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(54) Title: PEGYLATION OF A TRAIL LIGAND

(57) Abstract: The present invention reports polymeric conjugates of proapoptotic ligands of programmed death receptors Tumor Necrosis Factor (KillerTRAIL) for antitumor and/or anti-inflammatory therapy.

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PEGYLATION OF A TRAIL LIGAND

Technical field of the invention

The present invention relates to polymeric conjugates of KillerTRAIL to be used in antitumor and/or anti-inflammatory therapy.

Known art

The use of biotechnological drugs in the pharmaceutical sector represents the greatest innovation and therapeutic opportunity of the last decade. The compounds with a peptide and protein structure have a high selectivity and pharmacological activity, properties that make them therapeutic agents of first choice in the treatment of serious diseases such as tumors. However, despite the high pharmacological potential, the clinical use of these therapeutic agents is often limited by their inadequate physico-chemical and biopharmaceutical characteristics. The high structural complexity makes the protein macromolecules not very stable and easily inactivated by both physical, chemical and enzymatic processes. These processes can occur during the various stages of formulation development, or during storage or administration. The rapid elimination of these drugs from the systemic circulation, through various mechanisms including glomerular ultrafiltration and hepatic*uptake*, significantly reduces their bioavailability. The macromolecular character and the high hydrophilicity also prevent absorption through biological membranes and make their administration difficult. Finally, the intrinsic immunological characteristics of these macromolecules, such as immunogenicity and antigenicity, often represent an important limitation for their therapeutic use.

The biopharmaceutical problems that limit the use of protein drugs can be partially overcome by creating formulations that allow to improve their stability and, in some cases, to control their release rate, reduce side effects and optimize their therapeutic dose, with a consequent improvement in patient *compliance*.

Among the techniques used for the formulation of protein drugs currently known is the surface modification of proteins with biocompatible, biodegradable and soluble polymers, such as polyacrylates, polyalkylglycols, polysaccharides, polysialic acid, etc.

Components of thefamily of cytokines *Tumor Necrosis Factor* act as mediators of the immune system and inflammatory response. Their involvement in septic shock, autoimmune diseases and immune reactions related to transplant rejection has in fact been demonstrated (Wiley SR et al., Immunity. 1995; 3 (6): 673-82).

Among the members of TNF, the ligands Apo2 or Apo2L or TRAIL, (all the indicated terms are to be considered synonyms) and KillerTRAIL are mentioned as relevant for the present invention.

The ligand Apo2L *wt* that induces apoptosis on CD253 is a type of trans-membrane protein II of about 34 kDa, identified in the years 1995/1996 (Pitti RM et al., J. Biol. Chem. 1996; 271 (22) : 12687-90, Wiley SR et al., Immunity. 1995; 3 (6): 673-82). Apo2L or TRAIL is a protein consisting of an amino acid sequence of 281 aa in human form and has a molecular weight of approximately 32.5 kDa. TRAIL is characterized by a transmembrane domain, an extracellular C-terminal domain rich in cysteines and conserved among members of the same family and a cytoplasmic N-terminal domain that instead presents unique characteristics. Crystallographic studies have identified its structure as homotrimeric with three cysteine residues in position 230 of each sub-unit of the homotrimer, coordinated with each other through a Zn atom, which is important for the stability and biological activity of the trimer (Hymowitz SG et al., Molec. Cell. 1999; 4 (4): 563-71; Bodmer JL et al., J. Biol. Che., 2000; 275 (27): 20632-7).

The patent document WO2004001009 describes variants of the Apo2L/TRAIL protein and reports the derivatization with polyethylene glycol (PEG) performed on cysteines inserted in the chain of the variants of TRAIL in position 170 or 179 (indicated in the document respectively as PEG-R170C-Apo 2L and PEG -K179C-Apo 2L). PEGylation, which affects the

cysteine residues, is considered useful only if carried out on the cysteines inserted in the variants in position 170 and 179 in order to preserve the cysteine in position 230, considered fundamental for the biological activity and therefore not modifiable in order not to modify the homotrimeric structure and the corresponding activity of the protein.

This binding site has been shown to be important for protein activity. In fact, the substitution of cysteine 230 (Cys 230) determines an 8-fold reduction in apoptotic activity. Furthermore, removal of the coordinated metal from the homotrimer cystines causes a 7-fold decrease in affinity for DR5 and a 90-fold reduction in apoptotic activity. Furthermore, after the removal of the coordination Zn, the cysteines can be oxidized and Apo-2L dimers bonded with disulfide bridges are formed, which reduce apoptotic activity. The binding site of the coordination metal is hidden within the structure of the Apo-2L trimer and thus prevents its interaction with the receptor.

As reported in WO2004001009 and in the literature prior to it (Hymowitz SG et al., Biochemistry, 2000; 39 (4): 633-40; Bodmer et al., J. Biol. Chem., 2000; 275 (27): 20632-7), the bond with the divalent metal ion appears to be of fundamental importance to maintain the structure of the trimer and the stability of Apo-2L and the consequent biological activity. In document WO2004001009, therefore, a derivatization with PEG is carried out on the cysteines inserted in the polypeptide variants of Apo2L in order to maintain the trimerization and evaluate their therapeutic efficacy. The derivatizations with PEG considered particularly interesting are those carried out on the mutations in which the cysteine residues are in position C170 and C179.

The insertion of further cysteines in addition to the naturally present Cys 230, and their corresponding derivatization through PEG actually produce an improvement in the therapeutic activity, which however does not seem to be considered by the inventors to be optimal for use in the pharmaceutical field, as despite some potentially encouraging results, even if obtained by modifying the peptide chain of the native protein, no other

research published later has been carried out. In fact, making changes on the native protein chain by substituting one or more amino acids involves the *ab origine* manipulation of the genetic material. This situation complicates the processing and significantly increases the production costs and the risks of administration (in fact the metabolic characteristics and the potential adverse effects, especially hepatic, of the varied protein are not known). Furthermore, the improvement of the activity obtained through the changes reported in the aforementioned document is not considered such as to justify the risk of use.

Among all the recombinant versions of TRAIL a soluble form bearing a polyhistidine tag is known (Jo M et al., Nat. Med., 2000; 6 (5): 564-7; Nagata S, Nat. Med., 2000; 6 (5): 502-3).

The known compound monomeric human KillerTRAIL and present on the market (http://www.enzolifesciences.com/ALX-201-073/killertrail-protein-soluble-human-recombinant/) manufactured by E. *coli*, lends itself to being stabilized and conveyed after conjugation with biopolymers. This variant of KillerTRAIL is active on all human and murine receptors and on the osteoprotegerin receptor.

The ferry and therapeutic potentials of this ligand are manifold. However, the problem of its toxicity remains, linked to the aggregation of the protein after systemic administration, which often prevents the continuation of therapy due to the triggering of an immune response towards the same treatment (Le Blanc HN, Ashkenazi A, Cell. Death Differ., 2003; 10 (1): 66-75; Pan LQ et al., Sci. Rep., 2015; 5: 14872; Almasan A, Ashkenazi A, Cytokine Growth Factor Rev., 2003; 14 (3-4): 337 -48).

From the above considerations it is clear that the conjugation of KillerTRAIL with a polymer can bring benefits, but the complex structure of this protein and its mechanism of action make this approach difficult to implement.

The inventors have now found polymeric systems that simultaneously shield the protein by protecting it from enzymatic degradation and preventing its aggregation. It has also been unexpectedly

found that not only the new polymeric derivatives do not preclude the activity of KillerTRAIL but rather, they reduce its short and long term toxicity. Indeed, it has now been found by the authors that KillerTRAIL, conjugated with biopolymers, not only maintains the therapeutic activity of the native protein, but unexpectedly increases its circulatory half-life and prolongs the administration time.

Summary of the invention

The object of the present invention is therefore a method for obtaining KillerTRAIL polymeric conjugates in which the functionalization of the protein is performed at the level of the cysteine site Cys 230. In this way the targeting and therapeutic potential of this ligand is better exploited, significantly reducing the negative effects on specific tissues of the organism through a *drug delivery* strategyobtained through polymeric conjugation.

Another object of the present invention is a conjugate of KillerTRAIL which overcomes the disadvantages of the known art and is used for the treatment of tumors and autoimmune diseases.

Still another object of the invention are the pharmaceutical and diagnostic compositions comprising the polymeric conjugate of KillerTRAIL.

A further object of the invention is the use of the conjugate for the diagnosis of tumors and autoimmune pathologies. In fact, the cytokine TRAIL, as well as its receptors, is involved in both tumorigenesis (for example, ductal adenocarcinoma of the pancreas, neuroendocrine tumors, non-small cell lung cancer, large cell carcinoma, adenocarcinoma, carcinoma of the uterine cervix, mesothelioma, rhabdomyosarcoma, gliomas and their subtypes, chronic lymphoid leukemia) and in the development and progression of various autoimmune diseases (e.g., multiple sclerosis, autoimmune thyroiditis, Crohn's disease, active ulcerative colitis, autoimmune encephalomyelitis, atopic dermatitis, systemic lupus erythematosus , rheumatoid arthritis, autoimmune arthritis, autoimmune diabetes) (Lin WW, Hsieh SL, Biochem. Pharmacol., 2011; 81

(7): 838-47; Schaefer U et al., Front. Biosci., 2007; 12: 3813) . For this reason, the conjugate of the invention can be advantageously used both for the early diagnosis and in the treatment of pathologies in which the cytokine TRAIL is involved. The radiolabeled conjugate of the invention is therefore considered a useful tool in order to "follow" the same protein to the *target*, in this case the tumor tissue or the tissue affected by the autoimmune pathology and, to determine the pathological state and the subsequent therapeutic procedure. This is made possible by the presence of the polymer conjugated to the cytokine KillerTRAIL which prevents premature degradation and metabolization, without hindering or compromising *targeting* and therapeutic effect.

Still another object is a pharmaceutical or diagnostic *kit* comprising the KillerTRAIL polymeric conjugate, which can be administered in a hospital setting for the preventive diagnosis of such pathologies.

Further objects, objects and advantages will become apparent from the detailed description of the invention which follows.

Brief description of the figures

Figure 1. Synthesis scheme of mPEG-KillerTRAIL.

Figure 2. Reverse phase HPLC analysis (RP-C18) of the conjugation chemical reaction between KillerTRAIL and mPEG-maleimide at different incubation times (0, 4, 7 h). Key to the synthesis process: A) KillerTRAIL mono-PEGylated; B) KillerTRAIL bi-PEGylated.

Figure 3. SEC analysis of the purified derivative mPEG-KillerTRAIL at a concentration of 1 mg/mL. Legend: A) Refractive index; B) RALS; C) DP viscometer; D) LALS; E) UV.

Figure 4. Mass spectrum of the mPEG-maleimide structure (A, 2119.4 Da) and of the mPEG-KillerTRAIL conjugate (B, 24008.6 Da) by KillerTRAIL mass spectrometry**.**

Figure 5. SDS-PAGE of the conjugation reactions of KillerTRAIL with PEG 20 kDa as an indicative example). The reaction was performed under

reducing (RB) and non-reducing (NRB) conditions. KillerTRAIL was used as a reference standard to monitor the reaction process.

Figure 6. Cell viability inhibition *In vitro* and relative anti-tumor activity of KillerTRAIL and its derivative mPEG-KillerTRAIL on human colon cancer cells HCT-116 and human keratinocytes NCTC2544.

Figure 7. Pharmacokinetic profile of KillerTRAIL and its derivative mPEG-KillerTRAIL in blood and tissues: KillerTRAIL (A); mPEG-KillerTRAIL (B); plasma concentration of KillerTRAIL and its derivative mPEG-KillerTRAIL at different incubation times (C).

Figure 8. Biodistribution of KillerTRAIL (A) and the derivative mPEG-KillerTRAIL (B) in BALB/c nude mice implanted with HCT116 tumor cells (3 × 10^6 cells/ml) inoculated subcutaneously to favor the development of the tumor mass . (C) Summary graph of the percentage concentration of native protein accumulated in blood, liver and tumor mass of BALB/c nude mice implanted with HCT116 tumor cells (3 × 10^6 cells/ml) and treated with KillerTRAIL (A) and with the derivative mPEG-KillerTRAIL (B). KillerTRAIL (A) and mPEG-KillerTRAIL (B) were administered in the above-mentioned mouse models intra-peritoneally (ip) with a protein equivalent dose corresponding to 0.04 mg/kg of KillerTRAIL per day for 4 days.

Figure 9. anti-tumor activity *In vivo* of KillerTRAIL and mPEG-KillerTRAIL in mouse models implanted with HCT116 tumor cells. The *in vivo* antitumor activity was evaluated by measuring tumor growth for 20 days after treatment of mouse models with KillerTRAIL and mPEG-KillerTRAIL administered ip at the equivalent dose of 0.04 mg/kg/day.

Figure 10. Comparison between the activity data obtained by the present invention (panel A reproduced from Figure 9) and those contained in the patent WO2004001009A2 (panel A) relating to the PEGilata Apo-2L R170C variant and (panel B) relating to the PEGilata Apo- 2L K179C.

Figure 11. Mass spectra of the sequences relating to tryptic peptides, the sequences of which are reported in Table 1, obtained by hydrolysis in trypsin of the KillerTRAIL protein for MS-Digest analysis. Panel A and B: mass spectra of the sequences aa in position 125-130 (Table 1); CD panel:

mass spectra of the aa sequences in position 80-85 (Table 1); Panel EF: mass spectra of the aa sequences in position 56-61 (Table 1); GH panel: mass spectra of the aa sequences in position 5-12 (Table 1); Panel IJ: mass spectra of the aa sequences in position 170-177 (Table 1); Panel KL: mass spectra of the aa sequences in position 111-118 (Table 1); Panel MN: mass spectra of the aa sequences in position 28-38 (Table 1); OP panel: mass spectra of the aa sequences in position 39-50 (Table 1); QR panel: mass spectra of the aa sequences in position 159-169 (Table 1); ST panel: mass spectra of the aa sequences in position 143-155 (Table 1); UV panel: mass spectra of the aa sequences at position 85-97 (Table 1); Panel WX: mass spectra of the aa sequences at position 162-177 (Table 1); WZ panel: mass spectra of the aa sequences at position 20-36 (Table 1).

Figure 12. Mass spectra of the sequences relating to tryptic peptides, the sequences of which are shown in Table 4, obtained by hydrolysis of the KillerTRAIL protein in trypsin for MS-Digest analysis.

Figure 13. ESI MS mass spectrum related to the KillerTRAIL sample. Panel B: mass spectrum with high instrumental signal (TIC, *Total Ion Current*); Panel C: mass spectrum of the instrumental low signal protein (TIC, *Total Ion Current*); Panel A: molecular weight of the protein obtained following the deconvolution process applied to the mass spectra reported in panels B and C.

Detailed Description of the Invention

With the aim of overcoming the critical problems typical of therapeutic proteins such as rapid elimination, degradation and toxicity deriving from the formation of immune complexes, KillerTRAIL was derivatized in a single process of conjugation with a polymer chain.

Therefore, according to the present invention, the new molecular entity composed of the polyethylene glycol polymer and the single-stranded KillerTRAIL protein, linked by a stable covalent bond of the CS type on the cysteine in position 230 of the KillerTRAIL, is defined as the polymeric conjugate of KillerTRAIL. *Tumor Necrosis Factor* (KillerTRAIL) Enzo data

sheet (http://www.enzolifesciences.com/fileadmin/reports/Datasheet-ALX-201-073.pdf).

Tumor Necrosis Factor (KillerTRAIL) produced by Enzo Life Sciences, Inc. or Apo-2L, TNFSF 10, CD253 has a molecular weight of ~ 24kDa, calculated by the manufacturing company using the *Sodium Dodecyl Sulphate – PolyAcrylamide Gel ElectrophoresisSDS* orPAGE method (http://www.enzolifesciences.com/fileadmin/reports/Datasheet-ALX-201-073.pdf), and referred to the molecular weight of the commercial monomer. The KillerTRAIL protein is produced using as a cloning vector *E. coli*. The extracellular domain of the human TRAIL protein, which has the specific sequence constituted by aa 95-281, is fused in its N-terminal domain with a His-tag and a peptide-structured spacer.

The KillerTRAIL comprises the amino acid sequence aa 95-281 (SEQ.ID.N.
TSEETISTVQEKQQNISPLVRERGPQRVAAHITGTRGRSNTLSSPNSKNE KALGRKINSWESSRSGHSFLSNLHLRNGELVIHEKGFYYIYSQTYFRFQE EIKENTKNDKQMVQYIYKYTSYPDPILLMKSARNSCWSKDAEYGLYSIYQ GGIFELKENDRIFVSVTNEHLIDMDHEASFFGAFLVG 1) as official database Expasy.org.

The present invention therefore relates to the realization of a polymeric conjugate of the pro-apoptotic therapeutic protein of the exogenous type, the cytokine KillerTRAIL, which makes it possible to make its tertiary structure more stable. This cytokine induces apoptosis upon binding to pro-apoptotic transmembrane receptors.

The aim achieved by the present invention was to reduce the toxicity problems (hepatic) and low half-life shown by KillerTRAIL through a conjugation on a polymeric *backbone* at the level of Cys 230. The conjugation was obtained by binding with an alkoxy - preferably methoxy-polyethylene glycol (mPEG) derivatized with a thiol reactive group, preferably a maleimide. The molecular weight of the PEG was preferably of a magnitude comprised between 2-200 kDa, and preferably 10-50 kDa.

The process for obtaining the derivative according to the invention involves the thiol of the cysteine in position 230 of the peptide chain of KillerTRAIL with an alkoxy-PEG through the maleimide reactive group of the PEG. The reaction can take place in an aqueous environment in a single *step*, preferably after the prior purification of the native KillerTRAIL, for example by dialysis against PBS (pH 7.4) containing EDTA to allow the elimination of dithiothreitol (DTT) used as a preservative in the preservation of KillerTRAIL and in the case of the reaction, to avoid the spontaneous formation of disulfide bridges and aggregation between the protein monomers.

The alkoxy-PEG has the alkoxy group in alpha and is preferably the methoxy-PEG (mPEG) and the reactive group towards cysteine is in omega. The PEGs with reactive groups that can be used can be chosen from: PEG-Maleimide or mal-PEG, PEG-orthopyridine disulfide (OPSS-PEG), PEG iodine-acetamide (IA-PEG) and PEG-vinyl sulfone (VS-PEG) . Among these, mal-PEG is preferred which among all guarantees a stable, non-*cleavable bond in vivo*. Therefore the preferred conjugation group for KillerTRAIL is methoxy-PEG-maleimide conjugated on KillerTRAIL via the thiol -SH group of the amino acid cysteine in position 230.

It is clear that PEGylation can occur using different methods, through a series of conventional reactions. For example, an N-hydroxysuccinimide ester of a PEG (M-NHS-PEG) can be prepared from PEG-monomethyl-ether (which is commercially available from Union Carbide) by reaction with N,N'-dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS), according to the method of Buckmann et al. (Buckmann AF, et al., Macromol. Chem. Phys. 1981; 182 (5): 1379-84). Furthermore, a PEG terminal hydroxy group can be converted to an amino group, for example by reaction with thionyl bromide to form PEG-Br, followed by aminolysis with excess ammonia to form PEG-NH_2. The PEG-NH_2 is then conjugated to the protein of interest using standard coupling reagents, such as Woodward's K reagent. Furthermore, a terminal group PEG-CH_2OH can be converted into an aldehyde group, for example by oxidation with MnO_2. The aldehyde

group is conjugated to the protein by reductive alkylation with a reagent such as cyanoborohydride. Alternatively, activated PEGs suitable for use in the present invention can be purchased from a number of vendors. For example, Shearwater Polymers, Inc. (Huntsville, Ala.) sells methoxy-PEG-maleimide, MW 2000, as well as a methoxy-PEG succinimidyl carbonate and methoxy-PEG succinimidyl propionate. In any case, our choice fell on a more site-specific type of bond, as generally the cysteines (and therefore the thiol groups) are in a reduced quantity compared to the other amino acids and in this specific case KillerTRAIL only has a. In fact, it is preferable to avoid random bonds on endogenous proteins.

The PEGylated protein can be characterized by SDS-PAGE, gel filtration, NMR, peptide mapping, liquid chromatography-mass spectrophotometry *in vitro and biological assays*, all methods known to those skilled in the art. The polymeric complex according to the present invention was characterized by SDS-PAGE, colorimetric assays, Gel Filtration/Dimensional Exclusion chromatography with downstream tetradetector (DLS, viscometer, UV/Vis, Refractive index), high performance chromatography, MALDI-TOF mass and in vitro biological assays. The first phase of processing consists in the purification of the protein by dialysis (regenerated cellulose *cut-off* 10000 Da) to eliminate the solution in which the protein has been stored (Salts, ethylenediaminetetraacetic acid EDTA, dithiothreitol DTT) in which it is marketed. Subsequently, a treatment of the protein solution with 8 mM DTT alone was carried out at room temperature for 1 hour in order to avoid the spontaneous formation of disulfide bridges that would lead to the formation of the protein in the form of a trimer. To maintain the optimal conditions for the conjugate production, the protein solution from the DTT was further purified by desalting chromatography column. Under these experimental conditions the KillerTRAIL protein is found in monomeric form.

At this point the functionalized PEG and the KillerTRAIL protein thus treated and purified, is solubilized in aqueous environment in the presence of a molar excess of PEG (typically in a molar ratio 4/1 during the synthesis

phase of the derivative) in buffer, preferred is the phosphate buffer (pH 7.4) and are left under continuous stirring at room temperature, typically for about 7 hours. The reaction can be monitored by high performance chromatography (HPLC) in reverse phase (on column C18) from time t = 0 h and at time t = 7 h.

KillerTRAIL PEGylate is purified for example by gel filtration chromatography to eliminate unreacted PEG. At the end of the reaction process there will be a 1/1 molar ratio between PEG and native protein with an overall reaction yield equal to 70%.

KillerTRAIL PEGylate is then lyophilized and can be stored at a temperature of -20°C for subsequent uses.

The reaction takes place easily in an aqueous environment allowing the selective binding of the PEG with the thiol of the cysteine in position 230 of the peptide chain, by means of the preferred maleimide reactive group.

This reaction can take place in a single *step*, after the purification of the native KillerTRAIL by dialysis against PBS (pH 7.4) containing 0.05 mM of EDTA to allow the elimination of dithiothreitol (DTT), used as a preservative during the preservation of the protein.

The present invention therefore reports a KillerTRAIL polymeric conjugate capable of preserving at least 5% of the activity of the native protein, more preferably 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 110%, 120% or 130% of the activity of native, i.e. unconjugated, KillerTRAIL.

The polymer conjugated to the protein preferably has an average molecular weight (MW) between 200 dalton (Da) and 300000 Da, more preferably between 3000-100000 Da, even more preferably between 5000-80000 Da, even more preferably between 10000-40000 Da , and even more preferably between 10000-30000 Da, and more preferably 20,000 Da.

In preferred forms of the invention the polymer is selected from among those of the following group: poly (ethylene glycol) (PEG) of linear or branched structure*(branched)*,preferably monofunctional, preferably having a molecular weight (MW) average of between 2000 and 40000 Da

Da, more preferably between 2000 Da and 30000 Da and even more preferably between 5000 Da and 20000 Da. The most commonly used polyethylene glycols or PEGs are: mPEG-Maleimide 10000 Da, mPEG-Maleimide 20000 Da, mPEG-Maleimide 40000 Da, mPEG -Maleimide *branched* 10000 Da, mPEG-Maleimide *branched* 20000 Da and mPEG-Maleimide *branched* 40000 Da.

The polymeric conjugates obtained, of which the general name will be hereafter for simplicity and not for limitation mPEG-KillerTRAIL, can be used for all those pathologies in which the presence of programmed cell death receptors (Apoptosis), implicated in a series of tumor, immune, angiogenic and *turnover* bone pathologies was evaluated. KillerTRAIL can bind to five different receptors found on a variety of cell types: four membrane receptors and one soluble. Two of these membrane receptors, TRAIL-R1/death receptor 4 (DR4) and TRAIL-R2/death receptor 5 (DR5), act as agonist receptors, containing a cytoplasmic death domain through which KillerTRAIL can transmit an apoptotic signal. The other two membrane receptors, TRAIL-R3/*decoy receptor* 1 (DcR1) and TRAIL-R4/*decoy receptor* 2 (DcR2), act as antagonist/regulatory receptors, devoid of the death domain. In addition to these four transmembrane receptors, a soluble fifth antagonist receptor osteoprotegerin (OPG), responsible for, has been identified *turnover* bone. The pathologies to which we mainly refer are various types of solid and liquid tumors. The over-expression of the listed receptors is present in many pathologies listed in table 1 and, without constituting a limitation, these pathologies can be advantageously treated with the mPEG-KillerTRAIL according to the invention:

Table 1: Some pathologies whose cells over express death receptors related to the cytokine KillerTRAIL.

Leukemia
Breast cancer to
Cancer prostate
Cancer lung
Renal cancer
Pancreatic cell carcinoma
Alzheimer
Melanoma
Myelodysplastic syndrome
Lymphoma
Liver cancer
Randomiosarcoma
Glioma
Non-small cell lung cancer
Hepatitis B Virus
Hepatitis C Virus
liver steatosis non-dependent from taking alcohol
Fibrosis
Cirrhosis

Mesothelioma
Pulmonary Arterial Hypertension
Systemic sclerosis
Cervical carcinoma
Pulmonary fibrosis
Asthma
Flu syndromes
Atherosclerosis
Systemic lupus erythematosus
Multiple sclerosis
HIV
Amyotrophic lateral sclerosis
Cytomegalovirus
Rheumatoid arthritis
Myasthenia gravis

Among the most studied we report pancreatic carcinoma (ductal adenocarcinoma of the pancreas and neuroendocrine tumors, which originate from the cells of the islets of Langerhans), non-small cell lung cancer (squamous cell carcinoma, large cell carcinoma and adenocarcinoma that in turn is subdivided into sarcomatous and pulmonary lymphomas), carcinoma of the uterine cervix (squamous cell carcinoma and adenocarcinoma), mesothelioma (affects and differs in various tissues: lung (pleura), heart (pericardium), intestine (peritoneum) and testes (tunica vaginalis), rhabdomyosarcoma (embryonic and alveolar), gliomas and

related subtypes (glioblastoma, anaplastic astrocytoma, fibrillary astrocytoma, pilocytic astrocytoma, other astrocytoma, oligodendroglioma, ependymomas), the chronic lymphoid leukemia.

The conjugates produced can be used parenterally, together with vehicles such as water, buffer solutions, physiological solutions, stabilizers i (for example antioxidants), chelating agents (for example ethylenediamino tetracetic acid or EDTA), and can be produced under sterile conditions according to methods known to the skilled in the art. The term parenteral defines the type of administration by injection through the integuments or directly into the circulation (for example the intradermal route, the subcutaneous route, the intramuscular route and the intravenous route).

The conjugates produced can advantageously be administered in useful therapeutic doses, significantly increasing the efficacy of the therapy in terms of duration of the therapeutic effect (effect due to the reduction of *clearance* renal) and reduction of the formation of immune complexes (due to the steric hindrance of the polymer which prevents the aggregation of multiple protein molecules). The therapeutically useful doses will be defined by the specialist doctor also according to the correspondence of the individual patient. Furthermore, the conjugates produced allow, due to passive *targeting* phenomena, which exploits the natural tendency of some cells (liver, macrophages) to engulf foreign agents to the organism, the accumulation in the tumor tissue due to the EPR phenomenon (*Enhanced Permeation and Retention*) leading to dose decrease in healthy tissues. The conjugation with the PEG also allows the accumulation at the level of diseased tissues thanks to the increase in size but, above all, thanks to the increase in the plasma half-life of KillerTRAIL. The diseased tissue, be it inflamed or tumor, has the peculiarity of having more penetrable blood vessels (fenestrated, to allow the passage of nutrients and those recalled by the inflammatory process) and an amplified retention effect, the latter due to a pH more acid and an absence/loss of lymphatic drainage to retain necessary substances (EPR effect) (Greish K., J. Drug Target., 2007; 15 (7-8): 457-64). For this reason, the polymeric conjugate is naturally more likely

to reach diseased tissue than healthy tissue and compared to the native protein, which degenerates faster, will reach the target site in less quantity and only for a short period of time.

The pharmaceutical compositions comprising the polymeric conjugate PEG-KillerTRAIL, according to the invention, can be prepared by mixing this polymeric conjugate with adjuvants, excipients or stabilizers that meet the requirements for the excipients for pharmaceutical use present on the market (Remington's Pharmaceutical Sciences, 16th edition , Osol, A. ed. (1980)), in the form of lyophilized formulations, aqueous solutions or aqueous suspensions. The carriers, excipients or stabilizers used are preferably selected from: buffers such as Tris, HEPES, phosphate, citrate and other salts of organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride; benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; low molecular weight polypeptides (less than approximately 10 residues); proteins, such as serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides and other carbohydrates including glucose, mannose or dextrins; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; and/or non-ionic surfactants such as polysorbates or TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

Further examples of such carriers include ion exchangers, alumina, stearate of aluminum, lecithin, serum proteins, such as human serum albumin, buffers such as glycine, sorbic acid, potassium sorbate, mixtures of partial glycerides of saturated vegetable fatty acids, water, salts, or electrolytes such as protamine sulfate, disodium hydrogen phosphate, potassium hydrogen phosphate, sodium chloride, colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone and cellulose-based substances

.

Carriers for topical or gel forms include polysaccharides such as carboxymethylcellulose or methylcellulose, polyvinylpyrrolidone, polyacrylates, ethylene-propylene block polymers, PEGs and waxes. For all the administrations, the known conventional forms are suitably used. Such forms include, for example, microcapsules, nano-capsules, liposomes, patches, inhalation forms, nasal *sprays*, sublingual tablets and prolonged release preparations.

KillerTRAIL polymeric conjugates to be used for *in vivo* administration must be sterile. The sterility process is easily achieved by filtration through sterile filter membranes (0.22 µm), before or after lyophilization and relative reconstitution. The polymeric conjugates of KillerTRAIL are normally stored in anhydrous form, for example lyophilized, or in solution if used for systemic administration. If in lyophilized form, KillerTRAIL polymer conjugates are typically formulated in combination with other excipients used for their reconstitution (cryoprotectants such as, for example, mannitol, trehalose, sucrose, sorbitol, etc.) and with an appropriate diluent at the time of use. An example of a liquid formulation is a sterile, clear, colorless, unpreserved solution filled in a single-dose vial for subcutaneous injection.

The formulations can for example be placed in a container provided with a sterile access port, for example a bag or a vial of intravenous solution having a cap that can be pierced by a hypodermic injection needle. The formulations are preferably administered as repeated intravenous (iv), subcutaneous (sc), intramuscular (im) injections or infusions or as aerosols suitable for intra-nasal or intra-pulmonary delivery.

As regards the use of the invention for diagnostic purposes, it is possible to foresee the same synthesis with the use of the same radio protein labeled with ^{125}I (Xiang H et al., Drug Metab-Dispos., 2004; 32 (11) : 1230-8; Duiker EW. Et al., Br. J. Pharmacol., 2012; 165 (7): 2203-12) or ^{188}Re /^{188}W (Wang XG. Et al., Ther. Deliv., 2014 ; 5 (2): 139-47). In addition to the substances listed above, the radio-labeled PEGylated protein is added to the formulation to be administered so that it can be followed through the classic diagnostic imaging techniques used for functional

analyzes, nuclear magnetic resonance associated with positron emission tomography and computed axial tomography associated with positron emission tomography (MRN/PET and CT/PET respectively). Also according to what is reported in the literature (Xiang H. et al., Drug Metab. Dispos., 2004; 32 (11): 1230-8), the presence of the radioisotope can increase the stability in plasma and the therapeutic efficacy and this, reasonably, leads us to think that the invention in question, already in itself improving safety (fewer side effects due to the formation of aggregates and rapid metabolism), stability and efficacy, can further increase the positive characteristics of this drug, especially in the context of safety in therapy.

The compositions comprising KillerTRAIL may further comprise additional therapeutic agents such as: 5-fluorouracil, CPT-11, (Ashkenazi A et al., J. Clin. Invest., 1999; 104 (2): 155-62) and/or be administered in combination with ionizing radiation (Chinnaiyan AM et al., Proc. Natl. Acad. Sci. USA, 2000; 97 (4): 1754-9) and/or diagnostic agents if radiolabelled, such as ^{125}I, ^{188}Re, ^{188}W and related combinations.

It is important to highlight that, according to the literature reported in the previous paragraphs, the Apo-2L TRAIL protein and consequently also the KillerTRAIL variant, are proteins that exist in a trimeric form and as such act determining the therapeutic effect. However, the invention relates to the direct PEGylation of the single KillerTRAIL chain, resulting in the obtaining of a new macromolecular entity containing the PEG polymer (various molecular weights as indicated above) and a single protein chain. This new invention has proved to be an improvement over both the unconjugated trimeric KillerTRAIL and the PEGylated variants (R170C Apo-2l-PEG and K179C Apo-el-PEG) reported in patent WO2004001009A2. The improvement is to be understood on various points of view listed below:

1) No changes to the native KillerTRAIL chain are imposed.
2) The dosage used is significantly lower.
3) The therapeutic efficacy is maintained or increased even for a longer time.
4) Low or no mortality of the animal models used.

For those pathologies for which the positive effects of this therapy have been ascertained, the advantages of the invention are seen *primarily* in the increase in the plasma half-life of KillerTRAIL, due to the presence of the mPEG polymer which manages to protect the protein from degradation due to enzymes plasma. This leads to an increase in therapeutic activity over time and therefore to a decrease in doses or to an extension of the time elapsing between one dose and the next.

The decrease in the concentration per single dose or the decrease in the number of doses required, reduce the toxic effect of KillerTRAIL (hepatotoxicity, toxicity at the injection site and serious side effects due to the increase in stimulation of TNF receptors even in healthy tissues, such as MOF, *multiple organ failure*) and also increases *compliance* patient. All these positive effects improve the therapeutic approach of the previously listed disabling diseases.

The following examples are to be considered illustrative and not limitative of the scope of the invention.

EXAMPLES

Materials

The KillerTRAIL was purchased by Enzo Lifescience (Florence, Italy).

mPEG-maleimide (methoxy-N (maleimide (polyethylene glycol)) (MW 2-200 kDa) was purchased from Iris Biotech srl (Germany). Ethylene-diamino-tetraacetic acid (EDTA), phosphate buffer solution (PBS), kit Bicinconinic acid assay, *kits* TRAIL ELISA, picrylsulfonic acid, 5,5'-dithiobis (2-nitrobenzoate), acrylamide, bisacrylamide, ammonium persulfate, TEMED, Glycine, Coomassie Blu Brilliant, Bromophenol Blue, LC resins have been purchased from Sigma-Aldrich SpA (Steinheim, Germany). The dialysis membranes used in the experimental phase were purchased from Spectrum Laboratories Inc. (Eindhoven, The Netherlands). All aqueous solutions were prepared using deionized water (MilliQ-grade, 18.2 MSZ) obtained using the Millipore MilliQ system (MA, USA). All the solvents and

the remaining reagents used were selected from those specific for HPLC or with a degree of purity from those available in the catalog from Sigma-Aldrich (St. Louis, MO, USA) or by Carlo Erba (Milan, MI, Italy).

The conjugates produced were characterized by high performance chromatography both by the inverse phase technique (C18 RP-HPLC), in which the protein was found to be stable, and by the gel filtration technique (GF-HPLC), with the aim to monitor the progress of the reaction, determine its completion, quantify the yield and purify the derivatives obtained, according to what is reported in the following section.

The products were analyzed by various types of colorimetric assays (BCA, Habeeb, Bradford), to determine the concentration of the protein in solution and by assays at known concentration, to determine the PEG interference on these assays.

The same conjugates were analyzed by molecular exclusion chromatography combined with four detectors positioned in series (*Light scattering*, refractive index, viscometer and UV/Vis photodiode spectrometer), in order to obtain information on the molecular weight and on the arrangement in space. of the conjugate and the protein. Thanks to this method, data on the stability of the conjugated protein in solution and the purity obtained after dialysis were obtained.

In order to know the exact molecular weight, the conjugates were subjected to analysis by MALDI/TOF mass spectroscopy.

Evaluations by circular dichroism (CD) analysis allowed to highlight any changes in the molecular arrangements of the protein (tertiary structure) in the presence of PEG. This was monitored to assess whether the presence of PEG at that position of the amino acid chain would interfere excessively with the spatial conformation of the protein, completely preventing recognition by the receptor site.

Electrophoresis studies were carried out on the conjugates to confirm the chromatographic evaluations, under denaturing conditions, in order to prove the absence of dimers, trimers or protein aggregates in general.

The stability of the products was evaluated by comparing it with the stability of the protein in a buffered solution at predetermined times and at room temperature. The analyzes performed at physiological (pH 7.4, PBS) and pathological (pH 5, PBS) pH, are predictive of the behavior of the product during storage. Stability was also evaluated on biological fluids (plasma), with the aim of predicting the behavior of the drug in the bloodstream.

Subsequently, the products were tested *in vitro* on HTC116 liver cancer cells to obtain data on cytotoxic therapeutic activity (by cell viability assay with 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide or MTT) of the products compared to the native protein.

Analyses were developed *in vivo* on the derivatives described above, on mouse models (*xenographed*) implanted with human hepatic carcinoma cells to determine the improvement of pharmacokinetic parameters, biodistribution and therapeutic antitumor activity in terms of tumor reduction and increased product survival compared to native protein.

Example 1: Synthesis of the mPEG-KillerTRAIL conjugate

Having ascertained that the native KillerTRAIL protein can be identified and monitored by RPC18-HPLC, we moved on to the synthesis of the derivative which involved the use of the maleimide group of PEG for the selective binding with the thiol of the cysteine in position 230 of the above protein. The molar ratios taken into consideration are all those possible starting from 10/1 to ending at 0.01/1 between the mPEG-MAL/KillerTRAIL reagents, and vice versa. The reaction is maintained at pH 7.4 and the pH measurements were performed using pH indicator papers (Whatman®, Sigma Aldrich SpA, Steinheim, Germany).

Below is the reaction scheme (Figure 1).

Example 2: Colorimetric analysis using standard assays: Determination of the primary amino groups of the KillerTRAIL derivatives (Habeeb assay).

The quantitative determination of the amino groups of the mPEG-KillerTRAIL conjugate was calculated by Habeeb's colorimetric assay with 2,4,6-trinitrobenzenesulphonic acid (TNBS). TNBS is made to react under basic conditions with the amino groups of the samples to be analyzed and the chromophore that is formed is determined by spectrophotometry at the maximum wavelength (λ_{max}) of 420 nm.

The samples to be analyzed were prepared by dissolving the compounds in 975 µl of 0.1 M borate buffer, pH 9.3. 25 µl of TNBS (1% w/v in DMF) were subsequently added to each sample. The absorption at 420 nm of the sample is directly proportional to the concentration of amino groups whose concentration was previously calculated with a calibration line obtained from a solution with known concentrations of glycyl-glycine (Gly-Gly) or monomethoxy-PEG -amino (m-PEG-NH_2).

Example 3: Chromatographic analyzes.

High performance liquid chromatography **(HPLC)**

The chromatographic analyses were performed with a *High Performance Liquid Chromatography system* (Jasco, Tokyo, Japan), consisting of a PU-1580 binary pump system, a MD 1510 diode array detector (DAD) (Tokyo, Japan). A Jupiter C18 Phenomenex analytical column (250 × 4.6 mm, 5µm) was used for the RP-HPLC analysis. The eluents and buffer solutions were filtered with Millipore systems (Benford, MA, USA) equipped with 0.22 µm filters and were sonicated with the ultrasonic system Ultrasonic Cleaner mod. 5210 Branson (Dambury, USA) for at least 30 minutes before their use in chromatographic analysis.

The protein solutions of KillerTRAIL and derivatives were analyzed by RP-C18 HPLC and the elution profile was obtained by spectrophotometric determination at λ_{max} of 280 nm.

The mobile phase used for the evaluation of both proteins was H_2O and CH_3CN both containing 0.05% (v/v) of TFA. The chromatographic separation was carried out by gradient elution using the following mobile phases: H_2O/CH_3CN: at time (t) t = 0-90% (v/v) H_20 and 10% (v/v) CH_3CN;

at = 25 minutes 10% (v/v) H_2O and 90% (v/v) of CH_3CN; at = 30 minutes 90% (v/v) H_20 and 10% (v/v) of CH_3CN. The KillerTRAIL conjugation reaction was monitored until the final conjugation product was obtained (Figure 2).

Example 4: Gel Permeation Chromatography/Molecular Exclusion Chromatography (GPC/SEC).

The GPC/SEC technique was used to determine the molecular weight (MW) and MW distribution for different macromolecules, proteins, natural and synthetic polymers. The instrument used for this type of study was the Viscotek GPC/SEC TDAmax (Malvern Company, Viscotek Corp, Houston, USA), a system equipped with a Tetra Detector (TDA) including a Refractive Index (RI) which provides a accurate determination of molecular weight and specific increase of the refractive index (dn/dc), a *detector* UV/Vis (programmed for analysis at λ_{max} of 280 nm) that allows to study the chemical composition of the derivatives, a detector differential viscosimetric that provides a measure of the intrinsic viscosity of the derivatives and allows to determine the size, conformation and molecular structure of the derivatives, a double angle *Light Scattering* detector, one at 7° (*Low Angle Light Scattering* or LALS) to measure the Absolute MW also of the largest macromolecules, and one at 90° (*Right Angle Light Scattering* or RALS) to measure the absolute MW of small molecules. The GPCmax features an in-line degassing system to remove dissolved gases, allow for optimum pump performance, and improve baseline on all detectors.

The instrument is equipped with a double piston pump, a thermostatted autosampler, which has been equipped with two columns in series A2500 and A6000M (30 cm, 8 mm ID), and an automatic eluent sensor that stops the flow if it detects that the solvent supply is low avoiding damage to the columns.

The eluents used were ultra-pure water (90% v/v) and CH_3CN (10% v/v) at a flow rate of 0.7 ml/min. Two *standards* reference (PEG and Dextran) were used to calibrate the instrument. The data obtained were

processed using Omni SEC 4.7 software (Malvern Instruments, Ltd, Worcestershire, UK) and showed the presence of the PEGylated derivative of KillerTRAIL (Figure 3). The data concerning the molecular weight, the polydispersion index, the hydrodynamic radius, and the diffraction increase of the monoPEGylated derivative of KillerTRAIL and its relative PEG residues are reported in Table (Table 2).

Table 2. Analysis of the chemical-physical properties of mPEG-KillerTRAIL by means of the GPCmax SEC tetradetector Viscotek viscometer.

	Peak RV (ml)	**Mw (Daltons)**	**Mw /Mn**	**Rh(w) (nm)**	**RALS Area (mV/ml)**	**lals Area (mV/ml)**	
mPEG-KillerTRAIL	64,196	15,188	1,224	9,012	16.28	9.81	0147
residues of PEGylation	30,679	20.62	1,255	4,097	0.38	0.37	0,147

Peak RV: retention volume.

Mw: molecular weight.

Mw/Mn: polydispersion index.

Rh: Hydrodynamic radius.

RALS: right angle light scattering (90°measurament angle).

LALS: low angle light scattering (7°measurement angle).

dn/dc: differential increase of the refractive index.

Example 5: UV-Vis spectroscopy

The first preliminary evaluation of the KillerTRAIL protein was performed spectrophotometrically using a UV/Vis Lamda 25 Perkin-Elmer spectrophotometer (Northwolk, CT, USA) thus determining its known concentration. Starting from these considerations, a calibration line was constructed ($y = 4 * 10^{-8}x + 0.0213$; $R^2 = 0.9973$), to be used as a reference system for analyzing the derivatives of the native protein. The data reported in the literature (Expasy Data-protein, SIB Bioinformatics Resource Portal), showed that the molar extinction coefficient ε of the protein fraction 95-281 is 27390 (Abs 0.1% (1 g/l) = 1.274, assuming that all cysteine residues are in reduced form, λ = 280nm).

Example 6: MALDI-TOF mass spectrometry (*Matrix-Assisted Laser Desorption Ionization - Time of Flight*).

MALDI-TOF mass spectrometry is an analytical technique that allows to very accurately measure the molecular weight of macromolecules of biological interest and to determine their identity based on the mass/charge ratio, and has been used to characterize derivatives of KillerTRAIL. For analytical purposes only, the mPEG-MAL 2kDa polymer and the respective conjugate with the KillerTRAIL protein were analyzed. The obtained results showed that the KillerTRAIL protein was conjugated to the 2kDa mPEG chains through the maleimide residue leading to the formation of a stable final product with a yield of about 90% (Figure 4).

The spectra were produced with REFLEX instrumentation (AB Sciex 4800 plus MALDI-TOF Analyzer) equipped with an ion source *SCOUT* capable of operating in positive linear mode. The ions generated by a pulsed UV *laser beam* (nitrogen laser, λmax = 337 nm) were accelerated to 25 kV. A saturated solution of synapinic acid in acetonitrile/water (1: 1 v/v) was used as a matrix and mixed with the samples dissolved in 0.1% (v/v) aqueous solution of TFA in a ratio of 1: 1 v/v .

The data obtained from the MALDI-TOF analysis demonstrated the presence of the PEGylated conjugate with the protein under examination. The analysis was carried out on the m-PEG-MAL 2kDa polymer (Figure

4A) whose molecular weight was 2119.4 Da and on the conjugate (Figure 4B) mPEG-KillerTRAIL thus obtaining the absolute molecular weight of 24008.6 Da.

Example 7: Electrophoretic analysis SDS PAGE (Polyacrylamide gel electrophoresis).

Electrophoresis is an analytical technique that allows to separate charged molecules, on the basis of the different mobility in a given electric field. The proteins were denatured with sodium dodecyl sulfate (SDS), an anionic detergent that binds with great affinity to proteins (one molecule of SDS every two years or so) and gives them a net charge proportional to mass. In this way the effective charge of the polypeptide chains is negligible.

Since all proteins are denatured and have the same mass/charge ratio, the mobility of the molecules depends solely on the molecular weight and porosity of the gel, which acts as a molecular sieve.

A gel was constructed for the electrophoretic run of the protein samples, consisting of a 12% (w/v) *running gel* and a 4% (w/v) *stacking gel*. The instrument used was a Mini-PROTEAN® Electrophoresis System (Bio-Rad, Laboratories, Inc., USA) equipped with a vertical cell, powered by a power supply. The percentage of polyacrylamide of 10% (w/v) in the gel was used because it is suitable for the resolution of the protein derivatives to be analyzed. For the electrophoretic run, 10 µg of protein were loaded into a final *buffer* volume of 15 µl. Each sample was denatured with 15 µl of *sample buffer* consisting of: TRIS 250 mM, pH6.8; 2% (v/v) SDS, 25% (v/v) of β-mercaptoethanol as a reducing agent to break any sulphide bridges present; bromophenol blue 0.1% (w/v); 10% (w/v) of glycerol. The samples diluted in the *sample buffer*, before being loaded on the gel, were completely denatured by heat treatment at 100°C for at least 5 minutes. The electrophoretic run was carried out at a constant voltage of 100 mV for at least 1 h in a run buffer consisting of: 1 mM TRIS, pH 8.3; glycine 960 mM; SDS 0.1% (w/v); water. At the end of the stroke, to observe the separation of the proteins, the gel was immersed in a dye solution consisting of: 0.1%

(w/v) *Coomassie Brillant Blue*, 40% (v/v) ethanol, 10% (v/v) acetic acid. The acid-ethanol mixture is intended to precipitate the proteins on the gel and fix the added dye there. Finally, the gel was bleached to remove excess dye and highlight only the remaining protein bound. The obtained polyacrylamide gel was then analyzed using a Molecular Imageroptical system® (Chemi Doc TM, Biorad Laboratories Inc., USA). The Horizon 58 electrophoretic chamber (Life Techonologies Horizontal Gel Electrophoresis System, USA) was used for the electrophoretic evaluation.

The synthesis of KillerTRAIL derivatives was monitored by SDS-PAGE. This method highlighted the formation of the mPEG-KillerTRAIL conjugate (Figure 5) confirming the previously reported chromatographic data (HPLC and mass spectrometry), thus highlighting the formation of a therapeutic protein with a macromolecular structure with potential therapeutic activity.

Example 8: Analysis of biological activity *in vitro* of free protein and conjugated protein.

Cytotoxicity studies were conducted on all synthesized conjugates. Furthermore, biological activity and residual activity studies were performed on the KillerTRAIL conjugate alone.

Cell viability was assessed by MTT assay. The MTT salt is reduced in the presence of NADH (*nicotinamide adenine of nucleotide hydrogenase*) by cellular mitochondrial dehydrogenase, with formation of insoluble formazan crystals (blue in color). The cytotoxicity study of KillerTRAIL derivatives *in vitro* was evaluated using human colon cancer cells (HCT116) responsive to human keratinocyte cells (NCTC 2544 cells) not responsive to native protein. The cells were incubated (Guaire® TS Autoflow Codue water Jacketed-Incubator), in Petri dishes for cell culture (100 mm × 20 mm) at 37°C (5% CO_2) using D-MEM supplemented with glutamate, penicillin (100 IU/ml), streptomycin (100 µg/ml) and FBS (10%, v/v).

Having obtained an 80% confluence, the cells were detached with trypsin (2 ml), collected in a centrifuge tube containing 4 ml of culture

medium, further washed with 2 ml of PBS to remove the residual cells and finally transferred to a centrifuge tube. The cell suspension obtained was centrifuged at 1200 rpm at room temperature for 5 minutes with a Heraeus Sepatech Megafuge 1.0 (Heraeus Sepatech, Osterode/Harz, Germany). The *pellet* obtained was resuspended in an appropriate volume of culture medium (2 ml), seeded in 96-well plates (5×10^3 cells/0.1 ml) and incubated for 24 h at 37°C to favor surface adhesion of cell culture plates.

The culture medium was then removed and the HCT116 and NCTC 2544 NCTC 2544 cells were treated for 24, 48 and 72 h with a solution of KillerTRAIL (100 ng/ml) or with the PEGylated conjugate at the same protein concentration, in order to evaluate cell viability following treatment by MTT-*test*. Each plate presented untreated cells as a negative control.

After 24 h of incubation, a 10% (w/v) solution of tetrazolium salts solubilized in PBS (5 mg/ml) was added to each well and the plates were incubated again for 3 h. The cell culture medium was removed and the formazan salts (precipitated at the bottom of the well after oxidation) were dissolved with 100 µl of a mixture of DMSO/ethanol (1: 1 v/v), shaking the plates for 20 minutes at 230 rpm (IKA® KS 130 control, IKA® Werke GmbH & Co., Staufen, Germany).

The solubilized formazan salts were quantified with a spectrophotometer for microplates (BIO-RAD xMark ™, Bio-Rad Laboratories Srl, Segrate (MI), Italy) at a wavelength of 570 nm and 690 nm. Cell viability was evaluated by measuring the amount of formazan crystals obtained after the oxidation reaction which is directly proportional to the amount of cells still alive after treatment with the compounds (KillerTRAIL and its PEGylated derivative) reported above.

Cell viability was calculated using the equation below:

$$\textit{Cell viability (\%)} = (AbsT/AbsC) \times 100$$

where, *AbsT* is the absorbance of the treated cells and *AbsC* is the absorbance of the control (untreated cells) . The concentration of formazan is directly proportional to the cell viability, which is the result of the average of three different experiments ± the *standard* deviation.

The results obtained show that KillerTRAIL and its derivative mPEG-KillerTRAIL have antitumor activity against HCT116 cells (Figure 6). In particular, mPEG-KillerTRAIL decreases the cell viability of HCT116 cells by 50% at a concentration of 1.5 ng/ml. A reduction in cell viability of less than 20% was instead obtained by increasing the equivalent amount of KillerTRAIL in the PEGylated product to 2 ng/ml (Figure *6*). No effect of KillerTRAIL and its derivative mPEG-KillerTRAIL was observed on NCTC 2544 human keratinocyte cells as this cell line is an untransformed human cell line which is not sensitive to KillerTRAIL (Figure 6).

Example 9:evaluation *In vivo* of KillerTRAIL and mPEG-KillerTRAIL

The animals used for the experiment are male Sprague Dawley (SD) rats with an average weight of 220-250 g of 4-5 weeks and BALB/c nude mice (20-25g , 4-5 weeks, purchased from Charles River Laboratories (Germany). The animals were kept in the enclosure under continuous monitoring, divided into groups of 6-8, and subjected to 12 h light/dark cycles, with food and water *ad libitum* for 2 weeks. The procedures involving the use of animals and their welfare have been conducted in accordance with institutional guidelines that comply with national and international laws and policies (European Community Council Directive, of 24 November 1986, 86/609/EEC).

Example 10: Pharmacokinetic studies of KillerTRAIL and mPEG-KillerTRAIL

The pharmacokinetic characteristics of the KillerTRAIL protein and its conjugate were studied in mouse models (rats) after intraperitoneal (ip) administration. The animals were cannulated through the jugular vein one day before the experiments and divided into 6 groups (n = 5): 3 groups used for the treatment with KillerTRAIL and 3 groups for the treatment with mPEG-KillerTRAIL. The animals subdivided into the different groups reported above were administered with a dose of KillerTRAIL equivalent to 10 µg (0.04 mg/kg) or the molar equivalent of PEGylated conjugates of

KillerTRAIL in PBS (pH 6.0). The plasma samples taken at different incubation times, after ip administration, were centrifuged and stored at -80°C until the time of analysis. The amount of KillerTRAIL in rat plasma samples was evaluated using the commercial protein *kit* ELISA (BioSource International, Inc.). Pharmacokinetic parameters were calculated from plasma concentration profiles by non-compartmental model analysis. Pharmacokinetic data demonstrated that KillerTRAIL is rapidly removed from the systemic circulation after intraperitoneal (ip) administration, while the mPEG-KillerTtrail derivative remains more in the systemic circulation. The half-life ($t_{1/2}$) of KillerTRAIL and mPEG-KillerTRAIL has been reported in Table 3; and in particular $t_{1/2}$ of mPEG-KillerTRAIL is 28.3 times greater than$_{1/2}$ tof KillerTRAIL (Figure 7). All other pharmacokinetic parameters demonstrate that KillerTRAIL PEGylation increases the systemic circulation of the native protein and reduces its metabolism and elimination (Table 3 and Figure 7).

mPEG-KillerTRAIL accumulates more in organs and tissues than KillerTRAIL (Figure7). PEGylation increases the distribution of KillerTRAIL in the systemic circulation (Figure 7) and reduces its *uptake* by the organs of the reticulum endothelial system (RES) such as the liver, spleen, lungs, kidneys (Figure 7).

Table 3. Pharmacokinetic parameters of KillerTRAIL and its derivative mPEG-KillerTRAIL.

Pharmacokinetic parameters					
Formulations	$t_{1/2}$ (h)[a]	C_{max} (µg/ml)[b]	T_{max}(h)[c]	Vd (ml)[d]	AUC (µg/ml×h)[e]
KillerTRAIL	1.3±0.12	1.30±0.17	–	13.00±0.19	11.3±0.05

mPEG-KillerTRAIL	37.24±0.26	1.42±0.11	–	43.2±0.23	58±0.016

at1/2 = plasma half-life.

bCmax = maximum concentration.

cTmax = maximum time.

dVd = volume of distribution.

eAUC = area under the curve.

Example 11: Biodistribution *in vivo* of KillerTRAIL and mPEG-KillerTRAIL

The evaluation of biodistribution was performed on mice implanted (xenographed) with HCT116 tumor cells (3 × 10^6 cells/ml). Cells were inoculated subcutaneously into BALB/c nude mice. The tumor mass reached its optimal size after one week. The mice were treated for four days with a single dose (administered ip) of KillerTRAIL and its PEGylated conjugate, and were sacrificed 96 h after administration of the compounds. Tumors and organs (liver, lung, kidney, heart, brain, muscle, bone, pancreas, bladder, stomach, small intestine, colon, spleen) were surgically removed, collected, centrifuged and stored at -80°C until the time of analysis. The samples were extracted and the supernatants obtained were analyzed using a *kit* commercial ELISA for KillerTRAIL (BioSource International, Inc.).

For tumor *targeting in vivo* studies, guinea pigs were divided into groups of 8 animals and the analyzes were performed using the concentration value of the PEGylated derivatives within the tumor mass at different incubation times as a reference parameter (15, 30, 60, 240 min). The experimental analyzes were performed in duplicate both on the control mice and on the treated samples.

The experimental data obtained showed that KillerTRAIL and its derivative mPEG-KillerTRAIL are distributed in different organs and tissues in a time-dependent manner (0 - 240 min) and the distribution kinetics can

be used to evaluate the accumulation rate of the native protein and of its PEGylated derivative in tumor tissue (Figure 8). In particular, KillerTRAIL increases the amount of native protein accumulated in the tumor compared to mPEG-KillerTRAIL; while it reduces that of the native protein present in the bloodstream and in the RES organs due to the effect of PEGylation (Figure 8). This type of effect demonstrates a better biopharmaceutical of the mPEG-KillerTRAIL derivative than KillerTRAIL, while the better selectivity for tumor tissue of KillerTRAIL compared to its PEGylated derivative depends on the greater amount of free native protein able to rapidly bind to the KillerTRAIL receptors present in the tumor tissue. These results are in agreement with the data reported in the literature for other therapeutic proteins, monoclonal antibodies, enzymes, biologically active compounds and drugs conjugated to polymeric macromolecules (from Silva Freitas D et al., Bioconjug Chem., 2013; 24 (3): 456-63; da-Silva-Freitas D. et al., Protein Pept. Lett., 2015; 22 (12): 1133-9).

Example 12: Antitumor activity of KillerTRAIL and mPEG-KillerTRAIL.

The anticancer effect of KillerTRAIL and mPEG-KillerTRAIL was studied in mouse models implanted (xenogratransplanted) with HCT116 tumor cells. After 7 days from inoculation, the mice were treated for 7 days with KillerTRAIL and mPEG-KillerTRAIL (50 or 100 mg/kg/day, dose equivalent to KillerTRAIL). The native protein and its PEGylated derivatives were administered intravenously through the marginal vein of the animal's tail. The volume of the tumor mass was monitored for the next 24 days every 5 days. The volume of the tumor mass was calculated as a function of the longitudinal length (L) and the transverse diameter (W) of the tumor using the following formula:

$V = (L \times W2)/2$

In contrast, the tumor growth inhibition values (TGI) were calculated using the following equation:

$TGI = (1\text{-}TV\ sample/TV\ control) \times 100$

Where TV is the tumor volume.

The antitumor activity of KillerTRAIL and mPEG-KillerTRAIL was evaluated in mouse models implanted (xenografted) with HCT116 tumor cells. After tumor engraftment and subcutaneous development, the mouse models were treated ip with KillerTRAIL and mPEG-KillerTRAIL at the equivalent dose of 0.04 mg/kg/day. mPEG was used as a negative control. The development and growth of the tumor mass were monitored over time. The data obtained show that mPEG-KillerTRAIL significantly reduces the volume of the tumor mass after 20 days of treatment compared to the same dose of KillerTRAIL (Figure 9). At the same time, mPEG-KillerTRAIL reduces tumor growth by 74% compared to KillerTRAIL which, on the contrary, only reduces tumor mass by 37.5% (Figure 9). The improved *in vivo* antitumor activity of mPEG-KillerTRAIL compared to KillerTRAIL is linked to an improvement in the biopharmaceutical parameters of the native protein after its conjugation with mPEG. The PEGylation of KillerTRAIL modifies the half-life of the native protein and favors its accumulation in the tumor tissue. These values are similar to those already published in the literature that show the therapeutic advantages of bioactive PEG conjugates and their potential therapeutic applications (Pasut G., Veronese FM. Drugs Today (Barc), 2009; 45 (9): 687-95 ; Pasut G., Veronese FM. J. Control. Release, 2012; 161 (2): 461-72).

The data reported in the patent document WO2004001009 and relating to the PEGylation of the Apo 2L protein on cysteines inserted in the chain in position 170 or 179 show a dosage of 10 mg/kg, administered in two weekly doses for two weeks (therefore an overall quantity of compound equal to 20 mg/kg/week). The authors obtained a reduction in tumor volume increase, after 15 days, compared to the native protein, equal to about 57.6% for the PEGylated R170C-Apo 2L mutant and equal to about 6.2%, for the PEGylated K179C-Apo 2l. The data obtained by us, on the other hand, relate to a 7-day treatment with a pro-die dose of 0.04 mg/kg (therefore 0.21 mg/kg/week, about a tenth of what was used by the authors of the patent document WO2004001009 of the aforementioned treatment, the analysis of the mean tumor volume was performed by us for the

following 13 days. The data obtained show a reduction of the tumor at 15 days of 56% and at 20 days of 59% for the mPEG-KillerTRAIL conjugate compared to the native protein. It should be noted that the treatment was blocked on the seventh day and that the analysis was not performed beyond the twentieth because the control mice (towards which the reduction at 20 days is 74% for the PEG-KillerTRAIL compared to 37% of the native protein) and the mice treated with native KillerTRAIL died.

Considering these factors, it is evident that the mPEG-KillerTRAIL derivative object of this invention is better than that reported by the state of the art for the following reasons: the dosage used is 10 times lower and administered for a time equivalent to half of that reported in the literature, which presents an extension of the treatment more than 5 days more, the therapeutic yield in terms of reduction of the tumor volume is equivalent if not higher than one of the two conjugates reported in the patent WO2004001009A2, namely the PEG-R170C-Apo 2L, while it is significantly greater (by about 90%) than the second conjugate reported in the patent WO2004001009A2 or the PEG -K179C-Apo 2L.

These results are absolutely unexpected compared to what is reported in the patent WO2004001009 where the amino acid chain of Apo2L is modified by introducing a cysteine in position 170 or 179 thanks to the substitution of an arginine and a lysine respectively. This modification, according to the authors of WO2004001009, would have been necessary in order to protect the cysteine in position 230, considered fundamental for the trimerization necessary for the activation of the receptor system thanks to the ionic bond with a zinc ion. On the contrary, our studies have shown that the PEGylation on the Cys 230 residue increases the therapeutic action of the protein, without modifying the amino acid chain of the native protein in any way.

Please note that killerTRAIL (the polyhistidinehttp://www.enzolifesciences.com/ALX-201-073/killertrail-protein-soluble-human-recombinant/) is found to cause hepatic toxicity (apoptosis) due to tag. At the same time it appears to be much more

effective than the Apo2L protein, which in order to achieve the *in vitro* activity demonstrated on killerTRAIL, must be co-administered with a high dose of doxorubicin, which is also particularly toxic (Jo M et al., Nat. Med. 2000; 6 (1): 564-7). The effectiveness of PEGylation KillerTRAIL is due to the reduction of the hepatotoxic effect reported in the literature, which was confirmed by us by the reduction of the accumulation of KillerTRAIL in the liver tissue (Figure 8C). The *in vitro* activity study, object of this invention, on HCT 116 tumor cells, sensitive to the therapeutic activity of KillerTRAIL, has in fact highlighted a reduction in the cytotoxicity of the same native protein (KillerTRAIL) (Figure 6), which is however balanced by the greater permanence in the bloodstream after PEGylation, as demonstrated in the related pharmacokinetic studies (Figure 7).

The results obtained from a comparison with C170 and C179 of WO2004001009 are absolutely unexpected. The authors of WO2004001009 in fact modified the amino acid chain by introducing a cysteine in position 170 or 179 thanks to the substitution of an arginine and a lysine respectively. This modification, according to their studies, was necessary to easily PEGilate the protein while maintaining the cysteine present in position 230 unchanged for trimerization, necessary for the activation of the receptor system thanks to the ionic bond with a zinc ion.

We instead carried out the PEGylation directly on the Cys 230 and unexpectedly this procedure guaranteed an improvement action of the protein. As can clearly be seen from the comparison shown in Figure 10, in fact, the Apo 2L protein modified in position 179 (K179C-Apo 2L), graph C, Figure 10 B of WO2004001009 shows an activity comparable to the control and slightly lower than the thick PEGilated protein. The modification of the protein in the amino acid chain canceled the activity of the native protein and the PEGylation had practically no effect. The Apo 2L protein modified in position 170 (R170C-Apo 2L), graph B corresponding to Figure 10 A of the aforementioned document, on the other hand, appears to maintain the activity, even if much lower than the native protein and, in this case, the PEGylation improves its therapeutic characteristics by about 56%. On the

other hand, our invention, in which KillerTRAIL is not modified, maintains its activity at significantly lower dosages both in the non-PEGylated and PEGylated form, where the reduction in tumor volume is evident even after 15 days from the end of the treatment. In this case, PEGylation results in increasing its effectiveness by 60% (Figure 9). We must also highlight the differences in the timing of administration: in fact in the aforementioned patent the authors describe a weekly treatment of 20 mg/kg (in protein) divided into two doses, for two weeks, for a total of 40 mg/kg per the entire duration of the experiment. Considering the timing of administration, it appears that the animals were treated every 3 days for the duration of the volumetric monitoring of the tumor. Our treatment modalities instead provided for a daily dosage of 0.04 mg/kg (in protein) for 7 days (therefore equivalent to 0.28 mg/kg in all) and a monitoring of the tumor volume from time 0 (first inoculum) for the following 15 days after the seventh day of treatment, for a total of 21 days of volumetric monitoring. From this comparison the following results: our product is improved in terms of:

1. increased therapeutic efficacy;
2. noticeable increase in the time of therapeutic activity of the protein not accompanied by the administration of a dose.

It is fair to point out that the treatments did not last longer than 21 days as the control animals and those treated with the native protein did not survive, unlike those treated with the PEGylated protein mPEG-KillerTRAIL. According to what is reported in the invention, the conjugation with the PEG was directed on the only available cysteine, Cys 230, responsible for the trimerization. This procedure allowed not only to PEGilate the single monomer, but also to prevent trimerization. The experimental evidence relates to the MALDI-TOF mass spectroscopy study carried out on the conjugate obtained with PEG_{2000} Da, in which the absolute molecular weight of PEG (2119.4 Da) and of the monomer mPEG-KillerTRAIL- (24008.6 Da) is evident (Example 6, Figure 4). In fact, the molecular weight of the single filament of KillerTRAIL corresponds to about 22000 Da, as reported by the

manufacturer, as well as by the reference database Expasy.org. The fact that only the single filament has been PEGylated, presents us with a substantial difference with the patent WO2004001009, where the PEGylation of the homotrimeric form of KillerTRAIL is reported. This difference is also evident in activity and efficacy in an absolutely unexpected way, as already highlighted above.

Example 13: KillerTRAILCharacterization

Sequence KillerTRAIL

The amino acid sequence of KillerTRAIL Enzo Life has been partially solved as shown below:

TSEETISTVQ EKQQNISPLV RERGPQRVAA HITGTRGRSN TLSSPNSKNE KALGRKINSW ESSRSGHSFL SNLHLRNGEL VIHEKGFYYI YSQTYFRFQE EIKENTKNDK QMVQYIYKYT SYPDPILLMK SARNSCWSKD AEYGLYSIYQ GGIFELKEND RIFVSVTNEH LIDMDHEASF FGAFLVG (sequence of KillerTRAIL Protein protein (soluble) (human), (recombinant)).

The sequence experimentally obtained is comparable with the amino acid sequence of TRAIL aa 95-281 (1 SEQ.ID.N. TSEETISTVQEKQQNISPLVRERGPQRVAAHITGTRGRSNTLSSPNSKNE KALGRKINSWESSRSGHSFLSNLHLRNGELVIHEKGFYYIYSQTYFRFQE EIKENTKNDKQMVQYIYKYTSYPDPILLMKSARNSCWSKDAEYGLYSIYQ GGIFELKENDRIFVSVTNEHLIDMDHEASFFGAFLVG) as Expasy.org official database.

The amino acid sequence obtained experimentally, and further analyzed by mass analysis present in the commercial product, does not show any difference with the theoretical one of KillerTRAIL.

The average value relative to the theoretical molecular weight (MW) of TRAIL (sequence 95-281) was found to be equivalent to 21607.1 Da.

The protein sequence was mapped by MALDI analysis. The tryptic peptide mixtures were sub-digested with chymotrypsin and the identified peptides are highlighted in gray as reported in Table 4. In the tryptic peptide

mapping approach, the KillerTRAIL protein was characterized by MALDI analysis with a sequence coverage equal to 97.8%.

Table 4. The table shows the mass values identified in the MALDI spectra and assigned to the corresponding peptides in the KillerTRAIL protein.

Position of the Sequence	[MH] + (Yes)	Note
158-161	533.16	
111-115	668.25	Non-specific triptych
cut125-130	Tyr115714.35	
144-149	715.41	
134-137	724.30	
80-85	738.43	
22-27	742.35	
56-61	776.38	
98-109 158-163	793.39	
92-97	801.36	Non-specific triptych
Tyr91	70-76	852.48 Non-specific
triptych	Phe69	150-157 891.47 Non-Tyr149
specific150-157	triptych874.45	PYR GLU[1]
5-12	905.47	
28-36	925.50	
111-117	944.41	
57-64	978.44	
170-177	1009.42	

13-21	1037.56	PYR GLU[1]
13-21	1054.59	
111-118	1072.52	
90-97	1077.51	Non-specific triptych
cut56-64	Tyr891106.53	
147-156Non-	1126.55specific	triptych cut Tyr146
28-38	1138.56	
89-97	1240.57	Non-specific triptych
cut	Tyr88	147-157 1254.64 Non-cut Tyr146
specific98-107	triptych1265.61	
39-50	1277.61	
159-169	1293.62	
65-76	1367.76	
119-130	1440.72	
143-155	1509.68	
86-97	1607.72	
85-97	1735.81	
147-161	1768.81	Non-specific triptych
cut39-55	Tyr1461802.87	
162-177	1898.9	
20-36	1904.81	
140-157	2065.96	
71-88	2125.90	
88-103	2178.04	

131-149	2212.02	Non-specific triptych
cut70-88	Tyr1492239.06	
138-157	2280.99	Non-specific triptych
cut	Tyr137	162-181 2351.05 Non-cut Phe181
specific20-42	triptych2533.23	
140-161	2580.16	
162-184	2626.18	Non-specific triptych
cut99-119	Phe1842692.20	
111-133	2807.25	
108-130	2851.39	
86-107	2854.4	
162-187	2895.34	
65-89	2917.37	Non-specific triptych
cut108-133	Tyr893165.45	
57-85	3346.54	
77-103	3401.52	
77-107	3873.70	
65-107	5222.6	

[1]PYR GLU: Cyclization of the N-terminal Gln peptide.

The data reported in Table 4 indicate the mass/charge fragments that refer to the molecule weight and the relative amino acid sequences that have been identified by analyzing the KillerTRAIL samples. The identified peptides are highlighted in gray and correspond to the tryptic peptide sequences corresponding to a KillerTRAIL sequence, after digestion of the samples with chymotrypsin and identification by MALDI of tryptic peptides, equivalent to 97.8% of the analyzed sequence.

The data reported in Table 4 were obtained by the Mass Mapping technique which is a strategy used for the analysis of the primary structure of proteins. This technique allows, once the spectrum of a peptide mixture has been obtained, to interpret the different mass signals, attributing to the signals present on the spectrum, specific molecular weights related to tryptic peptide sequences, in order to verify and confirm the protein sequence or identify any mutations. The program allows, through the MS-Digest program, to insert the known sequence of the protein and obtain a list of all the possible peptides that can be generated by a hydrolysis of the protein, with an appropriate enzyme, with the relative molecular weights. The MS-Digest program may also present some possible modifications on some peptides such as oxidations or cyclizations if they have been previously set. The set of peptides found contributes to define the sequence “mapping” or the coverage of the amino acid sequence.

The data in Table 4 show the KillerTRAIL specific tryptic peptide sequences, with their relative molecular weight, as a function of the trypsin hydrolysis process. These data are confirmed and in accordance with the mass spectra obtained after digestion (Figures 11A - 11Z) which indicate the fragments obtained for the tryptic peptide sequences obtained by mass analysis of KillerTRAIL and the corresponding molecular weights (Table 4).

Hydrolysis of KillerTRAIL in trypsin

The sample was dried using a bench centrifuge (Savant Speed-Vac, Holbrook, NY) at a speed of 12,000 rotations per minute (rpm) for 4 h, at room temperature and subsequently resuspended in ammonium Bicarbonate (50 mM, pH 8.0)m and then digested with 37°C trypsin for 16 hours. The peptide mixture obtained, purified by C18 zip-tip (Sigma-Aldrich, Milan) with a nominal volume of 0.6 µl, according to the specifications reported by the supplier (https://www.sigmaaldrich.com/catalog/product/mm/ztc18s ? lang = it & region = IT & gclid = CjwKCAiAoOz-BRBdEiwAyuvA63lo4iMhL-FN0cDy1swOzw0hEKoUKAsIwkds6BVT-

jh79Q6ldqr4oBoCKIMQAvD_BwE) was dried in a bench centrifuge (Savant Speed-Vac, Holbrook, NY) at a speed of 12,000 rpm , at room temperature, for 4h, and then resuspended in 0.2% TFA. Finally, a MALDI-MS analysis of the tryptic digest was performed by MALDI TOF-TOF 4800 Plus mass spectrometer.

Hydrolysis of KillerTRAIL into Chymotrypsintryptic

The peptide mixtures, obtained by hydrolysis in trypsin, were further digested with chymotrypsin in Ammonium Bicarbonate (50 mM, pH 8.0) for 4 hours (E: S 1:30 w/w) to provide complete coverage of the amino acid sequence to be analyzed and identified by MALDI.

MALDI-MS by KillerTRAIL

MALDI-MS analyzes were performed on a MALDI TOF-TOF (AB Sciex) 4800 plus mass spectrometer and the obtained data analyzed using 4000 Series Explorer v3.5 software.

For the analysis of the KillerTRAIL samples, 0.5 µl of the peptide mixture was mixed with an equal volume of a-cyano-4-hydroxycinnamic acid as matrix (10 mg/ml in 0.2% trifluoro acetic acid (TFA) in 70% of acetonitrile), loaded onto the sample metal plate and air dried.

Mass calibration was performed using the *standard* mix provided by the manufacturer. MALDI-MS data were acquired over a mass range between 500 and 5600 m/z in positive-ion *reflector mode*. The data obtained were acquired and processed using the software provided by the manufacturer.

The mass spectra were manually interpreted by associating a specific peptide with each peak (Figure 11). The results of the mass spectra were then correlated with the values reported in the MS-Digest software which provides a list of all possible peptides that can be generated by hydrolysis of the protein with an appropriate enzyme with relative molecular weights. The process of assigning each signal to a specific peptide made it possible to obtain the effective coverage of the protein, attributing a number

of peptides to each individual sequence to obtain the best mapping of the protein.

The mass spectra of the tryptic peptides obtained relate the intensity with the m/z ratio in a mass range ranging from 500 to 5600 Da. The mass spectra of the individual tryptic peptides and the relative molecular weights were shown in Figure 11 and Table 4.

The identification of the peptides was performed on the basis of the mass values recorded in the spectra (Figure 12) and the data are summarized in Table 4.

Determination of the molecular weight of KillerTRAIL

300 µl of KillerTRAIL sample, equivalent to 833 pmol, was purified by reverse phase chromatography (RP-HPLC) on a Phenomenex Jupiter C4 column (250 mm × 2.0 mm, pore size 300 Å) with a linear gradient 10% to 95% solvent B (0.07% TFA in 95% acetonitrile) for 30 minutes, at a flow rate of 0.2 ml/min using an Agilent Technologies 1100 HPLC (Agilent Technologies, USA) system connected with a UV-Vis detector.

The chromatographic peak in Figure 12 refers to the analysis of the identified KillerTRAIL protein using an HPLC system connected to a UV-Vis detector at a wavelength of 220 nm. The chromatographic peak corresponding to the elution of a sample of KillerTRAIL protein after drying in a SpeedVac centrifuge and reconstitution in the mobile phase (0.07% of TFA in 95% of vinegar nitrile). The analysis shows that KillerTRAIL has a single peak with a retention time of 18,876 minutes, symmetrical, which is free of impurities and well defined after chromatographic analysis.

The sample eluted by HPLC analysis was then re-dried by bench centrifuge (Savant Speed-Vac, Holbrook, NY) at a rate of 12,000 rotations per minute (rpm) for 4 h, at room temperature, resuspended in 50 µl of ACN 50% v/v and HCOOH 0.2% v/v and analyzed by ESI-MS mass spectrometry using the Q-TOF Premier system (Waters, Milford, MA, USA) with ESI source by direct injection with a calibrated syringe at a flow rate of 10 µl/min. The mass

spectrum was acquired in a range from 800 to 2500 m/z. Raw data was processed using MassLynx 4.1 software (Waters, Milford, MA, USA).

In the obtained ESI MS spectrum, the sample showed an experimental molecular weight of 24013.91 ± 0.94 Da which corresponds to the KillerTRAIL protein (theoretical molecular weight: 21607.1 Da) with a mass difference of 2406.81 Da calculated difference between the two species (TRAIL and KillerTRAIL) is represented by the peptide *linker* and the His tag (Figure 13). The experimental difference of 2406.81 Da obtained by making the difference between the theoretical molecular weight of 21607.1 Da of the known sequence of TRAIL and the experimental molecular weight (24013.39 Da) of KillerTRAIL show that the amino acid sequence of TRAIL is present and stored in the KillerTRAIL (commercial Enzo Life product) and there are also a peptide *linker* and a His tag sequence that are specific to KillerTRAIL and are not present in the TRAIL. The presence of a peptide *linker* and His tag is confirmed by the ESI MS mass spectrum for direct injection which shows fragmentation peaks for functional group signals related to the amino acid sequence of TRAIL together with those of the peptide *linker* and His tag (Figure 13).

The peak in Figure 13A indicates the molecular weight of the protein (equal to 24013.91 Da) relative to KillerTRAIL consisting of the known sequence TRAIL, the Killer peptide and the His tag. The ESI MS analysis indicates that the peak corresponding to the molecular weight 24013.91 Da is the main one obtained from the mass spectrum and therefore characteristic of KillerTRAIL. This mass value is obtained through the deconvolution process of the high (Figure 1 3B) and low (Figure 13C) signal intensity mass spectra.

Following enzymatic digestion and LC-MS analysis, the peptide corresponding to KillerTRAIL can in turn be isolated and further fragmented in tandem mass spectrometry into the individual amino acids that compose it. The mass spectra shown in Figure 13B and Figure 13C contain the peptide *linker* and the His tag. To obtain the *linker* peptide sequence it is necessary to resort to further tandem mass spectrometry (MS/MS) experiments following enzymatic digestion, isolation of the peptide *linker*

and its subsequent fragmentation in MS/MS. The tandem mass spectrometry allows to fragment a single peptide using an appropriate collision energy which, together with the presence of an inert collision gas (generally Argon), causes the breaking of the peptide bonds providing the amino acid sequence of the peptide of interest.

CLAIMS

1) Polymeric conjugate of KillerTRAIL consisting of polyethylene glycol bonded with stable covalent CS type bond on the cysteine in position 230 of the single-stranded KillerTRAIL.

2) Polymeric conjugate according to claim 1 comprising the sequence of SEQ.ID.N1.

3) Polymeric conjugate according to any one of claims 1-2 wherein the polyethylene glycol (PEG) is an alkoxy- preferably methoxy-polyethylene glycol derivatized with a thiol reactive group, preferably a maleimide group.

4) Polymeric conjugate according to any one of claims 1-3 wherein the polyethylene glycol has a molecular weight comprised between 2-200 kDa, preferably 10-50 kDa.

5) Polymeric conjugate according to any one of claims 1-4 wherein the polyethylene glycol has the alkoxy group in alpha and is preferably the methoxy-polyethylene glycol (mPEG) and the cysteine reactive group is in omega.

6) Polymer conjugate according to any one of claims 1-5 which is a radiolabelled conjugate.

7) Polymeric conjugate according to any one of claims 1-6 for use in the medical and diagnostic field.

8) Polymeric conjugate according to any one of claims 1-7 for use in the diagnosis and treatment of the following conditions: tumors, autoimmune diseases, viral diseases.

9) Pharmaceutical and diagnostic composition comprising the polymeric conjugate according to any one of claims 1-8 and a pharmaceutically or diagnostically acceptable adjuvant.

10) Pharmaceutical and diagnostic composition comprising the polymeric conjugate according to any one of claims 1-8 in which the affections are selected from: multiple sclerosis, autoimmune thyroiditis, Crohn's disease, active ulcerative colitis, autoimmune encephalomyelitis, atopic dermatitis, systemic lupus erythematosus, rheumatoid arthritis , autoimmune arthritis, autoimmune diabetes; leukemia, breast cancer, prostate cancer, lung

cancer, kidney cancer, pancreatic cancer, Alzheimer's, melanoma, myelodysplastic syndromes, lymphomas, liver cancer, HBV Hepatitis B, HCV Hepatitis C, unassociated fatty liver disease, liver fibrosis tumor, liver cirrhosis , pneumonia, mesothelioma, asthma, infection, influenza virus, atherosclerosis, multiple sclerosis, HIV, lateral amyotrophic sclerosis, cytomegalovirus, myasthenia gravis, ductal adenocarcinoma of the pancreas and neuroendocrine tumors originating from the cells of the islets of Langerhans, lung cancer and non-small cell lung cancer, squamous cell carcinoma, large cell carcinoma, cervical cancer, squamous cell carcinoma, mesothelioma of the lung (pleura), heart (pericardium), intestines (peritoneum) and testicles (tunica vaginale), rhabdomyosarcoma (embryonic and alveolar), gliomas and their subtypes (glioblastoma, anaplastic astrocytoma, diffuse astrocytoma, pilocytic astrocytoma, other astrocytomas, oligodendroglioma, ependymoma), chronic lymphocytic leukemia.

11) Pharmaceutical and diagnostic composition comprising the polymeric conjugate according to any one of claims 1-8 and further comprising one or more therapeutically active agents selected from: 5-fluorouracil, CPT-11, doxorubicin, gemcitabine, paclitaxel, ionizing radiation; one or more radio-labeled diagnostic agents selected from ^{125}I, ^{188}Re, ^{188}W.

12) Pharmaceutical and diagnostic composition comprising the polymeric conjugate according to any one of claims 1-8 formulated for oral, parenteral, rectal, topical, vaginal, ophthalmic or inhalation use.

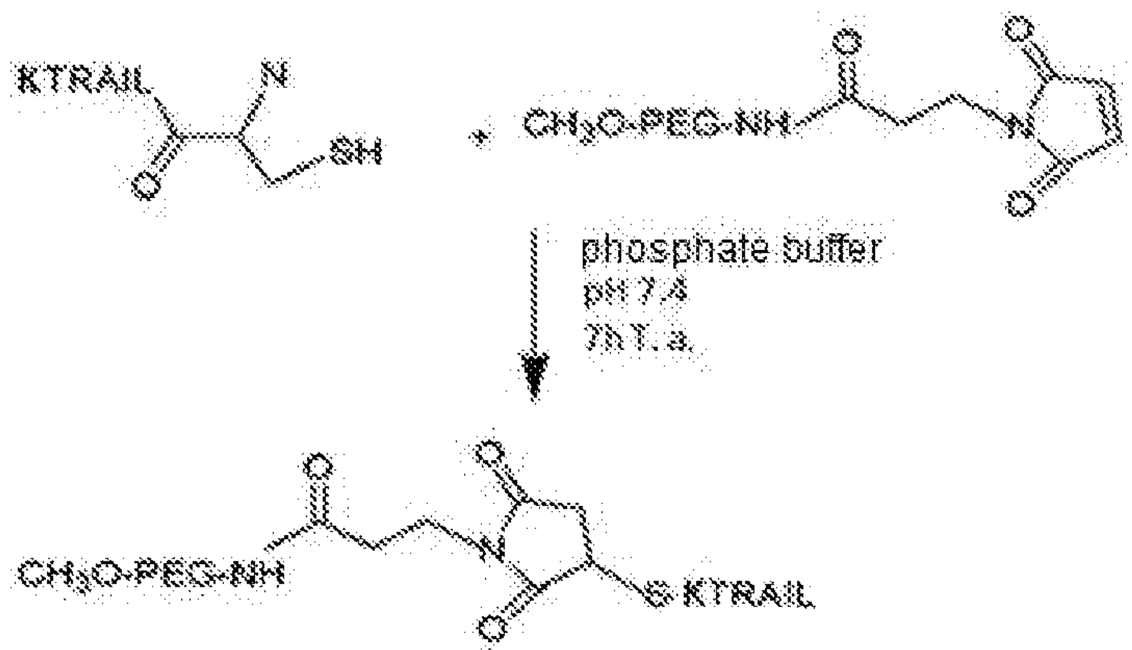


Figure 1

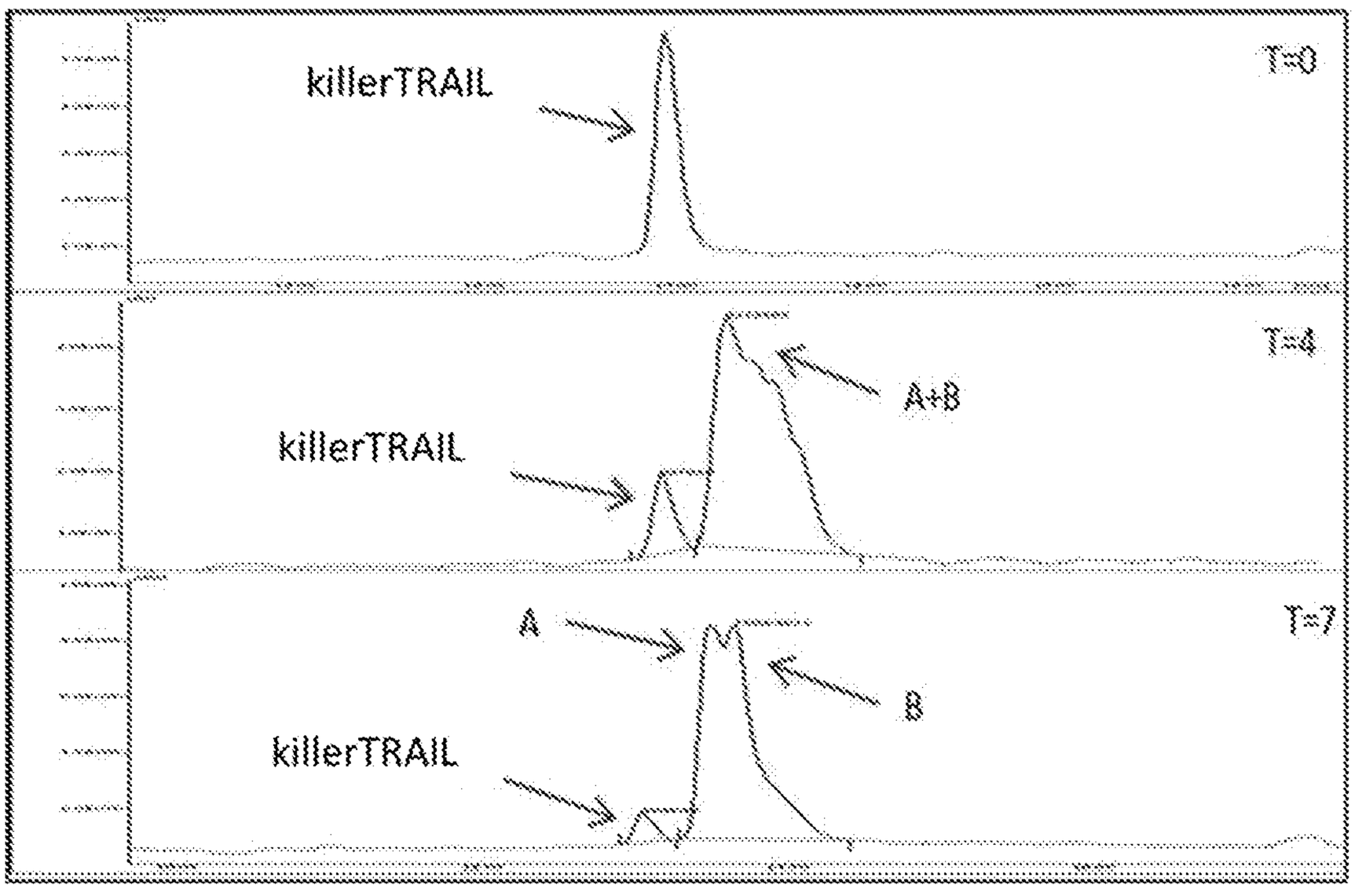


Figure 2

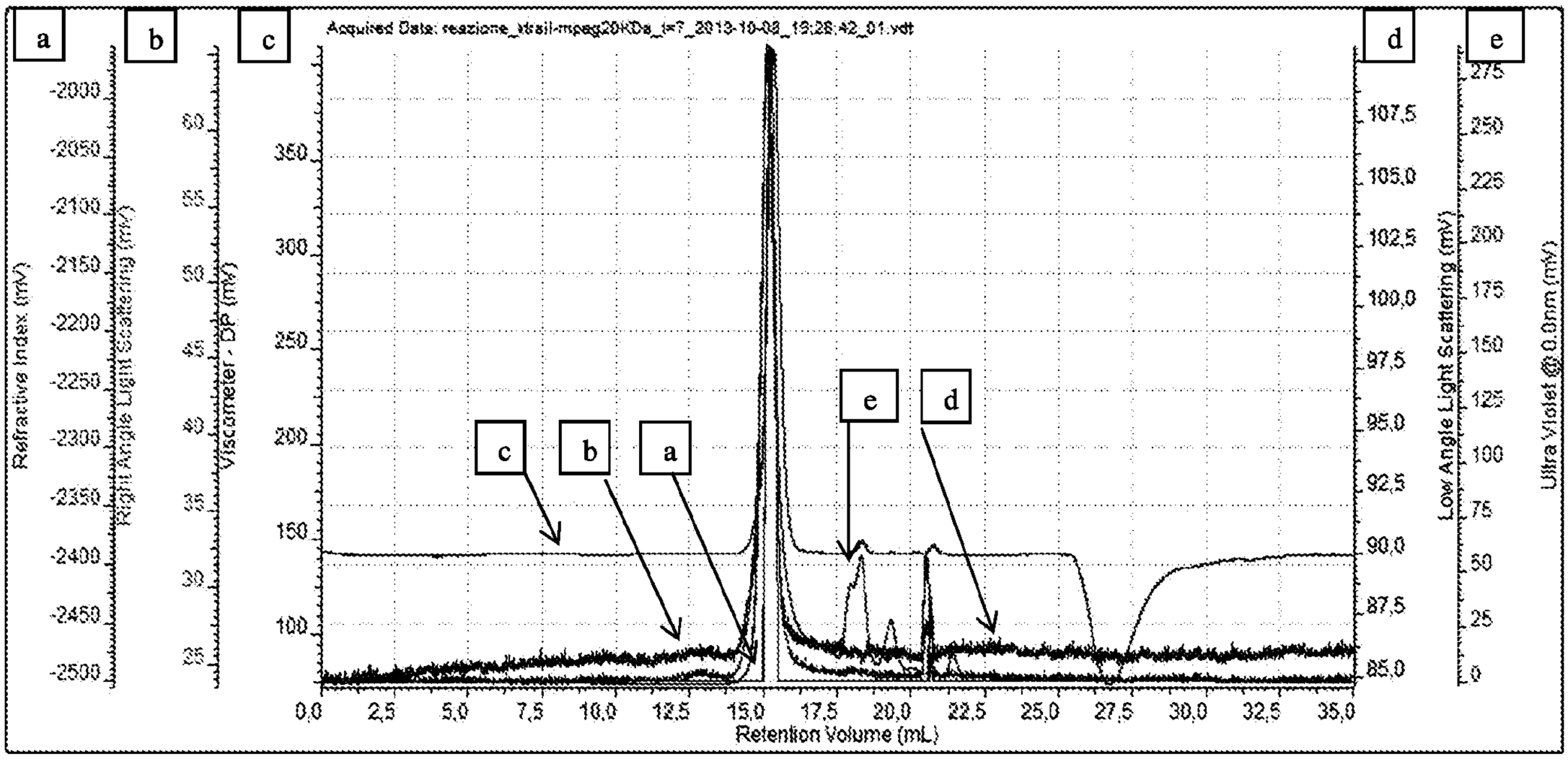


Figure 3

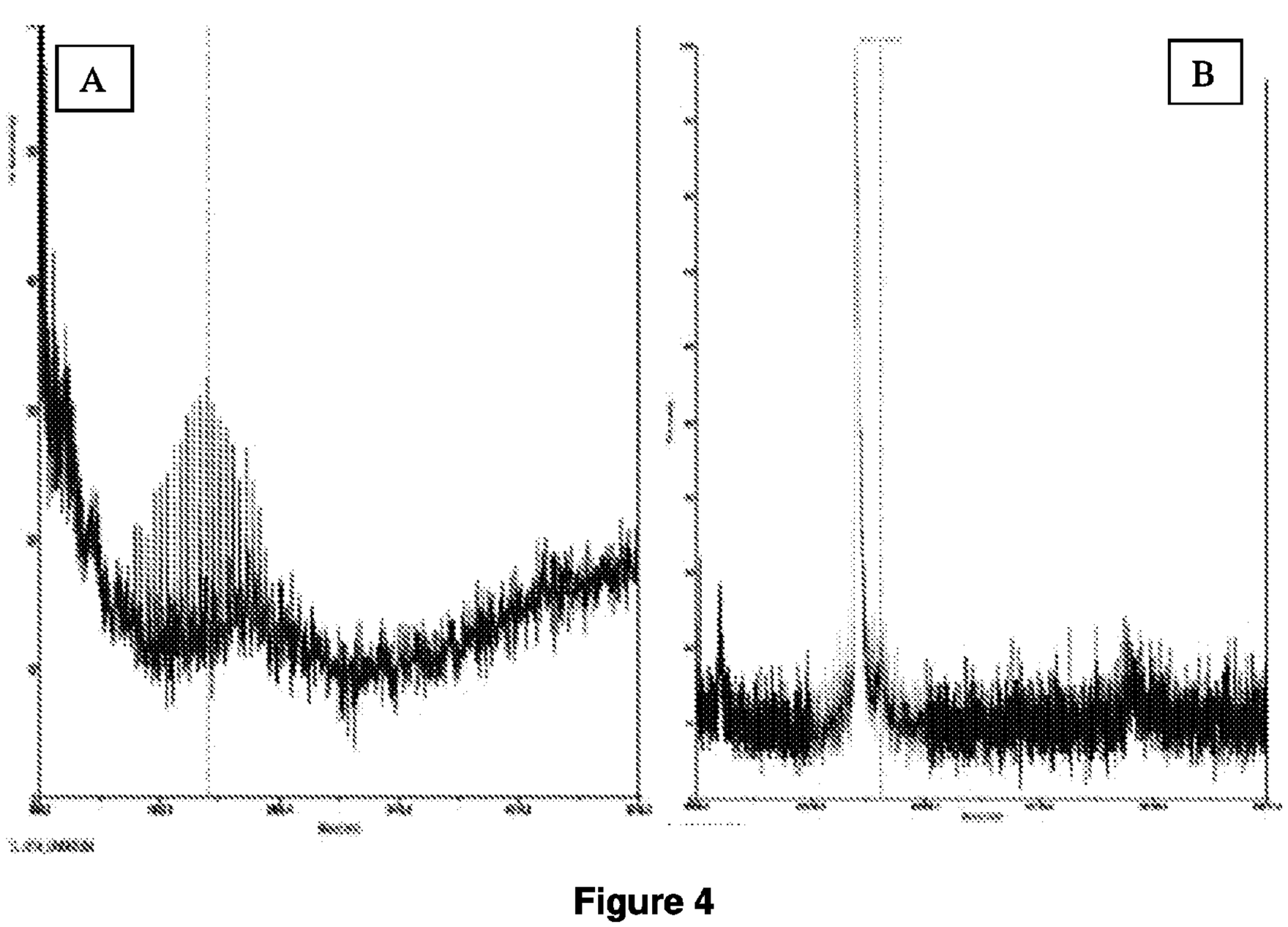


Figure 4

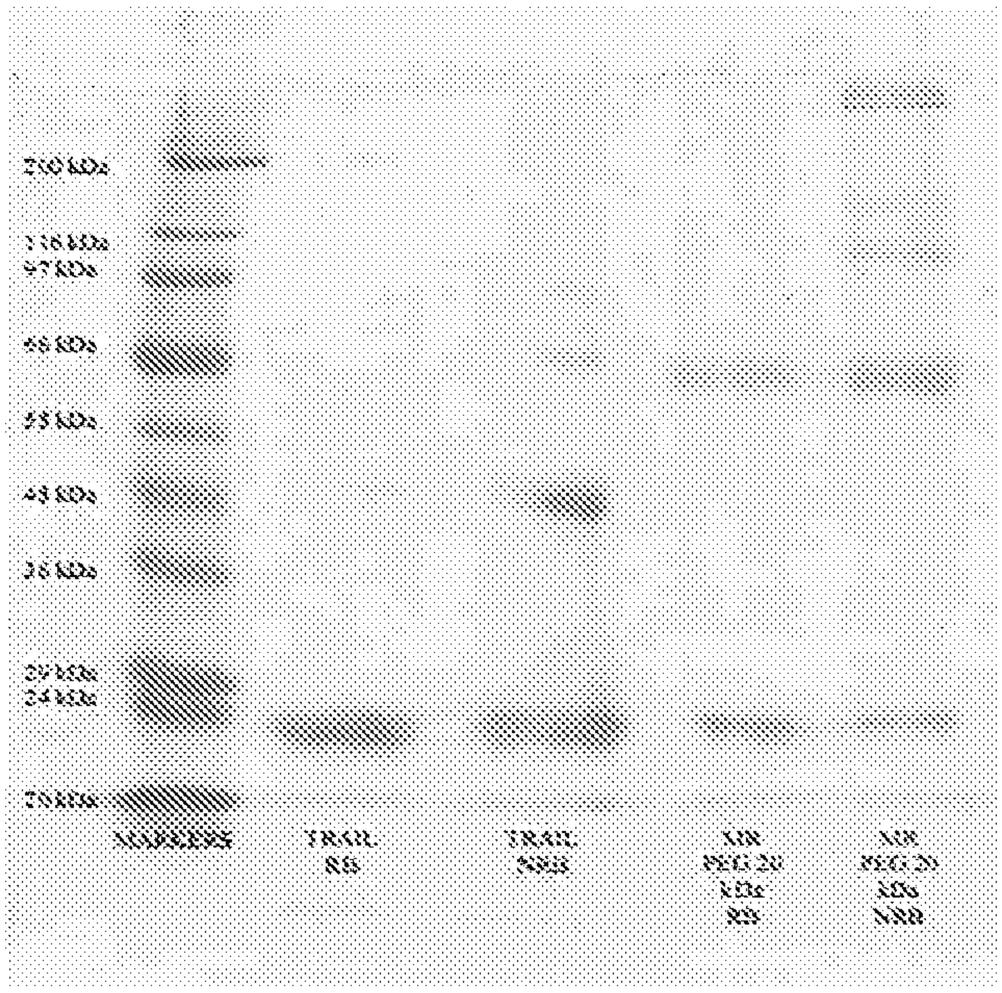


Figure 5

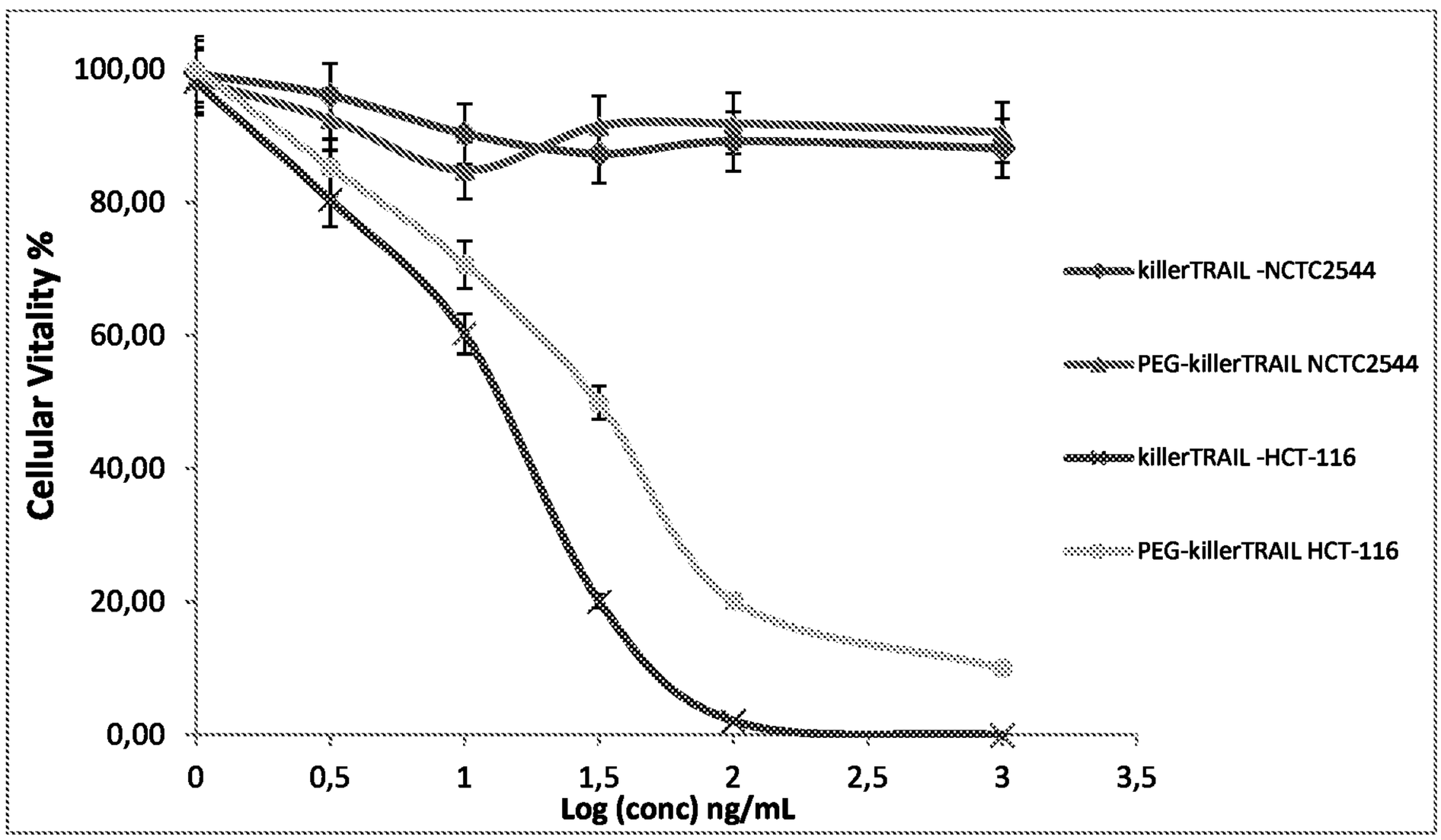
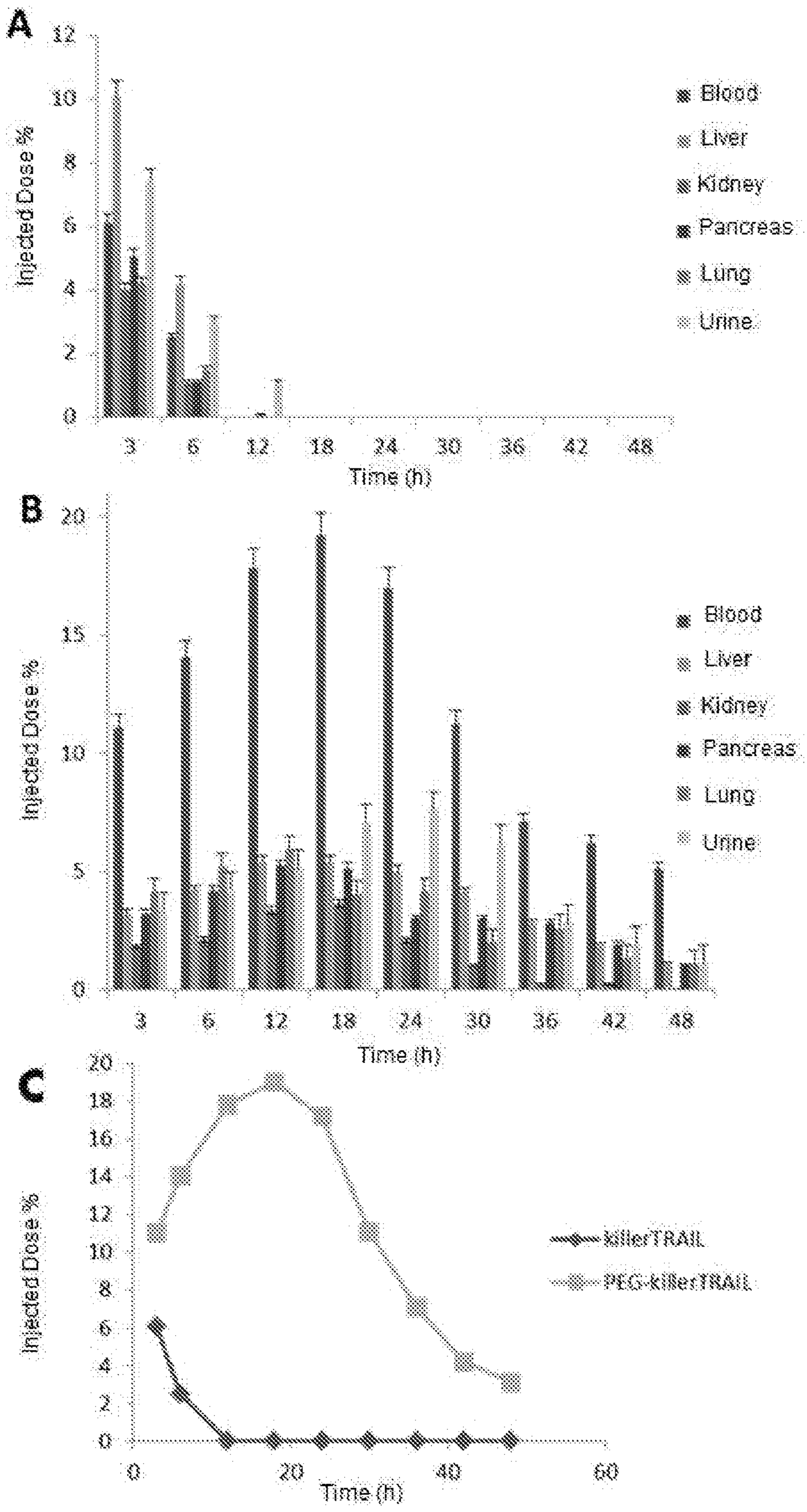


Figure 6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Injected Dose %
0
2
4
6
8
10
12
3
6
12
18
24
30
36
42
48
Time (h)
Blood
Liver
Kidney
Pancreas
Lung
Urine
B
Injected Dose %
0
5
10
15
20
3
6
12
18
24
30
36
42
48
Time (h)
Blood
Liver
Kidney
Pancreas
Lung
Urine
C
Injected Dose %
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2
4
6
8
10
12
14
16
18
20
0
20
40
60
Time (h)
killerTRAIL
PEG-killerTRAIL

Figure 7

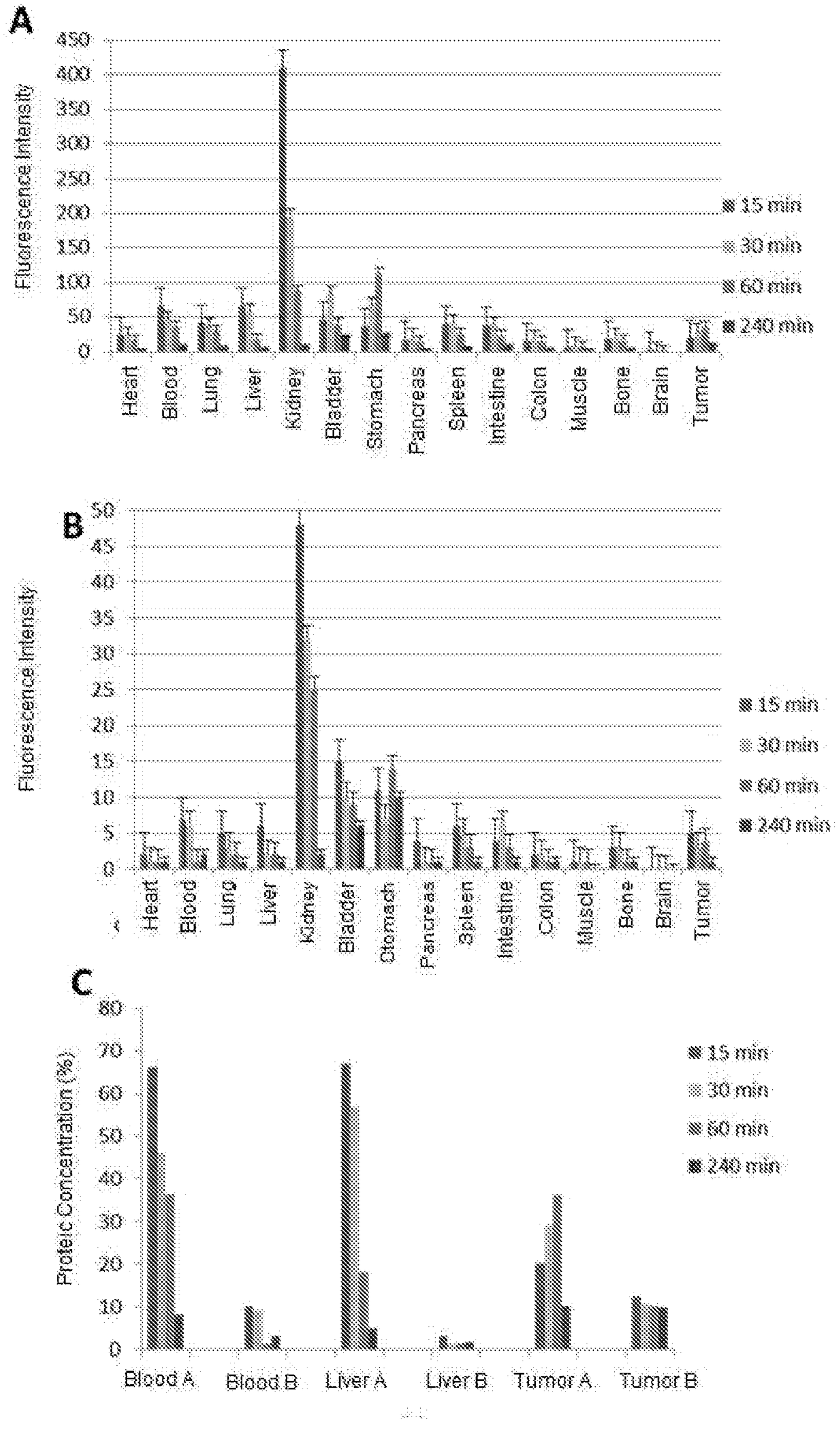

A
Fluorescence Intensity
450
400
350
300
250
200
150
100
50
0
Heart
Blood
Lung
Liver
Kidney
Bladder
Stomach
Pancreas
Spleen
Intestine
Colon
Muscle
Bone
Brain
Tumor
15 min
30 min
60 min
240 min
B
Fluorescence Intensity
50
45
40
35
30
25
20
15
10
5
0
Heart
Blood
Lung
Liver
Kidney
Bladder
Stomach
Pancreas
Spleen
Intestine
Colon
Muscle
Bone
Brain
Tumor
15 min
30 min
60 min
240 min
C
Protein Concentration (%)
80
70
60
50
40
30
20
10
0
Blood A
Blood B
Liver A
Liver B
Tumor A
Tumor B
15 min
30 min
60 min
240 min

Figure 8

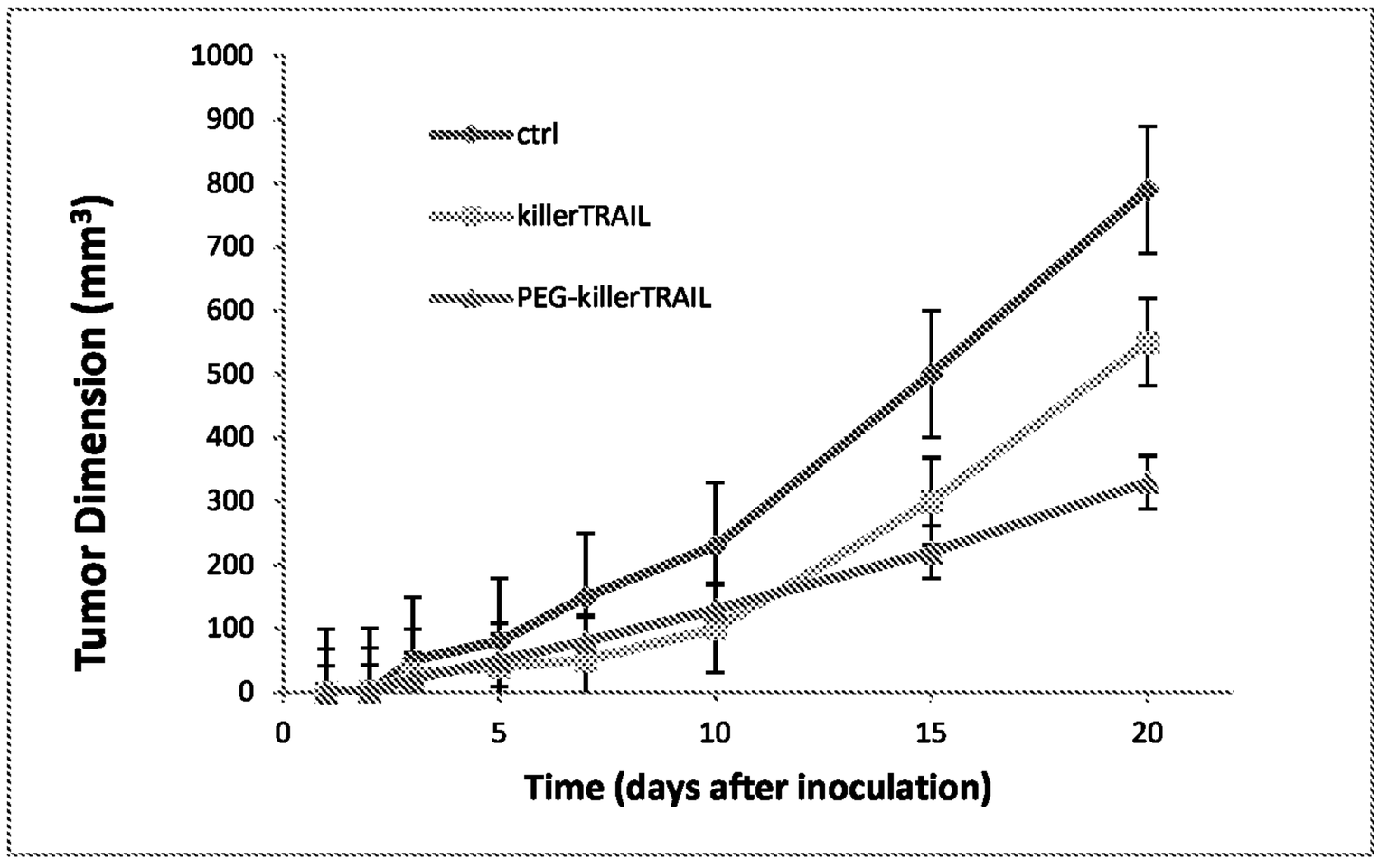

Tumor Dimension (mm³)
1000
900
800
700
600
500
400
300
200
100
0
0
5
10
15
20
Time (days after inoculation)
ctrl
killerTRAIL
PEG-killerTRAIL

Figure 9

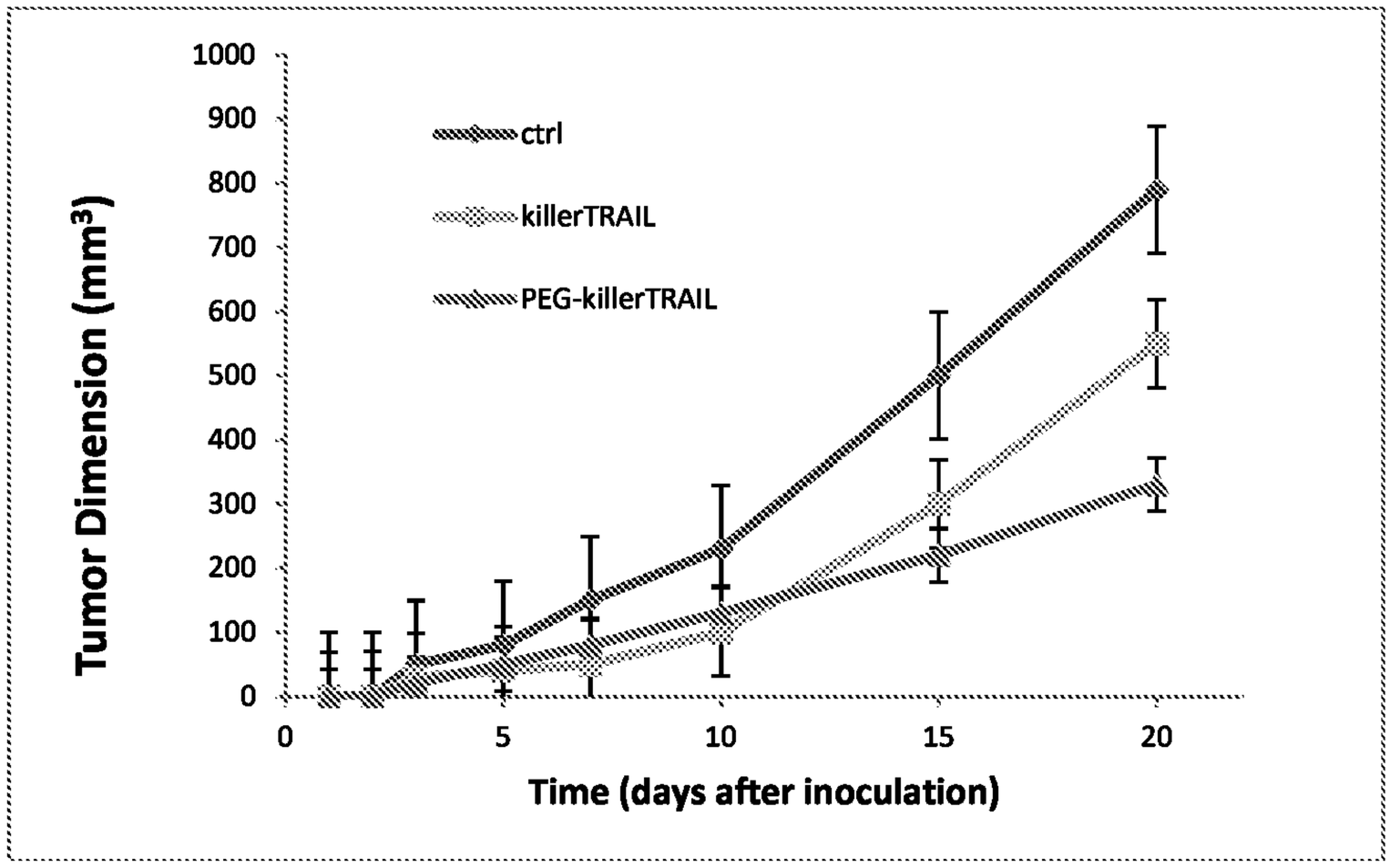


Figure 10A (reprinted from Figure 9 object of the invention)

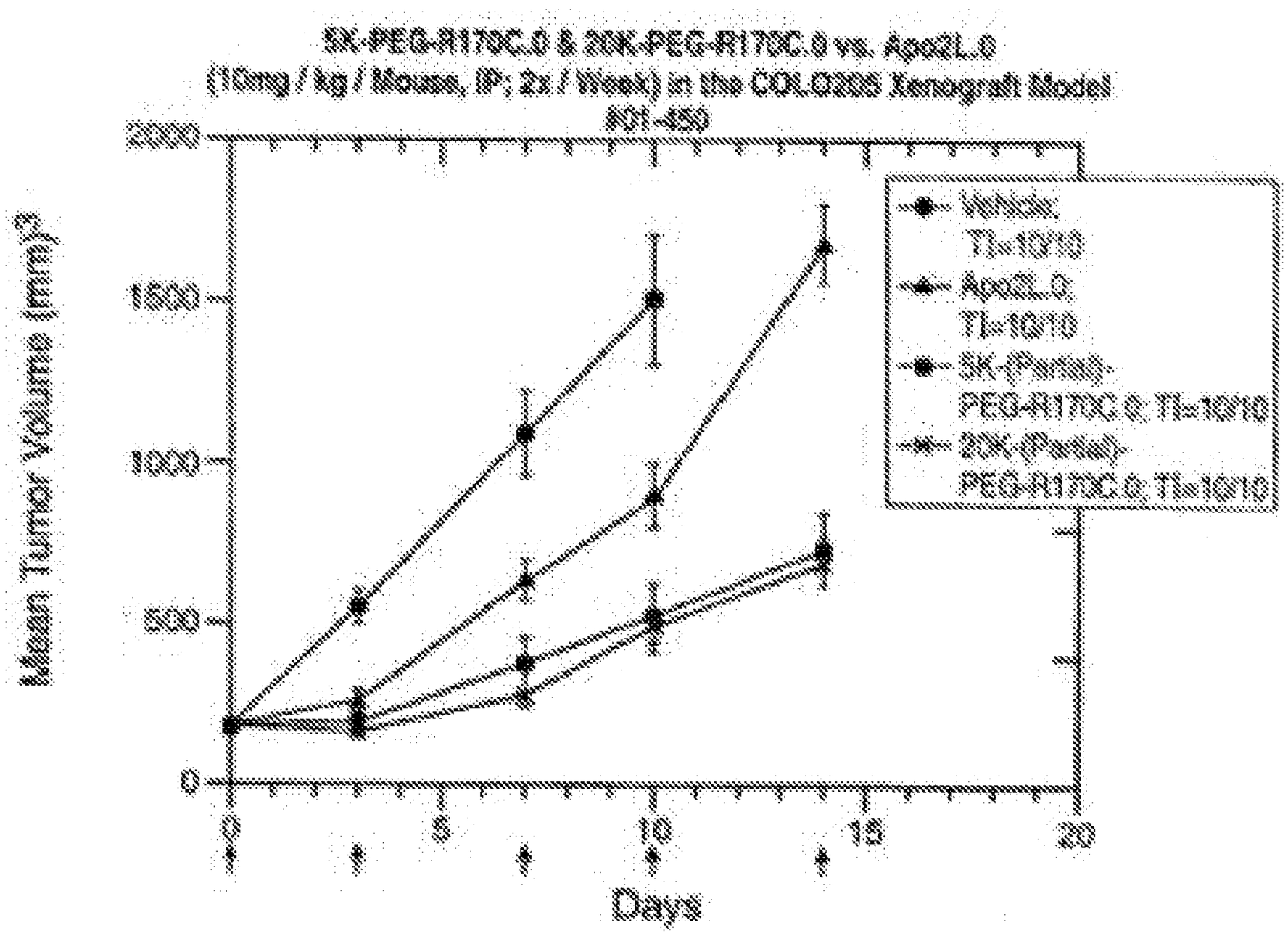


Figure 10B (reprinted from WO2004001009)

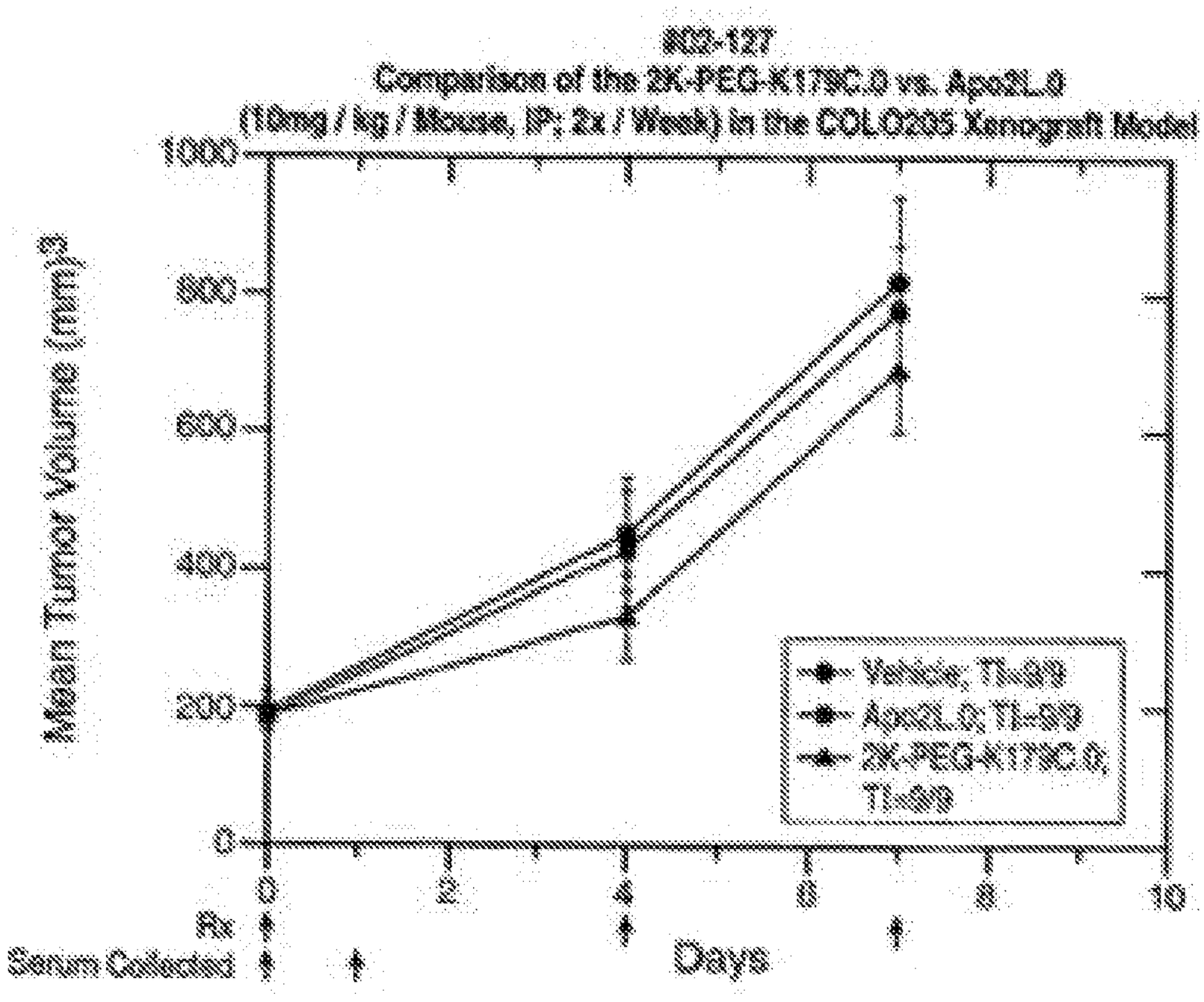


Figure 10B (reprinted from WO2004001009)

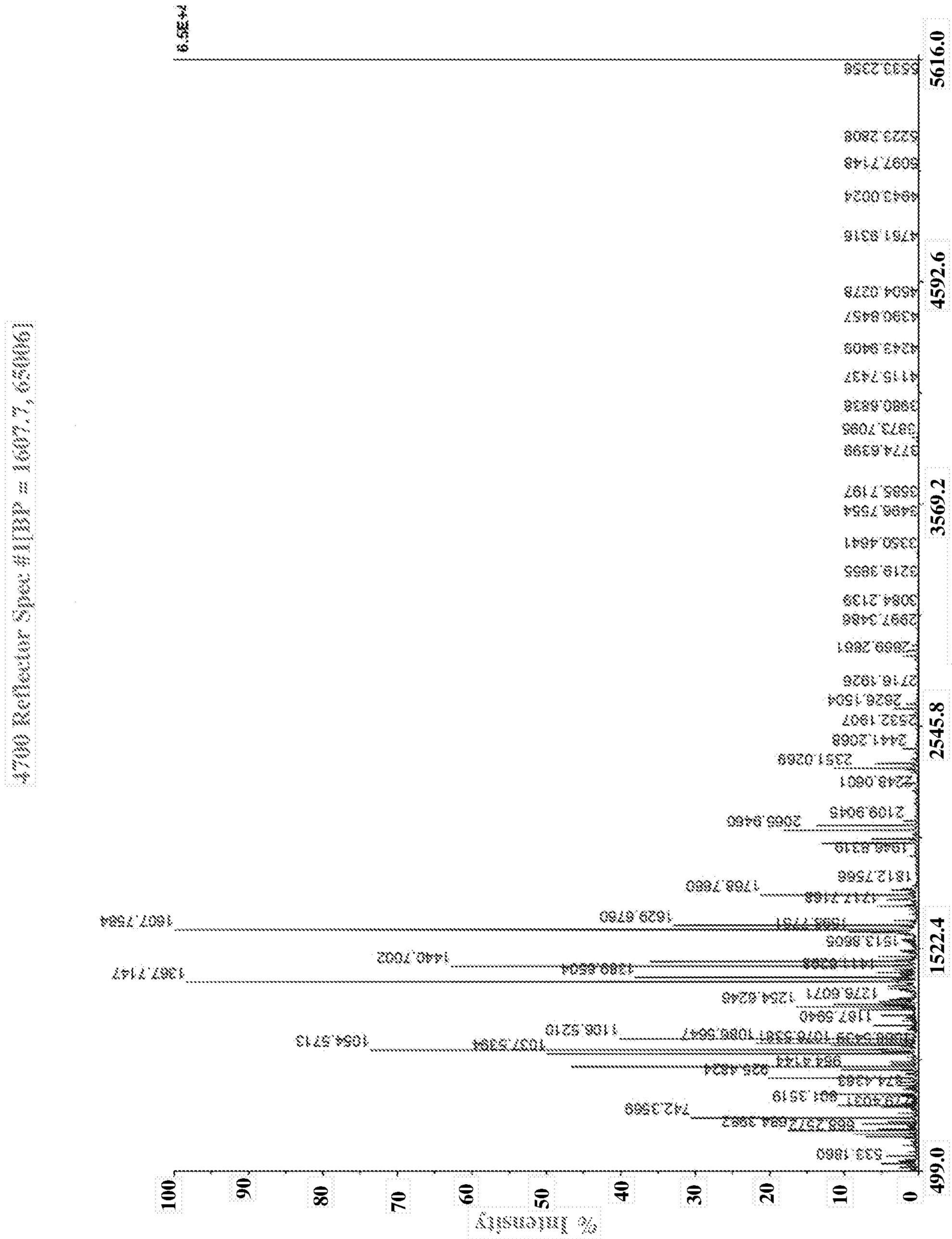


Fig. 11A

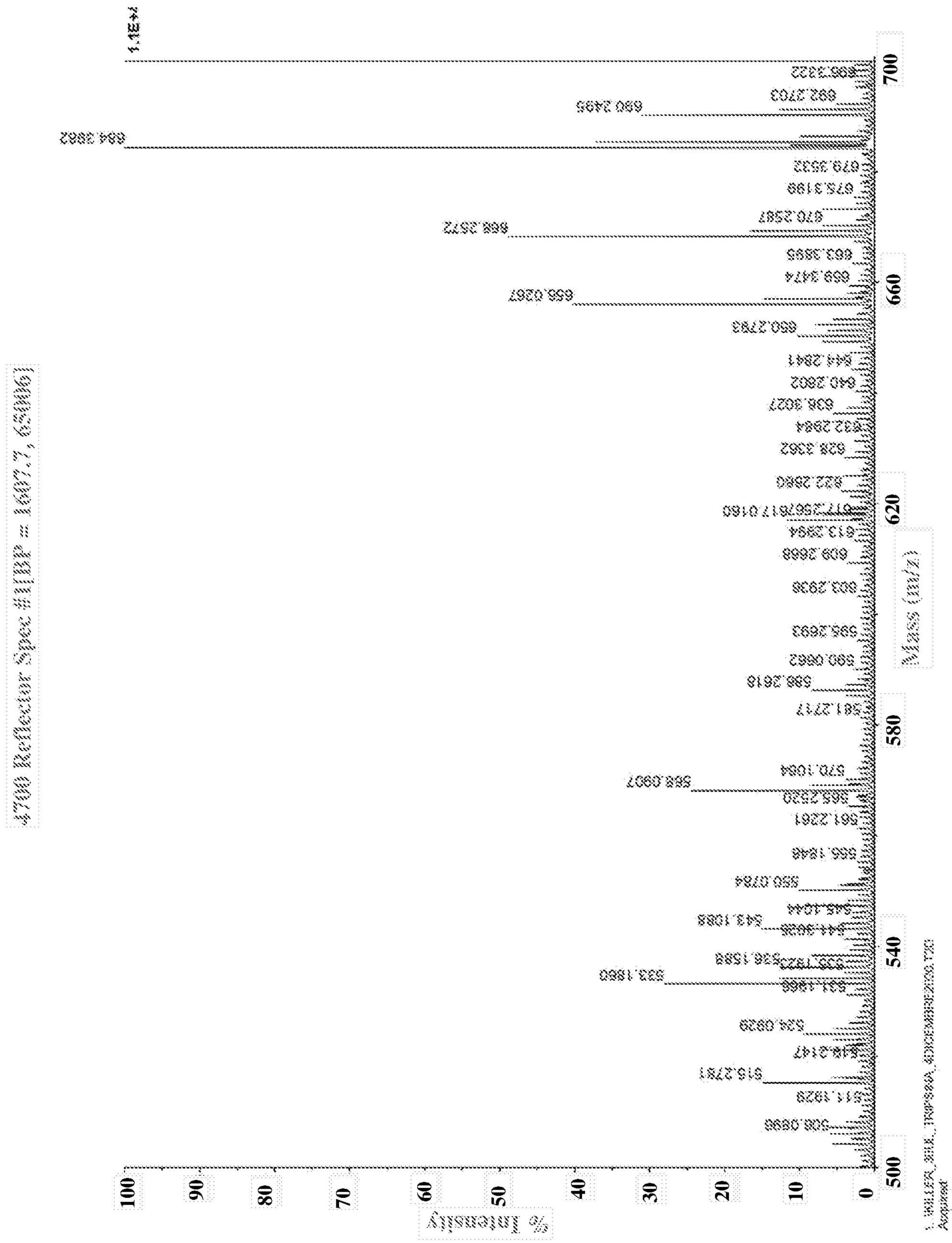

700
660
620
580
540
500
% Intensity
100
90
80
70
60
50
40
30
20
10
0
Mass (m/z)
1.1E+4
694.2862
692.2703
690.2495
686.3322
679.3532
675.3199
670.2587
668.2572
659.3474
658.0267
650.2783
644.2841
640.2802
638.3027
632.2894
628.3362
622.2860
617.2567/617.0160
613.2954
609.2668
603.2938
595.2693
590.0862
588.2618
581.2717
570.1064
568.0907
565.2520
561.2261
555.1848
550.0784
545.1044
543.1088
541.3036
535.1822/526.1588
533.1860
531.1868
524.0829
518.3147
516.2791
511.1929
508.0898

Fig. 11B

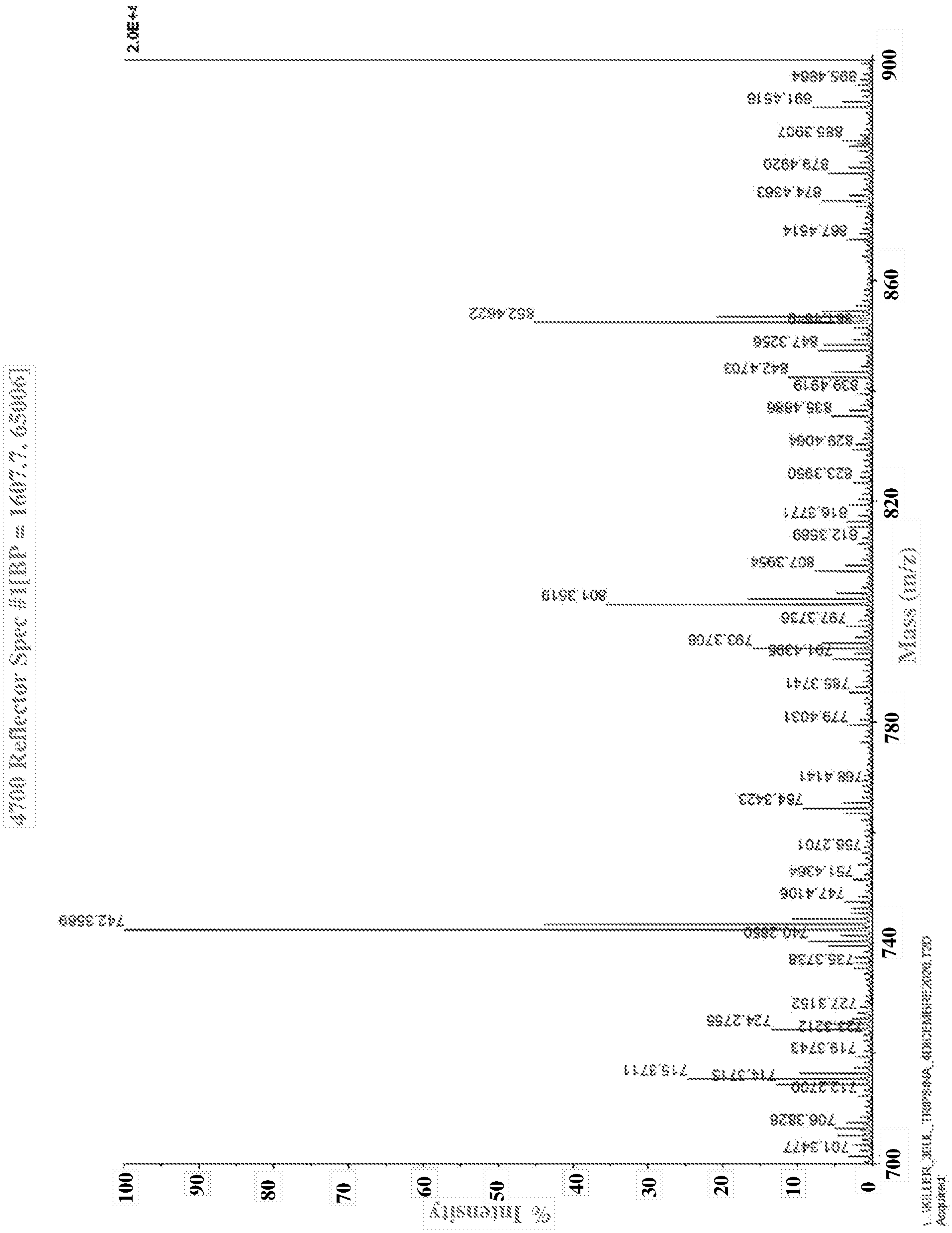

4700 Reflector Spec #1[BP = 1607.7, 65006]
2.0E+4
% Intensity
Mass (m/z)
100
90
80
70
60
50
40
30
20
10
0
700
740
780
820
860
900
701.3477
706.3826
713.3700
714.3718
715.3711
718.3743
724.2765
727.3152
736.3738
740.2880
742.3589
747.4106
751.4364
758.2701
764.3423
768.4141
779.4031
785.3741
793.3708
797.3736
801.3618
807.3954
812.3569
816.3771
823.3850
828.4064
835.4886
839.4918
842.4703
847.3256
852.4622
867.4514
874.4363
879.4920
885.3907
891.4518
895.4694

Fig. 11C

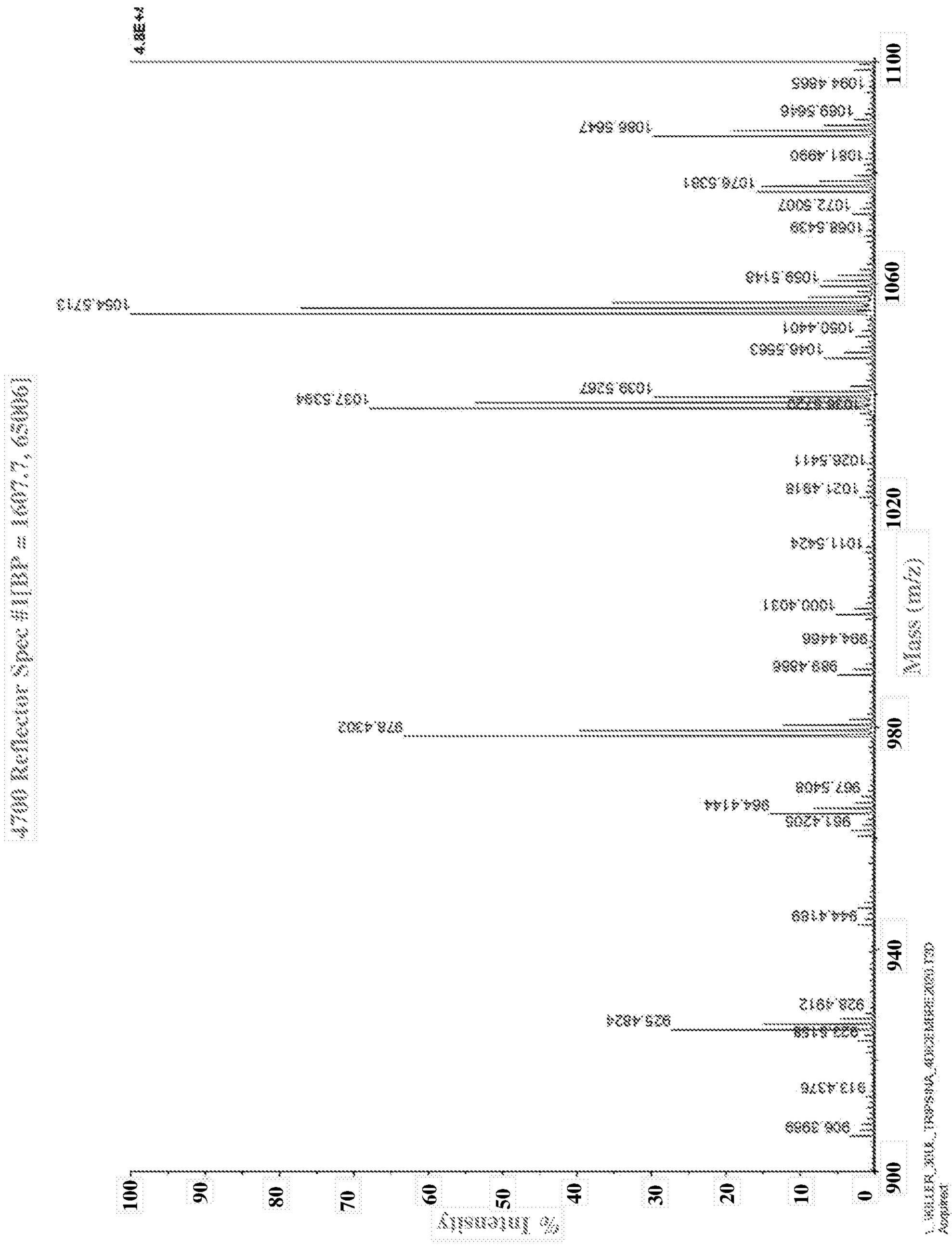

1100
1060
1020
980
940
900
% Intensity
Mass (m/z)
100
90
80
70
60
50
40
30
20
10
0

Fig. 11D

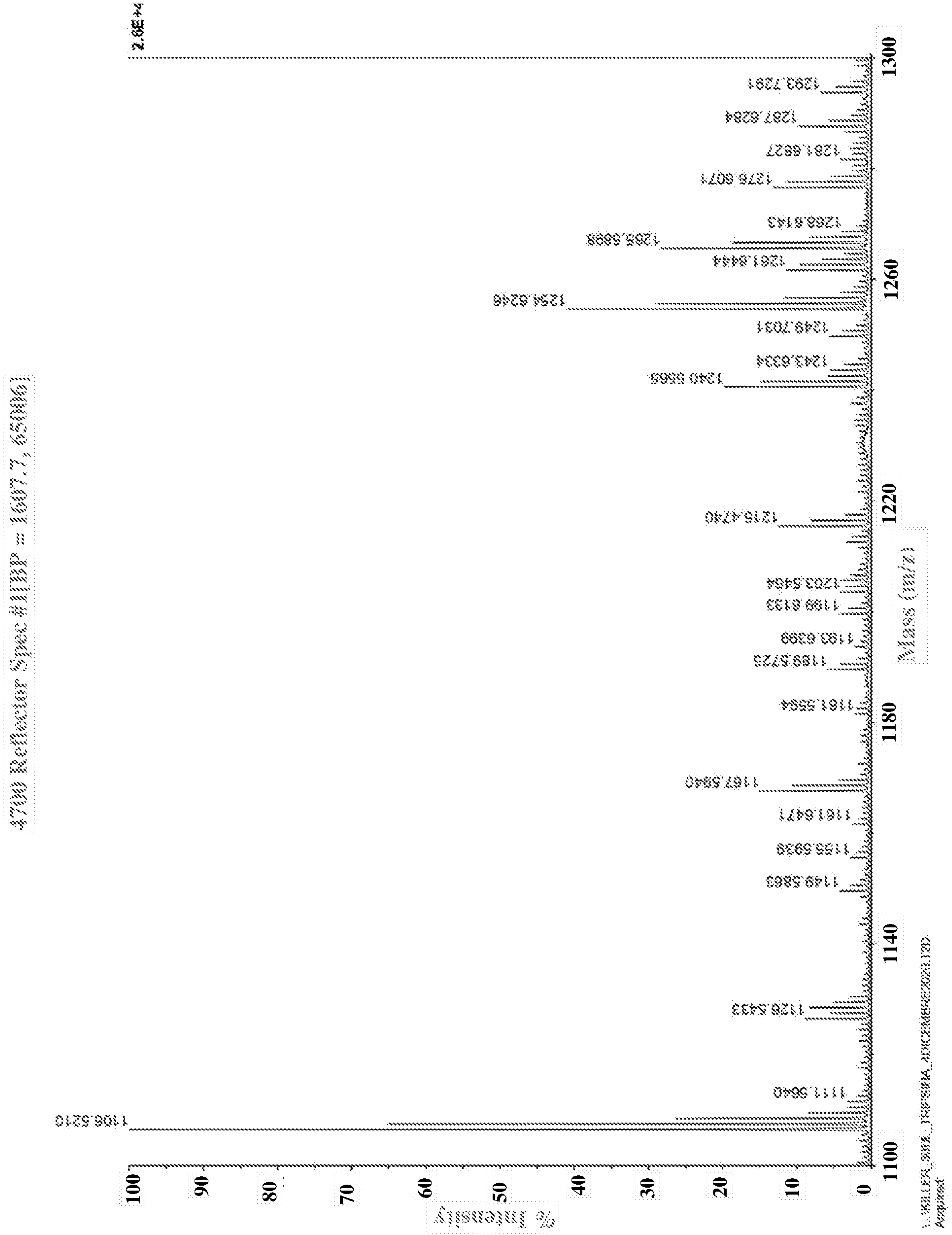

4700 Reflector Spec #1[BP = 1607.7, 65006]
2.6E+4
% Intensity
Mass (m/z)
0
10
20
30
40
50
60
70
80
90
100
1100
1140
1180
1220
1260
1300
1106.5210
1111.5640
1128.5433
1149.5863
1155.5938
1161.6471
1167.5840
1181.5504
1189.5725
1193.6399
1199.6133
1203.5464
1215.4740
1240.5565
1243.6334
1248.7031
1254.6246
1261.6444
1265.5698
1268.6143
1276.6071
1281.6627
1287.6284
1293.7291

Fig. 11E

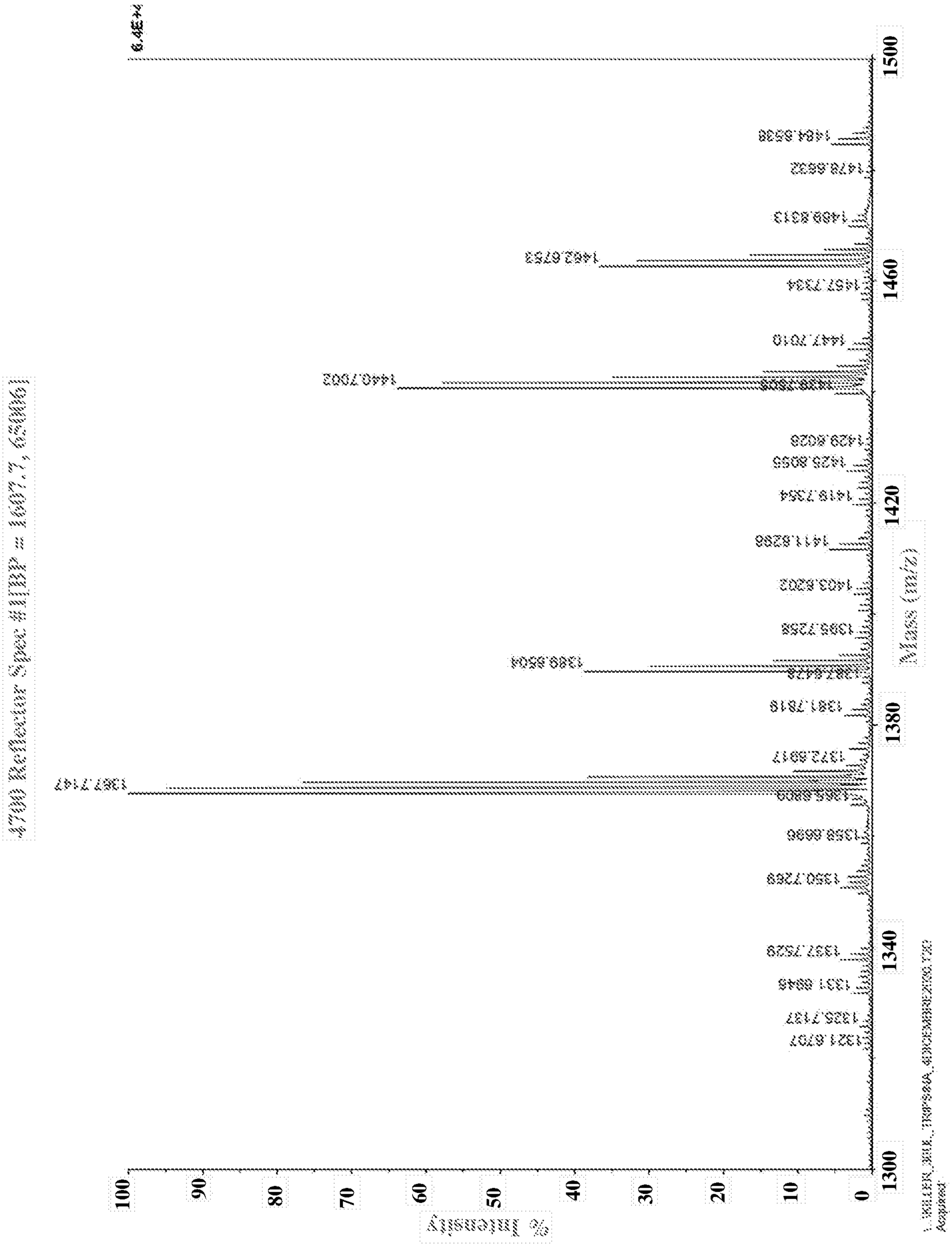

1500
1460
1420
1380
1340
1300
% Intensity
Mass (m/z)
1464.8538
1478.8632
1408.8613
1462.6753
1457.7334
1447.7010
1438.7606
1440.7003
1429.8026
1425.8055
1418.7354
1411.8286
1403.8202
1396.7258
1389.8504
1387.6428
1381.7818
1372.8017
1365.8809
1387.7147
1358.8696
1350.7269
1337.7529
1331.8946
1325.7137
1321.8707

Fig. 11F

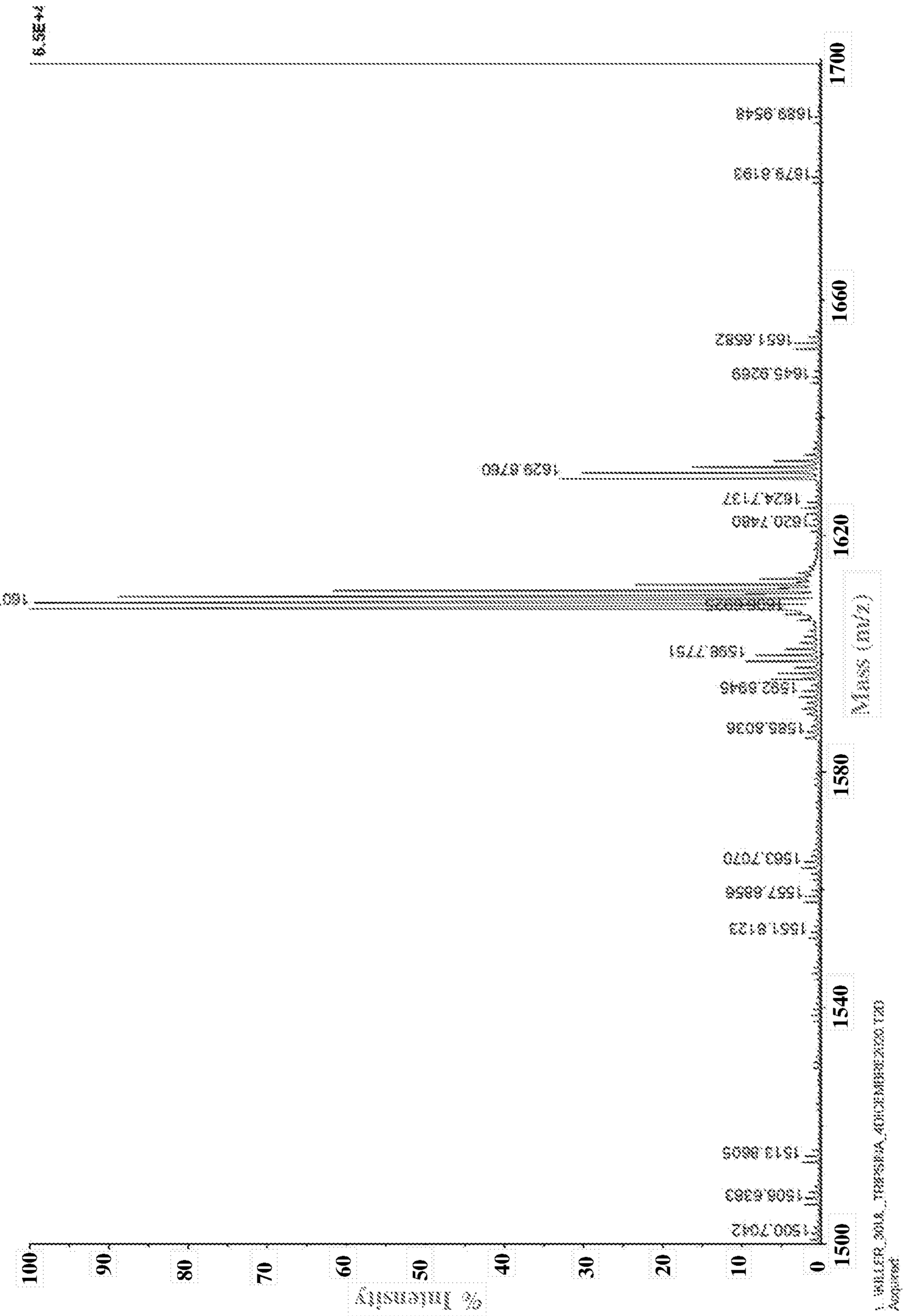

% Intensity
Mass (m/z)
1700
1660
1620
1580
1540
1500
100
90
80
70
60
50
40
30
20
10
0

Fig. 11G

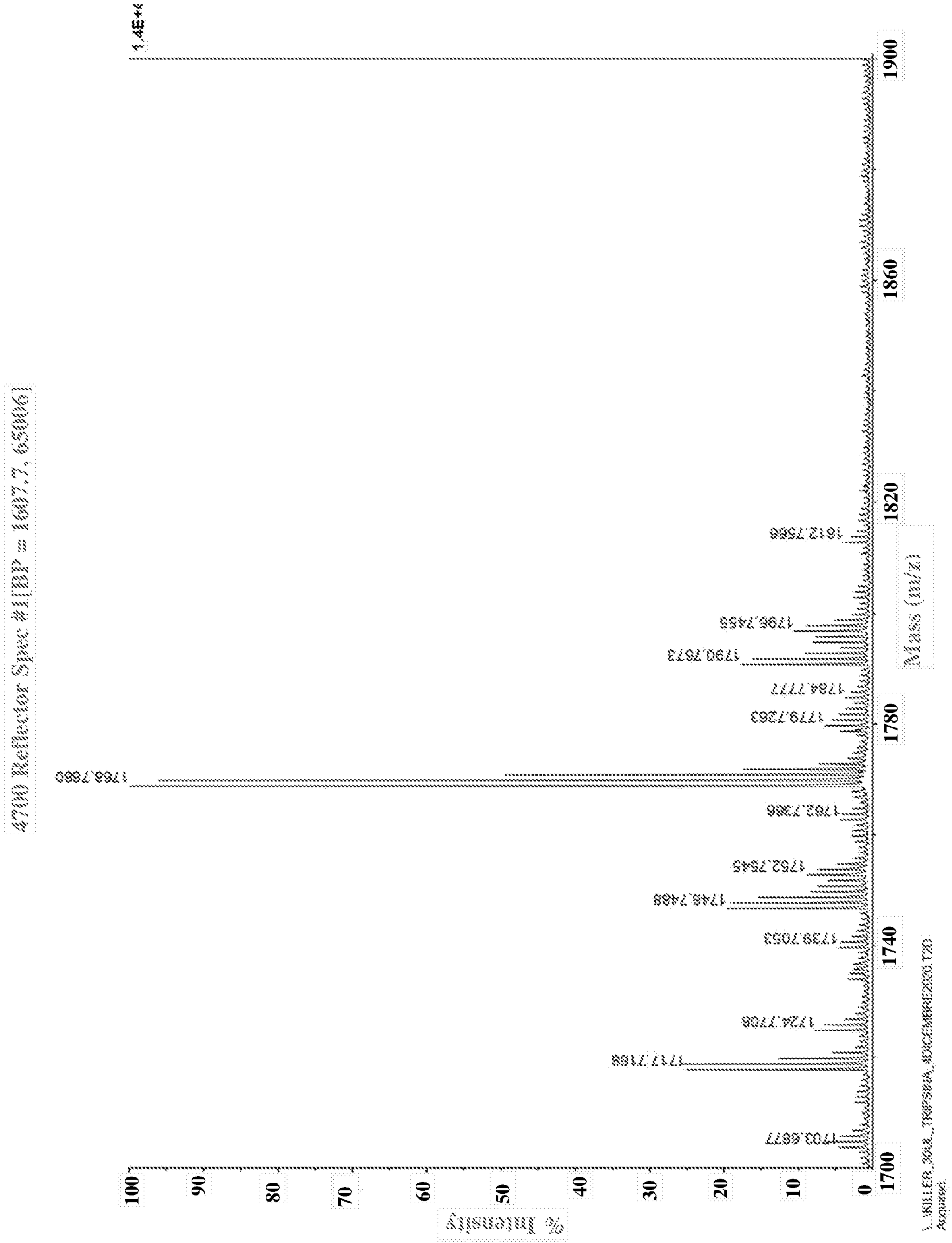

1.4E+4
1900
1860
1820
1780
1740
1700
1612.7586
1796.7455
1790.7673
1784.7777
1779.7263
1762.7386
1752.7545
1746.7468
1739.7053
1724.7708
1717.7168
1703.6877
1766.7660
m/z
% Intensity
100
90
80
70
60
50
40
30
20
10
0

Fig. 11H

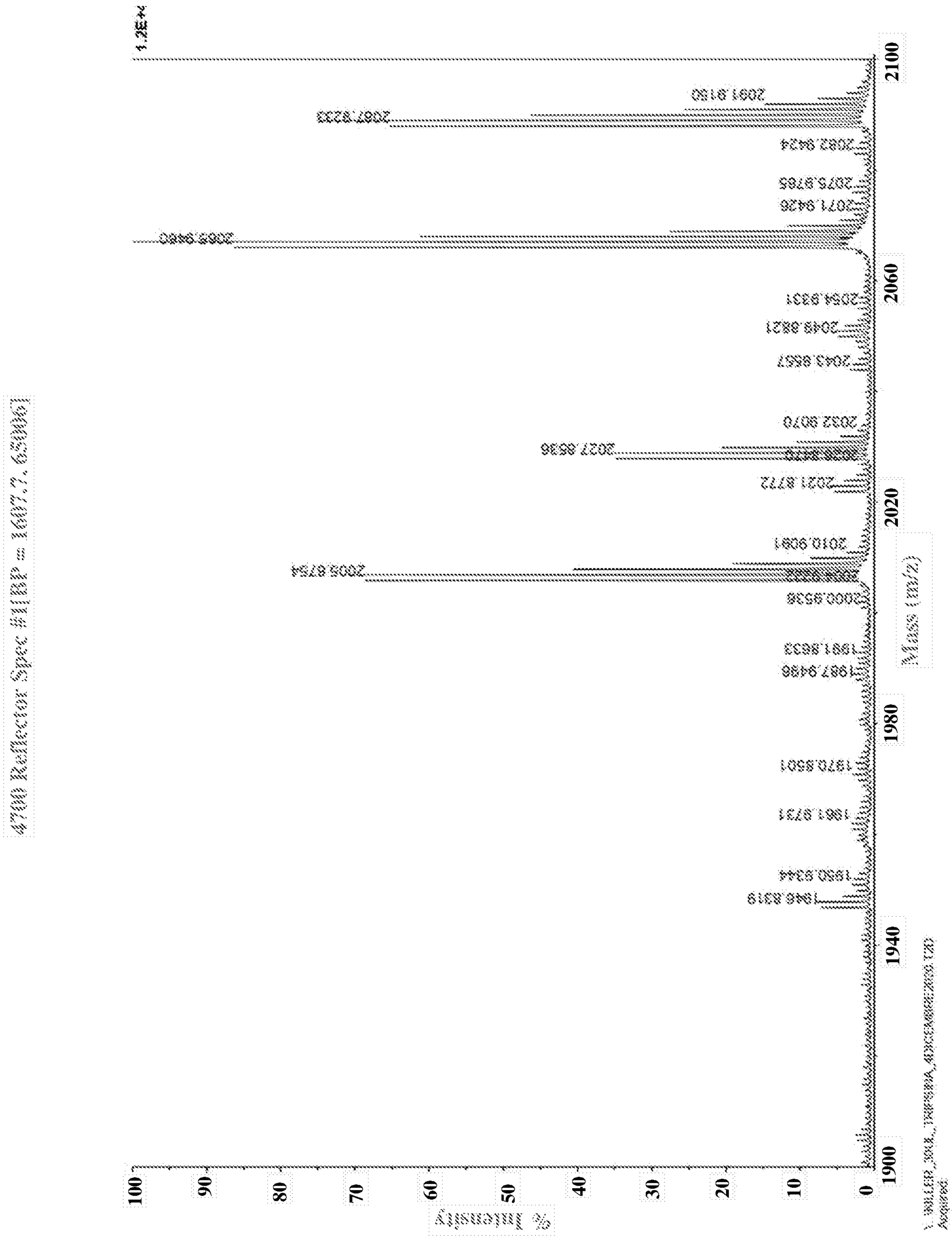

% Intensity
Mass (m/z)
2100
2060
2020
1980
1940
1900
2091.8180
2087.8233
2082.8424
2075.8765
2071.8426
2065.8480
2054.8331
2048.8821
2043.8537
2032.8070
2027.8536
2021.8772
2010.9081
2005.8754
2000.8538
1987.8486
1981.8633
1970.8501
1961.8731
1950.8344
1946.8319

Fig. 11I

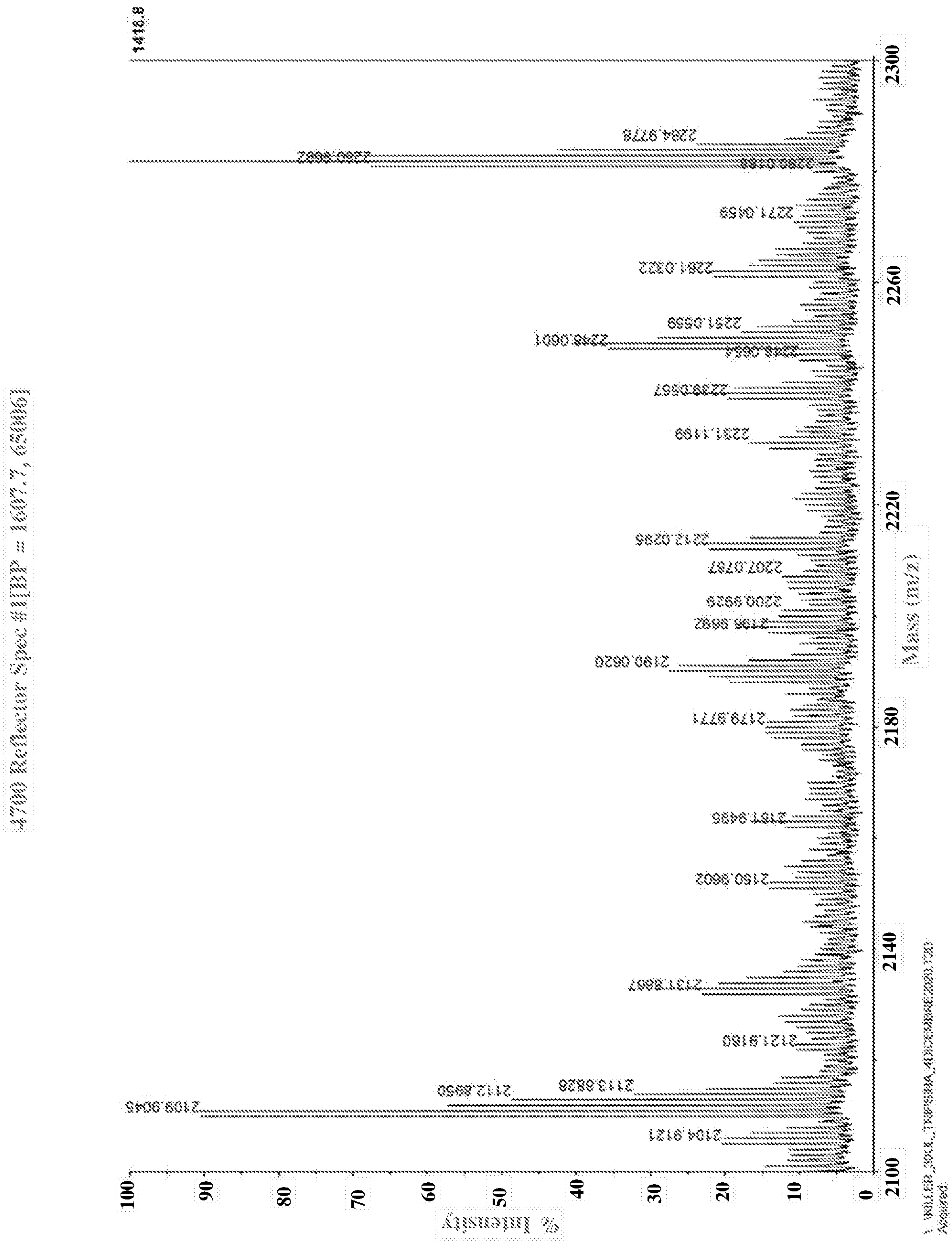

4700 Reflector Spec #1[BP = 1607.7, 65006]
1418.8
% Intensity
100
90
80
70
60
50
40
30
20
10
0
Mass (m/z)
2100
2140
2180
2220
2260
2300
2104.9121
2108.9045
2112.8850
2113.8828
2121.9180
2137.8867
2150.8602
2161.9485
2179.9771
2190.0620
2198.9682
2200.9929
2207.0767
2212.0296
2231.1189
2239.0557
2248.0601
2251.0559
2261.0322
2271.0459
2280.8682
2284.9778

Fig. 11J

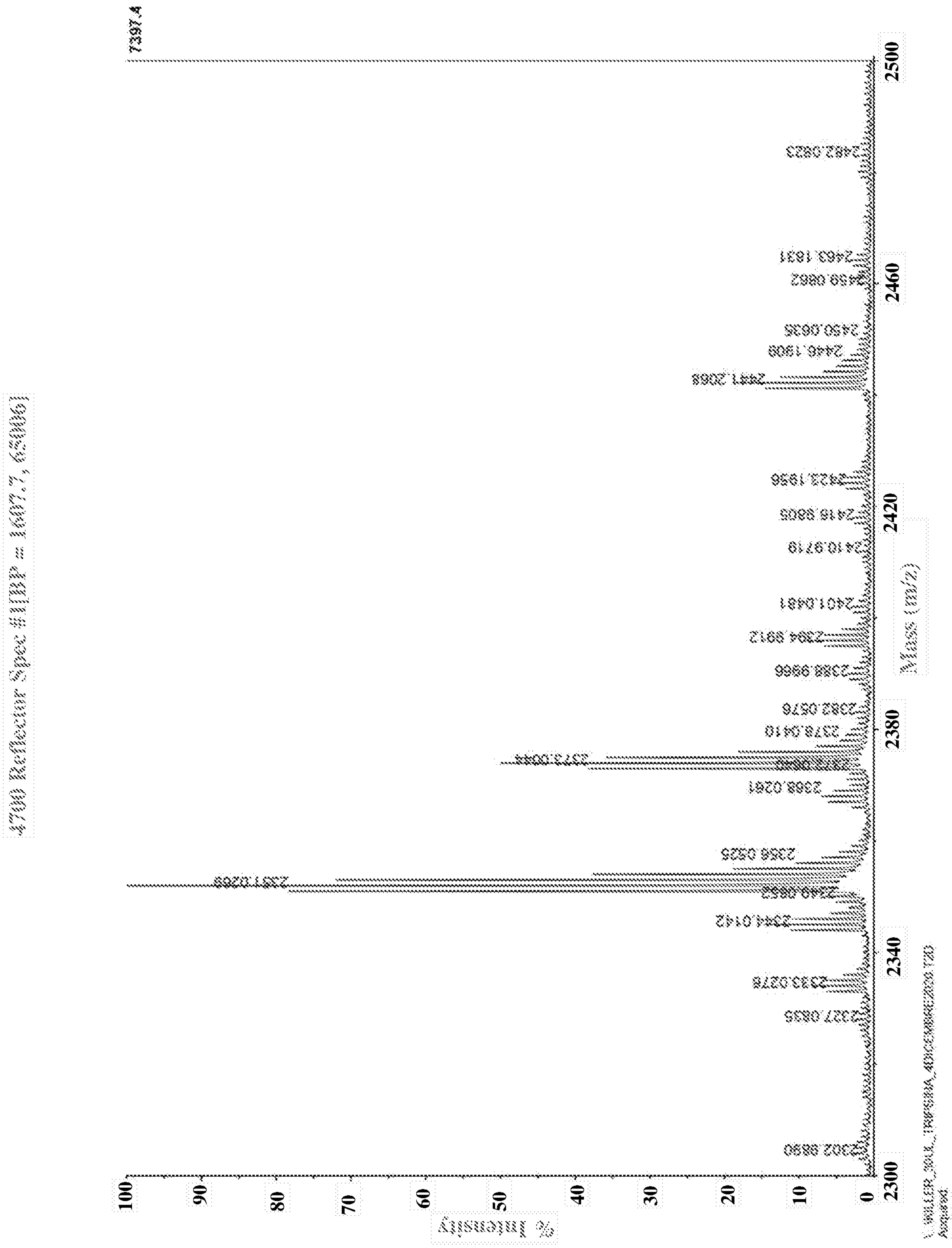

4700 Reflector Spec #1[BP = 1607.7, 65006]
7397.4
% Intensity
100
90
80
70
60
50
40
30
20
10
0
2300
2340
2380
2420
2460
2500
Mass (m/z)
2302.0890
2327.0835
2333.0278
2344.0142
2349.0852
2351.0268
2356.0525
2368.0261
2373.0044
2378.0410
2382.0576
2388.9866
2394.8912
2401.0481
2410.9719
2416.8805
2423.1956
2441.2068
2446.1909
2450.0635
2459.0862
2463.1831
2482.0823

Fig. 11K

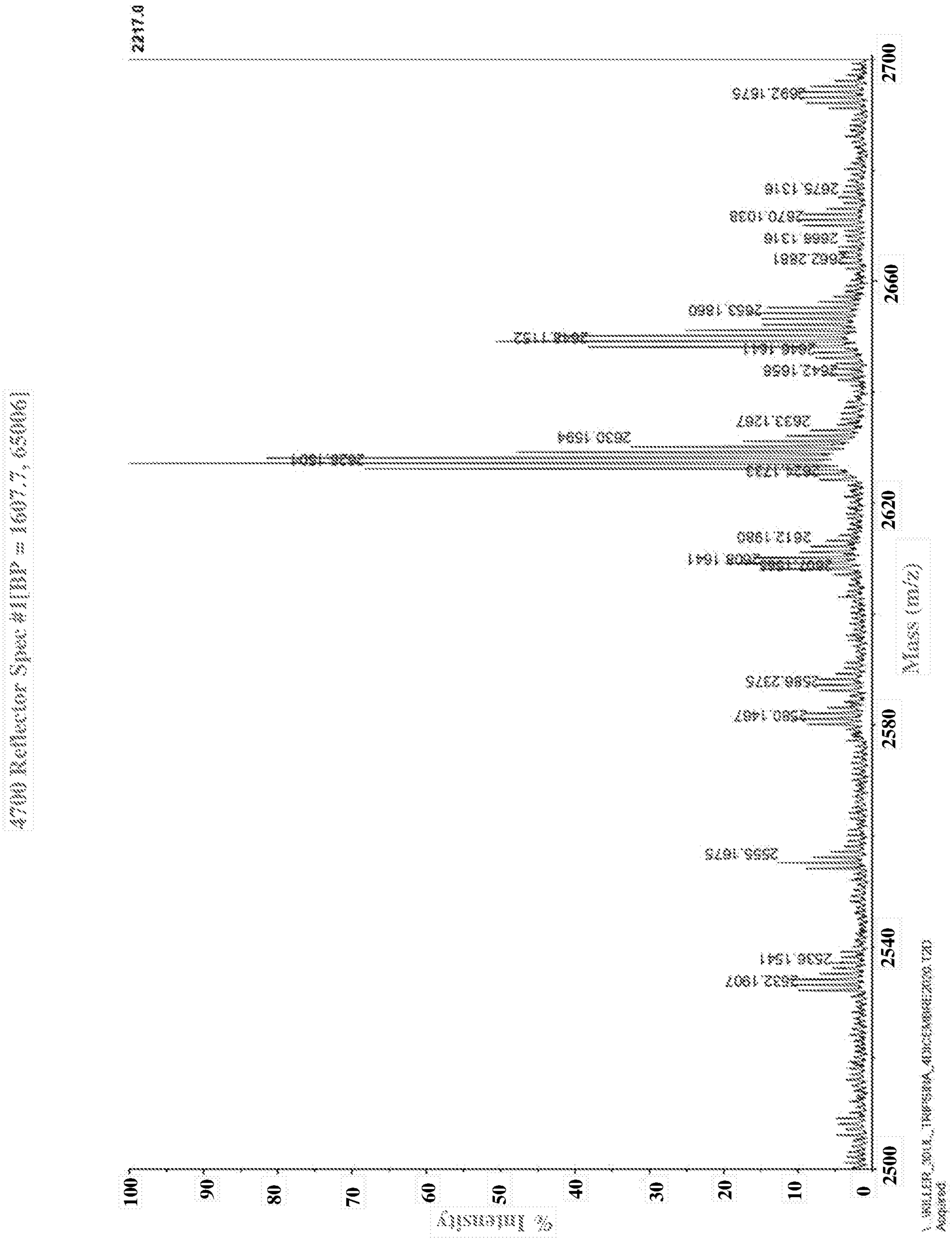

4700 Reflector Spec #1[BP = 1607.7, 65006]
2217.0
% Intensity
Mass (m/z)
0
10
20
30
40
50
60
70
80
90
100
2500
2540
2580
2620
2660
2700
2532.1807
2536.1541
2556.1675
2580.1467
2586.2375
2608.1641
2612.1980
2628.1604
2630.1594
2633.1267
2642.1656
2646.1641
2648.1152
2653.1660
2662.2881
2668.1316
2670.1038
2675.1316
2692.1675

Fig. 11L

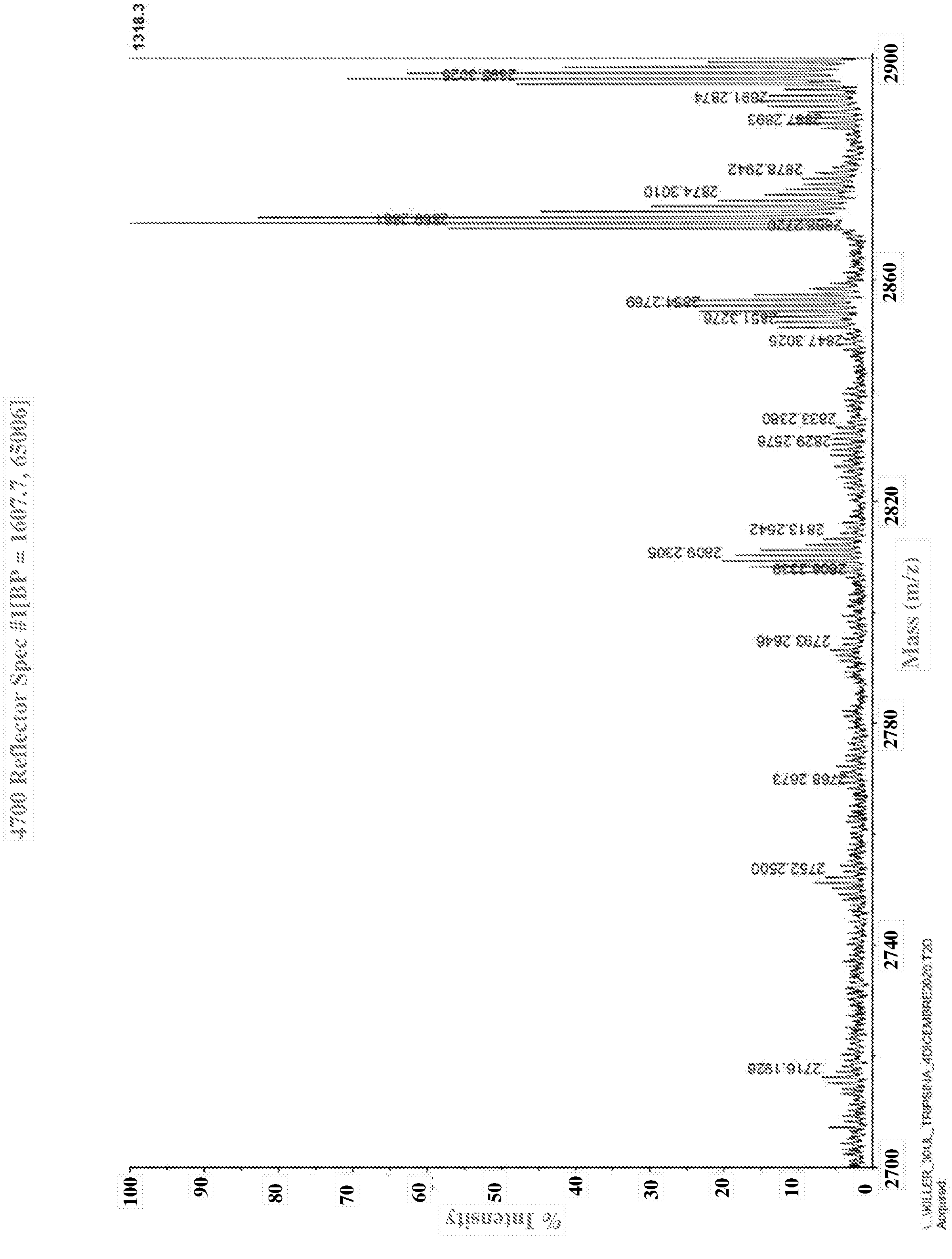

4700 Reflector Spec #1[BP = 1607.7, 65006]
1318.3
% Intensity
100
90
80
70
60
50
40
30
20
10
0
Mass (m/z)
2700
2740
2780
2820
2860
2900
2716.1828
2752.2500
2768.2673
2793.2646
2808.2305
2813.2542
2829.2576
2833.2380
2847.3025
2851.3278
2854.2769
2866.2720
2874.3010
2878.2942
2887.2803
2891.2874

Fig. 11M

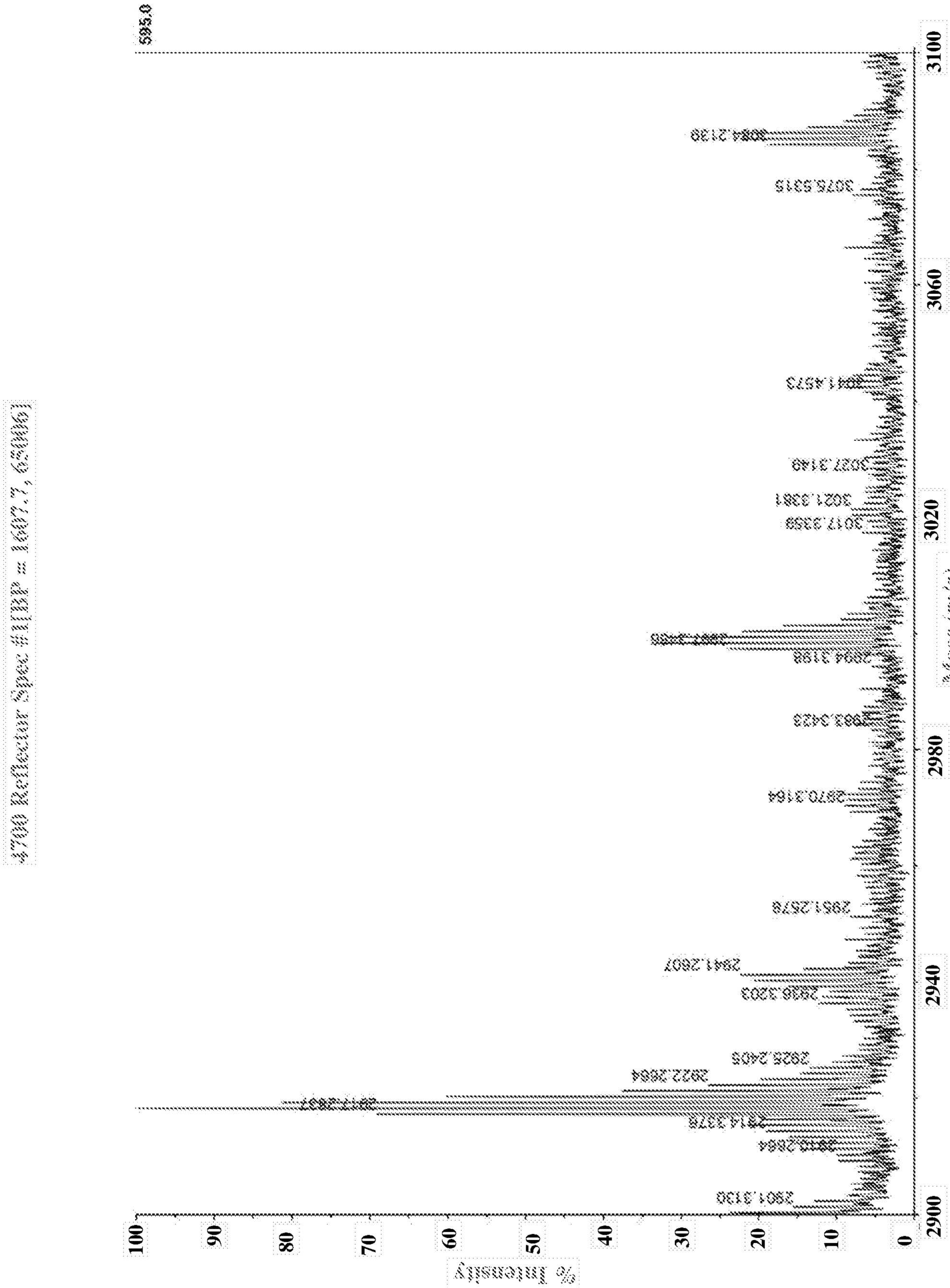

595.0
3100
3084.2138
3076.5315
3060
3020
3017.3359
3021.3381
3027.3149
3004.4573
3063.3423
3004.3188
2980
2970.3164
2961.2578
2941.2607
2940
2939.3203
2925.2405
2922.2664
2914.3378
2900
2901.3130
100
90
80
70
60
50
40
30
20
10
0
% Intensity
4700 Reflectron, Spec #1[BP = 1607.7, 65300.6]

Fig. 11N

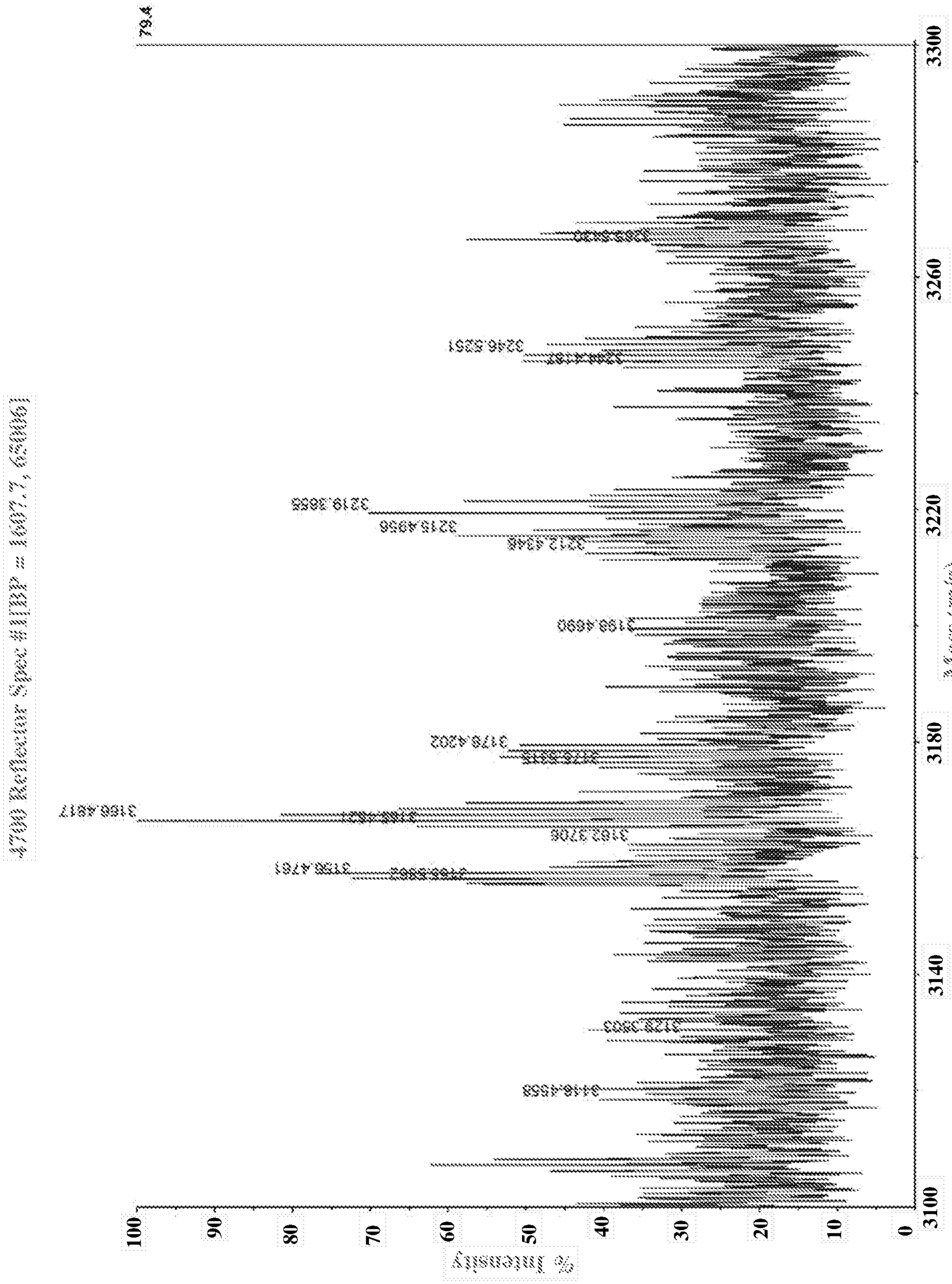

79.4
3300
3260
3220
3180
3140
3100
% Intensity
100
90
80
70
60
50
40
30
20
10
0
3246.6251
3319.3656
3215.4956
3212.6346
3198.4690
3178.4202
3166.4817
3162.3726
3128.3903
3416.4568
3178.4761

Fig. 110

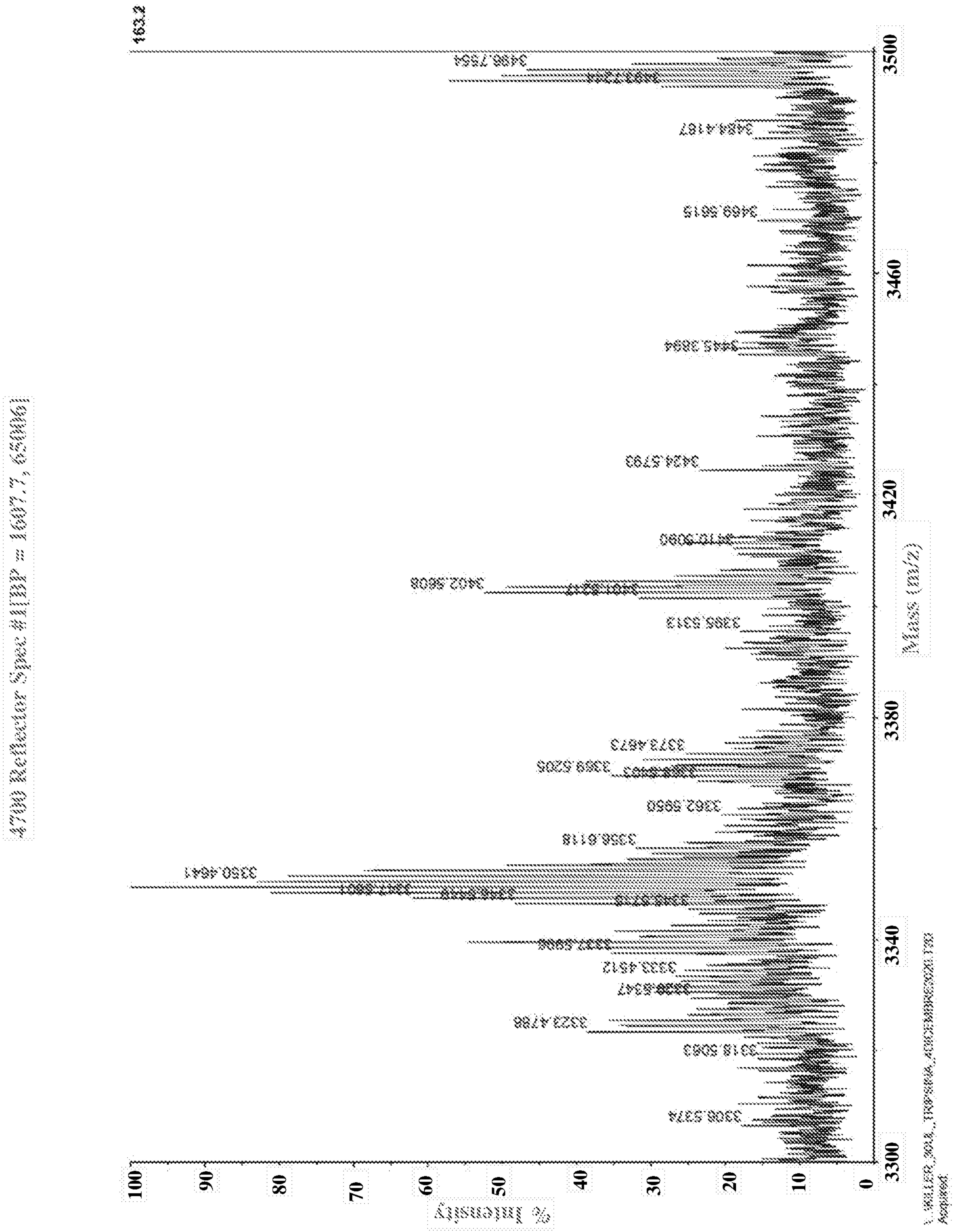

4700 Reflector Spec #1[BP = 1607.7, 65006]
163.2
% Intensity
100
90
80
70
60
50
40
30
20
10
0
Mass (m/z)
3300
3340
3380
3420
3460
3500
3308.5374
3318.5063
3323.4786
3328.6347
3333.4612
3337.6008
3350.4641
3356.6118
3362.5950
3369.5205
3373.4673
3395.5313
3402.5608
3410.5090
3424.5793
3445.3884
3469.5615
3484.4187
3498.7554

Fig. 11P

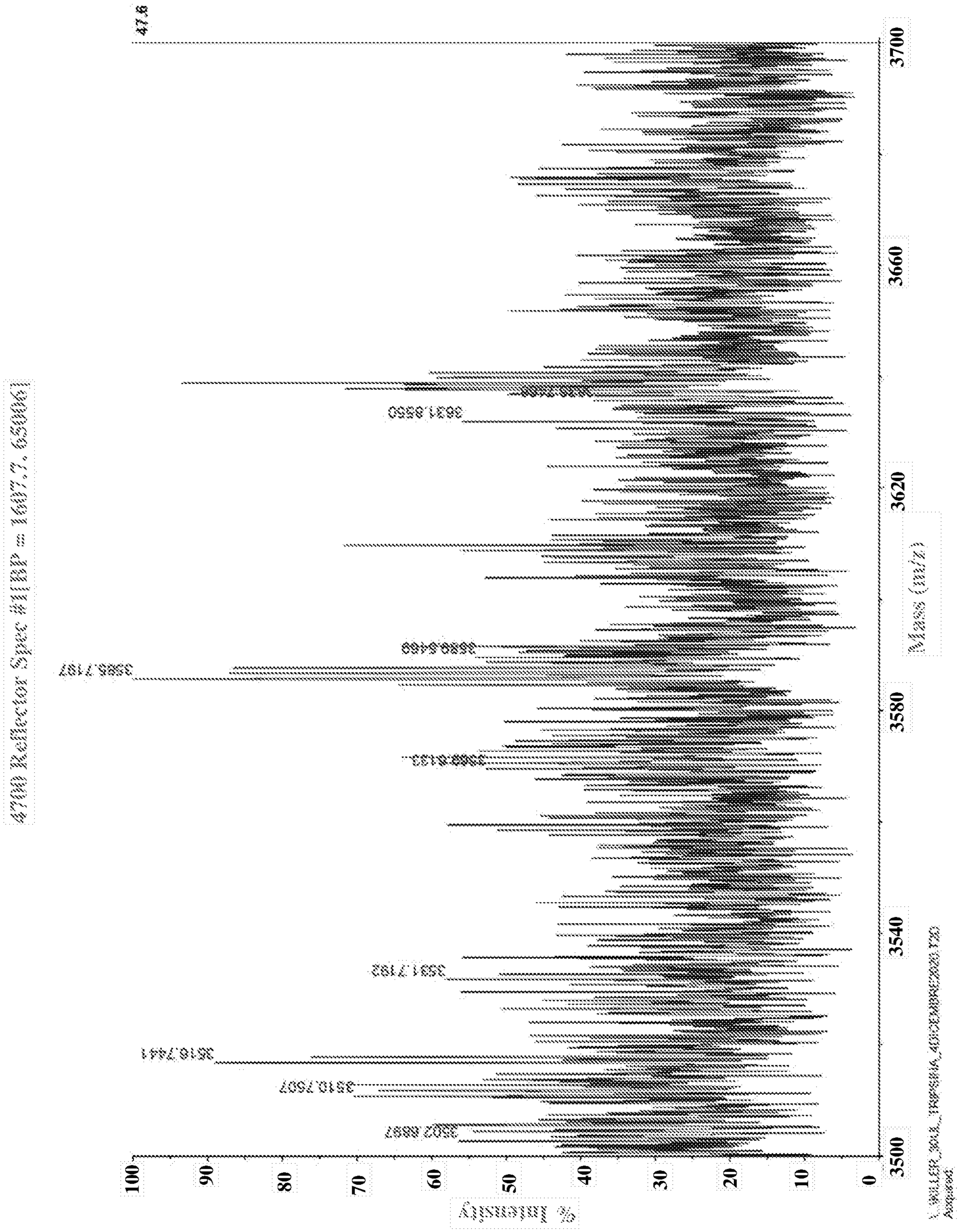

47.6
3700
3660
3620
3580
3540
3500
% Intensity
100
90
80
70
60
50
40
30
20
10
0
3502.0897
3510.7507
3510.7441
3541.7182
3588.6469
3588.7197
3588.8133
3631.8550
3638.3408
4700 Reflector Spec #1[BP = 1607.7, 65500K]

Fig. 11Q

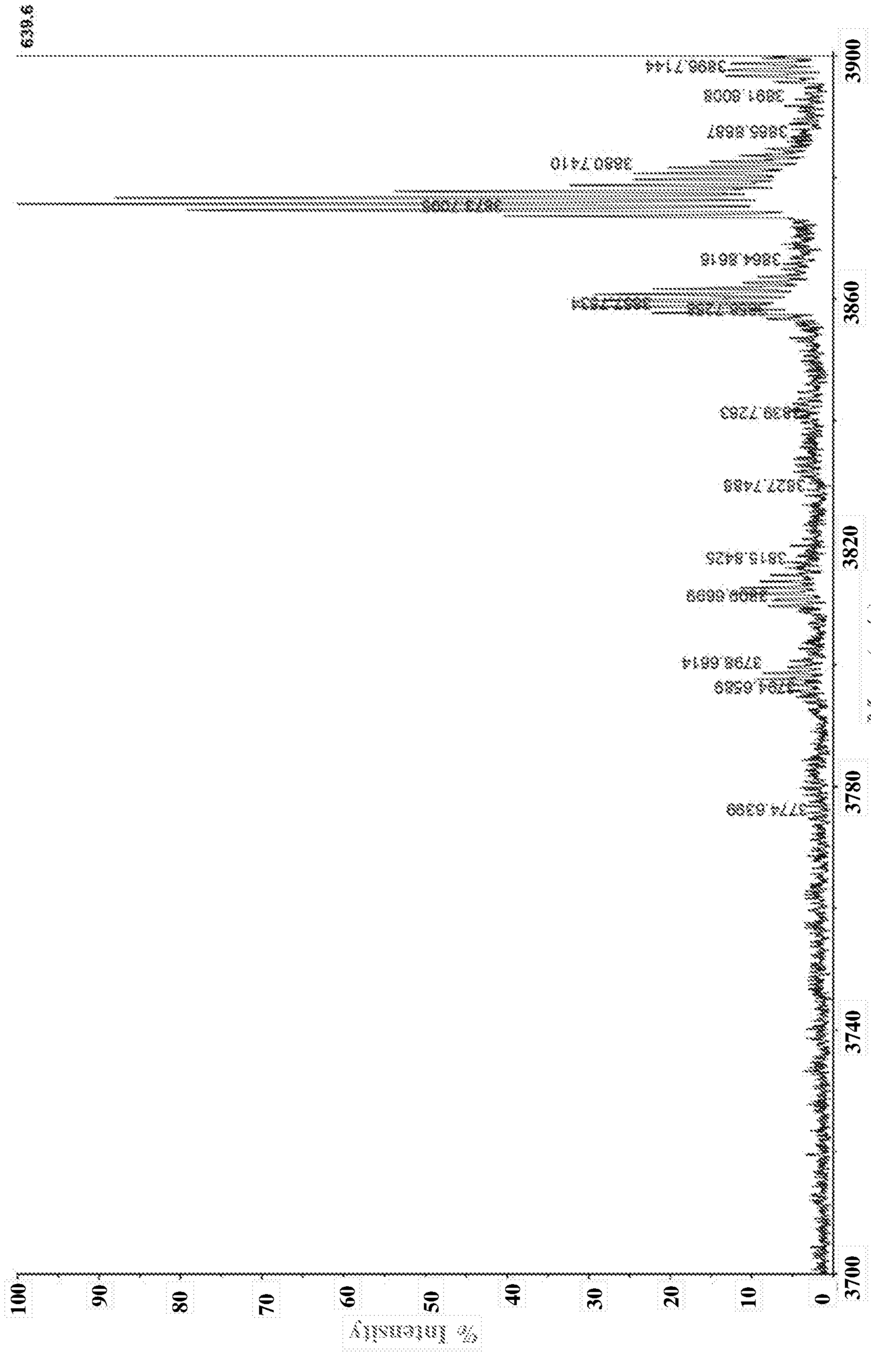

3900
3860
3820
3780
3740
3700
Mass (m/z)
% Intensity
3806.7144
3801.6008
3865.8687
3880.7410
3873.7095
3864.8616
3837.7334
3828.7263
3827.7489
3815.8425
3805.6689
3794.6589
3798.6814
3774.6389

Fig. 11R

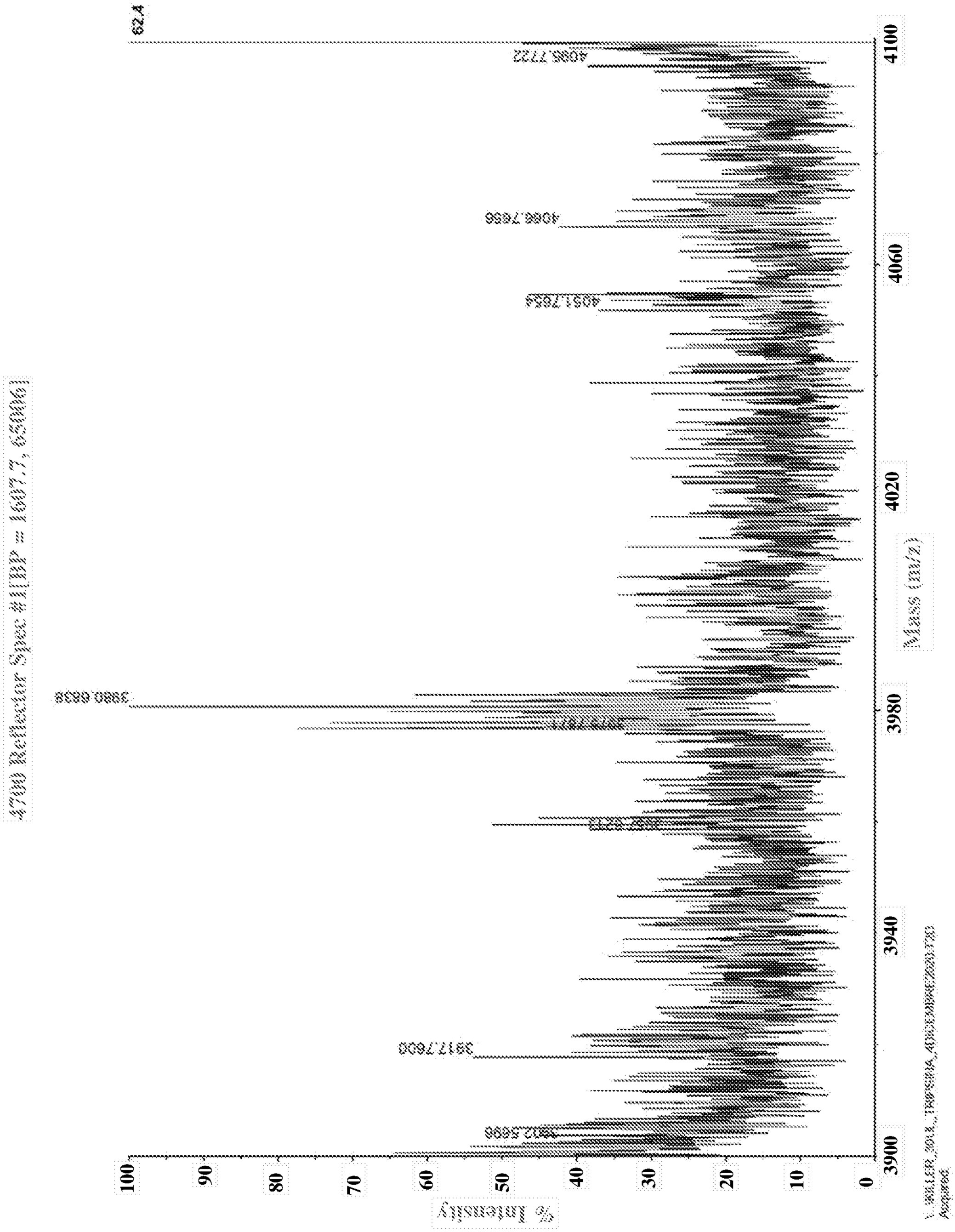

4100
4060
4020
3980
3940
3900
Mass (m/z)
% Intensity
100
90
80
70
60
50
40
30
20
10
0
4086.7722
4066.7656
4051.7659
3980.6638
3917.7609
3902.5696

Fig. 11S

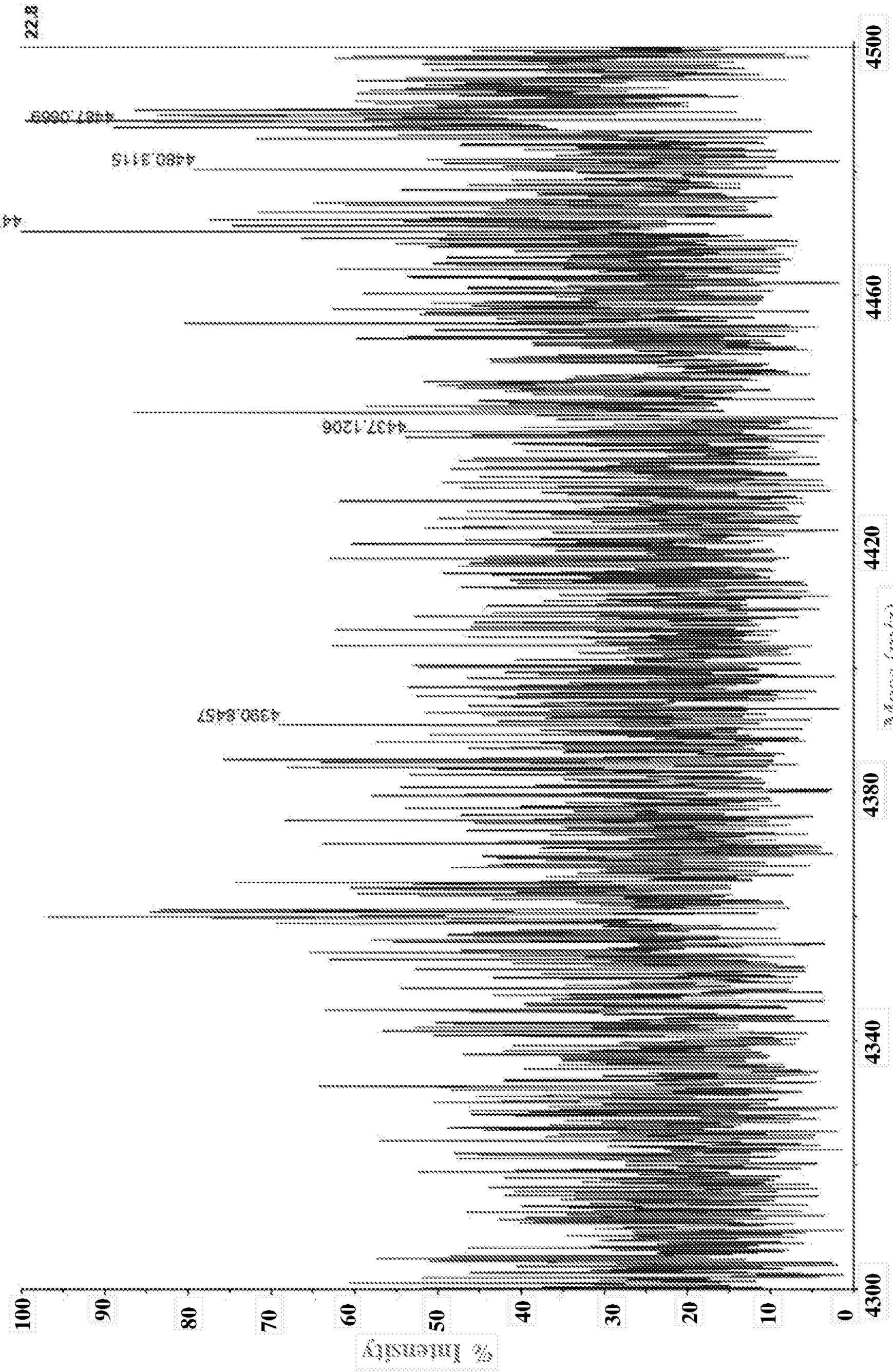

22.8
4500
4460
4420
4380
4340
4300
Intensity (counts)
4470.3340
4437.1206
4390.8457
4480.3115
100
90
80
70
60
50
40
30
20
10
0
% Intensity

Fig. 11T

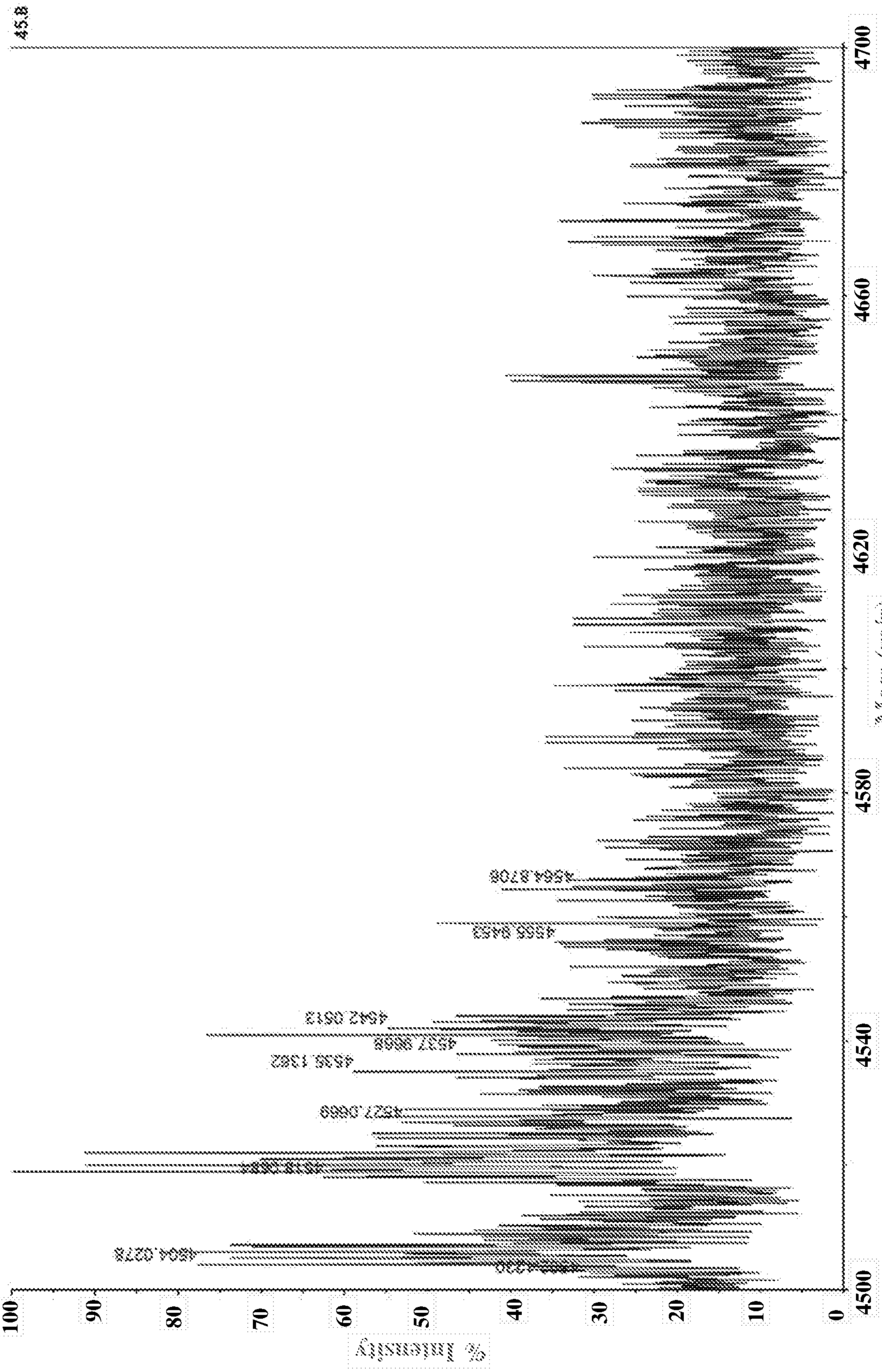
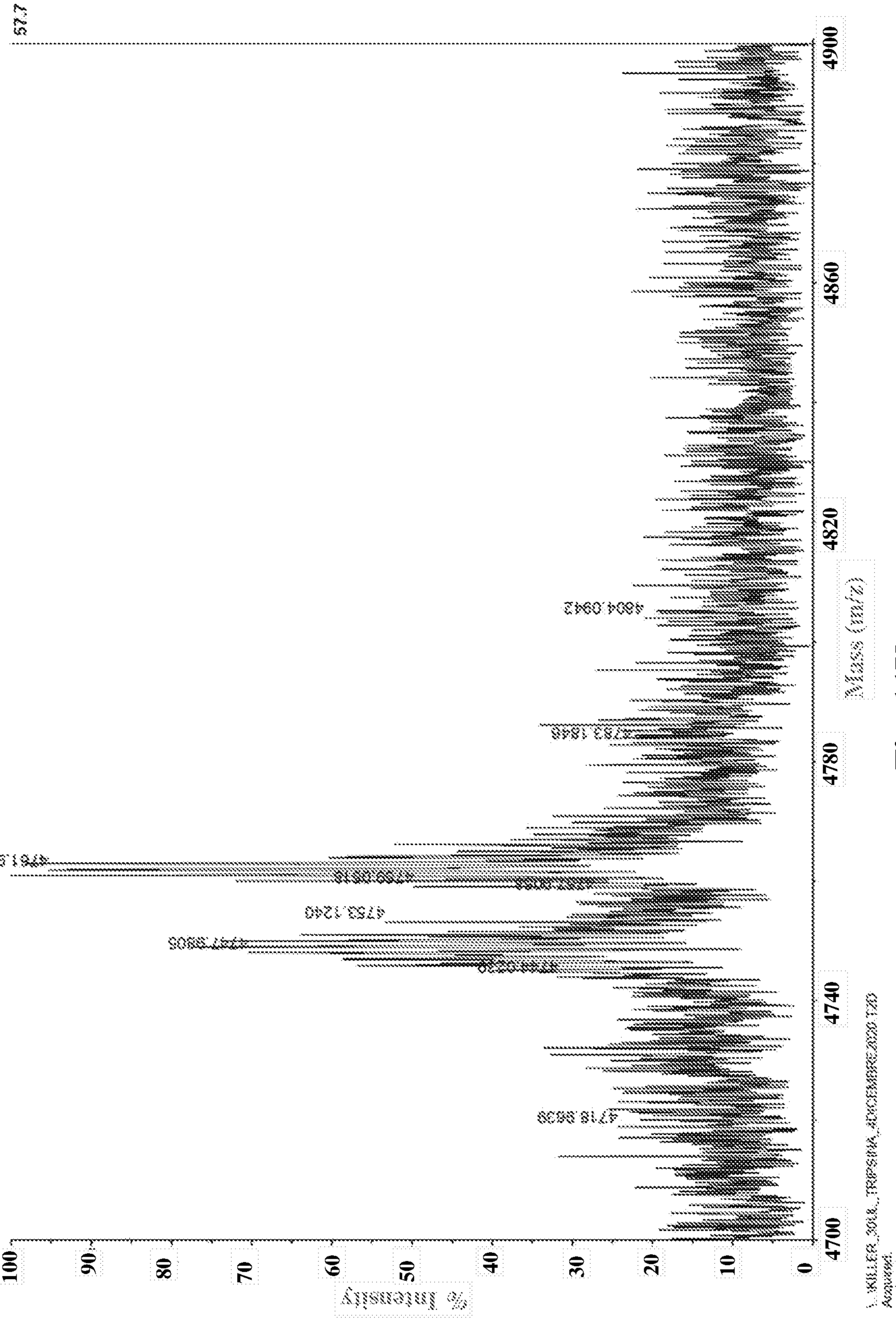

45.8
4700
4660
4620
4580
4540
4500
Mass (m/z)
% Intensity
100
90
80
70
60
50
40
30
20
10
0
4700 Reflectron Spec #1[BP = 16017.7, 4500.1]
4564.8708
4505.8453
4542.0613
4537.8868
4535.1362
4527.0669
4518.2662
4504.0278
4504.0230

Fig. 11U


4900
4860
4820
4780
4740
4700
% Intensity
0
10
20
30
40
50
60
70
80
90
100
4604.0943
4783.1846
4761.9318
4759.0518
4753.1240
4727.3906
4718.8630
Fig. 11V

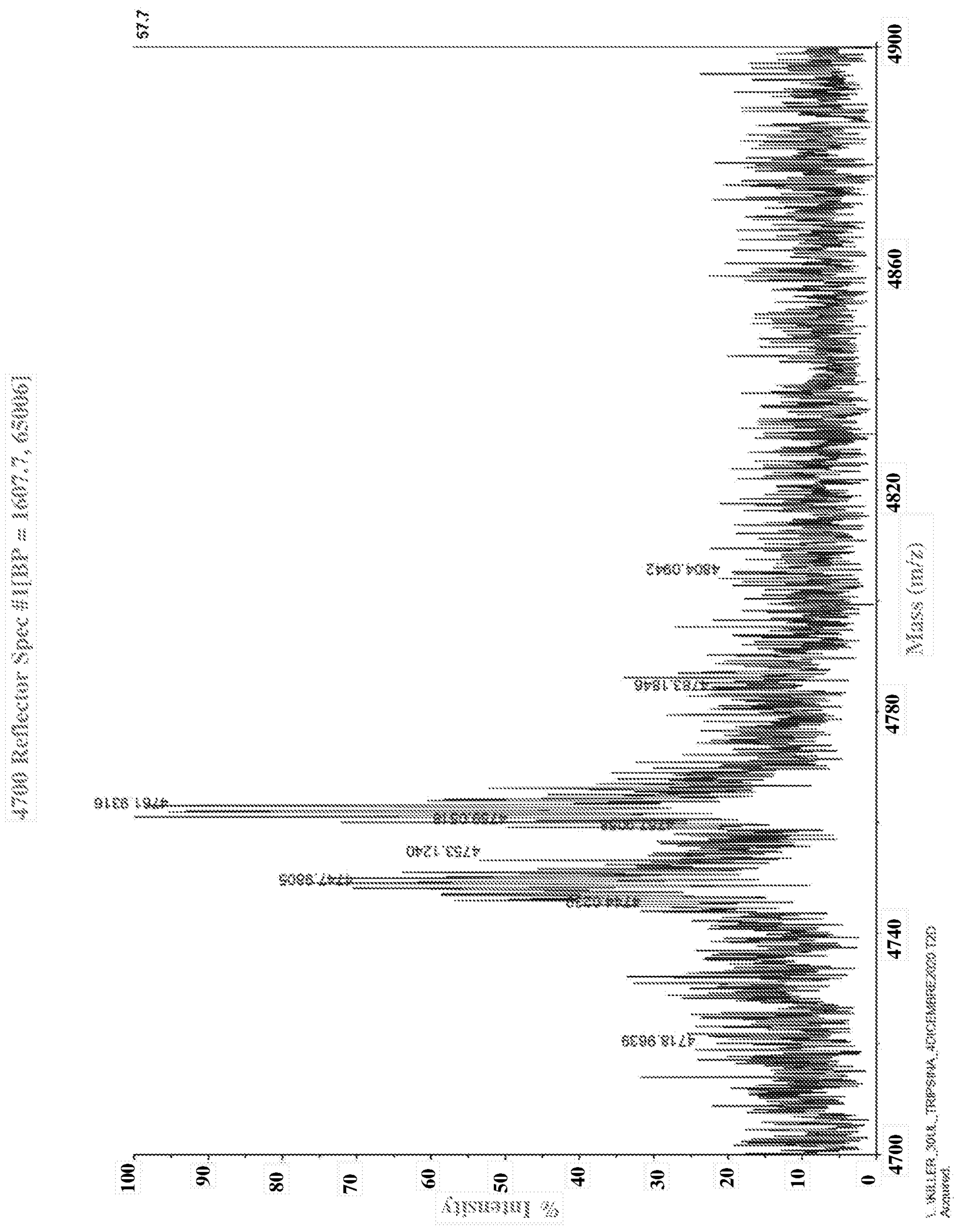

4700 Reflector Spec #1[BP = 1607.7, 65006]
57.7
% Intensity
100
90
80
70
60
50
40
30
20
10
0
Mass (m/z)
4700
4740
4780
4820
4860
4900
4718.9639
4753.1240
4761.9316
4783.1846
4804.0942

Fig. 11W

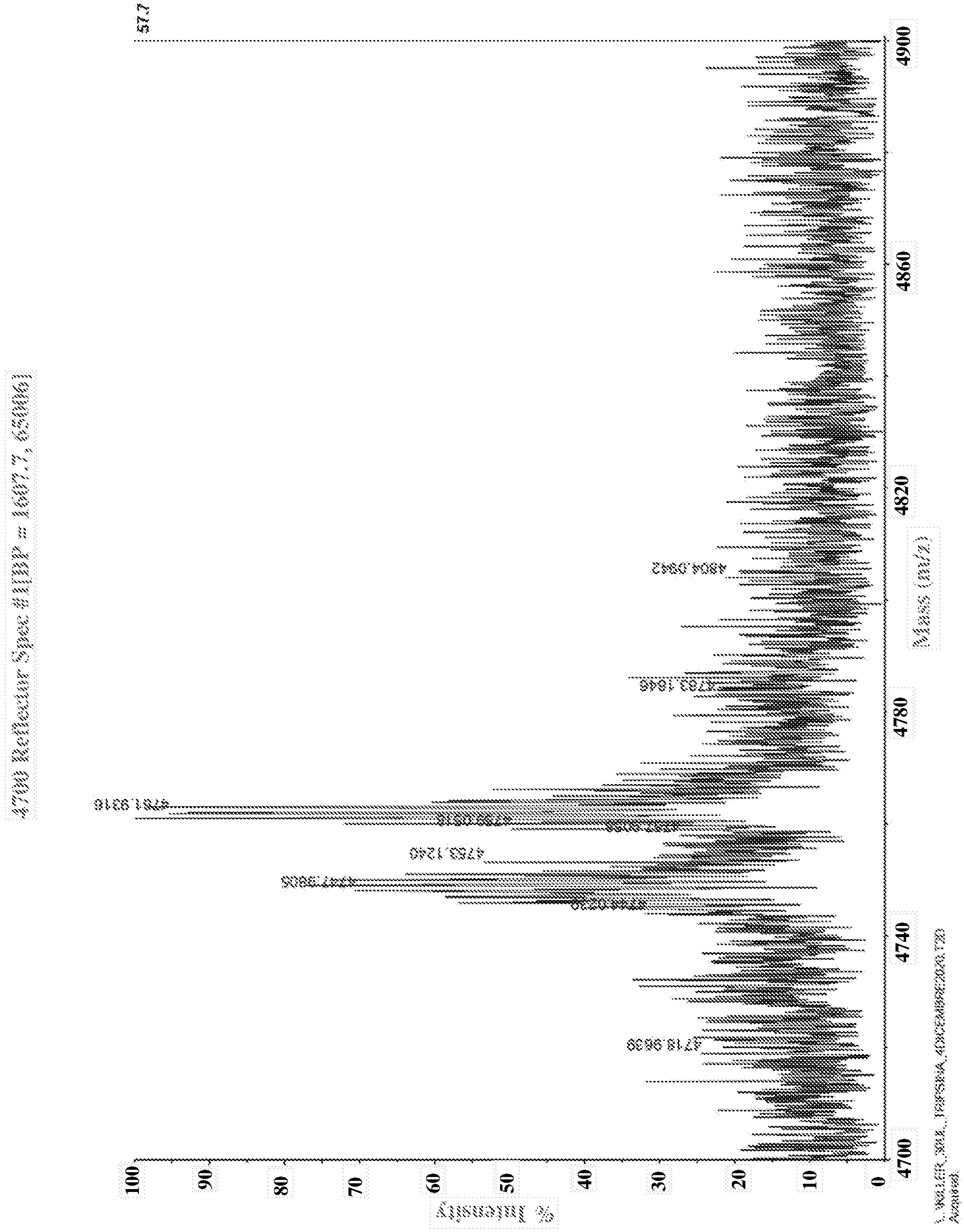

517
4900
4860
4820
4780
4740
4700
Mass (m/z)
% Intensity
100
90
80
70
60
50
40
30
20
10
0
4804.0942
4763.1846
4719.9839

Fig. 11X

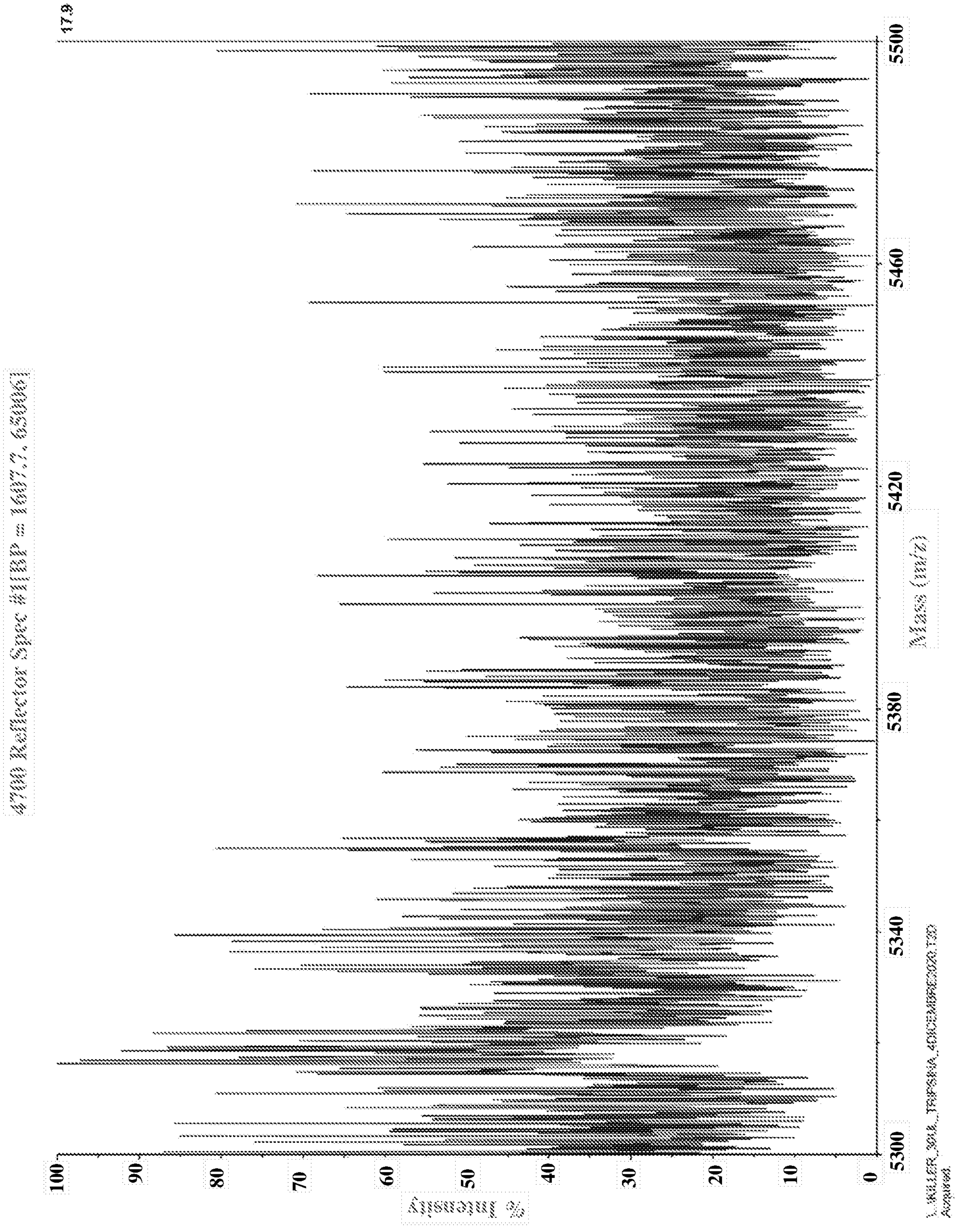

4700 Reflector Spec #1[BP = 1607.7, 65006]
17.9
% Intensity
100
90
80
70
60
50
40
30
20
10
0
Mass (m/z)
5300
5340
5380
5420
5460
5500

Fig. 11Y

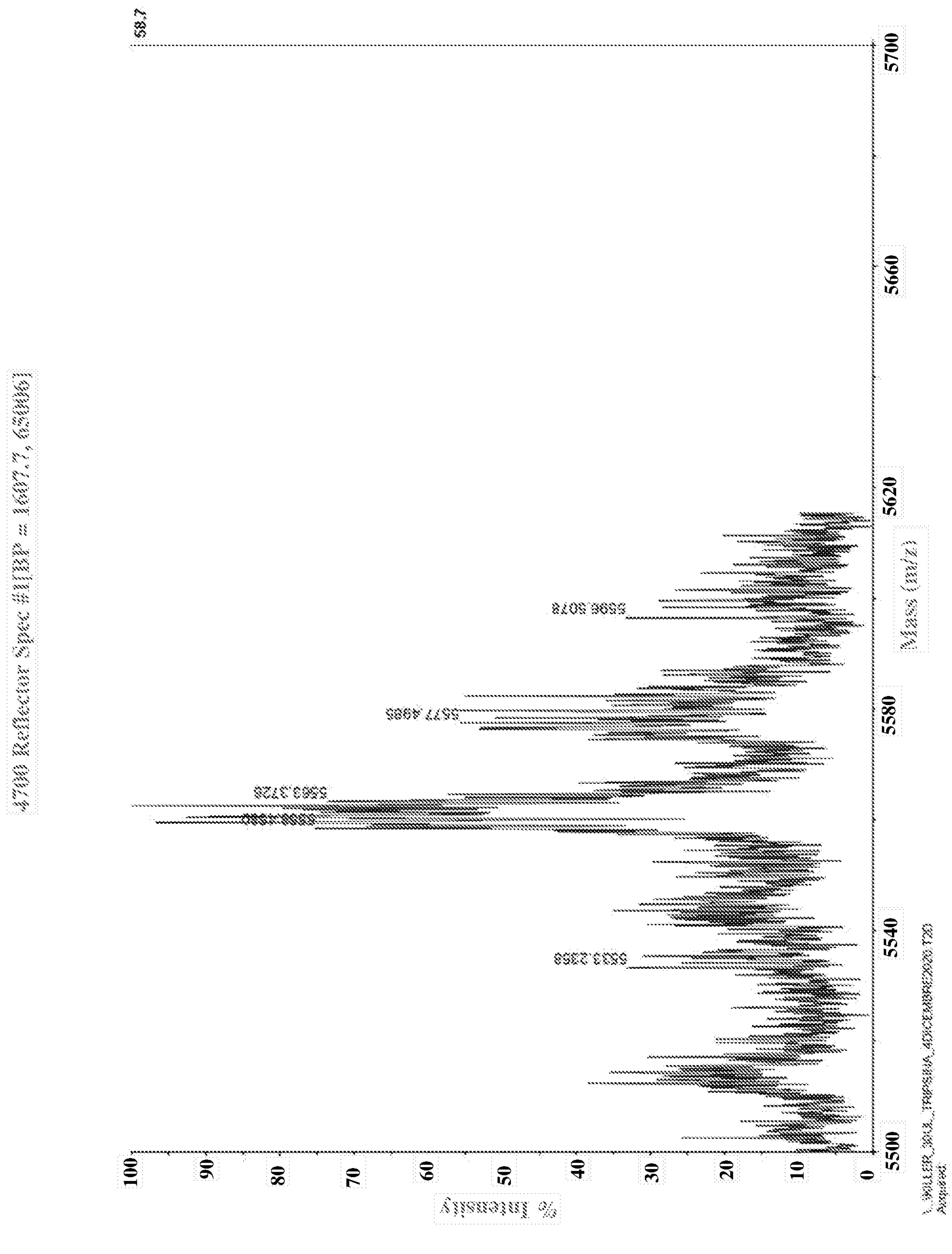

4700 Reflector Spec #1[BP = 1607.7, 65006]
58.7
% Intensity
100
90
80
70
60
50
40
30
20
10
0
5533.2358
5563.3728
5577.4985
5596.5078
5500
5540
5580
5620
5660
5700
Mass (m/z)

Fig. 11Z

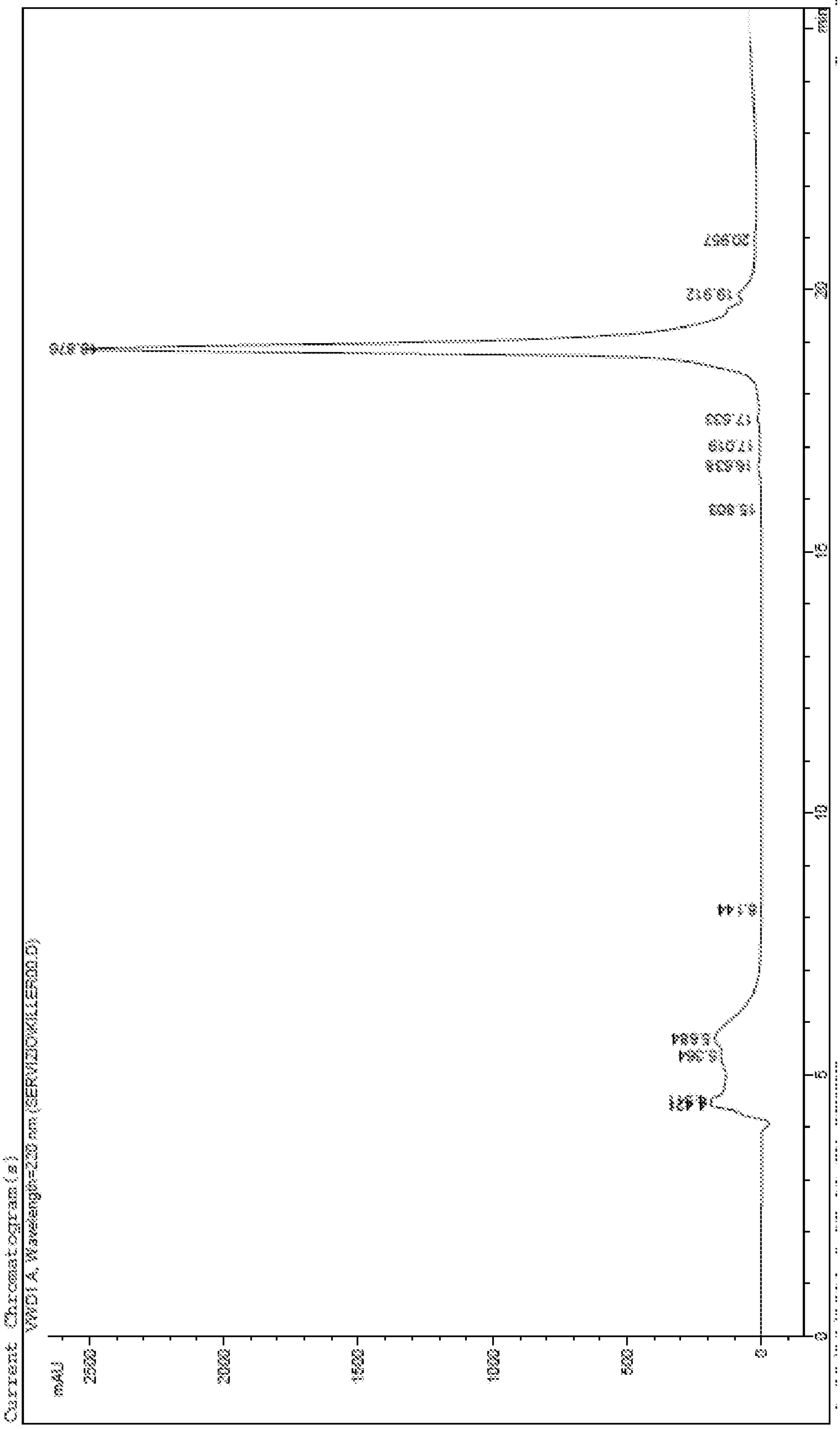

Current Chromatogram(s)
VWD1 A, Wavelength=220 nm
mAU
2500
2000
1500
1000
500
0
min
0
5
10
15
20
4.421
5.364
5.684
8.144
15.803
16.628
17.019
17.633
19.912
20.957

Figure 12

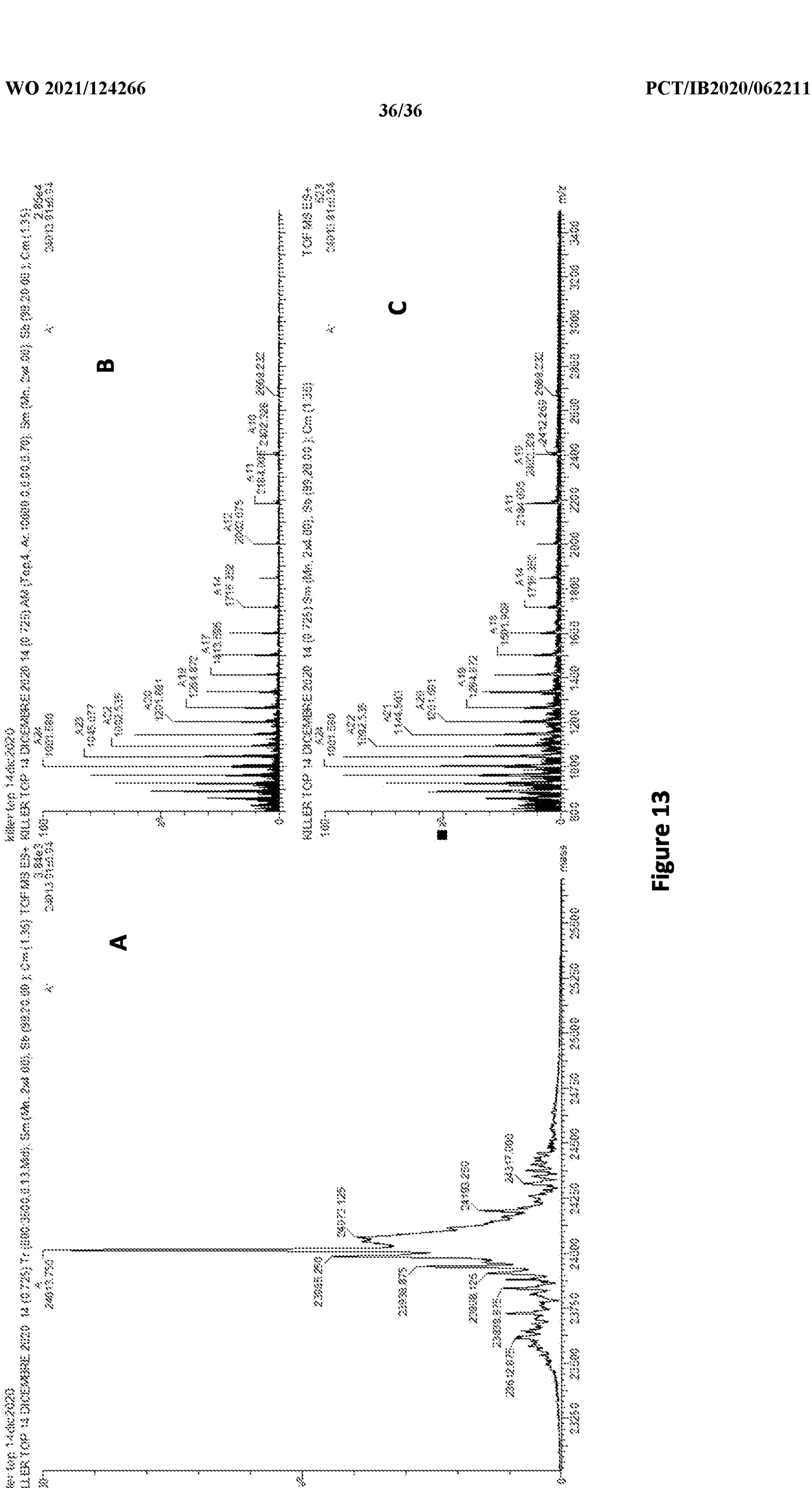


Figure 13

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2020/062211

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/60 A61P35/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2004/001009 A2 (GENENTECH INC [US]; HYMOWITZ SARAH [US] ET AL.) 31 December 2003 (2003-12-31) cited in the application page 2, line 25 - line 35; examples 8-10 -----	1-12
T	Anonymous: "PRODUCT DATA SHEET - KillerTrailTM Protein (soluble) (human), (recombinant)", , 5 May 2020 (2020-05-05), XP055720979, Retrieved from the Internet: URL:https://www.enzolifesciences.com/fileadmin/reports/Datasheet-ALX-201-073.pdf [retrieved on 2020-08-07] -----	

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 March 2021	07/04/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Mooren, Nicolai

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2020/062211

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2020/062211

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004001009 A2	31-12-2003	AU 2003247609 A1	06-01-2004
		AU 2009212834 A1	24-09-2009
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		WO 2004001009 A2	31-12-2003