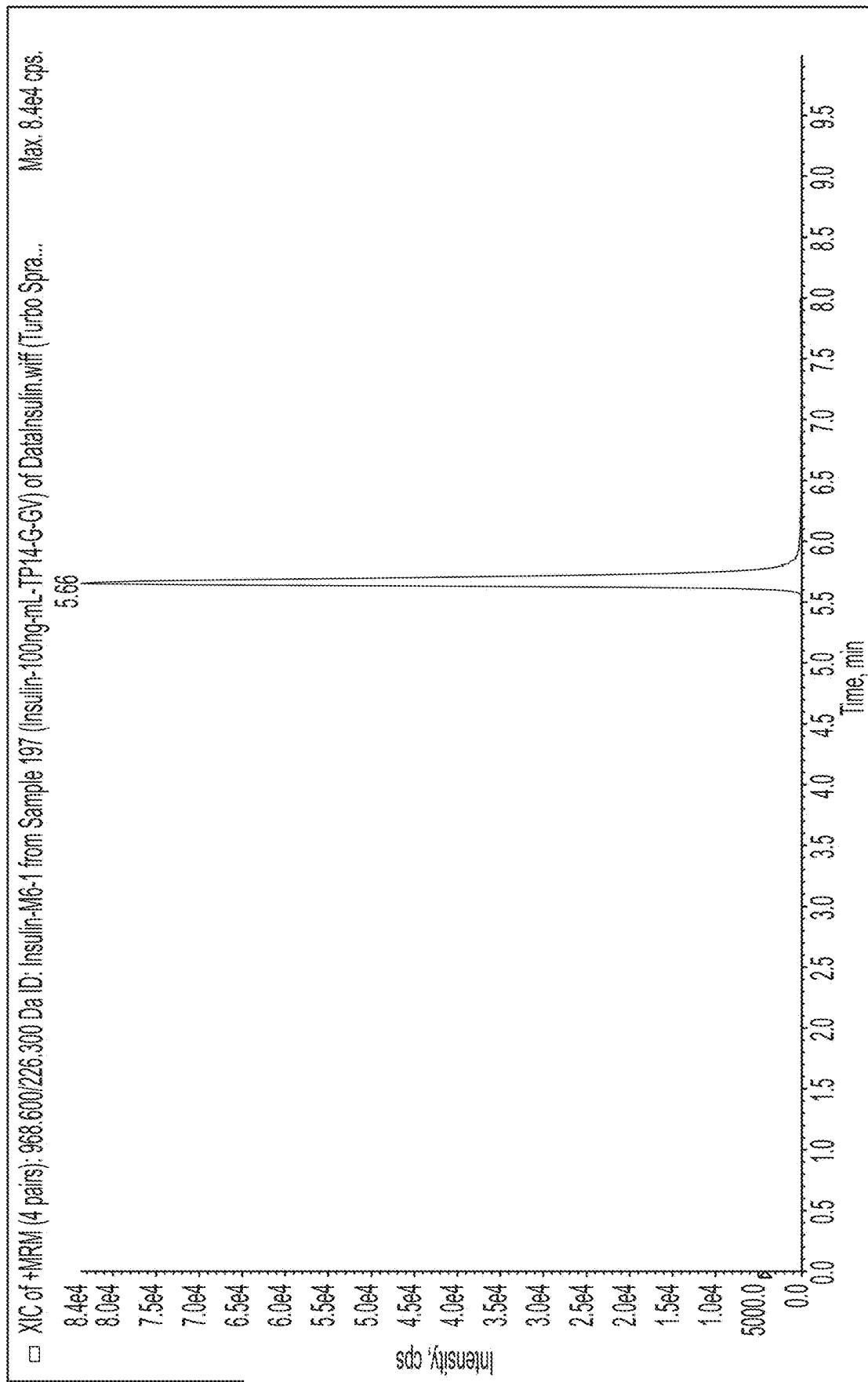


FIG. 2A

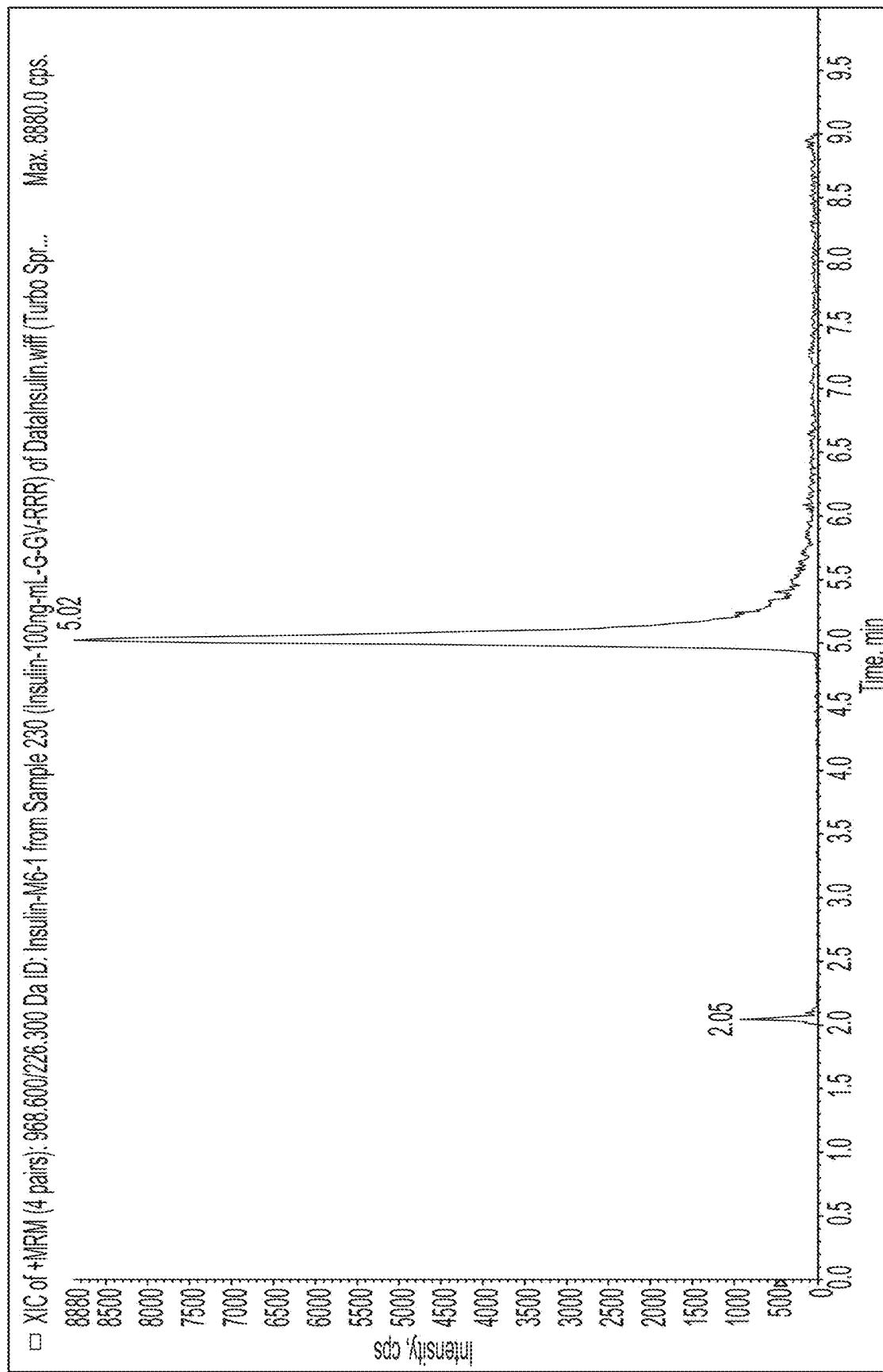


FIG. 2B

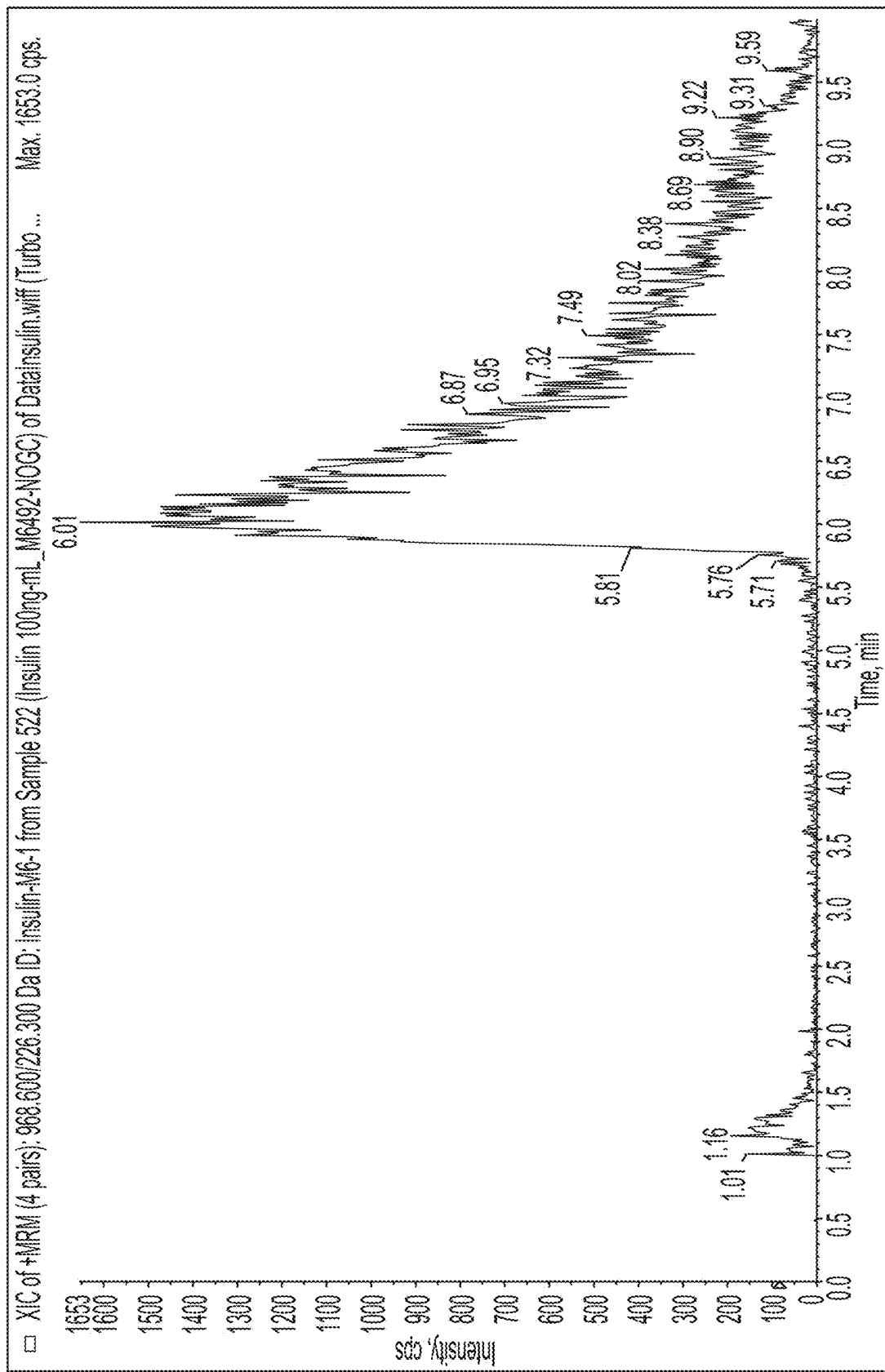


FIG. 3A

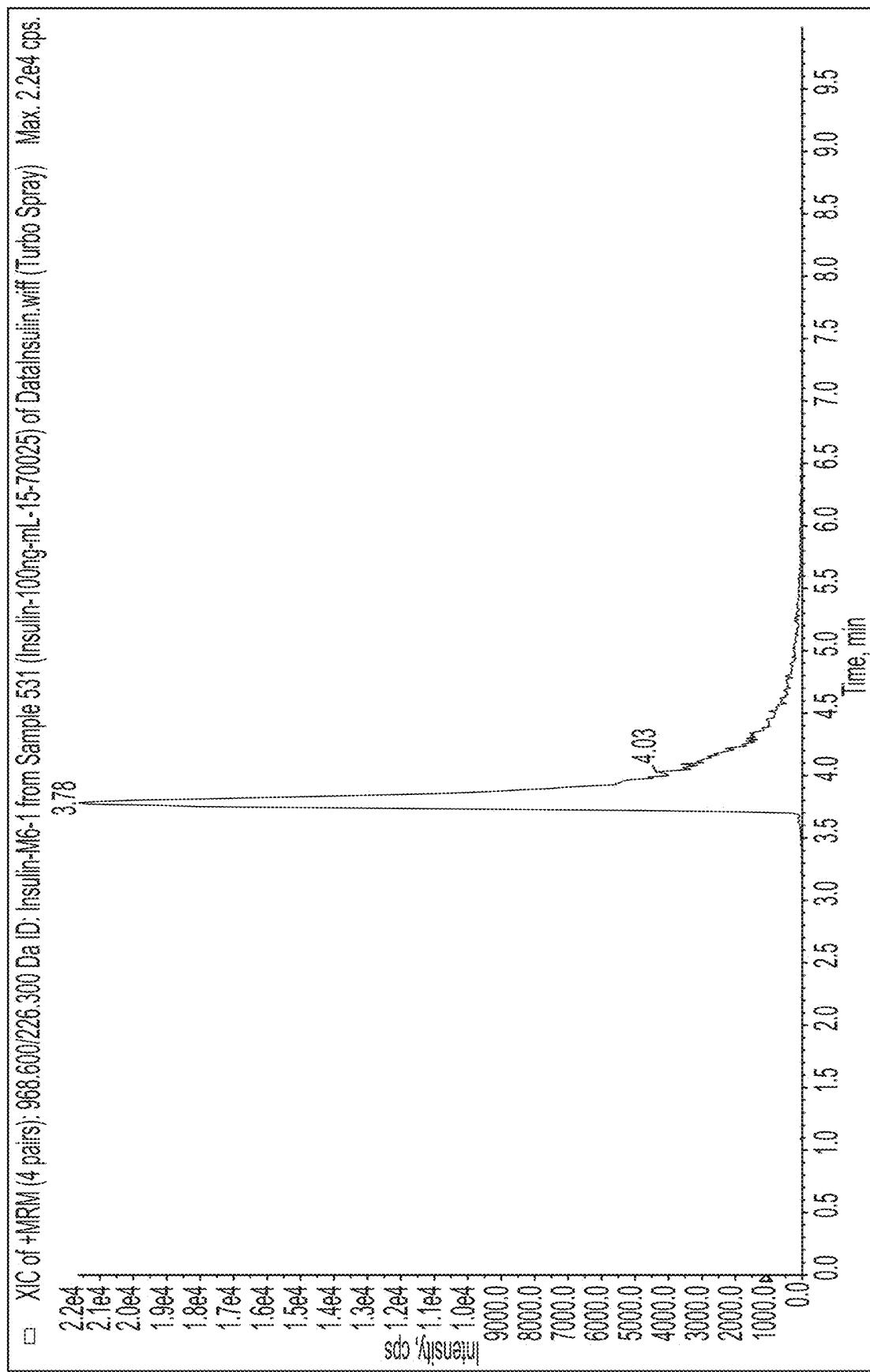


FIG. 3B

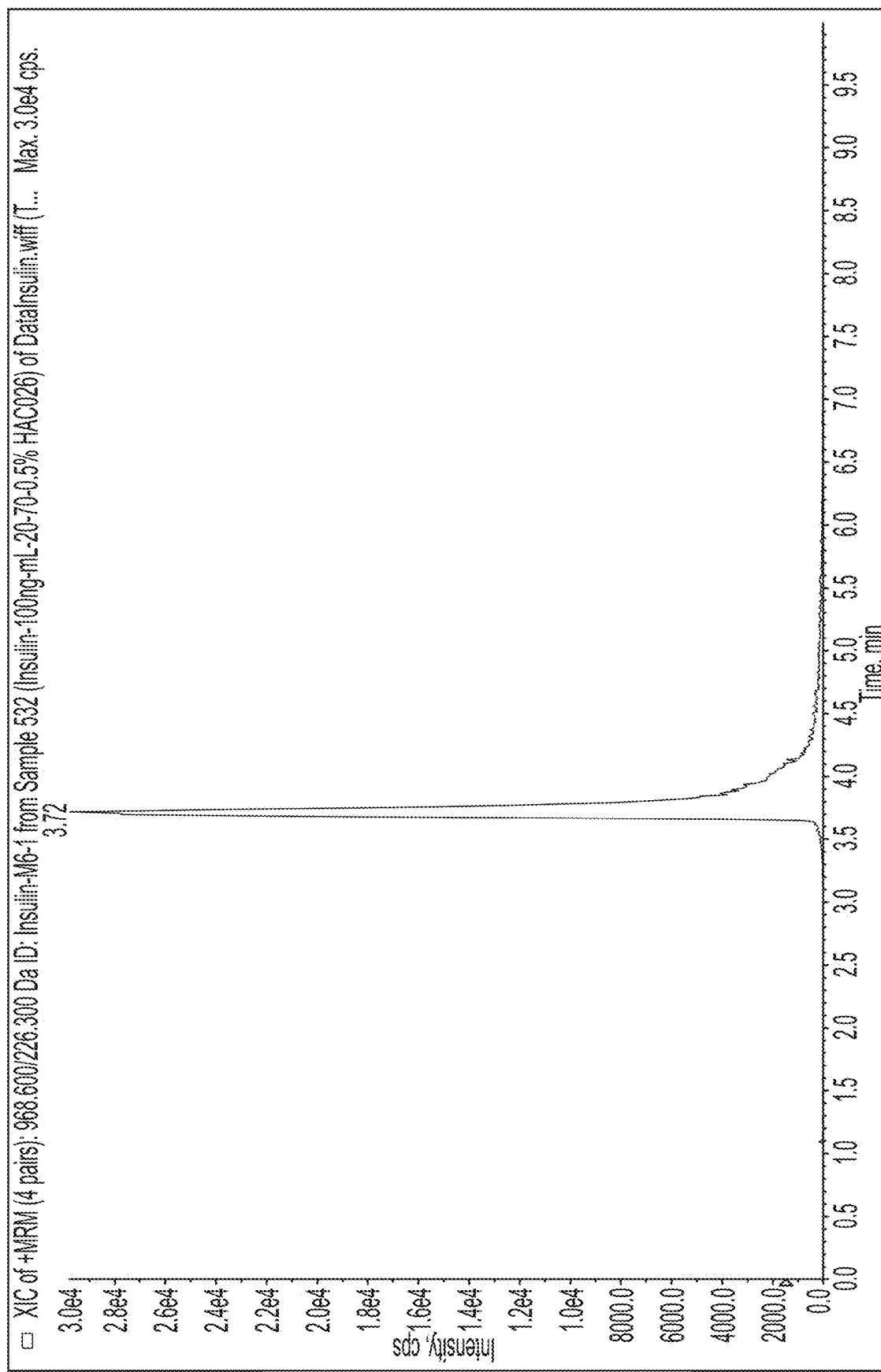


FIG. 3C

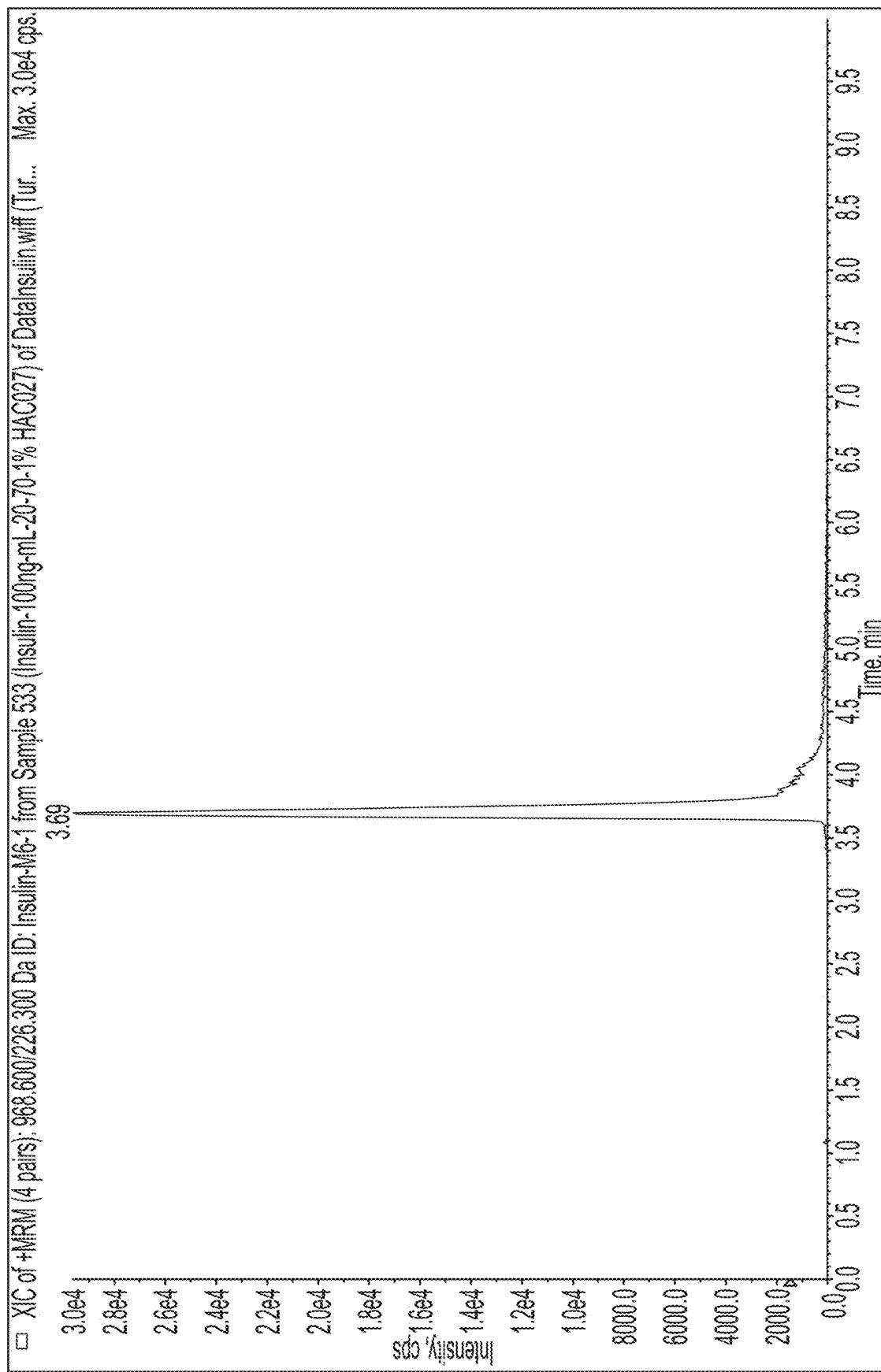


FIG. 3D

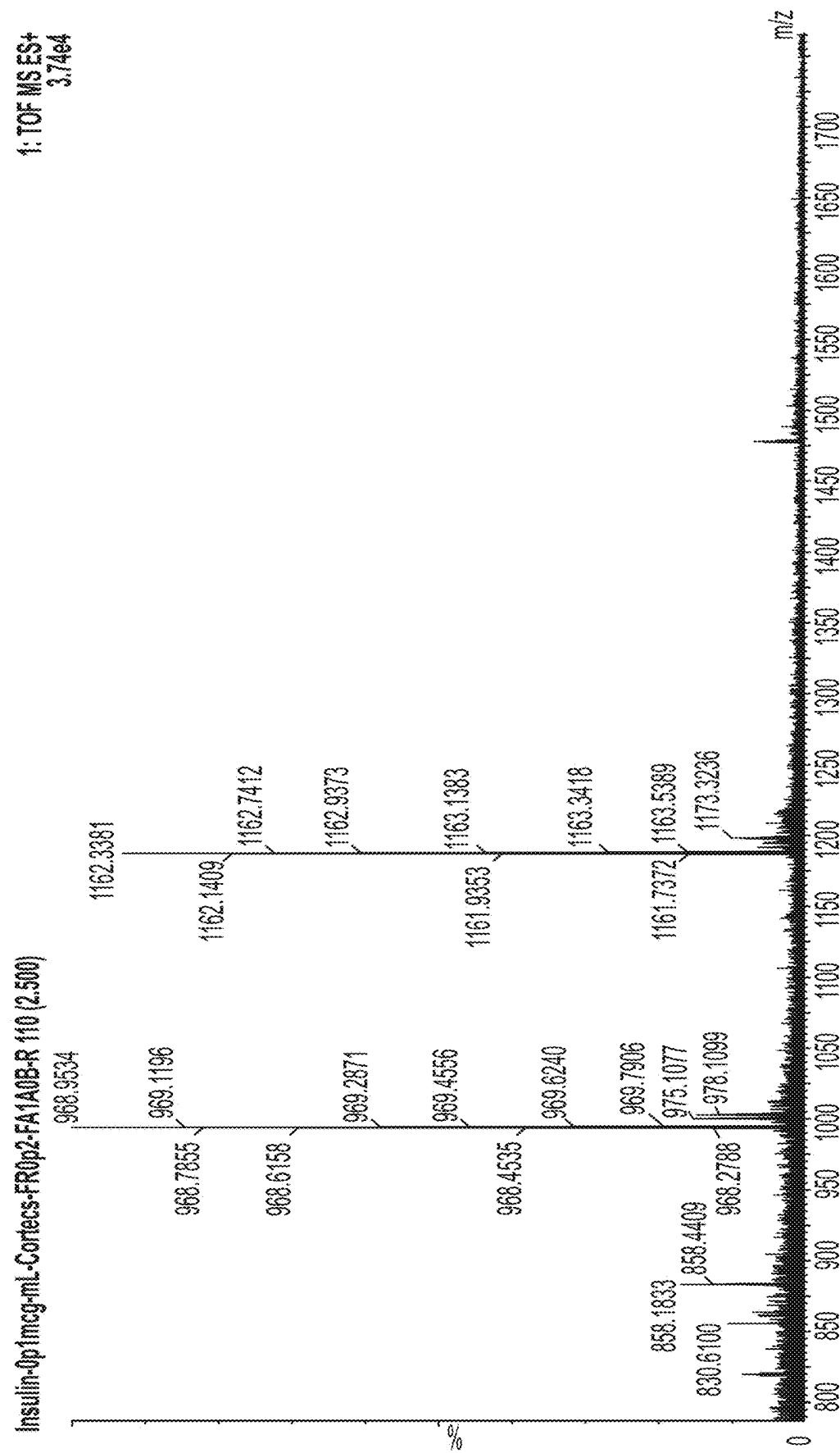


FIG. 4A

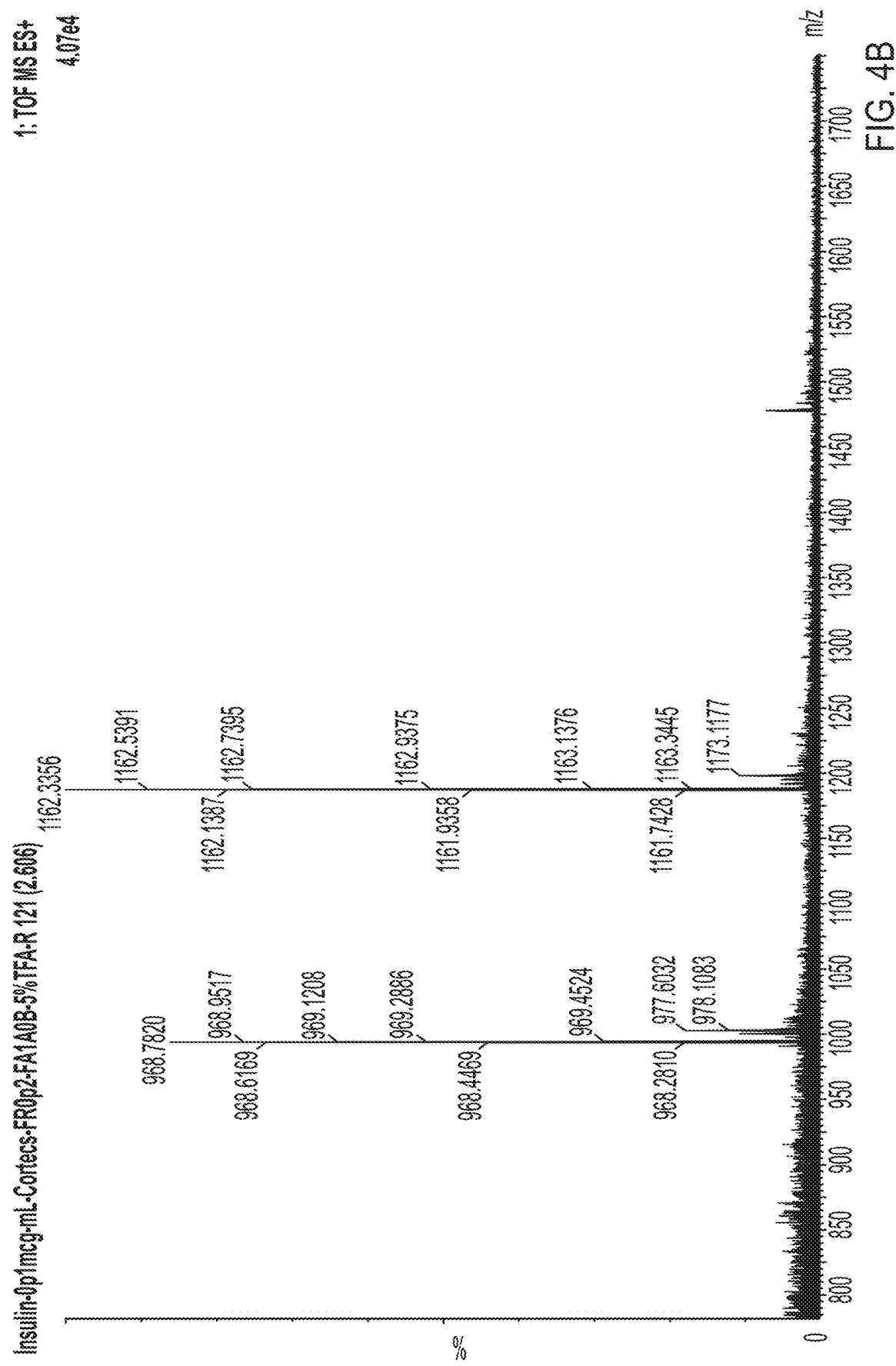
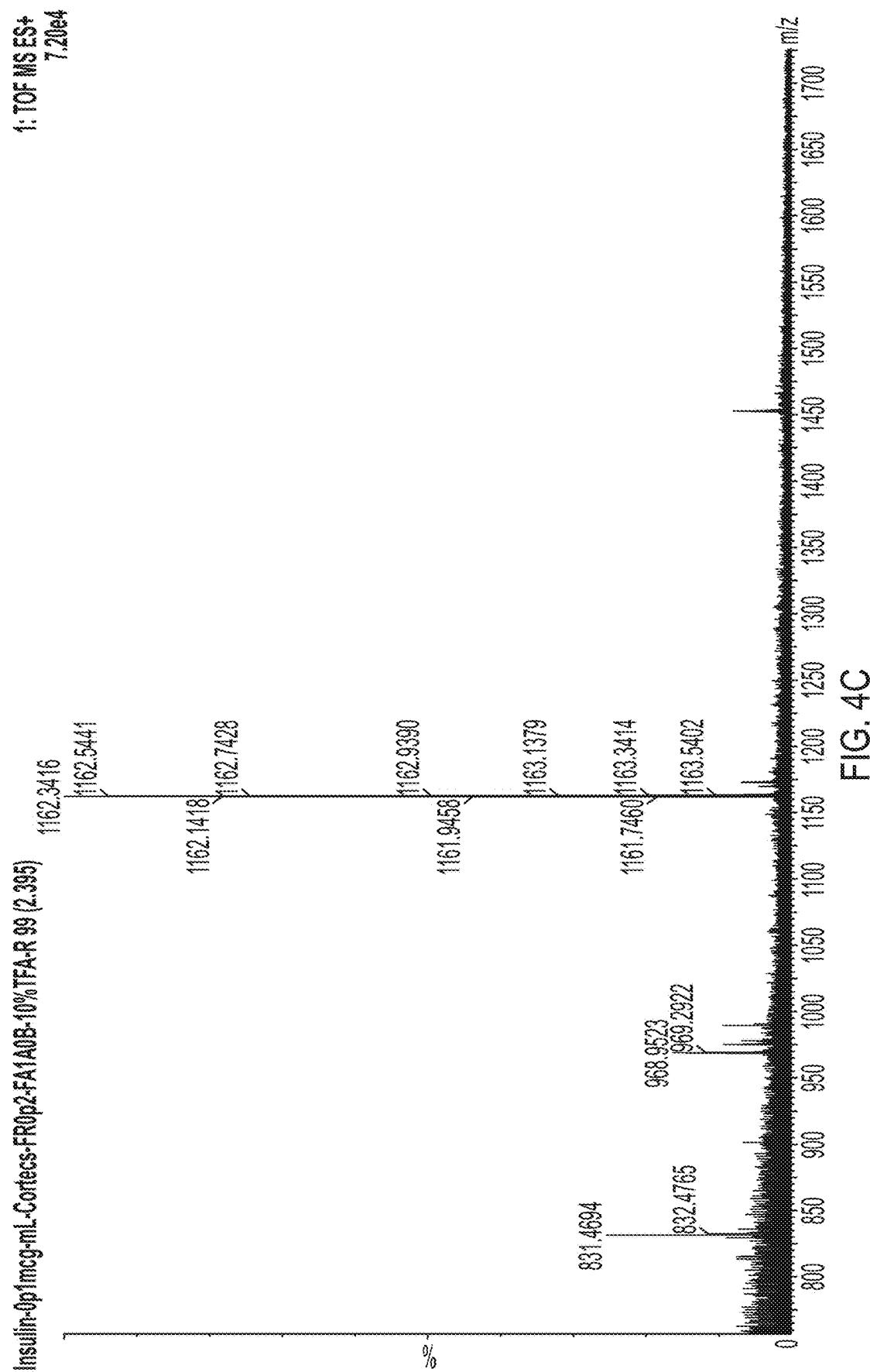


FIG. 4B



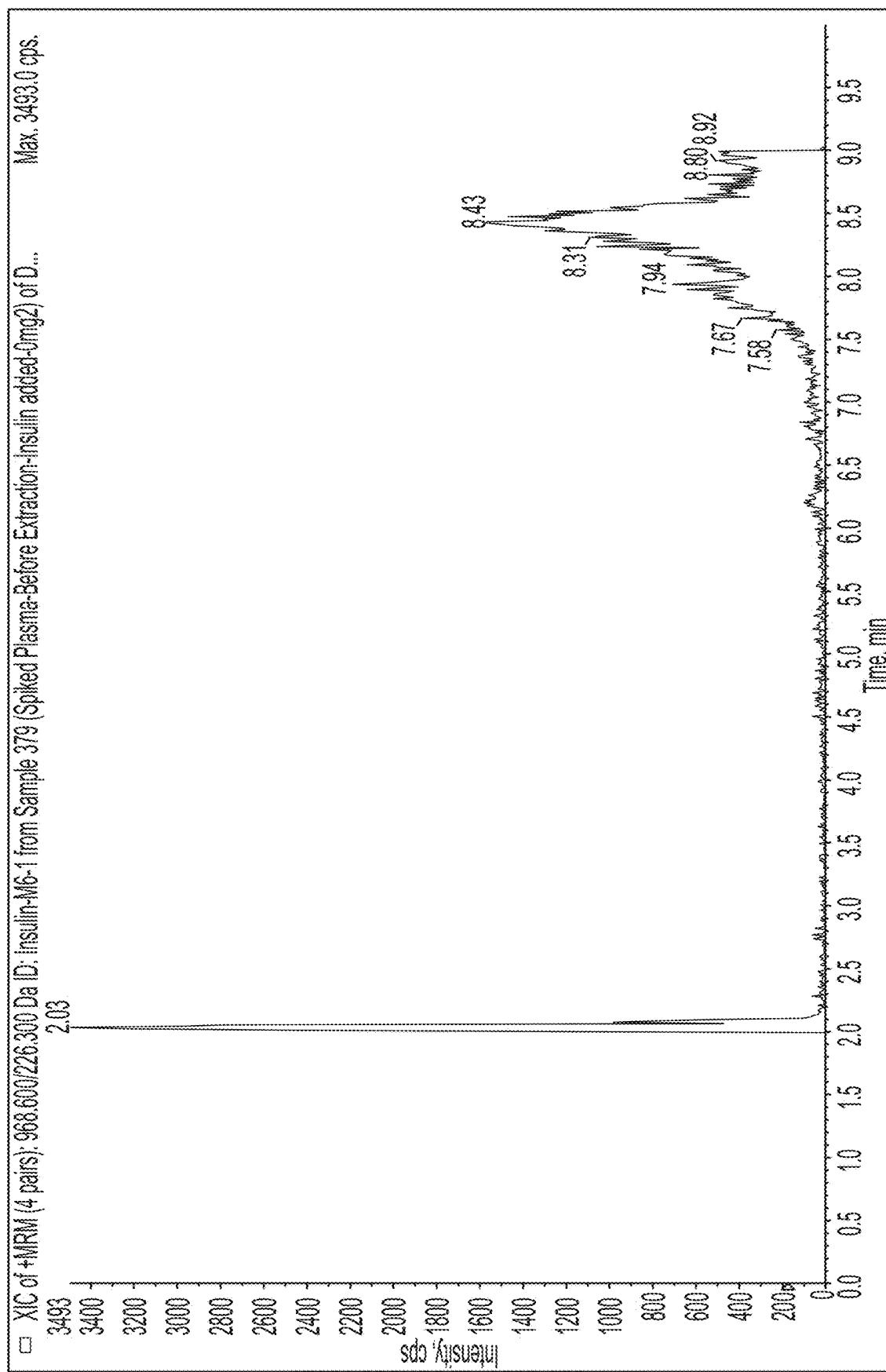


FIG. 5A

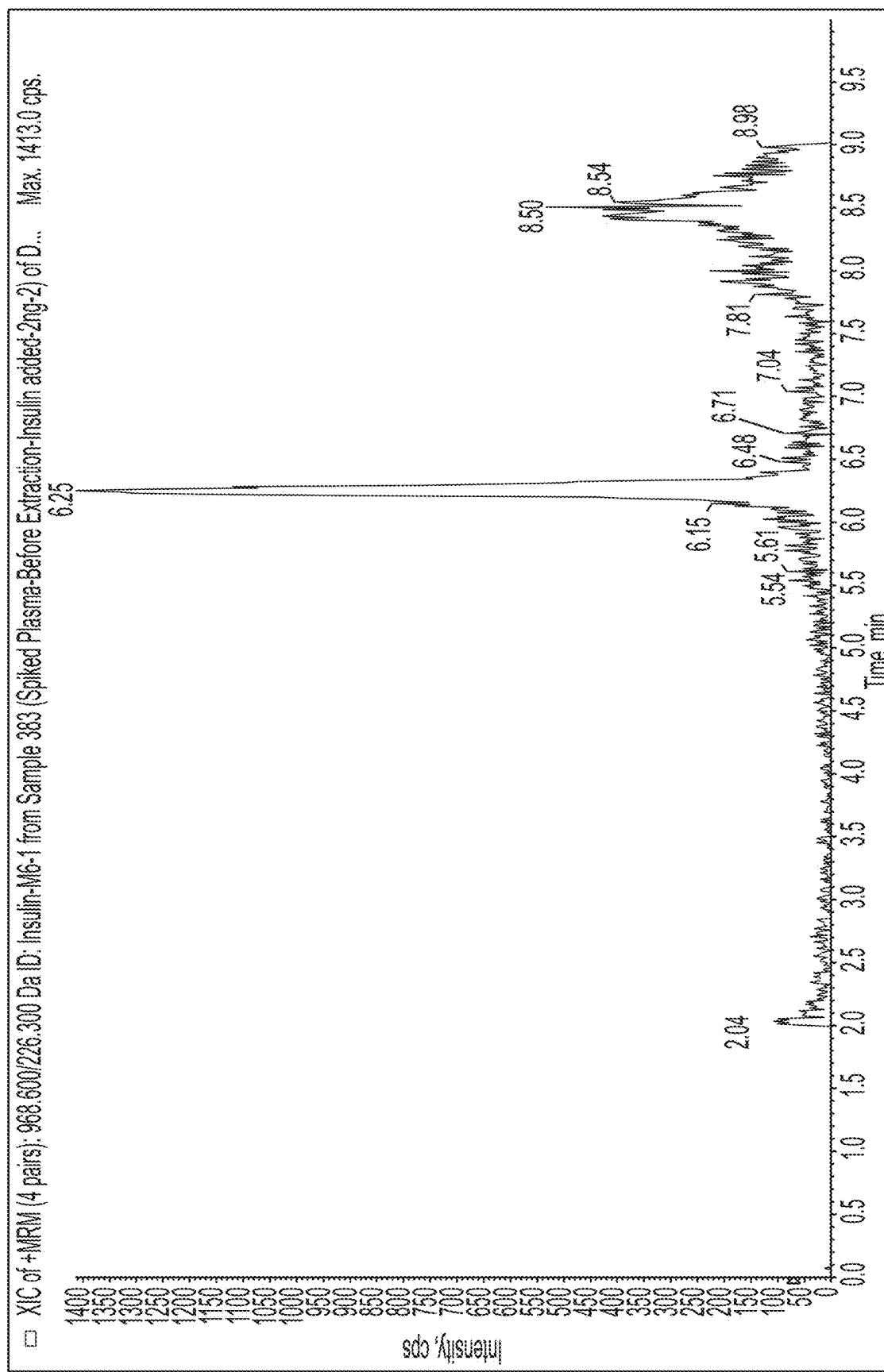


FIG. 5B

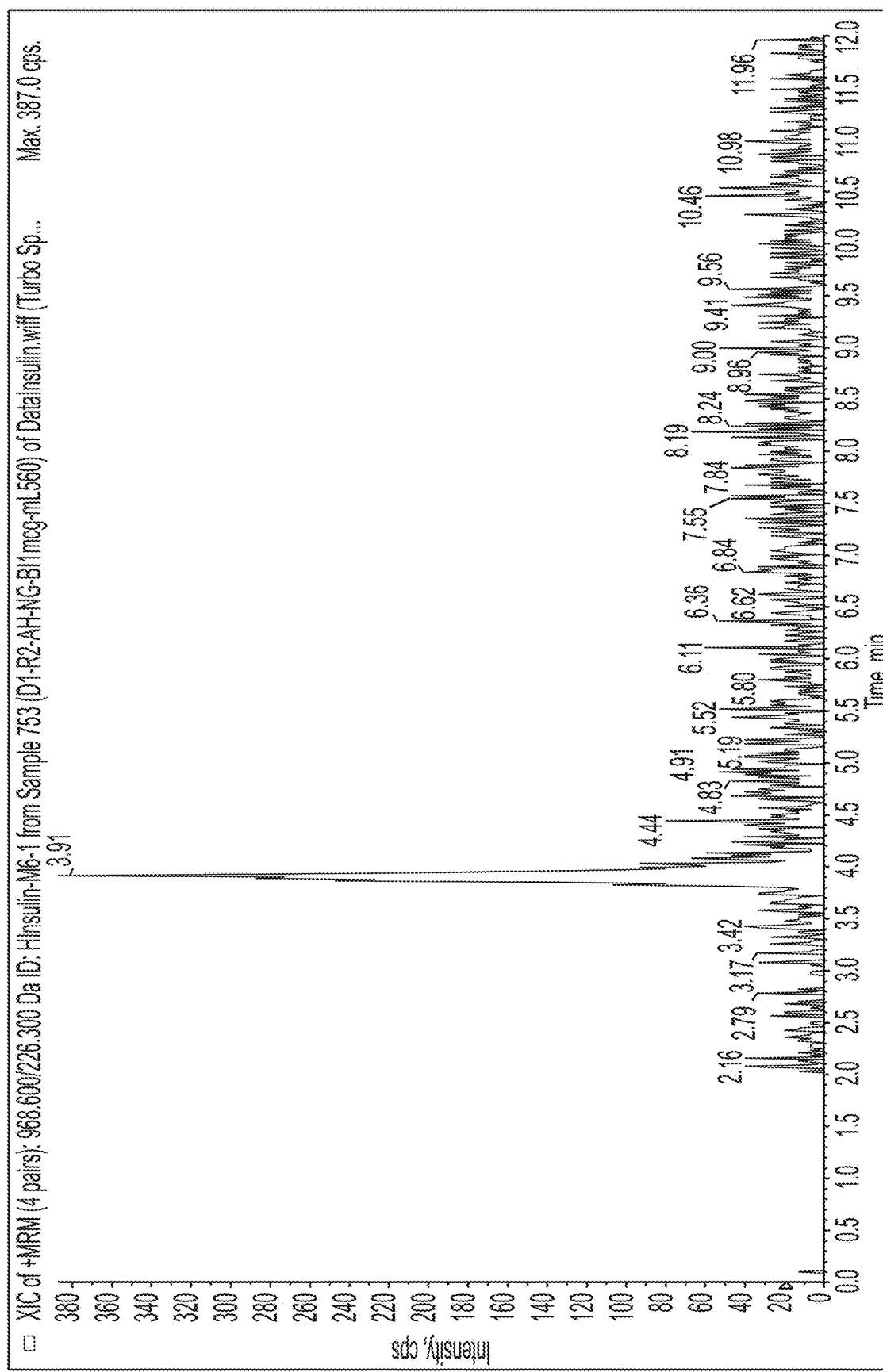


FIG. 6A

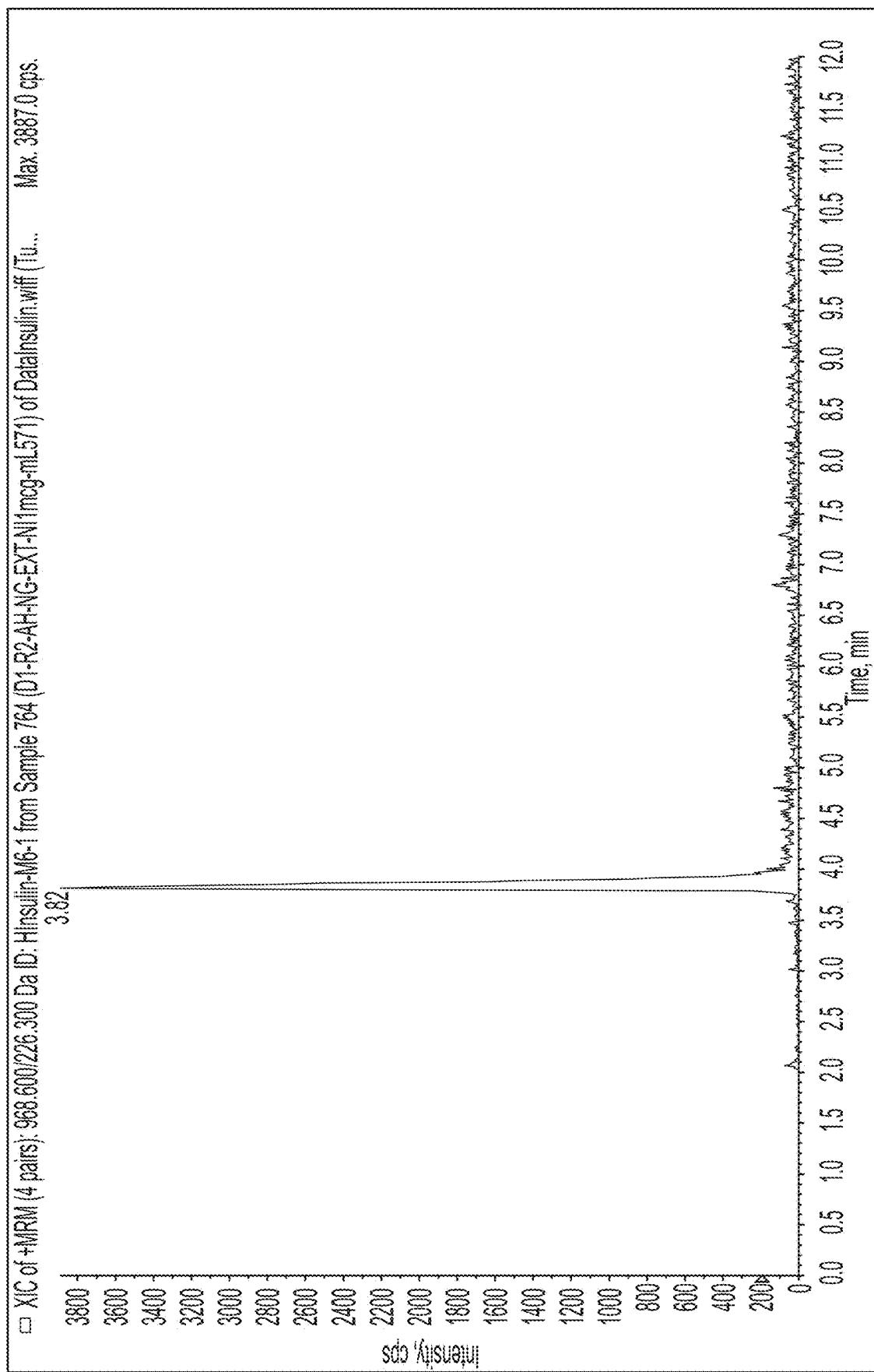


FIG. 6B

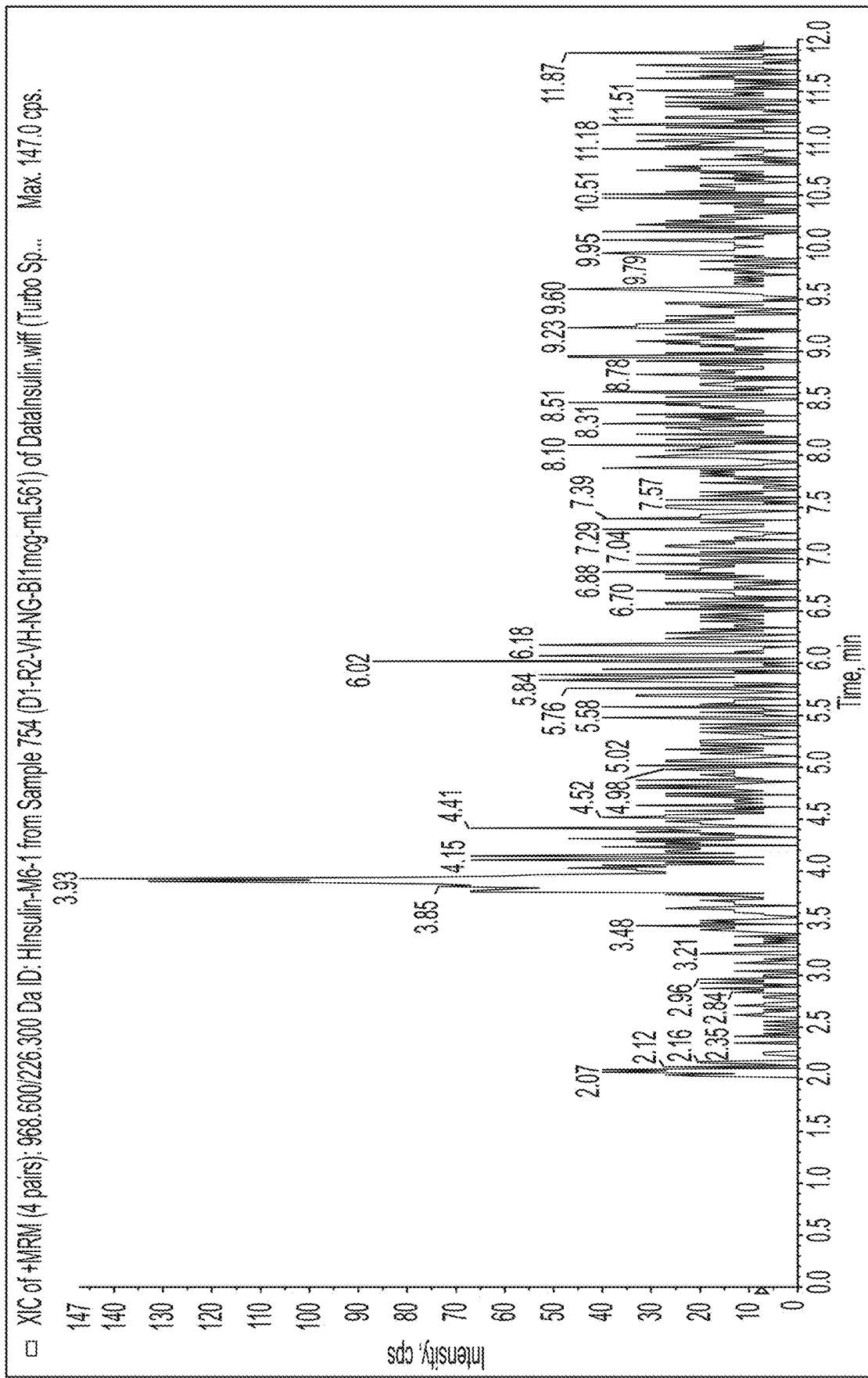


FIG. 6C

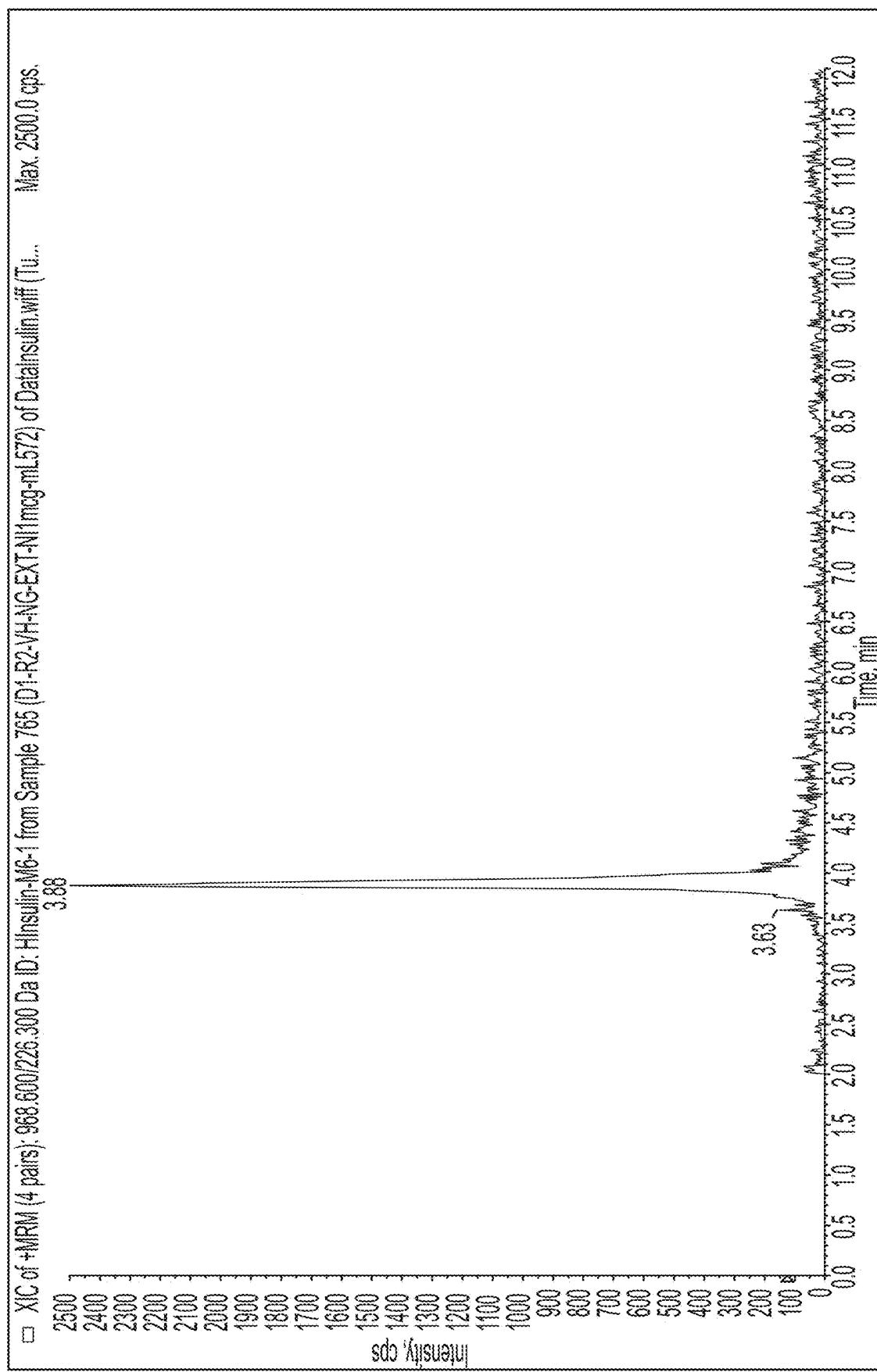


FIG. 6D

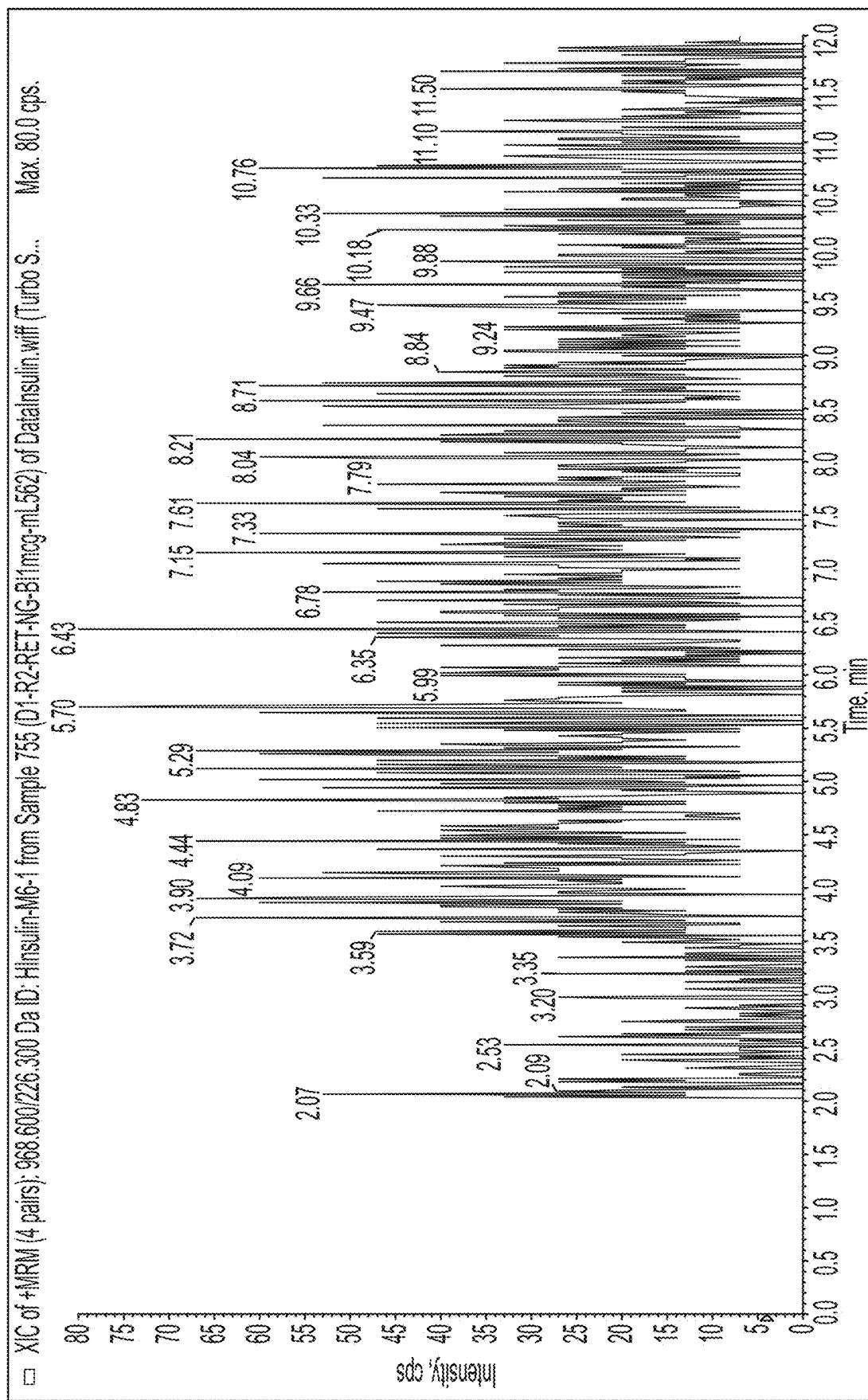


FIG. 6E

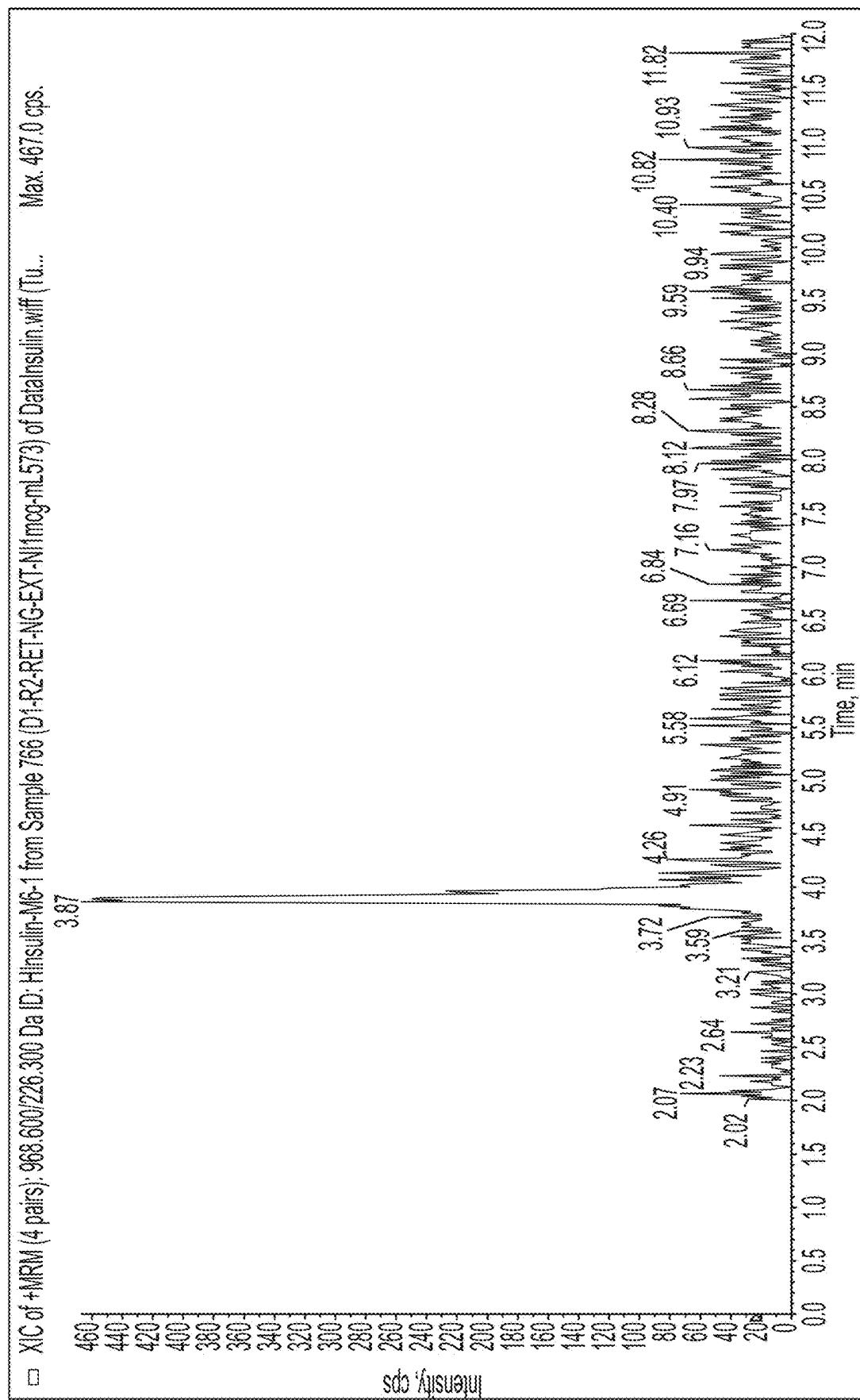


FIG. 6F

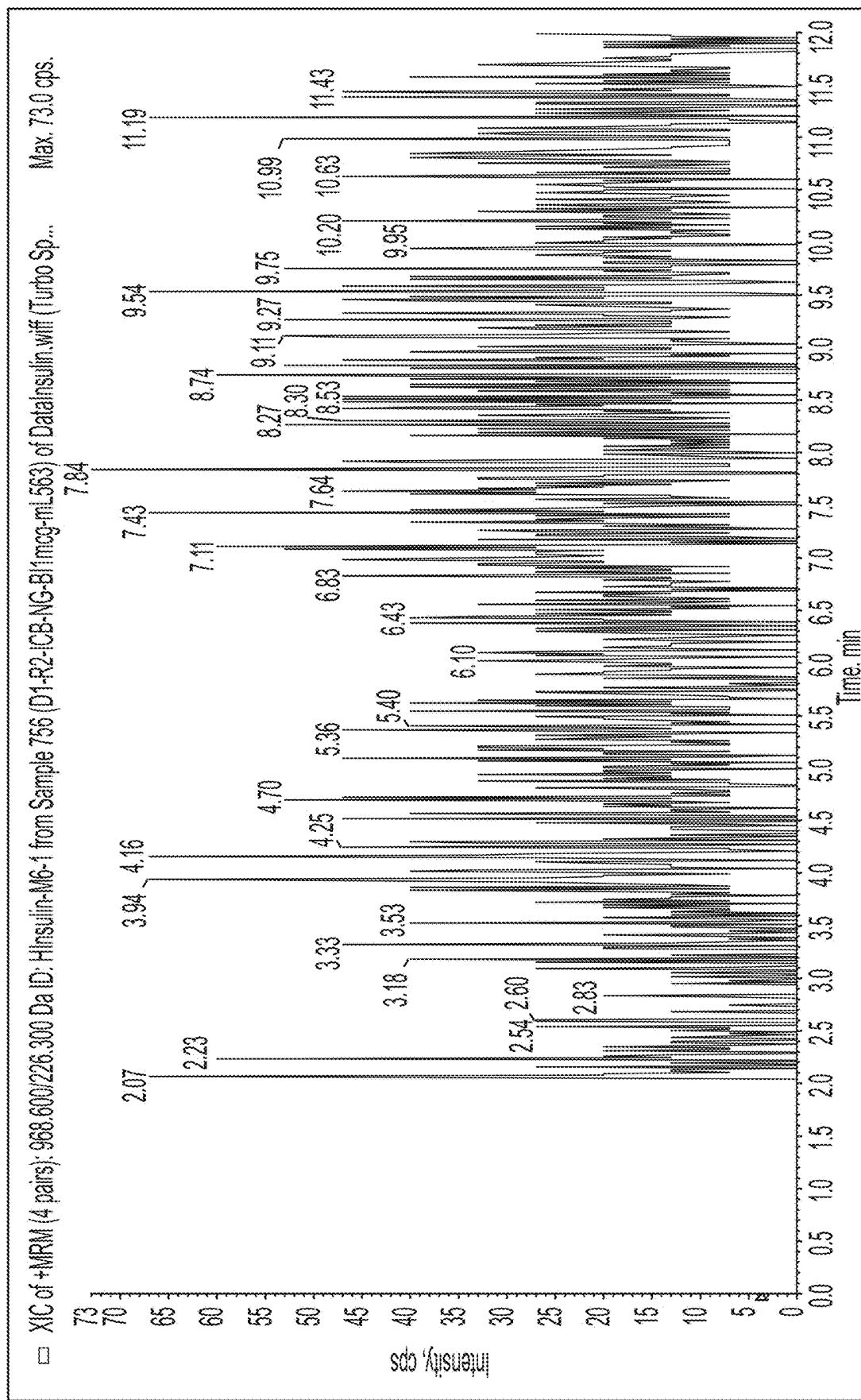


FIG. 6G

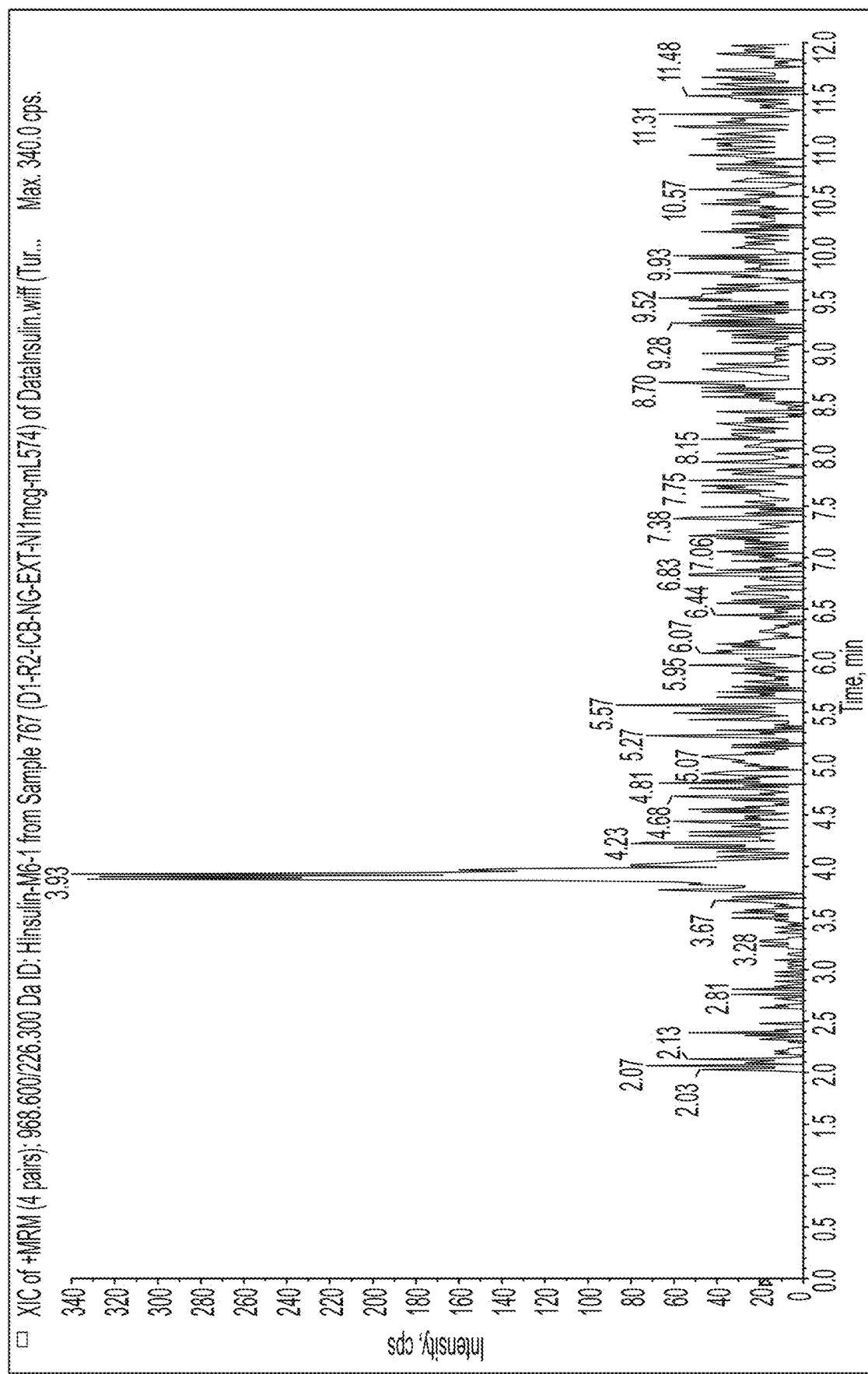


FIG. 6H

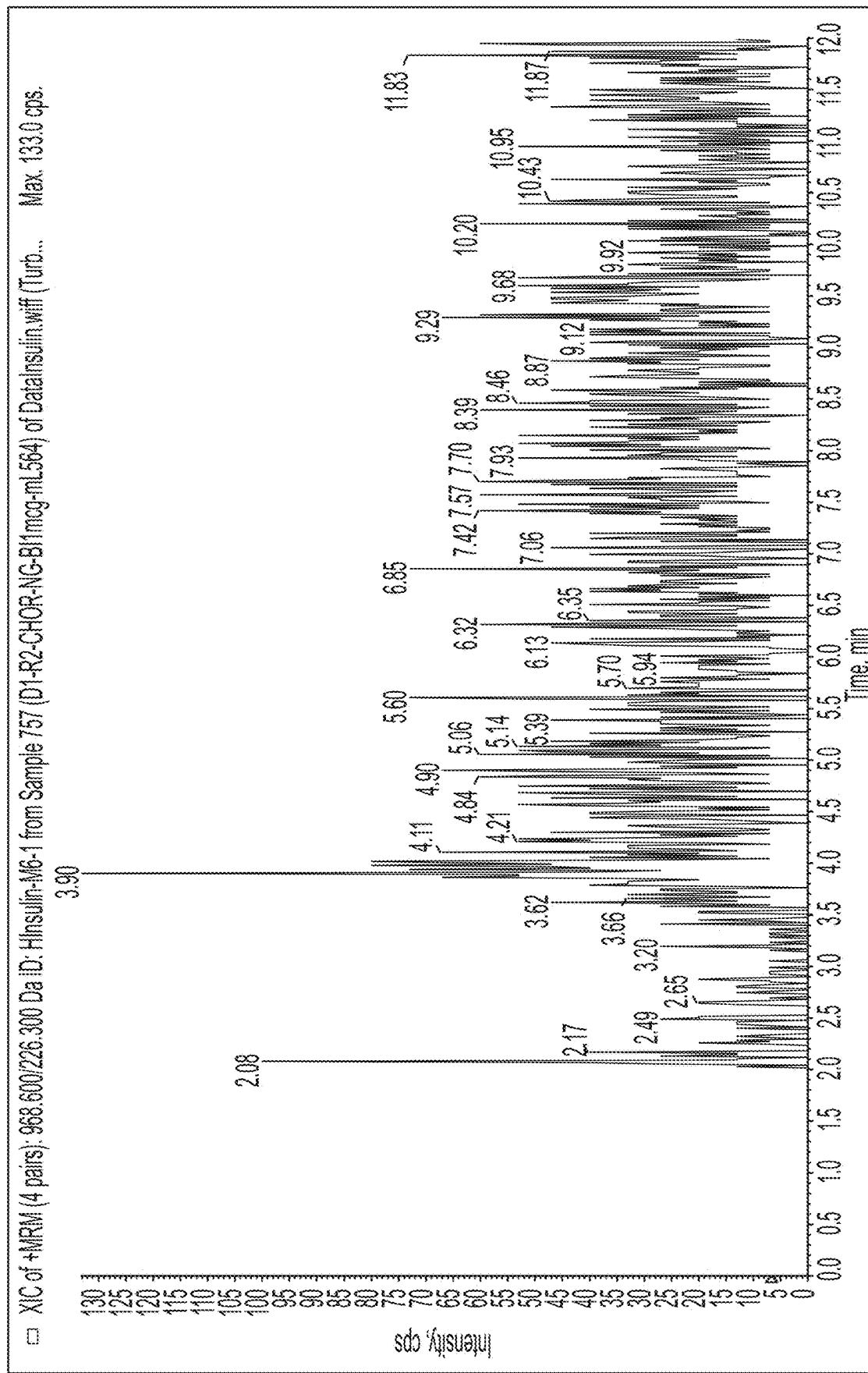


FIG. 6I

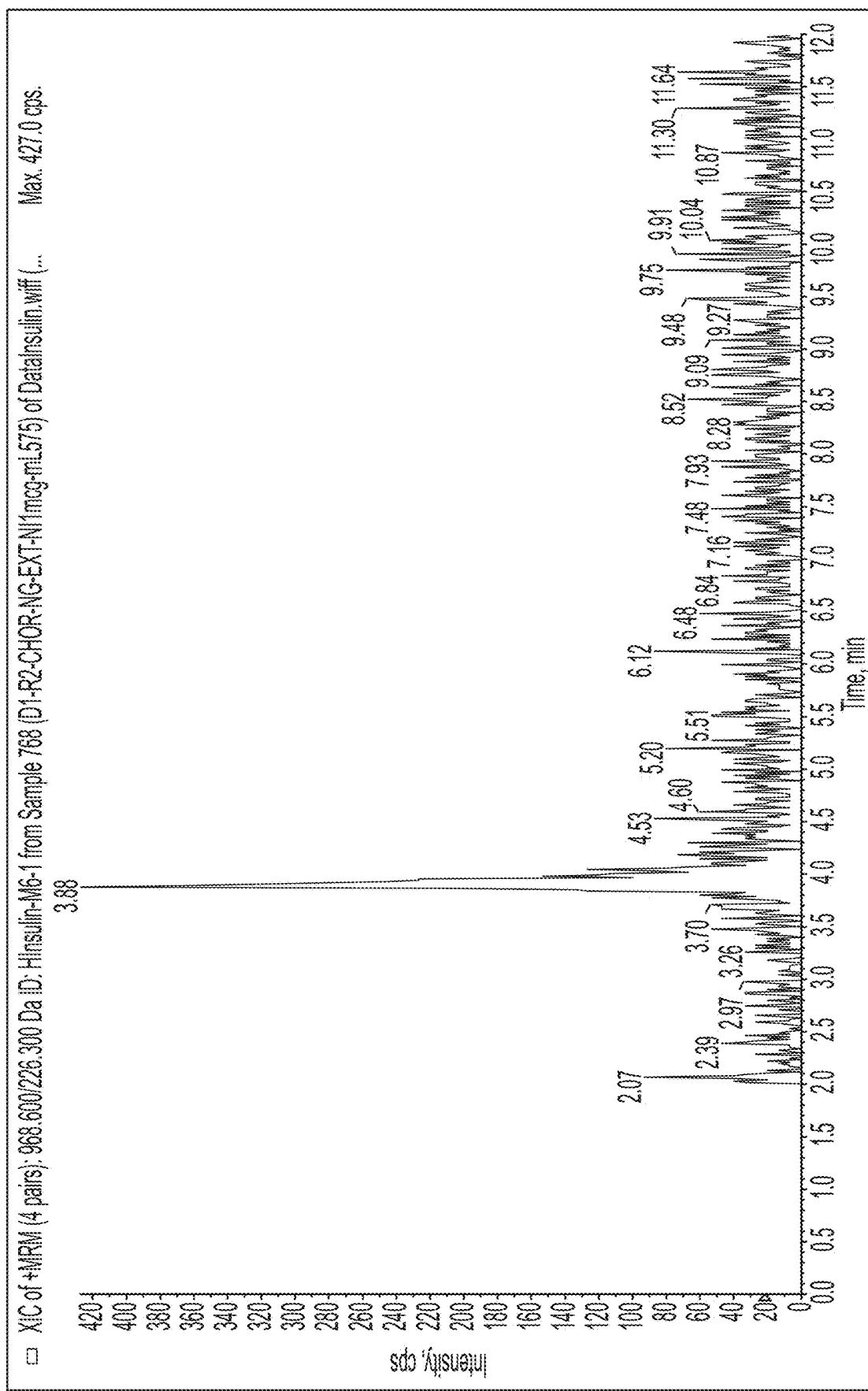


FIG. 6J

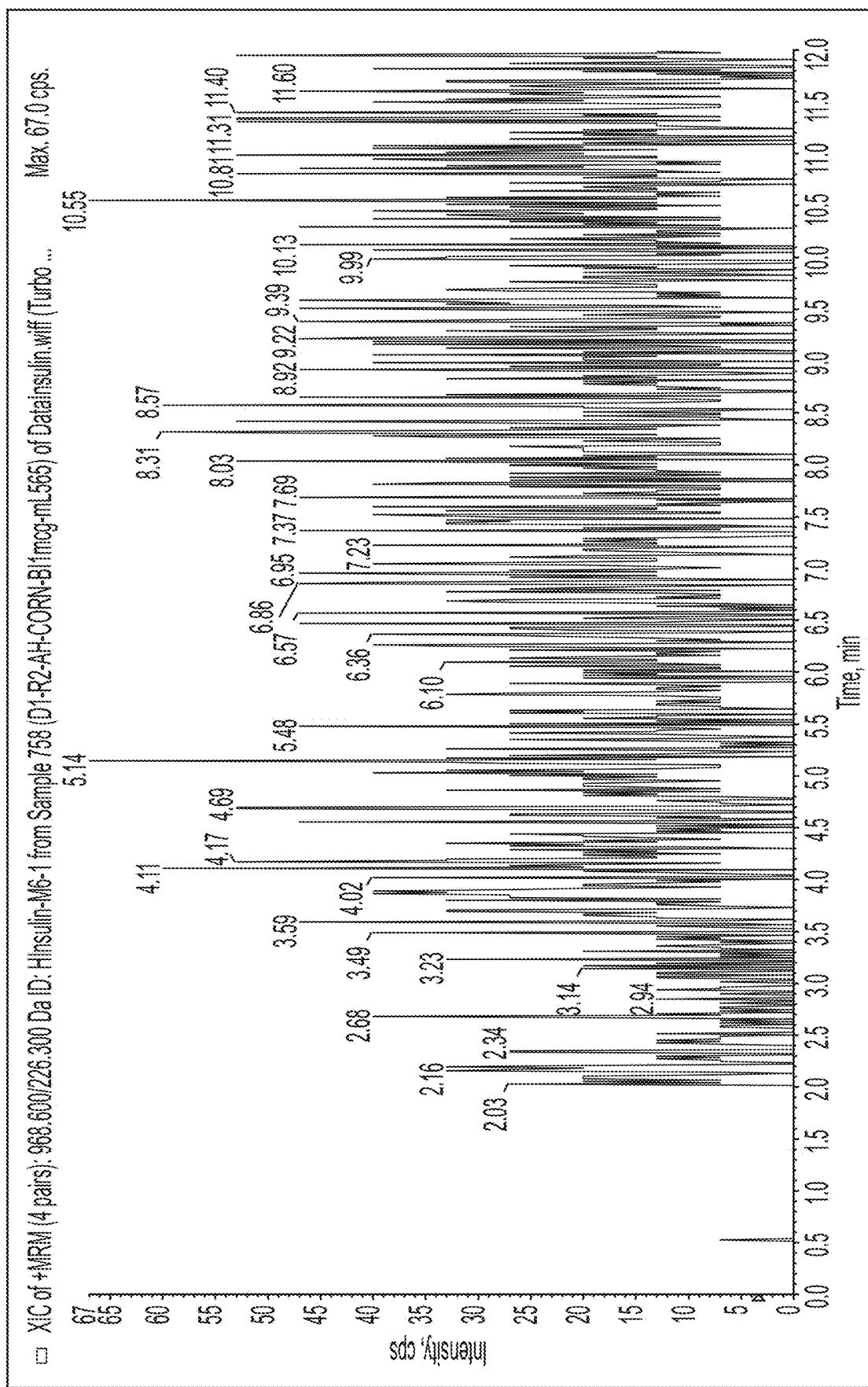


FIG. 6K

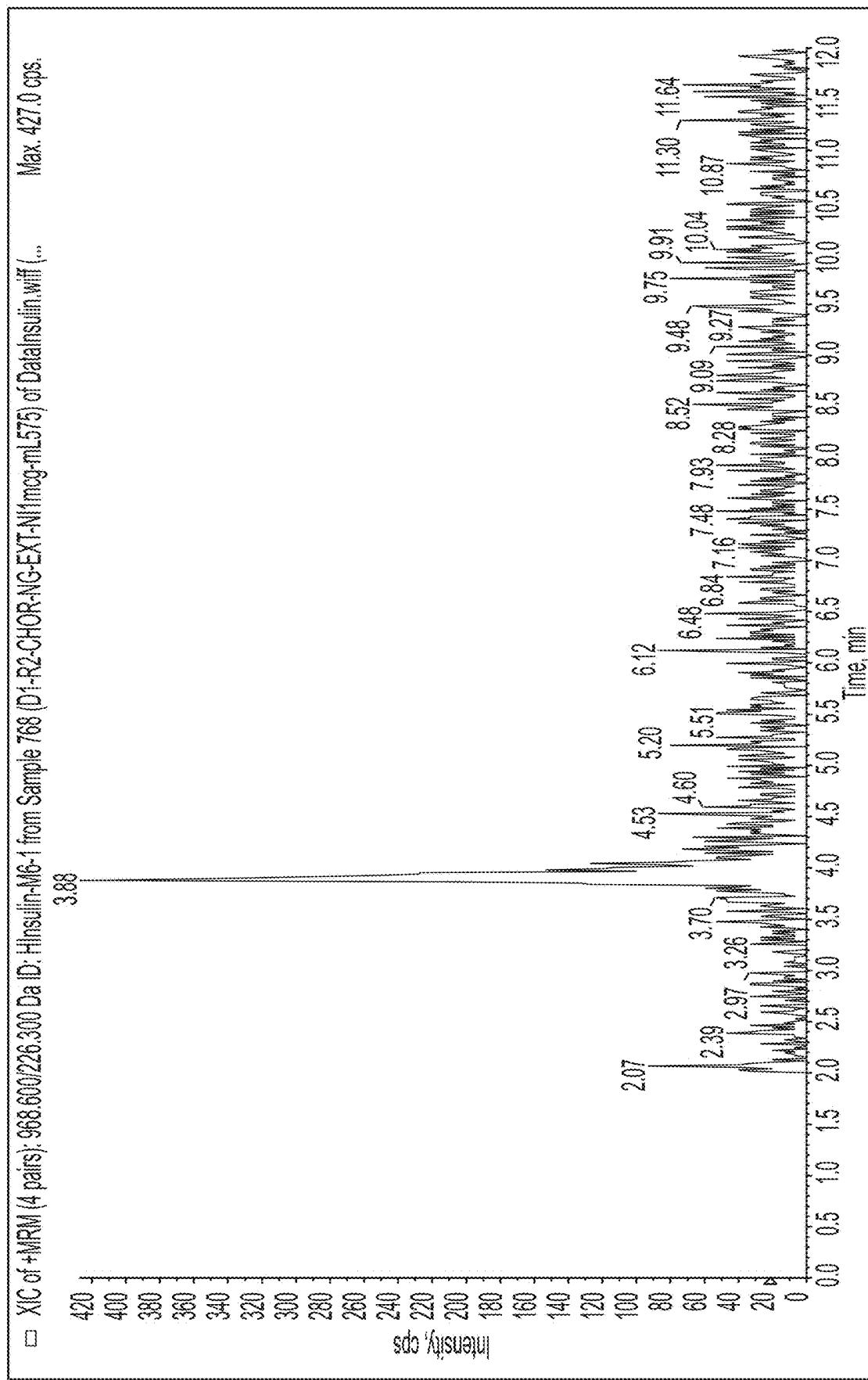


FIG. 6L

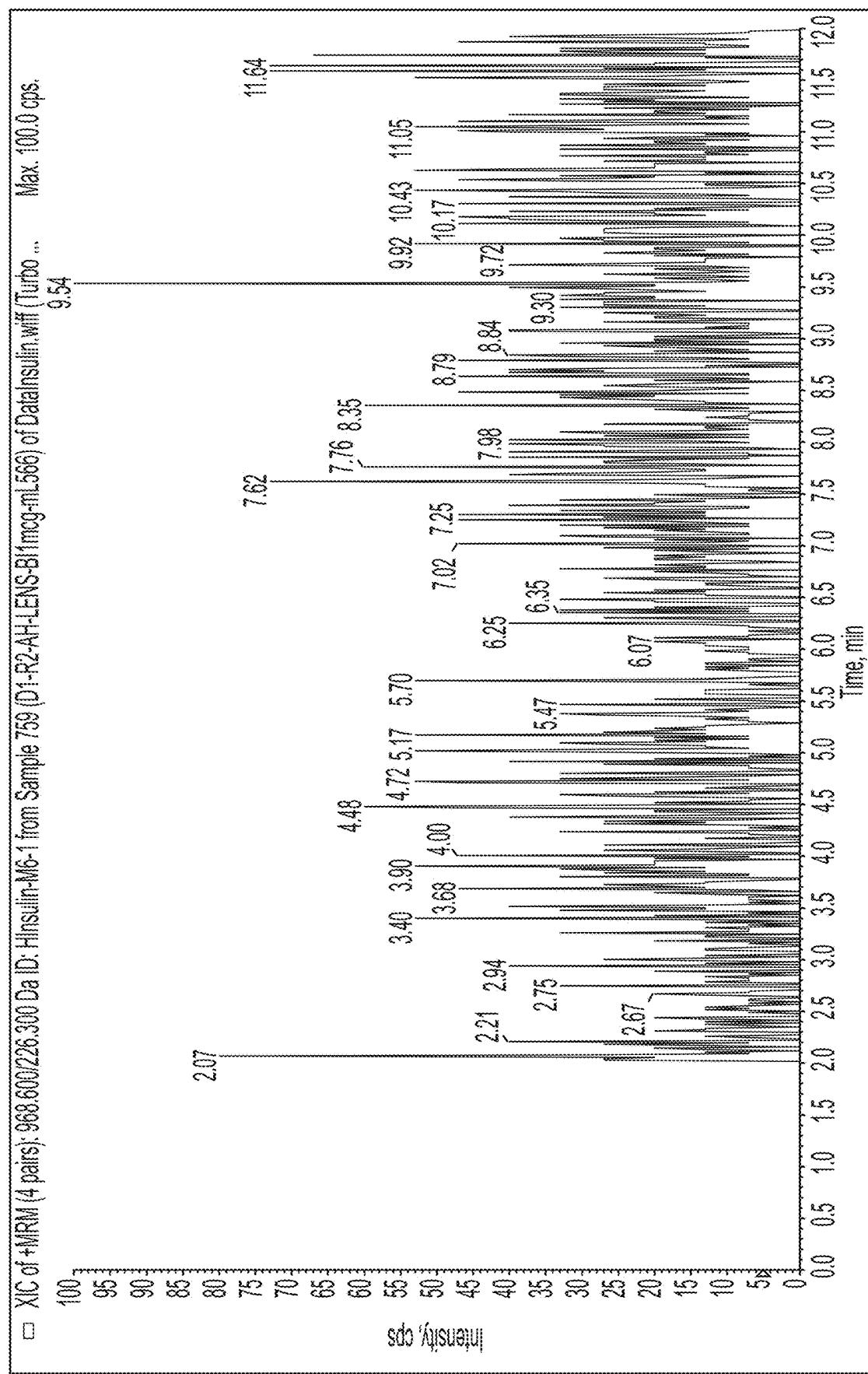


FIG. 6M

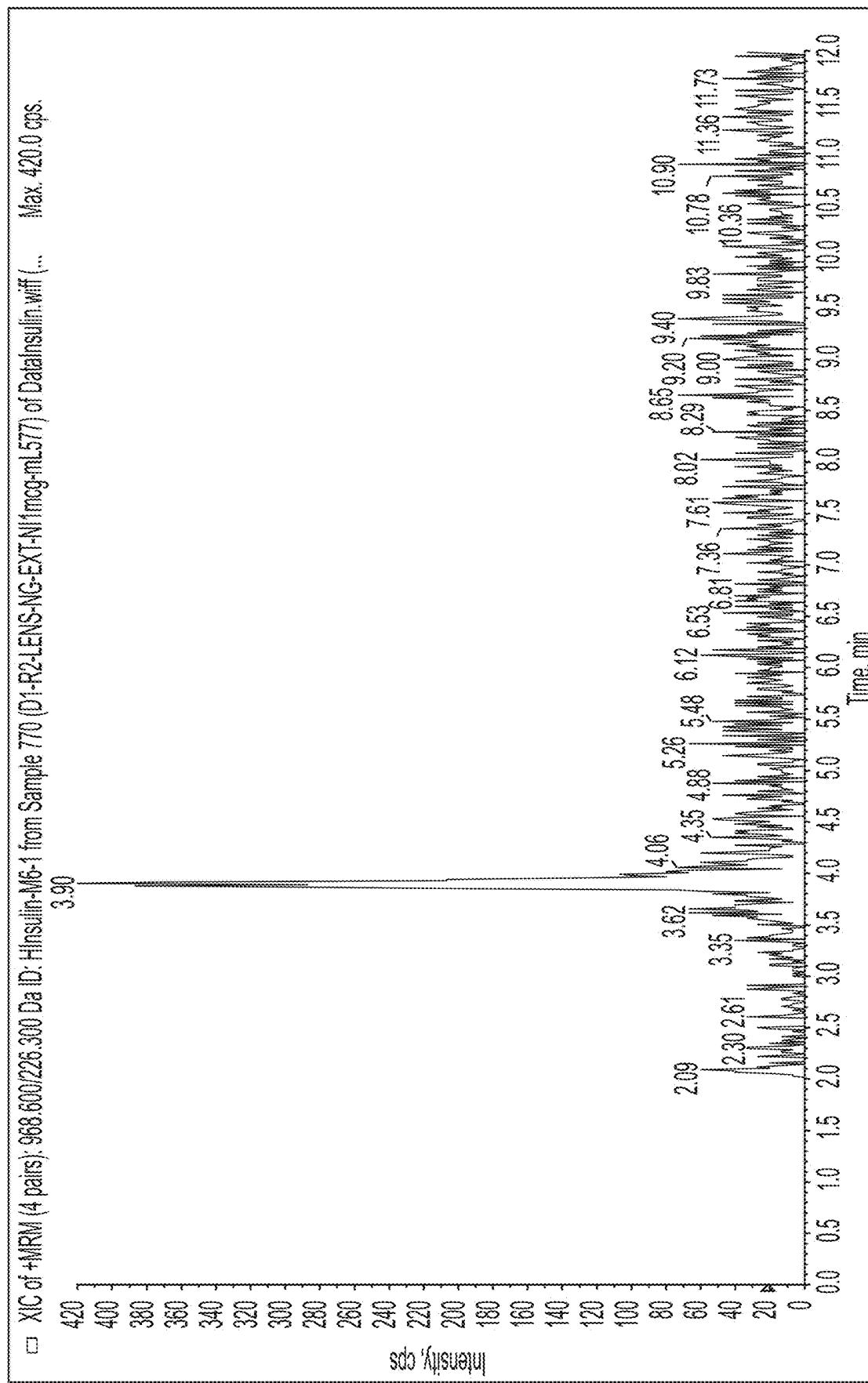


FIG. 6N

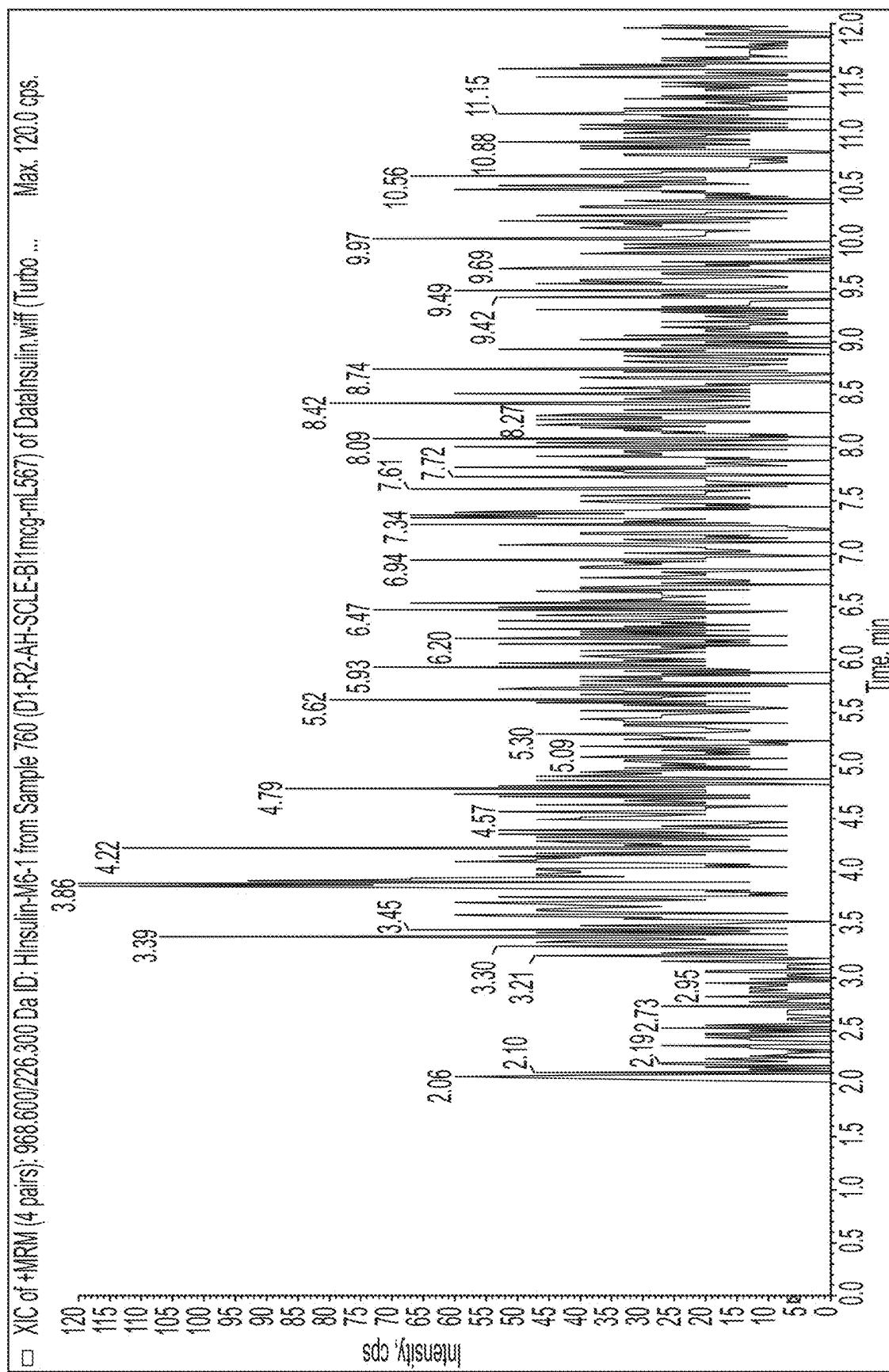


FIG. 60

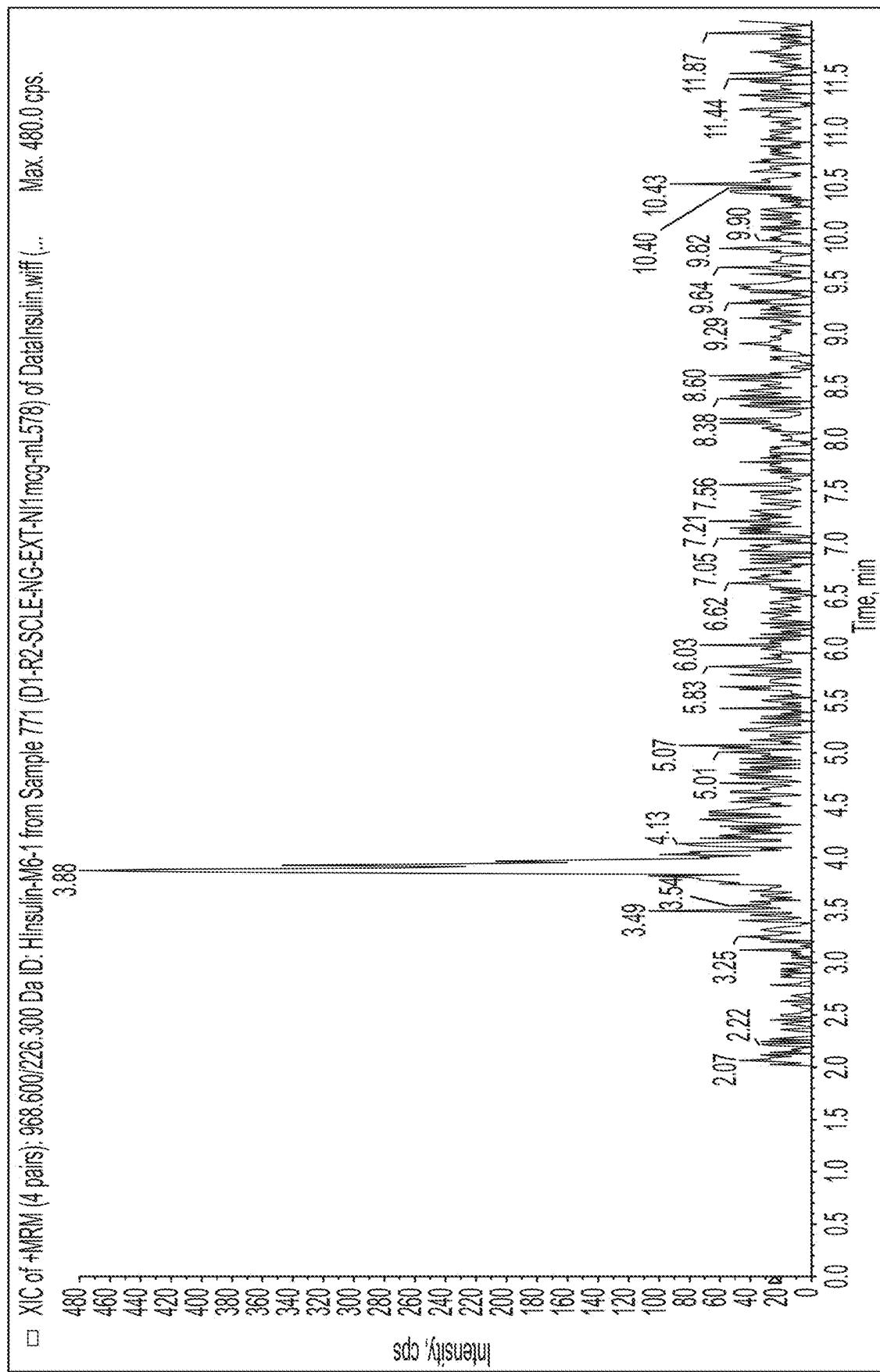


FIG. 6P

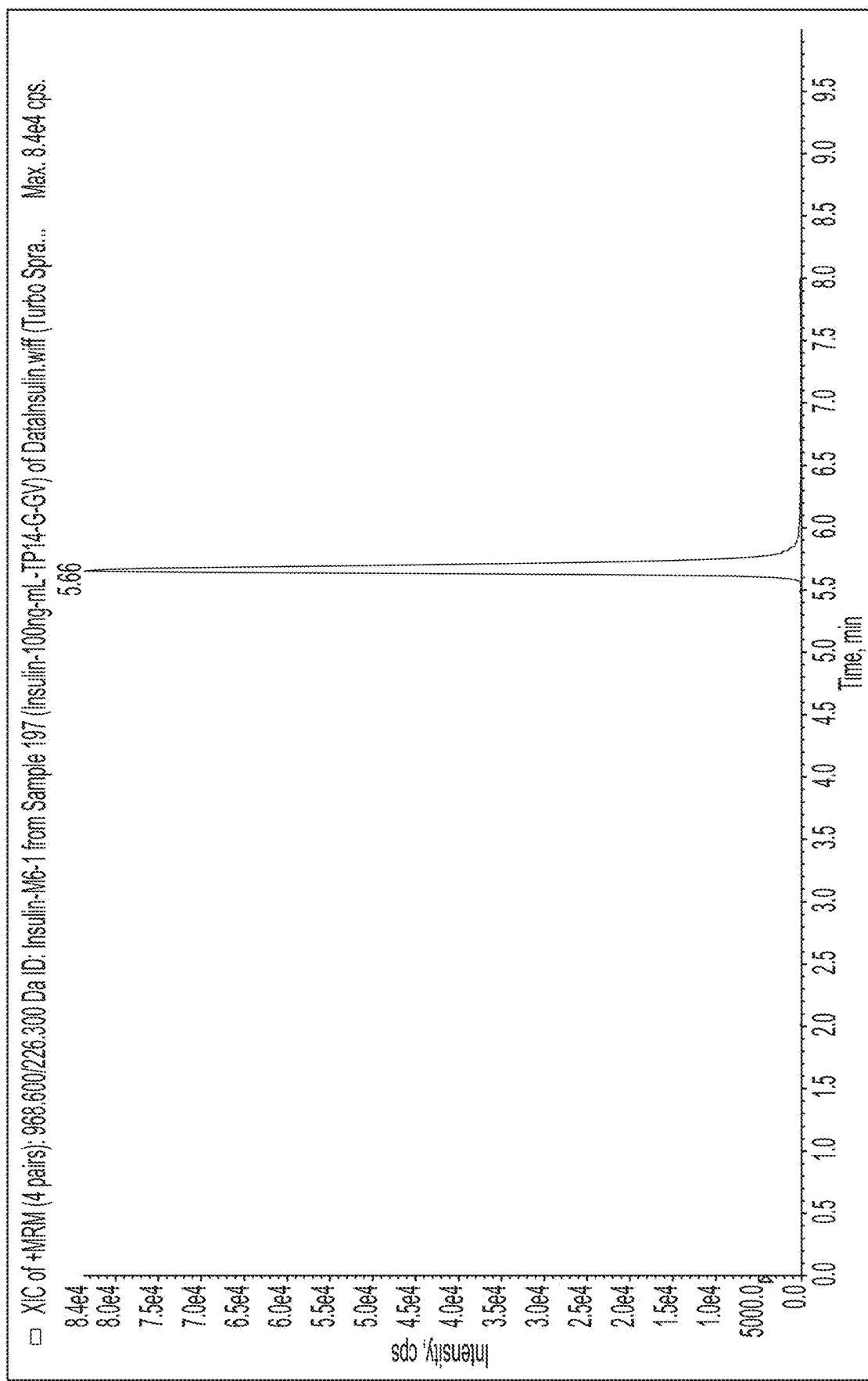


FIG. 7A

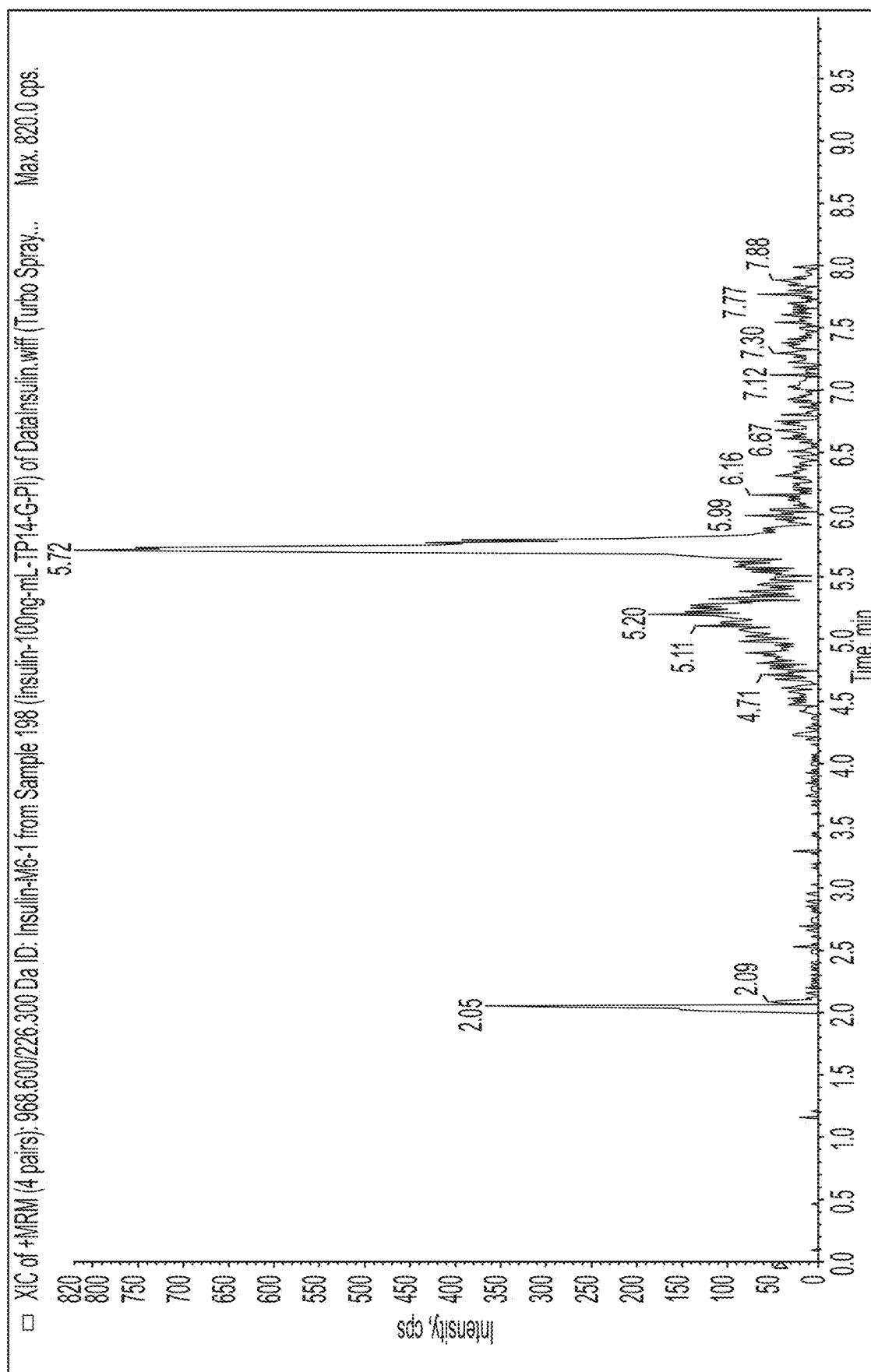


FIG. 7B

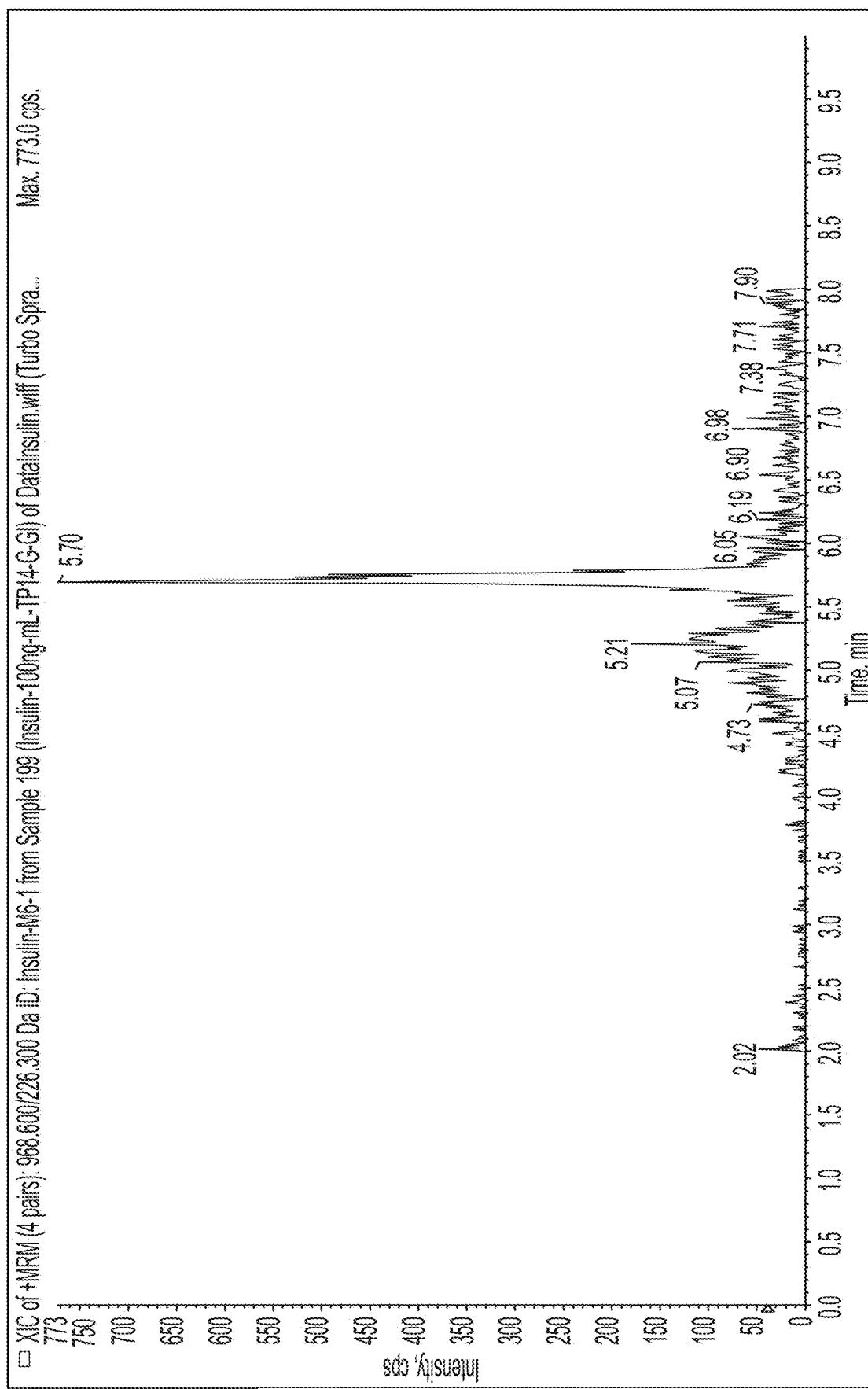


FIG. 7C

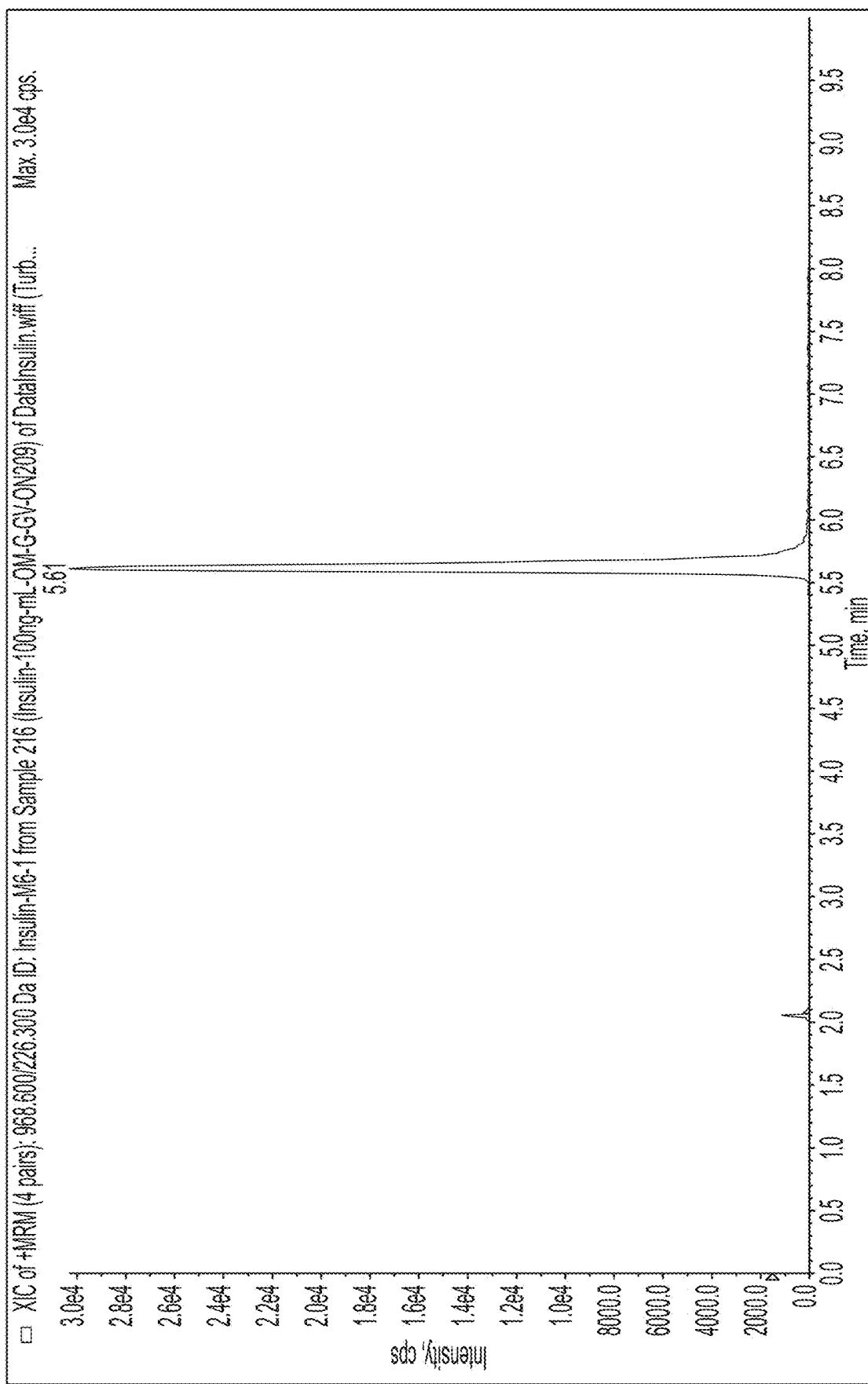


FIG. 7D

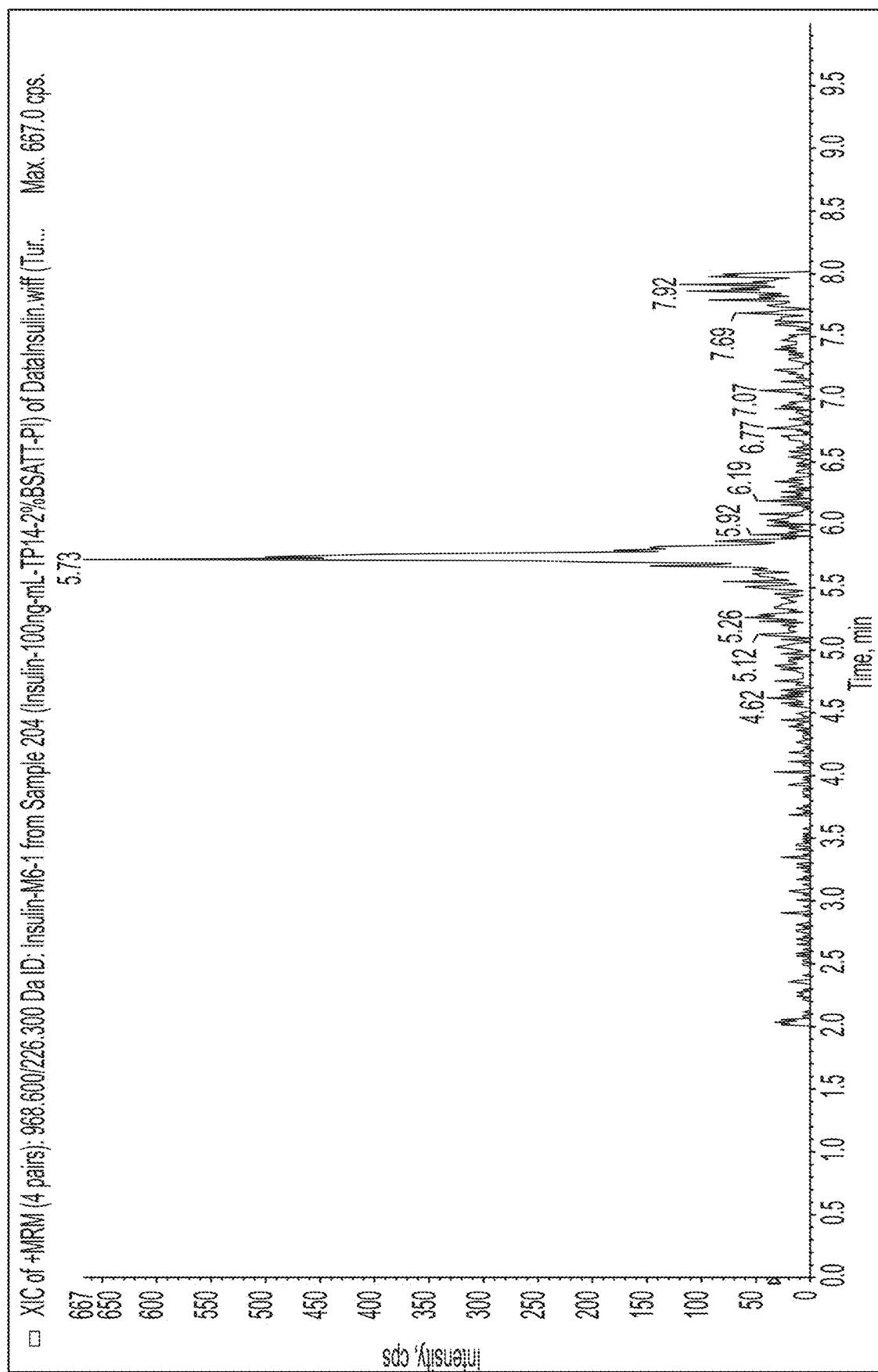


FIG. 7E

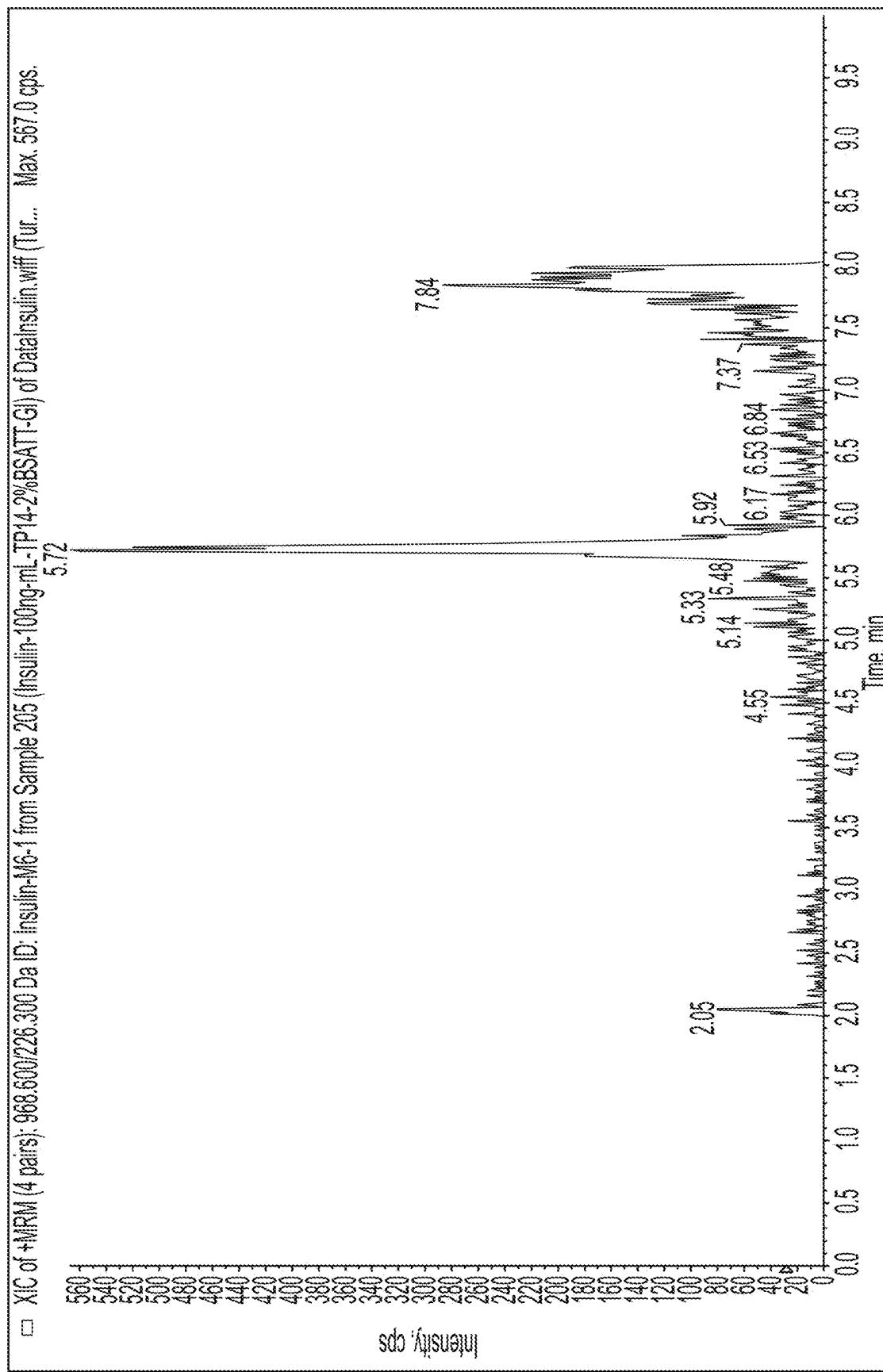


FIG. 7F

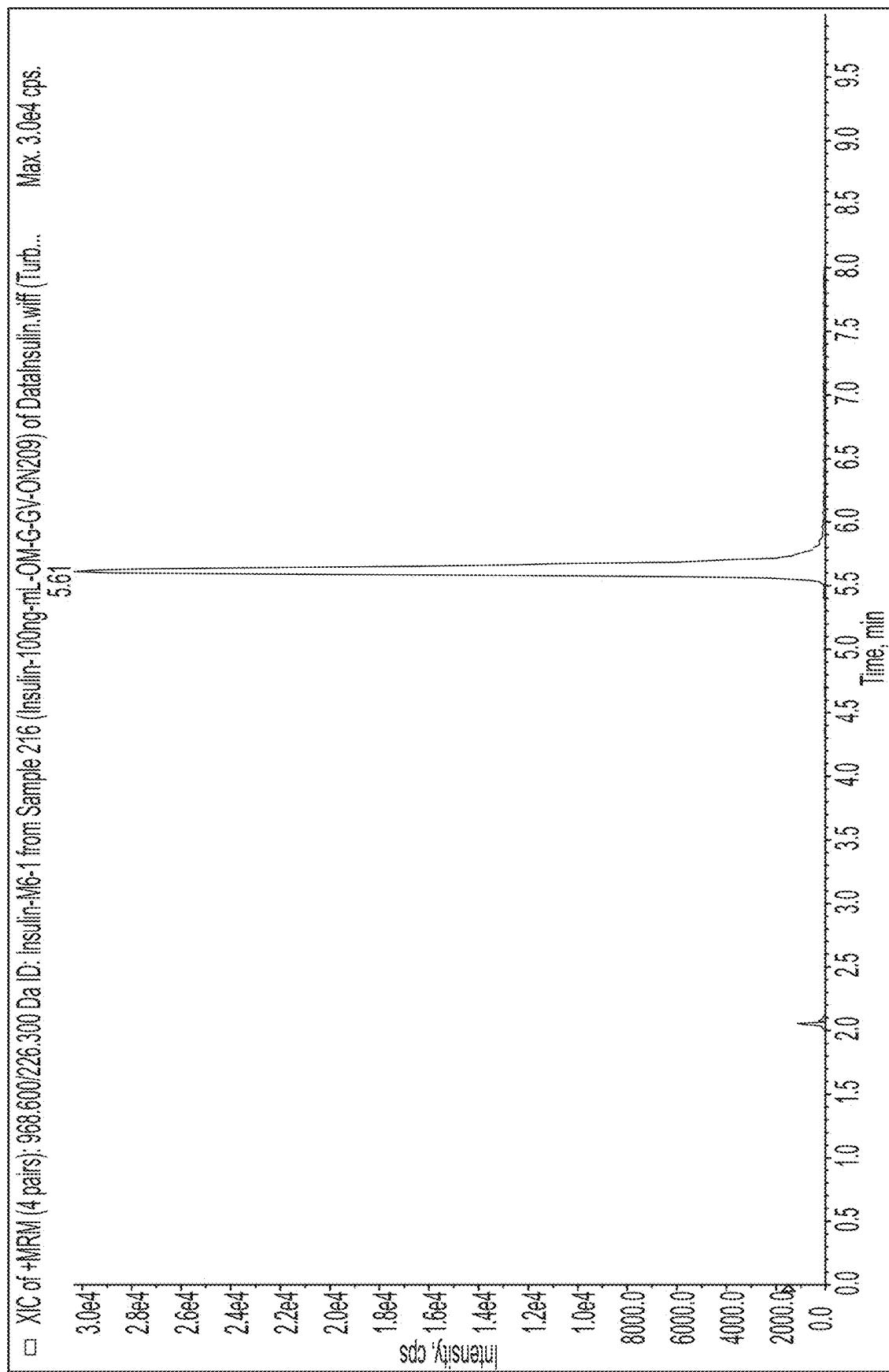


FIG. 7G

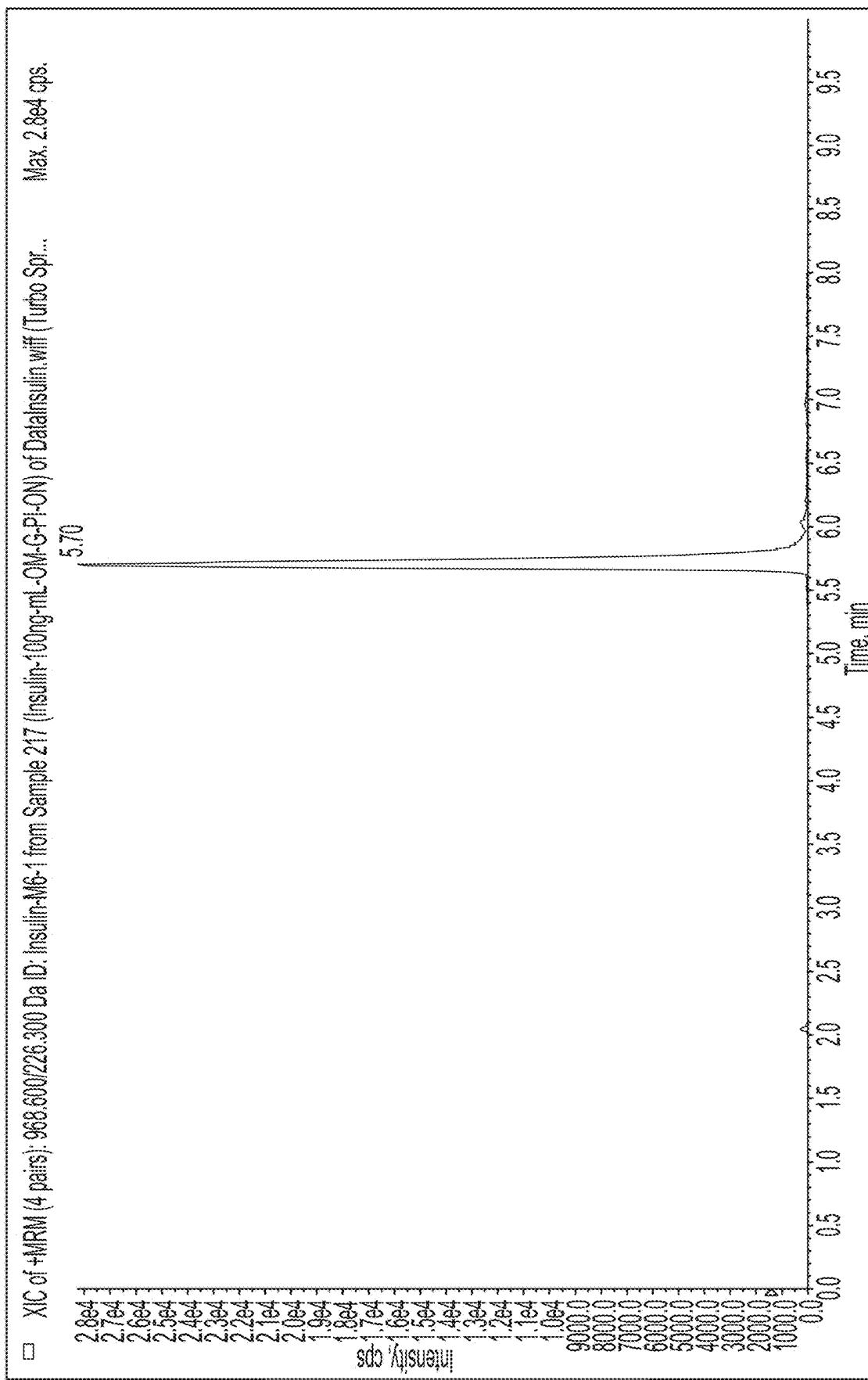
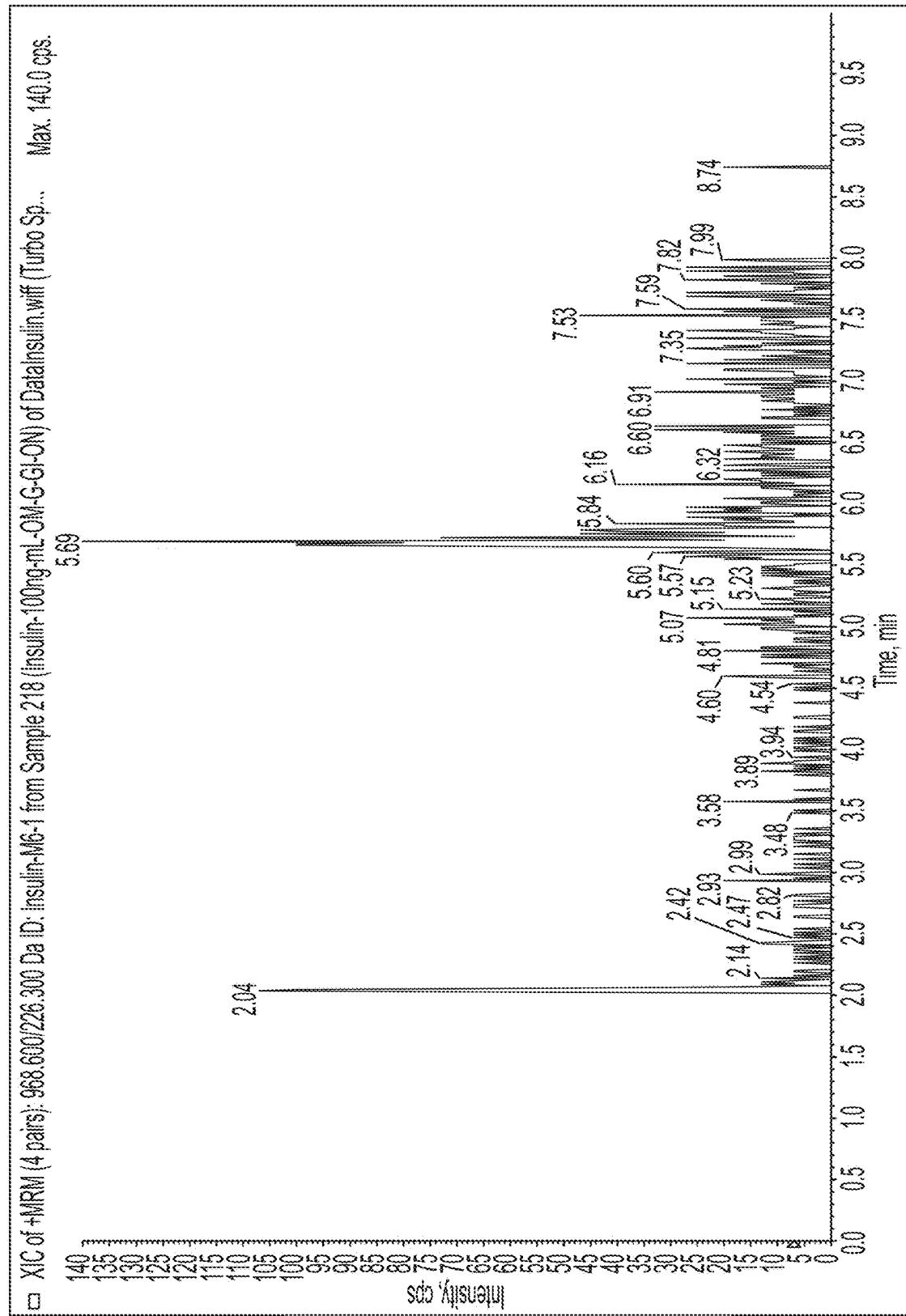


FIG. 7H



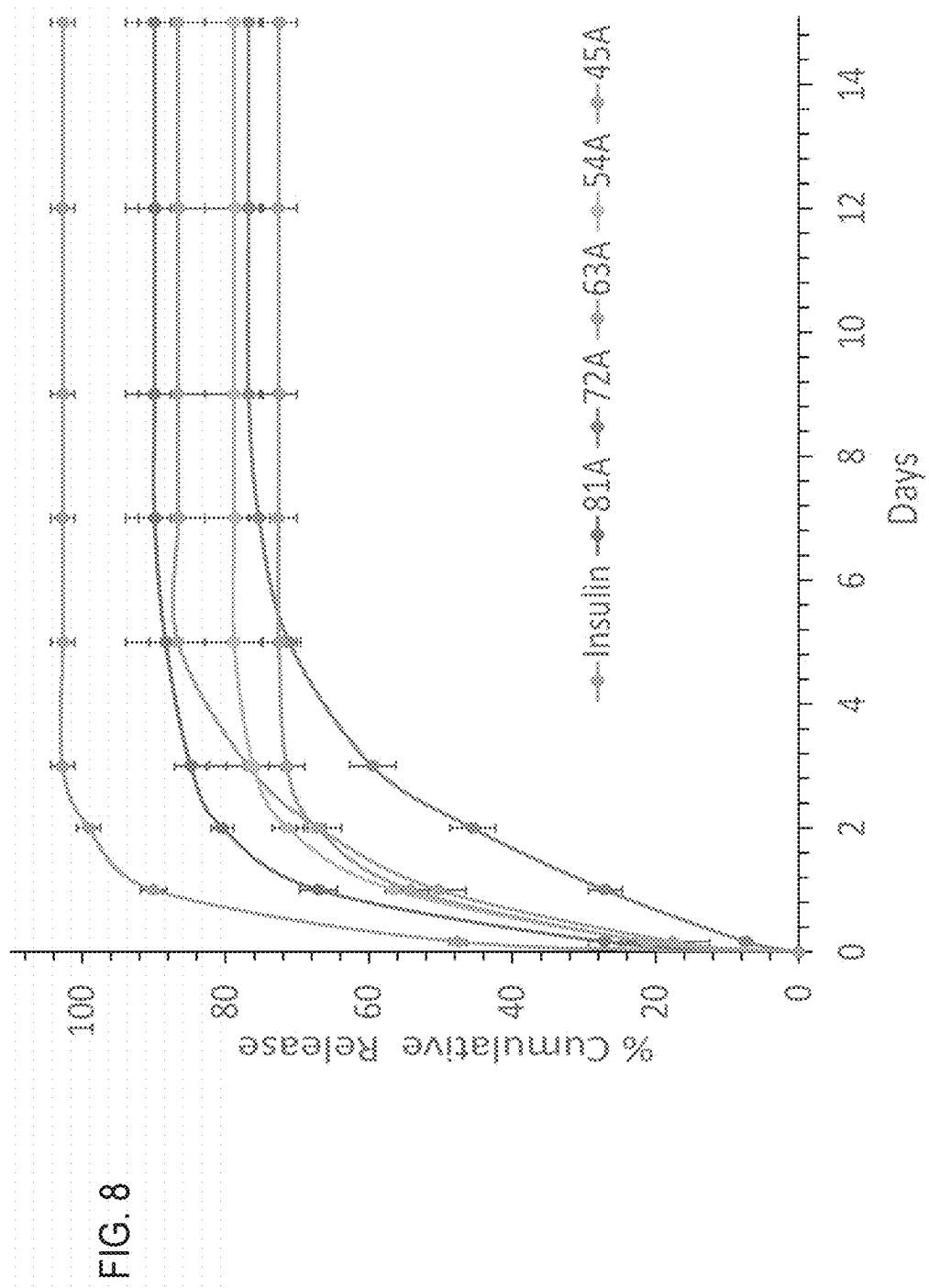


FIG. 9

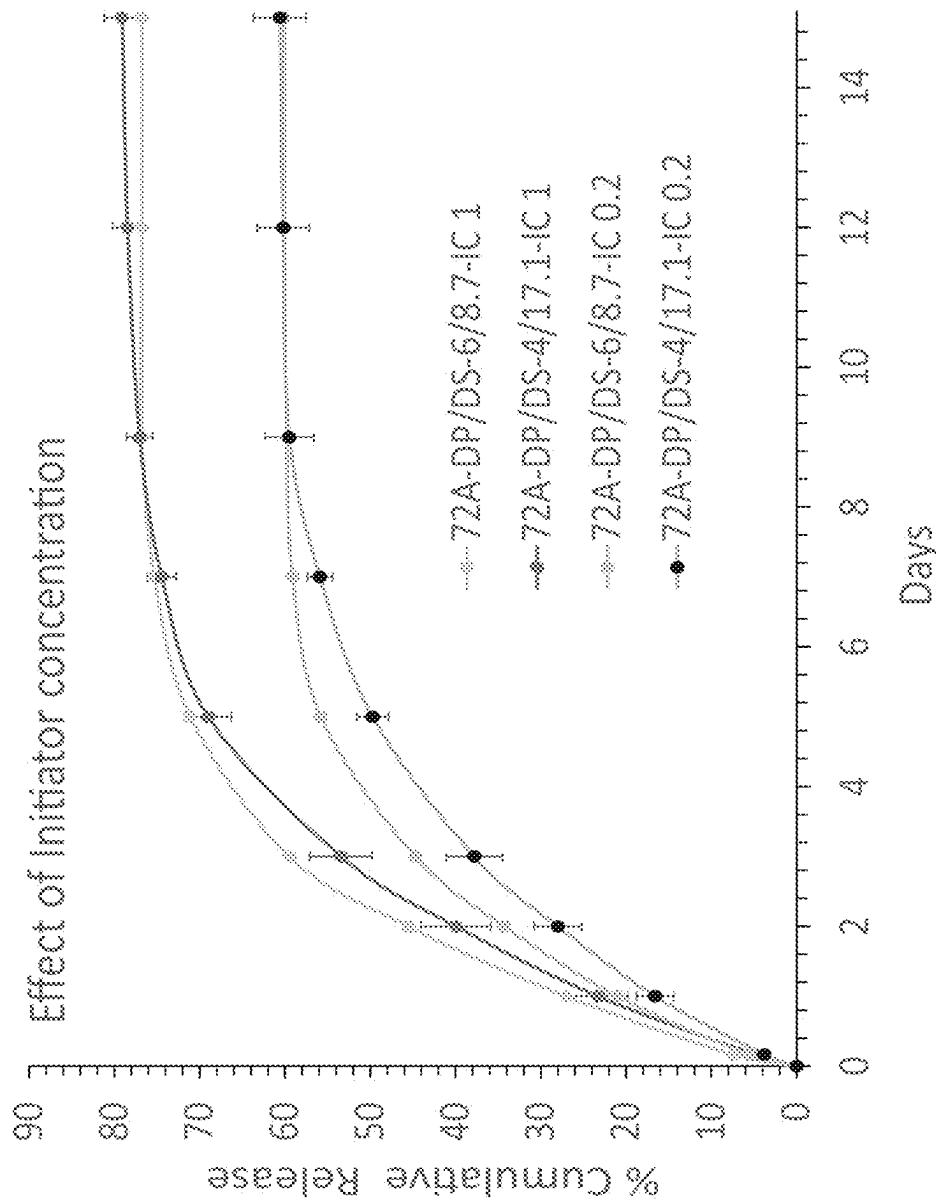


FIG. 10A

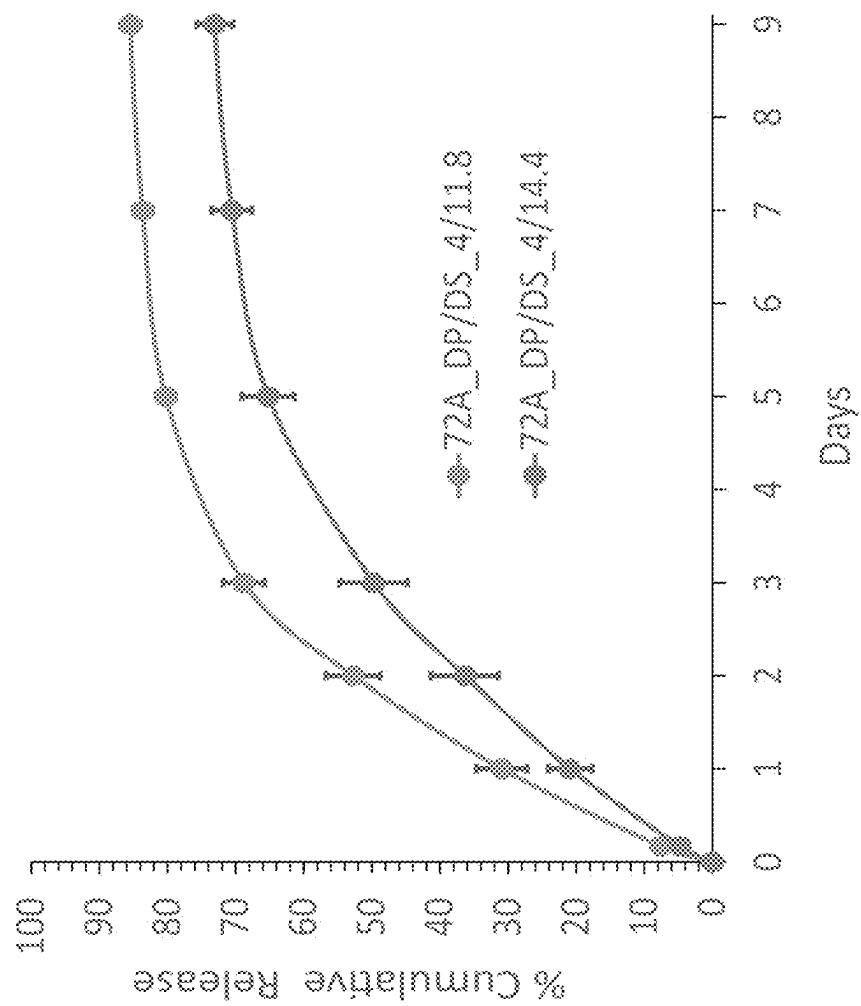


FIG. 10B

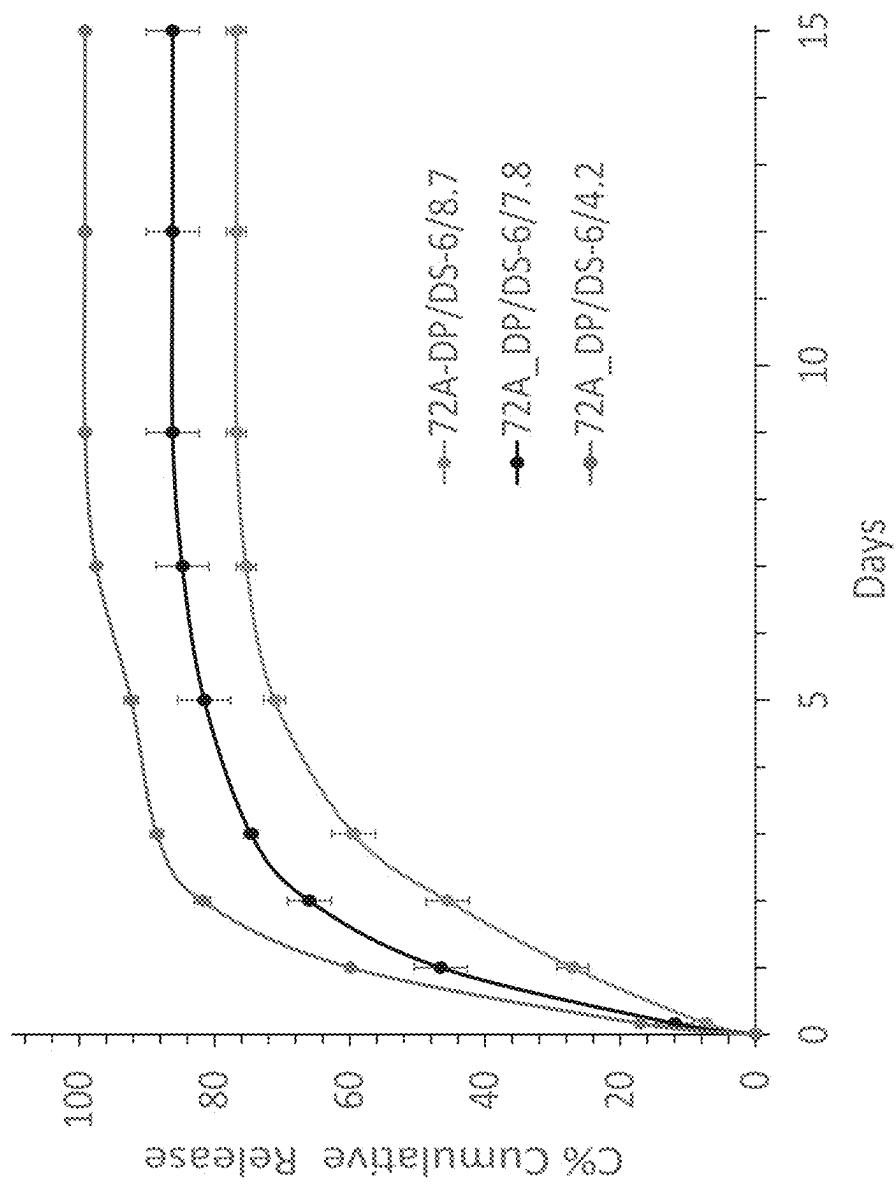


FIG. 11

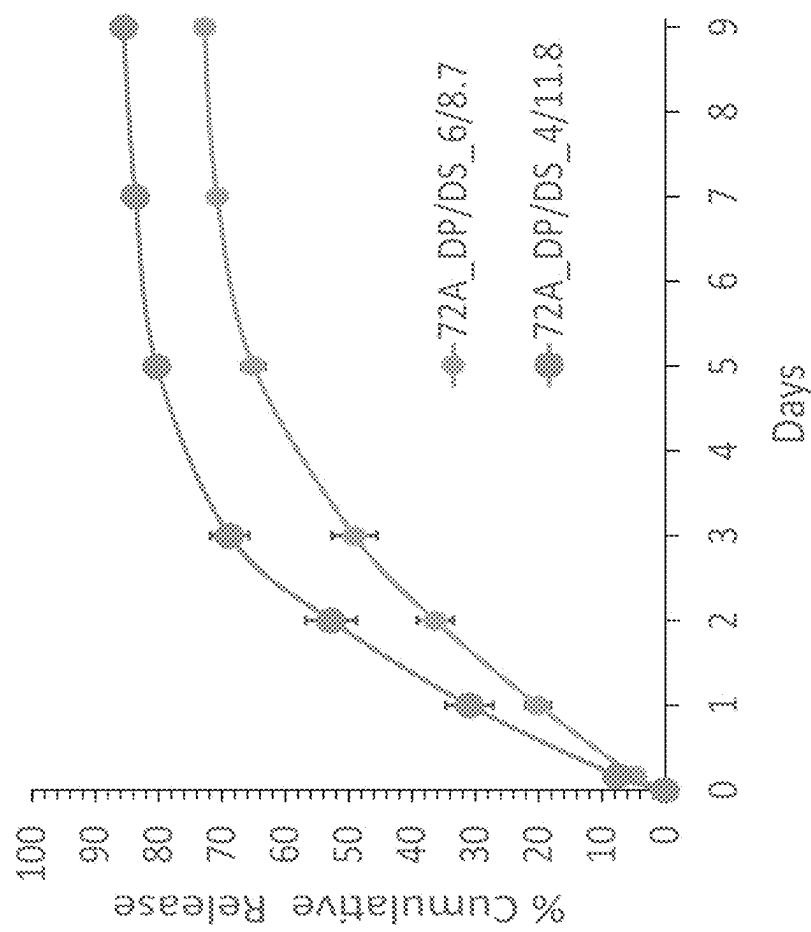
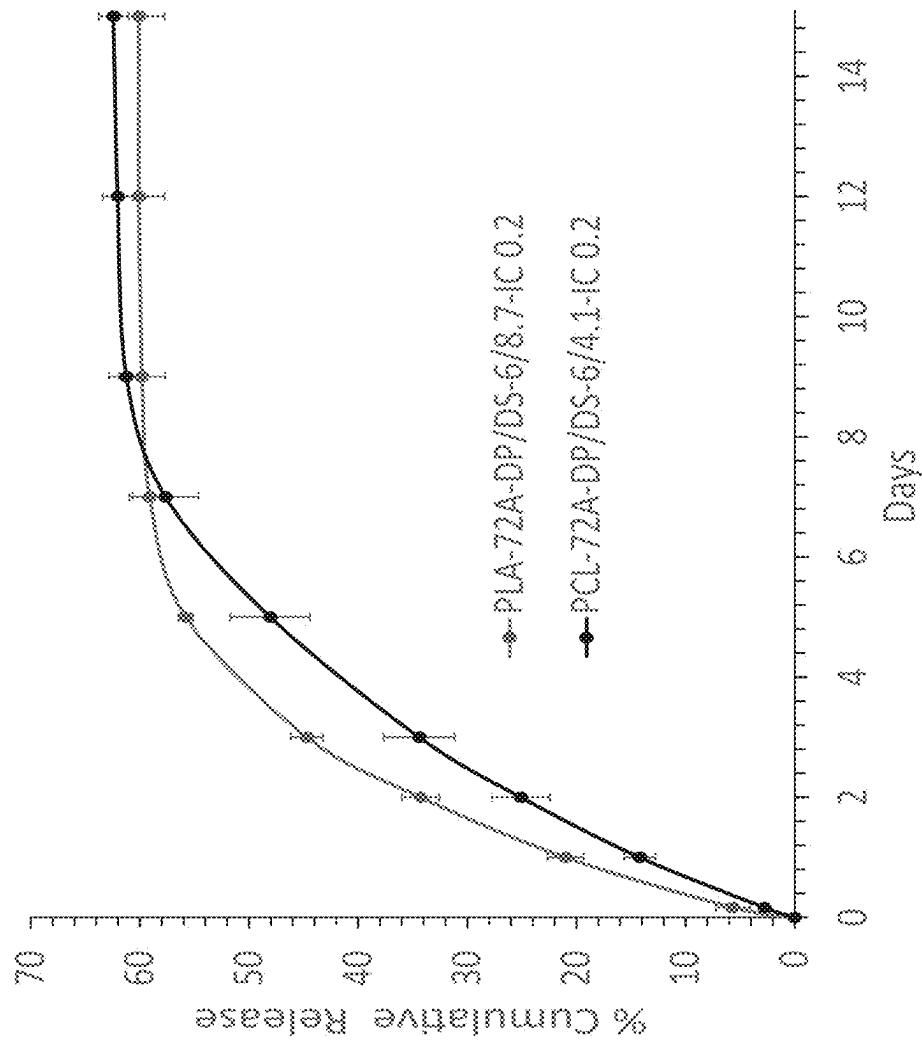


FIG. 12



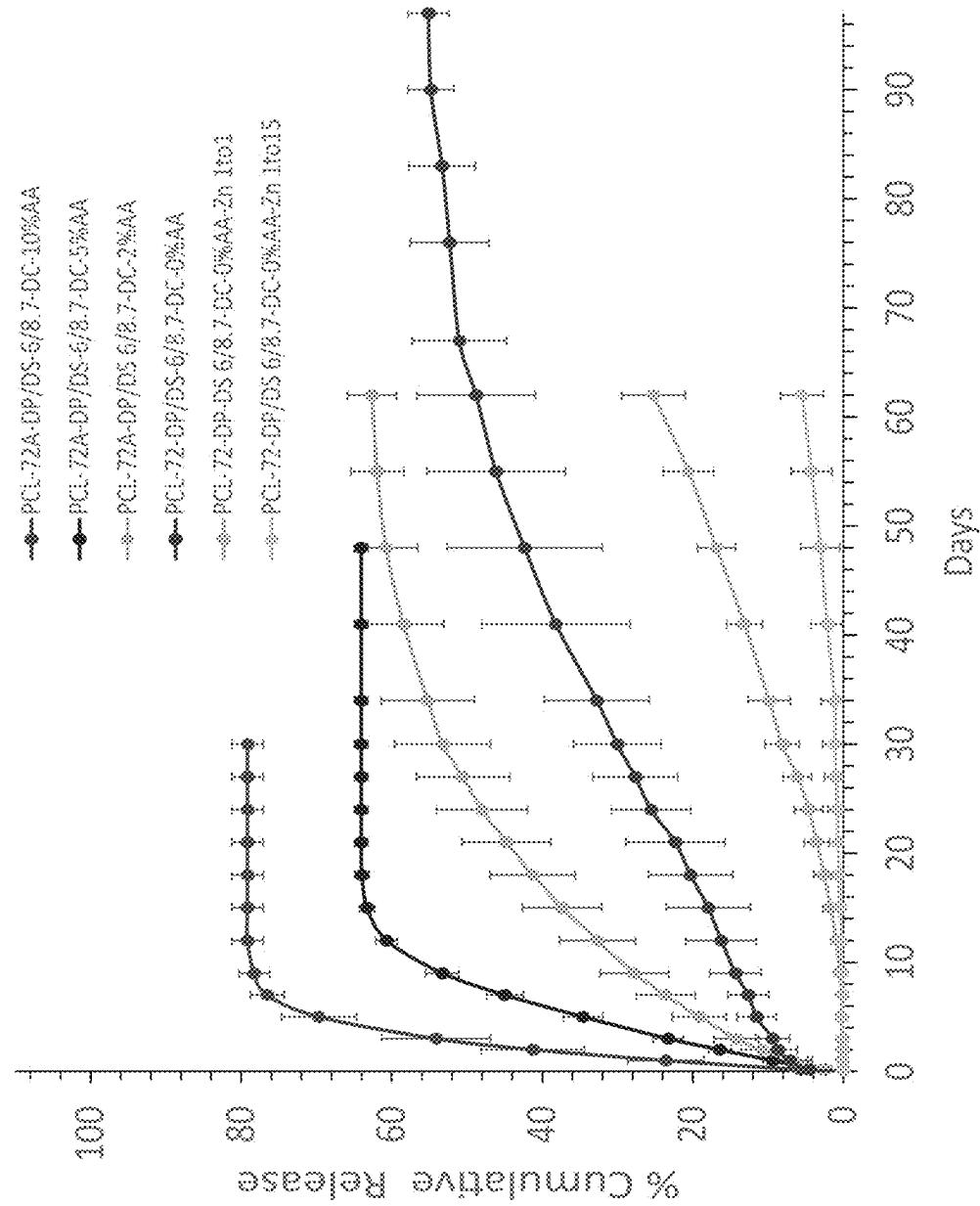


FIG. 13

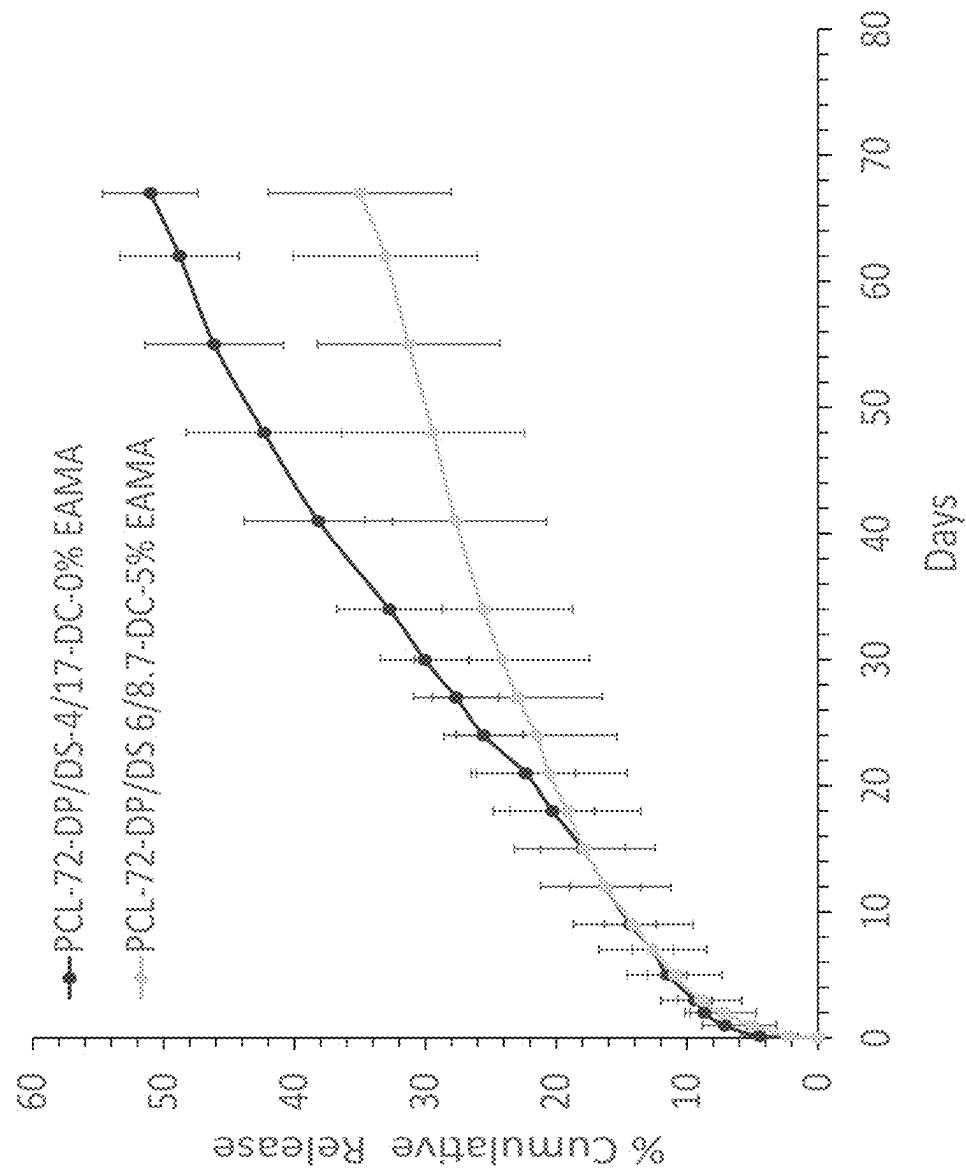


FIG. 14

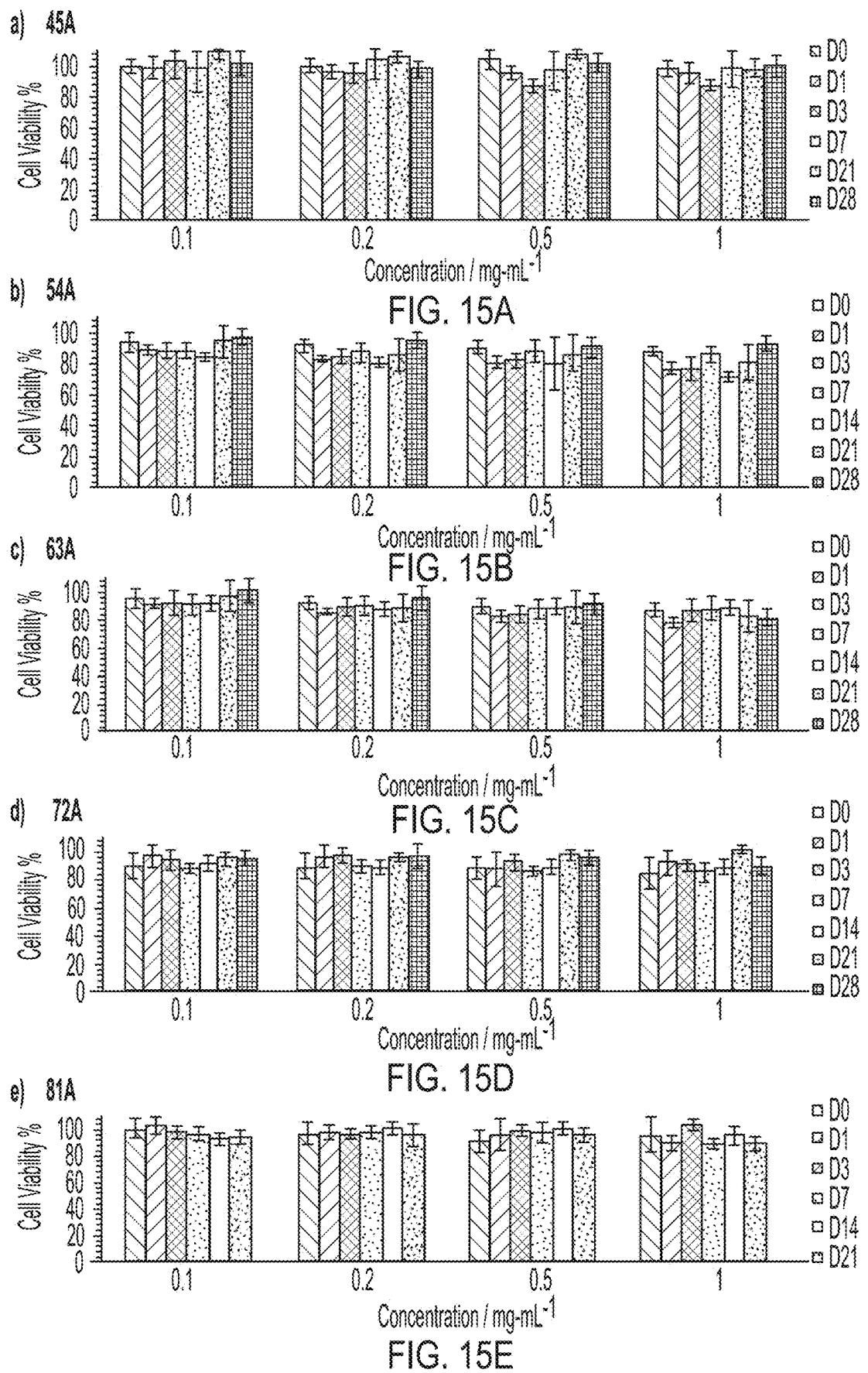


FIG. 16A

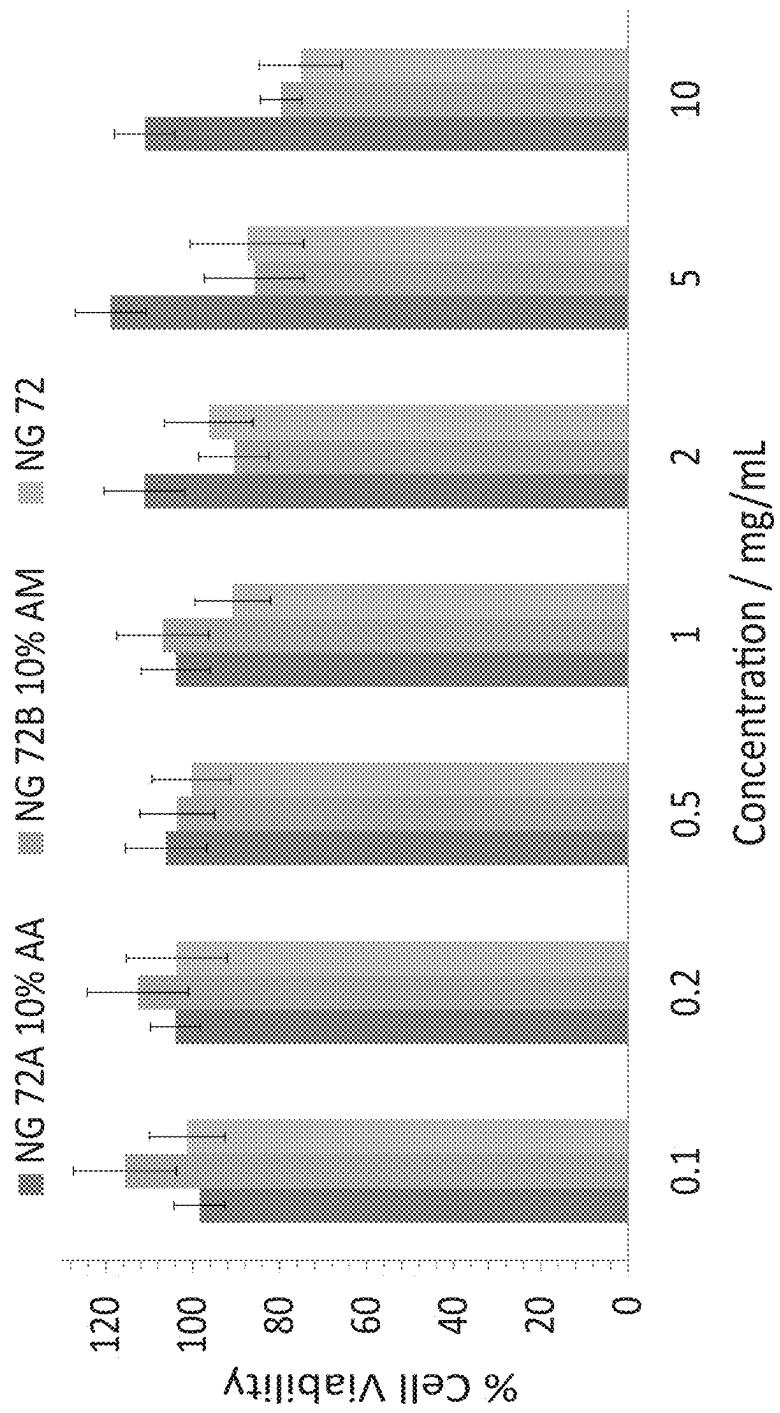


FIG. 16B

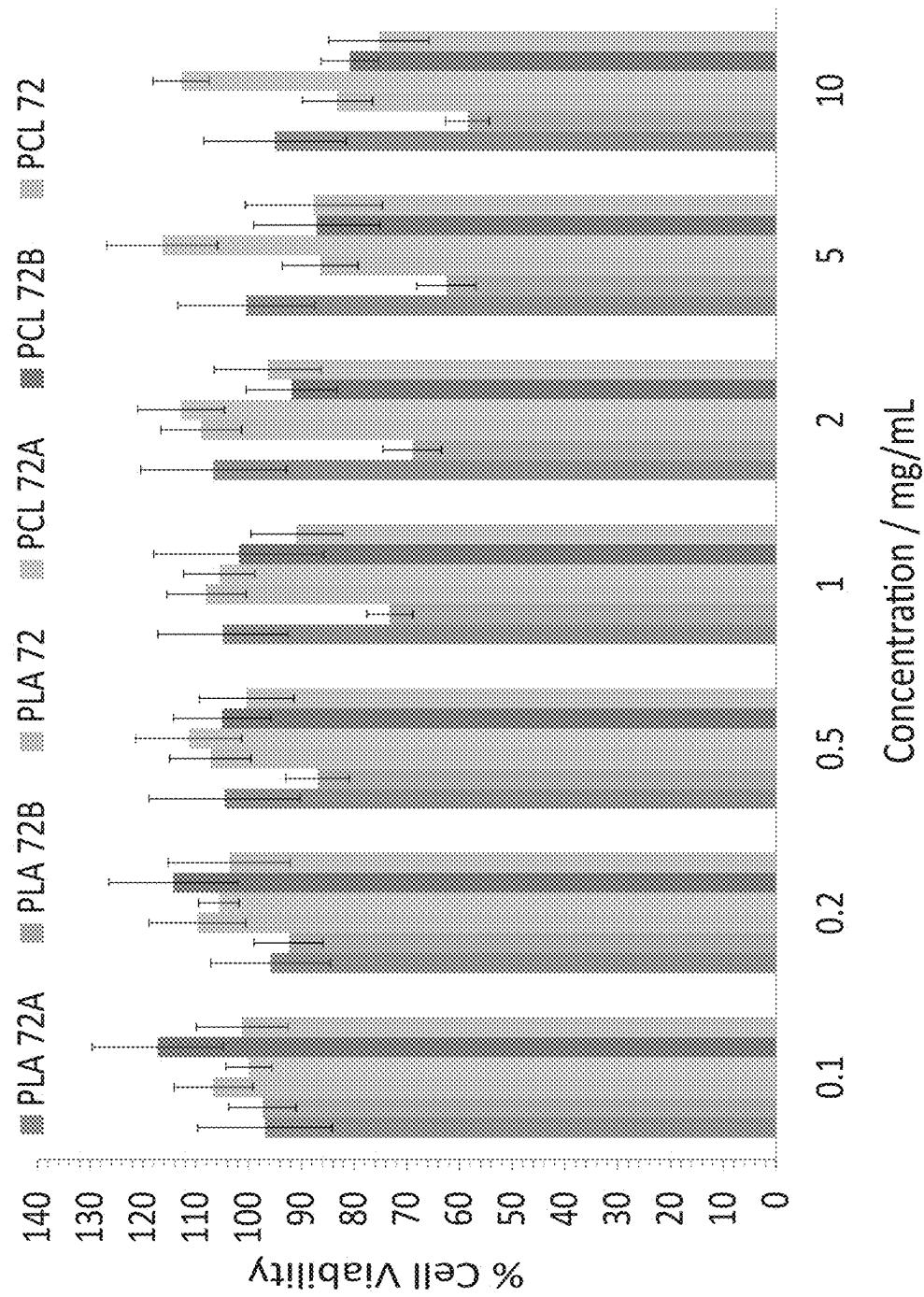


FIG. 17

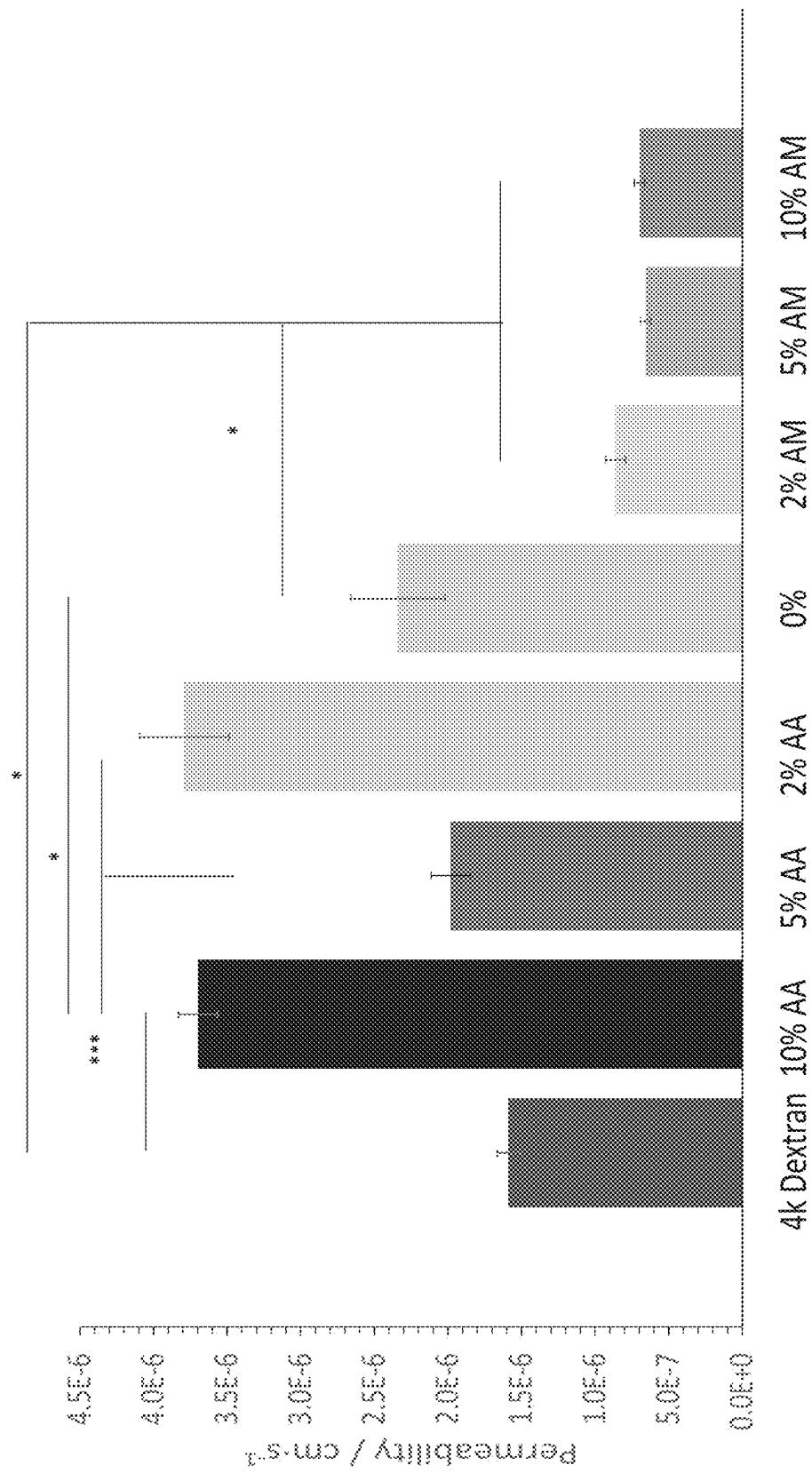


FIG. 18A

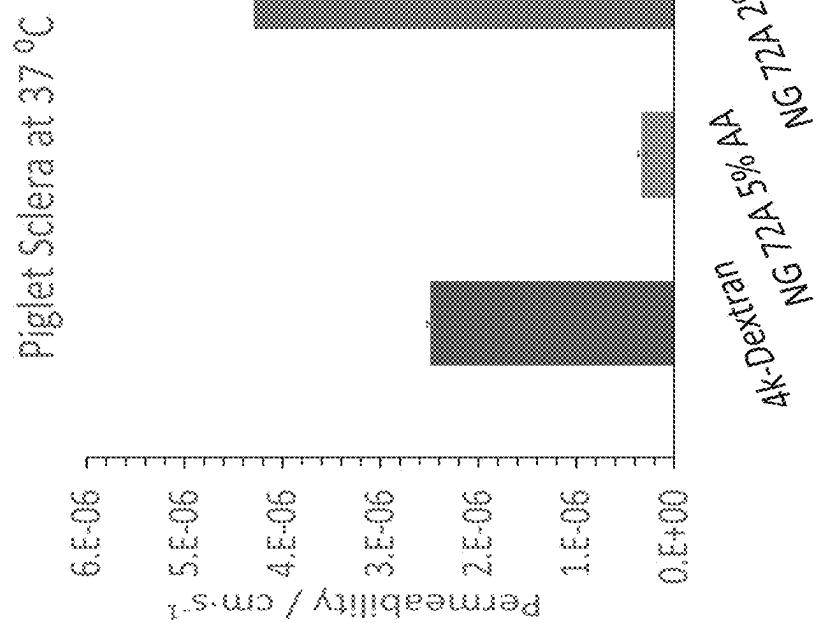


FIG. 18B

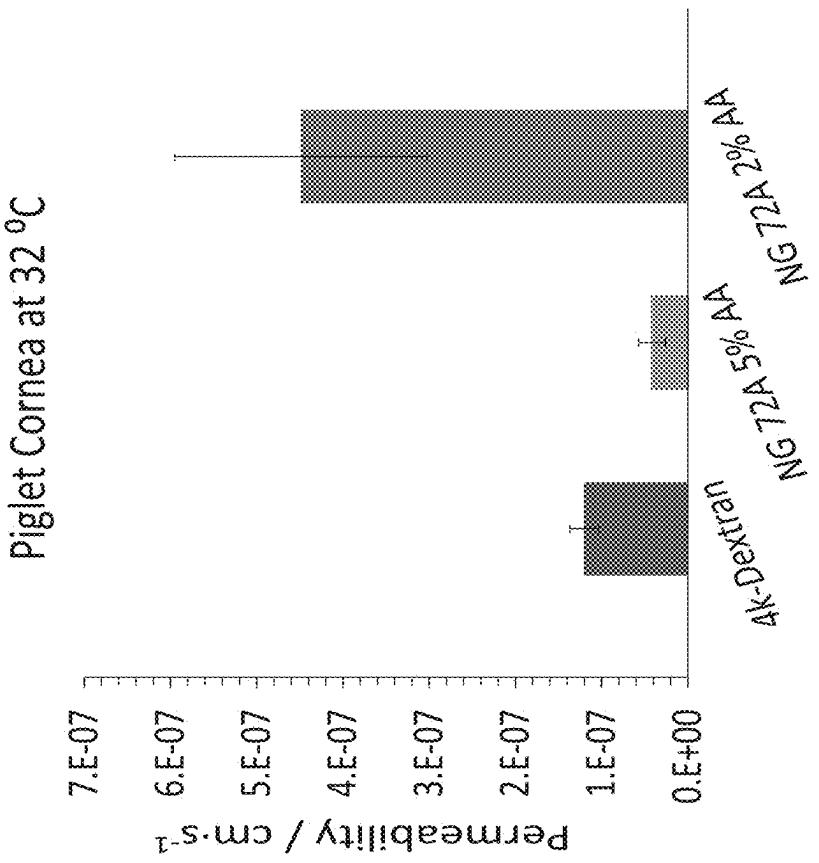


FIG. 19

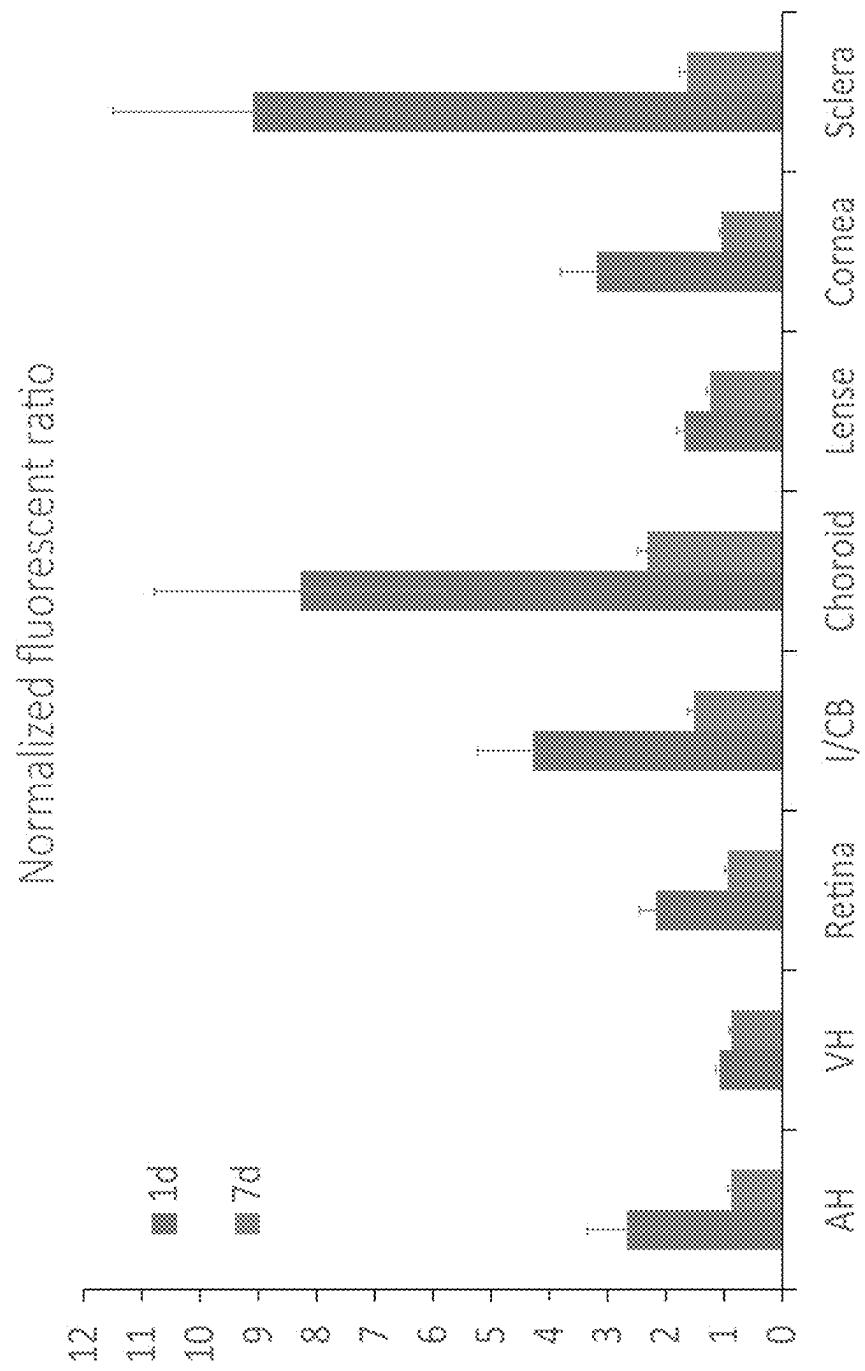
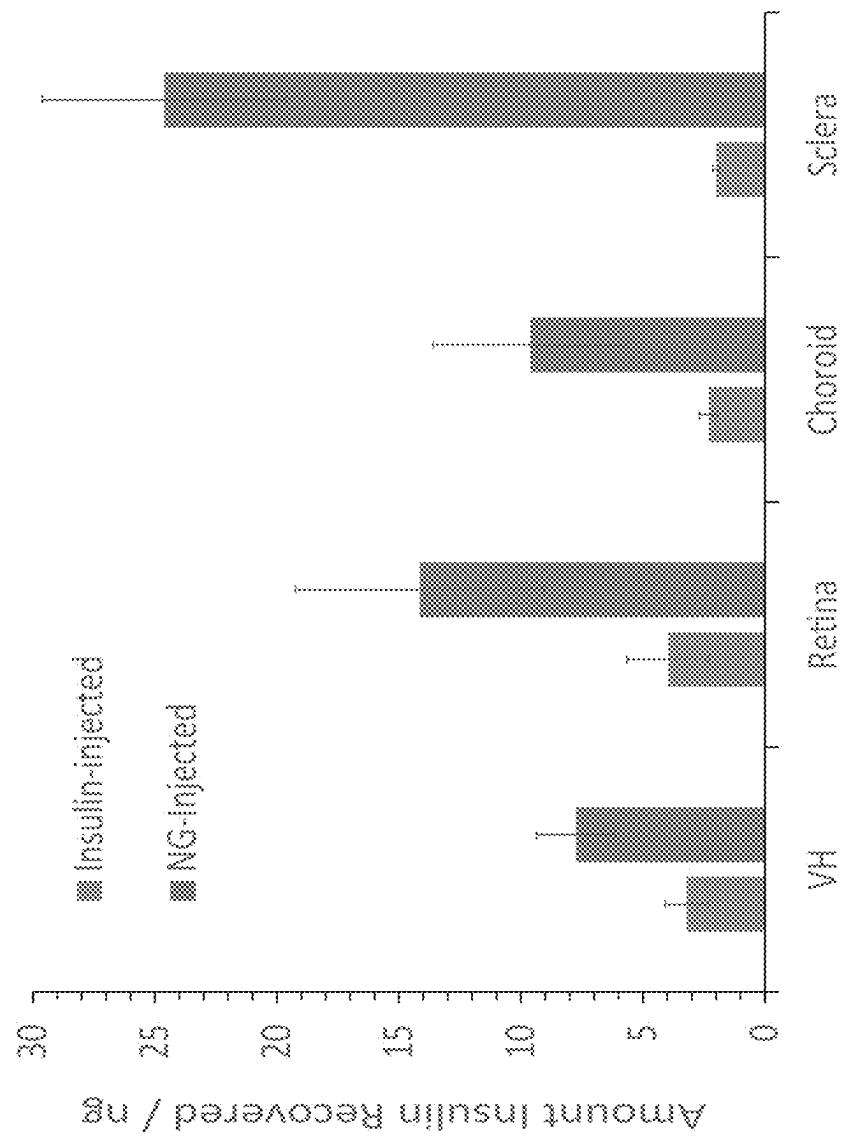


FIG. 20



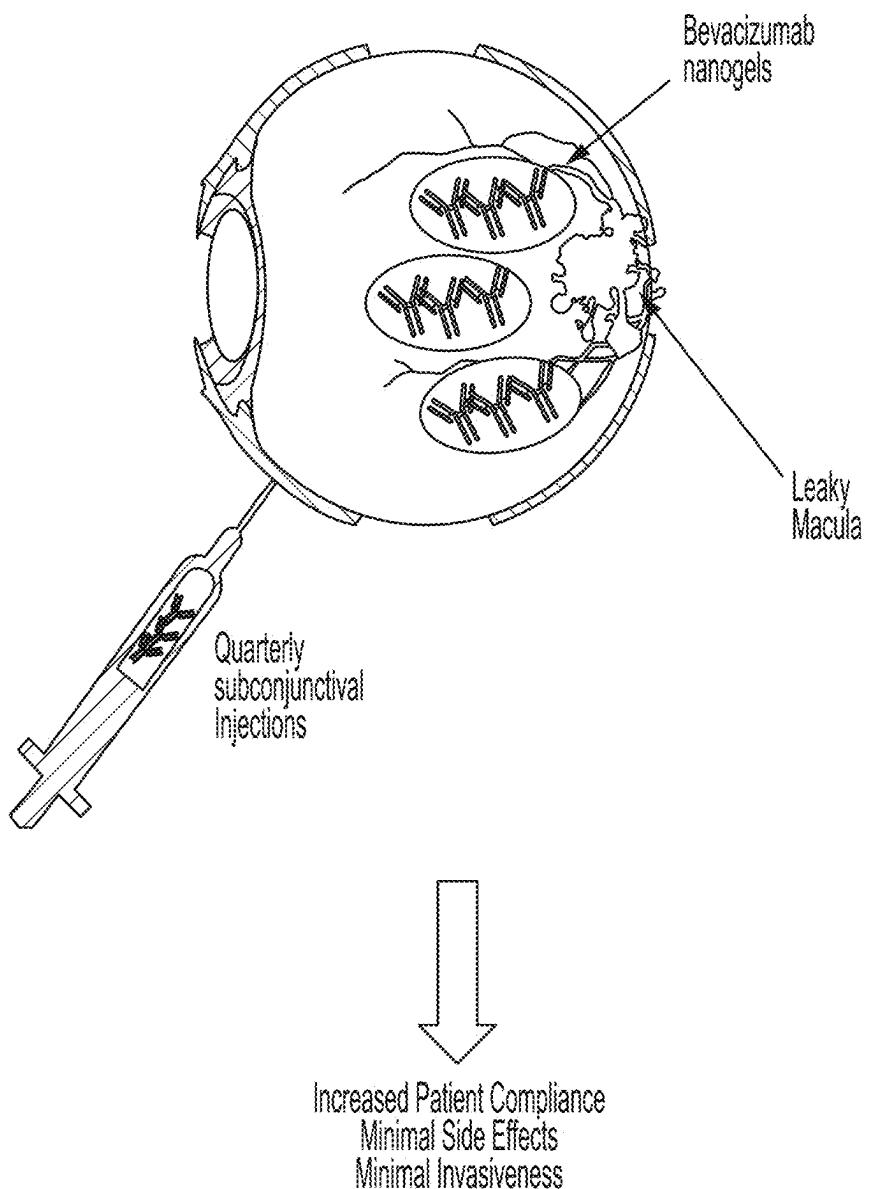


FIG. 21

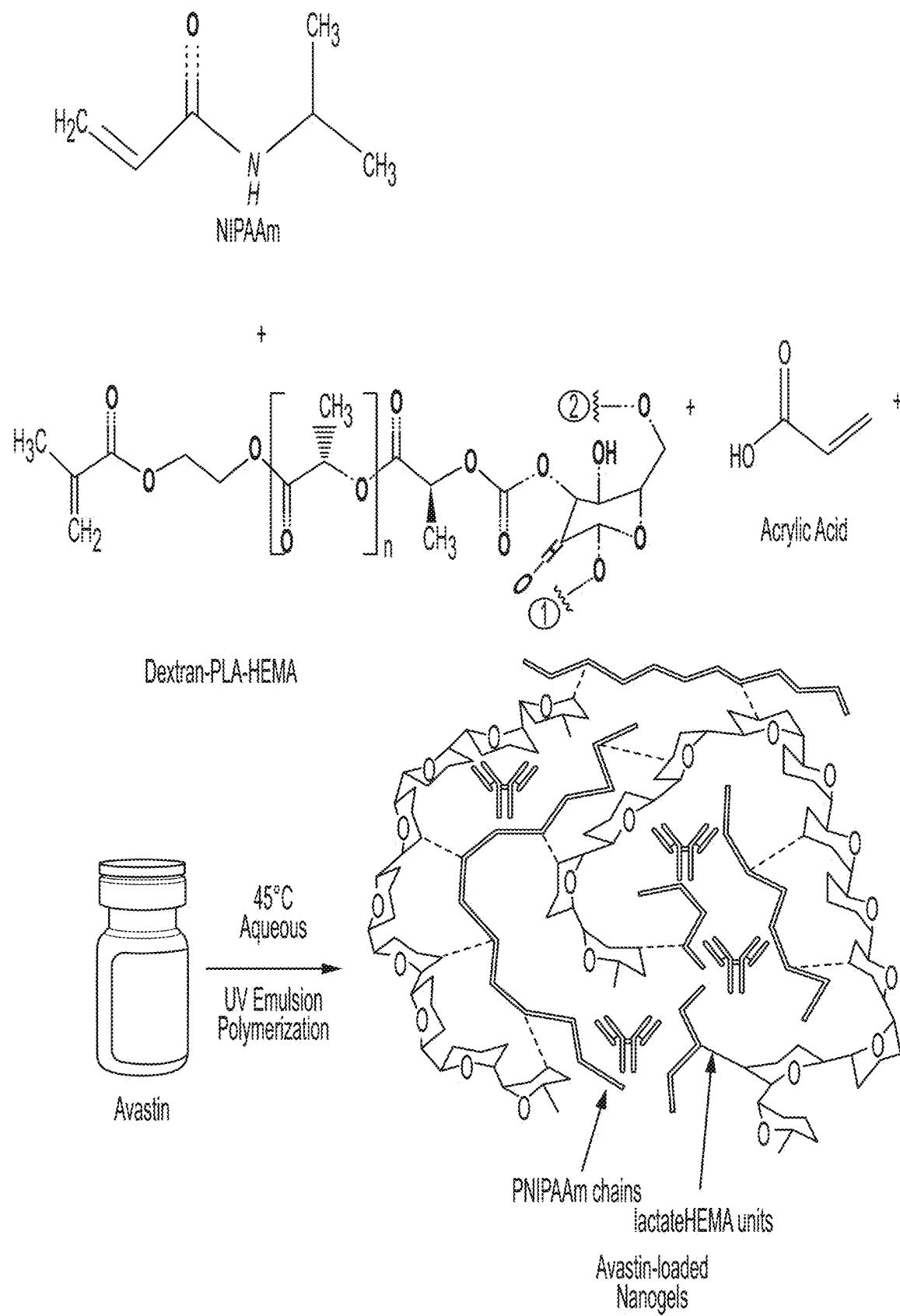


FIG. 22

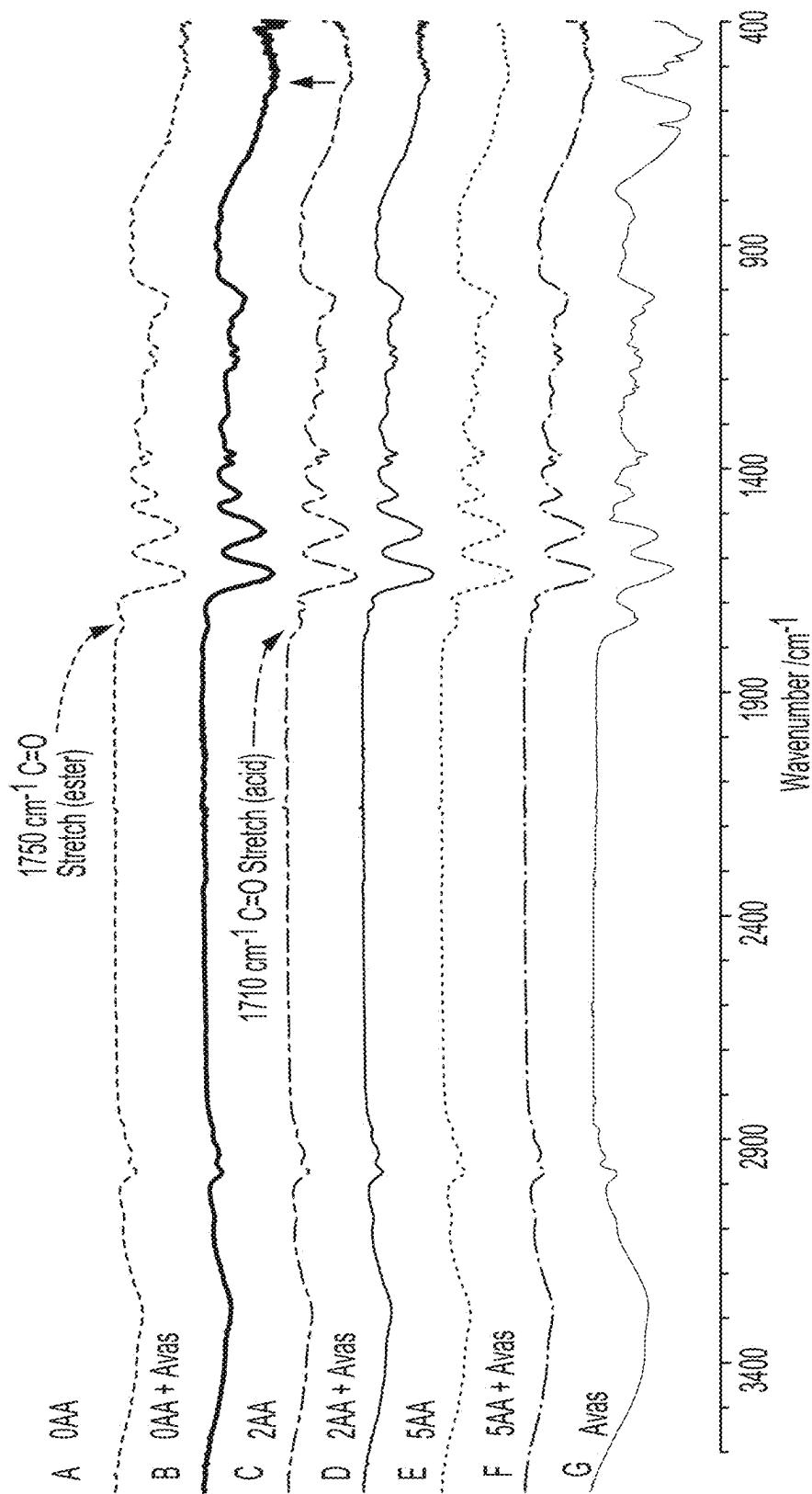
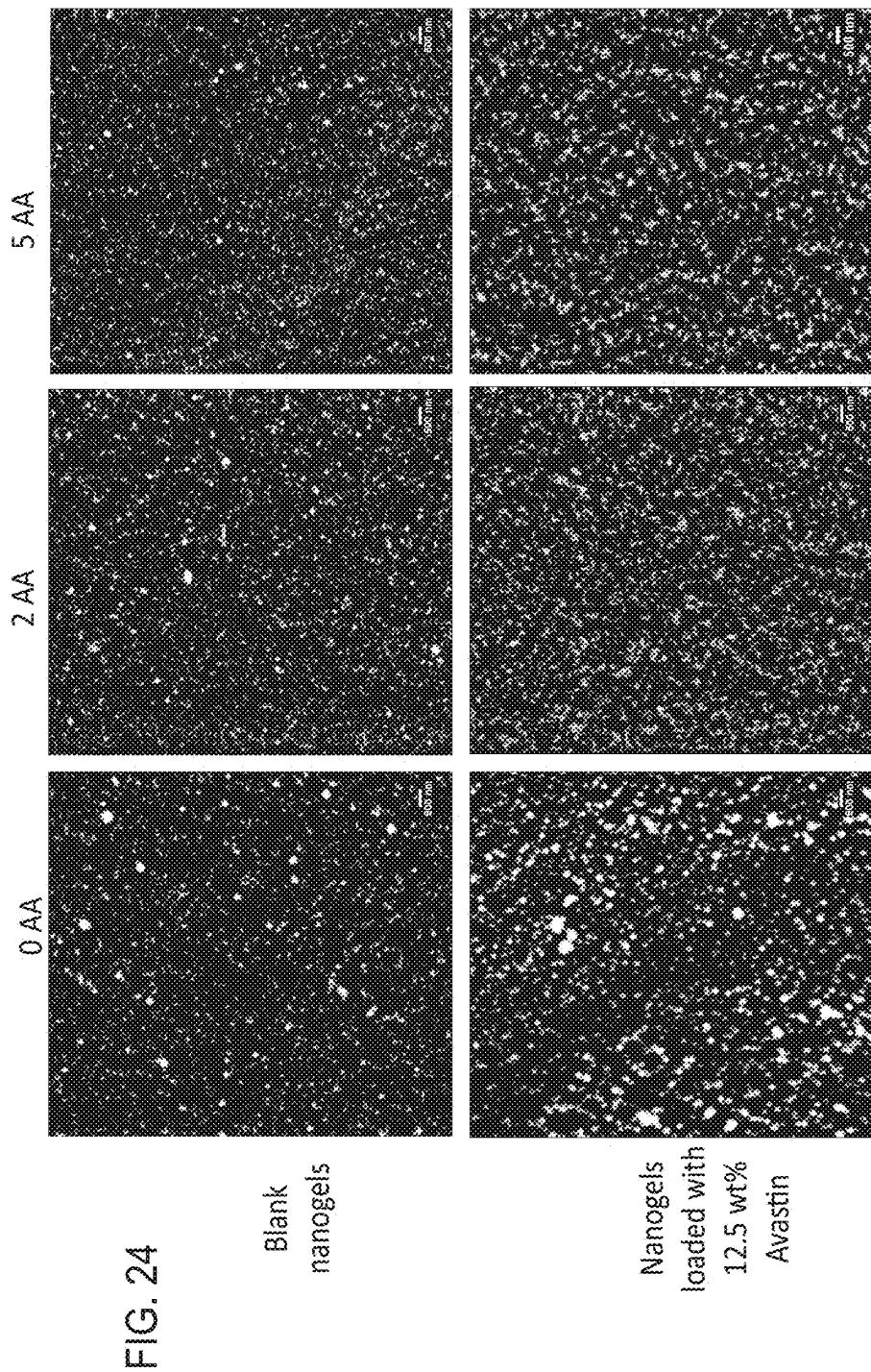


FIG. 23



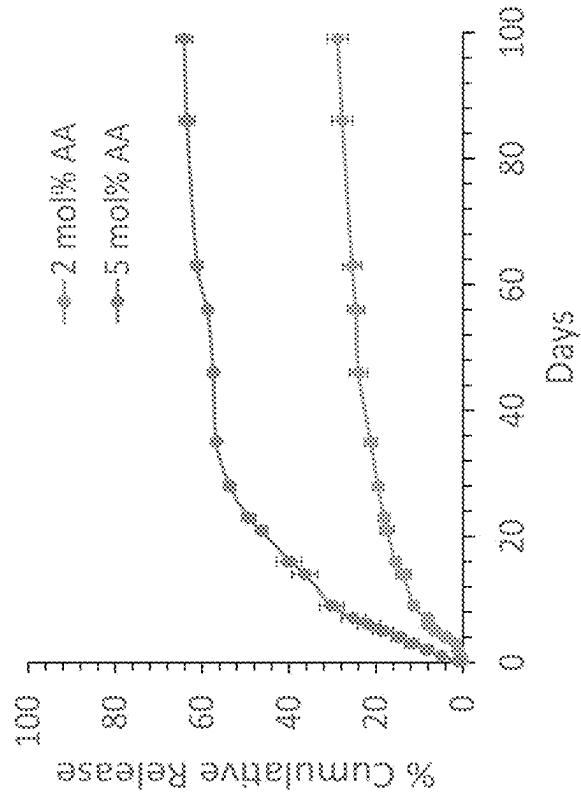


FIG. 25A

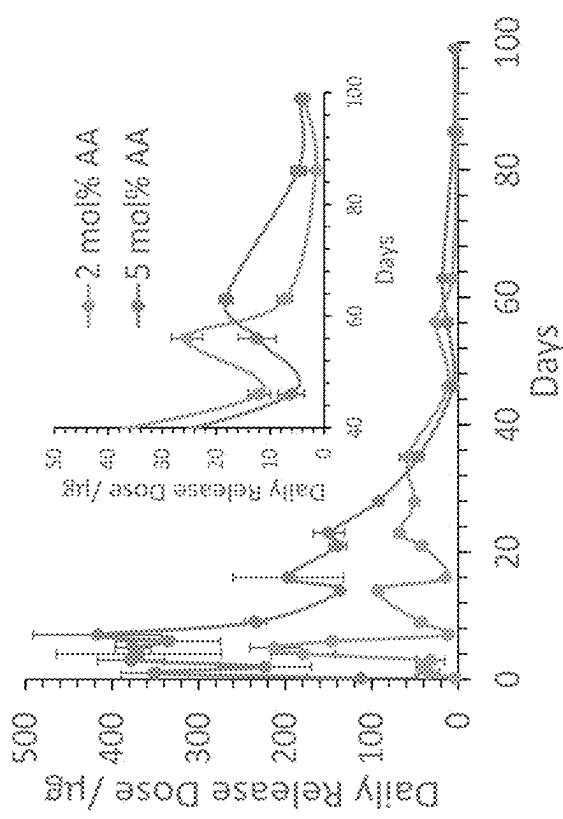


FIG. 25B

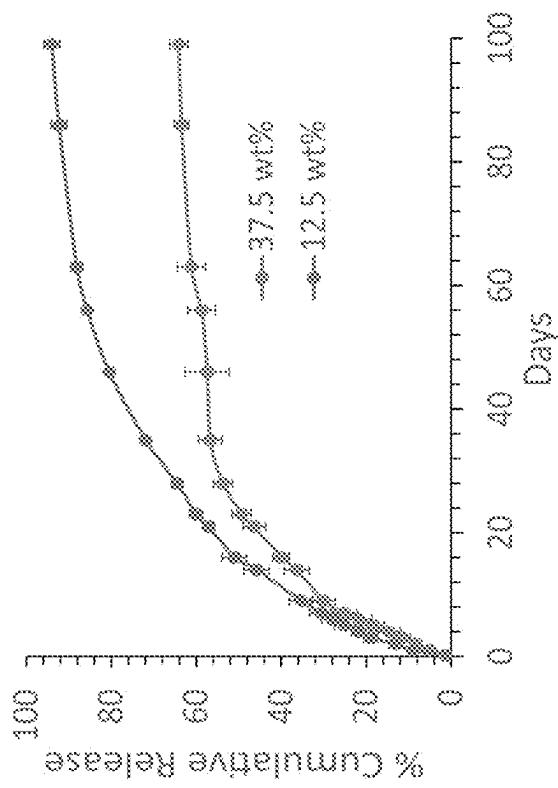


FIG. 25C

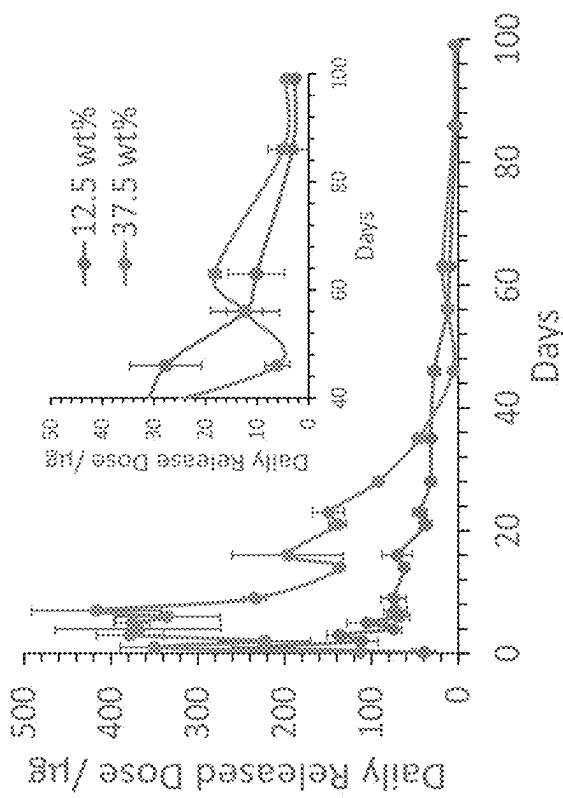


FIG. 25D

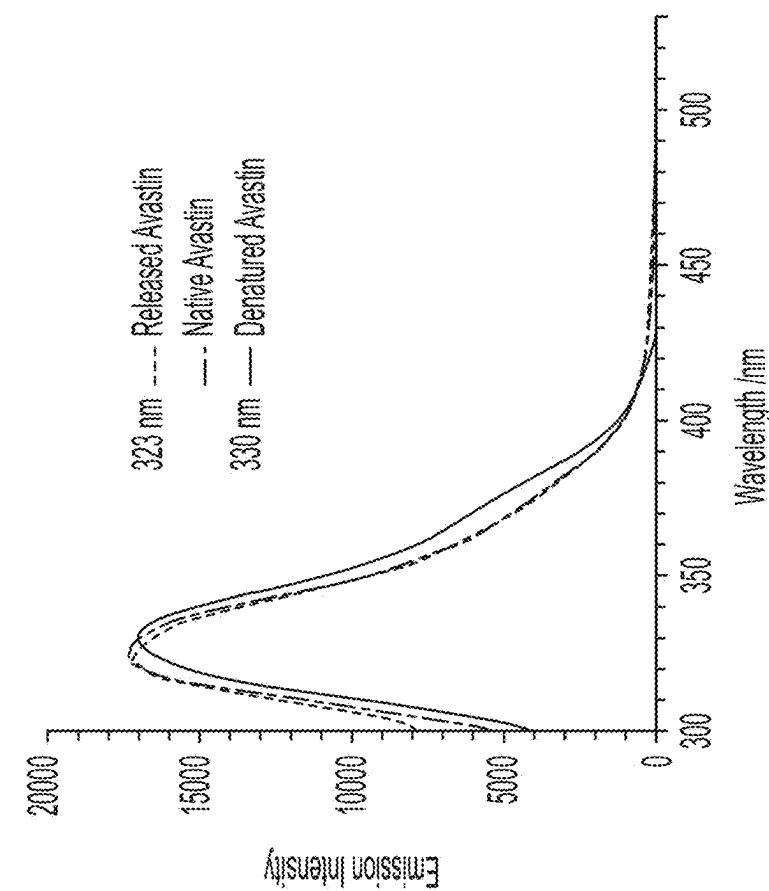


FIG. 26B

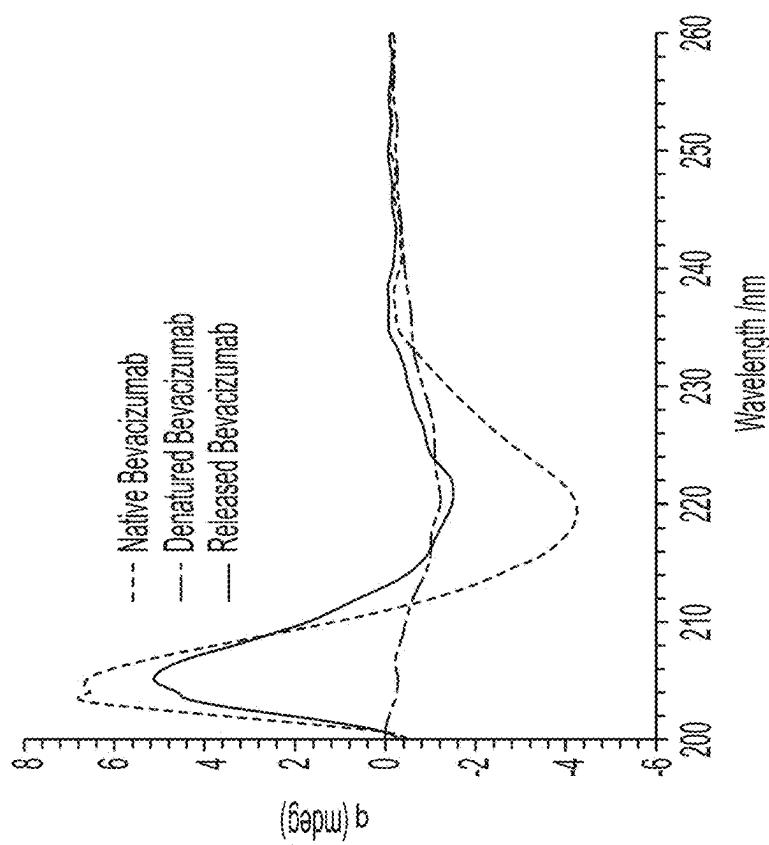


FIG. 26A

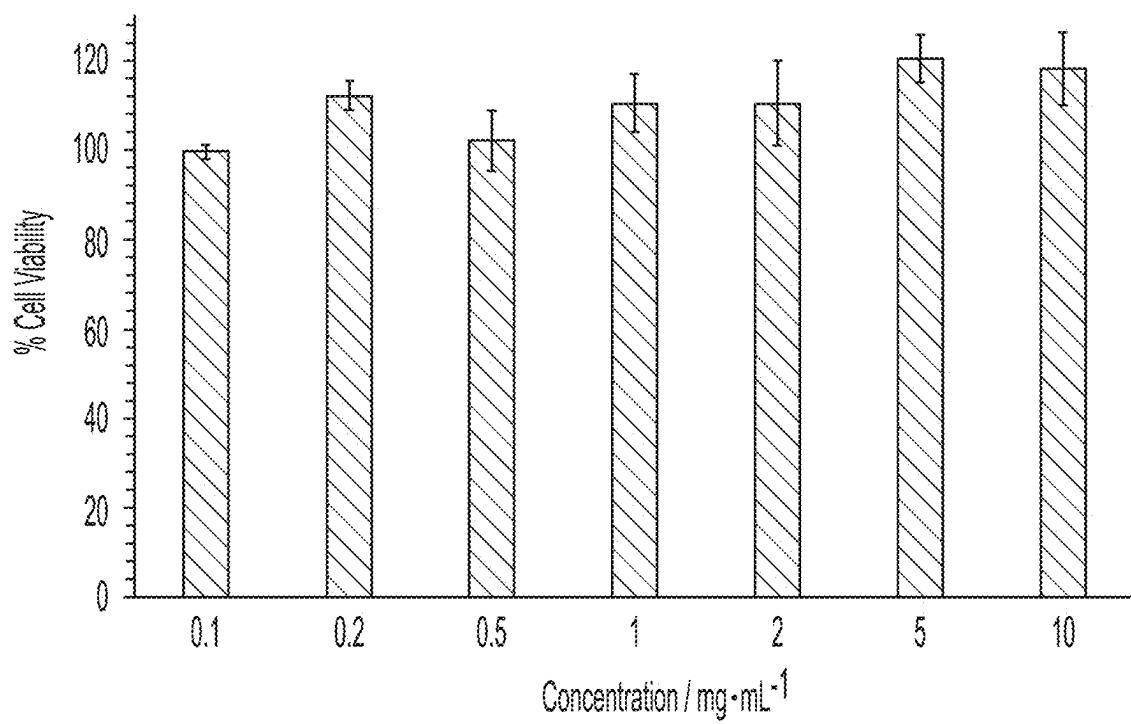


FIG. 27

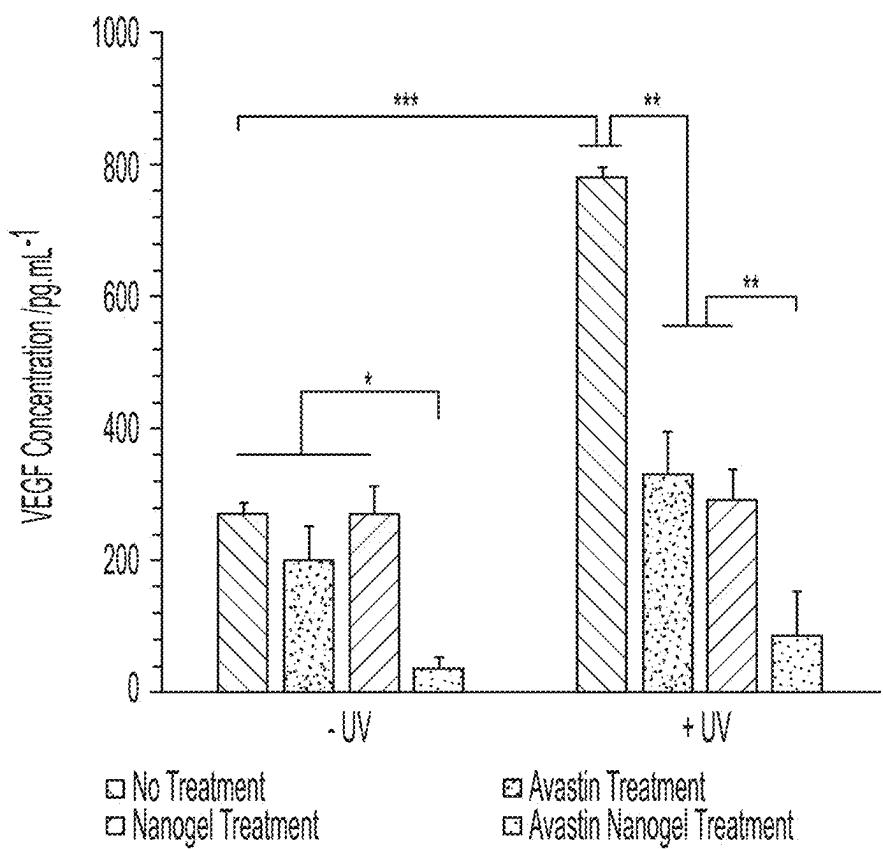
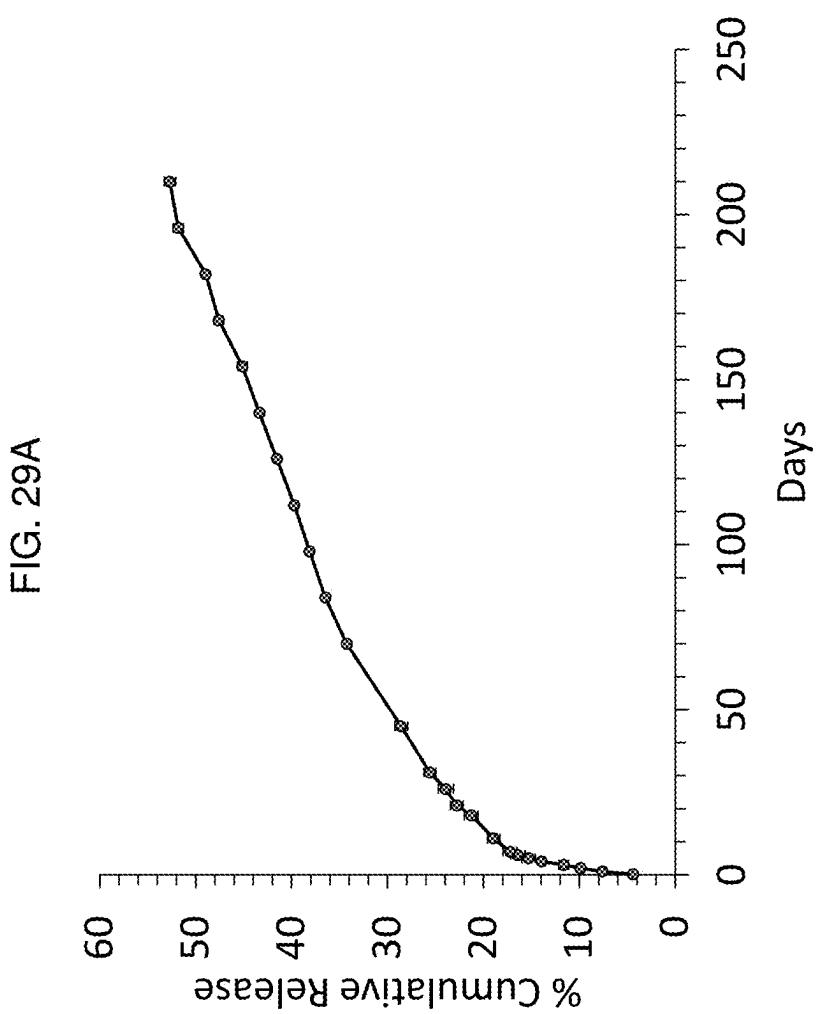
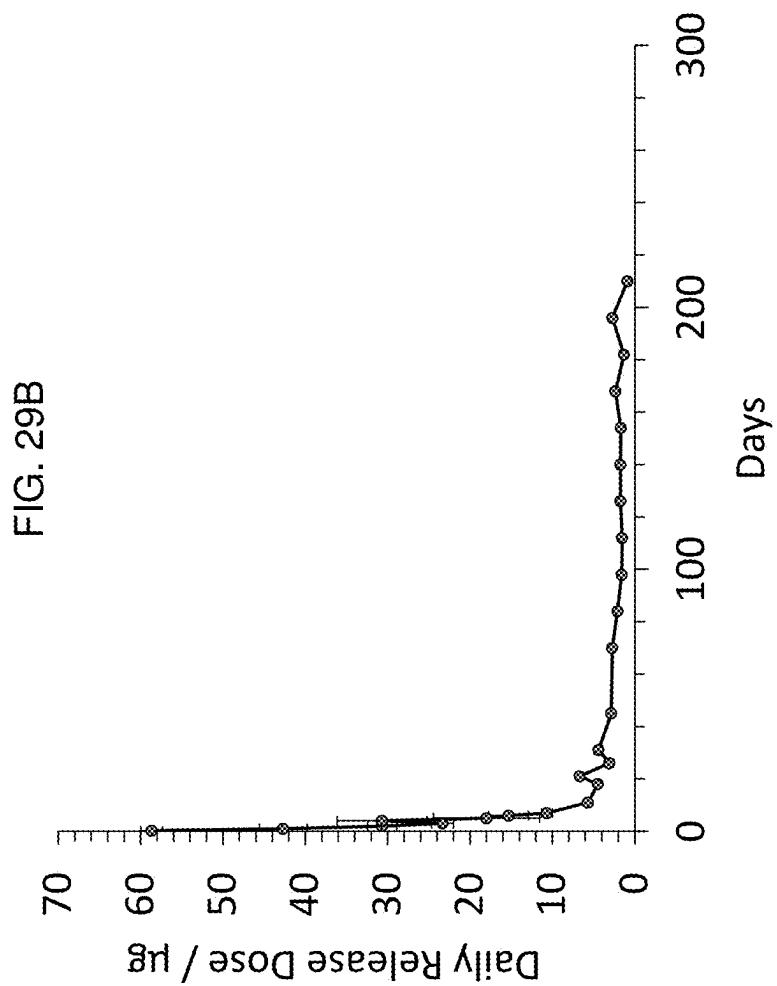


FIG. 28





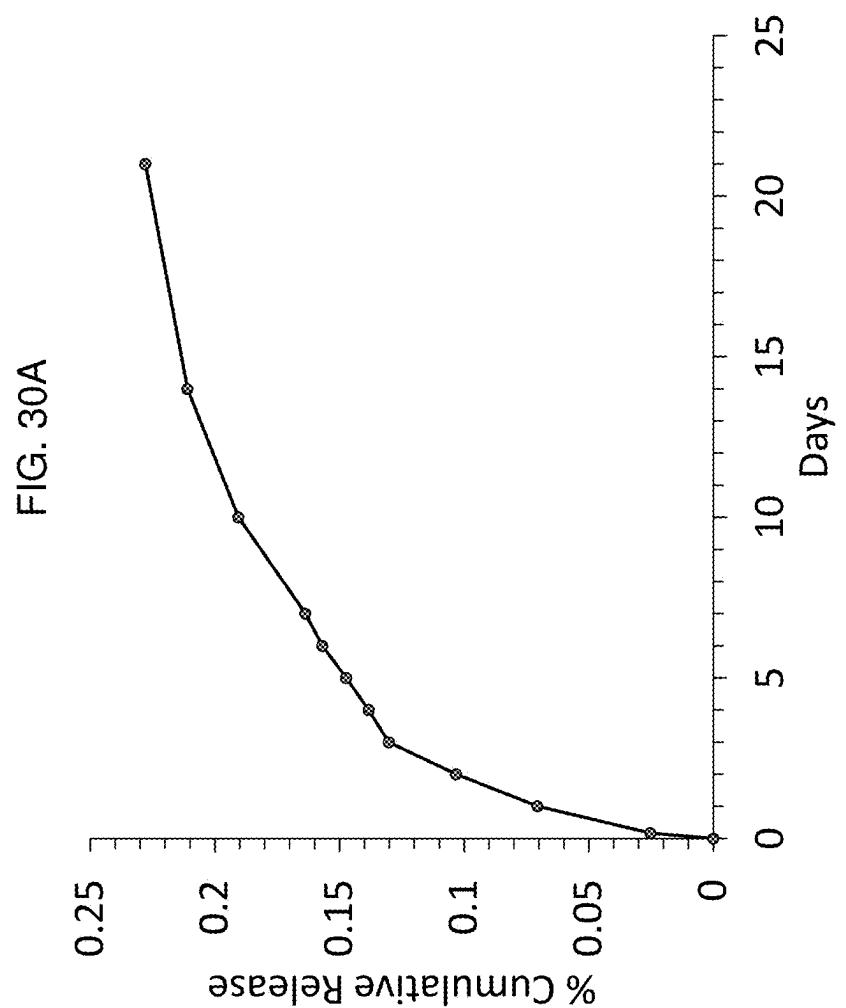
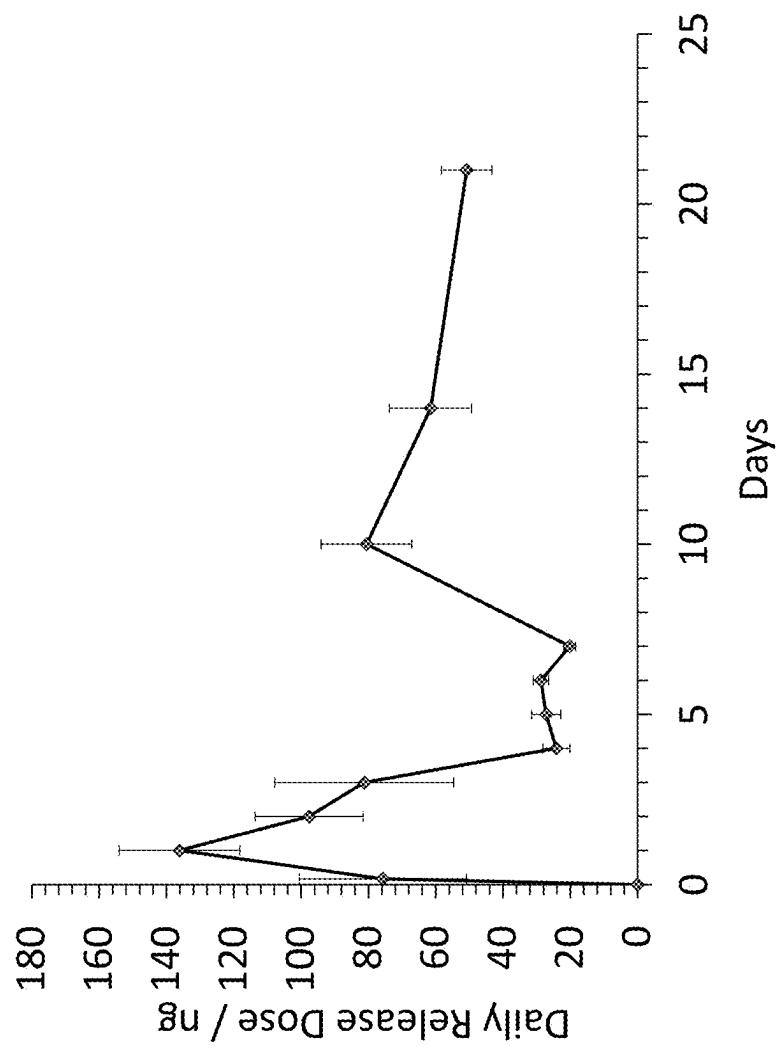


FIG. 30B



light of a suitable wavelength, depending on the initiator used. For Irgacure® 2959, light of about 365 nm is recommended for the reaction.

[0063] Additional components that can be included in the nanogels of this invention include components that modify the bulk and/or surface properties of the nanogel. Comonomer acrylic acid, 2 amino-ethyl methacrylate, 2-allyl meta-cresol, monomers containing carboxylic acid side group, monomers containing primary or secondary amine side group, or a combination thereof, for example, can be added during synthesis in order to modify the surface and bulk properties of the nanogels, modulate biologic release kinetics and amount from the nanogels, enhance the stability of biologics (use of 2-allyl meta-cresol), and/or increase cellular uptake and/or tissue penetration of the nanogels and/or biologics. Zinc salts, such as zinc sulfate, polyhydric alcohols, glycerol, sucrose, glucose, or trehalose, and the like, or a combination thereof, can be used as an additive to slow release of the biologic medicament from the nanogel. For example, zinc sulfate can be added during synthesis of the nanogel in a range of about 0.1 wt % to about 500 wt %, preferably about 1 wt % to about 500 wt %, and most preferably about 1 wt % to about 250 wt %.

C. Synthetic Methods

[0064] The components of the nanogel, including the macromer (i.e. Dex PCL-HEMA macromer or Dex-PLA-HEMA macromer), NiPAAm or another monomer, acrylic acid or other modifier (optional), the biologic medicament or medicaments, Irgacure® or another initiator, are mixed together in water. Initiator is added and the mixture is subjected to UV irradiation at 365 nm wavelength (for Irgacure®) and 1 W/cm² intensity for about 10-60 minutes under stirring at 200-300 rpm at about 45° C. See FIG. 1. The product then is subjected to dialysis for 5 hours against water, using a 50-300 kDa dialysis membrane, with frequent change of the dialysis water (every 0.5 h to 1 h) followed by lyophilization.

D. Modification of Nanogel Formulations

[0065] Comonomer acrylic acid, 2 amino-ethyl methacrylate, 2-allyl meta-cresol, monomers containing carboxylic acid side group, or a monomer containing primary or secondary amine side group, or a combination thereof is added together with the monomer and macromer before the UV-emulsion polymerization is initiated. The amount of these comonomers to be added is at molar ratio to the monomer at about 0.1 mol % to about 50 mol %, preferably about 1 mol % to about 30 mol %, and most preferably about 1 mol % to about 25 mol %. Zinc salts, such as zinc sulfate, polyhydric alcohols, glycerol, sucrose, glucose, or trehalose, and the like, or a combination thereof is added before the initiator is added in a range of about 0.1 wt % to about 500 wt %, preferably about 1 wt % to about 500 wt %, and most preferably about 1 wt % to about 250 wt %.

[0066] Nanogels synthesized with different macromers and/or different DP/DS values for the macromer are expected to behave differently and may exhibit different drug release profiles. Dex-PCL (poly- ϵ -caprolactone)-HEMA macromers versus macromers containing PLA produce different effects, which can be useful in modifying the release profile of the medicament to the patient. PCL is less

polar and exhibit a slower degradation kinetics than PLA and may better control drug release for long term use.

[0067] Generally, the actual DP value was greater than the theoretical DP value in a particular nanogel and the actual DS value was less than the theoretical DS value. As expected, however, the longer the reaction time, the higher the DS value and the yield. The DS values of PCL-HEMA were relatively less than the DS values of the corresponding PLA-HEMA oligomers showing that PCL is less reactive towards dextran than PLA. See the examples below for effects on the nanogels.

[0068] Nanogel synthesis yield and particle size increased considerably with increasing initiator concentration, particularly up to 1.0% and 1.5% of the initiator concentration for yield and particle size, respectively. The morphology, mechanical strength and stability of the nanogel product can be adjusted as follows: change chemical composition, adjust the relative amount of the components including the macromer, monomer and modifiers, and change the DP and DS of the macromer.

E. Loading of Nanogel Formulations

[0069] To load the nanogel, the biologic or biologics to be loaded are included in the synthetic mixture. Generally, a concentration of about 1×10^{-3} wt % to about 50 wt %, preferably about 1×10^{-3} wt % to about 50 wt %, and most preferably about 1×10^{-3} wt % to about 35 wt % can be used. The amount can easily be determined by the practitioner depending on the nature of the biologic(s), the dose required for a particular patient and disease or condition from which the patient suffers. See synthetic method, above. Quantitation of the loaded medicament in the nanogel can be determined using an ultra performance liquid chromatography (UPLC), mass spectroscopy, ELISA, and/or bicinchoninic acid assay (BCA) method. See Example 1, below.

[0070] The percentage synthesis yield is calculated as (the amount of nanogels obtained after lyophilization/theoretical weight of the nanogel $\times 100$). Percent biologic loading is calculated as (total amount of biologic loaded -- total amount of biologic recovered in the dialysis media)/total amount of biologic loaded $\times 100$). Release from the nanogels can be measured as shown in Examples, below.

F. Toxicity

[0071] The toxicity of the nanogels and their degradation product was investigated by MTT assay, as described herein. The nanogels exhibited very low cytotoxicity in all tests with cell viability more than 90% at concentration up to 2 mg/mL except for the nanogels made of PLA-containing macromer and positively charged 2-aminoethyl methacrylate. The nanogels made of PCL-containing macromer and negatively charged acrylic acid are not cytotoxic at concentration up to 10 mg/mL.

G. Biologics

[0072] The biologics that can be loaded into the nanogels of the invention for release to a patient include any protein, peptide, nucleic acid, or the like as known in the art. Preferably, it is contemplated that the nanogel invention is useful for administration to a patient of antibody medications, peptide hormones, RNA and DNA medications, and the like as are known to the person skilled in the art. Preferred examples of biologics are monoclonal antibodies,

growth factors, aptamers, peptide or protein hormones, nucleic acids (RNA or DNA), nucleotides, oligonucleotides, siRNA, mRNA, gene therapies, vaccine proteins and peptides, receptors, enzymes, ligands, hormones, gene and cellular therapies, blood products, biopolymers, natural polymers, biomolecules, biomacromolecules, polysaccharides, lipids, lipopolysaccharides, glycosaminoglycans (GAGs), steroids, nutrients, amino acids, protein kinases, cytokines, growth factors, differentiation factors, neurotrophic factors, stem cell factors, fusion proteins, carbohydrates, TNF inhibitors, interleukin inhibitors, B cell inhibitors, T cell inhibitors, and the like. Specific, non-limiting examples of suitable biologics are Humalog® (insulin lispro), Admelog® (lispro), Novolog® (aspart), Fiasp® (aspart), Apidra® (glulisine), Humulin® R (U-100) (regular human insulin (RHI)), Novolin® R (regular human insulin (RHI)), Humulin® R (U-500) (regular human insulin (RHI)), Humulin® N (neutral protamine Hagedorn (NPH)), Novolin® N (neutral protamine Hagedorn (NPH)), Lantus® (glargine (100 U/ml)), Basaglar® (glargine (100 U/ml)), Levemir® (detemir), Toujeo® (glargine (300 U/ml)), Tresiba® (degludec), Humulin® 70/30 (70% neutral protamine hagedorn and 30% regular human insulin), Novolin® 70/30 (70% neutral protamine hagedorn and 30% regular human insulin), HumaLog® Mix 75/25 (75% insulin lispro protamine and 25% lispro), HumaLog® Mix 50/50 (50% insulin lispro protamine and 50% lispro), NovoLog® Mix 70/30 (70% insulin aspart protamine and 30% aspart), Ryzodeg® 70/30 (70% insulin degludec protamine and 30% aspart), Humira™ (adalimumab), Herceptin™ (trastuzumab), Avastin™ (bevacizumab), Alymsys (bevacizumab), Mvasi (bevacizumab-awwb), Vegzelma (bevacizumab-adcd; CT-P16), Zirabev (bevacizumab), Lucentis® (ranibizumab), BYOOVIZ™ (ranibizumab-nuna), EYLEA® (afiblertcept), Botox™ (onabotulinumtoxinA), insulin like growth factor 1, insulin like growth factor 2, insulin-like growth factor-binding protein 3, TNF, anti-TNF α , TNF-bp, nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), glial derived neurotrophic factor (GDNF), neurotrophic growth factor, neurotrophic factor 3 (NT3), bone growth factor, osteoprotegerin (OPG), bone morphogenetic protein 2 (BMP2), bone-derived growth factor, platelet-derived growth factor (PDGF), interleukin, interleukin-1 (to 18) receptor antagonist (IL-1ra), interferon (alpha, beta, gamma), consensus interferon, erythropoietin, granulocyte-colony stimulating factor (GCSF), leptin (OB protein), heparin, fibroblast growth factor (FGF), transforming growth factor (TGF), platelet transforming growth factor, milk growth factor, endothelial growth factors (EGF), endothelial cell-derived growth factors (ECDGF), alpha-endothelial growth factors, beta-endothelial growth factor, vascular endothelial growth factor (VEGF), hepatocyte stimulating factor, plasmacytoma growth factor, 4-1 BB receptor (4-1BB), TRAIL (TNF-related apoptosis inducing ligand), artemin (GFRalpha3-RET ligand), BCA-1 (B cell-attracting chemokine), B lymphocyte chemoattractant (BLC), B cell maturation protein (BCMA), keratinocyte growth factor (KGF), thrombopoietin, megakaryocyte derived growth factor (MGDF), keratinocyte growth factor (KGF), BRAK, C-10, Cardiotrophin 1 (CT1), CCR8, thyroid stimulating hormone (TSH), sex hormone binding globulin (SHBG), prolactin, luteotropic hormone (LTH), lactogenic hormone, parathyroid hormone (PTH), melanin concentrating hormone (MCH), luteinizing hormone (LHb),

growth hormone (HGH), follicle stimulating hormone (FSHb), amphotericin B, Alzheimer vaccine, heparin, protein A, streptavidin, beta-galactosidase, beta-amyloid, eukaryotic initiation factor-4G, granulocyte macrophage colony stimulating factor (GM-CSF), novel erythropoiesis stimulating protein (NESp), thrombopoietin, tissue plasminogen activator (TPA), urokinase, streptokinase, kallikrein, collagen, gelatin, elastin, elastin-like-peptides, fibrin, silk, dextran, hyaluronic acid, celluloses, chitosan, alginate (alginic acid), and the like. All of these types of biologics are contemplated for use with the invention.

H. Diseases and Conditions

[0073] Any disease or condition that can benefit from longer term administration of a biologic is suitable for treatment using the invention described herein. For example, common diseases and conditions that are treated using biologics include, but are not limited to type 1 diabetes, type 2 diabetes, diabetic retinopathy, cancer, macular degeneration, age-related macular degeneration, fungal, seizure, stroke, depression, hepatitis C, glaucoma, dry eye, Alzheimer's disease, Parkinson's disease, neurological disorders, pain, temporomandibular joint disorders, opioid overdose, rheumatoid arthritis, and the like; or any disease or condition that would benefit from long-term sustained release of an active agent for treatment; and also injuries, fractures and/or other damage to tissues and organs that need regeneration of tissues. The invention can be used in treating any tissue or organ of the body, including but not limited to neurological, eye (including retina), brain, ear, temporomandibular joint, dental, oral, facial, blood, bone, cartilage, joint, heart and vascular system, lung, bronchus, skin, muscle, reproductive, liver, cancer, diabetes, pancreas, gastrointestinal tract, endocrine tissues or glands, kidney, breast, oral, head, neck, esophageal, thyroid, fat, muscle, gastrointestinal stromal, intrahepatic bile duct, bladder, colon, rectum, vaginal, prostate, testicular, pancreas, cervix, uterus, pleura, immune system, and the like.

I. Methods of Use

[0074] The nanogels are administered to a patient in a location such that the biologic will be released near the damaged or injured tissue, or the site of action of the biologic. Preferably, a solution containing the nanogels is injected into the appropriate tissue. The solution used and the injection volume will depend on the concentration of nanogels and the location of the injection. The practitioner is able to determine an appropriate route of administration and amount.

[0075] Nanogels preferably are prepared as a suspension in a solution of water, saline, water and ethanol co-solvent, water and dimethylsulfoxide (DMSO), or the like, as determined by the practitioner, at a concentration of about 0.01 mg/mL to about 1000 mg/mL, preferably about 0.1 mg/mL to about 500 mg/mL, and most preferably about 1 mg/mL to about 250 mg/mL. A dose of the nanogel dispersion generally is given at about 1 μ L to about 20 μ L, and more preferably about 5 μ L to about 10 μ L, depending on the tissue or area of the body to be injected. Thus, an individual dose generally is about 1 mg/kg to about 800 mg/kg.

[0076] Any tissue or organ in need of treatment by the biologic can be treated according to the invention. For example, nanogels can be administered to the eye for

treatment of macular degeneration. See FIG. 21. Other examples include, but are not limited to, type 1 diabetes, type 2 diabetes, diabetic retinopathy, cancer, macular degeneration, age-related macular degeneration, fungal, seizure, stroke, depression, hepatitis C, glaucoma, dry eye, Alzheimer's disease, Parkinson's disease, neurological disorders, pain, temporomandibular joint disorders, opioid overdose, rheumatoid arthritis, and the like; or any disease or condition that would benefit from long-term sustained release of an active agent for treatment; and also injuries, fractures and/or other damage to tissues and organs that need regeneration of tissues.

[0077] Doses are contemplated for administration as frequently as every two or three days, every week or two weeks, every three weeks, every month, every two months, quarterly, or semi-annually, however any dosage schedule is appropriate depending on the release rate, total amount of biologic in the administered biogels, and the condition of the patient.

5. Examples

[0078] This invention is not limited to the particular processes, compositions, or methodologies described, as these may vary. The terminology used in the description is for the purpose of describing the particular versions or embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the present invention, the preferred methods, devices, and materials are now described. All publications mentioned herein, are incorporated by reference in their entirety; nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

Example 1: General Methods

A. Determination of Yield and Loading.

[0079] The yield of the prepared nanogels was calculated gravimetrically after freeze-drying. The percentage of insulin loaded was determined indirectly after measuring the amount of insulin recovered in the dialysis media. The amount of insulin in the lyophilized dialysis media was determined using an ultra performance liquid chromatography (UPLC, Waters ACQUITY UPLC®, Waters Technologies Corporation, Milford, MA, USA) method (see insulin quantitation method below). The percentage yield was calculated as (The amount of nanogels obtained after lyophilization/Theoretical weight of the nanogel×100). Percent insulin loading was calculated as (Total amount of Insulin Loaded–Total amount of Insulin recovered in the dialysis media)/Total amount of insulin loaded×100).

B. Particle Size, Size Distribution and Zeta Potential.

[0080] The particle size, polydispersity index (PDI) and zeta potential of the freshly prepared nanocarriers were measured at a concentration of 1 mg/mL and 25 and 37° C. by using dynamic light scattering using Zetasizer Nano ZS (Malvern Instruments Ltd., Westborough, MA, USA) or ALV dynamic light scattering containing ALV-CGS-8F compact goniometer system, DPSS laser (660 nm, 50 mW), and ALV-5000/EPP multiple tau digital real time correlator

(ALV-Laser Vertriebsgesellschaft m.b.H., Langen, Germany). The sample dispersions were filtered through 0.45 µm syringe filters before the measurements. The nanogels were equilibrated at 37° C., for 10 minutes before starting measurement. Three replicates were performed for each measurement and reported as mean±standard deviation. Atomic Force Microscopy (AFM) with quantitative imaging mode (JPK NanoWizard 4a AFM QI Mode, Bruker, Billerica, MA, USA) was used to measure nanogel particle sizes by plating 10 µg/mL sample dissolved in water on mica substrate. Nanogels on mica were dried by nitrogen air, and when samples were rehydrated when mounted on the AFM and then equilibrated at 37° C. before being imaged. C14 probe was used, 2x2 um map imaged, and several AFM regions of interest (2-3 regions) were captured per nanogel formulation, and 100 total particles sized using ImageJ per formulation, quantities reported as mean±standard deviation.

C. Determination of Lower Critical Solution Temperature (LCST).

[0081] The LCST of the nanoparticles was determined by measuring the size the nanogels using dynamic light scattering (DLS) over a temperature range of 15° C. to 65° C., with 1° C. intervals. Between measurements, the samples were maintained for 3 minutes at that temperature.

D. Determination of the Thermodynamic Stability of Synthesized Nanogels.

[0082] To determine their thermodynamic stability, prepared nanogels are stored in PBS buffer at 37° C. for 4 weeks and the size of nanoparticles is measured at predetermined time intervals. The change in particle size with time is an indicator of nanoparticle stability.

E. MTT Assays

[0083] The toxicity of the nanogels and their degradation product was investigated by MTT assay, as follows: 150 µL of Adult Retinal Pigment Epithelial (ARPE-19) cells or human fetal retinal pigment epithelial (hfRPE) cells were seeded in each well of 96-well plates at 50,000 cells/well density. After 24 hours, 30 µL solution was removed from each well and replaced by 30 µL of medium containing different concentrations of nanogels loaded with and without biologics. After 48 hours, 50 µL of 5 mg/mL MTT was added to every well and incubated at 37° C. for 4 hours. Absorbance was read at 570 nm using a BioTek cytation 5 microplate reader, for all experiments after removing all the wells content and then dissolving the formazan crystals in 150 µL of DMSO.

F. Insulin Quantitation by UPLC.

[0084] During insulin release and stability experiments, measurements of insulin were conducted using an ultra-performance liquid chromatography (UPLC) system (Waters ACQUITY UPLC®, Waters Technologies Corp.) and a photodiode array (PDA) detector. The UPLC was fitted with a reversed phase UPLC column (ACQUITY UPLC® BEH 130 C18 Column 1.7 µm 2.1×100 mm column, Waters Technologies Corp.). A gradient solvent system comprising 0.1% formic acid and acetonitrile (0 min, 20%; 4.5 min, 40%; 5.0 min, 95%; 5.1 min, 98%; 5.5 min, 20%; 6.0 min, 20%) was used as a mobile phase. The flow rate was set at

0.4 mL/minute and the sample injection volume was 5 μ L. Insulin detection was made using the photodiode array detector in the range of 190-400 nm. During the run, the column was maintained at room temperature and the autosampler temperature was set at 15° C. Insulin stock solution (1 mg/mL) was prepared in PBS for the calibration curve and standard samples were prepared by serial dilution of the stock solution (2.5, 5, 10, 15, 20, 25, 50, 75 and 100 μ g/mL). For in vivo experiments, higher level of sensitivity was required and a UPLC-MS method is being developed.

G. Insulin Quantitation by LC-MS/MS.

[0085] ELISA has been used for quantification of insulin in biological tissues. It is a highly sensitive method but lacks the ability to distinguish different insulin variants. As a result, an appropriate LC-MS/MS method was developed for the quantification of insulin extracted from biological tissues. During method development, nonspecific insulin binding to different surfaces and poor insulin fragmentation resulted in poor method sensitivity and reproducibility. Different approaches have been proposed to deal with these problems. Simple approaches include appropriate choice of stationary and mobile phases, sample preparation solvents, and purification and concentration of the extracted insulin using solid phase extraction. Other alternatives include nano HPLC, immunoenrichment (e.g. Mass Spectrometric Immunoassay Technology), reduction of insulin disulfide bonds followed by analysis of the a or b chains, or enzymatic digestion of the insulin and peptide quantitation. However, these approaches require special equipment or instruments and longer processing steps.

[0086] A SCIEX QTRAP® 5500 LC-MS system coupled with an LC-20AD XR Shimadzu HPLC system (Shimadzu USA Manufacturing Inc.), with an SIL-20 AC_{xz} auto sampler and a CTO-20 AC oven, was used for to measure low concentrations of insulin extracted from biological samples. Bovine insulin was used as internal standard. A Waters CORTECS® UPLC® C18+, 1.6 μ m, 2.1×50 mm column was used as a stationary phase and a gradient solvent system comprising 1% acetic acid in water (solvent A) and of 1% acetic acid in acetonitrile (solvent B) was used as a mobile phase at a flow rate of 0.2 mL/min. The gradient system comprised 0.01 min-start; 0.5 min-20% B; 4.5 min-60% B; 7.0 min-60% B; 9.0 min-98% B; 9.5 min-98% B; 9.9 min-20% B; 11 min-stop. The column/oven temperature was set at 55° C. and the sample injection volume was set at 10 μ L. During the experiment 1% acetic acid in 50% methanol was used as a washing solvent.

[0087] Quantitation was conducted in a positive ionization mode and the optimized MS conditions include, curtain gas (CUR), 20 Psi; collision gas (CAD), 8 psi; ion spray voltage (IS), 5500 V; temperature (TEM), 600° C.; ion source gas 1 (GS1), 60 psi; ion source gas 2 (GS2), 60 psi; Entrance potential, 10 V, vertical knob, 1.5; horizontal knob, 5.5. The M6+(968.6 to 226.3 obtained at DP=176 V, CE=53 V, CXP=14 V) and M5+(1162.3 to 226.3 obtained at DP=251.1 V, CE=63 V, CXP=14 V) fragmentation ions were monitored for human insulin and the M6+(956.1 to 226.3 obtained at DP=176 V, CE=51 V, CXP=12 V) fragmentation ion was monitored for the internal standard bovine insulin.

H. Effects of Stationary and Mobile Phases on LS-MS/MS.

[0088] The choice appropriate stationary phase is important for the development of sensitive LC-MS technique for

quantification of peptides and proteins and few specialized columns have been used. As a result, the effects of C-18 Luna and C-18 CORTECS® columns were compared and the CORTECS® column gave a sharper peak that is about 10-fold stronger than the Luna HPLC column (FIG. 2). FIG. 2A provides an MS spectrum of 100 ng/mL insulin using CORTECS® UPLC column; FIG. 2B provides an MS spectrum of 100 ng/mL insulin using a Luna HPLC column. Peak intensity=8.8×10³. Peak intensity=8.4×10⁴. Increasing the mobile phase from 15%-40% to 15% to 70% tremendously improved the sharpness and the strength of the HPLC peaks (FIGS. 3A and 3B).

[0089] In addition, both peak sharpness and strength significantly increased when the percentage of acetic acid in the mobile phase increased from 0.2 to 1% (FIG. 3B, FIG. 3C and FIG. 3D). FIG. 3 shows the LC-MS/MS spectra of 100 ng/mL human insulin showing the effects of mobile phase gradient (varied 0.5 to 0.45 min) and percentage of acetic acid (HAC) as mobile phase moodier on peak sharpness and strength. Phase A=water; phase B=acetonitrile. FIG. 3A: Gradient, 15% to 40%; HAC, 0.2%; peak intensity=1.6×10³; FIG. 3B: Gradient, 15% to 70%; HAC, 0.2%; peak intensity=2.2×10⁴; FIG. 3C: Gradient, 15% to 70%; HAC, 0.5%; peak intensity=3.0×10⁴; FIG. 3D: Gradient, 15% to 70%; HAC, 1%; peak intensity=3.0×10⁴.

[0090] The effect of THF as a mobile phase modifier was also investigated. However, it did not increase the intensity of the insulin LC-MS/MS peak sharpness or strength. Interestingly, investigation of the fragmentation pattern using a Q-TOF, showed that it suppressed the M6+ ion especially at high concentration (FIG. 4). FIG. 4 shows the effect of percentage of THF as a mobile phase modifier on insulin ionization pattern. FIG. 4A: 0% THF; FIG. 4B: 5% THF; FIG. 4C: 10% THF.

I. Assessment of Method Specificity Matrix Effect and Sensitivity.

[0091] Although insulin sample extraction and purification involves protein precipitation steps, due the presence of similar peptides and other components, assessment of insulin in the tissue extracts might have problems of specificity, selectivity and matrix effect. The selectivity and specificity of the developed method in biological sample was assessed using plasma as a surrogate matrix and spiking it with insulin. At the retention times of both human and bovine insulins, no -MS/MS peak was detected indicating that the method is selective to insulin; FIG. 5 provides LC-MS/MS spectra of rat plasma (FIG. 5A) and rat plasma (FIG. 5B) spiked with human insulin.

[0092] Similarly, the selectivity and specificity of the method in the different eye tissue was investigated and no LC-MS/MS peak was detected within the retention time of both human and bovine albumins. However, the intensities of the spiked samples was significantly low compared to the non-spiked samples showing a significant matrix effect and sample loss during sample preparation. To minimize the matrix effect, the tissue extract was further subjected to solid phase extraction (SPE) and in all the ocular tissues isolated SPE significantly reduced matrix effect and improved method sensitivity (FIG. 6). FIG. 5 provides LC-MS/MS spectra of insulin extracted from different eye tissues obtained with and without solid phase extraction. Accordingly, a calibration curve describing the relationship between concentration and AUC was constructed using

DS values indicating the difference would be even higher if PCL macromer with high DS value was used.

Example 11. Effect of Bulk and Surface Modifiers with Different Charges and Zinc on Insulin Release

[0114] Unlike the effect of monomer to crosslinker ratio, the type and DP and DS of the macromer, insulin release from nanogels were significantly influenced by surface and bulk modifiers. Acrylic acid was used as nanogel modifier nanogels surface charge and membrane permeability but is also had significant effect on insulin release. Generally, using a smaller percentage of acrylic acid with respect to NIPAAm significantly enhanced insulin release from the nanogels and insulin release could be controlled from 9 days to 90 days, by simply decreasing the mole percentage of acrylic acid from 10% to 0% (see FIG. 13). This is particularly true that the isoelectric point of insulin is around 5.3 and at the pH where insulin release was investigated (7.2) insulin is negatively charged. Therefore, the negative charge in the nanogel repels the insulin and enhances its release. Contrarily, the positively charged 2-aminoethyl methacrylate significantly slows down insulin release even though the nanogels containing 2-aminoethyl methacrylate have higher DP and DS (which should slow down the release) than the neutral nanogels without charges (see FIG. 14). The error bars stand for standard error.

[0115] Zinc has also been commonly used in different insulin formulations for prolonged insulin action. Particularly it initiates insulin aggregation and slows down its release. Similarly, in our case, addition zinc sulfate during nanogel synthesis considerably slowed down insulin release (see FIG. 13). In addition, its effect was proportional to its concentration. Taken together, insulin release from nanogels is effectively controlled by varying the concentration of acrylic acid with respect to NIPAAm. Zinc sulfate considerably slowed down insulin release from nanogels with effect proportional to the amount of zinc used. 2 amino-allyl methacrylate slowed down insulin release.

Example 12. Toxicity Study

[0116] The nanogels were tested for cytotoxicity as well. All the nanogels were not cytotoxic to the ARPE-19 cells at up to 5 mg·mL⁻¹. The nanogels were tested for cytotoxicity to human adult (ARPE-19) and fetal retinal pigment epithelial (hfRPE) cells at nanogel concentrations of 0, 0.1, 0.2, 0.5, 1, 5, and 10 mg/mL using thiazolyl blue tetrazolium bromide (MTT) assay after 72 hours of incubation at 37° C. and cell seeding density 50,000 cells/cm² in 96 well plates. The toxicity of nanogels synthesized at different monomer to macromer ratios (81A, 72A, 63A, 54A and 45A) and their degradation products, obtained by incubating the nanogels in PBS for up to 4 weeks, was assessed in RPE cells (FIG. 15) by MTT assay. All of the nanogels were safe at a concentration of 1 mg/mL. In addition, the effect of the type of macromer and modifier on insulin toxicity was investigated using MTT assay. Generally, PCL based, negatively charged nanogels showed a relatively better safety profile than PLA based positively charged nanogels (FIG. 16).

Taken together, the nanogels were not cytotoxic to the ARPE-19 cells in all tests with cell viability more than 90% at concentration up to 2 mg/mL except for the nanogels made of PLA-containing macromer and positively charged 2-aminoethyl methacrylate. The nanogels made of PCL-containing macromer and negatively charged acrylic acid are not cytotoxic at concentration up to 10 mg/mL. FIG. 15 shows the percent cell viability after an MTT assay of RPE cells exposed to varying concentrations of nanogels synthesized at different monomer to crosslinker ratio. FIG. 16A and FIG. 16B show the percent cell viability after an MTT assay of RPE cells exposed to varying concentrations of nanogels synthesized using PLA and PCL based macromers and using acrylic acid (A) or 2 amino ethyl methacrylate (B) as nanogel bulk and surface modifiers.

Example 13. Fluorescent Labeling of Nanogels

[0117] 5-(4,6-Dichlorotriazinyl) Aminofluorescein (5-DTAF) labeled nanogels were prepared by dropwise addition of 3.3 mg 5-DTAF dissolved in 0.2 mL DMSO into 2 mL of 50 mg/mL blank nanogel dispersion in sodium carbonate buffer (0.1 M, pH 9), while stirring. The reaction was carried out at 4° C. overnight. 5-(and-6)-Carboxytrimethylrhodamine (5 (6)-TAMRA) labeled nanogels were prepared by Steglich Esterification. Briefly, 5.56 mg of 5 (6)-TAMRA (1 eq.), 4.3 mg DCC (1.1 eq.) and 9.8 mg 4-dimethylaminopyridine (DMAP)(5 eq.) were dissolved in dichloromethane and stirred at room temperature in the dark for 6 hours. Then the mixture was added dropwise, while stirring, to 185.8 mg nanogel dissolved in dichloromethane and was further stirred at room temperature in the dark overnight. Finally, the nanogels were dialyzed using a 1 KDa MWCO dialysis membrane against DI water for 24 hours, changing the dialysis media every 8 hours, and then lyophilized. The nanogel-dye conjugations were carried out under mild conditions to minimize degradation of the labile lactide bond of the nanogels.

Example 14. In Vitro Permeability of Nanogels

[0118] FITC-dextran (4 kDa) or DTAF labeled nanogels at 1 mg·mL⁻¹ were used for permeability studies. Transwells with 0.45 um pore size were used for the in vitro study and Valia-Chien diffusion cells were used for the ex vivo study. All the permeability studies were done over 4 hours.

[0119] The studies were performed as follows. ARPE-19 cells were seeded at a density of 50,000 cells/well on transwell inserts and cultured in DMEM-F12 medium containing 10% FBS and 1% penicillin-streptomycin (10,000 U/mL) at 37° C. with 95% humidity and 5% CO₂. The medium in the wells was changed every other day until maximum TEER value was obtained. 5-DTAF labeled nanogels made of DEX-PCL-HEMA with various charges were suspended in cell culture medium at concentration of 1 mg·mL⁻¹. Negative and positive controls were media without nanogels and FITC-labeled dextran with 4 kDa molecular weight. Transport experiments were conducted in the apical to basal direction at 37° C. for 4 hours. At selected time points 5, 15, 30, 45, 60 minutes and every 30 minutes

thereafter, 50 μL medium was taken from the basolateral chamber and replaced with 50 μL of fresh medium. Medium (50 μL) was taken from the apical chamber at the 4 hour time point as well. Aliquots were quantitated using Cytaction™ 5 (BioTek, Winooski, VT) at excitation and emission wavelengths of 485 nm and 528 nm, respectively. The permeability (P_0) of the nanogels across the monolayer was calculated by the following formula:

$$\text{Permeability Coefficient } (P_0) = \frac{\text{Flux } (\text{J})}{\text{Donor Concentration}} = \frac{(F_r/\Delta t)/A_d}{F_d/V_d}$$

where P_0 , F_d , V_d , F_r and A_d are the permeability coefficient, the basolateral fluorescence of the solute over Δt time, the fluid volume of the basolateral chamber, the apical fluorescence of the solute, and the surface area of the filter, respectively.

[0120] FIG. 17 shows that the permeability across the ARPE-10 cell membrane of neutral and negatively charged nanogels was higher but that of positively charged nanogels was lower than the 4 kDa dextran control. The permeability of negatively charged nanogels with 2 and 10 mol % AA was higher than that of the neutral nanogels and the nanogels with 5 mol % AA. The permeability results correlate well with the cellular uptake of the nanogels by ARPE-19 cells within 4 hours. The greater the amount of nanogels taken up the cells, the more permeable are the nanogels. Due to the binding interaction of the positively charged nanogels with the negatively charged cell membrane, the uptake and permeability of the nanogels were lower than the neutral nanogels. The nanogel particle size increases with increase the charge amount and the aggregation of the nanogels increase with decreasing the charge amount and increasing temperature from 25 to 37° C. Due to the balance of the size and charge, the nanogels containing 5 mol % AA had lower cellular uptake and permeability than the nanogels containing 2 and 10 mol % AA; and there is not much difference in permeability between the positively charged nanogels with 5 and 10 mol % AM.

Example 15. Ex Vivo Permeability Study

[0121] Nanogels made of DEX-PLA-HEMA with 2 mol % acrylic acid was more permeable than those with 5 mol % acrylic acid and 4 kDa dextran control across the ex vivo porcine sclera and cornea tissues. See FIG. 18A and FIG. 18B. The nanogel charge, particle size and composition played important roles on nanogel permeability across ocular barriers.

Example 16. In Vivo Ocular Nanogel Distribution

[0122] 5-DTAF labeled 72A nanogel was dispersed in PBS (20 mg/mL) and was injected subconjunctivally (20 μL) to the left eyes of anaesthetized SD rats (n=5) using a 25 gauge Hamilton™ syringe and needle. PBS was injected to the right eyes of the rats to be used as sham controls. After 1 or 7 days of injection, the rats were euthanized, their eyes were inoculated, and the different eye tissues (aqueous

humor (AH), vitreous humor (VH), cornea, lens, Iris/ciliary body (CB), sclera, choroid and retina) were collected in a 2 mL flat bottom Eppendorf™ microcentrifuge tube and were kept at -80° C. until tissue homogenization and nanogel extraction. Before homogenization, the tough tissues (lens, cornea and sclera) were ground by a CryoGrinder™ (OPS Diagnostics LLC) at cryogenic temperatures. During tissue extraction, all the tissues were kept on an ice bath and 230 μL cold PBS was added and the samples were homogenized (except the AH and VH) twice for 15 seconds using a rotor-stator (Bio-Gen PRO200 homogenizer, PRO Scientific Inc.) attached with a 5-mm flat bottom generator probe, at a medium speed, with 10-second intervals between the two homogenization steps. Between homogenization of different tissues, the homogenizing probe was rinsed in DI water, followed by 3 washes in 70% ethanol and a final rinse in DI water, and was wiped and dried.

[0123] Prior to the homogenization step, to offset the effect of dilution, sample loss & nanogel tissue binding, a fixed amount of rhodamine (TAMRA) labeled nanogel was added as an internal standard (20 μl , 2 mg/mL TAMRA labeled nanogel dispersed in PBS). The homogenate was then centrifuged for 5 minutes at 5000 rpm and the supernatant was put in a 96-well plate and the fluorescent intensity was read at excitation/emission wavelength of 492/519 nm (DTAF) and 541/565 (TAMRA) using a fluorescent reader (Cytaction® 5 Cell Imaging Multi-Mode Reader, BioTek Instruments Inc.). A calibration curve of the DTAF label was constructed at a concentration of 5 to 100 $\mu\text{g/mL}$ after adding 160 $\mu\text{g/mL}$ of TAMRA-labeled nanogel as an internal standard.

Example 17. In Vivo Nanogel Ocular Distribution after Subconjunctival Injection

[0124] An efficient tissue homogenization and nanogel extraction method was developed for the quantitation of nanogels in different ocular tissues. The nanogel was labeled with the fluorescent dye 5-DTAF (absorption/emission maxima of ~492/516 nm) to be able to track its distribution in the tissues. Nanogel recovery from the different ocular tissue homogenates spiked with 5-DTAF-labeled nanogel ranged from 76.6% to 88.2% (see Table 10, below). The nanogel recovery after tissue spiking before homogenization step was lower (49.6%-77.7%). To improve the recovery and reproducibility of the results, 5-(6) TAMRA-(absorption/emission maxima of ~557/583 nm) labeled nanogel was used as an internal standard. 5-(6) TAMRA-labeled nanogel was chosen over the free dye assuming that the nanogel undergoes similar degree of tissue binding and precipitation. In addition, the two dye-labeled nanogels had different absorption and excitation wavelengths and minimal quenching and interference during measurements occurs. Protein precipitation using organic solvent also significantly reduced the nanogel recovery, and the loss was higher with acetonitrile.

protein kinase, a cytokine, a growth factor, a differentiation factor, a neurotrophic factor, a stem cell factor, a fusion protein, a carbohydrate, a polysaccharide, a lipid, a lipopolysaccharide, a glycosaminoglycan, a steroid, a nutrient, a tumor necrosis factor (TNF) inhibitor, an interleukin (IL) inhibitor, a B-cell inhibitor, or a T-cell inhibitor, or a combination thereof.

3. The nanogel pharmaceutical composition of claim 2 wherein the biologic medicament is selected from the group consisting of a peptide hormone, an antibody, an aptamer, and an siRNA or a combination thereof.

4. The nanogel pharmaceutical composition of claim 1 wherein CLU is a hydrolytically or enzymatically degradable, crosslinked nanogel.

5. The composition of claim 1, wherein the initiator is 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone.

6. The composition of claim 1, wherein the monomer is N-isopropylacrylamide.

7. The nanogel pharmaceutical composition of claim 1 wherein the biologic medicament is released from the nanogel for at least 20 days.

8. The nanogel pharmaceutical composition of claim 1 wherein the biologic medicament is released from the nanogel for at least 30 days.

9. The nanogel pharmaceutical composition of claim 1 wherein the biologic medicament is released from the nanogel for at least 60 days.

10. The nanogel pharmaceutical composition of claim 1 wherein the biologic medicament is released from the nanogel for at least 90 days.

11. The nanogel pharmaceutical composition of claim 1 wherein the biologic medicament is loaded in the nanogels in aqueous solution with at least 50% loading efficiency.

12. The nanogel pharmaceutical composition of claim 1 wherein the biologic medicament is loaded in the nanogels in aqueous solution with at least 80% loading efficiency.

13. The nanogel pharmaceutical composition of claim 1 wherein the biologic medicament is loaded in the nanogels in aqueous solution with at least 95% loading efficiency.

14. The method of treating a subject in need of a biologic medicament, comprising administering to the subject a pharmaceutical composition of claim 1.

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