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Mass Spectrometry Analysis of Organ-Specific Cellular Extracts - Nanomized Organo Peptides: The Stability Study

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Abstract

Mass spectrometry (MS) has emerged as a pivotal analytical technique in the characterization of organ-specific cellular extracts, particularly in the study of nanomized organo peptides. This research investigates the stability of these peptides, which are critical for understanding their biological functions and therapeutic potentials. We performed a comprehensive stability study by isolating organo peptides from various tissues and analyzing their properties under different conditions using high-resolution mass spectrometry. Our findings reveal significant variations in stability profiles related to specific organ sources and environmental factors, including temperature and pH levels.

This study underscores the importance of mass spectrometry in profiling organ-specific cellular extracts, providing a foundation for developing effective therapeutic interventions. MALDI-TOF employs a rapid protein fingerprinting method, facilitated by proteolytic digestion of samples, which generates MS spectra that can be efficiently matched against databases. This approach enables quicker identification of candidates ranked by scoring methods, wherein proteins yielding a greater number of proteolytic peptides receive higher scores, indicating greater likelihood of identification. The performance speed of MALDI-TOF, often generating results in under a minute, contrasts sharply with the prolonged durations associated with LC-MS/MS runs, which can extend beyond thirty minutes, combined with extensive data parsing times. This study underscores the benefits of MALDI-TOF mass spectrometry in peptide identification, reinforcing its role as a highly efficient alternative to traditional LC-MS/MS techniques for organo peptide analysis. The implications of peptide stability on drug formulation and targeted delivery strategies are discussed, highlighting the necessity for further research in clinical applications.

Keywords

Peptides; Nano Organo Peptides (NOP); Mito Organelles (MO) Peptides; mass spectrometry; cellular therapy; regenerative medicine; peptide therapy

Introduction

Peptides are linear chains composed of amino acid residues linked by peptide bonds [1]. In contrast to proteins, which typically consist of 50 to 2000 amino acids and have average molecular weights ranging from 5.5 to 22 kDa, peptides contain fewer than 50 residues and are generally lighter than proteins [1]. Within cells, a distinct array of proteins and peptides is produced, each serving various roles in maintaining biological balance. Research has shown that short peptides are crucial in regulating transcription, facilitating the transmission of biological signals, and reversing genetic changes associated with aging [2]. These peptides function as signaling molecules and regulatory factors by interacting with DNA and histone proteins [3]. Additionally, the process of aging is significantly influenced by the regulatory effects of peptides on homeostasis, which are closely linked to the aging of cells, tissues, and organs.

Peptide therapy is designed to enhance the signaling strength received by cells, either by stimulating peptide production or activating normal signaling processes [3-6]. This helps rejuvenate and revitalize tissues, as well as the organism as a whole. While the peptide content in cells is generally similar, the specific function and structure of each cell determine the types of biologically active substances it contains. Additionally, certain biologically active substances are mainly synthesized or stored in particular tissues. Since peptide signaling and function are closely tied to cell type, peptide therapy often employs organ-specific extracts to target diseased or aging tissues. Due to their short length and low molecular weight, peptides can be mass-produced through biosynthesis and extraction, making them suitable for therapeutic use [3]. After years of research and global practice, MF-Plus has developed two products—Nano Organo Peptides (NOPs) and Mito Organelle (MO) Peptides—designed for revitalization therapy in both humans and animals [7].

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Nano Organo Peptides (NOPs) are 3 nm in size with a molecular weight under 10 kDa [3]. They are derived from mammalian stem cells and undergo a proprietary parallel-extraction process, which includes multiple ultrafiltration steps through specialized Millipore filters to obtain cellular material, referred to as molecular-level ultrafiltrates. This extraction method ensures that the ultrafiltrates are specific to the cell type from which they originate. Initially, NOPs are extracted from organ-specific cells, which contain substances with a high molecular mass. These are then separated through several ultrafiltration steps using micro-Millipore filters, allowing only molecules with a molecular weight below 10 kDa to pass through, ensuring peptide specificity. Due to their small molecular size and high solubility, NOPs can be administered both sublingually and via injectable routes, such as subcutaneous or intramuscular injection [3]. NOPs have been studied and used in a range of applications, including cosmetics [8] and regenerative organ repair [9].

Despite the numerous studies highlighting the therapeutic effectiveness of NOP peptides and the established procedures documented on obtaining them, little is known of the exact makeup of these formulations. Mass spectrometry (MS) has been shown to identify and quantify analytes in complex solutions (13) and therefore is thought to be able to identify the population of peptides derived from peptide cocktail formulations. The mass spectrometer produces a readout of peaks plotted in relative abundance against the mass-to-charge ratios. By comparing the experimentally obtained peaks to a database of known proteins, the peptides can be identified. This report details the experimental methods used and presents the results from mass spectrometry analysis of various EW peptides. The objective of this study was to assess the stability of NOPs stored at either 4 or 22 oC temperatures for one, three, and six months.

Materials and Methods

Eight samples of EW peptides were provided by the company for a 1, 3 and six months before analysis. Samples were provided from lot N-06 for the LPPSIMKE, and N-18A for the liver samples. All samples were kept at either 4 or 22oC throughout the duration of the experiment and were handled using good laboratory practices.

Sample Preparation

Samples were refrigerated in sterile bottles until used. The ThermoFisher Scientific BCA Protein Assay protocol was utilized to determine the protein concentrations of the unknown peptide solutions. Triplicate sample readings were obtained for the four unknown peptide solutions using the Tecan Infinite F200 microplate reader at a wavelength of 570 nm.

Preliminary Mass Spectrometry Data Preparation

Peptides were withdrawn from the sterile bottles at a volume of 30µg/mL and pipetted into auto column tubes. Each sample prepared for mass spectrometry (using the Waters Xevo G2-XS QTof) was diluted with deionized (DI) water to ensure the peptide concentrations remained below the mass spectrometer's overloading threshold. The dilution factor was based on the protein concentration determined from the BCA assay. Once samples were loaded in the columns, they were gently vortexed for 1-3 seconds to ensure proper mixing. A total of 26 columns, made up of three replicates per sample and two auto column tubes

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of 0.9% saline controls, were labeled appropriately loaded onto plates in the mass spectrometer. Sample name, test type, inlet file type, column position on plate, and injection volume of sample were all entered into the MassLynx software. A blank was run between samples to flush the lines in preparation for the next sample and to check for any potential contaminants. Once all samples were loaded and sample information was entered into MassLynx, the mass spectrometry automation program was started. Chromatograms were generated and stored for analysis using the MassLynx software. The samples selected for MADLI-TOF analysis due to its relatively higher protein concentration and more prominent chromatogram peaks.

Sample Preparation

Peptides from the Liver or LPPSIMKE samples were withdrawn at a volume of 30 µg/mL and pipetted into auto column tubes in an agarose gel. Sample mixtures were separated based on molecular weight using SDS-PAGE, and Coomassie™ blue staining was applied to visualize the proteins. A single protein band was excised from the gel and transferred into a low-binding, siliconized microcentrifuge tube. The proteins were de-stained by adding 100 µl of a 1:1 methanol-water solution and vortexing. The gel piece was then washed further by discarding the de-staining solution and adding 400 µl of water. Tubes were shaken for 15 mins at room temperature, and the de-staining step was continued until the gel became colorless (minimum of 3 repeats). 400µl of 100% acetonitrile was added to dehydrate the gel for 10 mins and dried by vacuum centrifugation after removal of the supernatant. Disulfide bonds were removed with the addition of 100µl of 10mM dithiothreitol (DTT) to the gel and then the gel was incubated for 45 minutes at 55°C. The solution was removed and 100µl of 55mM iodoacetamide was added to the gel and it was then incubated for an additional 30 minutes at room temperature in low light conditions, allowing trypsin to access cleavage sites. The solution was then added to 400µl of the gel and a wash solution (50% per volume acetonitrile, 25mM ammonium bicarbonate) was added. The gel was then incubated at room temperature and vortexed for 15 minutes three times. The gel was subsequently dehydrated with 400µl of 100% acetonitrile for 10 mins and dried in vacuum centrifuge after removal of the supernatant.

Enzyme Digestion

A protease trypsin solution with a pH 8, diluted 1:1000 with 25mM ammonium bicarbonate, was prepared and diluted to a final concentration of 10 - 20 µg/ml. Trypsin was added to the gel and it was incubated on ice for 1 hour. The solution was removed and replaced with 25mM ammonium bicarbonate to cover the gel. The gel was then incubated at 37°C overnight.

Peptide Extraction

The supernatant containing the peptides was transferred to a new microcentrifuge tube. A gel extraction solution, consisting of 50% acetonitrile, 1% trifluoroacetic acid (TFA), and 49% water, was added, and the mixture was incubated at room temperature and vortexed for 20 minutes. This solution was then combined with the peptide-containing supernatant in the new tube.

Analysis

10µl of a 1:1 solution of 0.1% TFA and 100% acetonitrile was added to 10µl of each sample. 1µl of DMP was added onto the MALDI plate. 1µl of sample solution was then applied with 1:1 0.1% TFA and 100%

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acetonitrile onto the same spot as the DMP and the spot was allowed to dry. The samples were run on MALDI in triplicate and the peaks were compared against a database to identify the key peptides and amino acids found in the samples.

Data Analysis

Data was analyzed using the chromatogram tool in MassLynx software. Replicates for each sample were analyzed together and chromatograms for each were generated. Batches 1 and 2 were matched to compare consistency across products. The mass of the most prominent three peaks was deconvoluted and the key peptides and amino acid components were determined against open-source databases.

Results

The analyses were conducted to assess the impact of temperature storage and duration of storage on peptide stability. Figure 1 presents MS data for the acidic peptide N-06 D extracted from liver samples stored at 4 or 22°C for one-, three-, and six- month period of time. The left panels (Retention time plots) display the intensity plotted against time, showing major peaks around three minutes for each time point. The one-month sample displays a strong and sharp peak at three minutes, which becomes less intense and broader by three months. After six months, MS data shows the peak continues to diminish in intensity, suggesting a degradation or reduced peptide concentration. There is also a secondary peak around 4.2 minutes, which becomes less defined over time. The right panels display the intensity plotted against the mass-to-charge (m/z) ratio (x-axis). A consistent peak around 1037 m/z is observed across all time points, indicating that some peptides or peptide fragments remain stable during the six-month storage period. However, changes between 700-1000 m/z, show peaks shifts which vary in intensity, indicating potential breakdown products or modifications over time. The spectra are more complex and higher in intensity at 1 month, with significant declines by six months. Overall, the data suggests that acidic peptides stored at 22°C experience degradation over time, with a clear reduction in both peak intensities and overall peptide stability by six months of storage (figure 1).

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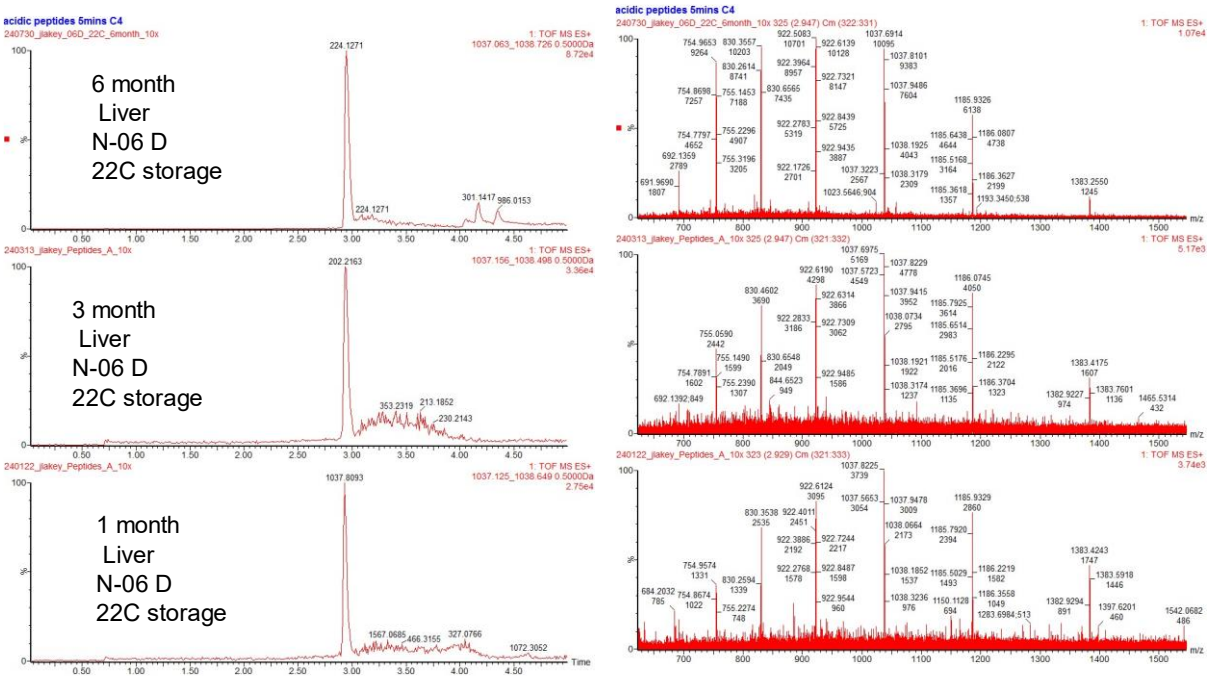

Figure 1: N-06A NOP assessed by MS stored at 4OC for six months.

The stability of a seven acidic peptide combination (N-18A) over time at room temperature analyzed by MS is presented in Figure 2. The mixed N-18A peptide was stored at 22°C for one, three- and six months. Retention time plots display the intensity plotted against time. At one month, a clear, sharp peak around three minutes, indicates a strong and stable peptide signal. AT 3 months, the peak remains prominent but appears to broaden slightly, and the intensity starts to diminish, suggesting a decrease in peptide stability. At six months, the main peak is still present, but the intensity is noticeably lower compared to earlier points, indicating further degradation or reduced peptide concentration. The right panels display the intensity plotted against the mass-to-charge ratio (m/z). Across all time points, there are consistent peaks around 1056 m/z, which indicates that certain peptide components remain stable over time. However, the 1-month data shows a more complex and intense spectrum with additional peaks in the lower m/z region (around 700-900 m/z), indicating a higher diversity of peptide fragments early on. By three months, some of these lower m/z peaks begin to weaken, and by six months, the spectrum shows a marked reduction in intensity, particularly in the lower mass range, suggesting degradation or loss of certain peptides. Overall, the data shows that N-18A stored at 22°C experiences progressive degradation over time, with a noticeable reduction in peptide stability and complexity by six months (figure 2).

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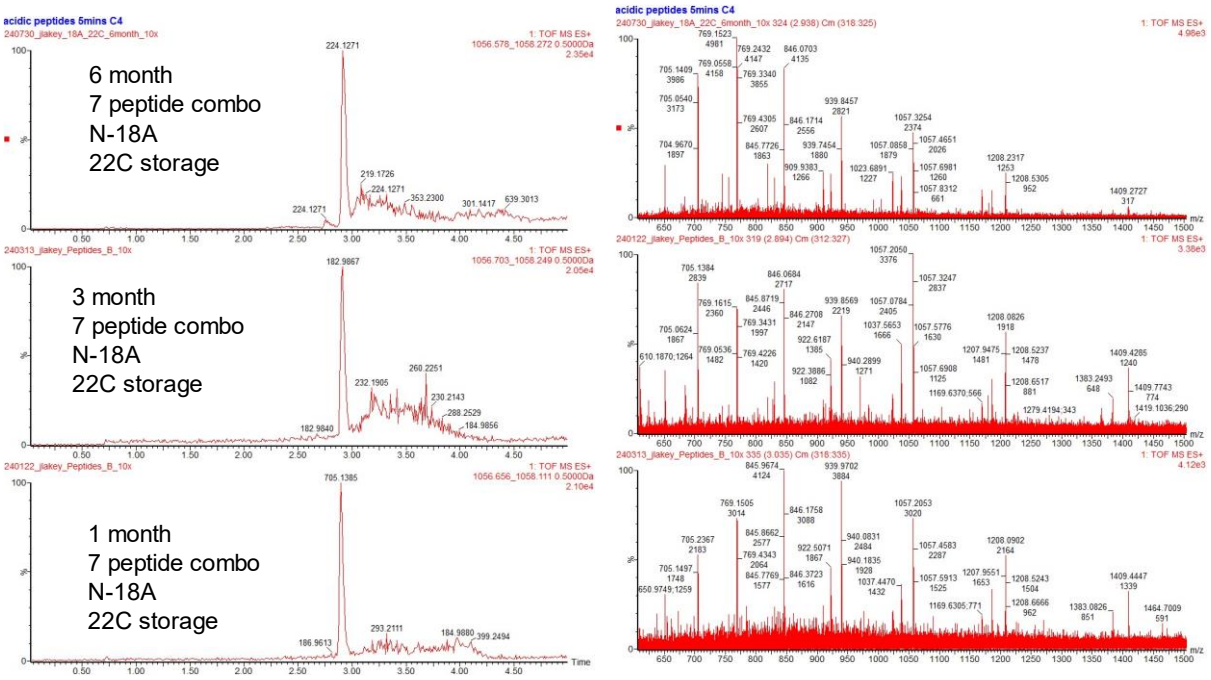

Figure 2: N-18A 7 peptide combination assessed by MS stored at 22OC for six months.

Figure 3 presents MS for a 7-peptide combination (N-18A) stored at 4°C over one- three-and six months. Left panels show intensity versus time, with a major peak around three minutes at each storage duration. The peak is sharpest at one- and three months. However, at six months, the peak intensity decreases and becomes less defined, suggesting some degradation or loss of peptide integrity over time. A secondary small peak around four minutes remains visible throughout, but its intensity also diminishes at 6 months. Right panels display the intensity versus mass-to-charge (m/z). Across all time points, a peak around 1057 m/z remains prominent, indicating the stability of a key peptide or peptide fragment in the mixture. However, changes are observed in lower m/z regions (around 650-850 m/z), where peak intensities and positions shift, suggesting peptide breakdown or modifications over time. Overall, the data shows that while some peptides in N-18A remain stable (e.g., around 1057 m/z), there is noticeable degradation and variability in the peptide profiles as the samples are stored for longer periods, particularly at the six-months (figure 3).

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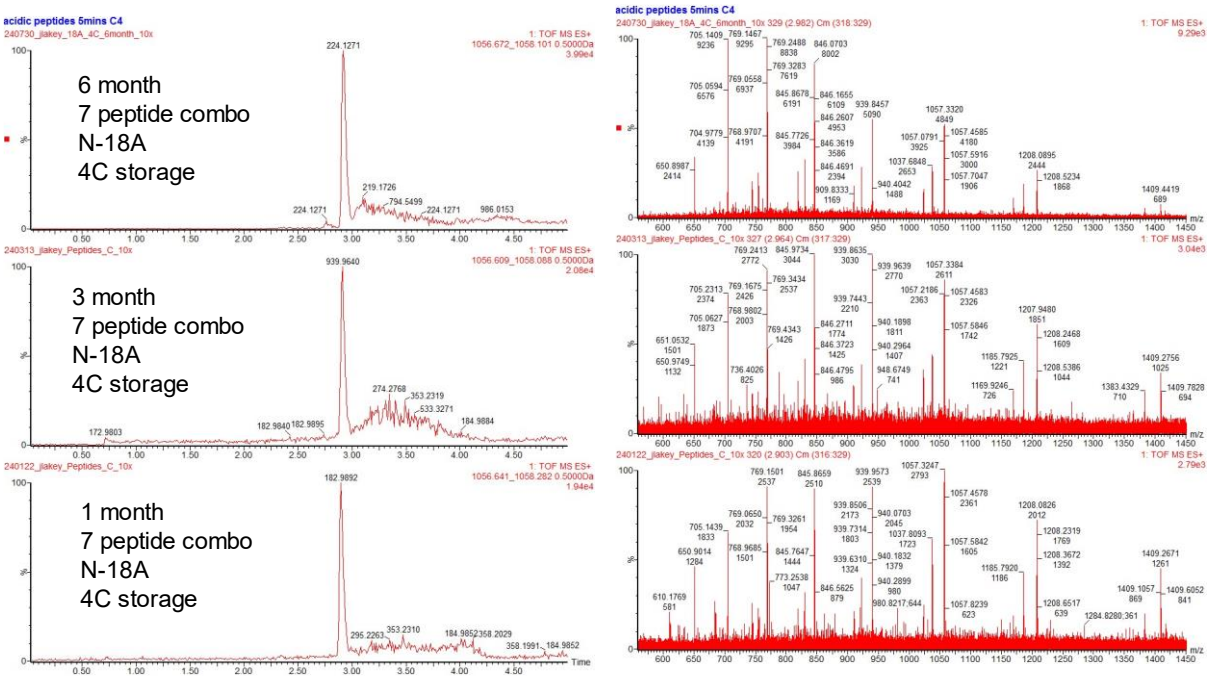

Figure 3: N-18A 7 peptide combination assessed by MS stored at 4OC for six months.

Figure 4 depicts MS comparing the stability of N-06D peptides in liver samples stored at 4°C over one-, three, and six months. Left panels show intensity plotted against time, highlighting peptide elution profiles for each storage duration. Across all time points, there is a prominent peak at approximately three minutes, though its intensity decreases from one month to six months, indicating a gradual loss in peptide abundance or stability over time. The peaks also become less defined and broader as storage time increases, especially at six months. Right panels display (intensity vs. m/z ratio), showing variations in the peptide mass-to-charge (m/z) profiles over time. The peak at 1037 m/z is consistently dominant across all times, suggesting the stability of N06D. However, changes in other peaks, especially between 700 and 1000 m/z, indicate alterations in the peptide composition or potential degradation products forming over time. In summary, the data suggests that while some peptides remain stable, there are notable changes in the peptide profiles over time (figure 4).

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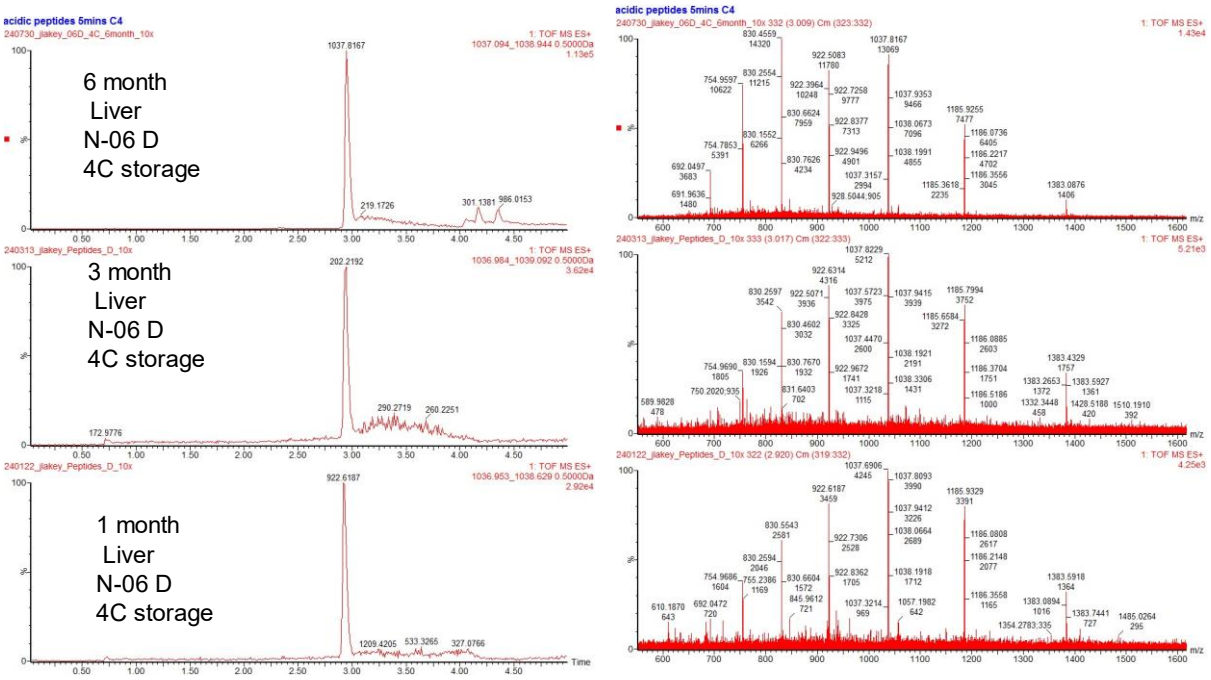

Figure 4: N-06A NOP assessed by MS stored at 22C for six months.

Discussion and Conclusion

Short peptides have been shown to play an important role in the modulation of transcription and transmission of biological information and have been found to decline in production during the natural process of aging [2]. Peptide therapy seeks to stimulate peptide production or restore normal signaling patterns by enhancing the strength of the signals received by cells. Since different tissues produce distinct peptides, the therapy uses organ-specific extracts to target aging or diseased tissues. The goal is to rejuvenate normal peptidergic signaling in these areas, thereby improving overall health and well-being. Through years of testing and development, European Wellness (EW) and MF-Plus has manufactured two products – Nano Organo Peptides (NOPs) – that are intended for use in peptide therapy in both humans and animals [7]. Despite numerous studies demonstrating the potential of NOPs in therapeutic applications such as cosmetics [8] and regenerative organ repair [9], little research has been done to investigate and identify the key peptides in these solutions. Mass spectrometry (MS) is a chemical analysis technique that enables the direct identification of molecules based on their mass-to-charge ratios and fragmentation patterns [14]. By comparing experimental MS data with that of well-established open-source databases, a determination can be made of the proposed identity of the molecules, peptides, or proteins found within a solution. Due to the low-cost and rapid application of MS in identifying the components of unknown solutions, our study employed MS as our primary method of identification.

Although our preliminary data relied upon LC-MS/MS based peptide sequencing techniques to produce chromatograms and deconvolute our data, we utilized MALDI-TOF MS identification techniques for our in-depth analysis due to a number of calculated benefits [15]. First, MALDI-TOF utilizes a protein fingerprinting method in which the sample is digested by a proteolytic enzyme such as trypsin and used to generate an MS spectrum that can be searched against a database. Matched hits are ranked according

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to a scoring method in which the candidate protein that contains more proteolytic peptides has a higher score and generally represents the protein/peptide that is most probable. The desirability of MALDI-TOF also includes the speed at which each run is performed– often less than one minute to obtain– and the speed at which analysis can be performed against a database. In contrast, data acquired through LC-MS/MS typically requires multiple hours to obtain due to the length of runs– sometimes requiring over thirty minutes for each run– and requires several hours to parse through the data to identify a potential candidate. Unlike MALDI-TOF, LC-MS/MS requires data to be manually sorted and compared against a database. This can be time consuming and won't necessarily guarantee the most accurate result.

The results of our study observed slight differences in peptide products between batches is likely due to the heterogeneous nature of cellularly-derived solutions and differences that occurred during the extraction process. Further research must be conducted to confirm or disprove this theory.

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Institutional Review Board Statement

Not applicable

Data Availability Statement

The data presented in this study are available in the study outlined.

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Conflicts of Interest

The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manusxcript, or in the decision to publish the results.

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