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SVEUČILIŠTE U RIJECI
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Sara Moržan

Sinteza peptida na čvrstom nosaču

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Mentor: Daniela Kalafatović, doc.dr.sc

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SUMMARY

Peptides are biomolecules composed of two to fifty amino acids, connected into chains through peptide bonds. They have a wide variety of applications in medicine, biology, chemistry and other biomedical-related fields because of their antimicrobial, antiviral and catalytic activities. Solid phase peptide synthesis (SPPS) is the method used for producing these compounds in the laboratory. It consists of a few basic steps, namely: deprotection, coupling, washing, acetylation, and cleavage.

This undergraduate thesis is focused on the Fmoc SPPS synthesis, analysis, and purification of the Ac-HREAKRTRVCYR-Am (Ac=acyl group; Am=amide group) peptide and provides summarized protocols for all used methods.

During the synthesis, difficulties regarding the removal of the protecting group and liquid chromatography-mass spectrometry analysis were encountered due to the high hydrophilicity of the peptide. For this reason, the aim was to find different ways to optimize the method for synthesis, characterization, and purification to achieve a peptide of high purity that could undergo different biocatalytic assays in the future.

Keywords: peptides, solid phase peptide synthesis

SAŽETAK

Peptidi su biomolekule sastavljene od 2 do 50 aminokiselina povezanih u lanac peptidnim vezama. Imaju širok u primjenu u medicini, biologiji, kemiji i ostalim srodnim biomedicinskim područjima zbog antimikrobnih, antivirusnih i katalitičkih sposobnosti. Sinteza peptida na čvrstom nosaču (*engl.* solid phase peptide synthesis; SPPS) metoda je koja se koristi za proizvodnju ovih spojeva u laboratoriju. Sastoji se od nekoliko osnovnih koraka: uklanjanje zaštitne skupine s aminokiselina, spajanja aminokiselina u lanac (*engl*. coupling), ispiranja, acetilacije i skidanja peptida s nosača (engl. cleavage).

Ovaj završni rad usmjeren je na Fmoc SPPS sintezu, analizu i pročišćavanje peptida Ac-HREAKRTRVCYR-Am (Ac=acilna skupina; Am=amidna skupina) i pruža sažete protokole za sve korištene metode.

Tijekom sinteze pojavile su se poteškoće s uklanjanjem zaštitne skupine i analize tekućinskom kromatografijom zbog visoke hidrofilnosti peptida. Iz tog razloga, cilj je bio pronaći različite načine za optimizaciju metoda sinteze, karakterizacije i pročišćavanja kako bi dobili peptid visoke čistoće koji može biti podvrgnut različitim biokatalitičkim testovima u budućnosti.

Ključne riječi: peptidi, sinteza peptida na čvrstom nosaču

CONTENTS

1. INTRODUCTION

1.1. Peptides and their composition

Peptides are short chains of amino acids connected through peptide bonds. In the 1900s, Emil Fischer, the father of peptide chemistry, was the first to introduce the concept of peptides and present protocols for their synthesis in the solution phase (1). In 1902 he used the name peptides for the first time, and he described them as small compounds, similar to proteins, but with a smaller molecular weight (2). The length of peptides can vary from two to fifty amino acids. Peptides are much smaller than proteins and they can be a small part of a protein chain. Amino acids are organic molecules, containing two functional groups, an amino ($-NH_2$) and a carboxyl (-COOH) group. In addition, each amino acid has a side chain, the R-group, attached to the alpha carbon. Amino acids differ one from another by their side chains which leads to changes in the peptide structure, size, polarity and solubility in water. Twenty natural amino acids are assembled into proteins during the process of translation. Amino acids are joined through peptide bonds formed between the amine group of one L-amino acid and the carboxylic group of another amino acid. The end of the peptide that contains the amine group is called the N-terminus while the end that contains the carboxylic group is called the C-terminus. In nature, peptides are synthesized from N-terminus to C-terminus. By convention, peptide sequences are written from N-terminus to C-terminus. However, using SPPS, peptides are synthesized from C-terminus to N-terminus (3).

1.2 Roles of peptides

Peptides have an important role in biochemistry, medicinal chemistry and physiology with activity against cancer, viruses and microbes because of their antimicrobial and antitumor effects (4). They can also mediate specific cellular responses in the cell: promote or inhibit angiogenesis, induce or protect against apoptosis, etc. (5). Many peptides function as hormones, neurotransmitters, cytokines and growth factors that affect different parts and pathways in the human body. Peptides can be used as synthetic antigens with a role in the preparation of polyclonal and monoclonal antibodies. Synthetic peptides can also help in epitope mapping to identify specific antigenic peptides used in synthetic vaccines or to determine protein sequence regions important for biological processes. Many well-known therapeutic agents, such as adrenocorticotropic hormone (ACTH), oxytocin, vasopressin and many more are peptide hormones (6).

1.3 Discovery of Solid Phase Peptide Synthesis

Solid phase peptide synthesis (SPPS) is used to synthetically make peptides. Two different solid phase strategies for peptide synthesis have been developed, both based on the N-terminal protection: fluorenylmethyloxycarbonyl/*tert*-butyl (Fmoc/*t*Bu) and *tert*-butyloxycarbonyl/benzyl (Boc/bzl). The Fmoc/*t*Bu strategy is more commonly used because it doesn't require repeated acidic treatments of the peptide and the use of hydrogen fluoride (HF) that is used in Boc/Bn peptide synthesis (7).

In 1963, Bruce Merrifield published a paper that described the first successful synthesis of an elongated peptide chain assembled on an inert solid support. Till then, the peptides were synthesized in the solution phase. This newly discovered technique was named solid phase peptide synthesis (SPPS). It took him three years to find a suitable support and appropriate chemical reagents

for it to work. He was awarded the Nobel Prize in Chemistry in 1984 for the discovery of SPPS. SPPS has many advantages, for example, excess reagents and byproducts can easily be washed away while the peptide chain stays intact on resin. A wide range of reagents can be used as solvents, and they are always used in excess to increase reaction rates and allow the reaction to be completed. SPPS opened the way for the use of synthetic peptides in chemical and biomedical fields (4).

1.4 Fmoc/*t*Bu SPPS

SPPS is a cyclic process with a few main steps: deprotection, washing, coupling, washing, and cleavage (Figure 1). Deprotection, washing, coupling and washing are repeated until all the required amino acids that form the peptide chain are added. After the formation of the peptide chain, acetylation of the N-terminus is optional, but not all synthetic peptides undergo this step. After successful SPPS, final cleavage is performed to remove the peptide from the resin.

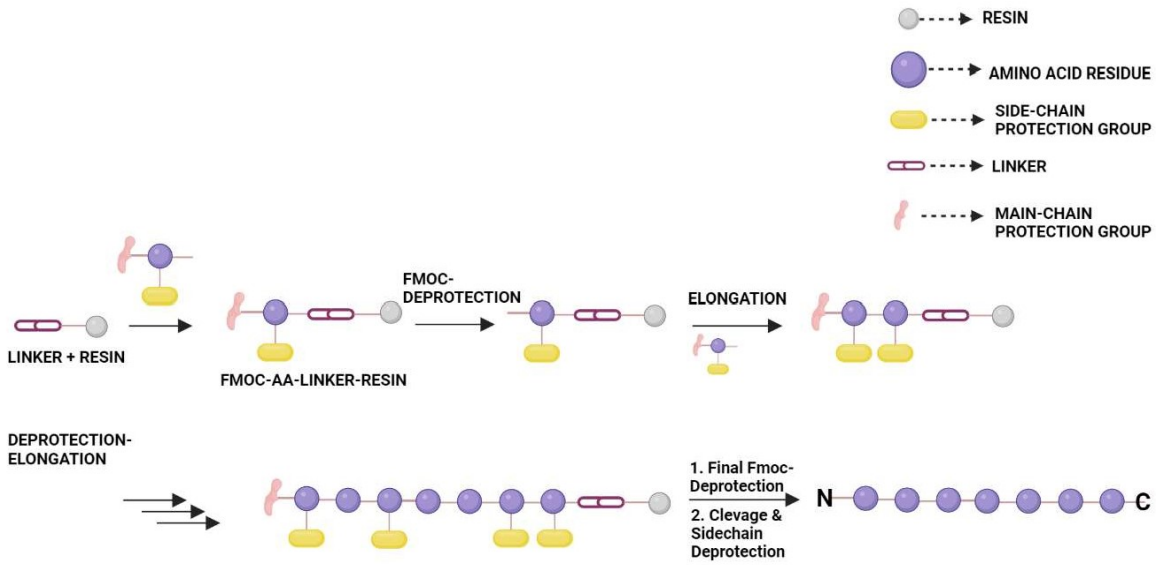


Figure 1. Schematic representation of the SPPS methodology illustrating its main steps: deprotection, washing, coupling and cleavage.

Side chains of some amino acids contain reactive functional groups such as hydroxyl, carboxyl or amine. Therefore, their protection is important to minimize the formation of byproducts. In addition, protection of the amino end of amino acids is very important to prevent undesirable side reactions, allowing the growth of the peptide chain. Protecting groups should be soluble in most common solvents, easy to remove, and with no side reactions (8). The most common protecting groups used in Fmoc/*t*Bu and Boc/bzl SPPS are: fluorenylmethyloxy carbonyl (Fmoc), *tert*-butyloxycarbonyl (Boc), trityl (Trt), *tert*-Butyl (*t*Bu) and 2,2,4,6,7-Pentamethyl-2,3-dihydrobenzofuran-5-sulfonyl (Pbf) (Table 1.).

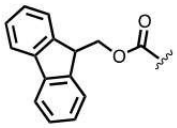
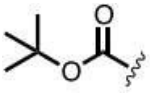
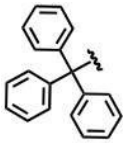
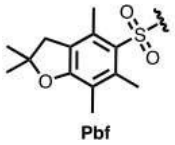
In Boc-based synthesis, protecting groups from the amine end are removed with trifluoroacetic acid (TFA). Using a stronger acid, hydrogen fluoride (HF), side chain protecting groups and the peptide on the resin can be simultaneously cleaved (9). This strategy requires protective groups that can tolerate repetitive TFA treatment. While in the Boc strategy, a hazardous acid TFA is used at every cycle, in the Fmoc strategy it is used only once, in the process of final cleavage.

Fmoc-based synthesis is mild, flexible, versatile and more widely used. In the Fmoc strategy, the protecting groups of the side chains and the amine end of amino acids present orthogonal chemistries where each type of protection is selectively removed, leaving the other type of protection intact. For the removal of the Fmoc, the piperidine base is used. Fmoc is stable in acidic conditions, hence side chain protecting groups and the cleavage of the peptide from the resin are simultaneously performed using strong acids, for example, TFA or HF (9).

When Fmoc is removed, the coupling is enabled. Coupling is achieved when the N-terminus of one amino acid reacts with the C-terminus of another amino acid, resulting in a formation of a peptide bond. After the peptide is elongated

to the desired length and constitution, the peptide is cleaved from the resin, purified and lyophilized.

Table 1. Protecting groups commonly used in solid phase peptide synthesis.

Protecting group	**Chemical structure**	**Protects side chain of amino acid**	**Resistant to**	**Labile to**	**Removed with**
Fmoc			Acid	Base	10-20% piperidine
*t*Bu		Ser, Thr, Tyr, Glu, Asp	Base	Acid	≥ 50% TFA in DCM
Boc		Lys, Trp	Base	Acid	≥ 50% TFA in DCM
Trt		Cys, Asn, Gln, His	Base	Acid	≥2% TFA
Pbf		Arg	Base	Acid	95% TFA

1.5 Steps of SPPS

SPPS allows the coupling of amino acids and the growth of a peptide chain on resin. This method enables the elimination of excess reagents and soluble byproducts after each reaction cycle while the peptide stays anchored to the resin. When the peptide is synthesized, it is cleaved from the resin using orthogonal conditions (9).

Solid support (*i.e.* resin) is a polymer functionalized with a linker molecule prior to SPPS, meaning that every solid support bead has a linker. There are three different types of resins: poly-styrene base, poly-ethylene glycol (PEG) grafted polystyrene and PEG resins without polystyrene (10). The linker allows the cleavage of the peptide from the resin after the completion of the synthesis (4). Linkers release the peptide in a form of either a peptide acid or peptide amide when treated with TFA (10). There are many different linker options, but the most used are Rink Amide and Wang linkers. Depending on the linker, the bond formed between the resin and the first amino acid is different, it can be an ester or an amide bond. Rink Amide resin is used when the desired peptide presents the amide group at its C-terminus. In this case, an amide bond is formed between an amino group of the linker and a carboxylic group of the amino acid. On the other hand, if the desired peptide has a carboxylic group on the C-terminus, an ester bond will form between the alcohol group of the linker and the carboxylic group of the first amino acid, hence the Wang linker is used in this case.

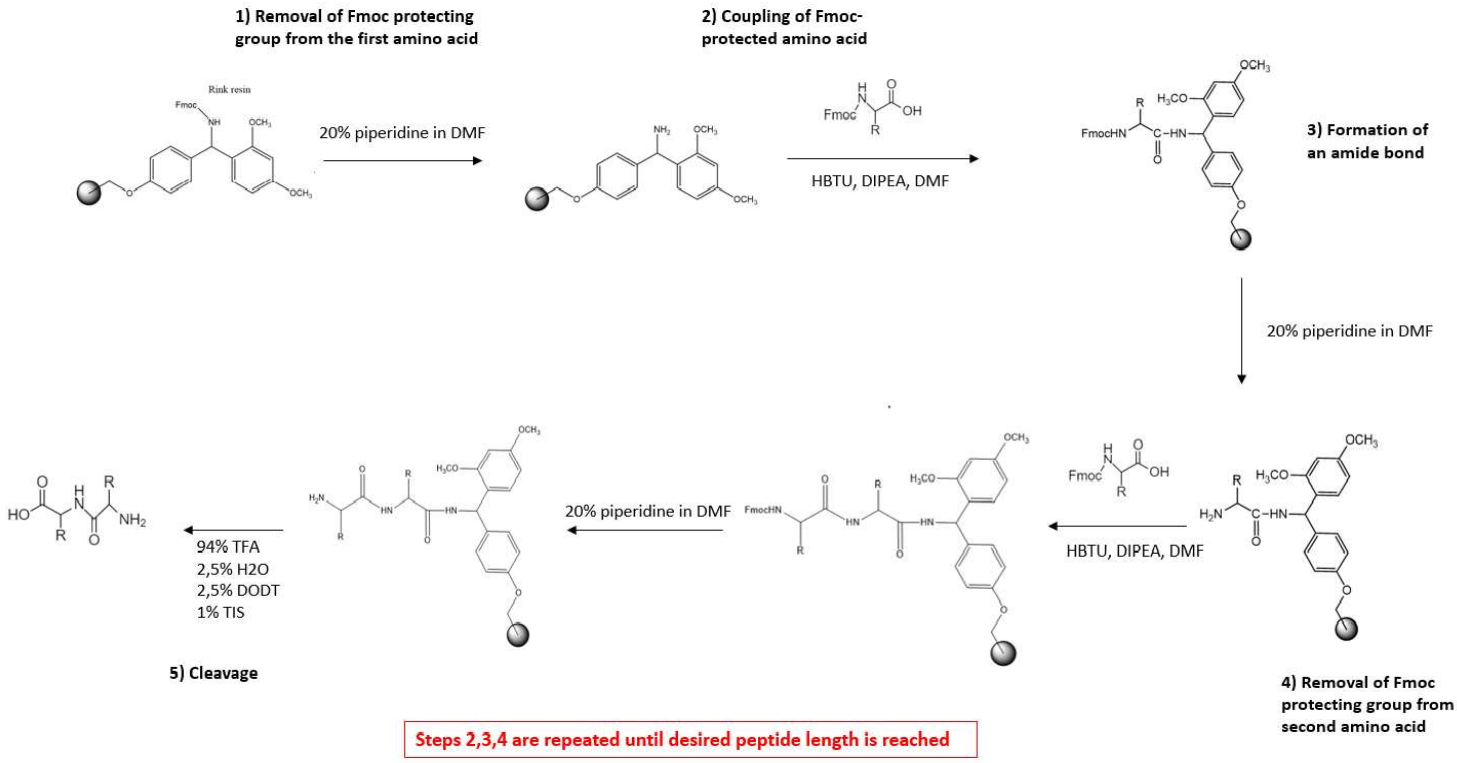
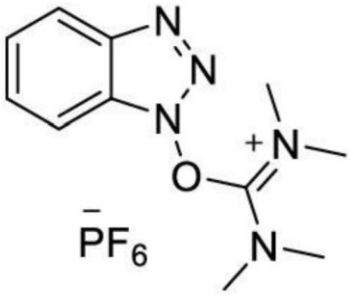


Figure 2. Schematic representation of the main SPPS steps on the Rink amide resin: deprotection of the amine, activation of the carboxyl end, amide bond formation and coupling.

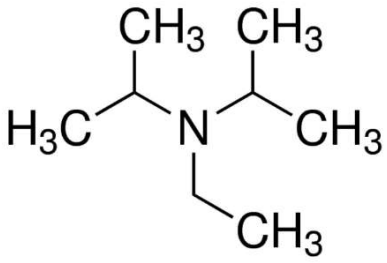
As shown in Figure 2, SPPS consists of a few different steps. The first step before starting the synthesis is to swell the resin. With swelling, the reactive functional groups are exposed to the surface of the resin, making them available for a reaction with the amino acid (4). The amine functional group on the linker is, just like all the amino acids, also protected with Fmoc. To allow for the chain growth, before adding the first amino acid and after every coupling, the Fmoc group must be removed with piperidine. After the deprotection step, the resin is washed with *N,N*-dimethylformamide (DMF) and dichloromethane (DCM) to get rid of the excess piperidine and any byproducts that may have been formed.

The coupling step consists of a carbonyl substitution reaction where the free amino group of the linker and the free carboxyl group of the amino acid form

an amide bond. The amino acid is added to the resin in a mixture with the coupling and activating agents presented in Figure 3, *N, N*-diisopropylethylamine (DIPEA) and *O*-(benzotriazole-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU). Carboxylic acid and carboxylate groups are normally not very reactive, and they should be activated to form the amide bond. For that reason, a combination of an activating agent HBTU and a coupling agent DIPEA is mixed with every amino acid (11). After coupling, the amino acid is now attached to the resin and byproducts are washed away with DMF and DCM. Before performing the next coupling, the coupled amino acid needs to be deprotected with piperidine because the Fmoc group is attached to the N-terminus. After deprotection, the free amine of the growing chain will react with the free carboxyl group of the new amino acid, forming another amide bond.



HBTU
Activating agent



DIPEA
Coupling agent

Figure 3. Chemical structure of HBTU and DIPEA.

If the peptide has a free N-terminus, under acidic conditions the amine group becomes protonated and can form a salt with TFA. The acetylation process neutralizes the positive charge on the free amino group which blocks further ionization and other modifications of the amino group (12). Sometimes, purified peptides must be free of TFA because otherwise, TFA could alter the results of biological assays. If the acetylation step is left out and the TFA salt

is formed the most common method to remove it is to use a stronger acid, such as hydrochloric acid (HCl) (13).

The cleavage removes all the side chain-protecting groups and the resin attached to the peptide. To cleave the peptide, a cleavage cocktail containing TFA, triisopropylsilane (TIS), 3,6-dioxa-1,8-octanedithiol (DODT), and H_2O is used. TFA is used for cleavage while TIS is a scavenger that "picks up" all the residues. During cleavage with TFA, side-chain protecting groups form carbocations that can react with amino acid side-chains that are rich with electrons, resulting in the formation of byproducts. To avoid this, scavengers trap carbocations, reducing the amount of unwanted byproducts (14). DODT is added to avoid cysteine oxidation as cysteine's thiol (-SH) group on its side chain is prone to the process of oxidation. When thiols oxidize, their chains form intermolecular and intramolecular disulfide bonds. They can also simply oxidize to sulfenic, sulfinic or sulfonic acid with exposure to air (15). As a result, peptides with two or more cysteines form cyclic peptides and dimers, complicating the whole SPPS process. This can be avoided by using DODT. After cleavage, the product needs further purification to remove all the byproducts that were not removed with the cleavage cocktail.

1.6. Peptide characterization

After synthesis, the crude peptide can be characterized by liquid chromatography-mass spectrometry (LC-MS) and matrix-assisted laser desorption/ionization (MALDI-TOF) to confirm its mass.

LC-MS is an analytical technique that combines the use of liquid chromatography for physical separation along with mass spectrometry for mass analysis. Liquid chromatography (LC) separates the sample into its components based on their hydrophobicity/hydrophilicity compounds while MS further analyzes their masses. LC-MS system has three components: a separation device, an interface and a mass spectrometer (16). The separation is based on the interactions of the sample with both the mobile and stationary phases. The stationary phase is usually packed with silica beads with attached hydrophobic chains. For peptide separation, long C_8 or C_{18} alkyl chains are commonly used. Solvents that serve as the mobile phase, denoted A and B, are added in varying concentrations over time. Solvent A is usually water while solvent B is usually an organic solvent such as acetonitrile (17). The sample first needs to be dissolved in solvent A and then injected into the column. The sample components are transported through the column by a mobile phase in a process called elution. As the components move through the silica beads, they bind to the alkyl chains. Depending on different properties each sample has, it binds to the column with different strengths. Solvent B gets gradually mixed with solvent A and pumped through the column and each sample is released from the column at a specific concentration of solvent B. Retention time (rt) is the time required for the sample to travel through the column. In reverse phase chromatography, the more hydrophobic the peptide is, its affinity for the stationary phase is stronger. This means that such peptides will stay on the column for longer and will have a longer retention time. After chromatographic separation, the LC and MS are coupled through an interface

resulting in the transportation of the components to the mass spectrometer. There, they are introduced into the gas phase, ionized at an ionization source and their mass is analyzed.

MALDI-TOF (MALDI=matrix assisted laser-desorption ionization; TOF=time of flight) is a type of mass spectrometry, where chemical compounds are ionized into charged molecules and identified based on their monoisotopic mass for singly or multiply charged ions. MALDI-TOF measures m/z ratio of ions based on the time required for an ion to travel the length of the flight tube and a characteristic spectrum is generated based on the TOF information. In the MALDI part of the procedure, a sample is first dissolved in a suitable solvent and then mixed with the matrix which protects it from breaking down into fragments during ionization (18). The sample-matrix mixture is then spotted on a MALDI plate and left to dry to a crystal. When a laser beam hits the sample-matrix crystal, the matrix absorbs the laser energy. This energy volatilizes the analytes in the sample and transfers protons from the matrix to the molecules in the sample resulting in the formation of ions. After desorption and ionization, the ions get separated in the mass analyzer based on their mass to charge (m/z) ratio. TOF utilizes an electric field to accelerate ions through a flight tube and then measures the time each ion takes to reach the detector. If the ions all have the same charge, their kinetic energy will be the same, meaning that the ions' velocity will depend only on their mass. Smaller molecules will reach the detector first and their peak will be shown first in the spectrum (19).

2. OBJECTIVES

The main objectives of this thesis are to synthesize the Ac-HREAKRTRVCYR-Am (Ac=acyl group; Am=amide group) peptide using solid phase peptide synthesis (SPPS) and characterize it using liquid chromatography-mass spectrometry (LC-MS) and matrix-assisted laser desorption/ionization (MALDI-TOF).

To optimize the synthesis of such peptides, it is important to determine possible limitations and problems that may occur during the process of synthesis. This peptide contains multiple arginines that can show complications during the deprotection of the side chains with TFA and the removal of the peptide from the resin. For that reason, it is important to optimize the method for successful synthesis, cleavage, and purification of this peptide so its biocatalytic properties can later be explored.

3. METHODS AND MATERIALS

3.1. Solid-Phase Peptide Synthesis (SPPS)

The peptide was synthesized using standard Fmoc/*t*Bu solid phase peptide synthesis (SPPS). The apparatus required for the synthesis was a syringe with a valve at the bottom connected to a vacuum separator and a vacuum pump. The peptide was synthesized on Rink amide resin (loading capacity of 0.78 mmol/g). First, the resin was swelled in DMF for 30 minutes. Using the vacuum pump, DMF was flushed out to a beaker. To remove the Fmoc deprotecting group from the N-terminus, 7 mL of 20% piperidine solution was added to the resin and left for 10 minutes on the shaker to mix. After Fmoc-removal the resin was washed six times, three times with DMF and three times with DCM.

In a Falcon tube, amino acid, HBTU and DIPEA were added in excess. Three-fold excess of amino acid, three-fold excess of HBTU and six-fold excess of DIPEA to 0,39 mmol resin. To allow the dissolution of the reagents, they were mixed using the vortex. If the amino acids were not completely soluble in DMF the samples were sonicated. After complete dissolution, the activated amino acid (with HBTU and DIPEA) mixture was added to the resin and placed on a shaker to mix for 45 minutes. The mixing initiates the chemical reaction and allows the coupling to happen. After amino acid coupling, the resin was washed three times with DMF and three times with DCM. Double coupling was performed for each amino acid to ensure the coupling was successful. Between each coupling, the resin was washed three times with DMF. After that the amino acid, HBTU and DIPEA mixture was added in the same quantity as for the first coupling. The list of used reagents and amino acids is presented in Tables 2 and 3.

Table 2. List of reagents and their amounts used for each step of the experiment.

Compound	**Mw (g/mol)**	**Mass (mg)**	**V (µl)**	**Purpose**
SPPS				
Resin (s)	/	500	/	Solid support matrix
HBTU (s)	379,24	428,9	/	Coupling agent
DIPEA (l) (ρ=0,76 kg/m^3)	129,24	/	400	Activating agent
Piperidine (l)	85,15	/	7000	The base for Fmoc removal
ACETYLATION				
Acetic anhydride (l)	102,09	/	350	Binds to the N-terminus
CLEAVAGE				
TIS (l)	158,36	/	100	Scavenger
DODT (l)	182,30	/	250	Prevents Cys oxidation during cleavage
TFA (l)	114,02	/	9400	Acid for protecting group removal

DIPEA was added in six-fold excess to 0,39 mmol of resin and its number of moles is $2{,}34 \times 10^{-3}$. The calculation for the required DIPEA volume:

$$\rho(DIPEA) = 0{,}76\ \frac{kg}{L} = 769\ \frac{g}{L}$$

$$n(DIPEA) = 6 \times 0{,}39\ mmol = 2{,}34\ mmol\ = 2{,}34 \times 10^{-3} mol$$

$$m(DIPEA) = n \times Mw = 2{,}34 \times 10^{-3} \times 129{,}25\ \frac{g}{mol} = 0{,}3024\ g$$

$$V(DIPEA) = \frac{m}{\rho} = \frac{0{,}3024\ g}{760\ \frac{g}{L}} = 3{,}98 \times 10^{-4} L = 398 \mu L \approx 400 \mu L$$

Table 3. List of amino acids and their respective amounts used in this experiment.

Amino acid	Mw (g/mol)	Mass (mg)
Fmoc-His(Trt)-OH	619,71	725,1
Fmoc-Arg(Pbf)-OH	648,77	759,1
Fmoc-Glu(OtBu)-OH	425,47	497,8
Fmoc-Ala-OH	311,33	364,3
Fmoc-Lys(Boc)-OH	468,54	548,2
Fmoc-Thr(tBu)-OH	397,46	465,03
Fmoc-Val-OH	339,39	397,1
Fmoc-Cys(Trt)-OH	585,71	685,3
Fmoc-Tyr(tBu)-OH	459,53	537,7

All the amino acids are added in three-fold excess to 0,39 mmol of resin. The number of moles for each amino acid is $1{,}17\,x\,10^{-3}$.

An example of a calculation for the required amino acid mass:

$$m(Fmoc-His(Trt)-OH) = n \times Mw = 1{,}17 \times 10^{-3} mol \times 619{,}71\ \frac{g}{mol} = 0{,}7251\ g = 725{,}1\ mg$$

3.2 Acetylation of the N-terminus

After the last amino acid coupling, the Fmoc deprotection needs to be carried out with 20% piperidine before beginning the acetylation. The acetylation mixture contained 5% acetic anhydride (350 µL), 8,5% DIPEA (595 µL) and 86,5 % DMF (6,055 mL). Everything was mixed in a Falcon tube using a vortex, added to the resin and shaken for 20 minutes. After 20 minutes the resin was washed three times with DMF and three times with DCM.

3.3 Cleavage

The cleavage was performed under different conditions. To confirm that the correct peptide sequence was obtained, a "mini cleavage" was performed first, where a small amount of resin was mixed with the "cleavage cocktail". The two different conditions were: 1) the cleavage cocktail containing 94% TFA, 2,5% H_2O, 2,5% DODT and 1% TIS, left on resin for 2 hours; 2) cleavage cocktail containing 95% TFA, 2,5% H_2O and 2,5% TIS, left on resin for 2 hours.

After characterization, the conditions for the final cleavage of the whole peptide from resin were determined. 10 mL of cleavage cocktail containing 94% (9,4 mL) of TFA, 2,5 % (0,25 mL) DODT, 1% (0,1 mL) TIS and 2,5% (0,25 mL) distilled H_2O was prepared and added to the resin. The cleavage cocktail was left on resin for 4 hours this time, after which the filtrate was collected. Next, the peptide was precipitated in 1 mL of cold diethyl-ether. The mixture was centrifuged for seven minutes at 12400 RPM and the diethyl-ether was decantated from the white pellet. The pellet was left in the fume hood for 15 minutes until all the diethyl-ether evaporated. The peptide was then dissolved in a 20% solution of acetonitrile in water and stored at -80°C.

3.4. Analysis

When the synthesis is finished, further analysis is required to confirm that the correct peptide is obtained. This characterization was performed using liquid chromatography-mass spectrometry (LC-MS) and matrix-assisted laser desorption/ionization (MALDI-TOF).

3.4.1. Matrix-assisted laser-desorption ionization (MALDI-TOF)

To determine the molecular mass of a peptide, a single-stage mass spectrometer, Bruker UltrafleXtreme MALDI-TOF was used. It enabled the identification of the peptide based on its monoisotopic mass for singly or multiply charged ions.

First, the sample was prepared by dissolving the peptide in 20% acetonitrile in water. The matrix and the peptide both need to be dissolved in the same solvent. The α-cyano-4-hydroxycinnamic acid matrix, was combined with 1mL of 20% acetonitrile as well. They were mixed in a 1:1 ratio. Then, 1 µL of the matrix and peptide mixture, was spotted on a MALDI plate and left to dry.

3.4.2. Liquid chromatography-mass spectrometry (LC-MS)

The lyophilized peptide was analyzed on Agilent Technologies LC MS equipped with a C18 AB column with an injection volume of 5 µL. A gradient system was used, consisting of A: water and B: acetonitrile. For LC-MS analysis a linear gradient was applied of A/B 99.9/0.1 to A/B 60/40 over 20 min. The flow was 0,3 mL/min, and detection was at 214 nm. Peak integration of the chromatogram between 5 and 20 min was performed. The LC-MS was performed twice, for both cleavage conditions mentioned in section 3.3.

4. RESULTS

The Ac-HREAKRTRVCYR-Am peptide (Ac=acyl group; Am=amide group) was successfully synthesized using the SPPS technique on Rink Amide resin. The average molecular weight was 1615,827 g/mol, calculated on a fragment ion calculator and confirmed by LC-MS and MALDI-TOF characterization. The chemical structure of the peptide is shown in Figure 3.

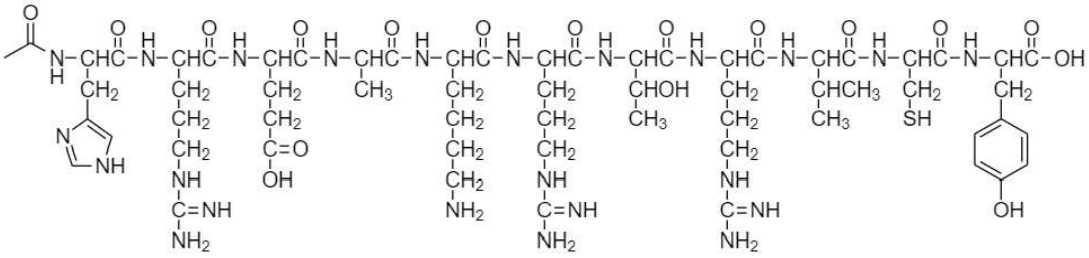


Figure 4. Chemical structure of Ac-HREAKRTRVCYR-Am peptide.

4.1 MALDI-TOF

MALDI-TOF was used to identify the peptide based on its monoisotopic mass for singly or multiply charged ions (Figure 5).

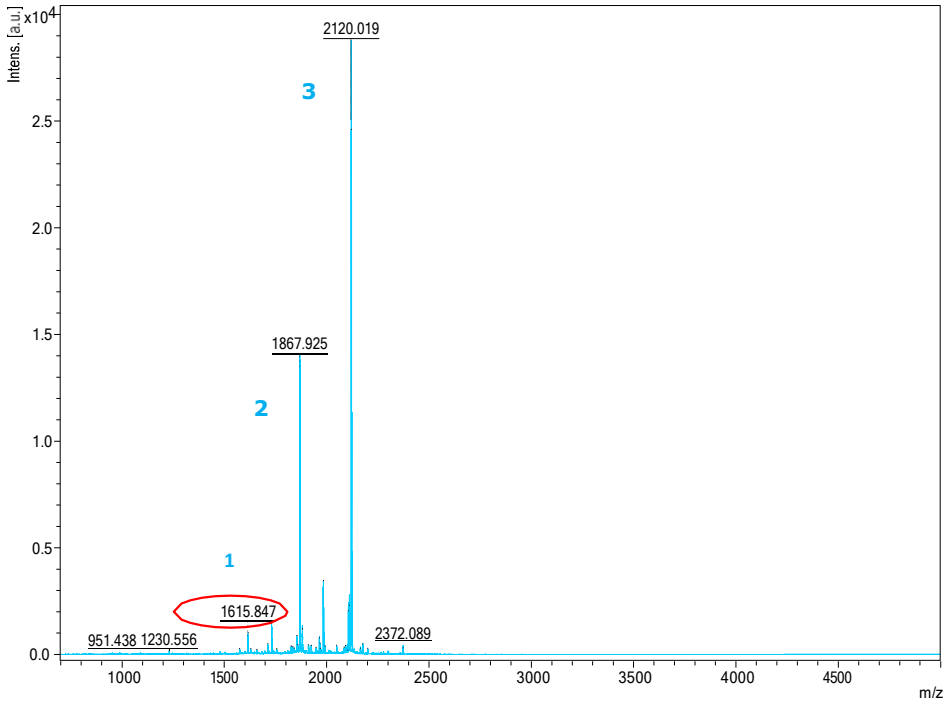


Figure 5. MALDI-TOF mass spectrum with three peaks of interest, labeled by numbers 1,2,3, of the peptide and its byproducts. The smallest peak (labeled 1) showing m/z=1615.847 g/mol is the synthesized peptide. The second peak (2) at m/z 1867.925 g/mol and the third peak (3) at m/z 2120.019 g/mol show the peptide with Pbf, Arg side chain protecting group, as a byproduct.

The x-axis indicates the m/z ratio of the ions observed while the y-axis indicates the ion signal intensity. The three main peaks, labeled 1 (m/z=1615.847 g/mol), 2 (m/z=1867.925 g/mol) and 3 (m/z=2120.019 g/mol) were identified, as shown in Figure 5. These are also the most intensive peaks and they correspond to the synthesized peptide. Peaks, 2 and 3, show a difference in molecular weight of 252,094 g/mol and double that value,

504,172 g/mol, compared to peak 1. This difference in the molecular weight corresponds to the molecular weight of Pbf (Table 4), a protecting group of arginine side chain, guanidine, with its molecular weight of 253,3 g/mol.

Table 4. The expected and measured mass of peptide and the byproducts. Pbf has a molecular weight of 253,3 g/mol.

	EXPECTED	**MEASURED**	**Δ M**
$[M+H]^+$	1615,827	1615,847	0,020
$[M+Pbf]^+$	1869,127	1867,925	1,202
$[M+2xPbf]^+$	2122,427	2120,019	2,408

4.2 LC-MS

Peptide characterization using liquid chromatography-mass spectrometry was performed in two steps. In the LC column, the molecules were separated based on their hydrophobicity/hydrophilicity. The retention time measured the time between the sample injection and the appearance of the peak after chromatographic separation. The MS analyzed the peptide mass and the results of the analysis are presented in Figure 6.

As mentioned in section 3.3, two different cleavage conditions were performed and the crude peptide was analyzed using LC MS. After the peptide was cleaved using the first condition of the cleavage cocktail, the LC trace showed a series of peaks that did not match the expected one, the peptide contained many byproducts.

In the second condition of the cleavage cocktail, DODT was left out to see if maybe the Cys dimerization affected the results by adding additional mass to the peptide. After the LC-MS analysis, better results were obtained, the detected molecular weight corresponded to the expected peptide mass of

1615.847 g/mol. Since the mass of the disulfide bridge was not detected on LC-MS and MALDI-TOF, it was concluded that Cys dimerization did not affect the formation of byproducts. The problem was in the time that the cleavage was left due to the presence of Arg and the difficulty of Pbf group removal.

The final "cleavage cocktail" contained DODT and was left on resin for 4 hours. The cleaved peptide was then analyzed and the LC trace in Figure 6a shows the peptide with a retention time of 2 minutes.

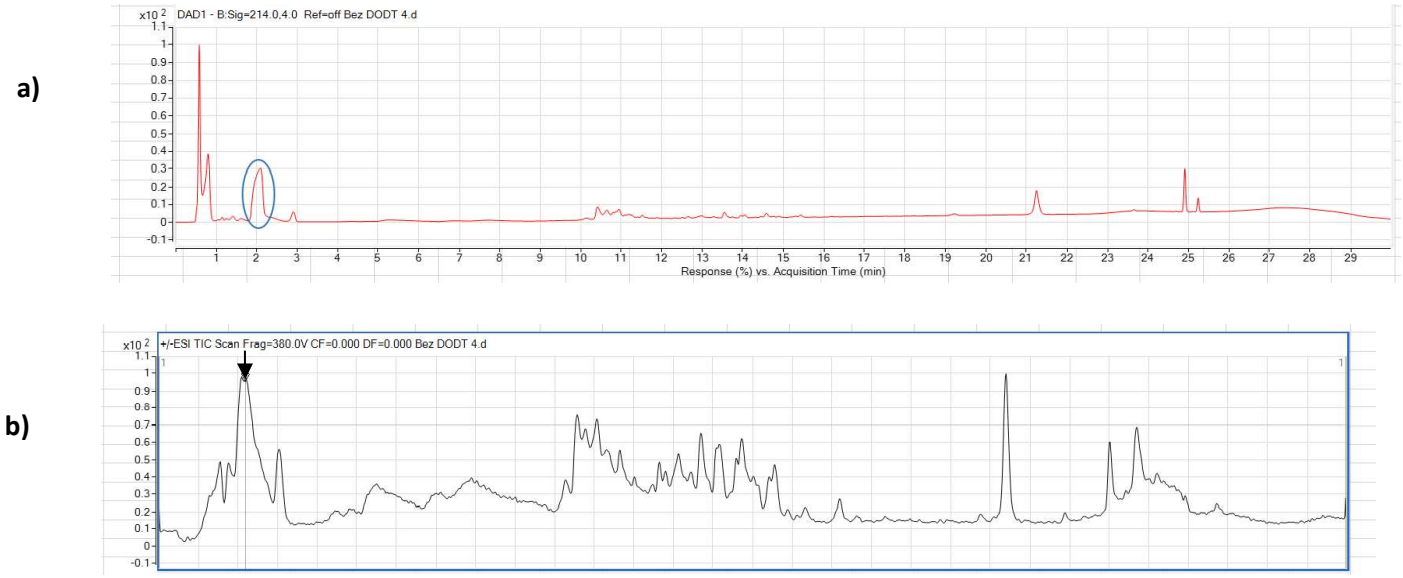


Figure 6. LC-MS data for the peptide at a detection wavelength of 214 nm. **(a)** LC spectrum of a peptide with R_t=2 min (circled), **(b)** The arrow indicates the peak for which the MS spectra is provided after electrospray ionization (ESI).

Then, the sample was analyzed according to its mass-to-charge ratio (m/z). After electrospray ionization (ESI) at 380 V, the representative peak was captured and displayed in the MS spectrum, as shown in Figure 7. Both, negative and positive ESI scans were done to confirm the peptide mass.

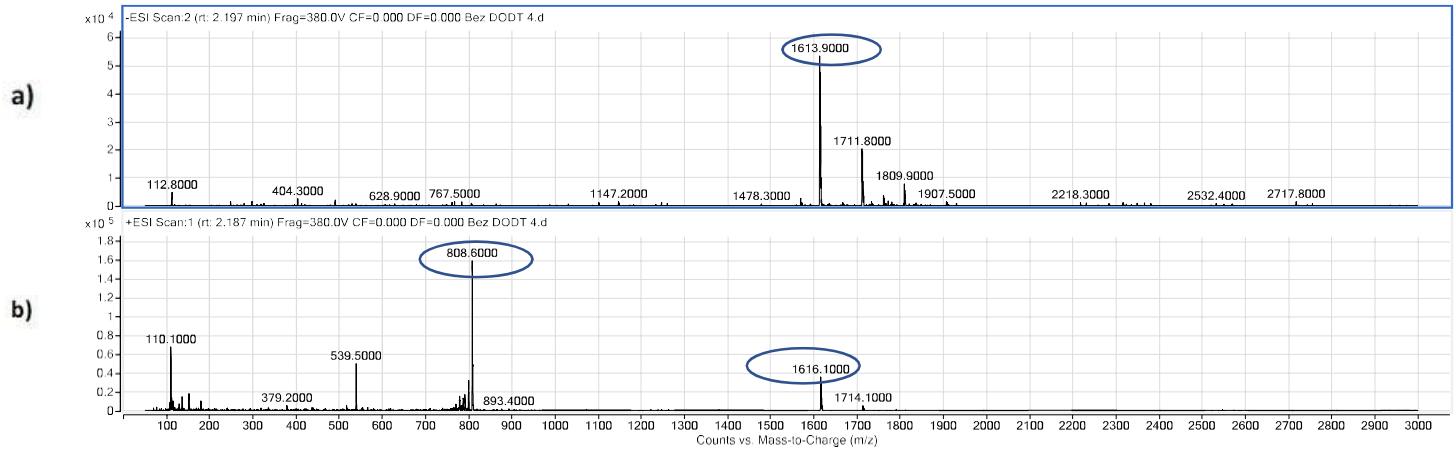


Figure 7. **(a)** Negative and **(b)** positive ESI scans of the peptide. The circled peaks correspond to: **(a)** R_t=2.197 min ($[M_w]$ =1613.9000) and **(b)** R_t=2.187 min ($[M+H]^+$ =1616.1000); $[M+2H]^{2+}$=808.600.

Table 5. The expected and measured peptide mass in -ESI and +ESI scans.

	EXPECTED	**MEASURED**	**Δ M**
[Mw]	1615,827	1613,900	1,927
$[M+H]^+$	1616,827	1616,100	0,727
$[M+2H]^{2+}$	807,914	808,600	0,686

The expected and measured peptide mass is presented in Table 5. The monoisotopic mass of the peptide is 1615.827 g/mol. A negative ESI scan (-ESI) with rt=2.197 min shows the negatively charged form of a molecule created when one proton is removed: $[M-H]^-$ = 1613.9000 m/z. On the other hand, a positive ESI scan (+ESI) with rt=2.187 shows the positively charged protonated molecule. In Figure 7b, two different peaks are detected in the spectrum, both belonging to the peptide. The smaller peak $[M+H]^+$ =1616.1000 m/z is the singly charged ion. The higher peak, $[M+2H]^{2+}$=808.600 m/z, corresponds to the doubly charged ion.

5. DISCUSSION

Using SPPS, the peptide chain Ac-HREAKRTRVCYR-Am was synthesized. This was confirmed using LC-MS and MALDI-TOF.

For this peptide, double coupling was performed for every amino acid by a second addition of the same amino acid. After the first coupling, the same amount of amino acid, HBTU and DIPEA is added, without removing the Fmoc. This ensures a successful attachment of each amino acid to the resin and is often used while working with arginine (Arg). Arg can be a challenge to incorporate into any peptide sequence as it is one of the largest and most sterically hindered amino acids. Arg has a bulky side chain, guanidine, and Pbf is typically used as its protecting group (20). Since this peptide sequence contained four Arg residues, double coupling had to be performed.

After the SPPS, the next step was the acetylation of the N-terminus of the peptide. By adding the acetyl group to the peptide, its properties, such as charge, hydrophobicity and molecular mass change. The free amino group has a positive charge and the acetyl group neutralizes that charge which blocks further ionization and other modifications of the amino group (12.)

The final step of the synthesis was to cleave the peptide from the resin. The cleavage removes all the side chain protecting groups and the resin attached to the peptide. Peptides that contain Arg require a longer time for the cleavage, about four hours or more compared to the standard two hours.

Out of six different peaks in MALDI-TOF mass spectrum, the three biggest ones confirmed the mass of the peptide, the ones labeled 1,2,3 in Figure 5: peak 1 with m/z=1615.847 g/mol, peak 2 with m/z=1867.925 g/mol and peak 3 with m/z=2120.019 g/mol. The difference in molecular weight between peaks 1 and 2 is the same as the difference between peaks 2 and 3; m/z= 252,094 g/mol. The difference between peaks 1 and 3 is 504,172, double the

value of 252,086. It was confirmed that peptide sequences with multiple arginine residues may cause a problem because of improper removal of side chain protecting groups, resulting in added mass. The molecular weight of Pbf is 253.3 g/mol and that concludes that the protecting group from Arg was not properly removed and caused the formation of byproducts.

With LC-MS analysis a few difficulties were encountered. The peptide sample was injected into a C_{18} column that acts as a stationary phase, commonly used in reverse phase chromatography. Then, the mobile phase was introduced into the column: an aqueous solvent, H_2O, and an organic solvent, acetonitrile. Solvents were added in varying concentrations over time. Since the inside of the column is hydrophobic, the samples have hydrophobic interactions with the stationary phase. Non-polar, hydrophobic compounds attach to the column and migrate down the column at a slower rate (21).

Hydrophilic peptides are either unretained by the C_{18} column or weakly bound to it, making them difficult to purify (22). The synthesized peptide is extremely hydrophilic, making it hard to have any hydrophobic interactions with the column. That explains why the peptide flows out early, with a retention time of 2 minutes. To increase retention of hydrophilic peptides a different approach should be used.

One way to optimize the LC-MS method would be to decrease the concentration of acetonitrile in the mobile phase. The method firstly used in this experiment consisted of water (A) and acetonitrile (B) applied from A/B 99.9/0.1 to A/B 60/40 over 20 min. Increasing the percentage of water and decreasing the percentage of acetonitrile by using A/B 99.9/0.1 to A/B 70/30 over 20 minutes should increase the peptide retention time. In future experiments, I would decrease the amount of acetonitrile and try out different concentrations of solvents in the gradient until the retention time increases.

If these modifications would not provide better results, a different technique that is more suitable for hydrophilic peptides should be used. Hydrophilic interaction chromatography (HILIC) may be a good choice as it is used for separating highly hydrophilic compounds that are too polar to be well retained by the column (23).

Unlike the hydrophobic C_{18} column used in LC-MS, the stationary phase used in HILIC is a silica gel modified with polar sorbents containing polar functional groups (e.g. 2-hydroxyethyl aspartamide, carbamoyl and diethylaminoethyl). Peptides retained by the HILIC column are eluted with a mobile phase of increased polarity. This can be accomplished by either decreasing the concentration of an organic solvent or increasing the concentration of an aqueous solvent in the mobile phase (24). The mobile phase forms a water-rich layer on top of the stationary phase and the separation is based on a different distribution of the analyte between the acetonitrile-rich mobile phase and the water-enriched layer on the stationary phase. The partitioning equilibrium shifts towards the water layer when the analyte is hydrophilic, meaning that the more hydrophilic the analyte is, the more it is retained on the column (25). For all these reasons, better results for peptide analysis may be achieved using HILIC.

Since in both MALDI-TOF and LC-MS spectra, peaks of highest intensity are the ones containing byproducts, further purification is needed. This would result in an increasing concentration of pure peptide.

6. CONCLUSION

In this thesis, the Ac-HREAKRTRVCYR-Am peptide was successfully synthesized using Fmoc SPPS strategy. The mass of the obtained peptide 1615,827 g/mol, was confirmed by liquid chromatography-mass spectrometry and matrix-assisted laser desorption/ionization.

Even though the synthesis was successful there were a few difficulties during the process. During LC-MS and MALDI-TOF analysis, multiple peaks containing the peptide with its byproducts were observed. This concludes that further purification is required to yield a peptide of high purity.

Due to the peptides' high hydrophilicity, a few difficulties during LC-MS analysis were encountered. The peptide was poorly retained on a reverse-phase C_{18} column and because of this, there is a need for a different approach to achieve better on-column retention. One way would be to decrease the concentration of acetonitrile in the mobile phase and if that would not work another option would be to try a different technique. In this case, HILIC could be suitable because it utilizes a polar stationary phase along with a polar mobile phase allowing the retention of polar analytes. Using HILIC, this peptide could be better retained on the column.

To conclude, the method for characterization of this specific peptide needs further optimization. The result should be a peptide of high purity that can be used in future studies for the assessment of its potential to catalyze ester hydrolysis. This peptide needs to go through the process of purification to increase its purity and different analysis techniques need to be explored to achieve a suitable retention time on the chromatographic column.

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Jezične vještine	• MATERINSKI JEZIK: hrvatski • Engleski: Slušanje C1 Čitanje C1 Govorna Produkcija C1 Govorna Interakcija C1 Pisanje C1 • Njemački: Slušanje B1 Čitanje B1 Govorna Produkcija B1 Govorna Interakcija B1 Pisanje B1
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