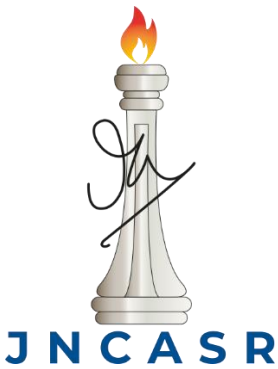
SUMMER RESEARCH FELLOWSHIP PROGRAM (SRFP)-2024

Synthesis of Bioactive Peptides using Solid Phase Peptide Synthesis for Alzheimer's Therapeutics



Submitted by

Neerav Sreekumar

SRFP – 2024

Under the supervision of

Prof. T. Govindaraju

Bioorganic Chemistry Laboratory

New Chemistry Unit

JAWAHARLAL NEHRU CENTRE FOR ADVANCED SCIENTIFIC RESEARCH

JAKKUR, BANGALORE

20th May, 2024 – 20th July, 2024

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CERTIFICATE

I hereby certify that the matter embodied in the report entitled **'Synthesis of Bioactive peptides using Solid Phase Peptide Synthesis for Peptide-based Alzheimer's Therapeutics'** is a bonafide record of the project work carried out as a part of the Summer Research Fellowship Program (SRFP-2024) under my guidance by **Mr. Neerav Sreekumar**, at the Bioorganic Chemistry Laboratory, New Chemistry Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India.

Prof. T. Govindaraju

Bioorganic Chemistry Laboratory

New Chemistry Unit, JNCASR

DECLARATION

I, **Mr. Neerav Sreekumar,** hereby declare that this project report entitled **'Synthesis of Bioactive Peptides using Solid Phase Peptide Synthesis for Alzheimer's Therapeutics'** is the result of the project work done by me under the supervision of **Prof. T. Govindaraju** at Bioorganic Chemistry Laboratory, New Chemistry Unit, Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bengaluru, as a part of the **Summer Research Fellowship Programme-2024** and that it has not been submitted elsewhere for the award of any diploma or degree.

Neerav Sreekumar

ACKNOWLEDGEMENT

I would like to express my heartiest gratitude to my supervisor Prof. T. Govindaraju for his generous acceptance to guide me during the summer project and his immeasurable support throughout the project.

I am genuinely thankful to Mr. Sabyasachi Mandal, New Chemistry Unit, JNCASR, for his valuable guidance throughout the project. My appreciation also extends to all my lab mates Dr. Soumik Dinda, Dr. Suresh R., Dr. Sumon Pratihar, Dr. Madhu R., Dr. Abu Sufian, Dr. Ashish Kumar, Dr. Saheli Sarkar, Mr. Shiva Prasad P. S., Ms. Dikshaa Padhi, Mr. Hariharan Moorthy, Mr. Milind Kumar Anand, Ms. Krithi K. Bhagavat, Mr. Jikesh Bhoi, Mr. Augustya Gupta, Ms. Shreyasri Sain, Ms. Piyasi Garai, Ms. Kavita N, Ms. Aparna, and my fellow summer interns, Mr. Chinmoy, Mr. Anjan, Ms. Unnati Jain, Ms. Aishani Sengupta, Ms. Debdatta Mandal for their support at every moment in this tenure. I would also like to thank JNCASR, Bengaluru, India, for giving me this chance to be a part of SRFP-2024. I thank my friends and family for being my constant source of support.

Sincerely,

Neerav Sreekumar

CONTENTS

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder marked by progressive cognitive decline, memory impairment, and behavioural changes.[1,2] The disease's pathology includes the formation of amyloid-beta (Aβ) plaques, neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein, synaptic dysfunction, neuronal loss, and neuroinflammation thanks to reactive oxidation species (ROS), which all contribute to making it a multifactorial disease. Peptides play a significant role in both the progression and potential treatment of AD. Essential peptides implicated in AD include Aβ, derived from the amyloid precursor protein (APP), and tau, a microtubule-associated protein.[1-4]

Peptide-based therapies offer innovative approaches to addressing AD pathology. These therapies prevent Aβ aggregation, enhance Aβ clearance, and reduce tau pathology. Other approaches include designing inhibitory peptides to prevent Aβ aggregation and developing immunotherapies and peptidomimetics to enhance Aβ clearance. Additionally, peptides that inhibit tau aggregation and modulate its phosphorylation can mitigate tau pathology. Despite the promise of peptide-based therapies, challenges such as effective brain delivery, stability, bioavailability, and specificity must be addressed.[5,6] Peptide-based therapies represent a promising avenue for AD treatment, targeting the disease's core pathological processes.

In this project, we have used solid phase peptide synthesis (SPPS), starting from a Rink Amide Resin and using Fmoc chemistry to synthesise various peptides. We also added an unnatural amino acid group to a particular number of peptides to preserve the peptide sequence's function and compare proteolytic enzymes' effect on the cell culture of modified peptides to unmodified peptides.

2. Details of Synthesized Peptides

1. **His-Lys (HK):** The HK peptide (His-Lys) has shown potential in AD research due to its copper-binding properties. HK peptide can help mitigate the metal-induced aggregation of amyloid-beta (Aβ) peptides by chelating copper ions, a hallmark of AD pathology. This reduction in Aβ aggregation can potentially decrease plaque formation and associated neurotoxicity. The HK peptide's ability to modulate metal homeostasis and reduce oxidative stress positions it as a valuable candidate for further investigation in AD therapeutic strategies.
2. **Gly-His-Lys (GHK):** The GHK peptide (Gly-His-Lys) has emerged as a promising candidate in AD research due to its multifunctional properties. GHK peptide exhibits a strong affinity for binding copper ions, which play a crucial role in the pathogenesis, which can potentially reduce amyloid-beta (Aβ) aggregation, thereby decreasing plaque formation and associated neurotoxicity. Additionally, GHK peptide has demonstrated antioxidant and anti-inflammatory effects, which can further mitigate oxidative stress and neuroinflammation, critical contributors to AD pathology. The neuroprotective and regenerative properties of GHK peptide make it a valuable candidate for developing novel therapeutic strategies for Alzheimer's disease.[7]
3. **Gly-Gly-His-Lys (GGHK):** By binding copper ions, GGHK peptide can help reduce the metal-induced aggregation of amyloid-beta (Aβ) peptides, a hallmark of AD pathology. This reduction in Aβ aggregation can potentially decrease plaque formation and associated neurotoxicity. Furthermore, GGHK peptide has demonstrated antioxidant and anti-inflammatory properties, which can mitigate oxidative stress and neuroinflammation, both of which are key contributors to AD.
4. **Asp-Ala-His-Lys (DAHK):** The DAHK peptide (Asp-Ala-His-Lys) has shown significant potential in AD research due to its copper-binding properties. By chelating copper ions, the DAHK peptide can help reduce the metal-induced aggregation of amyloid-beta (Aβ) peptides, a key pathological hallmark of AD. This reduction in Aβ aggregation can potentially decrease plaque formation and associated neurotoxicity. Additionally, the DAHK peptide exhibits antioxidant properties, which can further mitigate oxidative stress, another contributor to AD pathology.[8]
5. **Gly-His-His-Lys (GHHK):** Similar to GGHK and GHK, GHHK is known for its metal chelation properties and a positive response to neuro-inflammation and clearance of plaques, thus holding potential in tackling the multifactorial nature of AD. [9]

6. **Arg-His-Lys (RHK):** By chelating copper ions, RHK peptide can help reduce the metal-induced aggregation of amyloid-beta (Aβ) peptides, a hallmark of AD pathology. This reduction in Aβ aggregation can potentially decrease plaque formation and associated neurotoxicity. Additionally, RHK peptide exhibits antioxidant and anti-inflammatory properties, which can mitigate oxidative stress and neuroinflammation, both key contributors to AD, though limited research has been done on its properties.
7. **Phe-Arg-His-Asp (FRHD):** Existing research data on FRHD has been limited. FRHD peptide may interact with metal ions such as copper and zinc, which are implicated in the aggregation of amyloid-beta (Aβ) peptides, a hallmark of AD. By chelating these metal ions, FRHD could reduce Aβ aggregation, thereby decreasing plaque formation and associated neurotoxicity. Additionally, peptides with antioxidant and anti-inflammatory properties can mitigate oxidative stress and neuroinflammation, both significant contributors to AD pathology.[11]
8. **Other Peptides Synthesized:** HVDLSTLMS

The unnatural amino acid added to all the peptides was N-methyl glycine.

3. Methods

3.1. Synthesis of Peptide Chain using Rink Amide Resin

Attachment of the 1st amino acid to the insoluble polymeric support called resin is the first step. Resin serves as a solid phase support onto which peptide will be built. Resin is the solid phase insoluble support onto which peptides can be built. We use the Rink Amide Resin for our synthesis, allowing easy purification and filtration at each step. The amino acid is then covalently attached to the resin through the carboxyl group, leaving its amino group free to react in subsequent steps. Washing Rink amide resin with dichloromethane (DCM) is a crucial step in solid-phase peptide synthesis (SPPS) to facilitate resin swelling. Swelling is essential for ensuring efficient coupling reactions and high yields of the desired peptide. DCM is used because it is a highly effective solvent for swelling polystyrene-based resins, which include Rink amide resin. This swelling process increases the resin's surface area and pore volume, allowing better access of reagents to the reactive sites within the resin.[10,11]

We begin by taking 0.62 mmol of resin, and washing it with DCM twice, for 10 minutes, until the resin swells. Then, we wash the resin with DMF for 5 minutes, which is essential to remove soluble byproducts. We then deprotected the resin from the Fmoc group using 20% piperidine wash twice for 20 minutes. Unlike Boc-protected groups, Fmoc protection requires a mildly basic nature compared to a strongly acidic environment for Boc for resin deprotection (orthogonality), thus making it a preferable option for SPPS. After the piperidine wash, we finally washed the resin mixture with DMF 4 times, 6 minutes each, to prepare for the coupling reaction. We take 3.1 mmol of the required Fmoc-amino acid in a conical flask to start the coupling process. We proceed to add 3.3 mmol of HBTU which acts as a coupling agent for SPPS. HBTU is used to activate the carboxyl group of an amino acid. In the presence of a base (DIPEA), HBTU forms an active ester. This ester reacts with the amine group of the growing peptide chain, forming a peptide bond. We also add 3.3 mmol of HOBt to enhance the coupling and stabilize the intermediate ester. One of the primary reasons for adding HOBt is to minimize racemization during the coupling reaction. Racemization leads to the formation of D-amino acids, which can compromise the peptide's biological activity and structural integrity. HOBt forms an active ester with the carboxyl group of the amino acid, which is less prone to racemization than other activated intermediates. We dissolve this mixture in 15 mL DMF as coupling happens in the liquid state. As mentioned earlier, HBTU forms an active ester in the presence of DIPEA (base), so after dissolving, we add 1.5 mL of DIPEA. After the DMF wash, we add our amino acid solution and put the coupling reaction for 90 minutes in the instrument, using a 360-degree rotation wash.

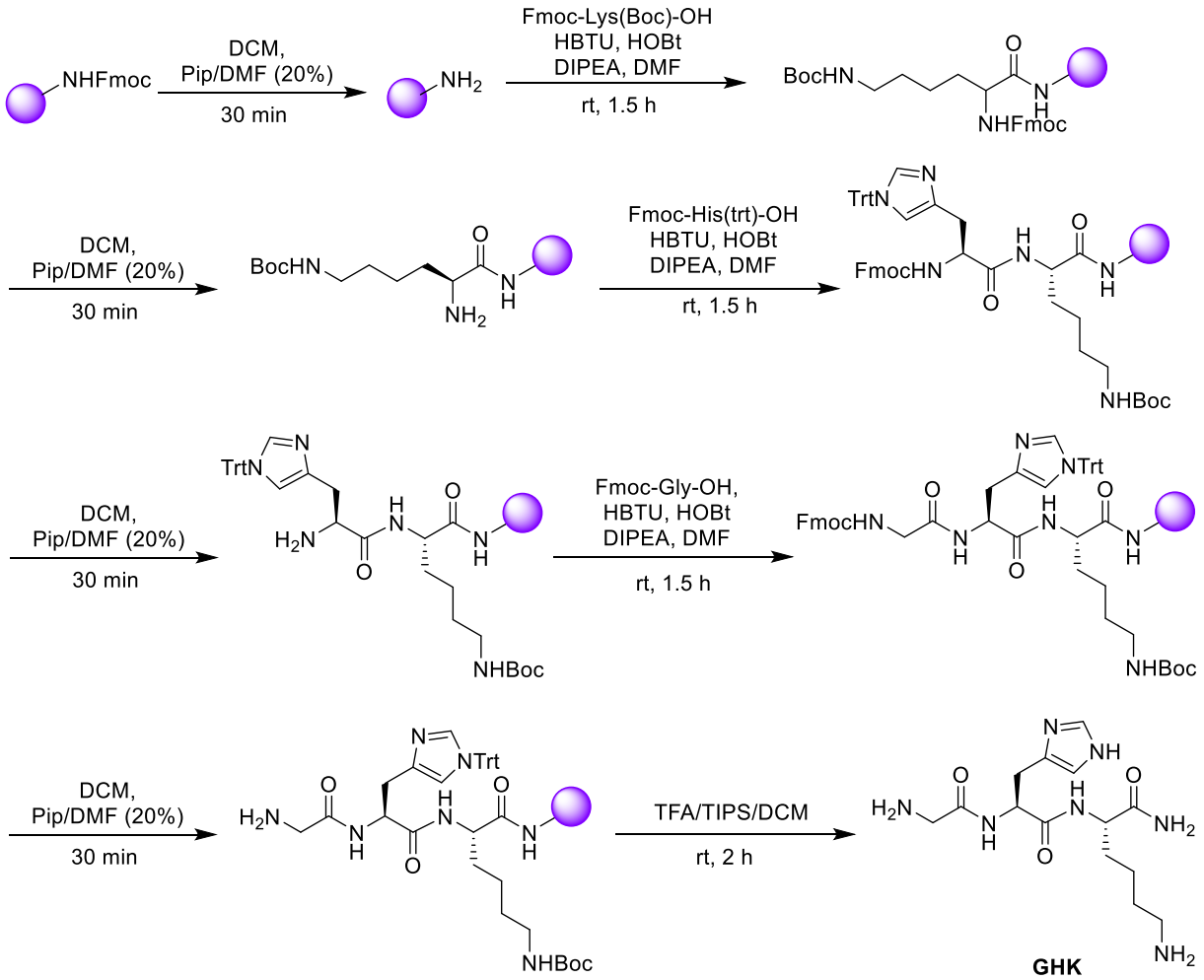


Figure 1. Schematic Diagram of SPPS reaction cycle

3.2. Kaiser Test

The Kaiser test is used to detect the nature of amines. We take 10 microlitres of each of our 3 reagents- Ninhydrin, Phenol, and Potassium Cyanide in an Eppendorf and heat the mixture to 96-100 degrees Celsius for 5 minutes. In the case of primary amines, the mixture turns dark blue. After adding piperidine, the mixture gets deprotected, and the primary amines give a dark blue colour, thus verifying Fmoc deprotection. However, no more primary amines are left after the coupling is completed. Thus, the Kaiser test shows no colour change upon heating for secondary and tertiary amines, helping us verify whether coupling was successful.

3.3. Cleavage of Peptide

In solid-phase peptide synthesis (SPPS), the cleavage process is crucial for releasing the synthesized peptide from the resin and removing protecting groups. This is typically achieved using trifluoroacetic acid (TFA) and scavengers such as triisopropylsilane (TIPS). The resin-bound peptide is first washed to remove residual solvents and reagents. Then, a cleavage cocktail,

primarily composed of TFA and scavengers, is added to the resin. The mixture is stirred at room temperature and incubated for 2 hours, depending on the complexity of the protecting groups. Scavengers are added along with trifluoroacetic acid (TFA) during the cleavage process in solid-phase peptide synthesis (SPPS) to protect the peptide from unwanted side reactions and to ensure the complete removal of protecting groups. TFA can generate reactive by-products that may attack and modify the peptide's side chains. Scavengers such as triisopropylsilane (TIPS) neutralize these reactive species, preventing damage to sensitive functional groups. This not only enhances the efficiency of the cleavage process by ensuring all protecting groups are effectively removed but also minimizes the formation of side products, leading to higher purity of the final peptide.

To prepare the cleavage cocktail of 5 mL, we need 95% TFA, 2.5% DCM and 2.5% TIPS. Thus, add 4.75 mL of TFA in a falcon tube and 0.125 mL of both DCM and TIPS (scavenger) and add it to the vacuumed mixture of peptide attached to the resin after deprotecting it using piperidine and washing it with DMF 4 times, 6 minutes each. Put the cleavage reaction for 2 hours, with an amplitude of 180 degrees. Meanwhile, store 40 mL of diethyl ether for precipitation in the fridge for cooling. After cleaving, wash the resin mixture with DCM and collect the peptide residue in the falcon with cold diethyl ether for precipitation. DCM is used because it acts as a solvent in which both the peptide and TFA cleavage mixture are soluble, facilitating the effective precipitation of the peptide in the presence of diethyl ether.

3.4. Centrifugation:

Centrifugation uses the principle of centrifugal force during rotation at high speeds to separate particles of different densities. In our mixture comprising of the solid peptide precipitated in diethyl ether, we use centrifugation to separate the solid peptide before checking its mass using MALDI-TOF and purification using HPLC. We set the centrifuge at 4 degrees Celsius to ensure the precipitate doesn't dissolve in the ether solution. We also weigh water in another falcon to balance the weight of the 1st falcon for centrifugation because we need equal masses during rotation to ensure that net angular momentum is 0 for it to rotate correctly. After setting the centrifuge at 4 degrees and 8000 rpm for 10 minutes, we collect the solid peptide after draining the diethyl ether. This is our peptide mixture, which we must purify further using HPLC.

3.5. High Performance Liquid Chromatography (HPLC):

High-Performance Liquid Chromatography (HPLC) for peptides involves separating, identifying, and quantifying peptides based on their hydrophobic properties.[12] The process starts with injecting the peptide sample (3 mL of peptide solution in MiliQ water) into the HPLC system, where a liquid mobile phase carries it, typically a mix of water and acetonitrile, through a column packed with a hydrophobic stationary phase, C18 reversed-phase column. As peptides travel through the column, they separate according to their interactions with the stationary phase; more hydrophobic peptides elute later, while less hydrophobic ones elute earlier. The separation is enhanced by gradient elution, where the solvent composition gradually changes. After separation, the peptides are detected by a UV detector (at 214 nm and 254 nm), which measures absorbance at specific wavelengths where peptides absorb due to their peptide bonds and aromatic amino acids. The resulting chromatogram, a plot of absorbance versus time, displays peaks corresponding to different peptides, enabling their identification and quantification. This method is crucial for determining peptide purity, mapping peptides for protein characterization, and ensuring quality control in peptide synthesis. We ran the HPLC setup for 21 minutes each for each sample and used acetonitrile and water gradients for the experiment. All the peaks that could have corresponded to the peptides were collected and were later verified for molecular mass using MALDI.

Method file used for HPLC purification

	Time (min)	Flow (mL/min)	%Water	%ACN
1	0	5.00	95	5
2	5	5.00	85	15
3	10	5.00	60	40
4	15	5.00	25	75
5	20	5.00	5	95
6	21	5.00	95	5

3.6. MALDI-TOF for Molecular Mass Verification:

MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-of-Flight) is a mass spectrometry technique used to determine the molecular mass of peptides by combining laser desorption and ionisation processes. In this method, the peptide is co-crystallized with a matrix material that absorbs laser energy, facilitating the ionisation of the sample without fragmentation when a laser is applied. An electric field accelerates the resulting ions, and their flight time to the detector is measured. This time correlates with the mass-to-charge ratio of the ions, allowing for the generation of a mass spectrum that reveals the molecular mass of the

peptide. MALDI-TOF is favoured for its ability to analyse biomolecules quickly and accurately, making it a powerful tool in peptide characterisation.

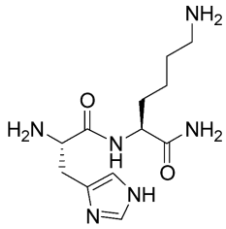
4. Conclusion:

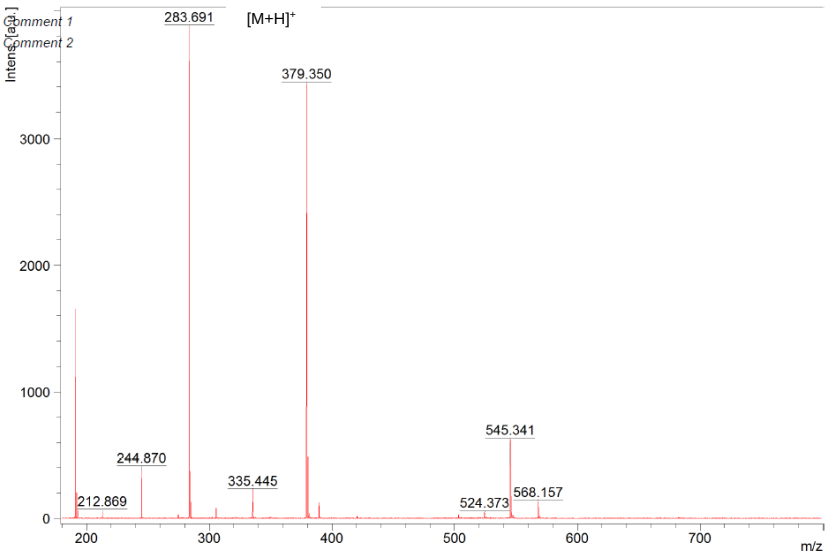
In this project, we have synthesised different peptides using different sequences of amino acids for cell culture studies. We started off with Rink Amide Resin, and using Fmoc-protected amino acids, we coupled the acids to form multiple peptides. Before cleaving the mixture to obtain the peptide, we removed half of the mixture to add an unnatural amino acid (Dimethyl Glycine) to compare peptides with and without the unnatural addition. The purpose of adding the unnatural amino acid is that the cells contain proteolytic enzymes that degrade the peptides during studies. Adding an unnatural group will make it difficult for the enzymes to detect the peptide sequence and protect it from degradation, thereby conserving the therapeutic properties of the peptide sequence during cell culture studies. The process of synthesis began by repeating stages of coupling and deprotecting until the sequence was formed, thereby cleaving the peptide by preparing a TFA-based cleavage cocktail and precipitating the peptide using diethyl ether, which was then obtained in the solid state using centrifugation. After the molecular masses of the peptides were verified using MALDI, we then purified the peptides using High-Performance Liquid Chromatography (HPLC).

Thus, we synthesised a list of peptides that have shown potential in tackling the multifactorial nature of Alzheimer's as an approach for peptide-based therapy for Alzheimer’s Disease.

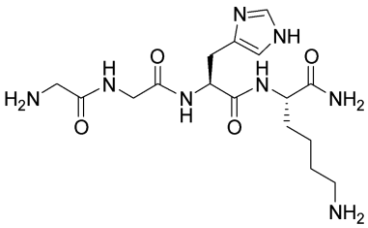
5. MALDI-TOF Figures for Molecular Mass Verification

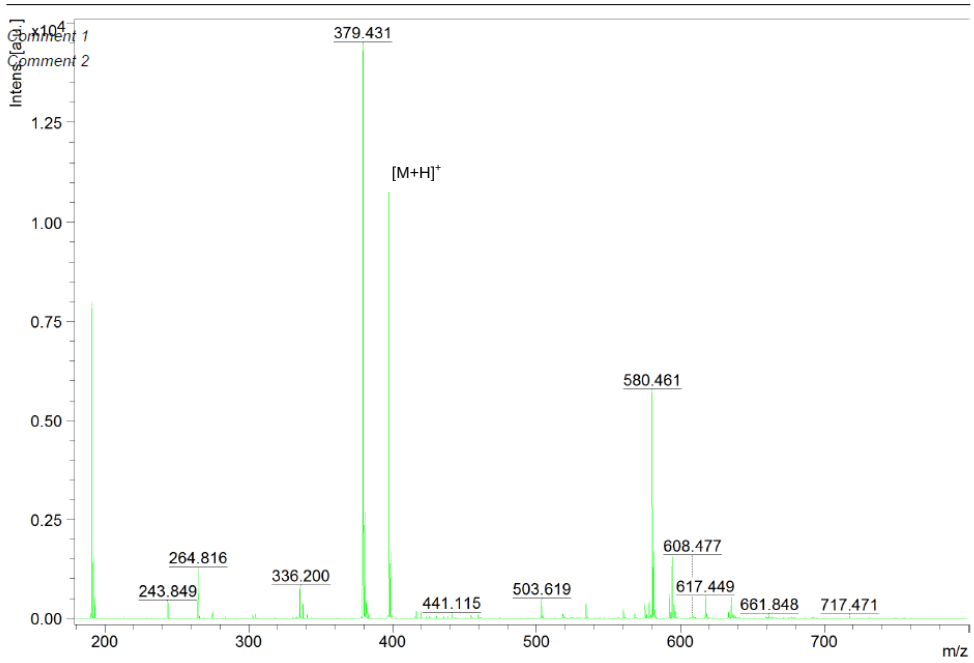
I. MALDI-TOF characterisation of HK [$C_{12}H_{22}N_6O_2$ M.W 282.3480]



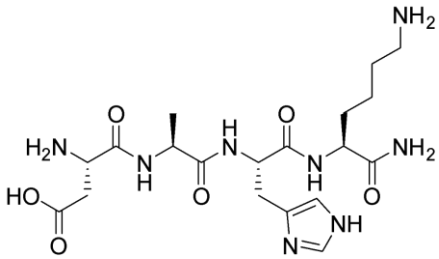


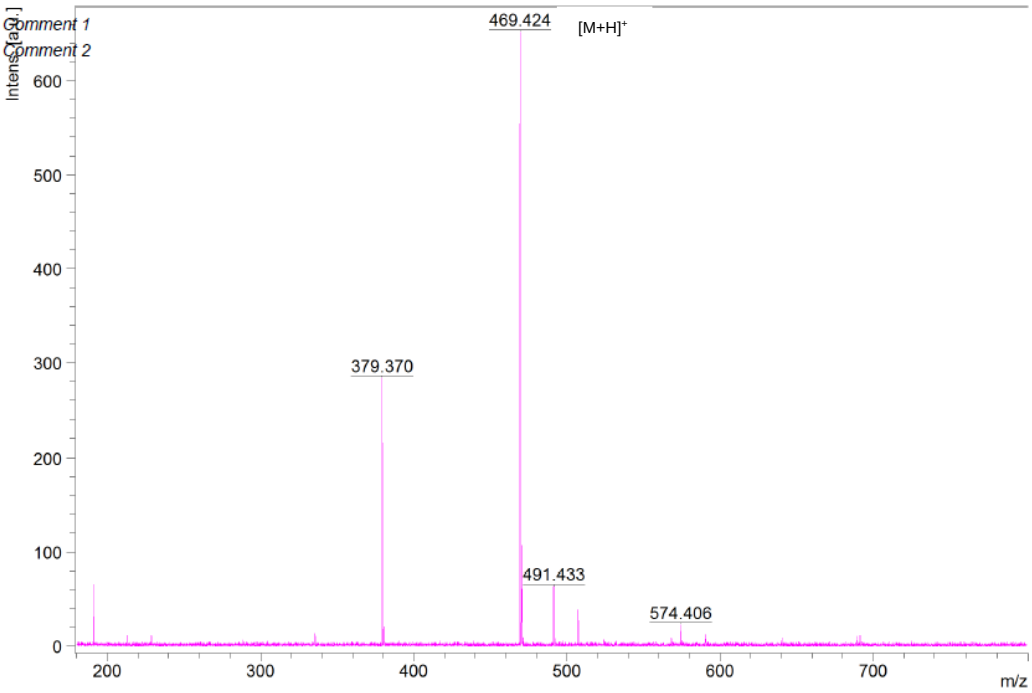
II. MALDI-TOF characterisation for GGHK [$C_{16}H_{28}N_8O_4$ M.W 396.4520]



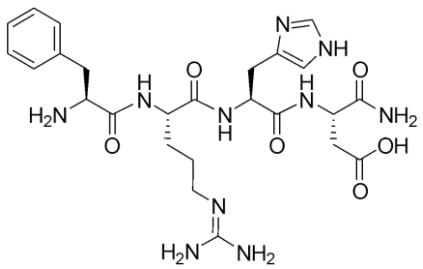


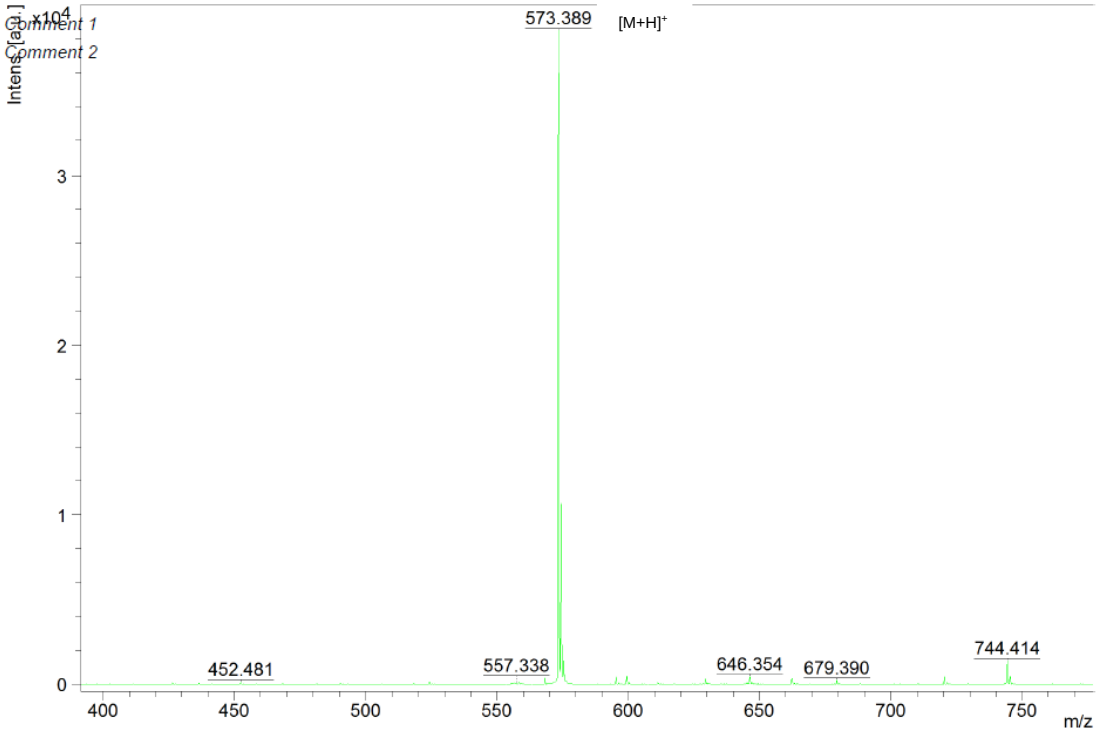
III. MALDI-TOF characterisation for DAHK [$C_{19}H_{32}N_8O_6$ M.W 468.5150]



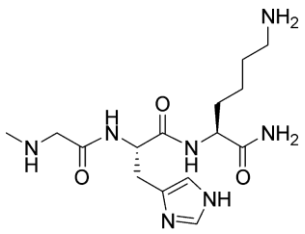


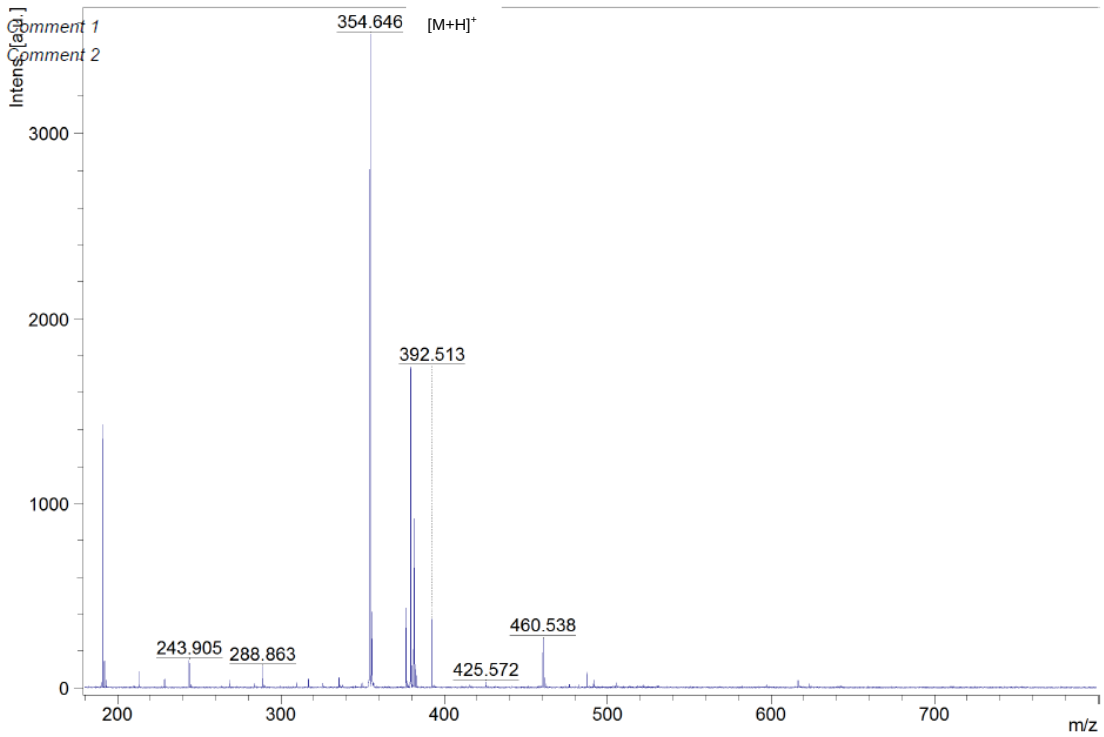
V. MALDI-TOF characterisation for FRHD [$C_{25}H_{36}N_{10}O_6$ M.W 572.6270]



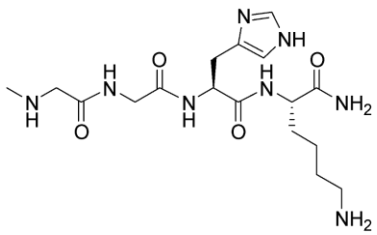


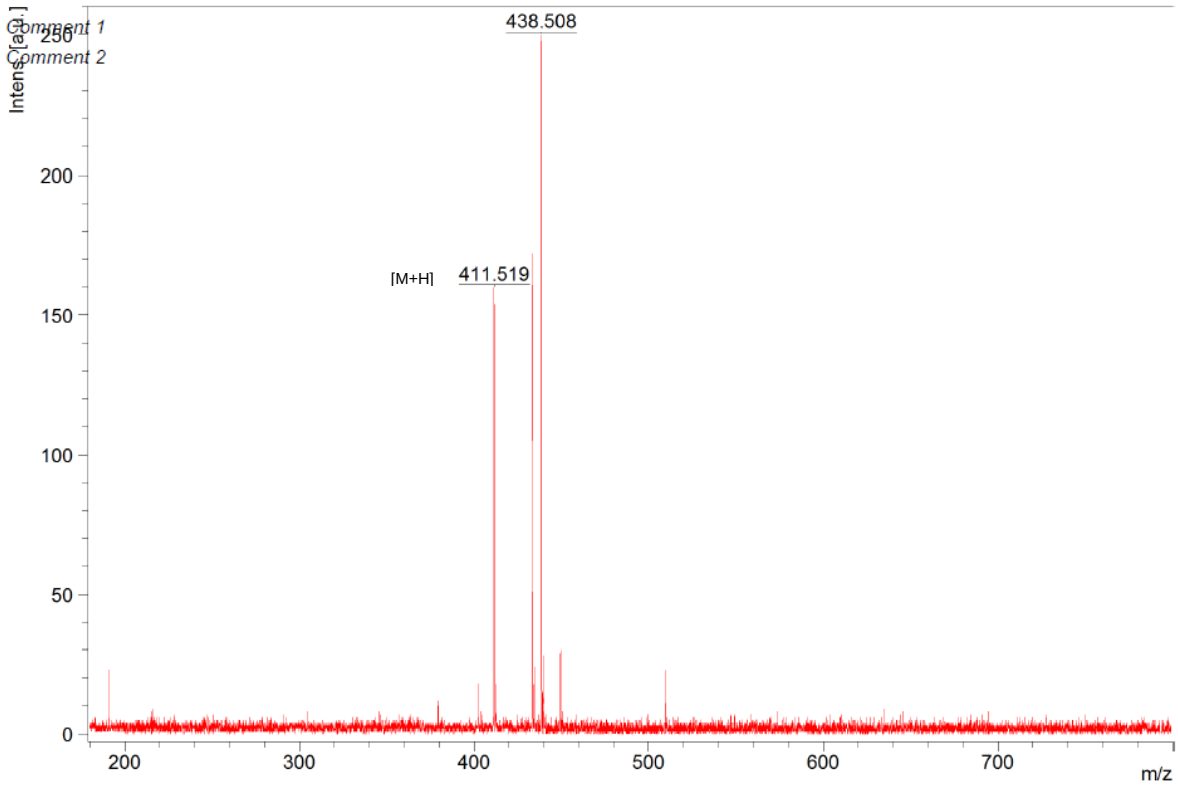
VI. MALDI-TOF characterisation for N-methyl glycine-HK [$C_{15}H_{27}N_7O_3$ M.W 353.4270]



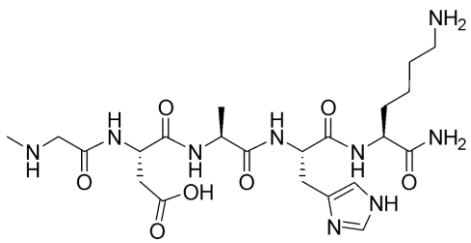


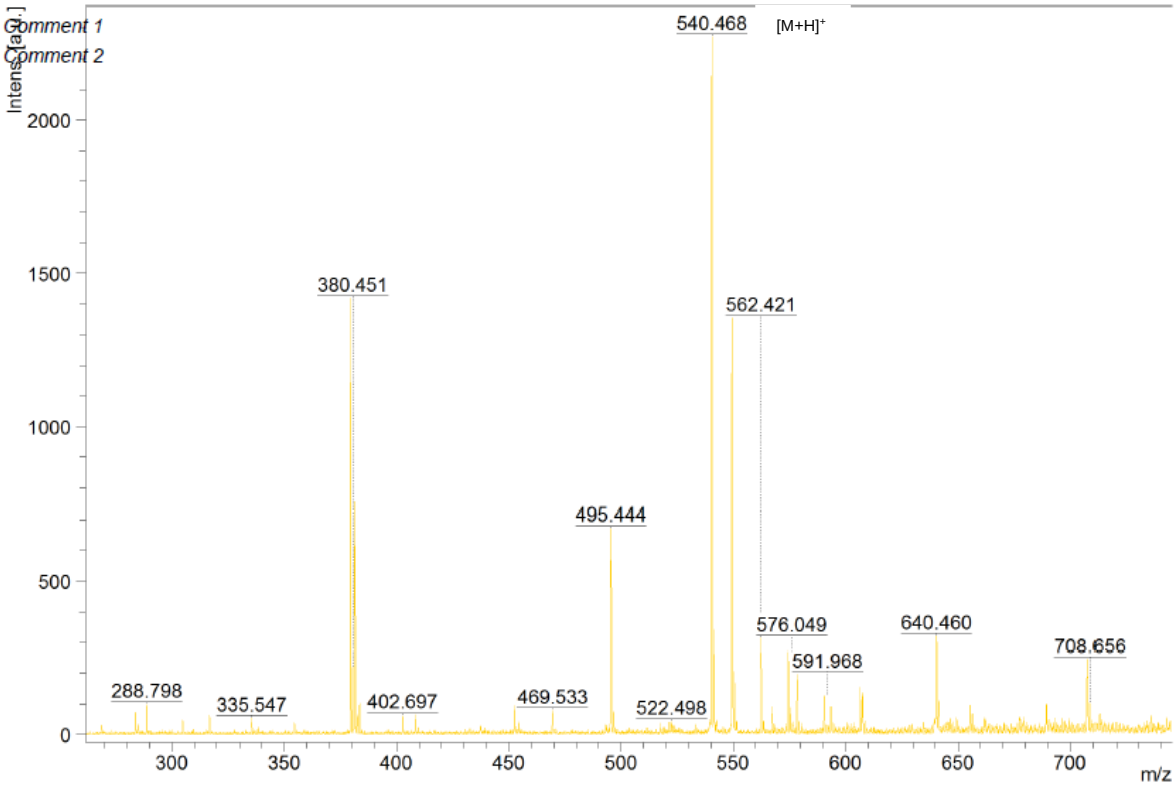
VII. MALDI-TOF characterisation for N-methyl glycine-GHK [$C_{17}H_{30}N_8O_4$ M.W 410.4790]



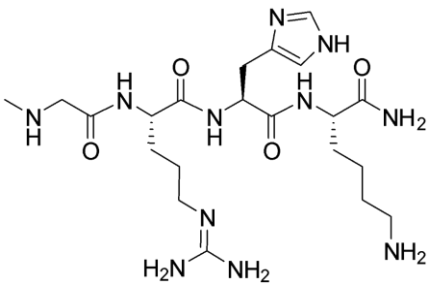


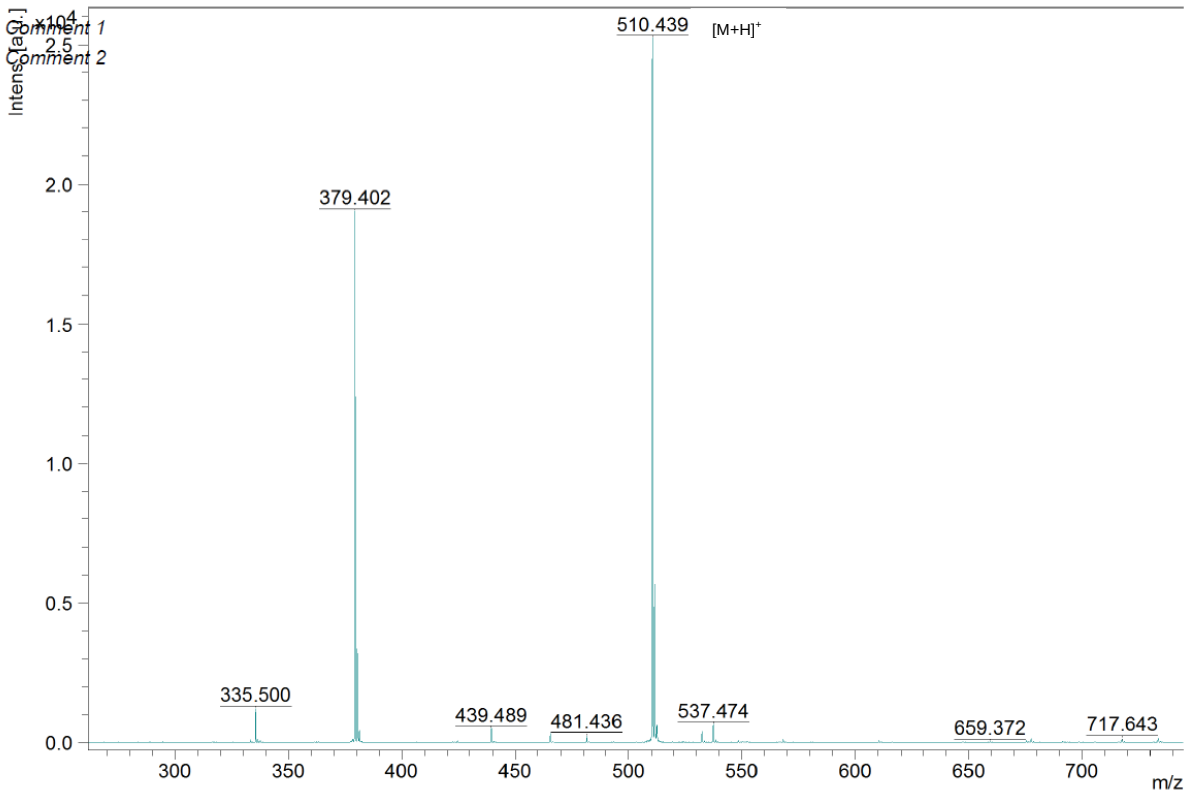
VIII. MALDI-TOF characterisation for N-methyl glycine-DAHK [$C_{22}H_{37}N_9O_7$ M.W 539.5940]



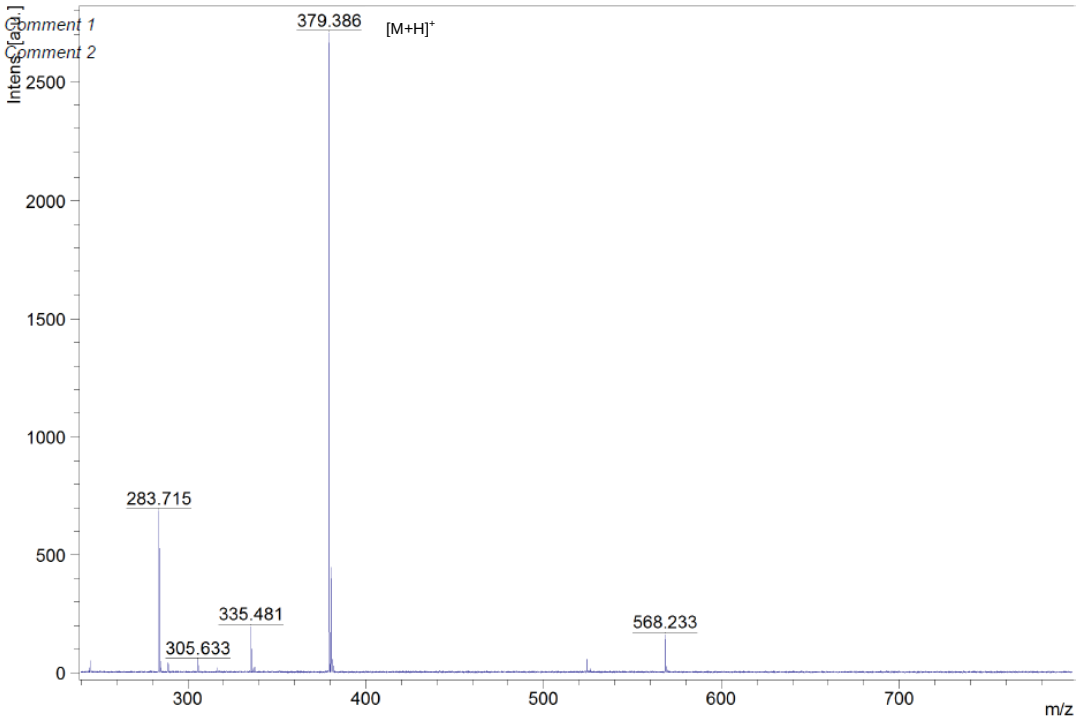


IX. MALDI-TOF characterisation for N-methyl glycine-RHK [$C_{21}H_{39}N_{11}O_4$ M.W 509.6160]

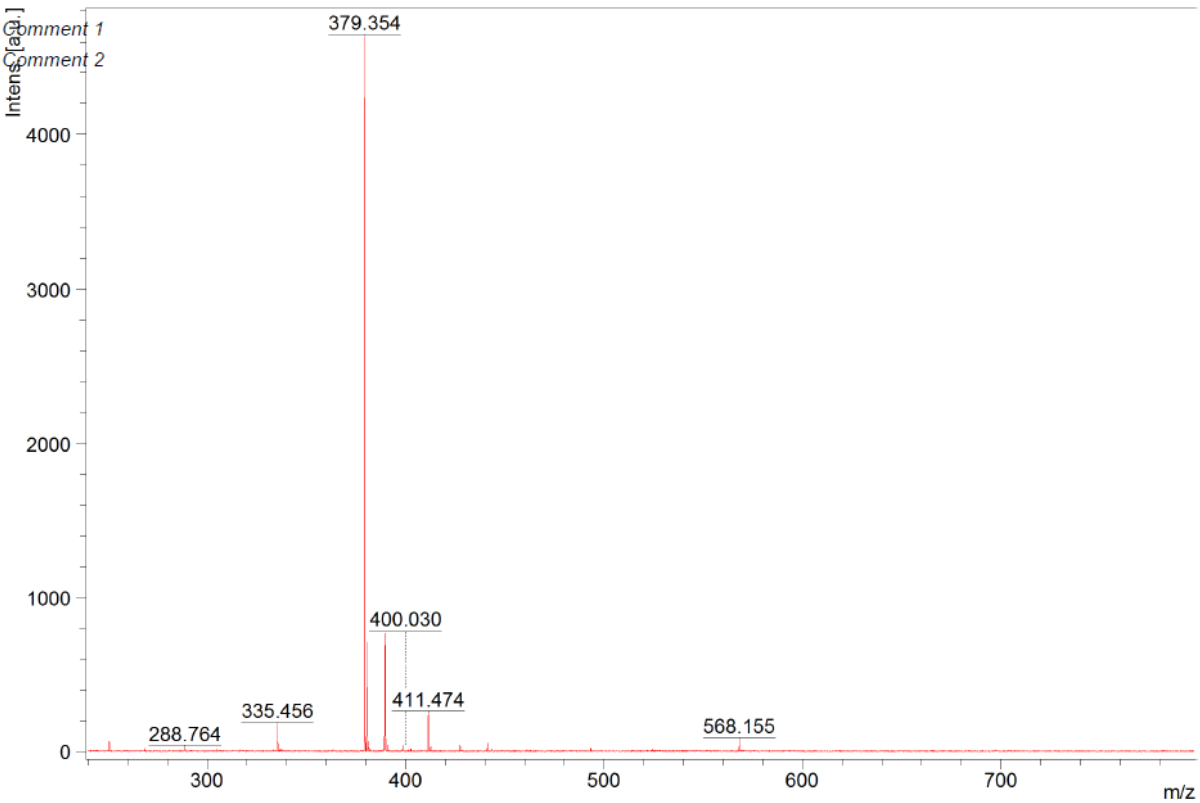




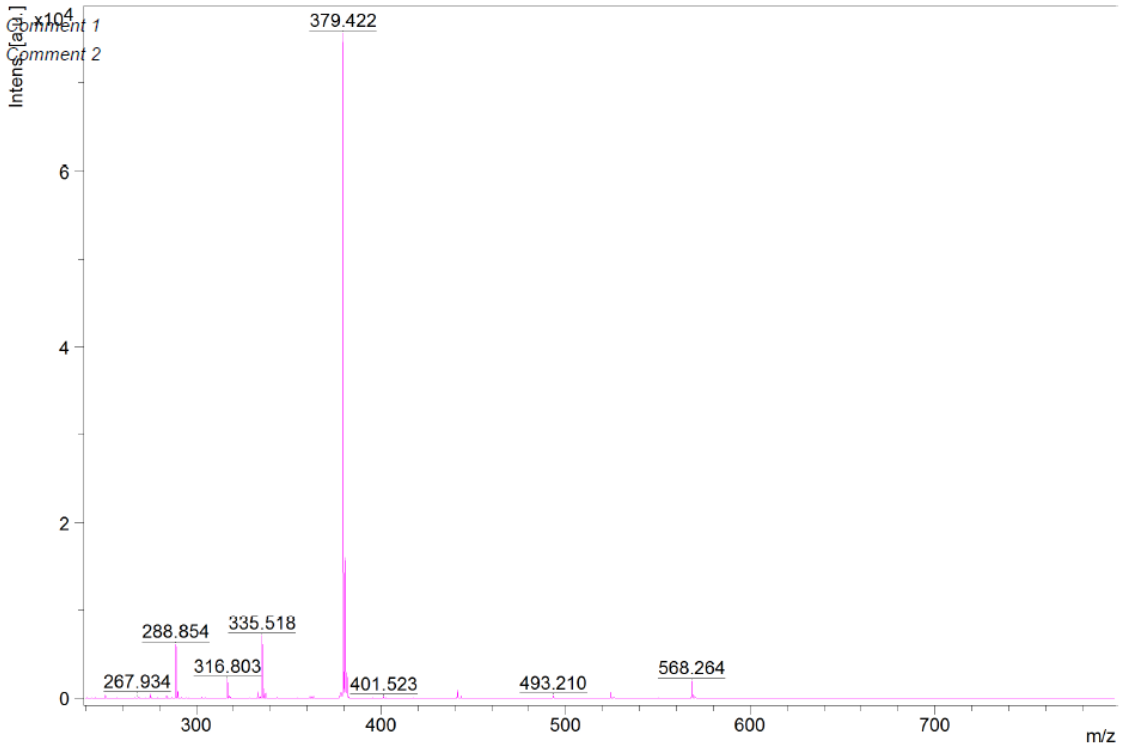
X. MALDI-TOF characterisation for HPLC HK Peak 1 [$C_{12}H_{22}N_6O_2$ M.W 282.3480]



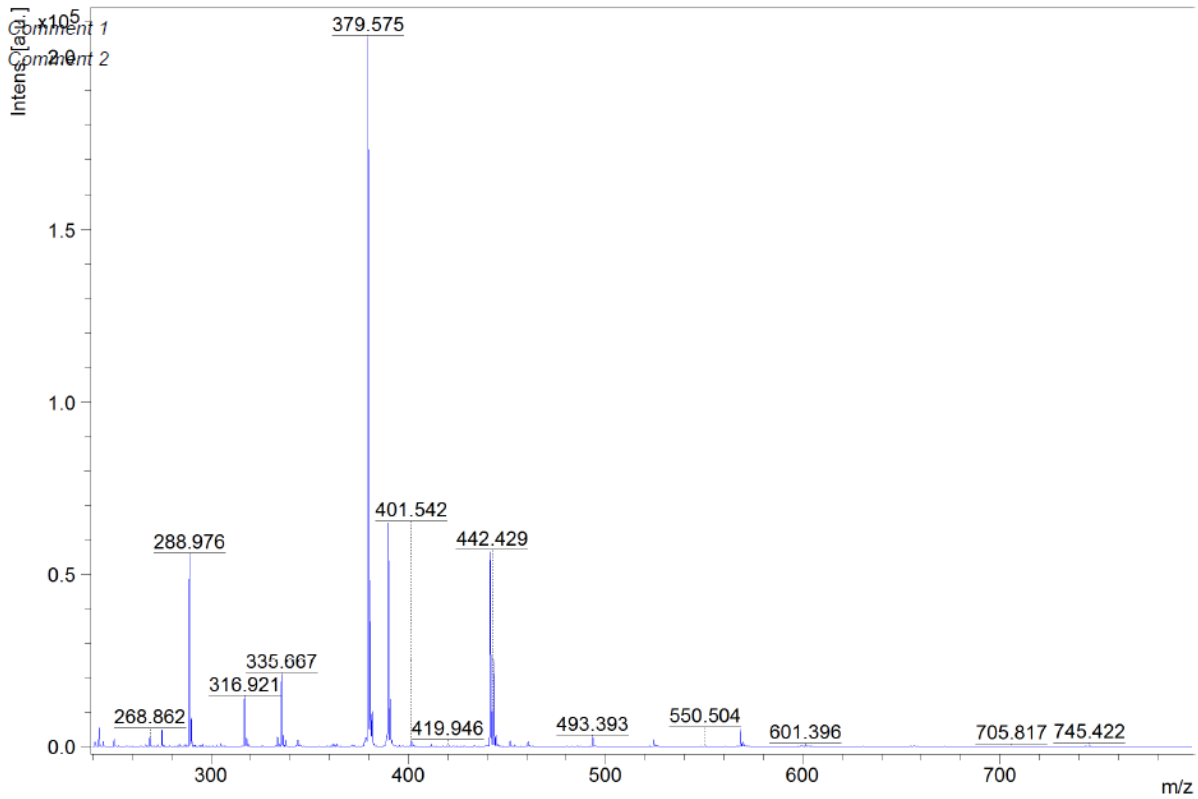
XI. MALDI-TOF characterisation for HLPC HK Peak 2 [$C_{12}H_{22}N_6O_2$ M.W 282.3480]



XII. MALDI-TOF characterisation for HPLC N-methyl glycine-HK Peak 1 [$C_{15}H_{27}N_7O_3$ M.W 353.4270]



XIII. MALDI-TOF characterisation for HPLC N-methyl glycine-HK Peak 2 [$C_{15}H_{27}N_7O_3$ M.W 353.4270]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419.946
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601.396
705.817
745.422
300
400
500
600
700
m/z

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