Large Scale Purification and Characterization of A21 Deamidated Variant- Most Prominent Post Translational Modification (PTM) for Insulins Which Is Widely Observed in Insulins Pharmaceutical Manufacturing

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Research

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Abstract

Background

The rapid growth of global diabetic population attracts the large scale manufacturing of insulin and analogues to meet the unmet demand. Biopharmaceutical development demands appropriate understanding of product variant, which are formed due to post-translational modification. Deamidation is the most common post-translational modification occurring in insulins and insulin analogues. Same time, the same variant is most prominent product variant occur during biopharmaceutical manufacturing process or during the storage. Often, one or the other deamidated variant is used as an impurity standard during in-process and final product analysis in the QC system. However, purification of large quantity of highly purified deamidated material is always being challenging due to very similar mass, similar ionic properties, and high structural similarity of the variant compared to the parent product

Results

In the present work authors tried to come up with a novel, easy and large scale process of purification of deamidated impurity with high purity from insulin. Anion exchange resin PolyQuat offers a high ionic capacity for both strong anion exchange and weak anion exchange species. Due to this unique functionality, PolyQuat offers enhanced performance and better selectivity than conventional ion exchange media, which enables to purify closely related A21 desamido variant from insulin. This single chromatography step can enrich the purity of desamido insulin to >96%. The PolyQuat elution pool was concentrated through the preparative RP-HPLC column in the bind elute mode. The RP-HPLC elution pool was precipitated and lyophilized in powder form. Detailed characterization of human insulin variant (+1 Da desamido) was carried out for its primary, secondary and tertiary structure through state-of-the-art mass spectrometry methods, circular dichroism and intrinsic fluorescence and was compared with human insulin. Intact, reduced and Peptide Mass Fingerprinting analysis identified an increase of 1Da mass in the peptide bearing A21 amino acid. We have also compared the extent of IR-B phosphorylation by A21 desamido insulin against human insulin reference standard. As biological functions are governed by protein structure, IR-B phosphorylation assay was performed to compare functional activity of AspA21 human insulin variant and human insulin. The comparative phosphorylation profiles and relative potency clearly suggested no impact of A21 deamidation on the functional activity of recombinant human insulin.

Conclusions

The present work reveals efficient scalable process to manufacture insulin desamido variant in the powder form with more that 60% recovery with 97% purity. This simplified process for manufacturing deamidated variant presented in this study can be handy for the insulin manufacturers to make large quantity variant standard and use for their process development as well as routine manufacturing process. Purified insulin desamido variant allowed detailed characterization of the species as well helps in using as an impurity standard for process monitoring in QC, which aids in robust control for insulin manufacturing.

Background

Post-translational modification (PTM) in proteins is an evolutionary response of the cellular organism for its regulation and is vital to increase functional diversity of the proteome to not only survive and grow but to be able to appropriately respond to an external stimulus. The PTMs lead to covalent addition of functional groups on proteins (on one or more amino acids), proteolytic cleavage of regulatory subunits or degradation of entire proteins [1-3].

These protein modifications include oxidation of methionine, N-acetylation, deamidation of asparagine and glutamine, variable glycosylation, and may lead to misfolding or aggregation, proteolysis or degradation [4]. In the recent times, due to large emphasis on the biologic drugs, these multifaceted PTMs have caught significant attention due to broad variability of their time course action that can be rapid such as phosphorylation to slower responses as in glycosylation [5].

Growing incidence of diabetic mellitus globally demands for large scale production of insulin and its analogues [6, 7]. While initial dependencies were on animal origin insulin, advances in recombinant DNA technologies made it possible to bring its first insulin product in 1980s. Human insulin is composed of 51 amino acid residues and has a molecular weight of 5807 Da. It consists of two polypeptide chains, A and B, linked together by disulfide bonds. Chain A has 21 amino acid residues and an intra-chain disulfide, which links amino acids A6 and A11. The chain B polypeptide has 30 amino acid residues. Both chains are connected by two inter-chain disulfide bonds between A7 and B7 and between A20 and B19 [8].

Deamidation in the protein chain leads towards most prominent product variant which can occur during biopharmaceutical manufacturing process or during the storage [9]. Likewise, the most common side reaction in manufacturing of human insulin is deamidation, which may occur in both acidic and neutral conditions [10]. This is because asparagine (Asn) and glutamine (Gln) are acid labile, meaning they can be hydrolysed under acidic conditions and become aspartic acid (Asp) and glutamic acid (Glu), respectively. The reaction can occur faster at ambient temperature. A typical deamidation reaction for an Asn residue is shown below in the Figure 1.

Primary sequence of human insulin contains three asparagine (Asn) residues at positions Asn^{A18}, Asn^{A21} and Asn^{B3}, and three glutamine (Gln) residues at positions Gln^{A5}, Gln^{A15}, and Gln^{B4}. Most prominent deamidation in insulin manufacturing occurs at amino acid Asn^{A21}, where the side-chain amide group undergoes hydrolysis to carboxylic group [9]. This occurs due to the presence of Asn^{A21} at the C-terminus of A-chain and is structurally most exposed in the three-dimensional space in comparison to the other Asn/Gln residues of insulin.

Even though advancement in process technology has significantly improved quality of insulin prepared from either biotechnological or chemical route, the Asn^{A21} deamidation has continued to be an unwanted side product during the manufacturing process and is difficult to separate from the parent product [11, 12] Hydrolysis of insulin has been studied during storage of various preparations at different temperatures. Insulin deteriorates rapidly in acid solutions due to extensive deamidation at residue Asn^{A21}. In neutral formulations deamidation predominantly takes place at residue Asn^{B3}, albeit at a substantially reduced rate leading to formation of a mixture of isoAsp and Asp derivatives. The rate of hydrolysis at Asn^{B3} is independent of the strength of the preparation, and in most cases the species of insulin, but varies with storage temperature and formulation [11-13].

According to the ICH Q6B Guidelines, product-related impurities include molecular variants of the desired product; for example truncated and other modified forms, aggregates, deamidations, precursors and certain degradation products arising during manufacture and/or storage. As per ICH Q6B guidelines, isolation and characterization of product-related variant is an important aspect of biopharmaceutical process validation [14]. It determines the properties of molecular variants that may differ in regard to its activity, efficacy and safety from the desired drug substance. Thus, the analytical activities concerning impurities in drugs are among the most important requirement in modern pharmaceutical analysis [15, 16]. Analytical monitoring of impurities in new drug substances is a key component of the recent guideline issued by the International Conference on Harmonization (ICH) [17]. The deamidated impurity must be minimized during the manufacturing in biopharmaceutical product, particularly from the regulatory context. These product-related variants need to be well understood in order to assess their impact on the safety and efficacy of the product. However, to achieve this, isolation of suitable quantity, often 100's of mg to gram, is required to perform a thorough characterization to identify the type of modification(s) and its functional activity. Additionally, deamidated variant is widely used in quality control laboratory for HPLC analytical method validation and routine analysis. The pure variant can be used as impurity reference standard (system suitability control) for routine analytical purposes for batch release and product quality monitoring. For confirmation (retention time identity) the "pure" variant can be added to the product sample (impurity spiking approach) and analyzed by analytical HPLC. The "pure" variant may subsequently be characterized by different analytical methods (e.g. LC-MS intact mass determination, LC-MS tryptic peptide mapping) for identifying the site of modification and its influence on structure and biological function. Andrasi et al. used capillary zone electrophoresis (CZE) for the separation of 10 different deamidated forms and studied different forms of deamidation for insulin [18]. Typically, multiple runs (e.g. CEX or SEC) or RP-HPLC on an analytical/semi preparative chromatography system are commonly performed and collected fractions from outlet are pooled to isolate common product related impurities [19]. However, this repetitive injection and collecting fraction process is extremely labour intensive and time consuming and may also lead to co-elution from the closely eluting species. Most of the times this enrichment is in the solution form, which will have limited shelf life and very inconvenient for usage purpose. USP monograph (USP43-NF38) also touched upon the process of enrichment of deamidated variant >5% by acidic treatment. However, often it is desired for the manufacturer to prepare large quantity of this variant with high degree of purity.

The deamidation of one amino acid leads to an addition of 1 Da mass to the molecular weight of a protein. Thus, highly similar mass and charge of this variant in comparison to the main product makes the purification of the product variant highly challenging. Reports are available to purify the antibody charge variants using preparative cation exchange chromatography and its characterization [20-22] but large-scale purification of insulin deamidated impurity remains challenging. In this report we have made an attempt to simplify repeated chromatography process to manufacture large quantity of purified A21 deamidated variant (level of >97% pure in analytical chromatogram) for usage as main impurity standard for recombinant human insulin testing [23]. Further, detailed characterization (physico-chemical and functional) showed comparable structure and functional properties of this variant corroborating earlier reports.

Results And Discussion

Preparation of A21 desamido variant enrichment

The RP-HPLC analytical profiles at different stages of the process were shown in Fig. 2 A-D. Purified Insulin drug substance obtained through recombinant technology when analysed through a reverse-phase HPLC method showed deamidated content of ~0.3%. In the starting, the minor deamidated peak is observed next to the insulin main peak in the analytical RP-HPLC chromatography (Fig 2.A). Deamidated impurity is the prominent impurity present in the final drug substance [24]. When the purified insulin drug substance was treated with 0.01N HCl and incubated at 45°C for a period of 24.0 hours, the drug substance degraded under such stress condition. It is known under such conditions the insulin will be predominantly degraded to A21 desamido and high molecular weight impurities [9]. As expected, the variant of interest, A21desamido insulin, was obtained at levels ≥15 % in the sample as identified by analytical RP-HPLC chromatography (Fig. 2.B). Thus, indicating the enrichment of deamidated peak under these degradation conditions. After attaining this level of desamido variant, first set of chromatography was performed using Anion Exchange Chromatography.

Chromatographic purification and final recovery of the purified product

Separation of deamidated impurity, which has similar charge and molecular mass w.r.t. human insulin, poses a significant challenge at the preparative scale. Due to difference in molecular mass of 1 Da, size exclusion chromatography is not suitable for separation of this variant from the main peak. Anion exchange resin PolyQuat offers a high ionic capacity for both strong anion exchange and weak anion exchange species. Due to this unique functionality, PolyQuat offers enhanced performance and better selectivity than conventional ion exchange media. The medium particle size (~30 micron) helps in separating products with minor differences in characteristics. A robust design space was established for the PolyQUAT chromatography through Design-Of-Experiments (DoE) based process optimization. Fig. 3A shows a representative preparative chromatogram obtained from medium scale purification from optimized process of deamidated product using PolyQuat resin in ÄKTA Explorer 100 system. In order to obtain sufficient resolution, a relatively shallow (30 CV) linear gradient was employed. As can be seen from the preparative chromatogram, two partially resolved peaks were obtained in the central portion of the gradient from 350 to 450 min of run time. Fractions analysis of small-scale trials showed that the 2^{nd} peak was the enriched portion of deamidated

product. In the medium scale repeated purifications, fractions were collected from 400 min onwards and subjected to analytical RP-HPLC analysis. Early-eluting fractions obtained from PolyQuat chromatography corresponded to higher levels of pre-peak (insulin) as determined by analysis. Later fractions enriched with deamidated impurity (>96% pure) were pooled together and subjected to analytical RP-HPLC. The analytical profile is shown in Fig. 2C and the purity as well as recovery of the anion exchange chromatography was 73% as captured in Table 1.

The PolyQuat elution pool was subjected to preparative RP-HPLC column in the bind elute mode. The purpose of RP-HPLC was to concentrate the already purified product and the preparative profile showed very sharp elution profile with highly concentrated purified product (Fig. 3B). At the end of RP-HPLC step the purity of deamidated impurity was obtained as 98% as measured through analytical RP-HPLC (Fig. 2D). The simple RP-HPLC step helped in concentrating the product by removal of additional salts, which aided in subsequent isoelectric precipitation step. There is a minute impurity improvement also attained in RP-HPLC purification step with 97% step recovery (Table 1).

The RP-HPLC elution pool was diluted to 14-16% solvent concentration, which helped for the isoelectric precipitation at pH 5.0. The precipitate was washed to remove the residual solvents. The recovery of the precipitation and centrifugation was found to be ~90%. The recovered precipitate was subjected to freeze drying to make the final purified deamidated impurity in the powder form with >97% purity (Table 1), which can be conveniently stored and used for the intended purpose.

Physicochemical characterization of the desamido variant

Detailed characterization of human insulin variant (+1 Da desamido) was carried out for its primary, secondary and tertiary structure through state-of-the-art mass spectrometry methods, circular dichroism and intrinsic fluorescence (Fig. 4, 5 and 7) and was compared with human insulin. Intact, reduced and Peptide Mass fingerprinting analysis identified an increase of 1Da mass in the peptide bearing A21 amino acid (Fig.4 and S1-S3). Further, MS/MS analysis of this fragment confirmed conversion of Asn^{A21} to Asp^{A21} (Fig. 5; Table 2). To understand, if the +1Da modification has any impact on its higher order structure, secondary and tertiary structure analysis was carried out using circular dichroism and intrinsic fluorescence spectroscopy. Secondary structure analysis using *far-UV* CD showed high similarity between profiles of A21 desamido variant and human insulin. Presence of minima at 208 nm and 222 nm confirms the prominence of helical structures in both proteins. Similarly, tertiary structure analysis by *near-UV* CD and intrinsic fluorescence showed comparable profile indicating similar globular folding for A21 desamido variant and human insulin (Fig. 7 A-C). This observation is in line with the published X-ray structures of human insulin, which shows that C-terminus A21 is solvent exposed and may not directly contribute in the proteins global fold.

Biological characterization

As biological functions are governed by protein structure, IR-B phosphorylation assay was performed to compare functional activity of Asp^{A21} human insulin variant and human insulin (Fig. 7D). Using engineered CHO-K1 cells overexpressing insulin receptor B (IR-B), we have compared the extent of IR-B phosphorylation by A21 desamido insulin against human insulin reference standard. Signalling through IR-B is a direct measure of insulin biological function. In terms of method sensitivity, this method can differentiate if the differences in active ingredients are more than 15%. As seen in (Fig. 7D), both A21 desamido insulin and human insulin reference standard showed a comparable receptor phosphorylation (EC_{50} of 63.47 ng/ml and 70.05 ng/ml respectively). The comparative phosphorylation profiles and relative potency clearly suggested no impact of A21 deamidation on the functional activity of recombinant human insulin (Fig. 7D and Table 3). As the potency of A21 species is comparable to that of the human insulin, it is possibly for the same reason, A21 content is cumulatively added for evaluation of assay for human insulin drug substance and drug product (USP monograph 43; Section: Insulin human).

Conclusions

Detailed mass characterization of the purified impurity confirmed deamidation occurred at A21 amino acid of the A-chain. Due to the structural location of A21 amino acid, the modification of this residue didn't impact the protein variant structural features w.r.t to human insulin as evaluated by CD and intrinsic fluorescence spectroscopy. Product variants such as (A21 desamido) is known to have comparable activity, safety and efficacy to the desired insulin product Brange et al. [25]. Extensive studies on the biological potency of desamido species in comparison to recombinant human insulin in cell-based assays reported that the biological activity of these species is similar to the parent molecule. Similarly, they reported from studies in rabbits that immunogenicity associated with deamidated insulin is comparable to the intact insulin. This was further corroborated by carrying out a comparative functional characterization of human insulin and A21 variant. Insulin Receptor-B (IR-B) phosphorylation assay is a direct measure of insulin mediated signalling cascade. *In-vitro* bioassay suggests that product variant A21 desamido insulin and human insulin have a comparable receptor phosphorylation indicating that the deamidation at A21 doesn't impact the site of interaction

Deamidation of biopharmaceutical products has always been a prime concern for the insulin (and insulin analogues) manufacturer and regulators. Deamidation of A-chain asparagine residue (A21) (Fig. 6) is one of the most common post-translational modifications occurring in human insulin produced using recombinant DNA technology and is also one of the major degradants of human insulin [9,11]. The pathway of deamidations are similar amongst different insulin analogues, albeit, with minor differences in the proportions of deamidated variants. Wherever possible, appropriate controls should be introduced to keep them within the safety specifications. As these variants contributes towards the degradation of the main product, it is important to understand the structural and functional relevance of these impurities in detail as per regulatory guidelines. As carried out in this study, the deamidated impurity for insulin analogues like insulin aspart, insulin glargine, insulin detemir can be isolated in large quantity, which can serve the intended purpose as explained above. For insulin and insulin analogues, deamidated impurity is widely used as one of the system suitability control in the HPLC assays in quality

control laboratory during analytical method validations and product release. Availability of pure desamido variant as lyophilized powder can provide requisite convenience in the QC system. This simplified process for manufacturing deamidated variant presented in this study can be handy for the insulin manufacturers to make large quantity variant standard and use for their process development as well as routine manufacturing process.

Materials And Methods

Chemicals and recombinant protein

Recombinant insulin drug substance of analytical purity of >99% pure containing deamidation variant of 0.3% was procured from Biocon Limited, Bangalore, India. Purified insulin drug substance was solubilized in 0.01N HCl targeting concentration of 4-10 mg/ml. Liquid solution was incubated at 45°C for minimum 24.0 hours. The sample was analysed in analytical HPLC to measure the level of enrichment of A21 deamidated variant in forced degradation condition. Processing of deamidated enriched material is pH adjusted to 7±0.1 using 1N NaOH. Conductivity of this solution was maintained at ≤ 3mS/cm and final load was filtered through 1.2 and 0.45µm filters After attaining this level, the material was used for feed for 1st step of purification through Anion Exchange Chromatography. The other regular laboratory chemicals and chemicals required for chromatography were procured from qualified vendors. The raw materials used for characterization studies were Hydrochloric acid (12N 37% Sigma Aldrich), Acetonitrile (J.T Baker (ACN)), Trifluoroacetic acid (Sigma Aldrich (TFA), C18 column (ACE), C8-column (Waters), Dithiothreitol (DTT), Glu-C (Endoproteinase, Roche), tris(hydroxymethyl) aminomethane (Sigma Aldrich (TRIS). Insulin phosphorylation in-vitro assay was performed using engineered CHO-K1 cells overexpressing insulin receptor B (IR-B).

Chromatographic media and instrumentation

Anion exchange chromatography was performed using Polyquat resin (Bakerbond XWP 500 Polyquat-35 from J.T.baker). RP-HPLC was performed with Kromasil C8 100Å 13µresin. Laboratory scale chromatography experiments were performed using a GE Healthcare ÄKTA Explorer 100 system. Anion exchange chromatography was performed using 1.6 cm & 5 cm diameter columns (XK-16 & HS50) obtained from GE healthcare, while RP-HPLC was performed using 2.12 cm & 1 cm Diameter prepacked columns (C8,13µ,100A°).

Anion exchange and Reversed phase chromatography

PolyQuat resin packed in the column was pre-equilibrated with 10 column volumes(CV) with at a linear velocity of ≤200cm/hr, with the buffer containing 20mM Imidazole, 10% Acetonitrile (Buffer pH was adjusted to 7.0±0.1 with HCl and conductivity was targeted to be 1-2mS/cm). Selection of Imidazole was done for its excellent buffering capacity at above mentioned pH range (7.0±0.1). The starting material (enriched deamidated material) was loaded onto the column with binding capacity of ≤6 grams of A21 impurity per litre of resin at a linear velocity of ≤200cm/hr. Once loading was completed, the column was washed with the 4 CV of same buffer at same flow rate. Product was eluted with 30 CV linear gradient from 0% to 100% of the elution buffer containing 20mM Imidazole, 200mM NaCl, 10% Acetonitrile at pH 7.0±0.1 with a target conductivity of 18-21mS/cm. While elution of the product was monitored at UV280 nm at preparative scale, eluted fractions were further analysed for its product quality in analytical RP-HPLC at UV220 nm. The fractions qualified for pre-determined acceptable criteria were pooled. Step recovery was also calculated by RP-HPLC analysis.

Elution pool from PolyQuat resin was diluted with purified water (having 10% acetonitrile) to target conductivity of ≤7.0mS/cm. pH was adjusted to 4.0±0.1 with 3.0 M glacial acetic acid. Load was filtered through 0.45- and 0.2-micron filter. Reverse Phase chromatography was carried out in pre-packed column using 0.05M Citrate buffer at pH 4.0±0.1 (Buffer A) and Acetonitrile (Buffer B) as organic modifier. The reversed phase chromatographic runs were carried out at a flow rate of ≤360 cm/h in loading, equilibration, washing and elution steps and monitored throughout at 280nm. Column was equilibrated with 4 CV of 10% buffer B (90% Buffer A). Column loading was performed with a binding capacity of ≤10 grams of A21 impurity per litre of resin and followed by washing with Column with ≥ 4 CV of same buffer. Product was eluted from the column using a step elution of 32% Buffer B (68% Buffer A). Elution fractions were collected and analysed in analytical RP-HPLC at UV220 nm. The fractions qualified for pre-determined acceptable criteria were pooled. Step recovery was also calculated by RP-HPLC analysis.

Precipitation and lyophilisation

RP HPLC elution pool was diluted with water for injection (WFI) to target the final concentration of acetonitrile in FFC (Feed for precipitation) in the range of 14-16%. pH was adjusted to 5.0±0.15 using 1.0N NaOH & it was mixed for 3-5 min at a tip speed of 0.42 m/s. Post pH adjustment, the material was left out for further settling & slurry was allowed to settle for > 60minutes. Slurry sample was further centrifuged for 30 minutes at 8000RCF maintaining the centrifuge temperature at 4±2°C. Centrifuged supernatant was decanted & slurry was further washed by adding 200L of WFI (24±2°C) per kg of A21 impurity. Slurry was allowed to settle for > 60minutes, post mixing (5 minutes of mixing at a tip speed of 0.42 m/s). Slurry sample was further centrifuged for 30 minutes at 8000RCF maintaining the centrifuge temperature at 4±2°C & final centrifuged slurry was made into a final homogenous suspension with mild mixing while adding the WFI (final slurry % targeted was in the range of 10-40% of A21 product). Final homogenous slurry suspension (also called as feed for lyophilization) was further dried up using the freeze-drying recipe to attain an efficient drying (target LOD was ≤10%).

Analytical methods for detection, concentration and purity measurements

Samples at each process stage were analyzed using a reversed phase high-performance liquid chromatography (RP-HPLC) Agilent 1100 &1200 series system (Agilent Technologies, Santa Clara , USA) fitted with an analytical (4.6 mm × 250 mm) symmetry C_{18}, 5 µm, 300 Å column (ACE technology). A gradient elution was performed at a flow rate of 1mL/min with solvent A (0.1% Trifluoroacetic acid in water) and solvent B (Acetonitrile). A gradient program was set as follows: 25% - 40% solvent B for 15 min and column was equilibrated with 25% solvent B for 3 min [26]. All the chromatograms were monitored under UV 220 nm. The retention time (RT) of product and each product related impurity peak was assigned as the time of elution from the column of corresponding product under the mentioned gradient program. The percentage purity of the target product as well as product concentration at all the stages of process were calculated using the HPLC peak area percentage as described in earlier work [26]

LC-MS analysis

a) Intact and reduced Mass analysis:

The A21 desamido Human Insulin variant was characterized and identified by state of art technique i.e. LCESI -MS (Thermo Scientific LTQ Orbitrap XL) with a top down approach. [27]The primary structure was determined by injecting the standard sample and the +1 Da variant in LC-MS analysis. Reverse-phase HPLC followed by electrospray ionization LC-MS (LC-ESI-MS; 5 µ C18-300Å 250 × 4.6 mm) was used to determine the intact and reduced masses of the drug product samples. Chromatographic separation of samples was carried out by using a reverse-phase C8 column (5 µ C8 250 × 4.6 mm), which was simultaneously ionized to give a mass to charge ratio (m/z) for particular species. The intact mass of variant was detected and a +1 Da was observed. Reduced masses of both samples A- and B-chains were obtained via dithiothreitol (DTT)-mediated reduction of disulfide bonds.

The difference in the standard protein chain mass and variant chain masses was observed and the site of +1 Da variant was identified.

b) Peptide mass analysis:

The variant was digested with Glutamyl endopeptidase (commercially available as Glu-C from Roche). The pH of the sample was adjusted to 7.0 with 1M tris(hydroxymethyl) aminomethane (Sigma Aldrich) and endoproteinase Glu-C enzyme is added in the enzyme-protein ratio of 1:25. The mixture of reaction was incubated at 37°C for three hours. Further, the disulphide bonds were reduced by using 1M DTT with 1:10 protein to DTT ratio and incubated for an additional hour. The variant was digested and reduced to individual fragments which were identified using LC-MS.

c) MS/MS analysis:

MS/MS spectrum based sequencing of polypeptides was done (CID) by using the nomenclature that was proposed by Roeptorff-Fohlmann-Biemann [28]. Based on the aforementioned theory, a polypeptide chain is thus sequenced to obtain the complete amino-acid sequence identity for the peptides. Software aided annotation of either *b* ions or *Y* ions are indicative of sequence coverage at amino acid level.

CD spectroscopy

Near (260-360 nm) and far (190-260 nm) UV circular dichroism (UV-CD) spectroscopy was used to study tertiary and secondary protein structures, respectively. Circular dichroism experiments were carried out on a Jasco J-815 spectrometer equipped with a Peltier-type cell holder. Spectra were recorded at 25°C with a scanning speed of 200 nm/min and a bandwidth of 0.1 nm in a quartz cell, with a path length of 0.1 cm. *Far-UV*CD spectra were recorded from 190 to 260 nm at a sample concentration of 0.3 mg/mL, whereas *near-UV*CD spectra were recorded from 260 to 360 nm at a sample concentration of 3 mg/mL. For each sample, 6 accumulations were performed, and baseline correction was applied.

Intrinsic Fluorescence

Intrinsic fluorescence measurements were performed on a Cary Eclipse fluorescence spectrometer (Agilent Technologies), and data were analyzed using Scan software version 1.2 (Agilent Technologies). Intrinsic fluorescence analysis was performed by excitation at 278 nm, and emission was scanned from 300 to 400 nm.

In-vitro Bioassay

CHO-K1 cells overexpressing insulin receptor B (IR-B) were cultured in DMEM F12 medium without serum. 20,000 cells/well/100 µL in culture medium were seeded in a 96 well flat bottom cell culture plate and incubated overnight at 37°C in 5% CO2 incubator. After overnight starvation, 100µl of drug dilutions were added to each well and incubated at 37°C in 5% CO2 incubator for 5-10 minutes. After incubation, drug dilutions were removed and washed with 100 µL of 1X ice cold DPBS followed by addition of 25 µL of 1X lysis buffer (supplied with Alpha SureFire INS-RB p-Y1150/1151 and Alphascreen Protein A kit (Perkin Elmer)). Samples were processed further by adding both acceptor beads and donor beads followed by measuring the alpha counts as per the kit protocol. The data was analysed using SoftMax Pro software.

Declarations

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Author contribution statement: **VS:** Conceived the idea, designed the study, Process data generation. **KS:** Characterization data generation. Madhu Kumar M S: Process data generation, data curation. **NV:** Characterization data curation, manuscript reviewing and editing. **NA:** Physicochemical characterization data generation. **NS:** Biological data generation. **SBP:** Biological experiment planning, data evaluation and manuscript review. **AK:** Characterization activities supervision and reviewing manuscript. **PH:** Conceptualization, overall supervision, reviewing data and writing the manuscript.

All authors read and approved the final version of the manuscript

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical declaration: This article does not contain any studies with human participants or animals performed by any of the authors.

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Tables

Table 1. Purification Table

	Purity (%)- Deamidated Insulin	Amount (gm) Deamidated Insulin	Yield (%)
Insulin Drug substance	0.3%	-	-
AIEX Load	16.65	2.82	73.5
AIEX Elution pool	96.52	2.08	
RP HPLC Load	96.94	1.79	97.3
RP HPLC Elution pool	98.13	1.74	
Precipitation	98.1	1.74	90.3
Insulin desamido (Drug Substance)	97.43	1.50	96

Table 2. Mass summary of A21 desamido human insulin peak and human insulin

Variant	Intact	Reduced		PMF				PMF (D+R)					
		Chain-A	Chain-B	F-1	F-2	F-3	F-4	F-1	F-2	F-3	F-4	F-5	F-6
	Average Mass(M+H)+			Monoisotopic Mass(M+H)+									
Human Insulin	5807.5	2383.92	3430.57	417.22	1116.54	1377.52	2969.2	513.16	417.22	1482.66	1116.54	867.39	1490.58
A21	*5808.5*	*2385.00*	3430.72	417.23	1116.58	*1378.56*	2969.32	*514.16*	417.23	1482.72	1116.58	867.43	1490.40

Note: Values shown in italics indicate modification of +1 Da w.r.t to human insulin control sample

Table 3. Comparison table of relative potency for A21 desamido insulin and human insulin IRB

Test Sample	Reference Standard	Individual Relative Potency	Average Relative Potency	% CV
A21 desamido insulin	Human insulin	0.868	1.00	12.87
		1.006		
		1.125		

Figures

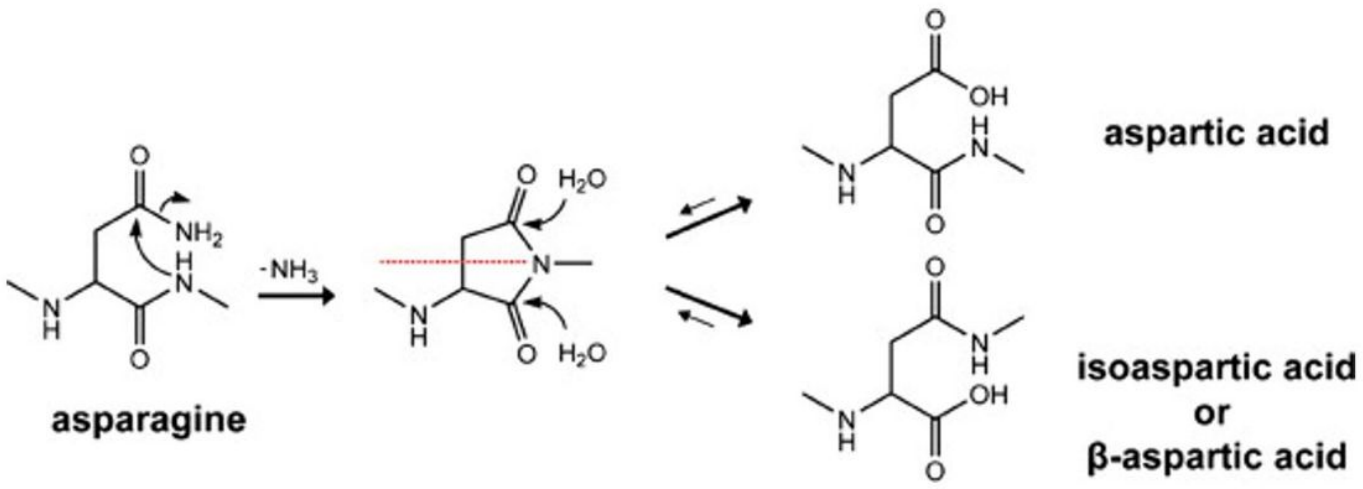
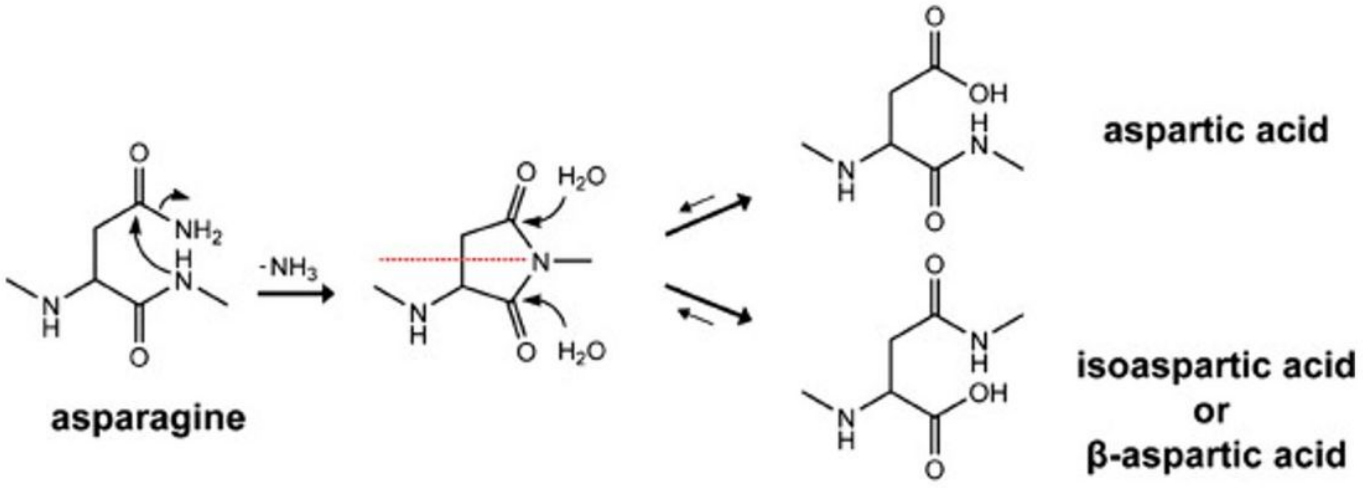


Figure 1

Schematic representation of deamidation of asparagine to Aspartic acid through Succinimide intermediate.



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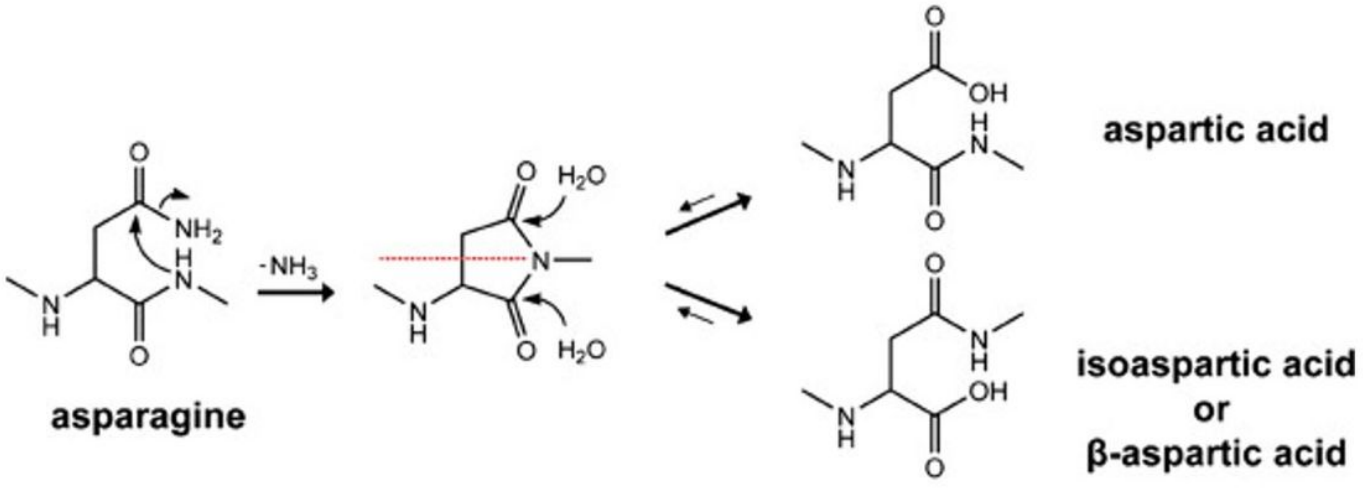

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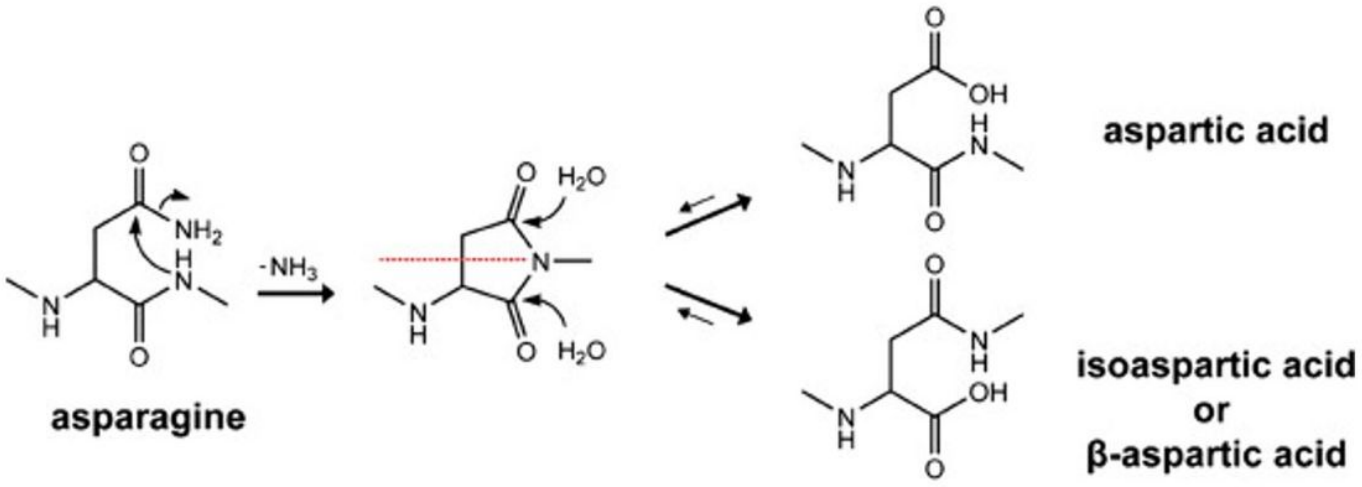

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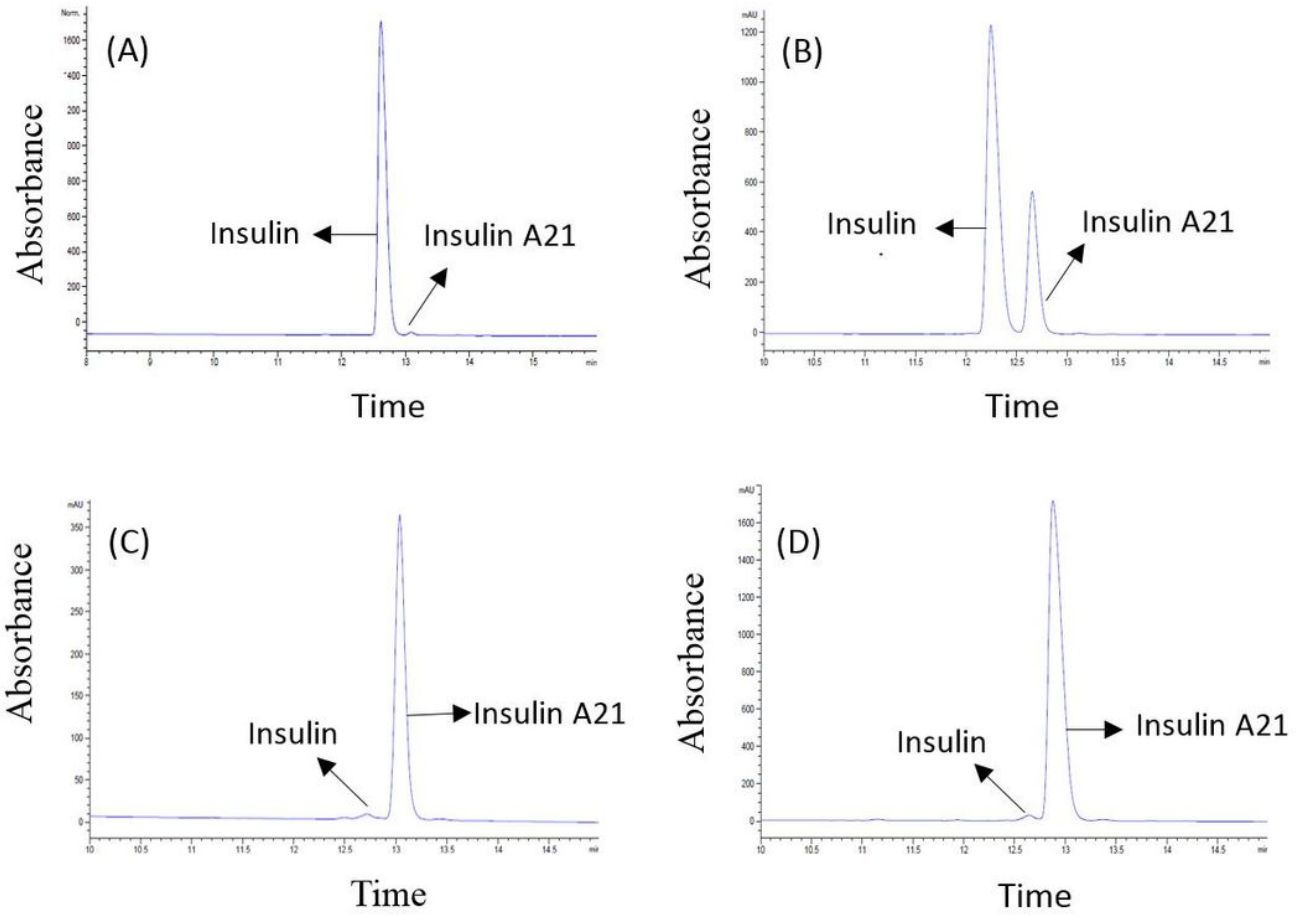
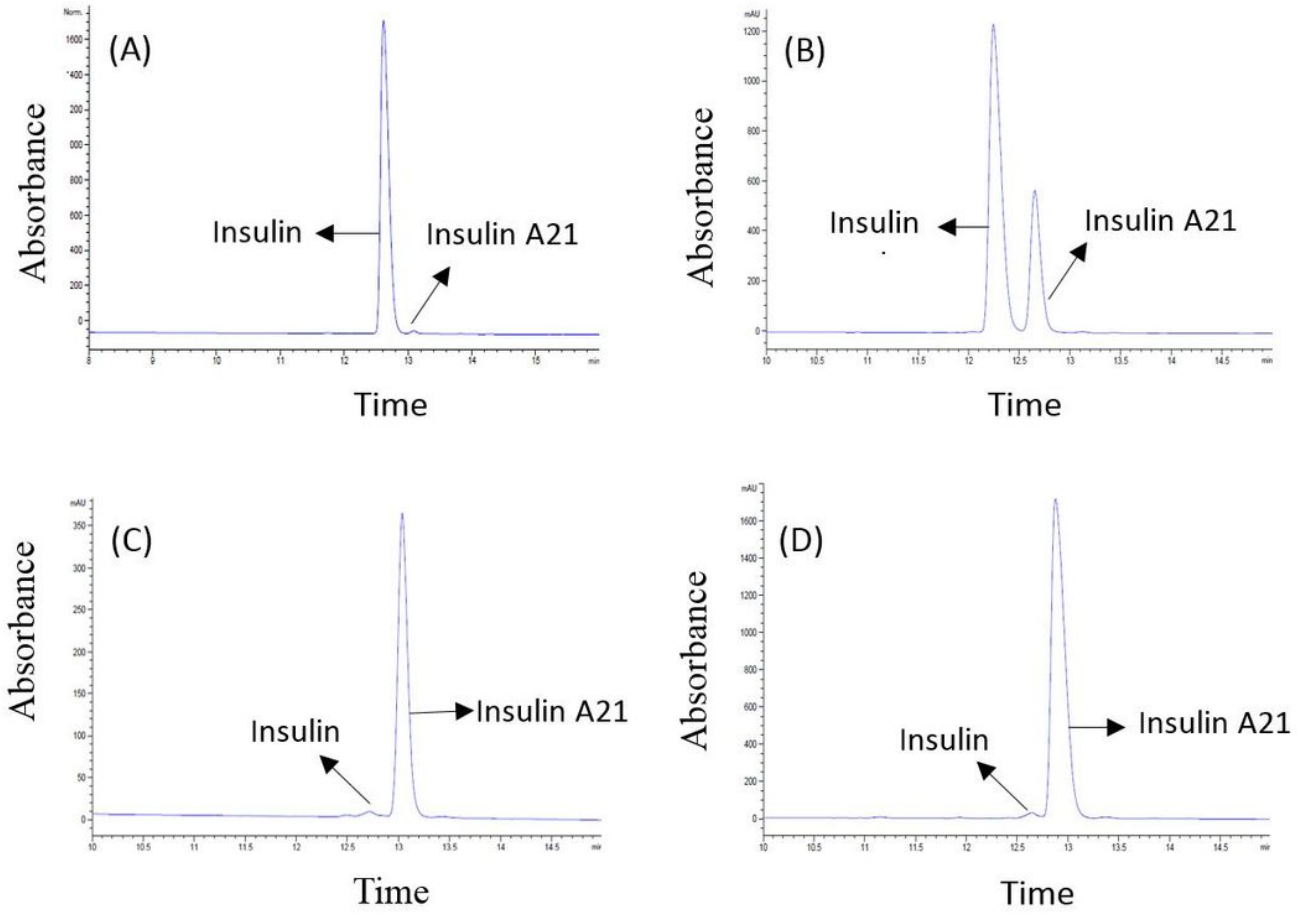


Figure 2

Analytical RP-HPLC profile at different purification stages, (a) solubilized insulin DS before degradation, (b)solubilized insulin after degradation (AIEX load), (c) AIEX elution pool product profile (after purification), (d) RP-HPLC elution profile product profile (after concentration). Insulin and desamido insulin peaks are marked in the pictures.



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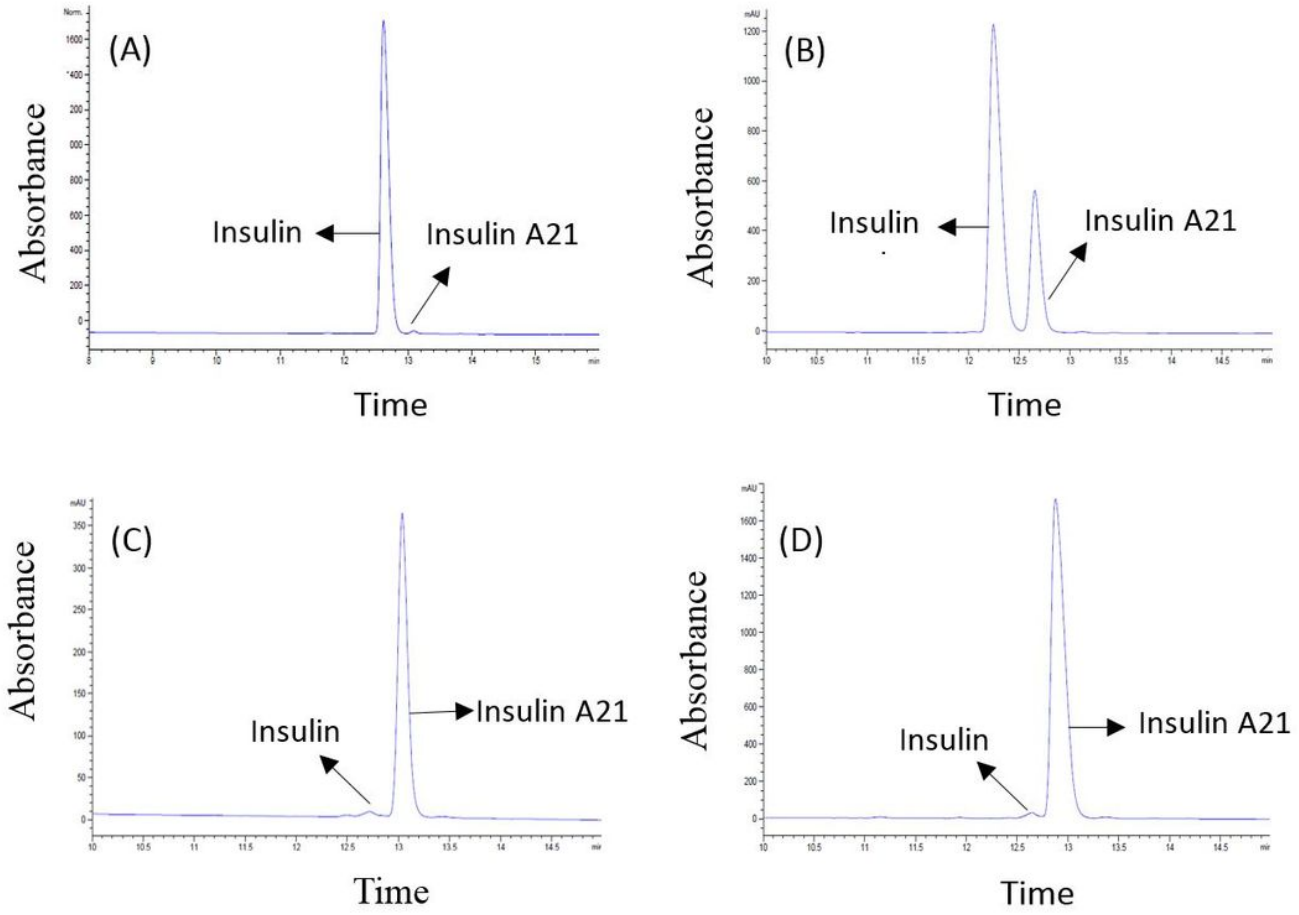

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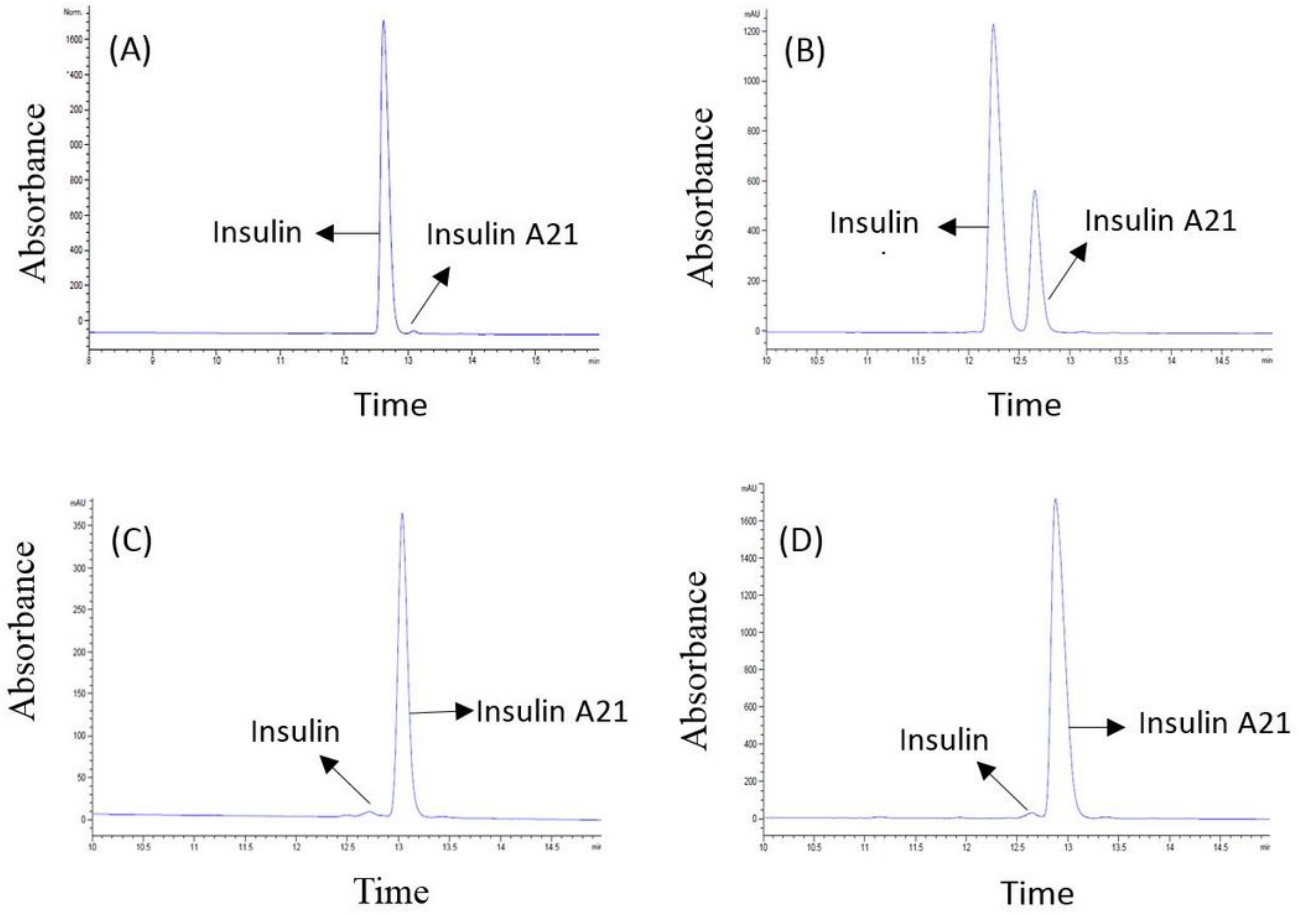

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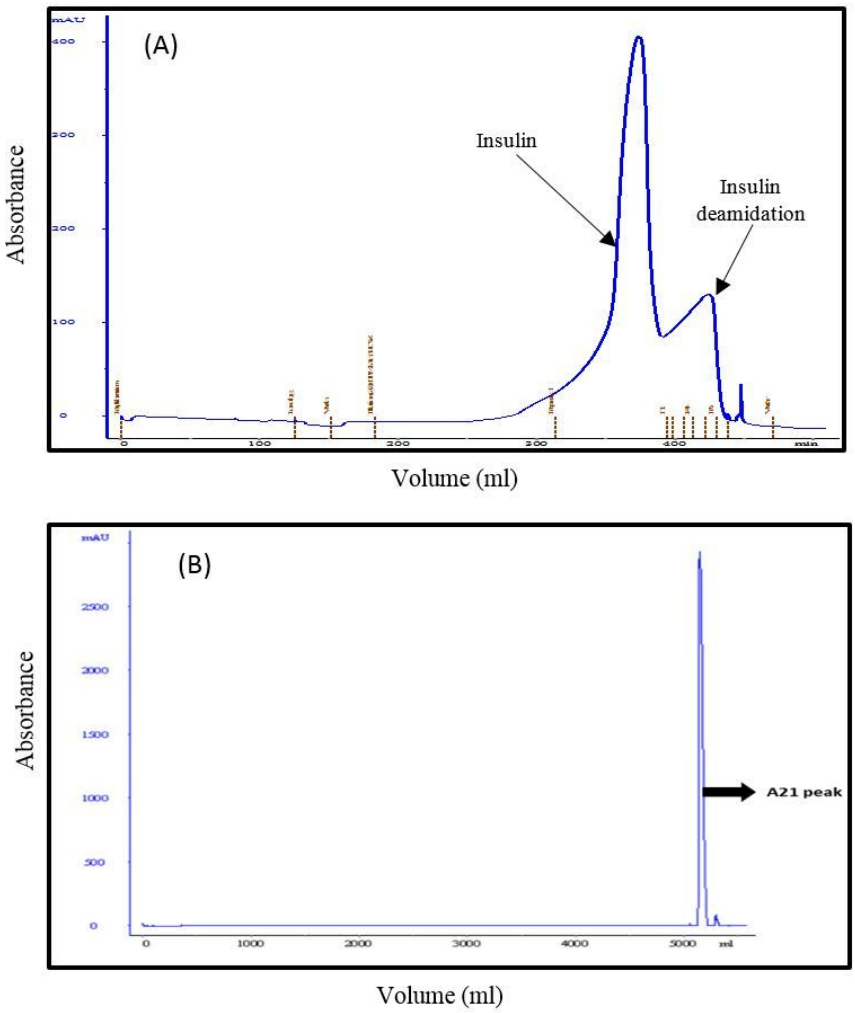

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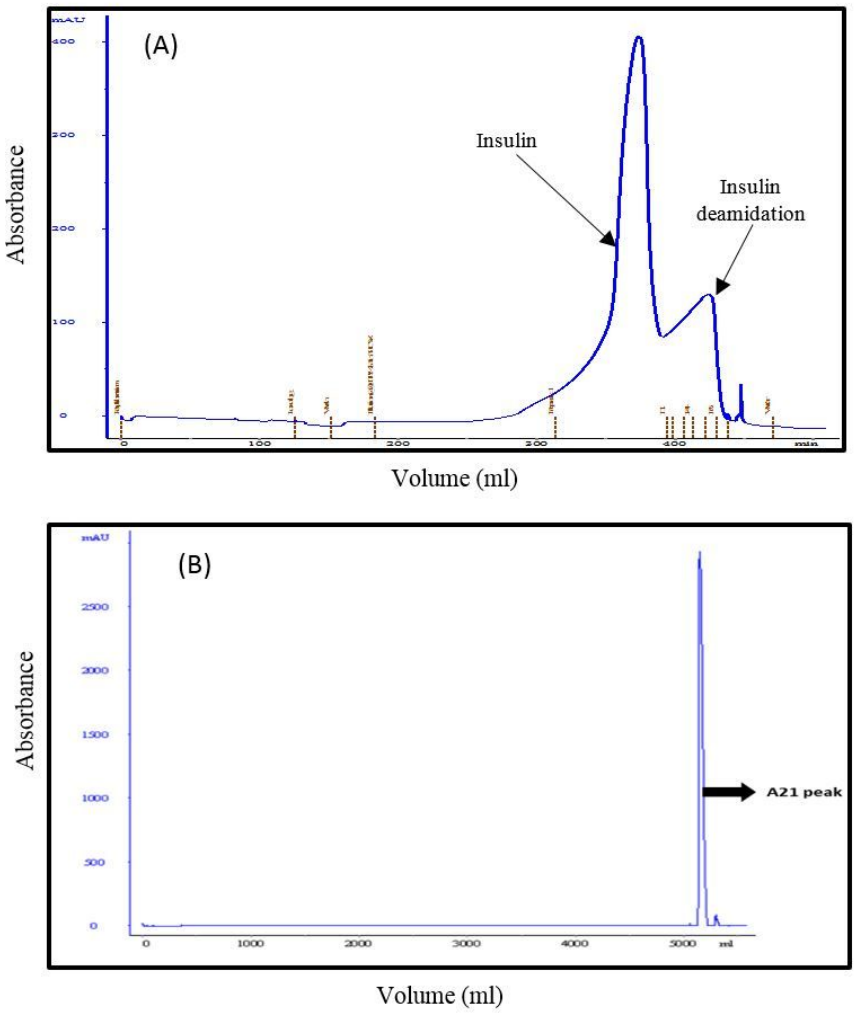

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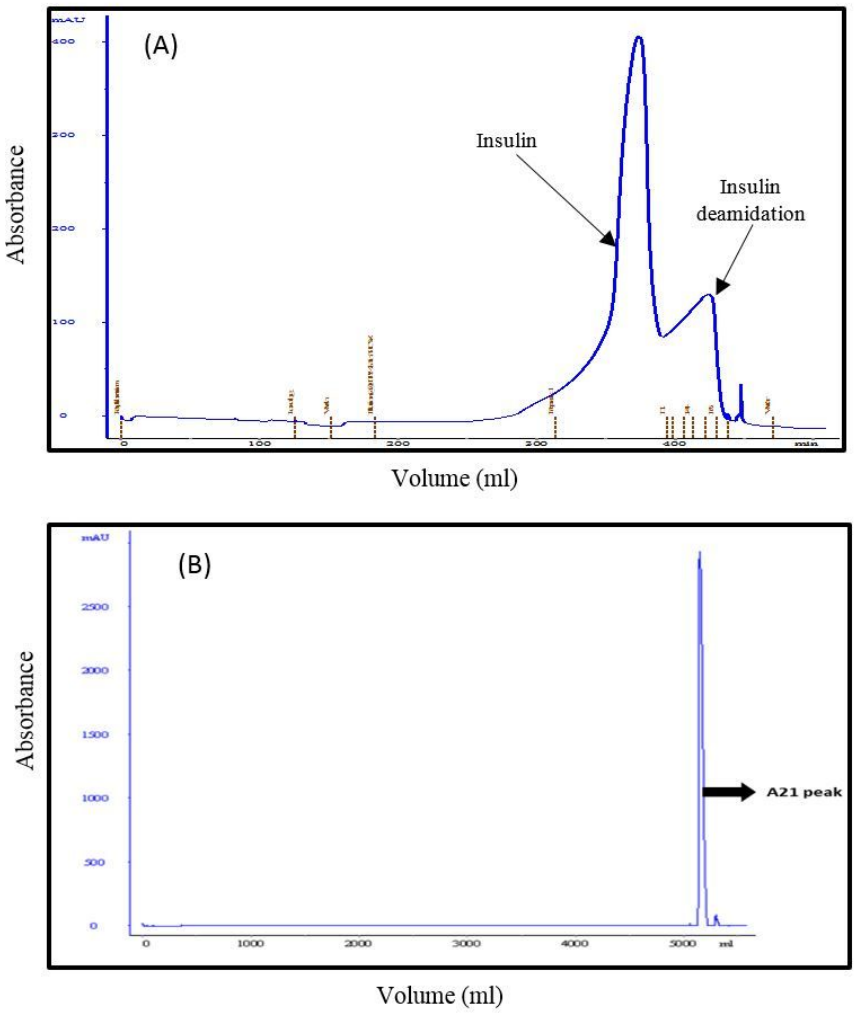

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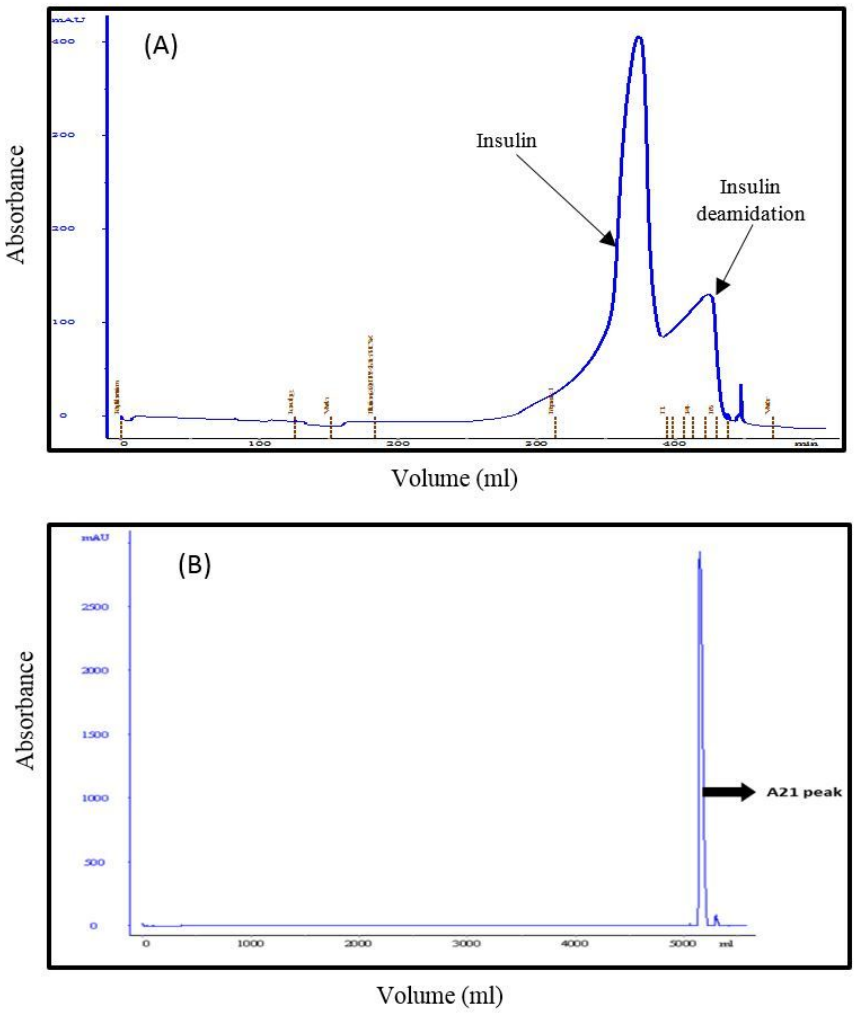

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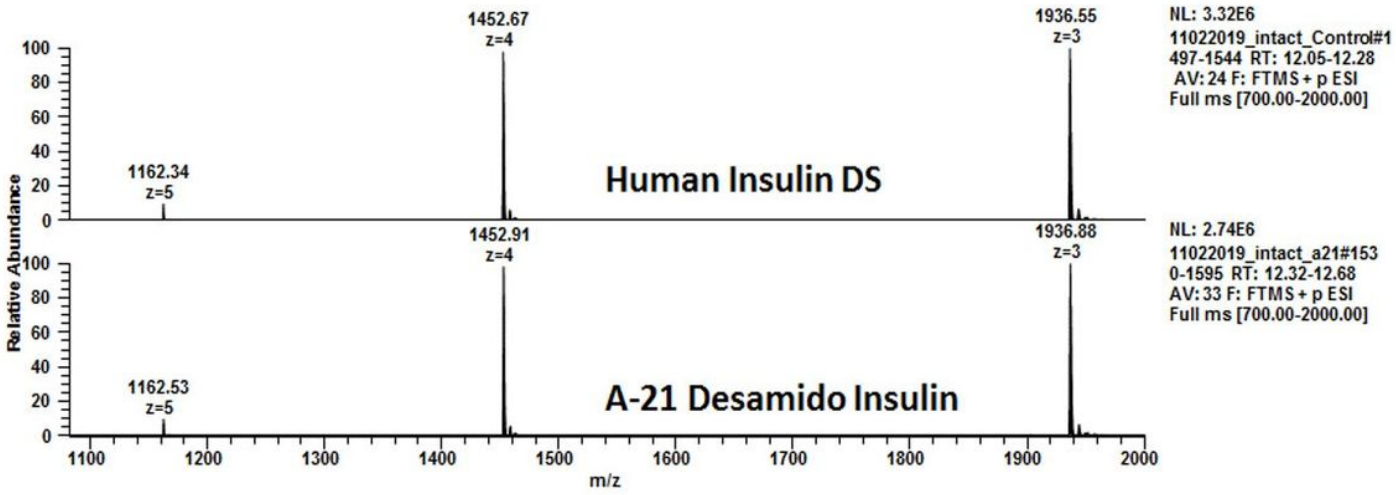

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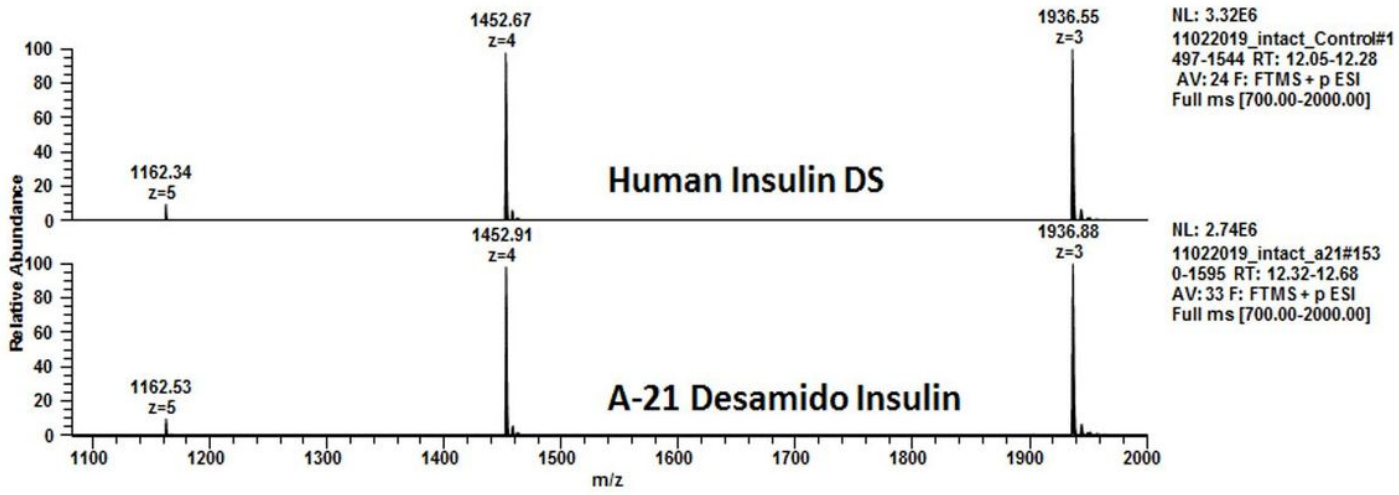

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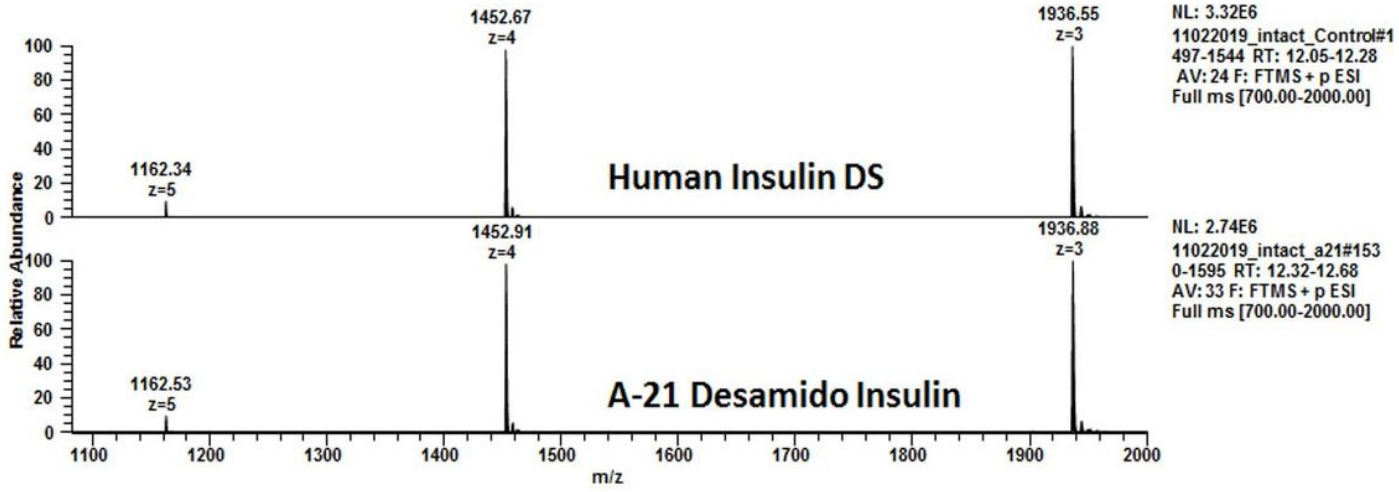

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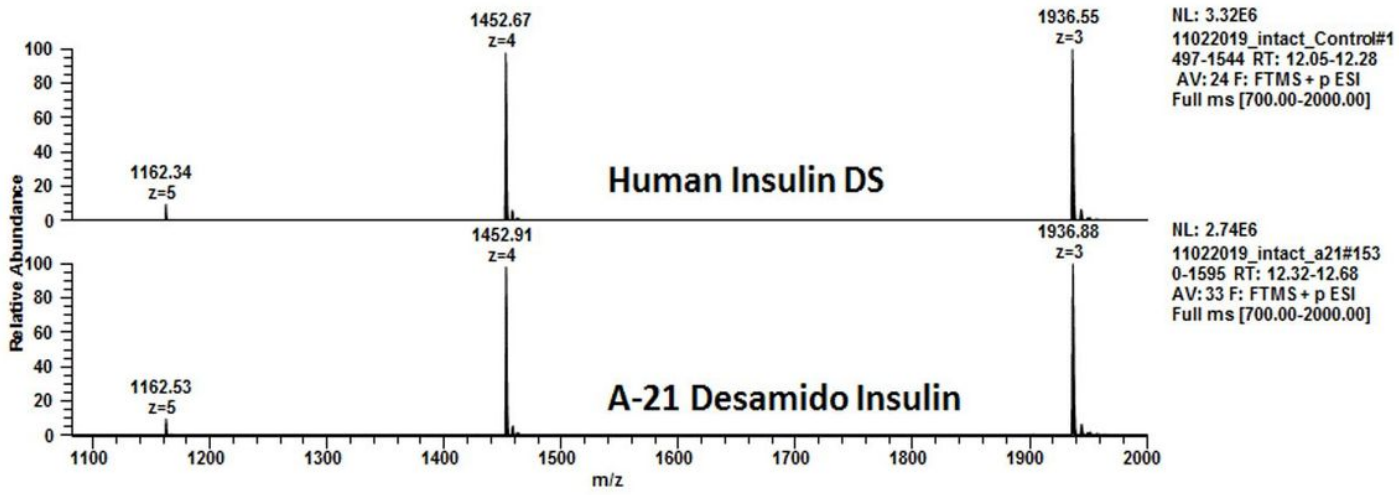

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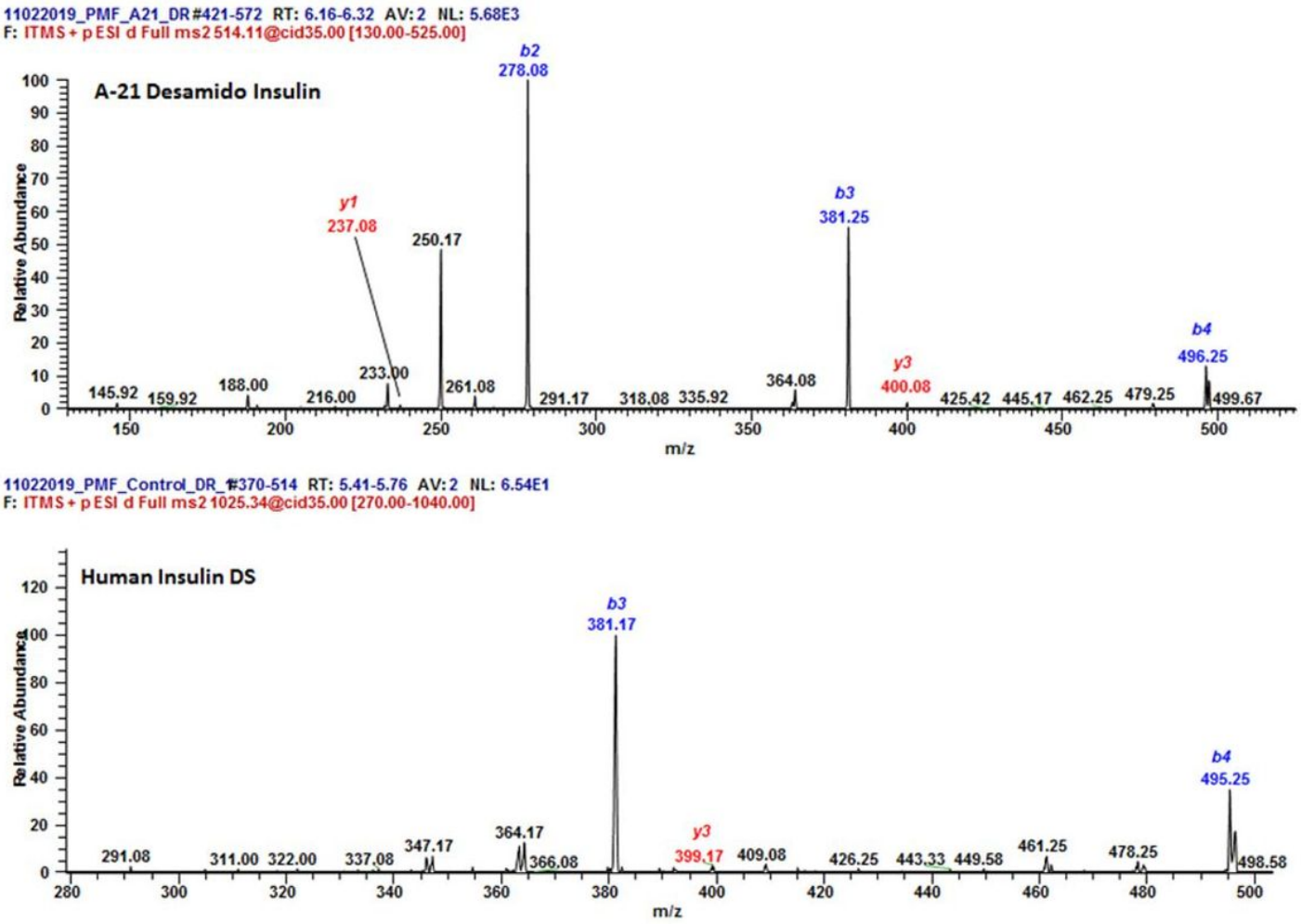

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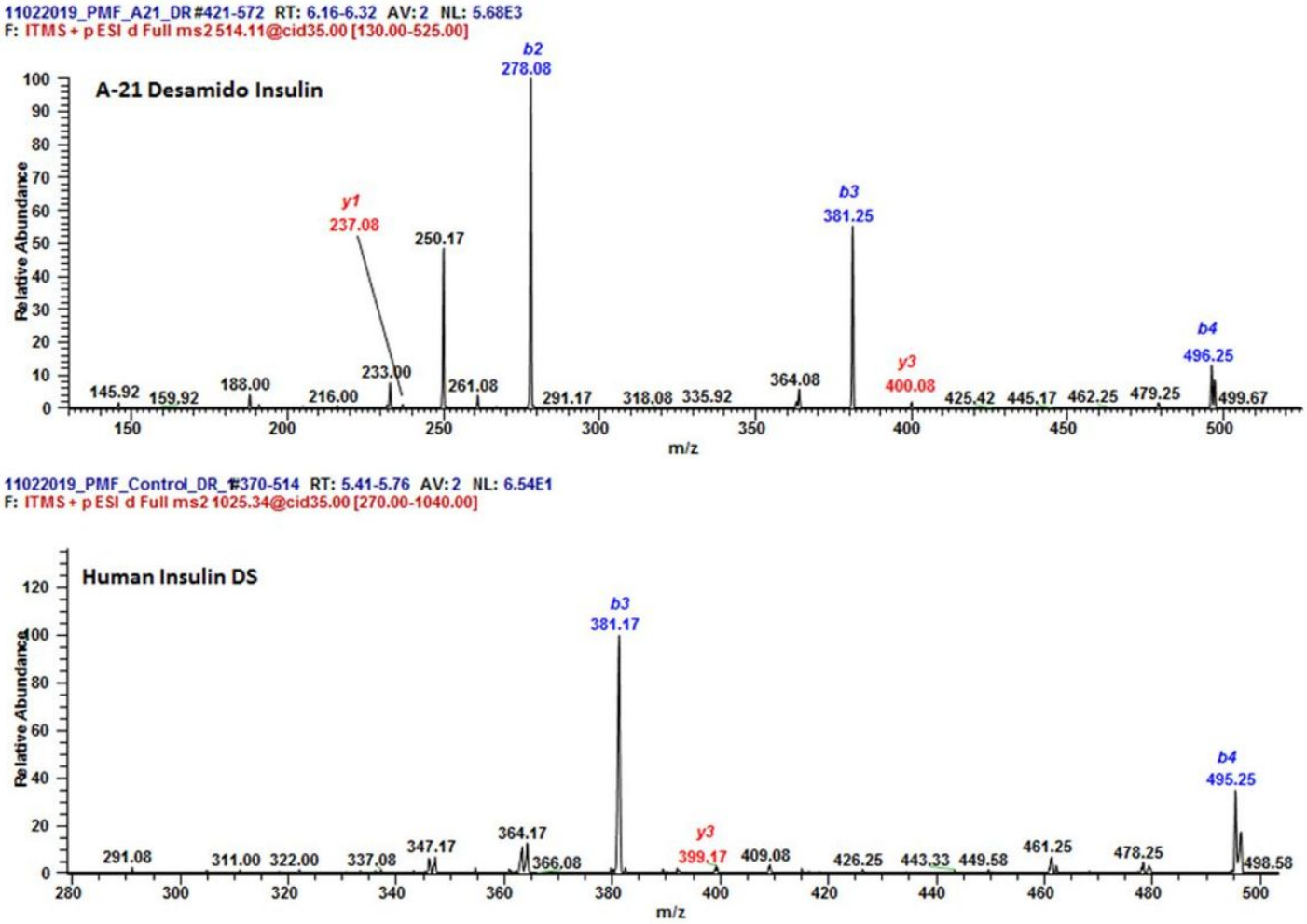

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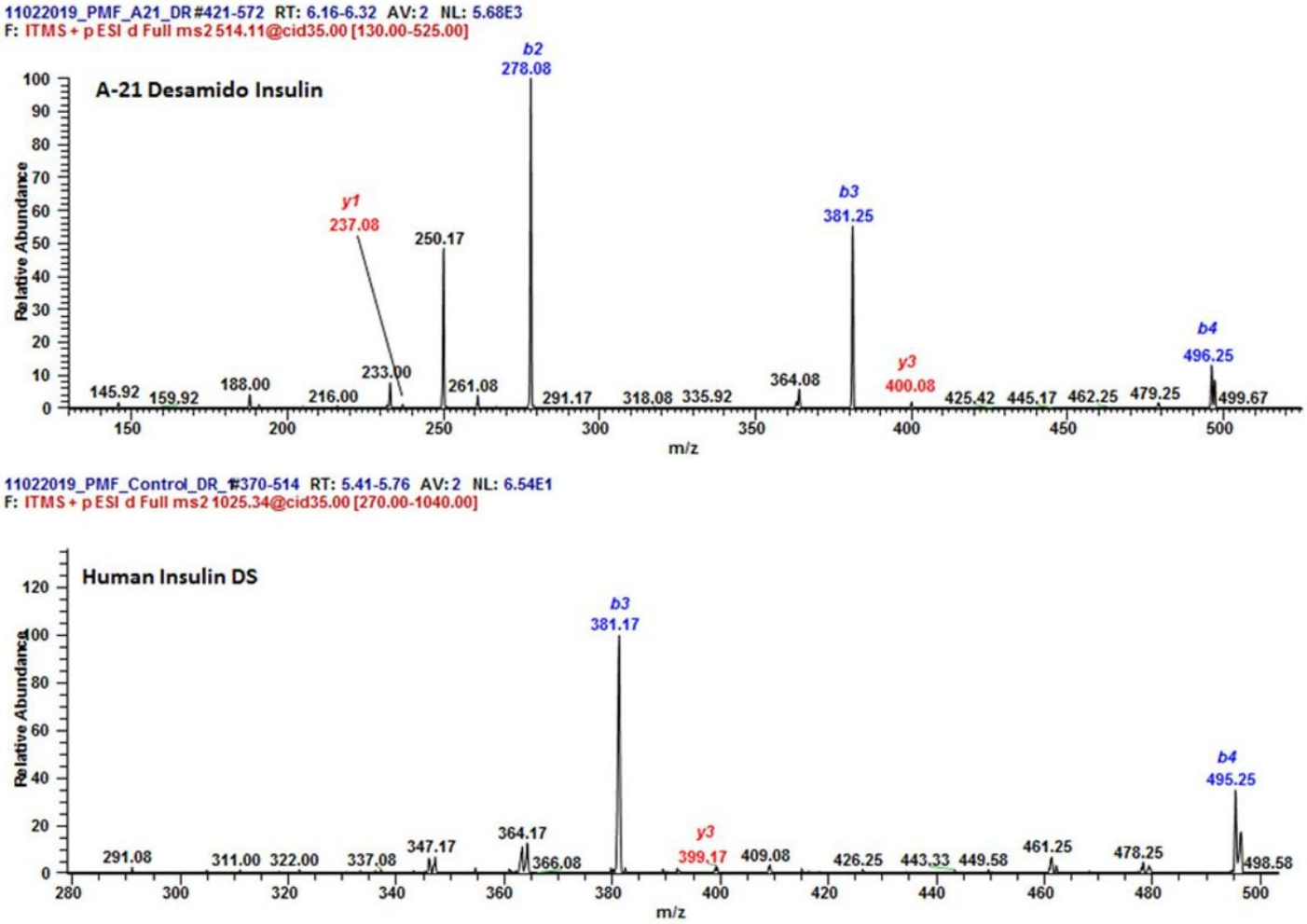

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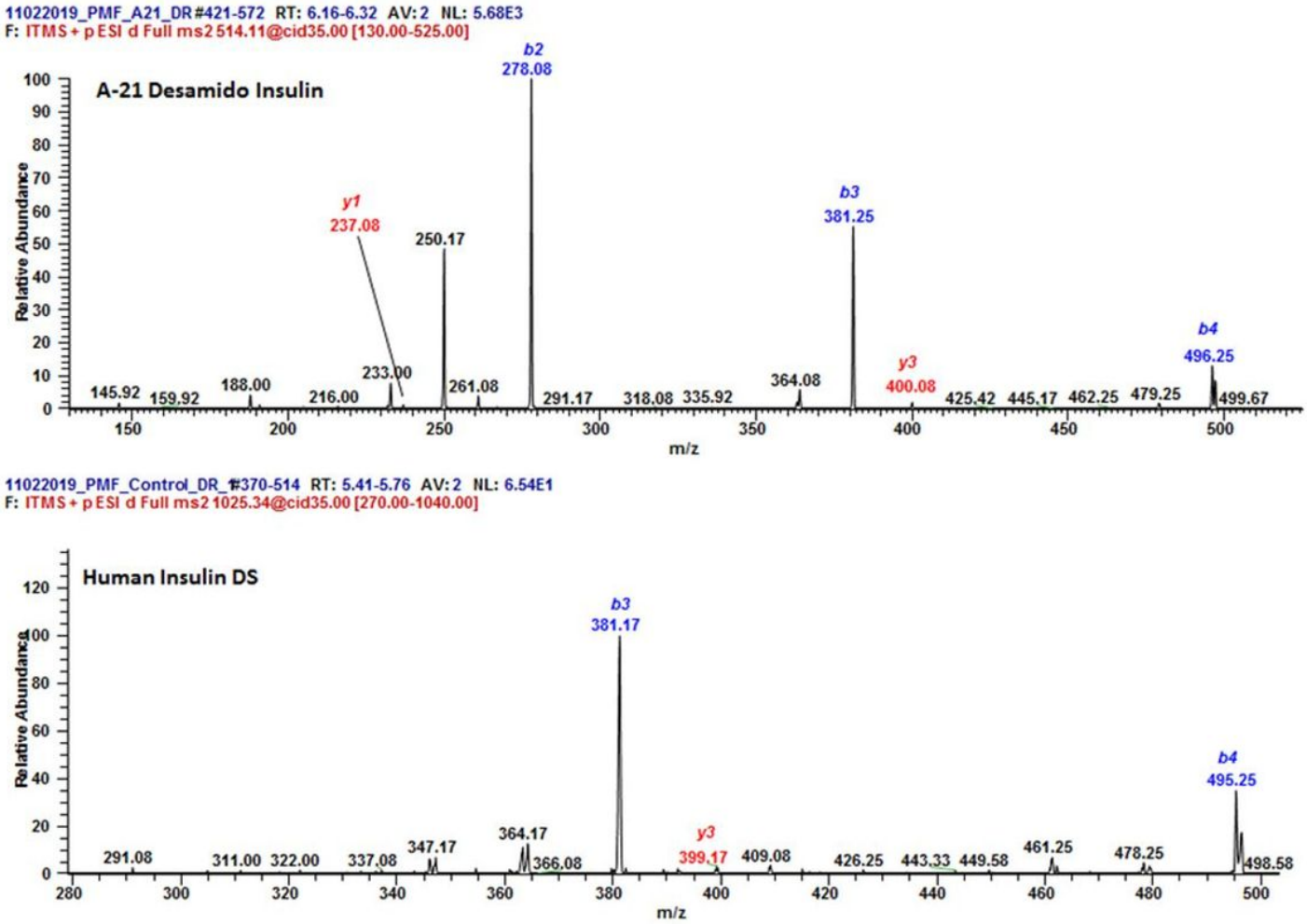

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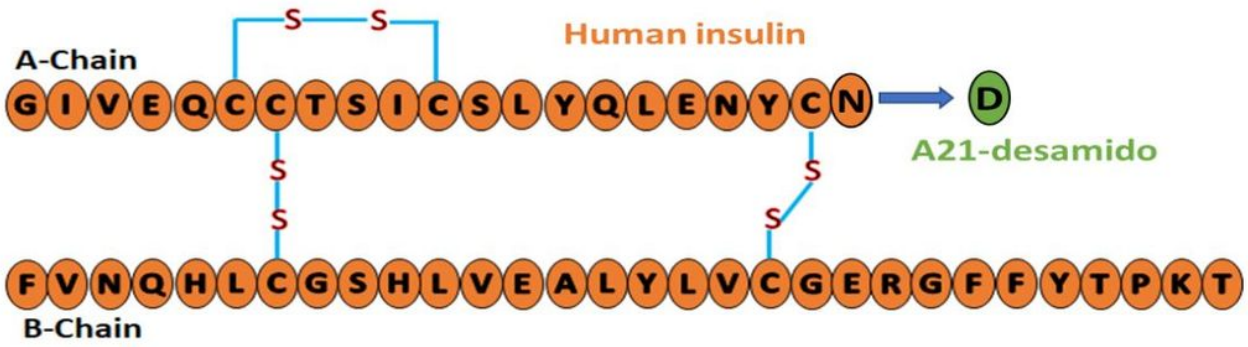

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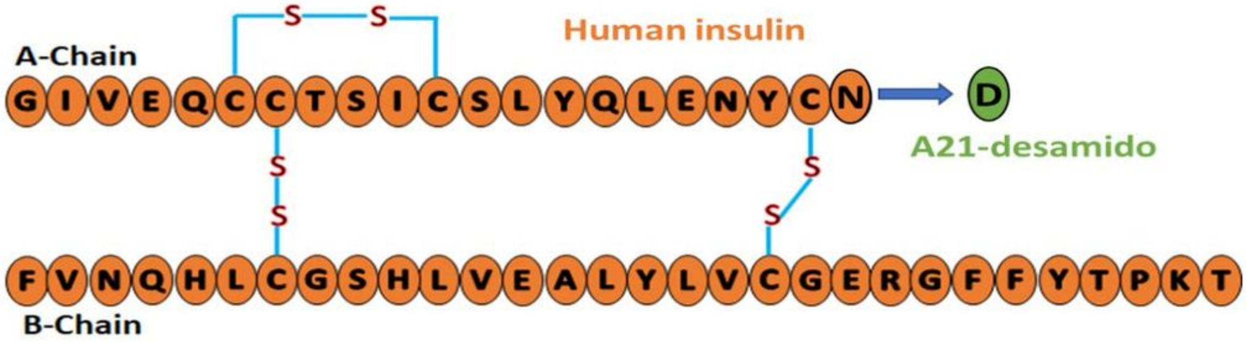

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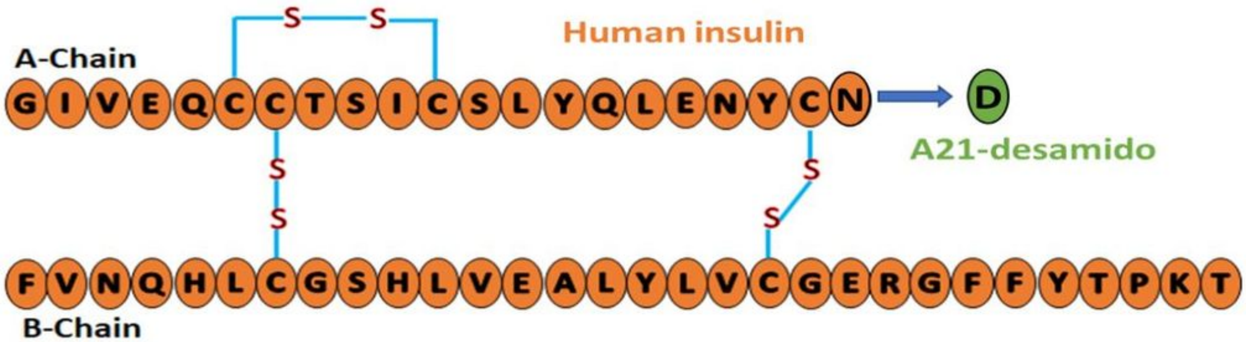

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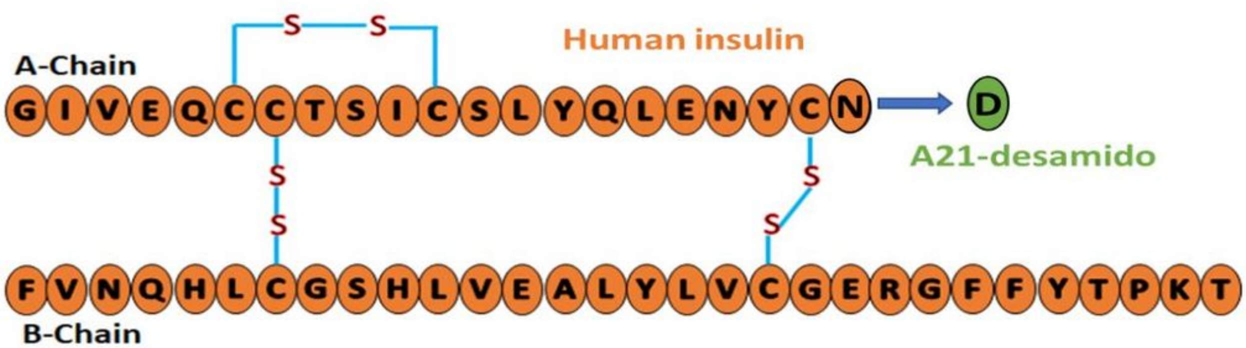

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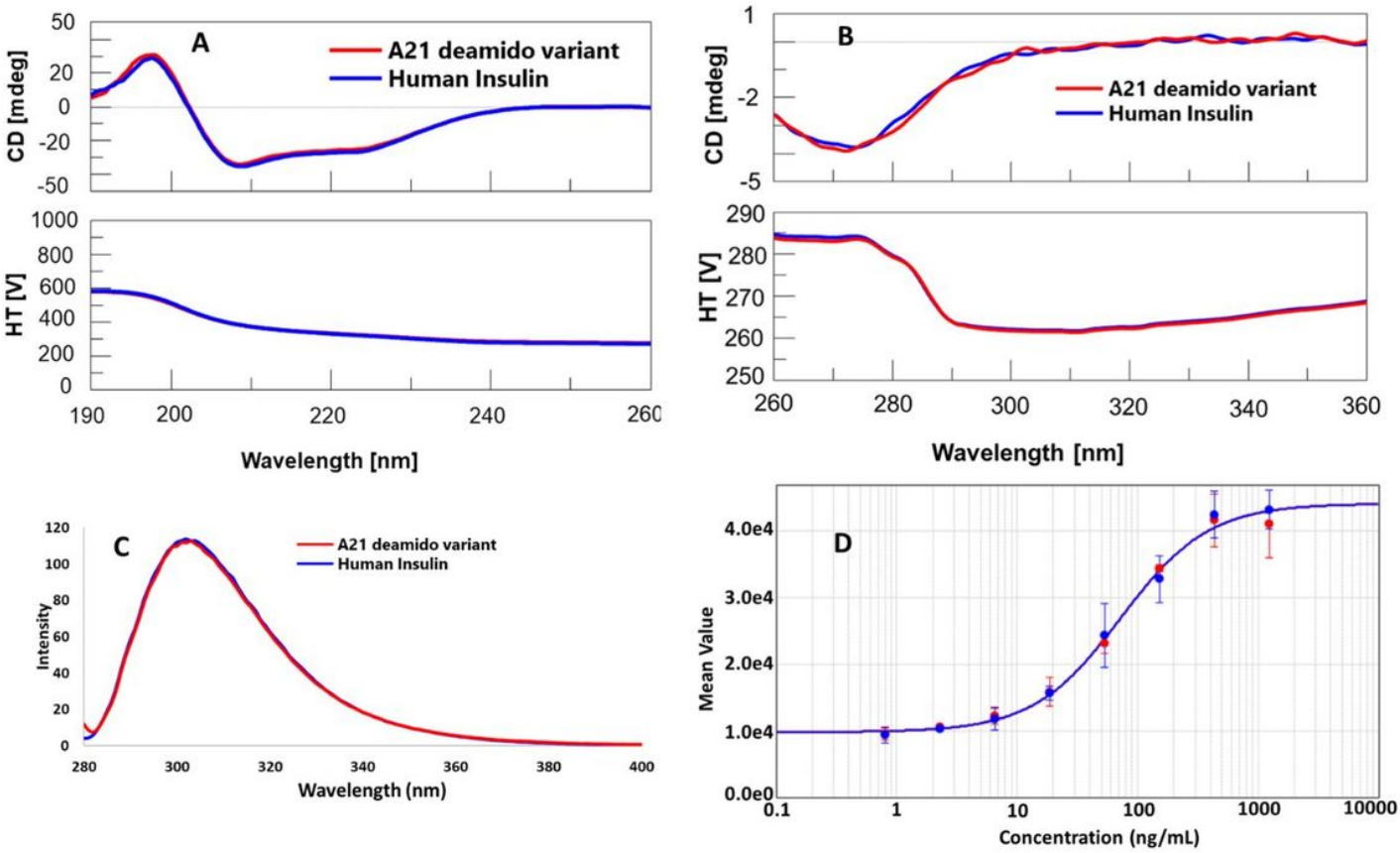

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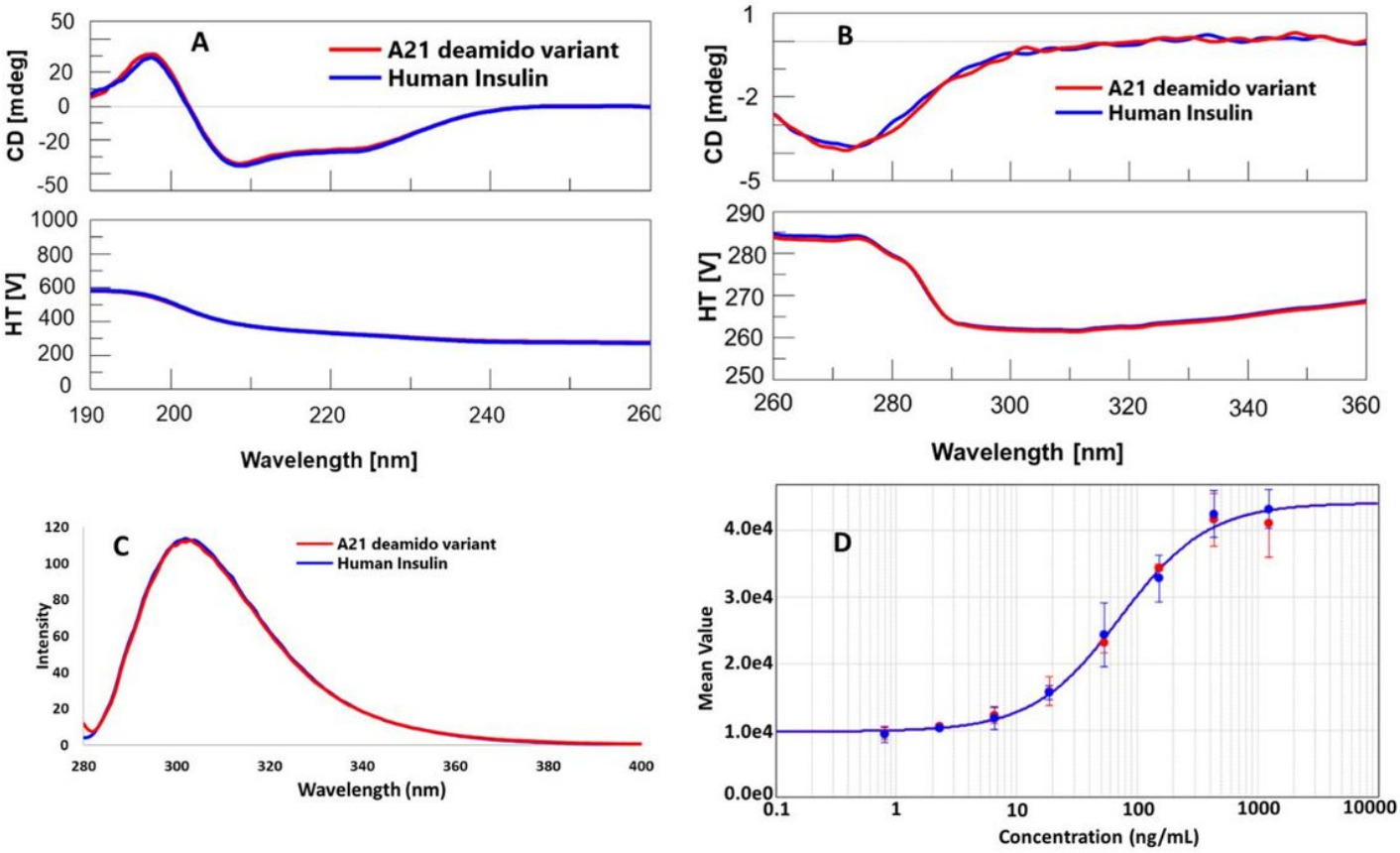

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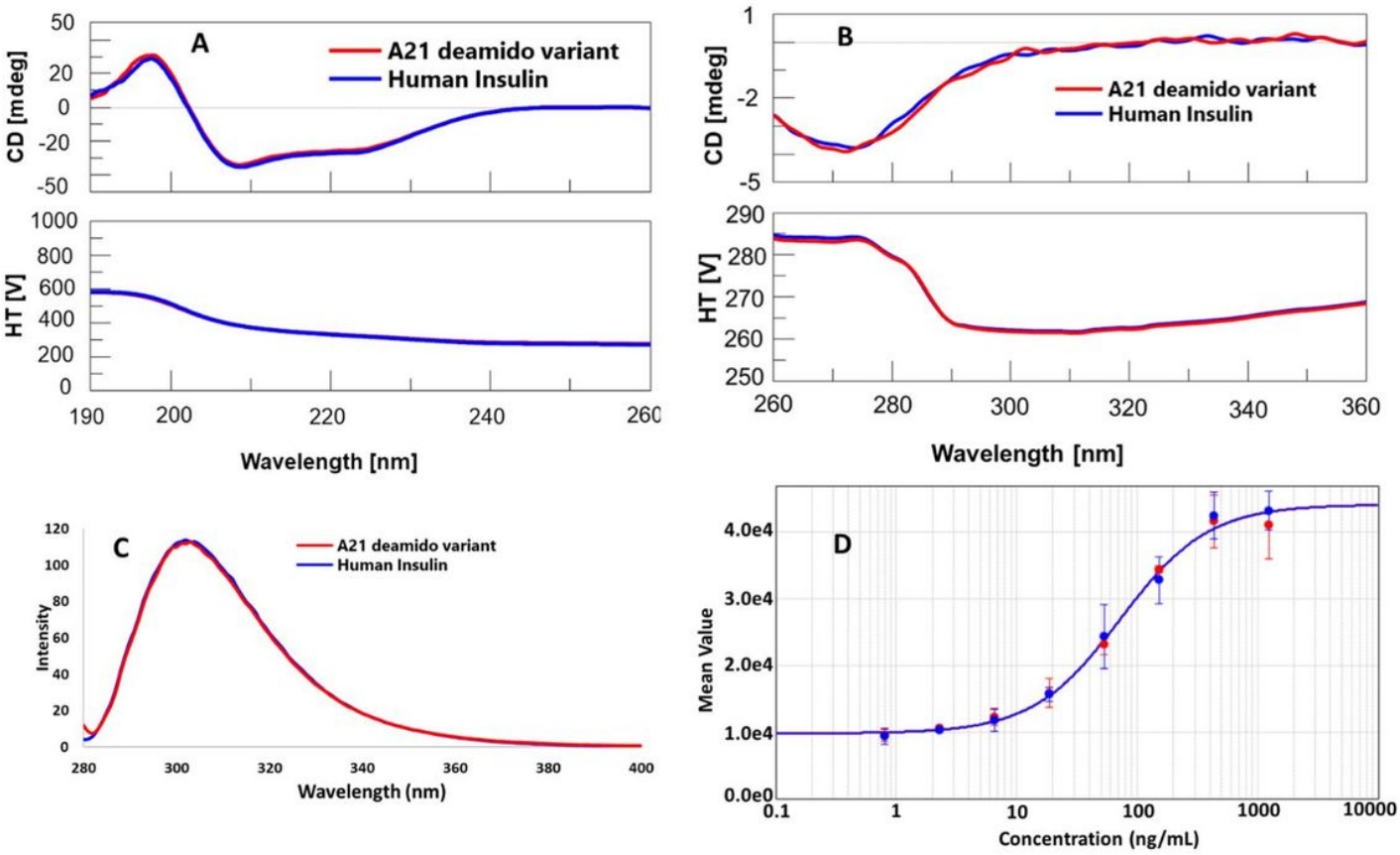

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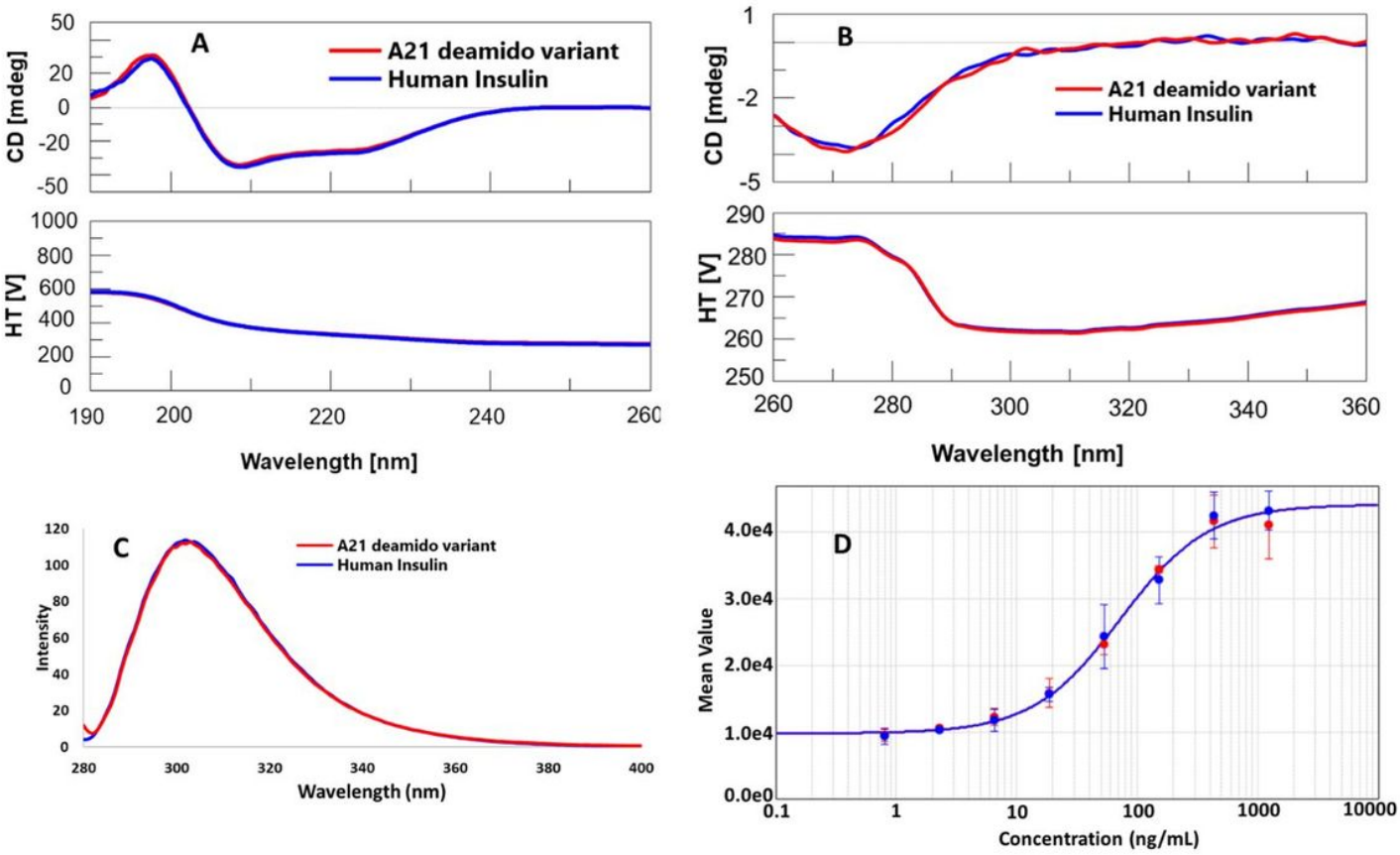

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