Clinical Medicine Insights
Received 16 Mar 2022 | Revised 30 Apr 2022 | Accepted 20 May 2022 | Published Online 8 June 2022

DOI: https://doi.org/10.52845/CMI/2022-3-2-2
CMI 03 (02), 297–303 (2022)

ISSN (O) 2694-4626

RESEARCH ARTICLE

Maldi-Tof Analysis of Mitochondrial Peptides

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Abstract
Short peptides are known to play an important role in modulating transcription, in transmitting biological information and in restoring the genetic alterations that occur with aging. This paper aims to describe a method of identifying the population of peptides within a peptide cocktail formulation. A sample of lung-derived Mito Organelle (MO) Peptides (LBS) of specific pathogen free (SPF) mammalian rabbits sourced from Charles River Labs were analyzed by mass spectrometry and chromatograms were generated for further examination. The experimentally derived peaks were compared between two batches of LBS MO peptides using MALDI-ToF mass spectroscopy. The following report outlines the experimental methods and the results from performing MALDI-ToF mass spectrometry on various peptides from the company European Wellness (EW).

Keywords: peptides, Mito Organelles (MO) Peptides, mass spectrometry, MALDI-ToF

Introduction

Peptides are linear polymers formed by a series of amino acid residues that are linked together through peptide bonds [1]. In comparison to proteins, which typically contain between 50 and 2000 amino acid residues and have a mean molecular weight between 5.5 and 22 kDa, peptides have fewer than 50 residues and a reduced weight. A unique set of proteins and peptides are produced in each cell line and these play a part in the regulation of biological homeostasis [2]. Peptides have been demonstrated to play an important role in modulating transcription, transmission of biological information, and in restoring the genetic alterations that occur with age [3, 4]. These peptides are signaling molecules that act as regulatory factors through their interaction with DNA and histone proteins. Moreover, the physiological process of aging is highly influenced by the peptidergic regulation of homeostasis. As part of the aging process, the frequency and strength of signals to the mitochondria declines, causing signals to be sent back to the nucleus that cause the arrest of cell proliferation and initiation of apoptosis (Haas, 2019; Akbari, Kirkwood, and Bohr, 2019) [5-6].

Peptide therapy aims to renew the strength of signals received by cells to either induce peptide production or renew normal signaling processes, thereby rejuvenating and revitalizing tissues as well as the organism as a whole [3, 4, 7-9]. Although the content of peptides is similar between cells, the function and morphology of each cell defines the contents of its biologically active substances and unique ultrastructures. Moreover, certain biologically active substances are predominantly synthesized or accumulated in specific tissues. Since the signaling activity and function of peptides is largely based on the cell type, peptide therapy utilizes organ-specific extracts to target diseased or aging tissue. Due to the short lengths of peptides (<45 residues) and their low molecular weights, their biosynthesis and extraction processes permit mass production and distribution for use in therapeutic treatments [3]. Through years of research and extensive global practice, MF-Plus has manufactured two products, Nano Organo Peptides (NOPs) and Mito Organelles (MO) Peptides, which are intended for use in both animals and humans as a revitalization therapy [10].

Mito Organo (MO) peptides are biologically extracted mixtures of cellular peptides that have predominantly mitochondria-specific functions [11]. Although cells of different organ systems have similar functions, variations in cellular functions between organs creates the differential expression of peptides, which can be utilized for various therapeutic purposes. MO peptides are organ-specific extracts that are aimed at revitalizing and rejuvenating mitochondrial activity, thereby regenerating cells and organisms as a whole [12,13].

Despite the numerous studies highlighting the therapeutic effectiveness of MO peptides, little is known of the exact makeup of these formulations. Matrix-assisted laser desorption/ionization time of flight (MALDI-ToF) mass spectrometry has been able to identify and quantify analytes in complex solutions and allows for highly sensitive, fast and high-throughput analysis [14,15]. In addition, it 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 mass-to-charge ratios. By searching the experimentally-derived peaks against a database of known proteins, it may be possible to identify the peptides. The following report outlines the experimental methods and the results of performing MALDI-ToF mass spectrometry on various EW peptides.

2. Materials and Methods

Two batches of LBS MO201901 EW peptides were suspended in saline solution upon collection. Formulations with the characterization LBS were extracted from lung samples in specific pathogen free (SPF) mammalian rabbits sourced from Charles River Labs. All samples were kept on ice throughout the duration of the experiment and were handled using good laboratory practices.

Sample Preparation

The ThermoFisher Scientific BCA Protein Assay protocol was utilized to determine the protein concentrations of the unknown peptide solution. Triplicate sample readings were obtained for the peptide solution using the Tecan Infinite F200 microplate reader at a wavelength of 570 nm.

Sample Preparation of LBS MO Samples

Peptides from the LBS MO samples were withdrawn at a volume of 30 µg/mL and pipetted into auto column tubes in an agarose gel. Sample mixtures were separated by molecular weight with SDS-PAGE and Coomassie™ blue staining to visualize the proteins. An individual protein band was cut out from the gel and placed into a low-binding, siliconized microcentrifuge tube. Proteins were then de-stained in the tube with 100µl of a 1:1 methanol and water solution and vortexed. The gel piece was further washed by removing the de-stained solution in the tube and adding 400µl of water. Tubes were shaken for 15 minutes at room temperature, and the de-staining was continued until the gel became colorless with a minimum of 3 repeats. After destaining, 400µl of 100% acetonitrile was added to dehydrate the gel for 10 minutes 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 then incubated for an additional 30 minutes at

room temperature under 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 three times for 15 minutes. The gel was subsequently dehydrated with 400µl of 100% acetonitrile for 10 minutes and dried in a vacuum centrifuge after removal of the supernatant.

Enzyme Digestion

A pH 8 protease trypsin solution 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 of 50% per volume acetonitrile, 1% trifluoroacetic acid (TFA) and 49% water was added, which was followed by incubation at room temperature and a vortex cycle of 20 minutes. This solution was combined with the supernatant from the peptides in the new microcentrifuge 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% 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 batch were analyzed together and chromatograms for each were generated. The masses of the three most prominent peaks were deconvoluted and the key peptides and amino acid components were determined against open-source databases.

3. Results

The concentration of peptides in each batch was initially determined via the ThermoFischer™ Scientific BCA Protein Assay (Figure 1). The average protein concentration of 252.0 µg/mL (±12.83µg/mL) and 260.6µg/mL (±32.23µg/mL) was determined for Batch 1 and Batch 2 respectively. There were no statistically significant differences between batches (p = ns).

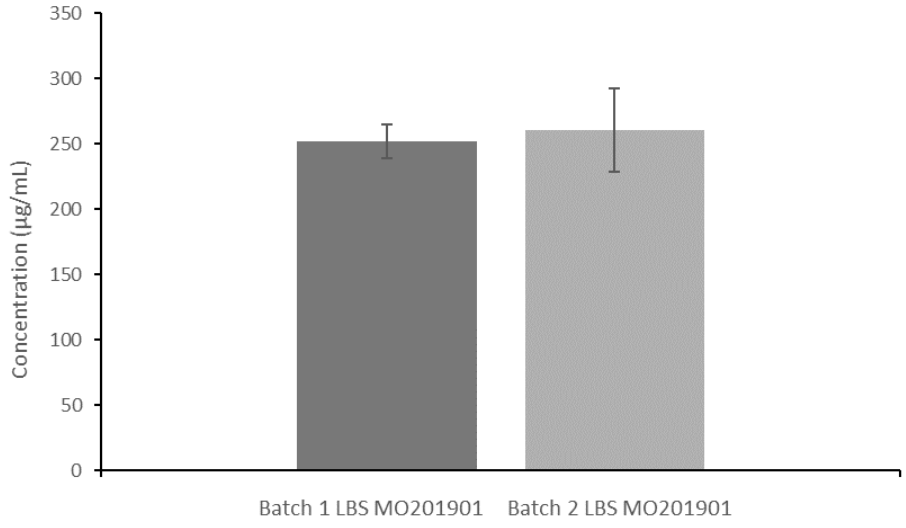

Figure 1. Average protein concentration (ug/mL) of Batch 1 and 2 LBS MO201901 peptides.

Standard error bars are included. No statistically significant differences were found between batches (p=ns).

The samples were then separated by molecular weight with SDS-PAGE and digested with the protease trypsin. Once prepared, the samples were run on MALDI-Tof in triplicate and chromatograms were generated (Figure 2). The deconvoluted masses of Batch 1 (Figure 3) and Batch 2 (Figure 4) of LBS MO show similarity in major protein components. The deconvoluted masses in Batch 1 represent five major components with sizes of 14,969 Da, 15,300 Da, 8,449 Da, 8,294 Da and 4,618 Da respectively. Batch 2 results showed six major protein masses of 14,969 Da, 15,301 Da, 8,294 Da, 8,449 Da, 5,436 Da, and 6,214 Da respectively.

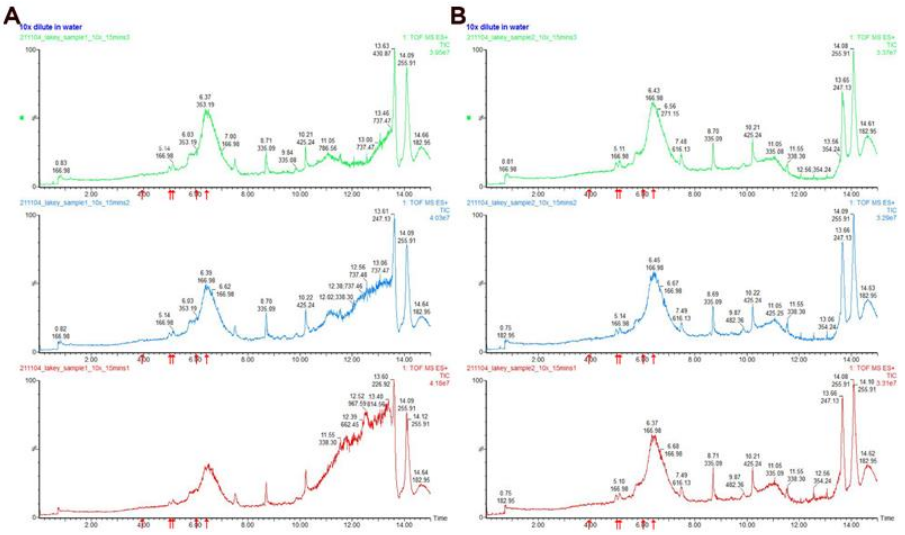

Figure 2. Chromatograms obtained from Batch 1 (A) and Batch 2 (B) of LBS MO201901.

Red arrows pointing upwards on the x-axis of each chromatogram identify peaks that correspond to differing peptide components in the solution. The green, blue, and red chromatograms associated with both Batch 1 and Batch 2 illustrate different runs of the sample.

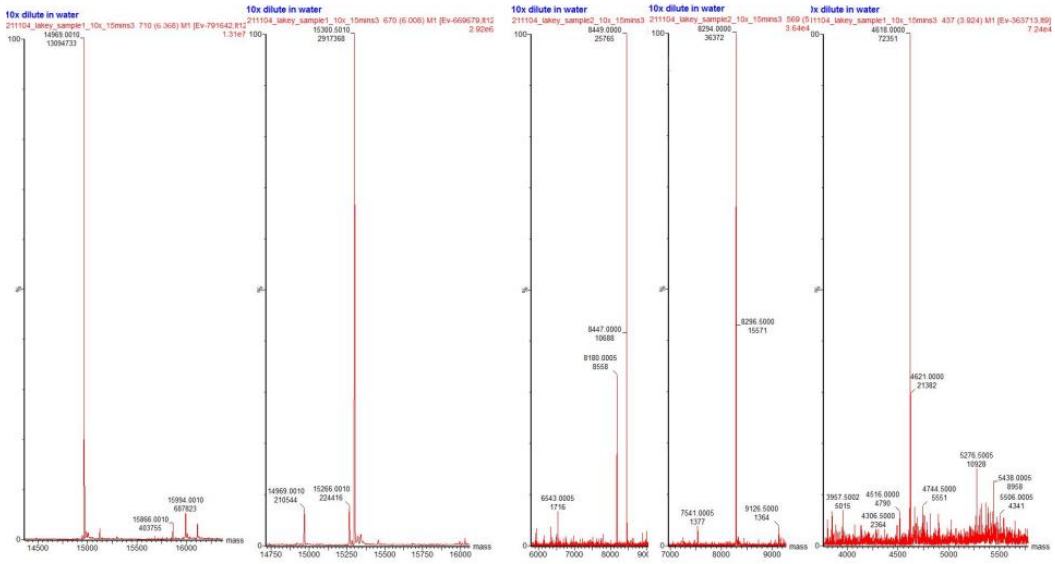

Figure 3. Deconvoluted mass spectrometry data obtained from LBS MO Batch 1.

Five peptide fragments are ordered from left to right in decreasing order of abundance within the sample. The relative sizes of peptides from left to right are 14,969 Da, 15,300 Da, 8,449 Da, 8,294 Da and 4,618 Da respectively.

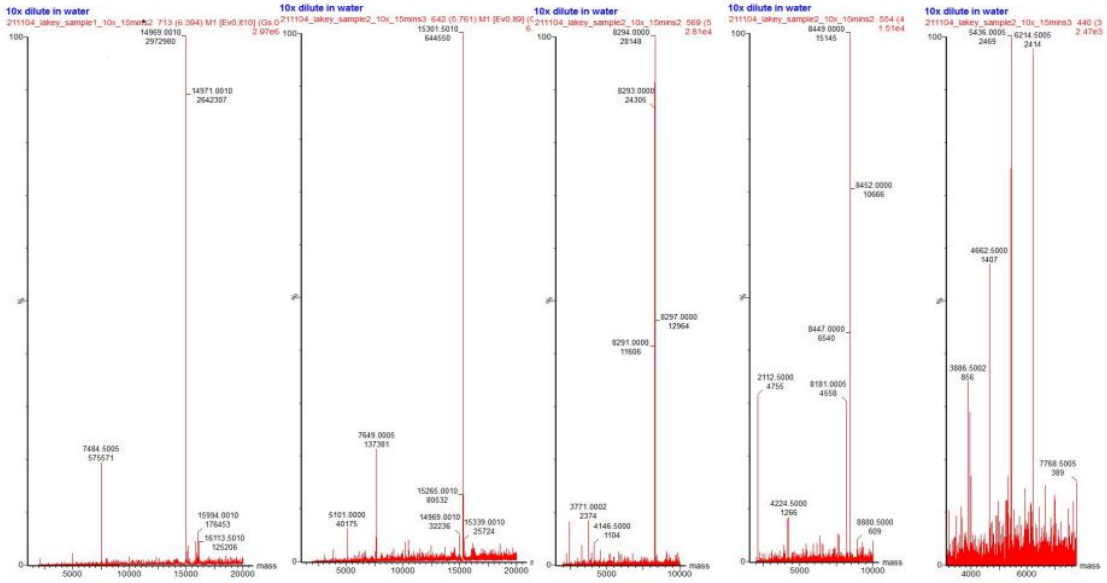

Figure 4. Deconvoluted mass spectrometry data obtained from LBS MO Batch 2.

Six peaks representing six major peptide fragments are ordered from left to right in decreasing order of abundance within the sample. The relative sizes of peptides from left to right are 14,969 Da, 15,301 Da, 8,294 Da, 8,449 Da, 5,436 Da, and 6,214 Da respectively.

4. Discussion

Short peptides are known to play a critical role in modulating the transmission and transcription of biological information and have been shown to decline throughout the natural process of aging [2]. Peptide therapy aims to either reinstate normal signaling patterns by renewing the strength of the signals received by cells or induce the cells to begin to produce peptides of their own [16]. Since different tissue types produce different peptides, peptide therapy utilizes organ-specific extracts to target aging or diseased tissue with the goal of revitalizing normal peptidergic signaling in these regions and improving overall health and wellbeing [17].

Through years of testing and development, European Wellness (EW) and MF-Plus have manufactured Mito Organelles (MO) peptides that are intended for use in peptide therapy in both animals and humans [18]. Despite numerous studies demonstrating the potential of MOs in therapeutic applications such as cosmetics [19] and regenerative organ repair [11], 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 [20]. By comparing experimental MS data with that of well-established open-source databases, the identity of the molecules, peptides, or proteins can be 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.

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 existing databases [21, 22]. Matched hits are ranked according to a scoring method in which the candidate protein that contains more proteolytic peptides has a higher score and generally represents the most probable protein/peptide. 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.

Conclusion:

The results of our study using MALDI-TOF to analyze the MO LBS sample indicated that there were five major peptide products of interest in Batch 1 and six major peptide products in Batch 2 (Figure 3 and 4). Following deconvolution, five

peptides were identified in Batch 1 (Figure 3) with masses of 14,969 Da, 15,300 Da, 8,449 Da, 8,294 Da and 4,618 Da. Batch 2 identified four of the same peptides– 14,969 Da, 15,301 Da, 8,294 Da, 8,449 Da in size– and two additional peptides of 5,436 Da, and 6,214 Da in size (Figure 4). 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 the identity of the peptides and discover their significance in peptide therapy.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

Author Contributions: Conceptualization, J.L., M.W., M.C., D.K., D.C.; methodology, B.K., M.A.; validation, B.K.; formal analysis, B.K.; investigation, A.W., A.G., B.K.; data curation, A.W., A.G., B.K.; writing—original draft preparation, A.W., A.G.; writing—review and editing, A.W., A.G., J.L.; visualization, J.L., M.W., M.C., D.K.; supervision, J.L., M.W., M.C., D.K., D.C., B.K., A.M.; project administration, J.L., M.W., M.C., D.K., D.C., B.K.; funding acquisition, J.L., M.W., M.C., D.K., D.C.. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by a grant to the University of California, Irvine from European Wellness Biomedical Group.

Institutional Review Board Statement: Not applicable

Data Availability Statement: The data presented in this study are available in the study outlined.

Acknowledgments: The authors wish to acknowledge the support of the Department of Surgery and the Core Mass Spectrometry laboratory of the University of California, Irvine for their support.

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 manuscript, or in the decision to publish the results.

References

1. Berg, J. M.; Tymoczko, J. L.; Stryer, L., Primary Structure: Amino Acids Are Linked by Peptide Bonds to Form Polypeptide Chains. In Biochemistry. 2002; 5.
2. Caputi, S.; Trubiani, O.; Sinjari, B.; Trofimova, S.; Diomede, F.; Linkova, N.; Diatlova, A.; Khavinson, V., Int J Immunopathol Pharmacol. 2019;33:2058738 419828613.
3. Khavinson, V. K., Lin'kova, N. S., & Tarnovskaya, S. I. Short Peptides Regulate Gene Expression. Bulletin of experimental biology and medicine. 2015; 162(2):288–292.
4. Apostolopoulos, V., Bojarska, J., Chai, T. T., Elnagdy, S., Kaczmarek, K., Matsoukas, J., New, R., Parang, K., Lopez, O. P., Parhiz, H., Perera, C. O., Pickholz, M., Remko, M., Saviano, M., Skwarczynski, M., Tang, Y., Wolf, W. M., Yoshiya, T., Zabrocki, J., Zielenkiewicz, P., … Toth, I. A Global Review on Short Peptides: Frontiers and Perspectives. Molecules (Basel, Switzerland). 2015; 26(2):430.
5. Haas R. H. Mitochondrial Dysfunction in Aging and Diseases of Aging. Biology. 2019; 8(2):48.
6. Akbari, M., Kirkwood, T., & Bohr, V. A. Mitochondria in the signaling pathways that control longevity and health span. Ageing research reviews. 2019;54:100940.
7. Klokol, D., Nallenthiran, L., Chan, M.K.S., et al. Cell therapy as the main stratagem of anti-aging and regenerative medicine. Europ J Pharm Med Res. 2019; 6(6):295–299.
8. Lee, A. C., Harris, J. L., Khanna, K. K., Hong, J. H. A comprehensive review on current advances in peptide drug development and design. International journal of molecular sciences. 2019; 20(10):2383.
9. De La Torre, B. G., Albericio, F. Peptide therapeutics 2.0. Molecules (Basel, Switzerland). 2020;25(10):2293.
10. Klokol, D.; Chan, M.; Wong, M.; Tullina, D.; Chernykh, V.; Wah, Y. K.; Rivera-Dugenio, J., Journal of Stem Cell Research and Medicine. 2017; 2(2):1-5.
11. Lee, C., Zeng, J., Drew, B. G., Sallam, T., Martin-Montalvo, A., Wan, J., Kim, S. J., Mehta, H., Hevener, A. L., de Cabo, R., & Cohen, P. The mitochondrial-derived peptide MOTS-c promotes metabolic homeostasis and

reduces obesity and insulin resistance. Cell metabolism. 2016;21(3):443–454.
12. Wu, D., Kampmann, E., Qian, G. Novel insights into the role of mitochondria-derived peptides in myocardial infarction. Frontiers in Physiology. 2021;12.
13. Krejcova, G., Patocka, J., and Slaninova, J. Effect of humanin analogues on experimentally induced impairment of spatial memory in rats. J. Pept. Sci. 2004;10: 636–63 9.
14. Hopfgartner G.r., Bourgogne E. Quantitative high-throughput analysis of drugs in biological matrices by mass spectrometry. Mass Spectrometry Reviews. 2003; 22(3):195–214.
15. Szaéjli, Emiélia et al. Investigating the Quantitative Nature of MALDI-TOF MS Molecular & Cellular Proteomics. 2008; 7(12):2410 - 2418
16. Bruno, B. J., Miller, G. D., & Lim, C. S. Basics and recent advances in peptide and protein drug delivery. Therapeutic delivery. 2013; 4(11):1443–1467.
17. Järvinen, T. A., May, U., & Prince, S. Systemically Administered, Target Organ-Specific Therapies for Regenerative Medicine. International journal of molecular sciences. 2015; 16(10):23556–23571.
18. Chan, M. K. S., Wong, M. B. F., Béguin, A., Teppone, M., Tukhvatullina, D., Klokol, D. In Efficacy of the MFIII placenta extracts softgels supplementation: a randomized double-blind placebo-controlled study, 2016.
19. Chan, M. K., Michelle, W. B., Klokol, D., Pong, H., Wolodymyr, C., International Journal of Current Medical and Pharmaceutical Research. 2017;3(1): 1278-1281.
20. Padoan A, Basso D, Zambon CF, et al. MALDI-TOF peptidomic analysis of serum and post-prostatic massage urine specimens to identify prostate cancer biomarkers. Clin Proteomics. 2018;15:23.
21. Singhal, N., Kumar, M., Kanaujia, P. K., Virdi, J. S. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Frontiers in microbiology. 2015; 6: 791.
22. Urban P. L. Quantitative mass spectrometry: an overview. Philosophical transactions. Series A, Mathematical, physical, and engineering sciences, 2016; 374(2079): 20150 382.

How to cite this article: Good, A. ., Wells, A. ., Katz, B. ., Alexander, M. ., Klokol, D. ., Chen, M. K. ., Wong, M. B. ., Cox, D. C. ., & Lakey, J. R. . (2022). MALDI-ToF Analysis of Mitochondrial Peptides. Clinical Medicine Insights, 3(2), 297–303. https://doi.org/10.52845/C MI/2022-3-2-3