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Characterization of Performance-Enhancing Peptides via Inlet Ionization on DART-TOF/MS

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Abstract

Consumers can purchase performance-enhancing peptides and cosmetic peptides online via portals similar to those for emerging or "designer" drugs – anonymously and at a low price. Such products are frequently marketed as being "for research purposes only," presumably in an effort to avoid legal ramifications; the sole non-medically-supervised use is to promote muscle growth or cosmetic enhancement. Though the sale of these peptides may not be legally prohibited, their identification remains of analytical concern. The detection of counterfeit peptide products, including those reportedly containing recombinant human growth hormone (rHGH), is of interest. These products may contain a different peptide or no active substance whatsoever. The authenticity of the peptides being sold to the public lead to health and safety, as well as legal, concerns.

In general, crime laboratories do not have protocols for the identification of peptides and large biomolecules. The gold standard of forensic drug analysis, gas chromatography-mass spectrometry (GC-MS), is typically limited to the analysis of small molecules that are readily vaporized at the inlet. High-resolution mass spectrometers capable of ambient ionization, on the other hand, are well-suited for the analysis of peptides. These instruments, including the JEOL AccuTOF, are becoming more prevalent in forensic laboratories as ambient ionization techniques, such as Direct Analysis in Real Time (DART), continue to gain popularity.

Through this project, we developed a protocol to analyze peptide samples utilizing a time-of-flight mass spectrometer through a variety of ionization methods. In many cases, this technique required no external ion source or additional equipment, theoretically enabling utilization with any mass spectrometer of appropriate mass analyzer range incorporating an atmospheric pressure inlet.

Various peptide standards and former case samples were successfully analyzed using matrix-assisted inlet ionization mass spectrometry. Additionally, we purchased and tested an array of peptides from an online vendor to determine their authenticity. To characterize peptides via molecular mass, samples were dissolved in 1:1 acetonitrile:water with 0.1% formic acid. The matrix compound 3-nitrobenzonitrile (3-NBN) was added to samples just before analysis. Utilizing matrix-assisted inlet ionization, electrospray ionization (ESI)-like spectra were obtained in a matter of seconds. Molecular masses were calculated using a mass spectral interpretation software package.

To further characterize the peptides, a simple enzymatic protein digestion procedure utilizing trypsin was performed. Matrix-assisted inlet ionization was utilized to analyze the resulting peptide fragments. Possible identifications for each peptide were assigned by comparing each digested spectrum against an online peptide database. Combined with the molecular mass attained from analysis of the intact peptides, identification of each peptide was made at a reasonable level of certainty.

Finally, peptide analysis was explored utilizing paper spray ionization. This ionization method was favored for extremely large peptides in which inlet ionization was not feasible. By utilizing both inlet ionization and paper spray ionization as complementary techniques, the vast majority of peptide samples that could be encountered in a crime laboratory could be readily characterized.

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Executive Summary

Consumers can anonymously purchase performance-enhancing peptides and cosmetic peptides online at low price through portals similar to those used for emerging or "designer" drugs. Such products are frequently marketed as being "for research purposes only", presumably in an effort to avoid legal ramifications; the sole non-medically-supervised use being to promote muscle growth or cosmetic enhancement. Though the sale of these peptides may not be prohibited, their identification remains an analytical requirement. The detection of counterfeit peptide products, including those reportedly containing recombinant human growth hormone (rHGH), is also of interest. These products may contain no active ingredient or, a different peptide. The authenticity of the peptides being sold to the public raises health and safety concerns for users.

Many crime laboratories do not have protocols for the identification of peptides and large biomolecules. The workhorse of forensic drug analysis, gas chromatography-mass spectrometry (GC-MS), is limited to the analysis of relatively small molecules that are readily vaporized at the inlet. High-resolution mass spectrometers capable of ambient ionization, on the other hand, are well-suited for the analysis of peptides. Moreover, such instrumentation is becoming more prevalent in forensic laboratories as ambient ionization techniques, such as Direct Analysis in Real Time (DART), continue to gain popularity.

Through this project, we developed a protocol to analyze peptide samples utilizing a time-of-flight mass spectrometer that is typically used for DART-MS analysis. In many cases, this technique required no external ion source or additional equipment aside from the mass spectrometer.

Utilizing matrix-assisted inlet ionization mass spectrometry, various peptide standards and case samples were successfully analyzed. We purchased an array of peptides from an online vendor to determine the authenticity of the products utilizing this technique. To determine the molecular mass of the peptides, samples were dissolved in 1:1 acetonitrile:water with 0.1% formic acid. The matrix compound, 3-nitrobenzonitrile (3-NBN), was added to the sample solutions until a noticeable amount of solid accumulated at the bottom of the sample vial. Approximately 5 µL of liquid sample, including some of the solid 3-NBN, was drawn up into a microliter pipette and introduced directly to the mass spectrometer inlet to initiate ionization. In a matter of seconds, electrospray ionization (ESI)-like spectra were obtained. Molecular masses were calculated using a mass spectral interpretation software package.

To further characterize the peptides, a simple enzymatic protein digestion procedure utilizing trypsin was performed. Matrix-assisted inlet ionization was utilized to analyze the resulting peptide fragments. Possible identifications for each peptide were assigned by comparing each digested spectrum against an online peptide database. Combined with the molecular mass obtained from analysis of the intact peptides, identification of each peptide was made at a reasonable level of certainty.

Finally, peptide analysis was explored utilizing paper spray ionization. This ionization method was favored for extremely large peptides in which inlet ionization was not feasible. By utilizing both inlet ionization and paper spray ionization as complementary techniques, the vast majority of peptide samples that could be encountered in a crime laboratory could be readily characterized.

ES.1 Project Goals

This project was proposed with the intent to improve forensic science services by developing an alternative method for the successful detection and identification of human growth hormone and other peptides. This was to be accomplished by developing a novel, cost-effective, and reliable method for characterization of targeted macromolecules in a typical crime laboratory. Because the popularity of high resolution mass spectrometers capable of atmospheric pressure ionization continues to increase, the project focused around this type of instrumentation, especially time-of-flight mass spectrometers combined with a Direct Analysis in Real Time (DART) ion source. The method needed to successfully meet two distinct requirements: ionization of the targeted compounds and detection of the ionized species. In order to successfully identify peptides and similar compounds, the method needed to be capable of measuring the molecular mass of the targeted compounds. To increase the level of certainty for identification, peptide fragments produced through enzymatic digestion could also be analyzed, producing a chemical fingerprint for each peptide analyzed. This digested product could then be compared against a database, or directly against a digested standard, depending on the degree of characterization necessary.

A final goal was to develop a spectral database of available peptides that could be shared amongst laboratories wishing to utilize the developed procedure.

ES.2 Methods Implemented During Project

Sample Preparation

Because this study was largely qualitative rather than quantitative, all peptide samples were prepared at roughly 1 mg/mL concentrations prior to analysis. Lyophilized peptide samples were dissolved in 1:1 acetonitrile:water with 0.1% formic acid (v/v) to the desired target concentration. Formic acid was added to the solvent to aid with dissolution of the peptides and to facilitate protonation during mass spectral analysis. The samples were analyzed utilizing paper spray ionization without further sample preparation. To analyze the samples via inlet ionization, solid 3-NBN was added to the dissolved samples just before analysis. 3-NBN was added to the samples until saturation of the matrix compound was noticeable and a small amount of material was visible at the bottom of the sample vial. The samples were analyzed via inlet ionization directly following the addition of the 3-NBN.

Further characterization was accomplished by enzymatically digesting the peptide samples with trypsin utilizing a published in-solution digestion protocol. Though the protocol calls for overnight incubation after the addition of the trypsin, useable digest data was obtained after only one hour of incubation with no further sample preparation. Analysis of the digested samples was accomplished via direct inlet ionization with the addition of 3-NBN in the same manner as described previously.

Instrumentation

Mass spectral analyses were carried out utilizing an atmospheric pressure ionization high-resolution time-of-flight AccuTOF JMS-T100LC mass spectrometer (JEOL USA, Peabody, MA).

Various ionization techniques were investigated during this project. As ESI-MS is a commonly accepted technique for analyzing large macromolecules such as peptides, the ESI source supplied with the AccuTOF mass spectrometer was used to collect initial reference spectra. If spectra of target peptides could be attained via electrospray ionization, it would be known that the mass spectrometer settings were adequate for the successful transport of generated ions through the mass analyzer to the detector. If signals were not observed utilizing the same mass spectrometer settings when other ionization techniques were utilized, it would indicate that ionization of the sample was not successful. The ESI reference spectra were thus used to evaluate the performance of the other ionization techniques discussed herein.

Data was ultimately acquired from 200 to 4500 m/z with an acquisition interval time of 0.5 ns and spectral recording interval of 0.3 sec. The atmospheric pressure interface was operated at the following potentials: orifice 1 = 90 V, orifice 2 = 5 V, and ring lens = 10 V. The temperature of orifice 1 was set to 200° C to facilitate evaporation of the solvent during sample introduction. The RF ion guide voltage was set to 2500 V to allow detection of the large-mass ions associated with charged macromolecules. Spectra were mass calibrated using an Ultramark 1621 spectrum that was collected via DART ionization during each sample analysis.

Ionization Methods

Due to the simplicity of the technique, matrix-assisted inlet ionization was the main ionization method that was investigated for this project. Introduction of liquid samples was accomplished by simply placing the opening of a micropipette containing the sample near the inlet of the mass spectrometer. Oftentimes, physical contact of the micropipette and the inlet was required. When this close proximity was achieved, the vacuum system of the mass spectrometer would rapidly draw the sample into the inlet, initiating the ionization process. Typically, 5 µL of sample was utilized for each analysis. Because sample introduction involves physical contact with the mass spectrometer inlet cone, the propensity for carryover is likely greater than with other techniques. Though minimal, contamination of the mass spectrometer inlet cone was observed; this was easily resolved by simply cleaning the surface of the inlet cone with a Kimwipe and methanol, and verifying its elimination prior to the next sample introduction.

Paper spray ionization, another relatively recent atmospheric pressure ionization technique which produces ESI-like spectra, was also investigated. Unlike inlet ionization, paper spray ionization utilizes a high voltage to induce charging of the liquid sample which results in ionization of target analytes. Since a high voltage is utilized, ionization efficiency is thought to be much higher than that of inlet ionization. For these reasons, it is hypothesized that analytes that prove difficult to ionize via inlet ionization may be ionized and analyzed via paper spray ionization.

Early paper spray studies were conducted utilizing a paper spray ionization source that was constructed in house by soldering a smooth copper-plated terminal clip to a length of insulated, high-voltage hook-up wire. The free end of the wire was soldered to a plug connector that was paired with the needle voltage port of the AccuTOF mass spectrometer. A Fisherbrand lab-jack was used to support and position the clip-end of the apparatus near the inlet of the mass spectrometer. 3000 V were applied to the filter paper utilizing the needle voltage power supply of the mass spectrometer during analysis. In collaboration with Dr. Robert Cody, JEOL USA designed and produced a commercial paper spray source for specific use with the AccuTOF mass

spectrometer. Later paper spray studies were carried out utilizing this new, commercially-available ionization source from JEOL USA and marketed as FilterSpray™.

Software/Data Analysis

Molecular masses of peptides were calculated manually using Microsoft Excel or automatically using MagTran, an automated charge state deconvolution program [1]. Peptide digestion data was processed using MagTran and Mass Mountaineer (RBC Software). Spectra of digested peptides were transformed to the $[M+H]^+$ domain and then converted to a centroided spectrum. To perform peptide mass fingerprint searches, the spectral data was exported as a text file and uploaded to MASCOT, an online search engine that utilizes mass spectrometry data to identify proteins from various databases based on primary sequence [2] to perform a peptide mass fingerprint search.

ES.3 Results

Because ESI-MS is commonly utilized to characterize peptides and proteins, the ESI source supplied with the AccuTOF mass spectrometer was used to collect reference spectra of various peptides prior to attempting other ionization methods. This process allowed for optimization of the mass spectrometer settings for the analysis of large molecules. This was necessary as the system is typically used for the analysis of relatively small molecules with masses less than 450 Da. Unfortunately, in order to utilize the ESI source, other ion sources typically need to be removed from the instrument before the ESI source can be mounted in place. Furthermore, ESI sources consist of tubing used to introduce a liquid sample which needs to be thoroughly flushed between samples to avoid carryover. When utilizing inlet ionization, there is no need to remove ion sources from the instrument and fewer parts are at risk of becoming contaminated during repeated analyses.

Early inlet ionization experiments were performed utilizing insulin (~5.8 kDa) and ubiquitin (~8.5 kDa). Both peptides were analyzed without the use of a matrix compound via inlet ionization, though low signal intensities were observed. When the peptides were reanalyzed incorporating the matrix compound, signal intensities increased significantly, roughly forty times for the analysis of insulin. Initially, no peptide-like signals were observed when attempting to analyze rHGH with this method. Further investigation revealed that not enough matrix compound was being utilized to facilitate efficient ionization. It later became common practice to simply saturate the solvated peptide sample with 3-NBN. When introducing the sample to the mass spectrometer inlet, solid matrix compound would be introduced with the solvated peptide. This resulted in very high intensity rHGH spectra. The experimental mass was calculated to be 22,124.8 Da, differing by less than one mass unit from the accepted value of 22,124 Da. In the same manner, the molecular weights of other peptides were determined with accuracy within 3 Da (typically less than 100 ppm) of the expected masses (Appendix A). Higher error in mass accuracy could be related to mass calibration of the spectra or unknown structural modifications of the peptides themselves. Nevertheless, the discrepancies are in the regime of a few protons, not full amino acids. Extensive validation of the method would likely increase mass accuracy.

Further peptide characterization was performed by analysis of peptide fragments produced through enzymatic digestion with trypsin. Data acquired for digested bovine serum albumin (BSA)

was processed and searched against MASCOT. In this case, MASCOT correctly identified the sample based on the digest data with a score of 96; anything over 70 is a significant match. Sequence coverage was found to be 33%, which is comparable to that of many reported MALDI-TOF methods of analysis. Utilizing a predigested BSA standard, Dr. Cody was able to obtain comparable results when performed at his laboratory in Peabody, Massachusetts. Other peptides were also digested and analyzed utilizing the same procedure. Myoglobin was correctly identified with a score of 70 with a 51% sequence coverage. Though not entirely necessary for successful identification of some substances, purification of the crude digest product with C_{18} ZipTips prior to analysis appeared to significantly increase the signal intensities of the peptide fragments. This was especially true for digested BSA.

Data acquired for digested rHGH could not be directly identified by MASCOT; however, several amino acid fragments were directly observed in the rHGH digest spectrum. When comparing the collected spectrum to a published reference, nine amino acid fragments specific to rHGH were identified. It is likely MASCOT was unable to identify the peptide because the software used to transform the collected spectrum to the single-charge domain was unable to do so correctly due to the spectral quality of the data. Further studies will be needed to determine the reason for this inability.

Further evaluation of the developed method was accomplished by analyzing 16 peptide samples that were obtained from an internet-based vendor. All 16 peptides were analyzed via direct matrix-assisted inlet ionization to determine the apparent molecular masses. Analysis of 13 of these samples produced results that were consistent with the advertised product; in other words, the products appeared to be authentic. The remaining three peptides did not produce the expected result. Further investigation is needed to determine the reason for the discrepancies.

Preliminary studies were conducted to evaluate the feasibility of utilizing paper spray ionization for the analysis of peptides within a forensic setting. Though not as simple as inlet ionization, paper spray ionization also results in ESI-like spectra in a matter of seconds. This ionization technique is suggested for samples in which inlet ionization lacks the appropriate efficiency to produce quality spectra since paper spray ionization shares many mechanistic similarities to traditional ESI-MS, which is quite amenable to biomolecular analytes by allowing investigation in the solution-phase; this may include peptides of much larger mass or peptides that include structural properties that prevent efficient ionization. Due to the ongoing collaboration with Dr. Robert Cody, JEOL USA developed considerable interest in paper spray ionization and produced a commercially available paper spray ionization source for the AccuTOF mass spectrometer, and marketed it under the name FilterSpray™. Early studies showed analysis of peptides, including rHGH, was successful when utilizing this technology.

ES.4 Conclusions

The main goal of this project was to develop a method for the identification of recombinant human growth hormone that could be easily incorporated into typical forensic laboratory procedures, preferably utilizing instrumentation already present within the laboratory. Due to the large mass of peptides and proteins, time-of-flight mass spectrometers are often utilized for analysis due to the extensive mass range of the mass analyzer. DART ionization sources are often coupled with time-of-flight spectrometers; therefore, this type of instrumentation was the ideal platform to utilize for the analysis of rHGH in this setting.

Direct inlet ionization and matrix-assisted inlet ionization with a time-of-flight mass spectrometer were found to be extremely well-suited for the analysis of rHGH and other large biomolecules. Dissolving the peptide samples in solvent and adding 3-NBN was the extent of sample preparation prior to instrumental analysis of intact peptide samples. Furthermore, 3-NBN was not even needed to attain signals for peptide samples weighing less than 8 kDa, though signals were improved with the use of the matrix compound. Molecular weights of intact peptides were easily obtained by deconvolution of the resulting ESI-like spectra, either manually or through mass spectrometry software packages.

Though a molecular weight may give insight into the identity of an unknown peptide sample, it is hardly a definitive means to identification. For this reason, further characterization was conducted through the analysis of peptide fragments produced by enzymatic digestion of the unknown peptide sample. A simple procedure utilizing trypsin as the protease of choice was effective in systematically cleaving the intact peptide samples. Molecular masses of the peptide fragments were easily obtained through inlet ionization analysis and mass spectral deconvolution, essentially producing a chemical fingerprint of the analyte. This, along with the molecular weight determined from the analysis of the intact peptide, can lead to an identification of fairly high certainty.

Many forensic drug identification laboratories, especially those performing evidentiary analysis for law enforcement agencies, typically do not have procedures in place for the successful identification of peptides and large biomolecules. Such labs mainly rely on GC-MS for the identification of unknown samples. When peptide samples are submitted for analysis, typical laboratory procedures are followed, normally resulting in negative results since peptides are not volatilized in the GC-MS inlet. Results are typically reported as no compounds detected. This is mainly due to the samples not being suitable for analysis when performed utilizing typical procedures. No useful information is provided to the submitting agency. The procedure developed through this research would allow laboratories to successfully detect and characterize peptides and other large biomolecules, potentially resulting in an identification that would otherwise not be possible.

1. Introduction

1.1 Statement of the Problem

The presence of performance-enhancing peptides has become more pervasive, regardless of geographic location. In December 2005, the Drug Enforcement Administration (DEA) led a steroid enforcement action known as "Operation Raw Deal." They targeted traffickers who imported raw materials to manufacture anabolic steroids. 11.4 million steroid dosage units were seized, as well as 242 kilograms of raw steroid powder from 56 steroid preparation labs across the United States [3].

In 2008, one manila envelope labeled "HGH #54" was reportedly by the Oklahoma State Bureau of Investigation, Forensic Science Center [4]. The envelope contained 10 vials with a white substance; the lab was unable to identify the substance, however. In the same year, the DEA Mid-Atlantic laboratory received a submission of 20 clear glass vials alleged to be freeze-dried rHGH; they were only able to presumptively identify it [5]. Since 2011, the Harris County Institute of Forensic Sciences Drug Chemistry Laboratory, in Houston, Texas, has received multiple cases of suspected rHGH for analysis. However, analysis in those cases was not performed due to the lack of an available instrumental method.

Human growth hormone is an endogenous protein consisting of 191 amino acids, with a molar mass of approximately 22 kDa. Along with growth hormone releasing peptides (GHRPs), such as ipamorelin, CJC-1295 and rHGH active fragments, various types of peptides are reportedly abused as performance enhancing drugs in cycling, bodybuilding, and other sports. As the majority of these substances sold are not fully characterized, there is a potential for harmful physiological effects. Additionally, except in rare circumstances, these substances are neither prescribed, nor utilized under medical supervision. There is a lack of quality control as these substances are not produced in regulated environments. There are reported instances of commensal skin microorganisms in unregulated peptide samples which pose additional health concerns [6].

This problem has continued in recent years, as the internet has become a convenient portal to purchase them, often internationally, at prices affordable to the public. Several analytical methods have been employed to detect the presence of rHGH in athletes [7-8]; however, these methods involve specialized equipment not generally available in controlled substance identification laboratories and not directly applicable for detection in bulk material.

Direct Analysis in Real Time-Time of Flight (DART-TOF) mass spectrometry has become more common in forensic laboratories. These units operate similarly to MALDI-TOF, in that both utilize soft ionization techniques and TOF mass analyzers. Unlike MALDI, DART typically ionizes only small or medium-sized molecules [9-20]; this has prevented its application to large biomolecules. However, inlet ionization is an emerging technique which generates multiple-charged ions and brings large biological molecules within the analyzable range. This technique can be utilized on a TOF with a slight modification. Inlet ionization may bring bulk protein analysis within reach of forensic laboratories. In addition, literature review indicated that inlet ionization on protein/peptide is highly sensitive, and preliminary data in our lab demonstrated successful measurement of intact protein using this technology. Following intact protein analysis, we proposed to conduct enzymatic digestion with the resulting fragments profiled utilizing inlet ionization.

We also proposed to investigate paper spray ionization (PSI) for the analysis of proteins and peptides. PSI is an ambient ionization technique that provides the advantage of combining

extraction and ionization in one step. A piece of filter paper can be used to collect the sample to which appropriate solvent is applied. A direct current is applied through a metal clip causing the analytes to be ionized as a spray is directed from the pointed tip of the filter paper towards the mass spectrometer inlet. When compared to electrospray ionization (ESI), the ionization mechanism involved with PSI is expected to be nearly identical [21]. Matrix-assisted inlet ionization and paper spray ionization are proposed to be a reliable and cost-effective means for a laboratory already equipped with atmospheric pressure inlet TOF instrumentation to characterize performance enhancing peptides in forensic settings.

1.2 Relevant Literature Review

Human growth hormone (HGH) is an endogenous protein consisting of 191 amino acids, with a molar mass of approximately 22 kDa, that is secreted by the pituitary gland [22]. HGH stimulates height and tissue growth as well as the metabolism of minerals, proteins, carbohydrates, and lipids. The United States Food and Drug Administration (FDA) has approved the prescription and use of several different trade names of recombinant HGH for injection (i.e., Genotropin, Norditropin, Humatrope, and Omnitrope) with the nonproprietary name somatropin.

Recombinant HGH (rHGH) is legitimately prescribed for adults and children suffering from growth hormone deficiency and related conditions. Access to pharmaceutically-produced rHGH is highly controlled, is only prescribed for adults and children suffering from growth hormone deficiency, and is generally considered an expensive therapy. However, numerous websites market rHGH for sale inexpensively promoting the abuse of this substance among the general public. In addition to inherent health risks with rHGH abuse, a study by Graham, et al. [6] showed a large proportion of these "underground" performance-enhancing products contain microbiological contamination leaving users at risk of receiving infectious diseases. In addition to rHGH, many websites sell and promote the use of peptide-based HGH secretagogues, such as ipamorelin, that are growth hormone releasing peptides (GHRP-2, GHRP-6, CJC-1295, and others). The C-terminal fragment of HGH, referred to as HGH 176-191, is marketed by these websites as a weight loss-promoting substance. It is reasonable that controlled substance identification laboratories should have the ability to identify and report the contents of these types of substances regardless of the validity of these claims.

While not generally considered high-volume compared to cocaine and marijuana trafficking, steroids and HGH-related peptides have a substantial presence in the underground drug trade. In 2007, a joint effort between the U.S. Drug Enforcement Administration (DEA), the Food and Drug Administration (FDA), and the US Postal Inspection Service, dubbed *Operation Raw Deal*, targeted the global trade of illicit rHGH and other growth factors, as well as anabolic-androgenic steroids. The operation led to the dismantling of 56 underground steroid laboratories as well as the discovery that close to 11.4 million steroid and rHGH dosage units had originated in China [3]. Despite the successes of such operations, the black market presence of rHGH in the United States remains a cause for concern. In 2008, the DEA Mid-Atlantic laboratory reported receiving a submission of 10 clear glass vials alleged to be freeze-dried rHGH that they were only able to presumptively identify it. In 2011 alone, the Harris County Institute of Forensic Sciences Drug Chemistry Laboratory, in Houston, Texas, received multiple cases of suspected rHGH for analysis. With no validated instrumental method, analysis in these cases was declined.

Anecdotal information on “legal drug” and bodybuilding websites indicate that the abuse of rHGH, as well as designer peptides like ipamorelin [23], GHRP-6 [24], CJC-1295 [25], and the 176-191 fragment of HGH [26], continues to rise as “legal” alternatives to anabolic-androgenic steroids [27]. Identification of these substances in the forensic crime laboratory is needed, but this area has seldom been investigated because of limited instrumentation and unsuitable technologies.

Wisniewski, Rees and Chege published one forensically valid method of identifying rHGH in 2009 [28]. However, as written, the method is specific for rHGH, requiring adaptations for smaller peptides as well as other proteins. Additionally, this method requires a very high concentration of rHGH sample (several hundred micrograms per milliliter) for digestion and whole protein analysis. Obtaining such a large amount of rHGH as a certified reference material is extremely costly [29]. The method makes use of an ion trap LC-MS, an instrument that is seldom available in forensic controlled substance identification laboratories.

In contrast, peptide/protein analysis is routinely performed on complex samples like whole cell and biological tissues within the proteomics community. Mixtures are usually separated by gel electrophoresis or high-performance liquid chromatography (HPLC) [30]. After separation, protein characterization is typically achieved using two different approaches: top-down and bottom-up [31-35].

In the top-down approach, intact proteins are ionized directly using high-resolution mass spectrometry (MS), and are then dissociated by a MS/MS spectrometer such as Fourier-transform ion cyclotron resonance (FTICR)-MS, linear ion trap-Orbitrap and linear ion trap-FTICR-MS [31]. This approach covers an entire protein sequence, and post-translational modifications are better preserved [35]. However, wide application of this method has yet to be realized due to incomplete understanding of protein fragmentation patterns, lack of development of suitable MS activation methods/instrumentation for efficient MS/MS data acquisition, and a lack of suitable database search tools [36]. In addition, this instrumentation is generally not found in forensic laboratories.

In the bottom-up approach, the separated protein is first subjected to proteolytic digestion, most commonly, with trypsin. Trypsin has well-defined specificity and hydrolyzes only the peptide bonds in which the carbonyl group is followed either by an arginine (Arg) or lysine (Lys) residue, or when Lys or Arg are N-linked to aspartic acid (Asp). Trypsin digestion gives middle ranged peptides ideal for MS analysis [37].

After enzymatic digestion, the generated peptides can be analyzed by two methods. The most commonly used method is called peptide sequencing, or sequence tag [38-39]. The peptides are subjected to collision-induced dissociation (CID) in an MS/MS system, and the resulting amino acid-specific fragments can be used to derive amino acid sequences of the peptides, leading to the identification of proteins via a database search. The typical MS/MS system used in this method includes 3D and linear ion trap [40-41] quadrupole-TOF [42] Orbitrap [43], FTICR-MS [33] and other hybrid MS/MS spectrometers (linear ion trap-Orbitrap [44], linear ion trap-FTICR-MS [45]). Again, these cost-prohibitive instruments are seldom found in the forensic setting.

The other method is referred to as peptide mass fingerprinting (PMF) [46-52]. It measures the mass values of the peptides resulting from enzymatic digestion, and then compares them to a database with predicted mass values based on a theoretical digestion. The most commonly used instrument for PMF is Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry, or MALDI-TOF [53-54]. MALDI is a soft ionization technique where the protein/peptide samples are first co-crystallized with a small organic matrix, which usually has a conjugated aromatic ring structure and can absorb at the wavelength of the laser to become ionized [55-56]. The ionized matrix molecules in turn ionize the large target molecules to predominately

produce singly-charged quasi-molecular ions, which can be detected with the time-of-flight mass analyzer (TOF). MALDI-TOF is an excellent tool to measure large, fragile biomolecules. However, as most target analytes in a forensic chemistry laboratory are substantially smaller, generally 400 m/z or less, the likelihood of MALDI-TOF instrumentation being utilized in this setting is low.

Direct Analysis in Real Time – Time of Flight mass spectrometry (DART-TOF) results in data similar to that of MALDI-TOF and has found increasing applications in the forensic science community. In the United States, more than a dozen of DART-TOF instruments are in operation in the forensic community, and the number is growing. The proposed mechanism for DART is to ionize molecules by metastable helium [57]. Unlike MALDI, DART can only ionize relatively small molecules ($m/z < 1500$) while protein and peptide analysis well exceeds that range, if singly charged. Fortunately, the TOF mass spectrometer can be utilized with other ionization techniques as it is not limited to only DART ionization.

An emerging technology, called "inlet ionization," may be used to address this problem. Inlet ionization can produce multiply-charged ions, bringing large biological molecules within a detectable range. In addition, it allows total transfer of ionized species and is highly sensitive. Most importantly, a DART-TOF system can be easily converted for inlet ionization, making it an excellent choice for protein characterization in a forensic setting, at little cost. Moreover, the techniques discussed herein should also be amenable to any mass spectrometric system incorporating an atmospheric pressure inlet and mass analyzer of appropriate mass range, further increasing the number of analytical laboratories that could incorporate such techniques with existing equipment.

The first inlet ionization method, laserspray ionization (LSI), was introduced by Trimpin et al. in 2010 [58-59]. The mechanism is thought to be similar to MALDI, but the ions produced are multiply charged, and the ionization is believed to occur from laser ablation of a matrix/analyte mixture, inside the ion transfer capillary before entering the first vacuum stage [59]. Later, McEwen et al [60] found that a laser is not required in the ionization process, and the matrix can be a solvent such as water, acetonitrile, methanol, and mixtures thereof [61]. This solvent assisted inlet ionization (SAII) introduces the analyte solution directly into the heated inlet, without using laser or voltage, promising simple ionization with improved sensitivity compared to ESI [61]. In addition, the matrix can be used to help the ionization without a heated inlet [62].

In ESI, even with the nanospray technology, 80% to 90% of the produced ions were estimated to be lost in the transfer from atmospheric pressure to the first vacuum region [63-64]. This ion loss is prevented in SAII, resulting in increased sensitivity for small molecules and a variety of peptides and proteins [65]. SAII can be achieved by simply inserting one end of a fused silica column (with coating removed) into the ionization inlet, and the other end to the sample in solution. The vacuum in the mass analyzer will generate a flow rate, which is influenced by the internal diameter and length of the fused silica, temperature, and the distance it protrudes inside the inlet. Alternatively, a syringe pump could be utilized to control the flow rate [66], or a conventional HPLC system can be interfaced with the fused silica [67]. Since this technique is still in development, it can be easily adapted into the DART-TOF system.

Another emerging ambient ionization technique is paper spray ionization. This technique has been applied in the analysis of biofluid and explosive samples [68-72]. A triangular paper with a sharp tip is used as the substrate. The sample in solution is loaded onto the substrate and the electrospray is produced by applying a high voltage to the paper. An advantage of PSI is that the paper substrate can effectively remove interfering components such as inorganic salts and other

excipients that do not dissolve in the spray solvent [73]. In addition, PSI combines extraction and ionization in one step which results in shorter analysis times [70].

1.3 Rationale for the Research

Currently, detection and identification of rHGH and other performance enhancing peptides is a difficult task in forensic laboratories. Although protein analysis is routinely performed on complex samples in the proteomics community, the cost-prohibitive instruments are seldom in a forensic setting. The proposed method could provide a sensitive and forensic-friendly protocol that can be utilized by forensic laboratories by measuring the exact mass of intact proteins via inlet ionization without the need for a specialty ionization source. Though the acquisition of a high-resolution mass spectrometer solely for this purpose is likely not practical for most forensic laboratories, this proposed method presents a means for a value-added analytical service beyond the typical mass range employed for laboratories already equipped with suitable instrumentation. The high molar masses of proteins typically require the use of a time-of-flight mass spectrometer because of its extensive mass range; however, the range is typically still limited to around 2500 m/z. Inlet ionization can produce ESI-like spectra consisting of various multiply charged species of the same intact peptide, which are observable within the mass range of the analyzer. Molecular weights of intact peptides can then be calculated from the various charge states observed.

An enzymatic digestion of these peptides can be performed and the resulting peptide fragments can be profiled. Using an online database, the peptides can be compared to a library and be successfully characterized. Finally, both intact and digested peptides can be analyzed and characterized using paper spray ionization. Once the project is complete, we expect the forensic science community to have a standardized, cost-effective method to analyze and characterize various protein and peptide samples.

2. Methods

Materials and Reagents

Acetonitrile, bovine serum albumin, and 3-nitrobenzonitrile (3-NBN) were purchased from Sigma-Aldrich (St. Louis, MO). Formic acid and C_{18} ZipTips were purchased from EMD Millipore (Billerica, MA). Trypsin was purchased from MP Biomedicals (Santa Ana, CA). Filter paper used for paper spray was purchased from Whatman (Maidstone, England). High voltage (5 kV) hook up wire was acquired from Alpha Wire (Elizabeth, NJ) and copper plated solder terminals from RadioShack (Forth Worth, TX). For inlet ionization sample introduction, 5µLWiretrol micropipettes were purchased from Drummond Scientific (Broomall, PA). Water used for solvents was purified using a Milli-Q water purification system (EMD Millipore, Billerica, MA). Urea, dithiothreitol, and a Coomassie (Bradford) protein assay kit were purchased from Thermo Scientific (Waltham, MA), ammonium bicarbonate from Acros (Geel, Belgium), and 2-iodoacetamide from Tokyo Chemical Industry (Toshima, Kita-ku, Tokyo, Japan).

An array of peptides samples was purchased from Peptide Sciences (www.peptidesciences.com). An attempt was made to purchase similar peptides from American Science Labs (www.americansciencelabs.com); however, the seller refused to fulfill the order.

Unknown samples that were suspected of being peptides were also acquired from various evidentiary submissions received by the Harris County Institute of Forensic Sciences.

Protein/Peptide Screening

A Bradford protein assay solution was used to perform a simple color test on suspected peptide samples. The premixed solution contained the dye Coomassie G-250 which initially exhibits a brownish-green color. Upon interaction with peptides or proteins, the absorbance of the dye shifts and the color becomes a royal blue. To perform the test, approximately 1 mL of Coomassie solution was placed in a test tube or microcentrifuge tube. A small amount of the lyophilized peptide was then added directly to the solution and allowed to incubate for approximately 10 minutes before checking visually for color change.

Sample Preparation

Lyophilized peptide samples were dissolved in 1:1 acetonitrile:water with 0.1% formic acid (v/v) to a target concentration of 1 mg/mL. Formic acid was added to the solvent to aid with dissolution of the peptides and to facilitate protonation during mass spectral analysis. If necessary, the samples were gently heated to further solvation. The samples were analyzed utilizing paper spray ionization without further sample preparation. To analyze the samples via inlet ionization, 3-NBN was added to the dissolved samples just before analysis. Solid 3-NBN was added to the samples in excess until saturation of the matrix compound was noticeable and a small amount of material was visible at the bottom of the sample vial. The samples were analyzed via inlet ionization directly following the addition of the 3-NBN.

Instrumentation and Parameters

Analyses were carried out utilizing an atmospheric pressure ionization high-resolution time-of-flight AccuTOF JMS-T100LC mass spectrometer (JEOL USA, Inc., Peabody, MA, USA). The atmospheric pressure interface was operated at the following potentials: orifice 1 = 90 V, orifice 2 = 5 V, and ring lens = 10 V. The temperature of orifice 1 was set to 200° C to facilitate evaporation of the solvent during sample introduction. The RF ion guide voltage was set to 2500 V to allow detection of the large-mass ions associated with charged macromolecules. Data was acquired from 200 to 4500 m/z with an acquisition interval time of 0.5 ns and spectral recording interval of 0.3 sec. When paper spray ionization was performed, a voltage of 3000 V was applied to the filter paper utilizing the needle voltage power supply of the mass spectrometer. The mass spectrometer was mass calibrated using an Ultramark 1621 (Alfa Aesar, Ward Hill, MA) spectrum that was collected via DART ionization during each sample analysis.

Ionization Techniques

As ESI-MS is a common and accepted technique used to analyze large macromolecules such as peptides, the ESI source supplied with the AccuTOF instrument was used to collect initial reference spectra. The reference spectra were later used to evaluate the performance of the other ionization techniques discussed herein. Other techniques are being investigated due to the tedious steps required to analyze samples via ESI. One major concern with ESI is assuring that the tubing and piping are free of contaminants that may be present from previously analyzed samples. In addition, other ion sources typically need to be removed from the mass spectrometer in order to mount the ESI source, time and effort that can be avoided with techniques discuss herein.

For inlet ionization experiments, Wiretrol micropipettes were used to introduce 3-5 μL aliquots of liquid sample directly into the mass spectrometer inlet. Introduction of the sample was accomplished by simply placing the opening of the micropipette near the inlet opening. This oftentimes required physical contact of the micropipette and the inlet cone (see Figure 2-1). When this close proximity was achieved, the vacuum system of the mass spectrometer rapidly drew the sample into the inlet, initiating the ionization process. Because sample introduction involves physical contact with the mass spectrometer inlet cone, the propensity for carryover is likely greater than with other techniques. Though minimal, contamination of the mass spectrometer inlet cone was observed on occasion; this was easily resolved by sampling cleaning the surface of the inlet cone with a Kimwipe and methanol. To ensure the inlet cone was free of contamination, a solvent blank containing 3-NBN was analyzed. If residual analyte remained on the surface of the inlet cone, it would be readily ionized and detected, indicating further cleaning was needed.

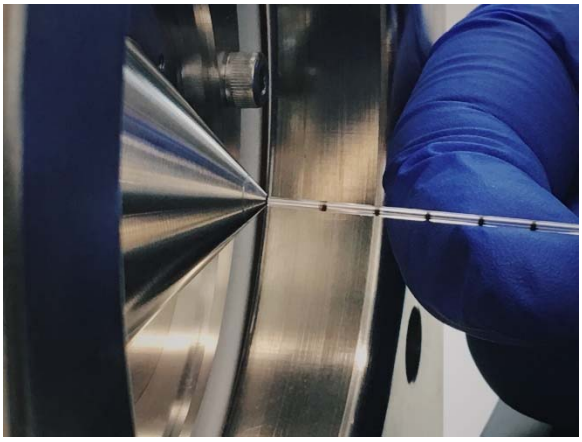


Figure 2-1. The photo above depicts a typical inlet ionization sample introduction. The vacuum system of the mass spectrometer draws the liquid sample into the inlet when the pipette in brought into close proximity of the inlet.

For early paper spray studies, an ionization source was constructed by soldering a smooth copper-plated terminal clip to a length of insulated, high-voltage hook up wire. The free end of the wire was then soldered to a plug connector that paired with the needle voltage port of the AccuTOF. A Fisherbrand lab-jack was used to support and position the clip-end of the apparatus near the inlet of the mass spectrometer. Later, paper spray studies were carried out utilizing a

commercially-available FilterSpray™ ionization source purchased from JEOL USA. No significant difference in performance was noted between these two devices.

A small triangular piece of filter paper was positioned in the electrode clip with one of the triangular points aimed at the mass spectrometer inlet. Because typical qualitative filter paper contains an abundance of salts, hardened ashless filter paper was utilized in order to minimize salt adduct formation. With the mass spectrometer set to acquire and voltage being supplied to the dry filter paper, a 5-10 µL aliquot of sample was applied to the filter paper with a disposable pipette. Upon wetting the filter paper, the electrical circuit between the terminal clip and the mass spectrometer inlet is closed, allowing current to flow. Due to the electrical potential difference, the charged liquid sample migrates through the filter paper towards the mass spectrometer inlet. As sample is expelled from the triangular tip of the filter paper, mechanisms similar to those observed with ESI take place as ionization occurs. Data is then collected as the resulting ions are sampled by the mass spectrometer. The filter paper was replaced and the terminal clip was cleaned after each analysis.

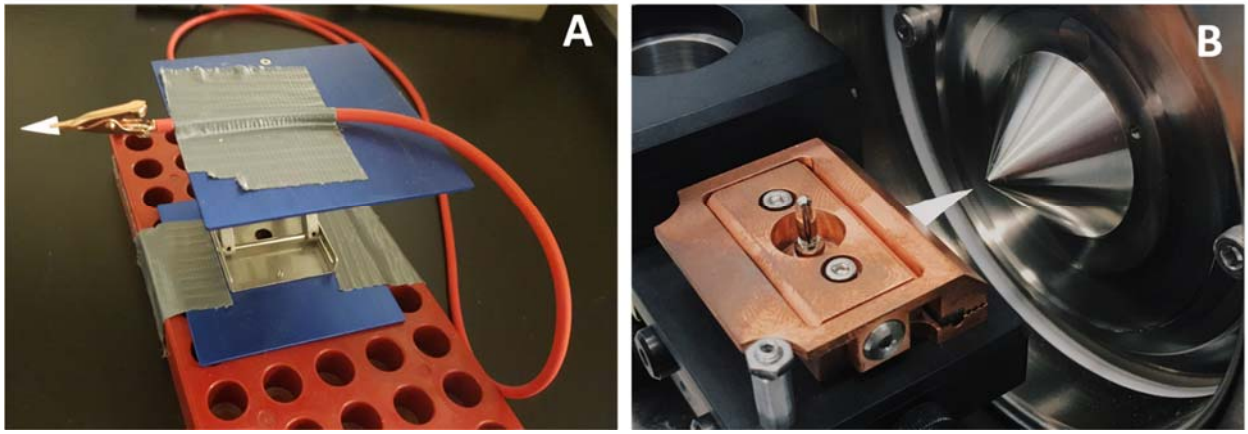


Figure 2-2. (A) The initial paper spray ion source that was built in-house and (B) the JEOL FilterSpray™ ionization source mounted to the interface of an AccuTOF mass spectrometer with filter paper triangle in position for analysis. A plastic safety housing is placed over the copper terminal of the FilterSpray™ source which also connects the voltage supply to the center pin.

Enzymatic Digestions

Further characterization was accomplished by enzymatically digesting peptide samples with trypsin utilizing an in-solution digestion protocol obtained from Scripps Center for Metabolomics and Mass Spectrometry [74]. This protocol involved reducing the peptide sample with dithiothreitol followed by alkylation with 2-iodoacetamide. Other alkylating agents, such as iodoacetic acid, could also be used, keeping in mind the cysteine modification will vary slightly depending on which alkylating agent is utilized. Though the protocol calls for overnight incubation after the addition of the trypsin, useable digest data was obtained after only one hour of incubation with no further sample preparation. Analysis of the digested samples was accomplished via direct inlet ionization with the addition of 3-NBN in the same manner as described earlier.

Because trypsin cleaves peptides after the amino acids lysine and arginine, or when either is followed by a proline, specific peptide fragments will be produced upon digestion. Typically, fragmentation of chemical species is accomplished by the instrument as a means of characterization or identification. Such fragmentation utilizing an AccuTOF mass spectrometer, or similar instrumentation, is not possible for large, stable molecules. To overcome this barrier, peptides can be digested enzymatically prior to instrumental analysis in order to obtain fragmentation information.

Data Analysis

Molecular masses of peptides were calculated manually using Microsoft Excel or automatically using MagTran, an automated charge state deconvolution program [1]. Peptide digestion data was processed using MagTran and Mass Mountaineer (RBC Software). Spectra of digested peptides were transformed to the $[M+H]^+$ domain and then converted to a centroided spectrum. The spectral data was then exported as a text file and uploaded to MASCOT to perform a peptide mass fingerprint search. MASCOT server is a free an online search engine that utilizes mass spectrometry data to identify proteins from various databases based on primary sequence [2].

3. Results

The procedure developed for this project is comprised of three distinct steps: **(i)** a presumptive color test to determine if an unknown sample could likely be a peptide/protein, **(ii)** analysis of the intact peptide to determine the molecular weight, and **(iii)** further characterization of the peptide via analysis after enzymatic digestion.

Presumptive Testing

Even though the analysis of the intact peptide via matrix assisted inlet ionization is a very rapid analytical technique, justification would likely be needed to perform such an analysis since it is substantially different from methods utilized for the analysis of a typical drug sample. A Coomassie protein assay solution was chosen as the preferred method for presumptive testing of suspected peptide samples. Typically used for staining proteins after electrophoretic separation in a polyacrylamide gel, a solution of Coomassie Brilliant Blue dye was found to be well-suited for use as a simple spot test for peptide samples.

A positive result for the presence of a peptide is indicated by the solution turning a brilliant royal blue color, hence the name of the compound. Common interfering substances, namely detergents, were also tested utilizing this assay to determine if false-positives would be a concern. When detergent was added to the Coomassie solution, an evident color change to turquoise was observed. The royal blue color observed with a positive result was easy distinguished from the turquoise negative result by visual means. If desired, the solution could be quantitated utilizing standard UV-Visible spectrophotometry and a series of standard concentrations of a known protein, typically bovine serum albumin in prepackaged assay kits. It should be noted that this method would only be an estimate of the concentration since the sample being quantitated would

need to react with the Coomassie Blue dye in the same manner as the standard protein being utilized for the assay. Different peptides would exhibit varying degrees of absorption depending on the exact interaction between the peptide and the dye. To perform a true quantitation, the standards used to produce the calibration curve would need to be the same peptide being quantitated. Nevertheless, a positive presumptive test would justify deviating from the standard analytical procedures in order to treat the unknown sample as a peptide.

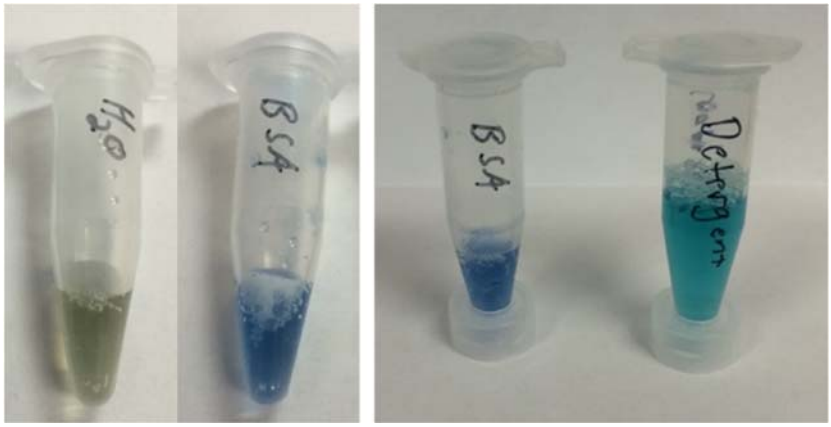


Figure 3-1. The photo on the left shows the color change of the Coomassie assay solution from the initial green to royal blue upon addition of solid bovine serum albumin. The figure on the right shows the distinct difference between the royal blue color of a positive spot test and the turquoise color that results from reaction with an interfering detergent.

Matrix-Assisted Inlet Ionization

After positive presumptive testing, analysis was performed to determine the molecular weight of the unknown peptide. This was found to be easily accomplished via direct inlet ionization. Depending on the mass of the peptide, a matrix compound may be necessary to facilitate successful ionization of the peptide. Early experiments were performed utilizing the small proteins insulin (~5.8 kDa) and ubiquitin (~8.5 kDa). Both peptides were analyzed without the use of a matrix compound via inlet ionization, though low signal intensities were observed. When the peptides were reanalyzed incorporating the matrix compound, signal intensities increased significantly, roughly forty times for the analysis of insulin. Once repeatability of the analysis was achieved for these two peptides, attempts were made to analyze higher mass peptides utilizing the same procedure.

Initially, only small amounts of the matrix compound 3-NBN were used in a variety of prepared samples. However, many of these tests resulted in no peptide-like signals observed. At that point, we believed we needed to modify the instrument parameters to successfully transport the generated ions through the mass spectrometer; however, it was possible that the ions were simply not being generated. To determine the source of the issue, analysis was performed by

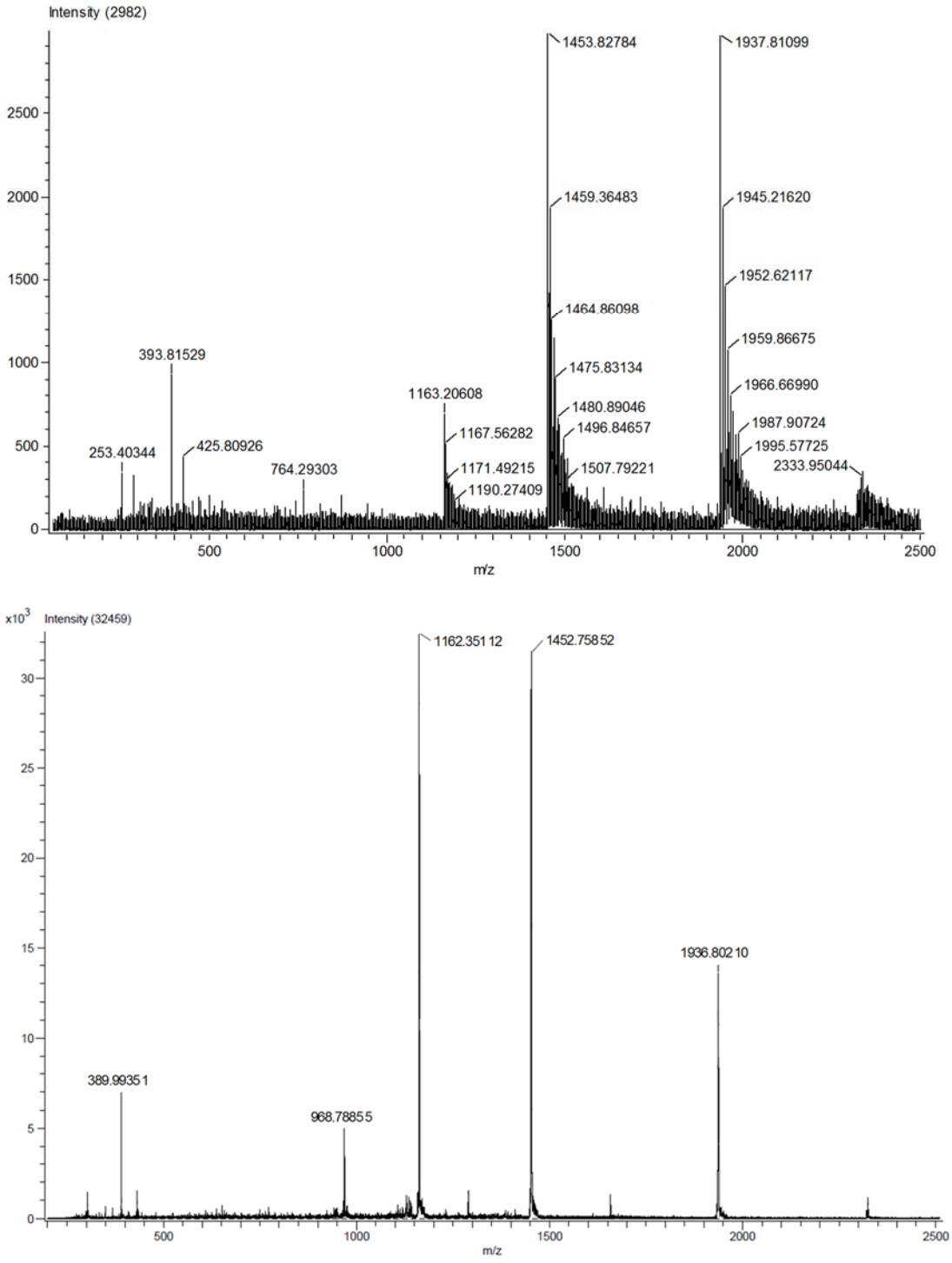


Figure 3-2. The top spectrum depicts the signal obtained from analysis of insulin (~345 µM) without the addition of 3-NBN while the bottom spectrum (~100 µM) illustrates the noticeable increase in signal intensity when 3-NBN is utilized.

adding additional matrix compound. Saturating the solvated peptide sample with 3-NBN was found to provide the best quality spectra, specifically when sample was introduced to the mass spectrometer inlet with a visible amount of solid 3-NBN included.

Due to the high price of human growth hormone standard, the enzyme trypsin was initially investigated as a surrogate protein target due to the similarity in molecular mass (~23 kDa) and low cost. Similar to other samples, trypsin was detected via inlet ionization with 3-NBN added, but with very low and unreliable signal intensities. When the amount of 3-NBN was increased to the point of saturating the solution, significantly higher signal intensities were achieved for a trypsin sample. Thus, the combination of instrument parameters and the amount of matrix compound used markedly increased the quality of the obtained spectra. With the successful detection of the trypsin standard, we demonstrated that peptides with similar mass to rHGH could be ionized via matrix-assisted inlet ionization and the generated ions were successfully navigating the mass spectrometer to the detector. As with ESI mass spectra of peptides and proteins, spectra obtained utilizing inlet ionization produced a series of peaks indicative of various charge states of the same intact peptide. The molecular weight of the peptide was easily determined through deconvolution of the spectra after mass calibration with Ultramark 1621. Deconvolution is possible either manually or through automated software packages. In the case of rHGH, the experimental mass was calculated to be 22,124.8 Da, differing by less than one mass unit from the accepted value of 22,124 Da. (It should be noted that most literature reports the mass of peptides to the nearest whole number.) In the same manner, myoglobin and insulin standards were analyzed resulting in experimental weights of 16,951 Da and 5,808 Da, respectively. In both cases, the experimental values differed from the accepted values by, at most, one mass unit, with accepted values being 16,951 Da and 5,807 Da, respectively.

Preliminary limits of detection for rHGH, myoglobin, and insulin were determined by serial dilution and inlet ionization analysis. All of these peptides could be detected at levels as low as 1 μM (approximately 6-22 μg/mL depending on the specific peptide). Further investigation would be needed to determine more definitive limits of detection for this system. Though the detection limits are high when compared to other ambient ionization techniques with similar instrumentation, instrument contamination was of minimal concern as it was easily resolved when necessary. Nevertheless, these limits of detection are much lower than any method of sampling performed in a typical drug analysis laboratory.

Enzymatic Digestion and Analysis

Initially, bovine serum albumin (BSA) was utilized to evaluate the feasibility of further characterization through enzymatic digestion of intact peptides. BSA was chosen due to the large molecular weight (~65 kDa) and low cost. The trypsin digestion procedure utilized recommended an overnight incubation period at 37 °C. Surprisingly, useable digestion data was obtained via inlet ionization with as little as an hour of incubation time at room temperature.

After digestion of BSA, the crude liquid was analyzed via matrix-assisted inlet ionization without further sample preparation in the same manner as with intact peptides. The raw data for the digested BSA sample was processed and then searched against the MASCOT Peptide Mass Fingerprint online service. When the appropriate parameters were set, MASCOT correctly identified the digested peptide as BSA with a statistical score of 96; anything over 70 being a significant match. Analysis of the digested BSA resulted in an overall sequence coverage of 33%.

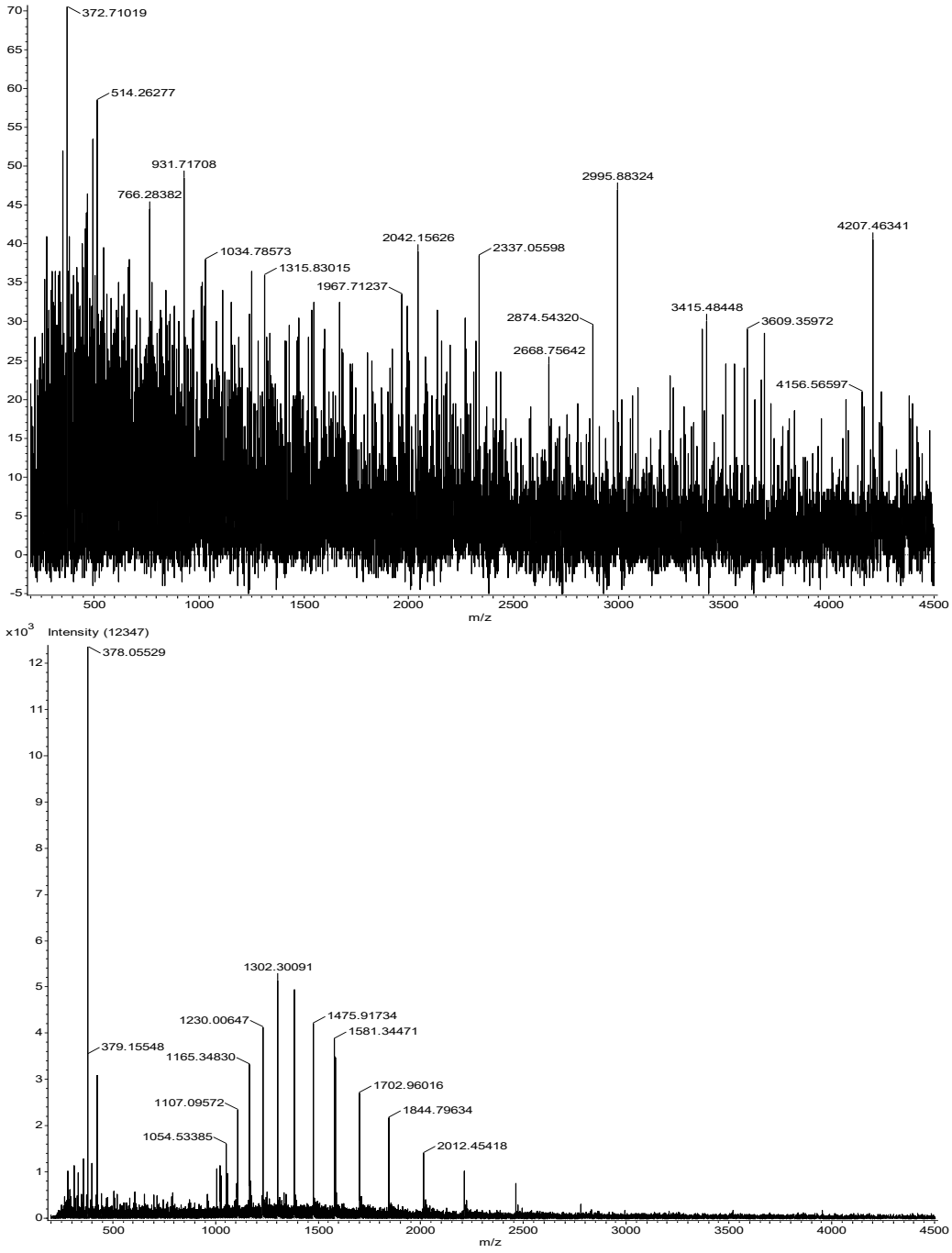


Figure 3-3. The top spectrum depicts the lack of signal obtained from analysis of rHGH without the addition of 3-NBN. The bottom spectrum depicts the ESI-like spectra obtained for the same rHGH sample when reanalyzed utilizing a saturated amount of 3-NBN.

This level of sequence coverage is comparable to that of many reported MALDI-TOF methods of analysis, considered by many to be the gold standard of mass spectral proteomic analysis. This success demonstrated the ease of use and utility of this method of analysis.

To evaluate if sample clean-up and desalting improved the quality of data for digested peptide samples, C_{18} ZipTip purification was performed per the manufacturer instructions after digestion of the peptide sample. The ZipTips were used to remove the excess urea and other non-peptide species prior to direct analysis via inlet ionization analysis. Purification utilizing ZipTips is similar to that of HPLC in that the selective elution of analytes from the C_{18} resin can be controlled with appropriate solvent selection. The excess species that remain after the digestion procedure were thought to skew or complicate the resulting mass spectra if not removed prior to analysis. The signal intensity for the BSA digestion product was greatly improved by using C_{18} ZipTip purification. MASCOT identified the peptide as being BSA with a score of 70 and 36% sequence coverage. Though not entirely necessary, purification prior to analysis would most likely be preferred if incorporating digestion into a working laboratory procedure.

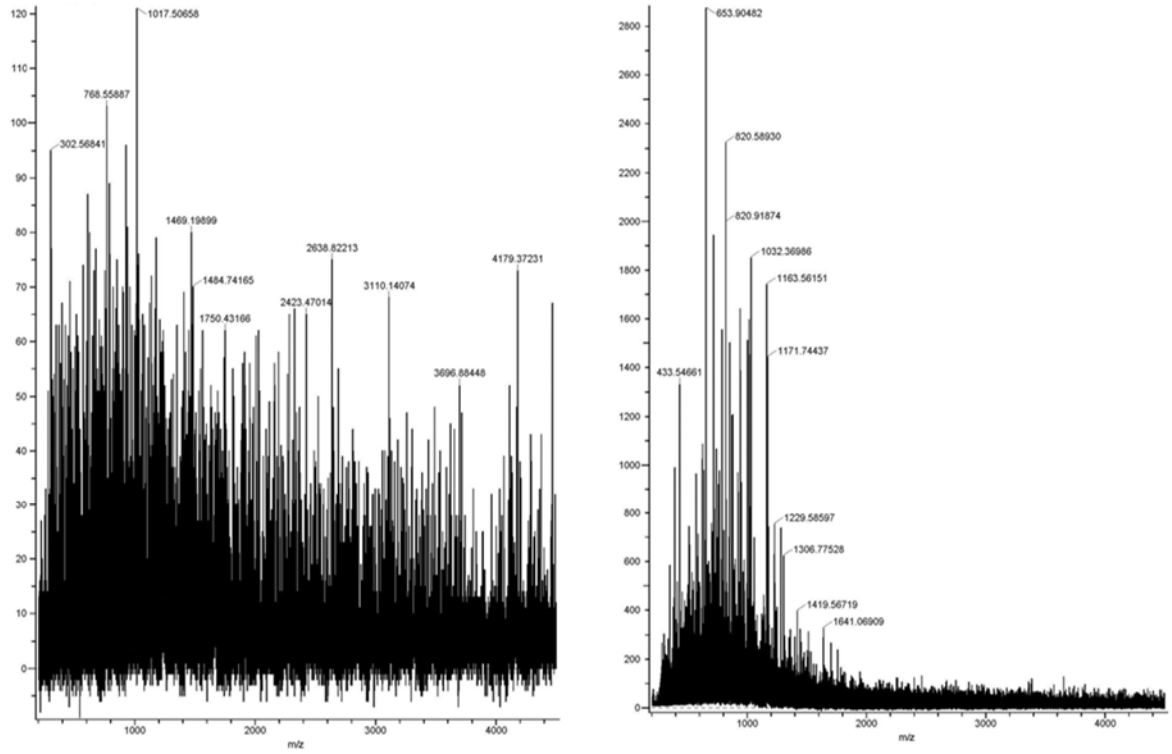
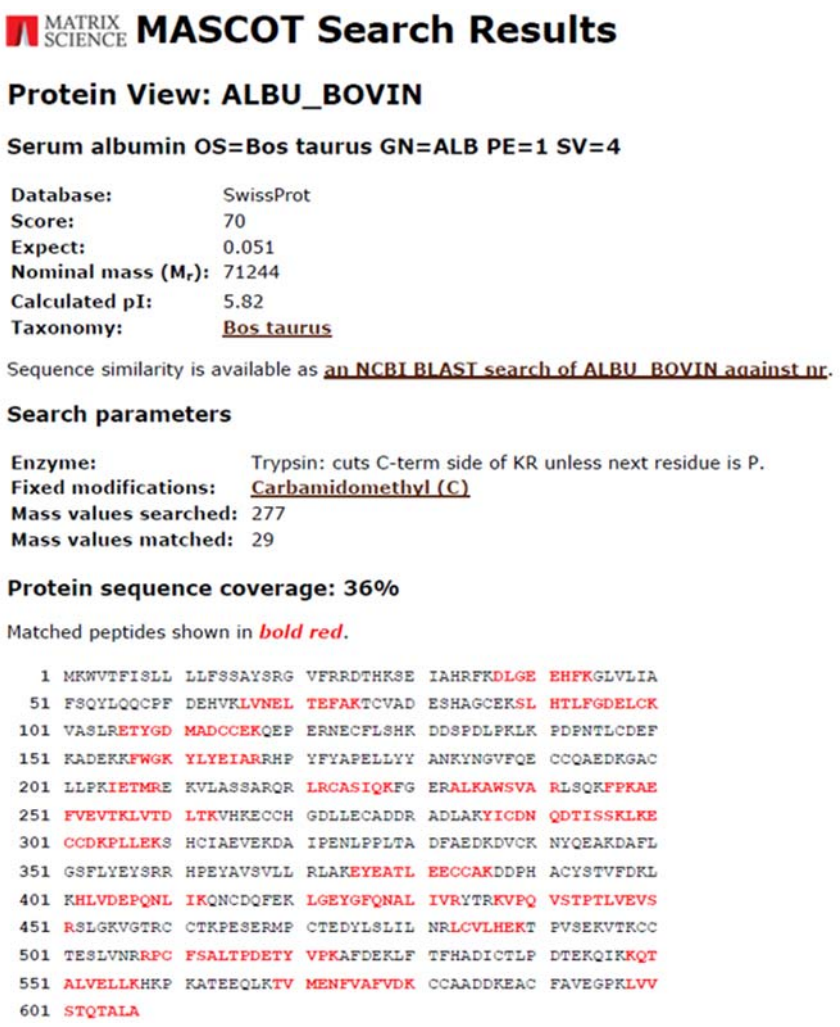


Figure 3-4. The spectrum on the left displays the results of analyzing a BSA digestion product immediately following the completion of the digestion protocol. In the spectrum on the right, the BSA digestion product was treated using a C_{18} ZipTip prior to mass spectral analysis. Though still a weak signal, a multitude of ions can be distinguished from the baseline after C_{18} ZipTip treatment.

Myoglobin was also digested to determine the robustness of this method. It was found that myoglobin is relatively resistant to trypsin digestion. In order to successfully digest the intact peptide, it first had to be denatured. This was accomplished by heating the peptide in a heat block at 95 °C for 30 minutes prior to performing the digestion procedure. With denaturing, MASCOT correctly identified myoglobin with a score of 70 and 51% sequence coverage.

Data acquired for digested rHGH could not be directly identified by MASCOT; however, several amino acid fragments were observed in the rHGH digest spectrum. When comparing the



MATRIX SCIENCE **MASCOT Search Results**

Protein View: ALBU_BOVIN

Serum albumin OS=Bos taurus GN=ALB PE=1 SV=4

Database:	SwissProt
Score:	70
Expect:	0.051
Nominal mass (M_r):	71244
Calculated pI:	5.82
Taxonomy:	Bos taurus

Sequence similarity is available as an NCBI BLAST search of ALBU_BOVIN against nr.

Search parameters

Enzyme:	Trypsin: cuts C-term side of KR unless next residue is P.
Fixed modifications:	Carbamidomethyl (C)
Mass values searched:	277
Mass values matched:	29

Protein sequence coverage: 36%

Matched peptides shown in ***bold red***.

```
  1 MKWVTFISLL LLFSSAYSRG VFRRDTHKSE IAHRFKDLGE EHFKGLVLIA
 51 FSQYLQQCPF DEHVKLVNEL TEFAKTCVAD ESHAGCEKSL HTLFGDELCK
101 VASLRETYGD MADCCEKQEP ERNECFLSHK DDSPDLPKLK PDPNTLCDEF
151 KADEKKFWGK YLYEIARRHP YFYAPELLYY ANKYNGVFQE CCQAEDKGAC
201 LLPKIETMRE KVLASSARQR LRCASIQKFG ERALKAWSVA RLSQKFPKAE
251 FVEVTKLVTD LTKVHKECCH GDLLECADDR ADLAKYICDN QDTISSKLKE
301 CCDKPLLEKS HCIAEVEKDA IPENLPPLTA DFAEDKDVCK NYQEAKDAFL
351 GSFLYEYSRR HPEYAVSVLL RLAKEYEATL EECCAKDDPH ACYSTVFDKL
401 KHLVDEPQNL IKQNCDQFEK LGEYGFQNAL IVRYTRKVPQ VSTPTLVEVS
451 RSLGKVGTRC CTKPESERMP CTEDYLSLIL NRLCVLHEKT PVSEKVTKCC
501 TESLVNRRPC FSALTPDETY VPKAFDEKLF TFHADICTLP DTEKQIKKQT
551 ALVELLKHKP KATEEQLKTV MENFVAFVDK CCAADDKEAC FAVEGPKLVV
601 STQTALA
```

Figure 3-5. Results obtained from MASCOT for the mass spectral results of a bovine serum albumin digest product analyzed via matrix-assisted inlet ionization.

collected spectrum to a published reference [75], eight amino acid fragments specific to rHGH were identified out of a total 12. It should be noted that one additional detected fragment was not mentioned in the reference but is predicted based on the amino acid sequence. Incomplete reduction or alkylation of the intact rHGH prior to digestion could account for the fragment not

being detected in the reference spectrum. Further research will be needed to determine the reason the fragment was detected in this study but not in the reference spectrum. Nevertheless, it is likely MASCOT was unable to identify the peptide because the software used to transform the collected spectrum to the single-charge domain was unable to do so correctly due to the spectral quality of the data. Further studies will be needed to determine the reason.

Table 3-1. Observed rHGH Fragments After Tryptic Digestion

Start-End AA Position	Observed $[M+H]^+$	Experimental M (Da)	Expected M (Da)	ΔM (Da)	Peptide Fragment
135-140	693.36	692.35	692.38	0.03	R.TGQIFK
173-178	764.56	763.55	763.41	-0.14	K.VETFLR
71-77	844.41	843.40	843.47	0.07	K.SNLELLR
1-8	930.41	929.40	929.52	0.12	<FPTIPLSR
9-16	979.31	978.30	978.49	0.19	R.LFDNAMLR
169-178	1253.41	1252.40	1252.60	0.20	K.DMDKVETFLR
116-127	1359.51	1358.50	1360.66	2.16	K.DLEEGIQTLMGR
146-158	1486.66	1485.65	1488.67	3.02	K.FDTNSHNDDALLK
20-38	2342.86	2341.85	2341.12	-0.73	R.LHQLAFDTYQEFEEAYIPK

It should also be noted that in-solution digestion typically involves stepwise reduction and alkylation steps prior to digestion utilizing trypsin. These steps convert cysteine residues of the peptide to the stable S-carboxyamidomethylcysteine (CAM) adduct. Doing so breaks potential disulfide bonds and prevents such bonds from reforming. If no cysteine residues are present in the peptide structure, this step can be omitted. Unfortunately, this procedure most likely would be performed on an unknown sample in which no structural information is known. Even if no cysteine residues are present, the reduction and alkylation steps do not appear to have any significant impact on the digestion outcome. To test this procedure, an rHGH sample was subjected to reduction and alkylation, omitting the digestion step with trypsin. When this sample was analyzed, a strong signal for the rHGH-CAM adduct was obtained. The experimental molecular weight of 22,356.8 Da was very similar to the theoretical value 22,356.11 Da; therefore, we concluded the reduction and alkylation steps of the digestion protocol were successful.

Authenticity of Peptides Purchased Online

Further evaluation of the developed method was accomplished by analyzing 16 peptide samples that were obtained from an internet-based vendor. All 16 peptides were analyzed via direct

matrix-assisted inlet ionization to determine the apparent molecular masses (See Appendix A). Analysis of 13 of these samples produced results that were consistent with the advertised product; in other words, the products appeared to be authentic. The remaining three peptides did not produce the expected result. One likely possibility for the discrepancies is that the vials did not contain the compound that was listed on the labeling. The mass obtained for CJC-1295 DAC was indicative of CJC-1295 without DAC, also known as Mod GRF (1-29). It is possible that the DAC group (Drug Affinity Complex) was cleaved from the main peptide structure during analysis; however, due to the soft ionization nature of this method, this is unlikely. These peptides could be purchased from additional sources to determine if results are consistent. Further investigation is needed to determine the specific reason for the discrepancies.

Analysis of Unknown Case Samples

In 2011, evidence was submitted to the Harris County Institute of Forensic Sciences which contained a multitude of suspected steroids and 28 vials labeled as "Jintropin Somatropin." No analysis was performed at the time due to lack of suitable instrumentation for the analysis of peptides and proteins. The court case was disposed without requiring analysis of these samples, which were subsequently donated to the laboratory for research purposes. After successfully utilizing matrix-assisted inlet ionization mass spectrometry for characterization of various peptide standards during this project, the technique was used in an attempt to identify the contents of the 28 vials.

Analysis of one vial did not produce mass spectra typical of rHGH. In fact, the spectra contained only two distinct peaks. It was initially believed that a contaminant was present that was masking the characteristic signal for rHGH or another larger peptide. However, after analysis of the data, we concluded that the two peaks were the singly and doubly charged molecular ions of one distinct analyte. Using the mass-to-charge ratio of the singly charged molecular ion, the isotopic molar mass of the unknown peptide was found to be 1023.53577 Da, which is consistent with Melanotan II. Melanotan II is available for purchase from online marketplaces; this supports the possible identification.

Another vial from the same submission was analyzed and produced very different results, though all 28 vials within the submission appeared identical. The spectrum for the second vial was not as clean as the first; however, one peak was much more prominent. A molecular weight of 872.46825 Da was calculated by using the prominent, singly charged ion. Compared to the peptides that were purchased online, the calculated mass led to a possible identification of GHRP-6. All other vials tested from this submission, as well as other later submissions, have indicated Melanotan II or GHRP-6. To date, no sample submitted to the Harris County Institute of Forensic Sciences has been identified as rHGH, the compound indicated on most of the labeling.

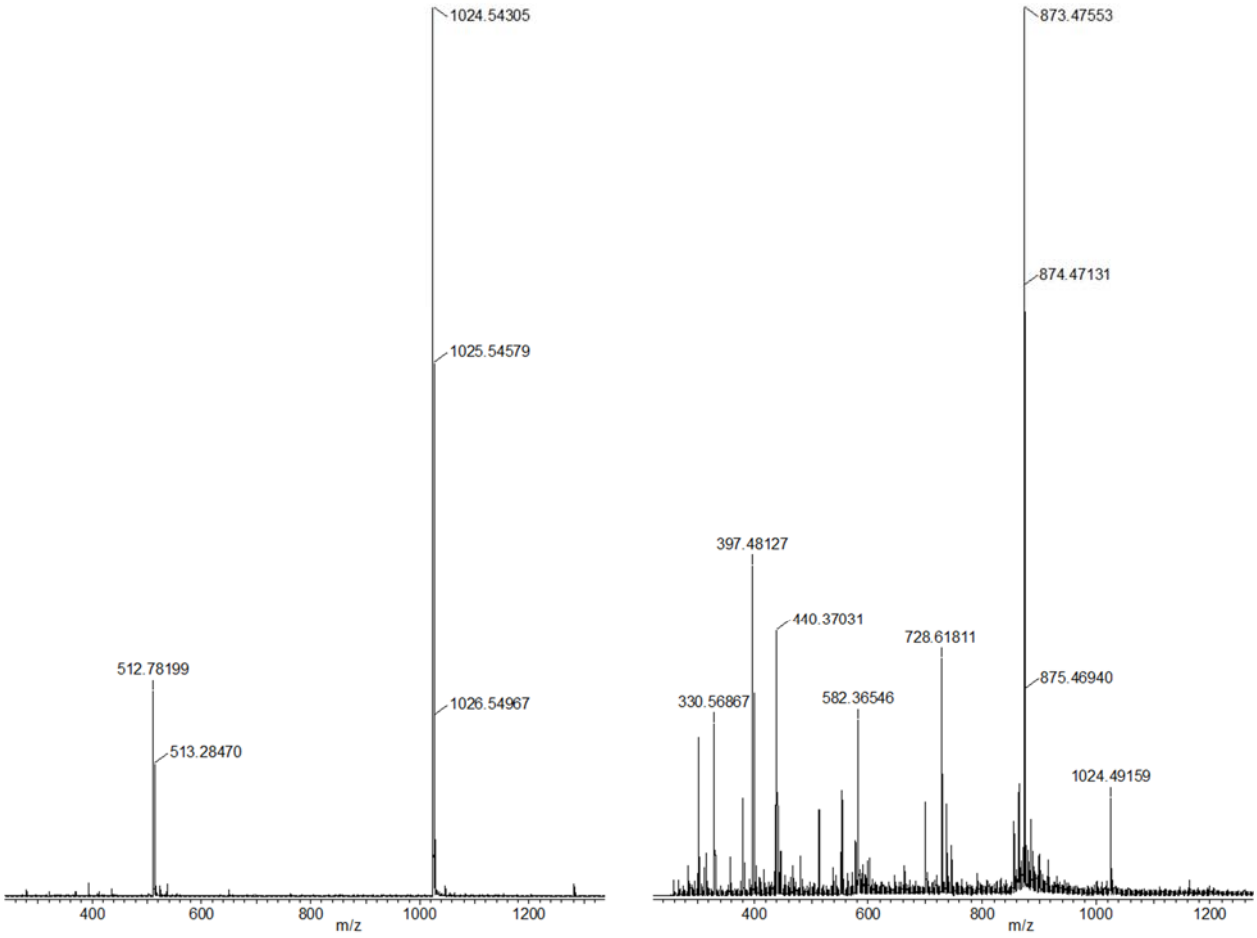


Figure 3-6. The two spectra above show the results of matrix-assisted inlet ionization of two vials of "Jintropin" that were submitted to the Harris County Institute of Forensic Sciences. Neither spectra indicated rHGH but rather Melanotan II (left) and GHRP-6 (right).

Paper Spray Ionization

Preliminary investigation for the analysis of intact peptides and peptide digests via paper spray ionization was conducted utilizing the JEOL FilterSpray® ionization source. Analysis of intact hexarelin and rHGH was successfully executed. The observed signal for rHGH was readily apparent and deconvolution of the mass spectrum resulted in a calculated molecular mass of 22,123.8 Da. Since references for rHGH typically only report the molecular mass to the nearest whole number, it is difficult to calculate the true error in the calculated mass. However, assuming the true molecular mass of rHGH is 22,124 Da, the calculated mass obtained utilizing paper spray ionization may in fact be closer to the reported value than that observed with matrix-assisted inlet ionization, 0.2 Da (9 ppm) versus 0.8 Da (36 ppm), respectively.

An rHGH standard was subjected to trypsin digested as described previously. Analysis via paper spray ionization was performed following sample clean-up utilizing C_{18} ZipTips. Though the resulting mass spectrum was somewhat similar in appearance to that collected during matrix-assisted inlet ionization of digested rHGH, some peptide fragment ions observed with inlet

ionization were not observed with paper spray ionization. However, two highly abundant fragment ions that were present in the inlet ionization data were also observed in the paper spray ionization spectrum. Moreover, the signal intensities for these fragments were roughly three times higher during paper spray analysis. Certainly, additional experiments are needed to determine the significance of these results; however, preliminary data obtained during this proof of concept study indicate that paper spray ionization may be more suitable for the analysis of digested peptide samples in terms of ionization efficiency.

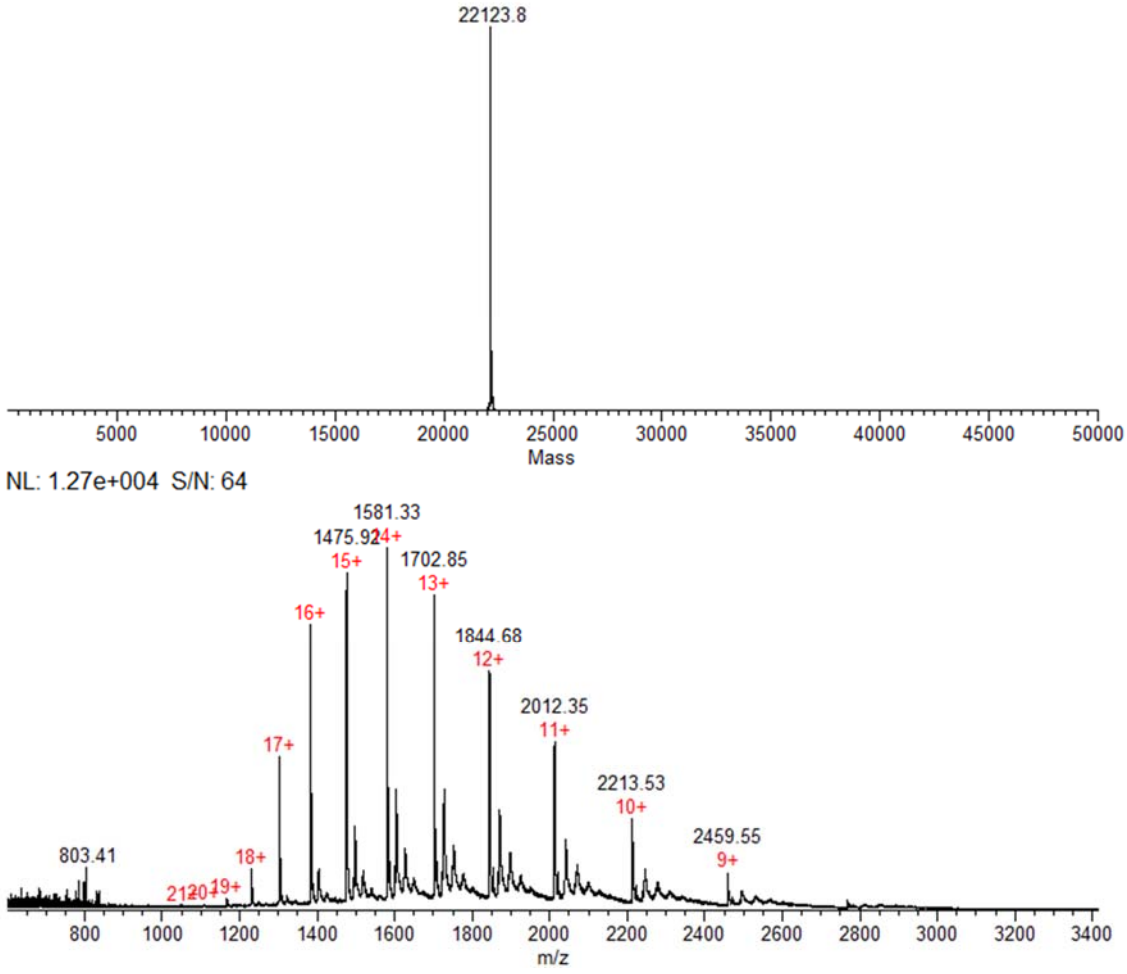


Figure 3-7. The mass spectrum of rHGH (bottom) was collected via paper spray ionization utilizing the JEOL FilterSpray® ionization source. Deconvolution of the mass spectrum (top) resulted in a calculated molecular mass of 22,123.8 Da.

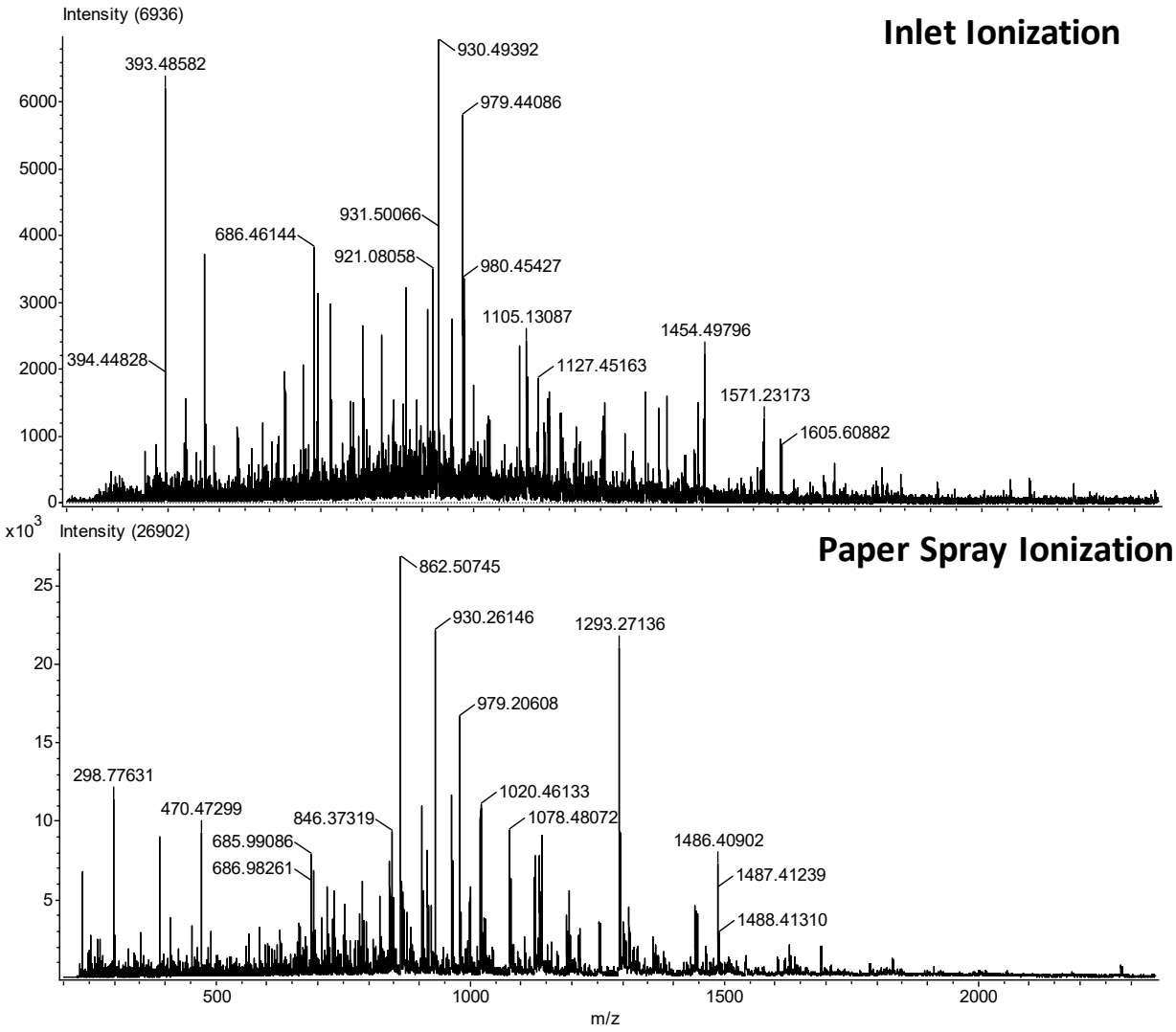


Figure 3-8. The top spectrum depicts the results of analyzing an rHGH digestion product following the completion of the digestion protocol utilizing matrix-assisted inlet ionization after sample clean-up with C_{18} ZipTips. The bottom spectrum depicts the results of analyzing an rHGH digestion product following the completion of the digestion protocol utilizing paper spray ionization after sample clean-up with C_{18} ZipTips.

4. Conclusions

4.1 Discussion of Findings

The main goal of this project was to develop a simple method for the identification of rHGH and other peptides that could be easily incorporated into typical forensic laboratory procedures, preferably utilizing instrumentation already present within such a laboratory. Due to the large mass of peptides and proteins, time-of-flight mass spectrometers are typically utilized to analyze such compounds due to the extensive mass range of the mass analyzer. Such mass

spectrometers are typical for systems sold for use with DART ionization sources. Because the popularity of JEOL AccuTOF systems and other atmospheric pressure inlet mass spectrometers continues to increase, this type of instrumentation was an ideal platform to utilize for the analysis of rHGH.

Direct inlet ionization and matrix-assisted inlet ionization with a time-of-flight mass spectrometer were found to be extremely well-suited for the analysis of rHGH and other large biomolecules. Spectra of intact peptides were obtained within a matter of seconds with little-to-no sample preparation. Dissolving the peptide samples in solvent and saturating with 3-NBN was the extent of sample preparation prior to instrumental analysis. Furthermore, 3-NBN was found not to be needed to attain signals for peptide samples weighing less than 8 kDa, though signals were improved with the use of the matrix compound. Molecular weights of intact proteins were easily obtained by deconvolution of the ESI-like spectra, either manually or through mass spectrometry software packages.

To increase the level of confidence in identification of unknown samples, intact peptides were subjected to enzymatic digestion utilizing trypsin. It should be noted that while exact mass spectra are highly specific with regard to elemental composition of a molecule, this method gives no information regarding the primary structure of a protein, that is, the sequence of amino acids. Enzymatic hydrolysis of a protein via a protease, such as trypsin, yields a predictable set of fragments, providing more information about the sequence; however, peptide sequencing remains the only definitive means of determining sequence identity.

4.2 Implications for Policy and Practice

Many forensic drug identification laboratories, especially those performing evidentiary analysis for law enforcement agencies, typically do not have procedures or suitable instrumentation in place for the successful identification of peptides and large biomolecules. Labs mainly rely on GC-MS for the identification of unknown samples. When peptide samples are submitted for analysis, typical laboratory procedures are followed, normally resulting in negative results since peptides are not volatized in the GC-MS inlet. Many times, such samples are reported indicating no compounds were detected. This is mainly because such samples are not suitable for analysis utilizing typical procedures. This gives no useful information to the requesting agency and can be misleading. The procedure developed through this research would provide laboratories the capability to successfully detect and characterize peptides and other large biomolecules, potentially resulting in an identification that would otherwise not be possible.

As mentioned previously, this method does not sequence the peptide, leaving the possibility of sequence variants as interfering substances. However, this limitation is not unique to the technique described here. Alternative methods such as MALDI-TOF and LC-MS/MS also have this limitation, raising the question of what constitutes a confirmatory technique. SWGDRUG guidelines [76] consider mass spectrometry a Category A technique (having the highest discriminating power), but has exceptions for when solely molecular weight information is provided. It could be argued that the additional identifying information provided by digestion and analysis, when compared to a standard, is sufficient to overcome this limitation, yielding this method a true Category A technique. When combining this with the Coomasie Blue color test indicating the presence of a protein (a Category C technique), this method may satisfy the criteria for true confirmation, rather than simply additional characterization, of the protein in question.

Accordingly, we urge caution in reporting when applying this method to casework to properly communicate limitations of the data.

4.3 Implications for Further Research

One aspect of this research that was not fully investigated was limits of detection utilizing the developed method. Preliminary results showed that all peptides analyzed could be detected at concentrations as low as 1 µM. Because forensic drug identification laboratories typically receive bulk powders and liquids for analysis, it would be highly unlikely that an analysis would need to be performed on a sample below the concentrations that were investigated in this study. Nevertheless, determining the limits of detection for any technique is necessary to fully evaluate the method.

Though rHGH was fully characterized through analysis of the intact peptide and digested fragments, enzymatic digestion of all acquired peptides was not completed. Most of the acquired peptides actually were of relatively low molecular weight with short amino acid sequences, many of which did not contain the amino acid residues necessary for digestion by trypsin. In order to characterize by enzymatic digestion, alternative proteases could be investigated that would be amenable to a wider range of small peptides. Trypsin was utilized here as proof of concept.

Because the focus of this project was analysis via inlet ionization due to the simplicity of the technique, only preliminary studies utilizing paper spray ionization were investigated. However, due to the ongoing collaboration with Dr. Robert Cody, JEOL USA found sufficient interest in paper spray ionization and produced a commercial paper spray ionization source for the AccuTOF mass spectrometer marketed under the name FilterSpray™. Further studies utilizing this ionization source for peptide analysis would be of great interest.

While it was initially desired to produce a compendium of digested data, enzymatic digestion was not possible for many of the peptides that were available for purchase through online vendors. Additionally, the MASCOT database, which is already freely available, serves the same library purpose for preliminary identification, short of comparison to an analytical standard. It appears as though most online vendors offering peptides for consumer purchase carry the same array of products. Knowing this, published molecular weights may be all the information needed to assign a preliminary identification when peptide samples are submitted as evidentiary material. As with other types of analysis, comparison with an analytical standard would be desirable for casework.

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6. Dissemination of Research Findings

Presentations by Project Personnel at Scientific Conferences/Meetings

1. Vircks, K. E.; Zavala, J. M.; Cody, R. B.; Samms, W. C.; Kahn, R. Characterization of Performance-Enhancing Peptides Via Ambient Ionization Time-of-Flight/Mass Spectrometry (TOF/MS). *68th Annual Scientific Meeting of the American Academy of Forensic Sciences, Las Vegas, NV*, **Feb. 27th, 2016.**

2. Vircks, K. E.; Zavala, J. M.; Wang, Y.; Cody, R. B.; Samms, W. C.; Kahn, R. Rapid Analysis of Peptides and Proteins Utilizing Matrix-Assisted Inlet Ionization Mass Spectrometry. *69th Annual Scientific Meeting of the American Academy of Forensic Sciences, New Orleans, LA*, **Feb. 15th, 2017.**

3. Vircks, K. E.; Zavala, J. M.; Wang, Y.; Cody, R. B.; Samms, W. C.; Kahn, R. Rapid Peptide Analysis Utilizing Matrix-Assisted Inlet Ionization and Paper Spray Ionization Mass Spectrometry. *2018 NIJ R&D Symposium, Seattle, WA*, **Feb. 20th, 2018.**

7. Acknowledgements

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Appendix A: Matrix-Assisted Inlet Ionization Peptide Spectral Results

*Molecular masses shown in this appendix were calculated using MagTran mass spectral software that limits numerical values to two decimal places.

Table of Contents

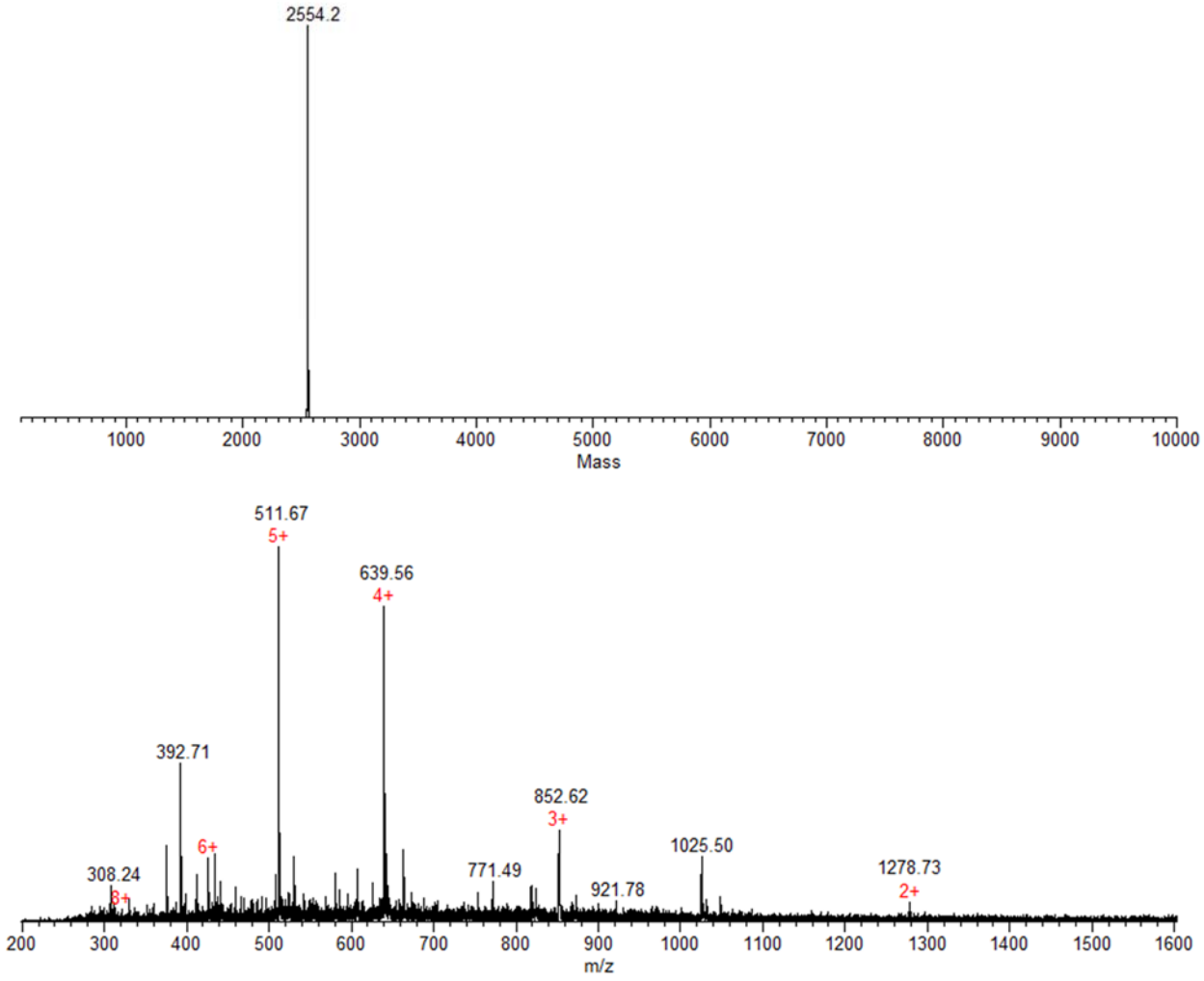
A1. Table of Results

Peptide	Expected Mass (Da)	Observed Mass (Da)	ΔM (Da)	ΔM (ppm)
Adipotide	2553.51	2554.2	-0.69	270
AOD9604	1815.88	3297.20*	N/A	N/A
BPC 157	1418.7	1418.75	-0.05	35
CJC 1295 DAC	3645.02	3367.40*	N/A	N/A
Follistatin 315	34.7 kDa	Inconclusive	N/A	N/A
Fragment 176-191	1815.88	1816.05	-0.17	94
GHRP-2	817.43	817.5	-0.07	86
GHRP-6	872.44	872.51	-0.07	80
Gonadorelin	1181.57	1182.71	-1.14	965
Hexarelin	886.46	886.51	-0.05	56
Ipamorelin	711.39	711.35	0.04	56
Melanotan 1	1645.84	1645.77	0.07	43
Melanotan 2	1023.54	1023.54	0	0
MGF (C-terminal)	2865.5	2556.10* and 2848.10*	N/A	N/A
PT-141	1024.52	1024.44	0.08	78
TB-500	4960.49	4962.8	-2.31	466
				*Expected Mass Not Obtained

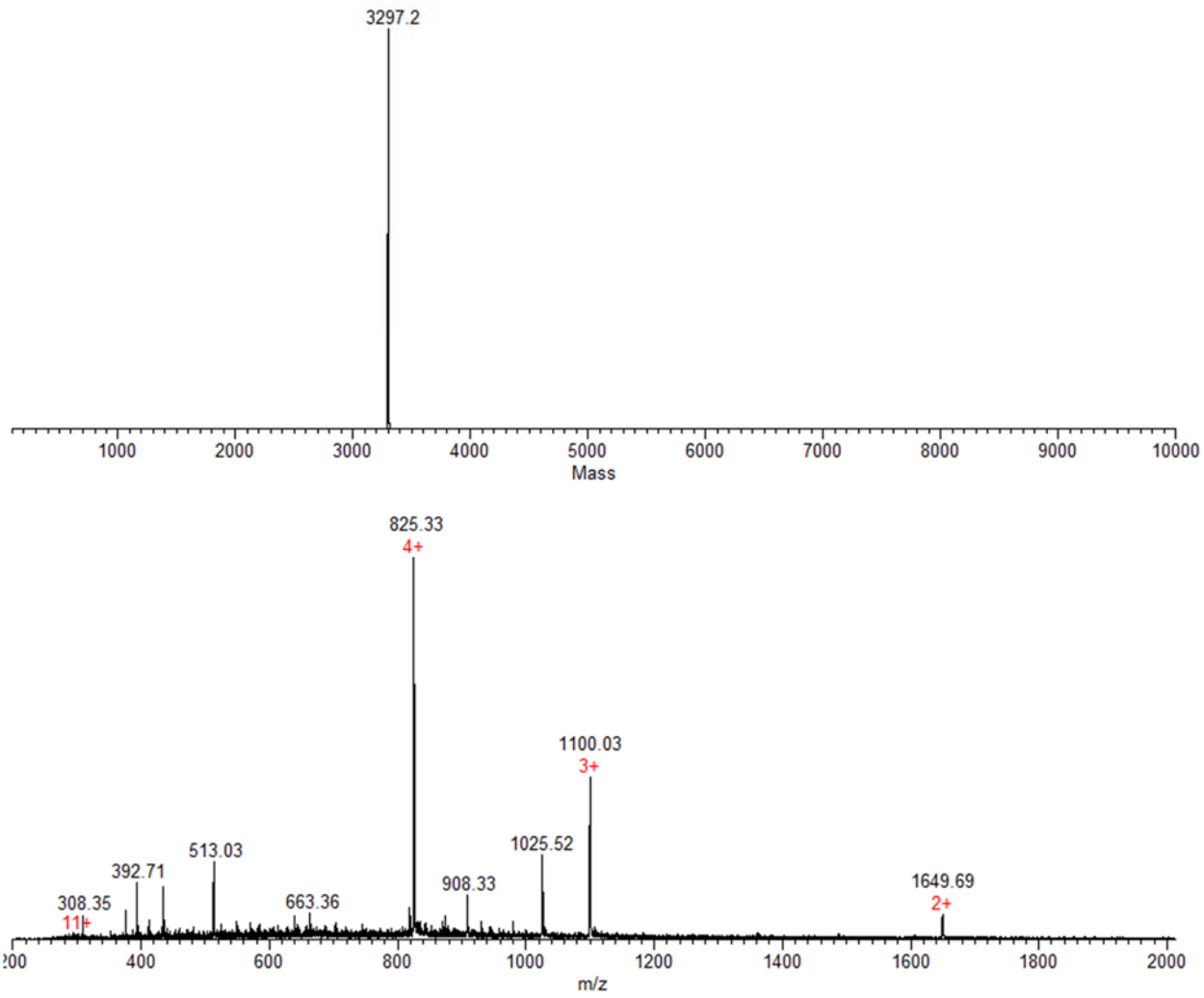
Reproducible spectra could not be obtained for Follistatin 315. Further attempts will need to be made to characterize this specific sample.

Higher error in mass accuracy could be related to mass calibration of the spectra or unknown structural modifications of the peptides themselves. Nevertheless, the discrepancies are in the regime of a few protons, not full amino acids. Knowing this study was more of a proof of concept, extensive validation of the method would likely increase mass accuracy.

A2. Adipotide

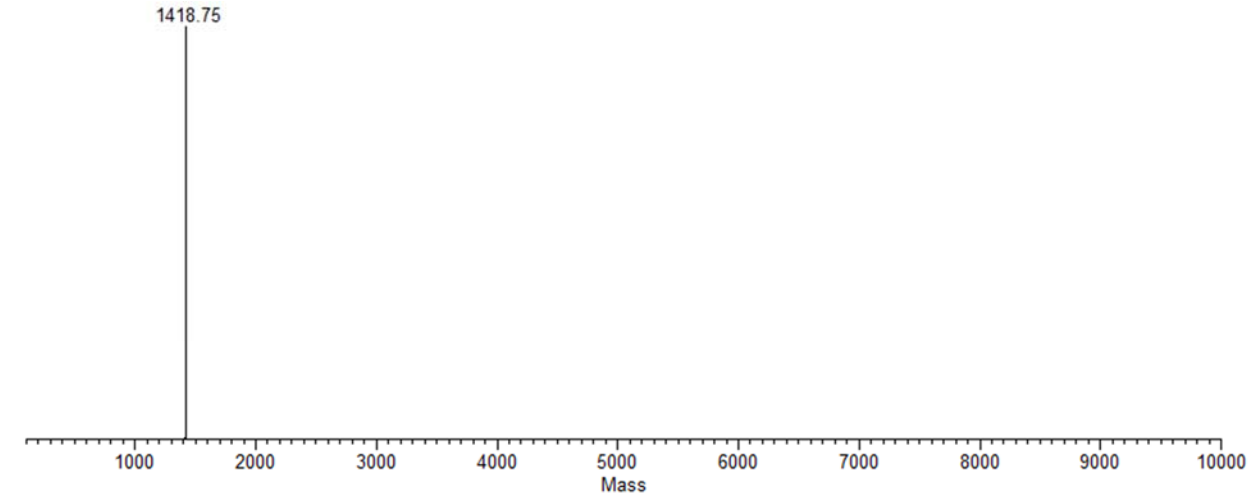


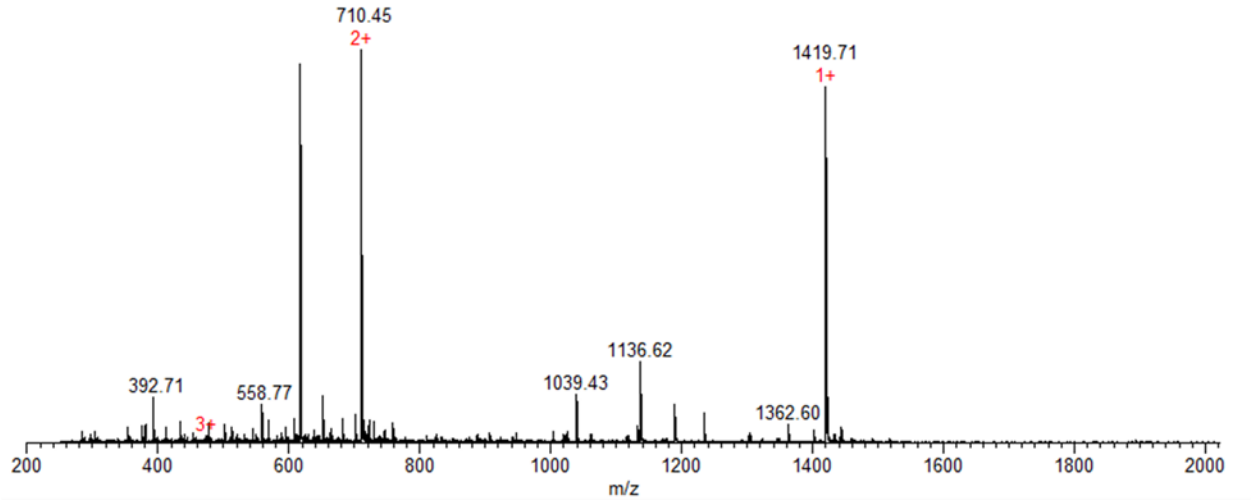
A3. AOD9604



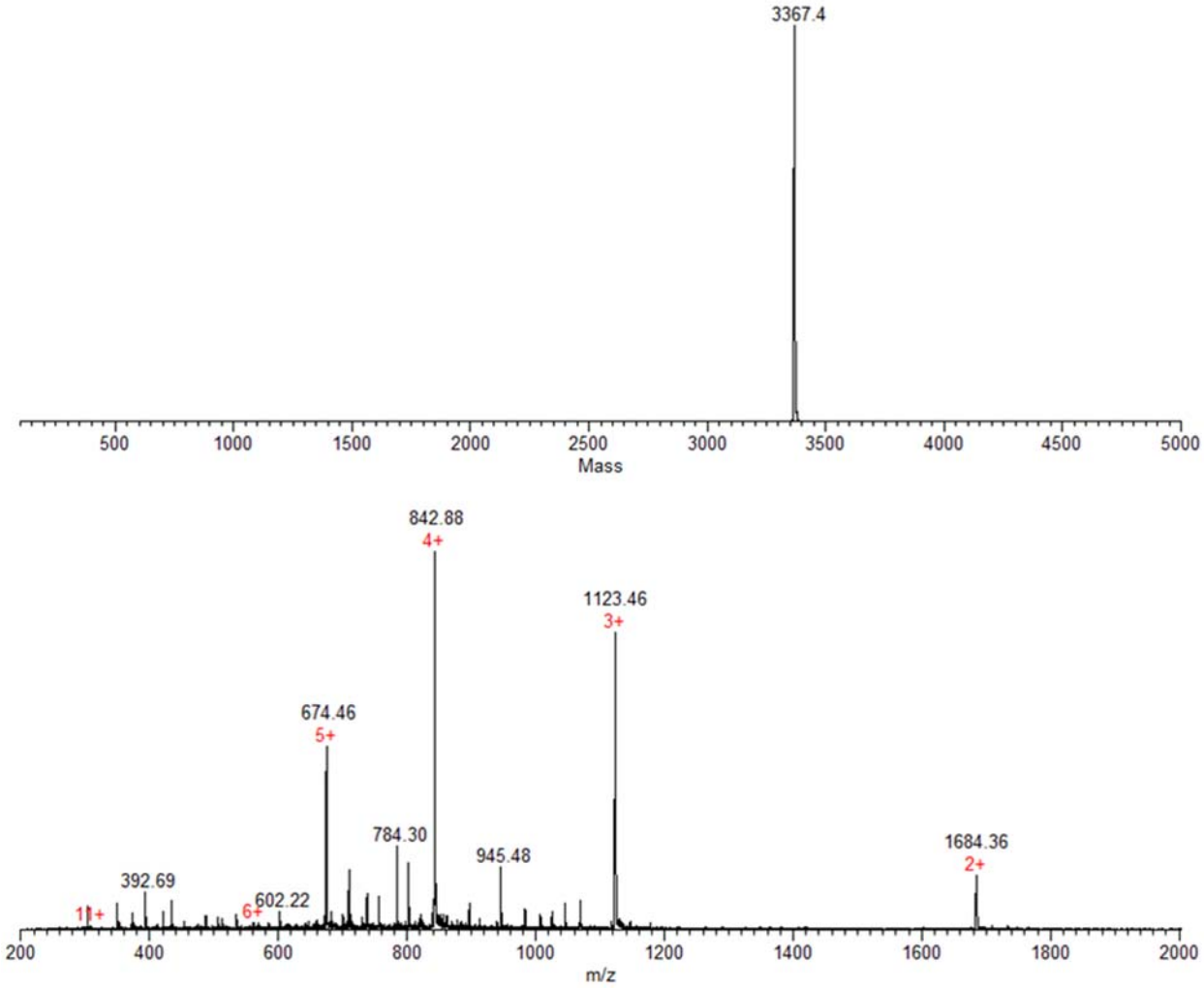
*The expected mass was not obtained (see A1 – Table of Results)

A4. BPC 157


1418.75
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
Mass

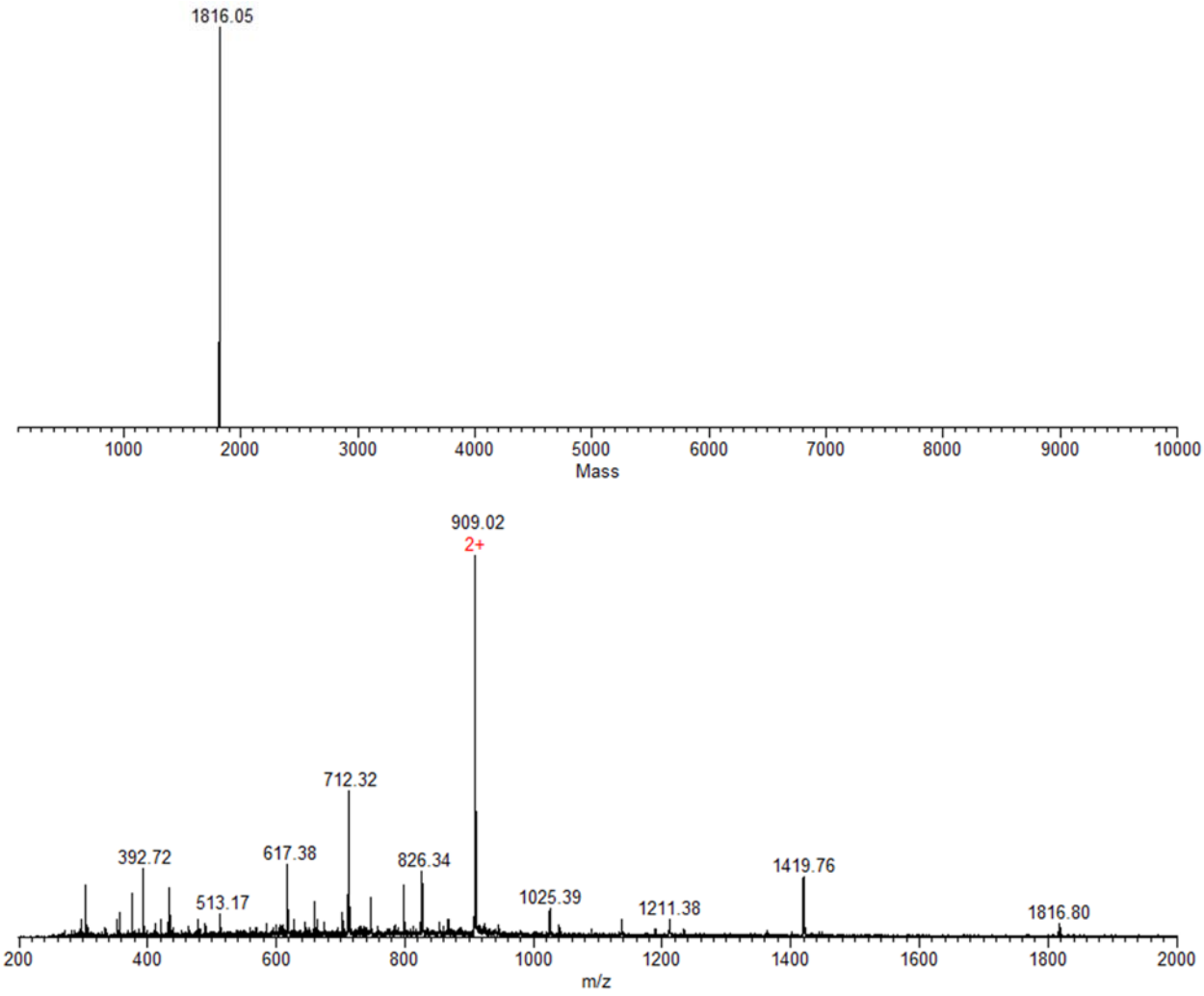

710.45
2+
1419.71
1+
1136.62
392.71
558.77
1039.43
1362.60
3+
200
400
600
800
1000
1200
1400
1600
1800
2000
m/z

A5. CJC 1295 DAC

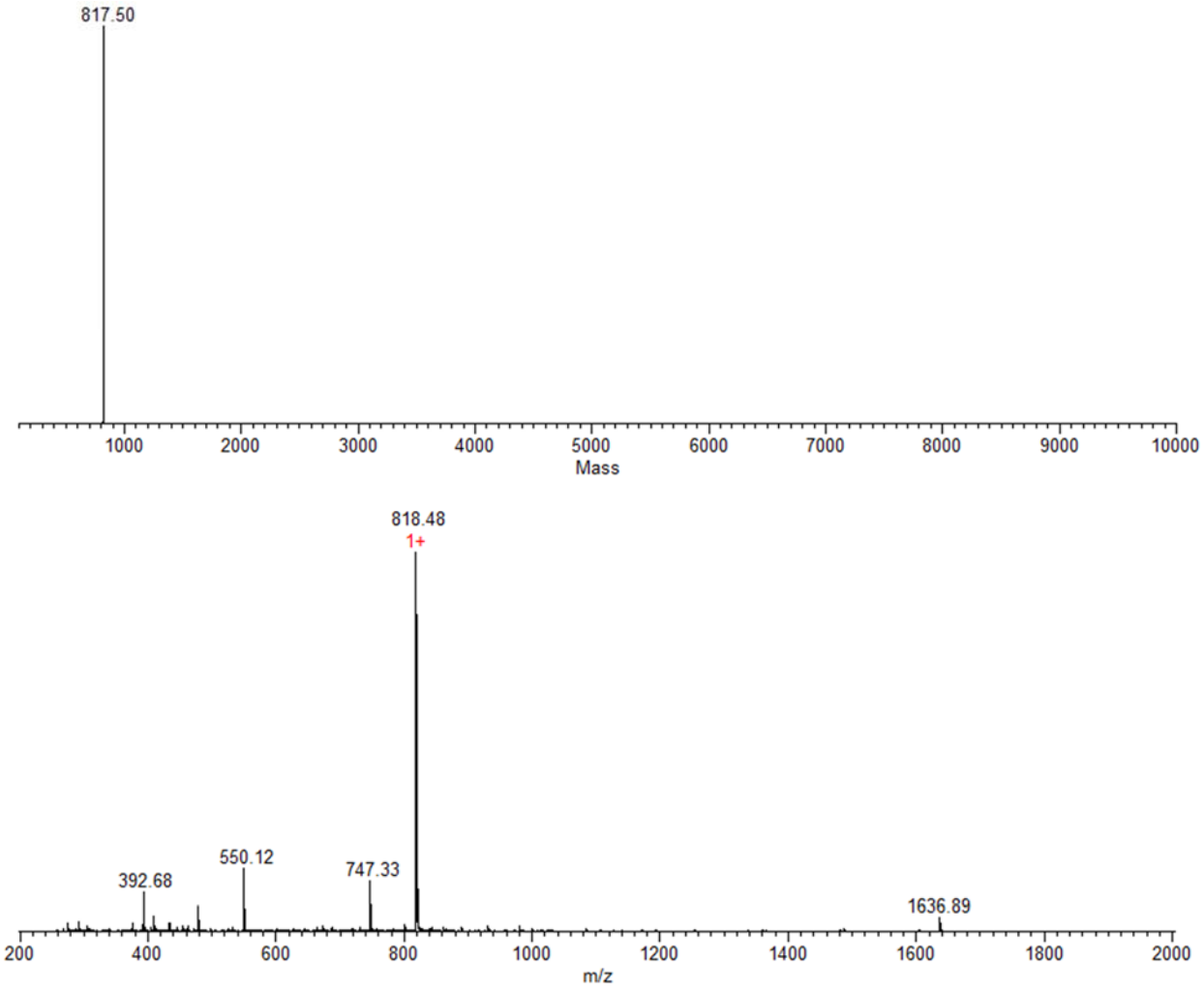


*The expected mass was not obtained (see A1 – Table of Results). This sample was interesting in that the mass obtained through analysis indicates the presence of CJC 1295 (without DAC). Further investigation is needed to interpret the findings for this sample.

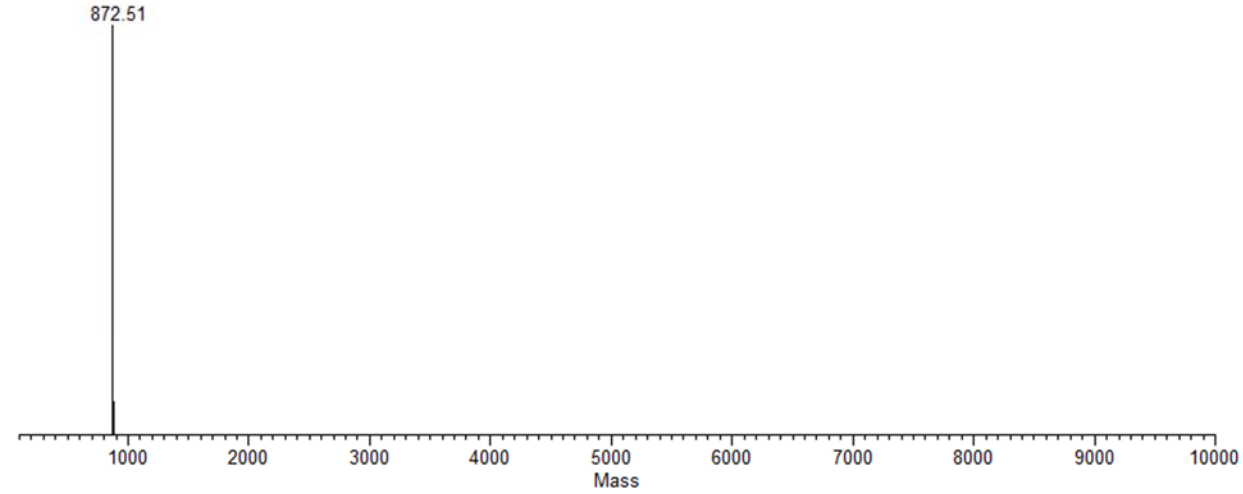
A6. Fragment 176-191

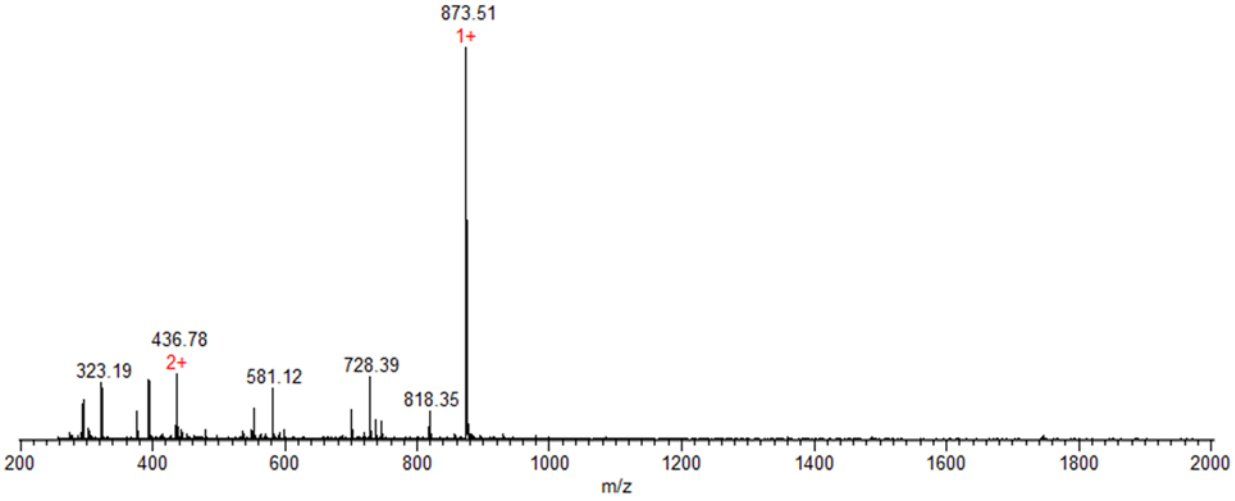


A7. GHRP-2

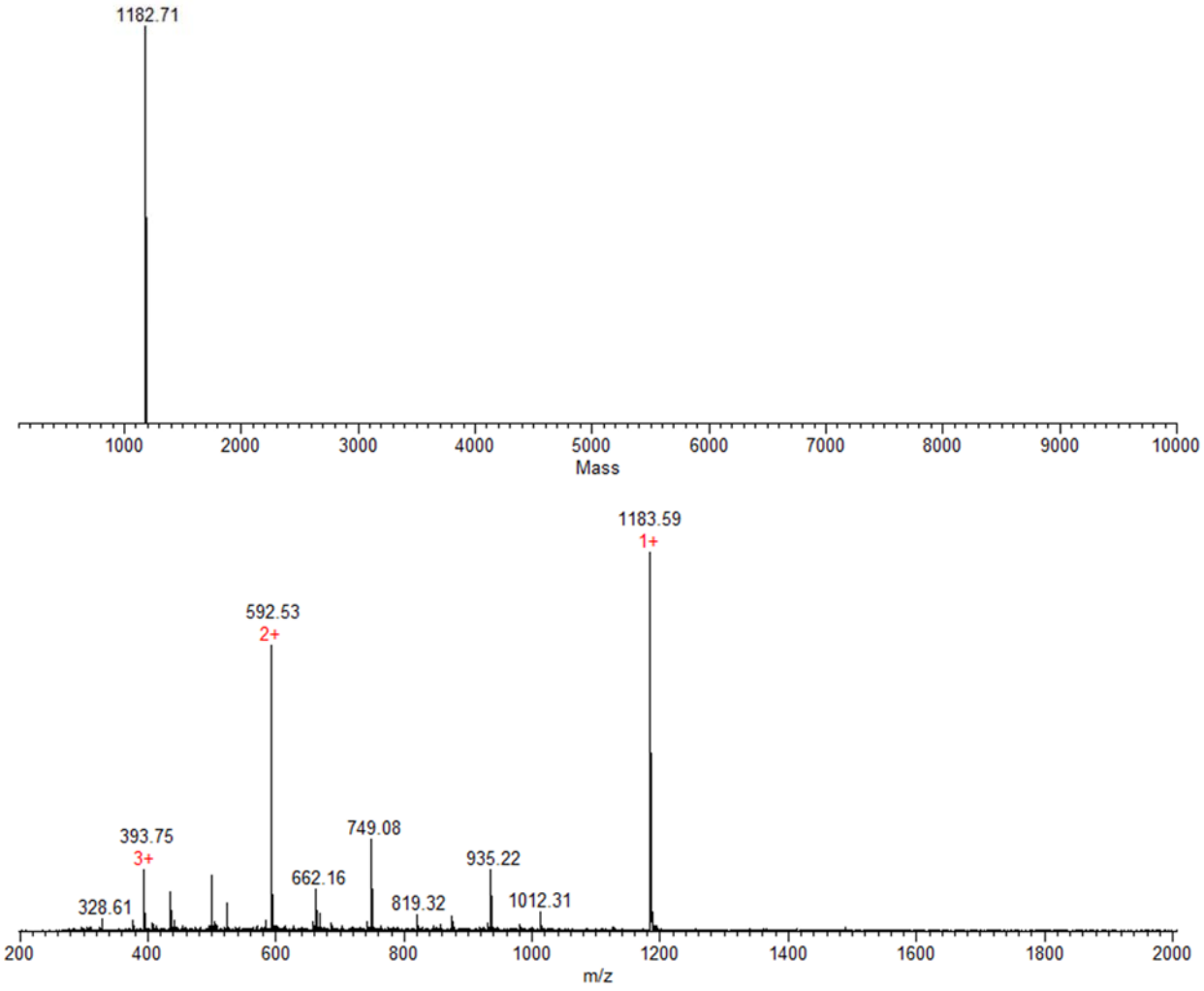

817.50
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
Mass
818.48
1+
392.68
550.12
747.33
1636.89
200
400
600
800
1000
1200
1400
1600
1800
2000
m/z

A8. GHRP-6

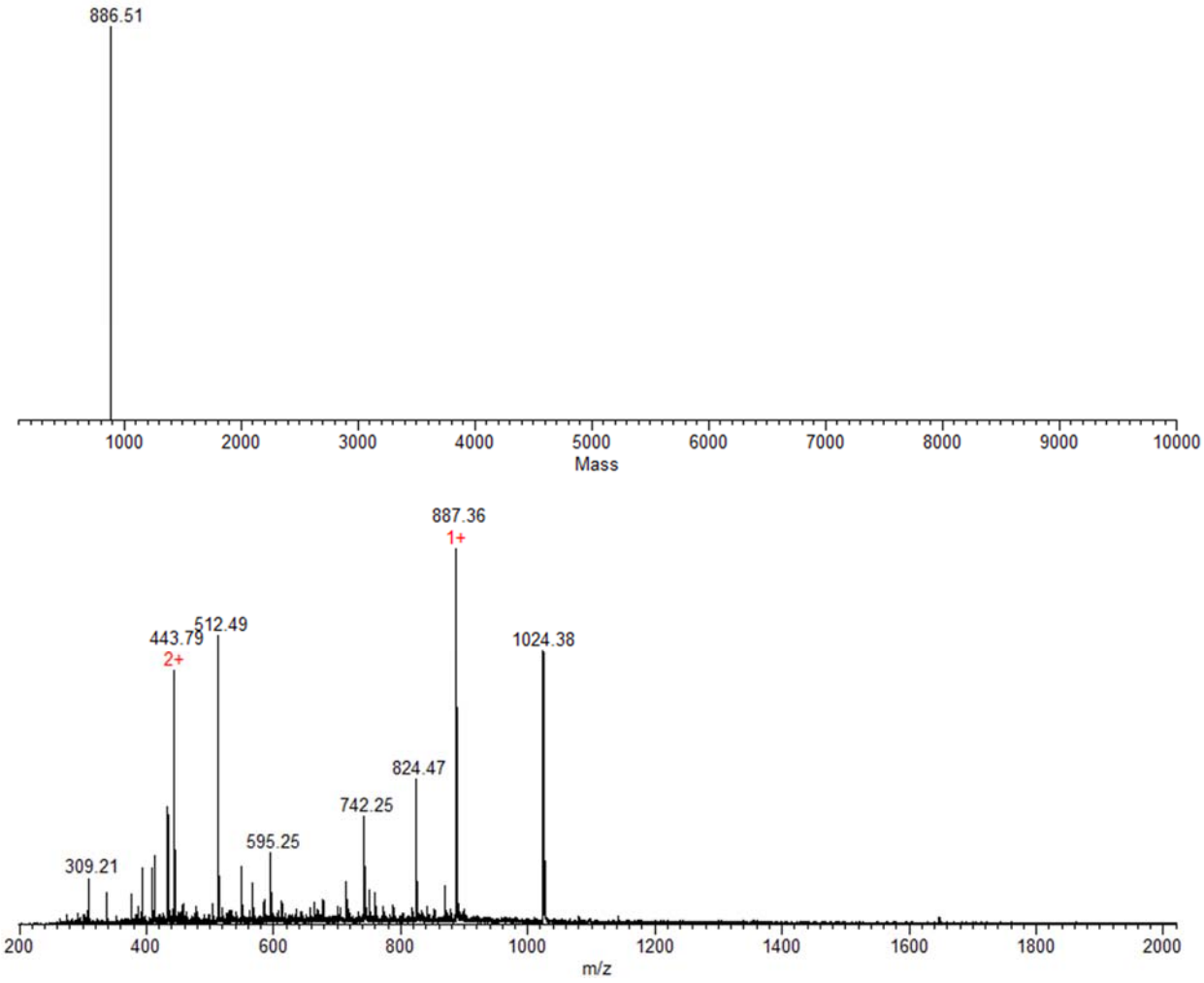

872.51
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
Mass


873.51
1+
436.78
2+
323.19
581.12
728.39
818.35
200
400
600
800
1000
1200
1400
1600
1800
2000
m/z

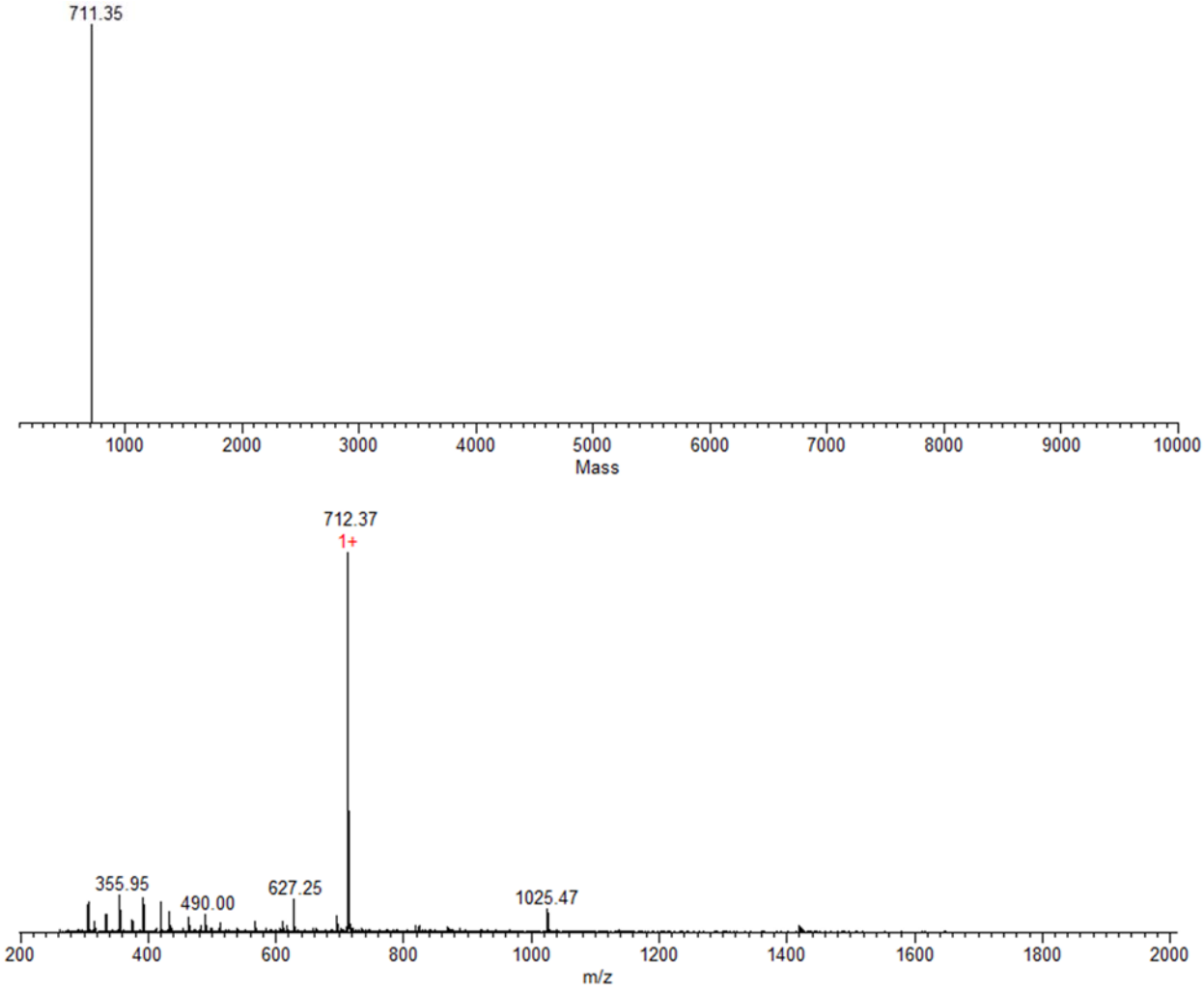
A9. Gonadorelin



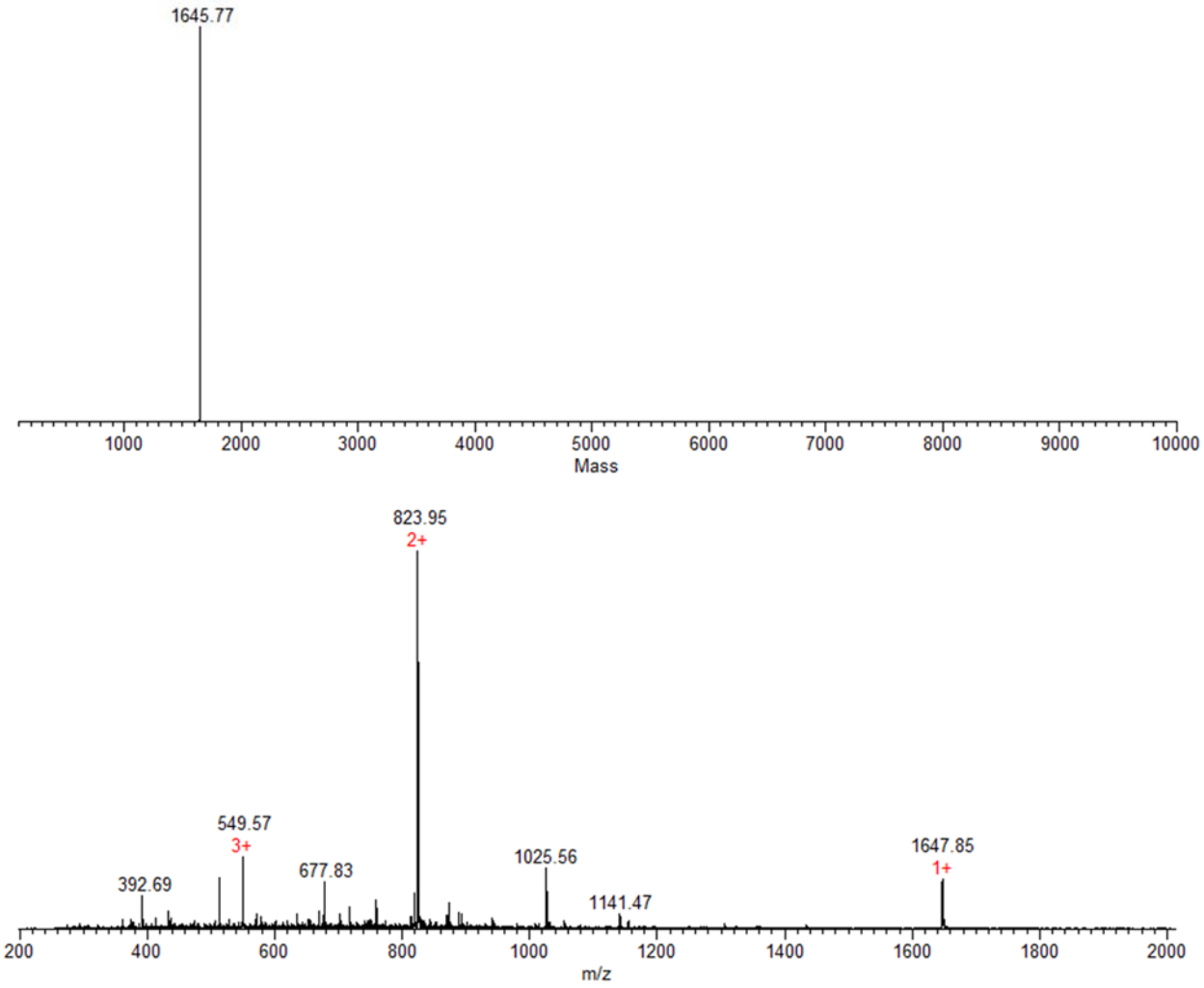
A10. Hexarelin



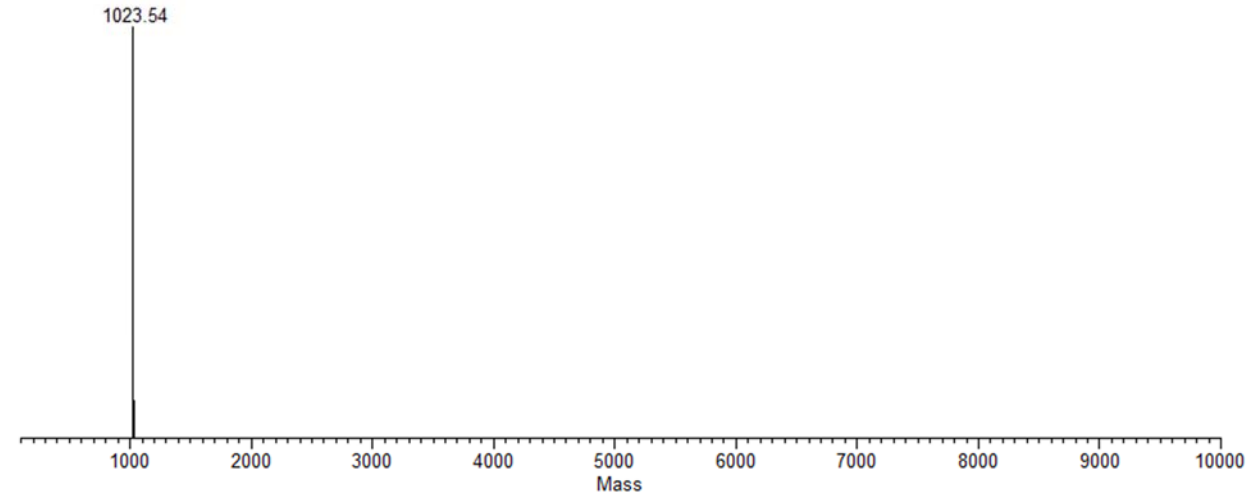
A11. Ipamorelin

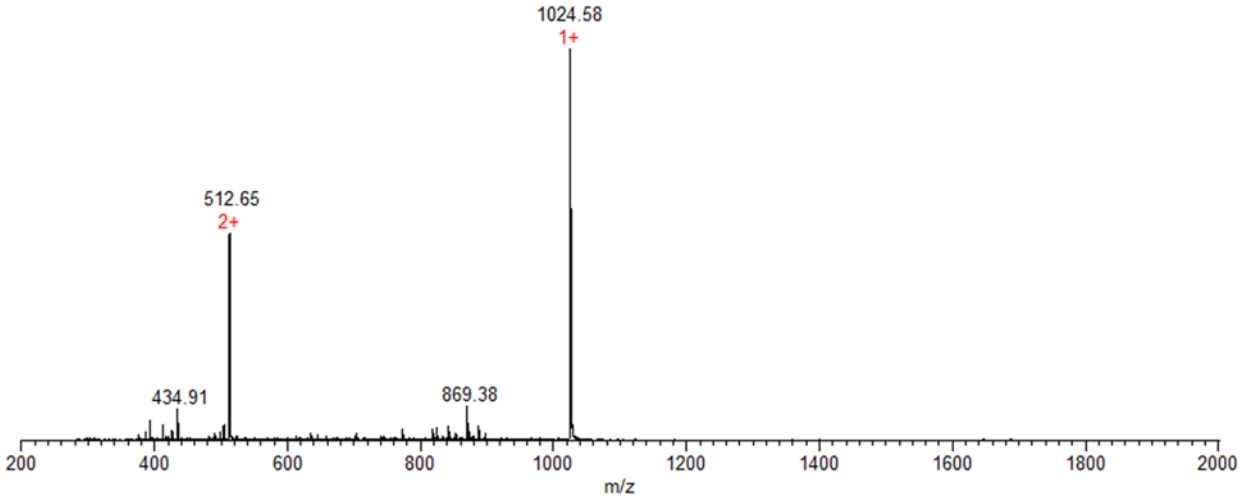


A12. Melanotan I

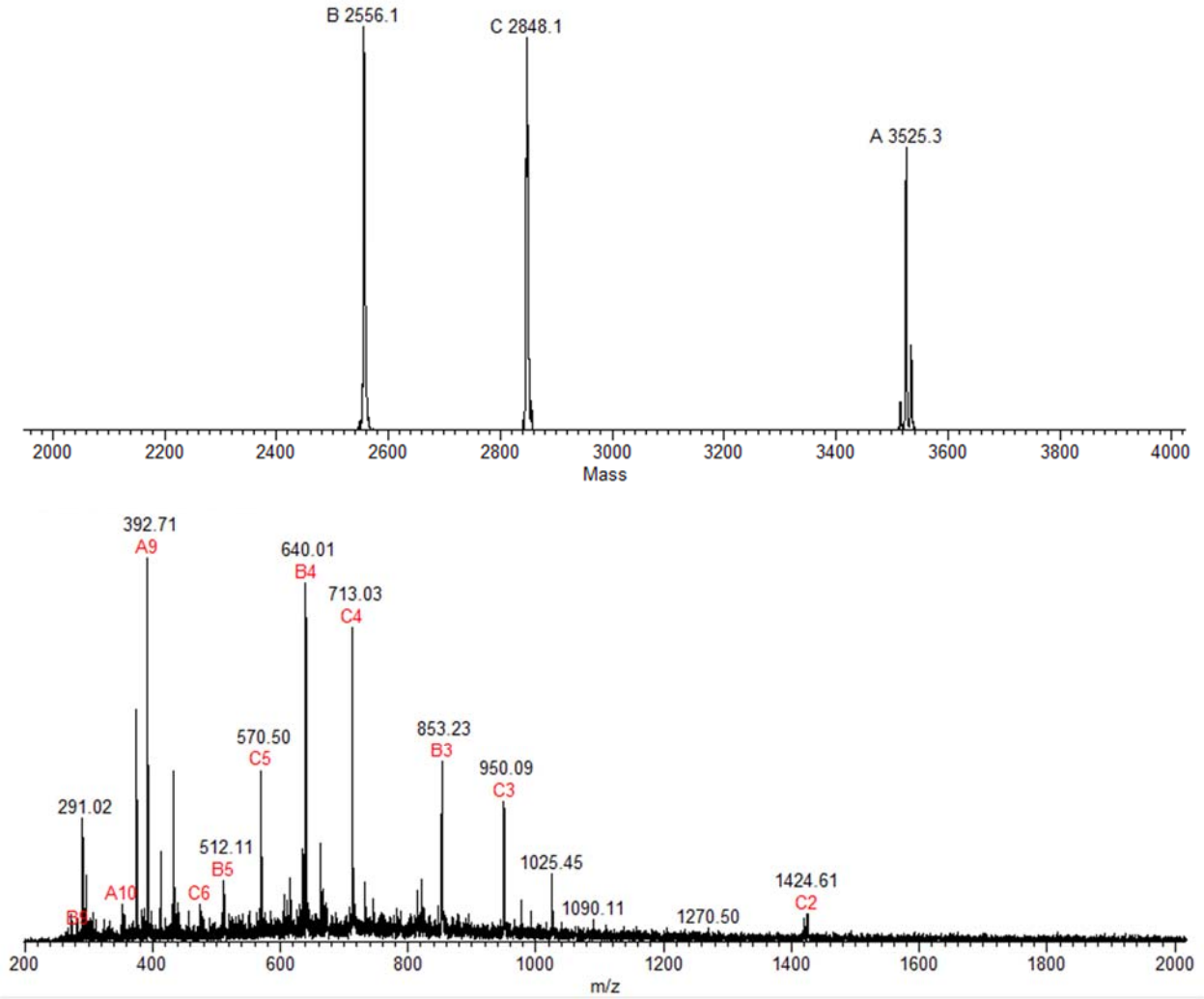


A13. Melanotan II



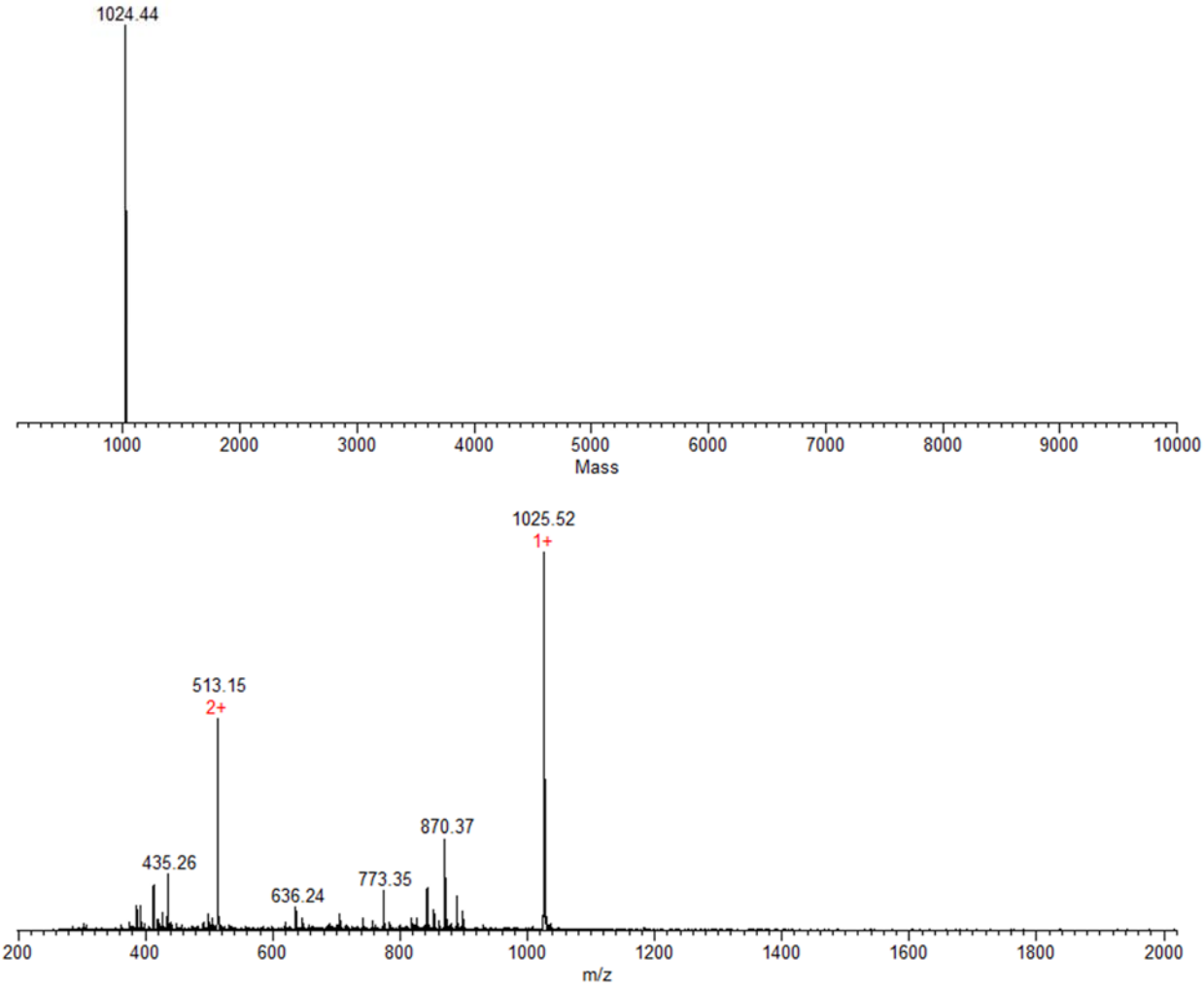


A14. MGF (C-terminal)

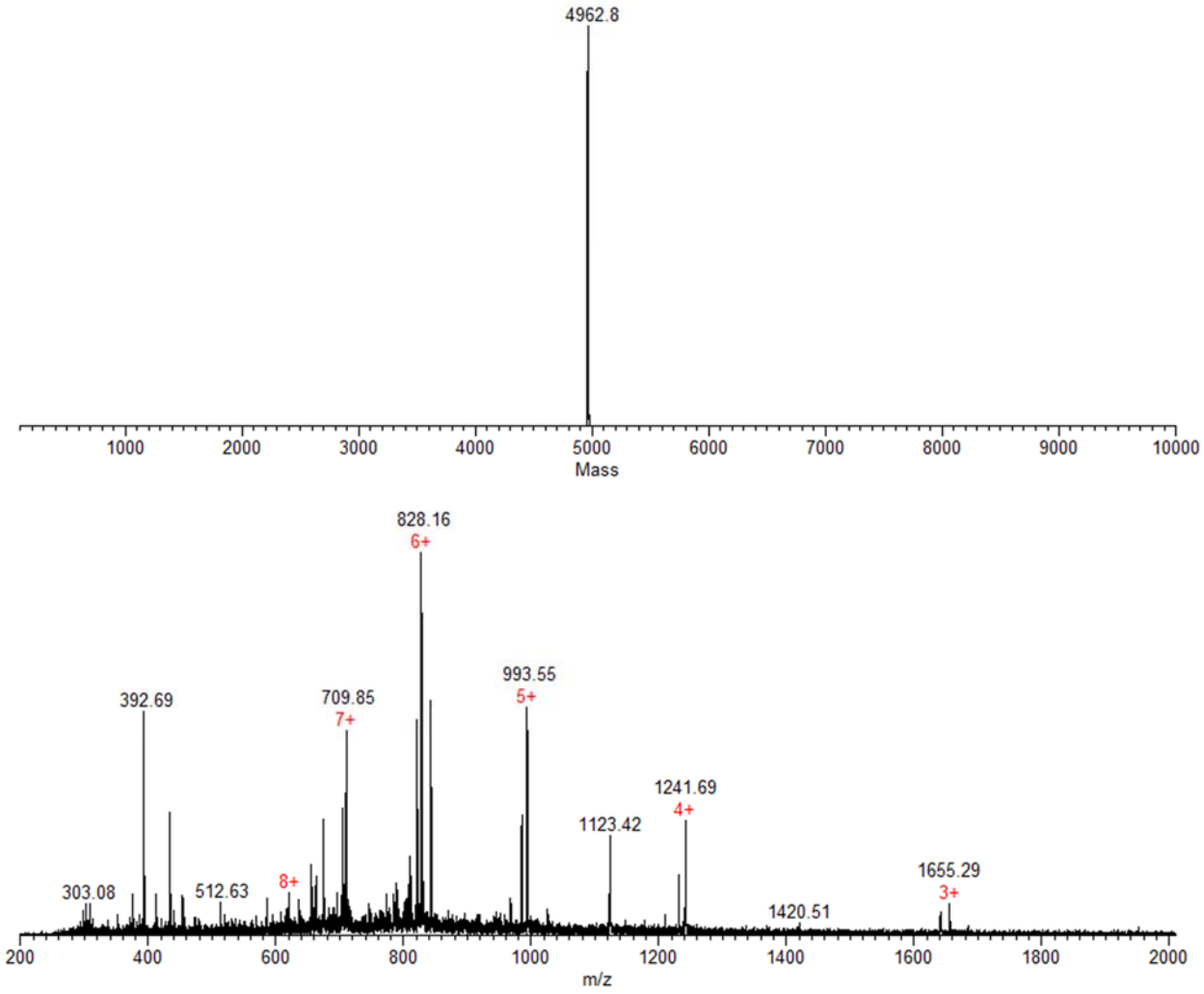


*The expected mass was not obtained (see A1 – Table of Results)

A15. PT-141


1024.44
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
Mass
1025.52
1+
513.15
2+
870.37
435.26
773.35
636.24
200
400
600
800
1000
1200
1400
1600
1800
2000
m/z

A16. TB-500


4962.8
1000
2000
3000
4000
5000
6000
7000
8000
9000
10000
Mass
828.16
6+
993.55
5+
392.69
709.85
7+
1241.69
4+
1123.42
303.08
512.63
8+
1655.29
3+
1420.51
200
400
600
800
1000
1200
1400
1600
1800
2000
m/z