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Treball Final de Grau

Development of an HPLC-DAD method for the analysis of a pharmaceutical product composed of ten peptides
Desenvolupament d'un mètode HPLC-DAD per a l'anàlisi d'un producte farmacèutic format per deu pèptids

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M’agradaria agrair a la Judit Benito, tutora del treball des de l’empresa, per tots els aprenentatges que he assolit durant aquest període i per donar-me l’oportunitat de desenvolupar el meu treball final de grau sobre un projecte tan interessant. També agraeixo a la Núria Serrano, tutora del treball des de la universitat, pels seus consells i implicació en el treball des del primer dia.

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REPORT

IDENTIFICATION AND REFLECTION ON THE SUSTAINABLE DEVELOPMENT GOALS (SDG)

To ensure a more promising future for the habitants of our planet, the United Nations approved the 2030 agenda in 2015. This agenda is composed of 17 Sustainable Development Goals (SDG) with the purpose of eradicating poverty, promoting economic growth, ensuring quality education, health and social protection. These goals can be grouped in five broad areas, known as the five Ps: people, prosperity, planet, peace and partnership. This work contributes to three of these Sustainable Development Goals.

First, SDG 3: Good Health and Well-being. This work presents the optimization process of an analytical method used to determine the content of ten peptides in a pharmaceutical product. This step is crucial during the development of new medicines to study the stability of their compounds and degradation products. The synthesis of new medicines plays a crucial role in improving healthcare and the quality of life for people. Therefore, this project contributes to ensure that the quality standards of medicines are accomplished.

Second, SDG 9: Industry, Innovation and Infrastructure. This project encourages technological innovation and continuous improvement in an important industry such as the pharmaceutical sector. The creation and improvement of analytical methods using established techniques like HPLC enhance the quality and research of pharmaceuticals production. The development and accomplishment of new projects facilitate the designing and construction of more sustainable working areas and industries.

Finally, SDG 12: Responsible Consumption and production. The development of an analytical method favours reliable results using the optimal conditions. This helps prevent the overuse of chemical reagents stablishing more sustainable experiences in research laboratories. Moreover, the appropriated recycling system for each residue is applied through all the experimental procedures.

CONTENTS

1. SUMMARY

Since their introduction for therapeutic purposes approximately a hundred years ago, peptides have played an important role in the pharmaceutical industry, and their development has been reflected in the growing production of peptide-based pharmaceutical products. Especially in the recent years, the pharmaceutical industry has experienced an exponential increase in the use of these molecules, and investment in research and development of these drugs is expected to increase in the coming years. These trends can be associated to the numerous advantages peptides present for therapeutic uses and their ease of synthesis.

In this project, a high-performance liquid chromatography (HPLC) preexisting method coupled with diode array detector (DAD) for peptide analysis has been optimized. The objective has been to improve the method parameters to achieve a better separation and detection through ultraviolet absorption of ten peptides that are part of a pharmaceutical formulation. This method is essential for the quantification of the content of the compounds and to analyse potential degradation products.

The optimization of the HPLC-DAD method has been based on the study and adjustment of the specific parameters that affect directly the separation, detection and quantification of the analytes. In this project, a suitable chromatographic column and mobile phase were selected to enhance the chromatographic performance. Afterwards, essential parameters such as the gradient and the column temperature were optimized to achieve a proper separation and quantification. Finally, an individual assessment of the optimal detection wavelength for every peptide was performed.

The final version of the studied method shows important improvements respect the initial one. Peak splitting, peptide separation, quantification and quality parameters such as the resolution, theoretical plates and asymmetry factor are the principal aspects that have improved during the development of the method.

Keywords: Peptide, HPLC-DAD, pharmaceutical product, quality parameters.

2. RESUM

Des dels inicis del seu ús amb finalitats terapèutiques fa aproximadament cent anys, els pèptids han ocupat un paper important a la indústria farmacèutica, i el seu desenvolupament s'ha fet notar en el creixement de la producció de productes farmacèutics basats en pèptids. Ha estat especialment en els últims anys que aquesta indústria ha experimentat un increment exponencial en l'ús d'aquestes molècules i es preveu que la inversió en recerca i desenvolupament d'aquests fàrmacs sigui major en els pròxims anys. Aquests fets es poden associar al conjunt d'avantatges que presenten els pèptids per funcions terapèutiques i a la seva facilitat de síntesi.

En aquest projecte, s'ha dut a terme l'optimització d'un mètode preexistent per cromatografia de líquids d'alta eficiència (HPLC) amb detector de sèrie de díodes (DAD) per l'anàlisi de pèptids. L'objectiu ha estat millorar els paràmetres del mètode per tal d'aconseguir una millor separació i detecció mitjançant l'absorció ultraviolada de deu pèptids que formen part d'una formulació farmacèutica. Aquest mètode és essencial per la quantificació del contingut dels compostos i analitzar possibles productes de degradació.

L'optimització del mètode HPLC-DAD s'ha basat en l'estudi i ajust dels paràmetres específics que afecten directament a la separació, detecció i quantificació dels analits. En aquest projecte, primerament s'ha seleccionat una columna cromatogràfica i una fase mòbil més apropiades per millorar l'eficiència cromatogràfica. Seguidament, s'han optimitzat els paràmetres essencials per aconseguir una bona separació i quantificació com són el gradient i la temperatura de la columna. Finalment, s'ha fet un estudi de la longitud d'ona de detecció ideal de cada pèptid individualment.

La versió final del mètode estudiat presenta avenços importants respecte a l'inicial. El desdoblament dels pics, la separació dels pèptids, la quantificació i els paràmetres de qualitat com la resolució, els plats teòrics i el factor d'asimetria, són els principals aspectes que han experimentat una millora durant el desenvolupament del mètode.

Paraules clau: Pèptid, HPLC-DAD, producte farmacèutic, paràmetres de qualitat.

3. INTRODUCTION

3.1. PEPTIDES IN THE PHARMACEUTICAL INDUSTRY

Peptides are short-chain biomolecules of up to fifty amino acid residues and often stabilized by disulphide bonds. They have highly specific biological activities depending on their primary sequence and conformational structure. Among these activities, the cell signalling role is very common in most peptides and is based on the translation and delivering of biochemical messages that trigger structural, molecular and cellular effects [1]. The signalling activity takes place in key physiological processes including defence mechanisms, immune response, growth regulation, homeostatic control and reproductive functions [2].

Amino acids, the fundamental structural units of peptides and proteins, are composed of an amino group, a carboxylic group, a hydrogen atom and a variable side chain, which differentiate all the amino acids. In figure 1, a structure of an amino acid can be appreciated. Depending on the size of the amino acid chain, peptides have different nomenclatures. Two amino acids linked by a peptide bond is called dipeptide. Until ten peptides, it is denominated oligopeptide, and polypeptides can contain between approximately ten and a hundred amino acids [3] . When the polypeptide chain adopts a defined three-dimensional structure, is referred as a protein. There are twenty different natural amino acids codified in DNA, which are the base of peptides and proteins. However, artificial peptides can be obtained through the use of recombinant microorganisms, transgenic plants and chemical synthesis [4].

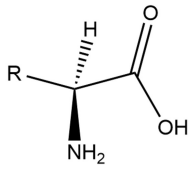

Figure 1. Structure of an amino acid.

Peptides have been used for therapeutic applications since the beginning of the 20th century. Insulin, a peptide hormone, was used for the first time in 1921 for the treatment of diabetes mellitus, a disease with a high mortality rate during these years [5, 6]. After this achievement, peptide-based products have been in constant development and nowadays play an important role in the pharmaceutical industry.

Initially, peptide-based medicine production had to deal with different difficulties that stopped its promising progression such as manufacturing difficulties and investigation high costs. Until 1990, only ten pharmacological products based on peptides were developed by chemical synthesis. However, by the year 2000, the number increased to forty and more than eighty were undergoing clinical trials at various stages [4]. Peptides can be used worldwide for the treatment of many diseases that affect millions of people. They can have therapeutic uses in cancer treatments, metabolic disorders, cardiovascular diseases, autoimmune syndromes and viral infections among other diseases.

Several factors contribute to explain why peptides are so interesting in the pharmaceutical sector. For example, when the sequence, structure and interaction of some oncogenic proteins are known, the appropriated therapeutic peptides can inhibit these interactions by using a sequence from the interaction domain. Moreover, peptides have an easy production process and there exist many ways of modifying their chemical sequence like chemical synthesis or molecular biology techniques [7].

Peptides have more similar properties to drugs than recombinant proteins or whole pathogen vaccines. Therefore, the investigation for this kind of vaccine delivery will keep increasing in the pharmaceutical industry [8]. Other advantages of using peptides for therapeutic purposes are their high potency of action, high target specificity and selectivity, low toxicity, low accumulation in tissues and high biological and chemical diversity. Nevertheless, they have some disadvantages. Peptides are not metabolically stable because they can undergo oxidation and hydrolysis of their peptide bonds, they have poor membrane

permeability, poor solubility and rapid clearance. Moreover, oral administration of peptide drug products is very challenging and these kinds of products have earlier expiration dates and stricter demands for storage conditions [6, 9]. For this reason, constant investigation in peptide-based medicines is being carried out to take advantage of the benefits of therapeutic peptides and find the way to counter their disadvantages.

3.2. ANALYTICAL TECHNIQUES FOR PEPTIDE SEPARATION

During the development of a peptide-based medicine, an analytical method able to identify and separate all the peptides that constitute this drug must be established. This analytical method is essential to ensure the identity, stability and purity of the peptides considered and to monitor potential degradation products.

Without an optimal analytical method, a pharmaceutical product with an incorrect composition of the peptides or a reduced therapeutic efficacy could be administered compromising patient safety. Moreover, accurate quantification of the peptides is needed to ensure that the product meets the quality control requirements and the regulatory standards.

3.2.1. High-Performance Liquid Chromatography

High-Performance Liquid Chromatography (HPLC) is a widely used experimental technique to separate analytes in a multi-component mixture based on their different affinity between the liquid mobile phase and the solid stationary phase [10]. This technique can be used in a width range of fields such as pharmaceutical, environmental and forensics [11].

Depending on the type of sample, different kinds of chromatography exist. Normal phase and reversed phase chromatography are based on the principle of hydrophilic and hydrophobic interactions. Ion exchange chromatography hinges on the attraction between charged molecules and charged functional groups bound to the stationary phase [12]. Size exclusion chromatography separates molecules by size [13] and bio-affinity chromatography separation is based on specific reversible interaction of proteins with ligands [14].

The popularity of HPLC is a consequence of the advantages it provides. This technique offers a highly specific and accurate analytical approach for a variety of complex substances, high resolution and high-speed separation of the compounds. It can handle macromolecules and has high adaptability due to the wide range of stationary phases and column dimensions. Moreover, this technique works at high pressures, handling up to 400 bars. One of the characteristics that has provided a bigger improvement in the analysis of multicomponent mixtures is the development of highly efficient micro particulate bonded phases [14]. Nevertheless, HPLC also has some disadvantages like the difficulty in separating certain protein-specific antibodies and very complex samples, and the difficult selection of the conditions when a chemical is not selective to the stationary phase [15].

The most used HPLC technique is reversed-phase (HPLC-RP) due to its selectivity and sensitivity for a vast number of analytes. Moreover, because of the capacity to separate compounds based on their hydrophobicity or lipophilicity, is widely used in pharmaceutical analysis [16, 17]. The hydrophobic functional group of the stationary phase in HPLC-RP analyses is made of modified silica-based material. Although there are a wide variety of commercially available columns, the most used are the octadecylsilane (C18) and the octylsilane (C8). On the one hand, C18 columns are usually utilized for nonpolar compounds. On the other hand, C8 columns are used for the analysis of highly polar molecules [18].

In HPLC-RP, a combination of hydrophilic and hydrophobic mobile phases is usually considered. Knowing that the stationary phase is apolar, a decreasing polarity gradient is commonly applied to promote the elution of the less polar compounds. This is helpful to increase the eluotropic strength, which is the efficiency of the mobile phase to move the analytes through the chromatographic column and achieve a quicker elution of the compounds. The eluotropic strength is unique for every solvent while using the same stationary phase [19].

Reversed-phase chromatography was used for the first time with the purpose of separating peptides in 1976 when a method for the quantitative analysis of oxytocin and vasopressin in individual rat pituitaries was developed [17, 20]. In HPLC-RP the hydrophobic part of the peptide interacts with the hydrocarbon chain of the stationary phase [21].

3.2.2. Ultra-High-Performance Liquid Chromatography

Ultra-High-Performance Liquid Chromatography (UHPLC) is a chromatographic technique which comes from the evolution of HPLC. The differences in fundamental working conditions compared to HPLC, allow UHPLC to achieve improved results. First, with UHPLC is possible to work at higher pressures compared to its predecessor. While HPLC systems can handle pressures of up to 400 bars, UHPLC instruments can work in a pressure range between 1200 and 1400 bars [22]. Moreover, the chromatographic columns used for this system are usually shorter reducing the time of analysis.

The particle size of the columns is smaller compared to HPLC. While HPLC columns have usually a particle size of between 3 and 5 µm, UHPLC ones can work with particles smaller than 2 µm. This characteristic plays a key role to achieve results with better resolution, greater theoretical plates values, more symmetric peaks and an improvement on their quantification.

Another significant characteristic of UHPLC is the low consumption volumes of mobile phase due to the lower flow rates needed to achieve similar separations compared to HPLC. This provides outstanding benefits in the reduction of the usage of organic solvents and laboratory material contributing to more sustainable analytical practices [23].

Nevertheless, some disadvantages of this technique are the more demanding sample preparation to avoid obstructions in the column, a more strict maintenance of the instrument and elevated costs due to the need of materials capable of supporting high pressures. Reversed phase UHPLC is widely used in biomedical investigation and the pharmaceutical industry. It accomplishes efficient separation of peptides and the small particle size allows good resolutions between peptides with similar physicochemical properties [24,25].

3.2.3. Capillary electrophoresis

Capillary electrophoresis is an analytical technique useful to separate complex samples [26]. The fundamental of this technique is the differential migration of charged species under an electric field [27]. The electrophoretic mobility is greater for highly charged and small-sized ions, while neutral particles remain unaffected. Therefore, atomic radius, charge and viscosity are crucial factors for capillary electrophoresis. There exist two kinds of mobility, electro-osmotic flow and electrophoretic mobility [28]. Some of the advantages that this technique provides are the high efficiency of the assay, short separation times, high resolution and low sample consumption [29]. Nevertheless, capillary electrophoresis is not as robust as other separation techniques and is not recommended for compounds with small concentrations because the low volume of injection can difficult their detection.

Capillary electrophoresis has high potential for the analysis of biopharmaceuticals. Some capillary electromigration separation techniques have been developed for the analysis of complex samples in life sciences areas such as clinical chemistry and metabolomics. Separation methods using capillary electrophoresis are very effective for therapeutic peptides because their modification often induces a change in their net charge [30].

3.3. DETECTORS FOR PEPTIDE ANALYSES

After the separation of the compounds is achieved, they are detected by a suitable detector. The concentration of the sample analysed is proportional to the intensity of the electric signal that reaches the detector, therefore analytes can be quantified [31]. Several detectors are widely used for separation techniques. However, the most commonly used for peptide analyses are explained below.

3.3.1. Diode array detector (DAD)

The diode array detector (DAD) is a widely used detector, which measures the absorbance through the full UV-Vis wavelengths range generating three-dimensional spectral data. First, a lamp generates polychromatic light directed through a flow cell. Then, the analytes and the eluting medium absorb light at characteristic wavelengths. The transmitted light is spread into a spectrum and then directed through a photo-sensitive array of diodes. Finally, the spectrum is compared with a reference one of the solution without the analyte. A few years ago, diode array detectors from different analytical instruments had issues of reproducibility, sensibility and

resolution. Nevertheless, nowadays these problems are solved for these detectors after constant improvement in photodiode technology [32].

It has high applicability for studies of samples containing different peptides because it allows an analysis of the absorbance at different wavelengths for each compound. This is essential to determine the optimal conditions to achieve a correct detection of all the peptides. The peptide bond absorbs light at wavelengths between 180 and 220 nm due to the transition $\pi \rightarrow \pi^*$, therefore, many analyses are focused on this range of wavelengths [33]. The acquirement of the UV spectra at different wavelengths for the same sample allows the study of the peak purity which can be helpful in case of peptides with similar retention times [34].

3.3.2. Mass spectrometry (MS)

A highly used technique for analysing ionisable compounds is mass spectrometry (MS), which can be coupled to an HPLC system to work as a detector. In this case, the mass-to-charge ratio of charged molecules is measured. First, some of the functional groups of chemical compounds are ionized using an ion source such as electrospray (ESI) or atmospheric pressure chemical ionization (APCI), among others. After that, ions are separated according to their mass-to-charge ratio through an analyser. There exist many analysers depending on the purpose of the assay. Some examples are quadrupole filters, time-of-flight and ion trap mass analyser. Finally, the ions are counted in the detector [35]. This detection technique is well-known for its high sensibility and specificity.

Because peptides are ionisable compounds, mass detectors coupled with separation techniques are widely used to analyse complex peptide-based samples. In mass spectrometry, when complex samples are analysed, low abundance compounds may not be ionised due to competitive interactions, which could lead to erroneous identification or quantification. For this reason, when a separation technique is employed, the prior separation of analytes at different retention times before entering the detector ensures the ionization of the molecules. In general, techniques such as LC/MS permit the measurement of many more peptides [36].

3.3.3. Fluorescence detector (FLD)

Another commonly used detector is fluorescence detector (FLD). Light at a specific wavelength excites the molecules of the sample, which then emit photons at a different wavelength. These photons are directly proportional to the concentration of the molecule in the sample. Therefore, quantitative analysis can be performed. Fluorescence detectors offer many advantages including their sensibility, which is from ten to a thousand times greater than in UV detectors [37].

Nevertheless, despite being very common in techniques such as HPLC, they are less used than the UV and mass detectors because of the requirement that analytes must contain native fluorescence or have been treated with fluorescence derivatives [38]. In the case of peptide analysis, they can only be used for peptides that emit fluorescence. Therefore, analyses of peptide-complex samples using this detector are limited. However, for analyses of peptides that contain amino acids such as tryptophan, tyrosine or phenylalanine, which exhibit natural fluorescence, is highly recommended.

3.4. QUALITY PARAMETERS FOR SEPARATION TECHNIQUES

The analysis of the quality parameters provides information about the performance of the assay. This is fundamental to perform some modifications on the analytical method and improve its efficiency. Next, the main quality parameters analysed in separation techniques are explained according to the US Pharmacopeia (USP) criteria [39].

- The retention time (t_R) refers to the elapsed time between the moment in which the sample is injected to the instrument and the appearance of the maximum peak response representing each compound. This parameter is characteristic for every different compound if the same chromatographic conditions are used. Therefore, it is an important parameter for the identification of the compounds. Nevertheless, since many compounds can share similar retention times it is not an unequivocal identification parameter.

- The number of theoretical plates (N) is an indicator of the performance of the column. Higher theoretical plates values indicate high resolving power. A theoretical plate represents a single equilibrium step between the stationary and the mobile phases [40]. Once the data is obtained, the following formula can be used to achieve the manual calculation:

$$N = 5.54\left(\frac{t_R}{W_h}\right)^2$$

Equation 1: Calculation of the theoretical plates number, where t_R is the retention time for the peak and W_h is the peak width at half-height

- The resolution (R_s) between two adjacent peaks in a chromatogram gives information about the separation achieved among them [41]. Therefore, high resolution indicates better separation of the compounds and good analytical precision, which means that peaks are less overlapped. Resolution is calculated using the following equation:

$$\mathrm{R}_s = \frac{1.18(t_{R2} - t_{R1})}{W_{h1} + W_{h2}}$$

Equation 2: Calculation of the resolution between two peaks where t_R are the retention times and W_h are the peak widths at half-height.

- The asymmetry factor (A_s) evaluates the shape of peaks. When the asymmetry factor is 1.0, there is complete symmetry. In cases where $A_s > 1.0$, the peak is tailing and, on the contrary, when $A_s < 1.0$, the peak is fronting. Suitable values of asymmetry are needed to ensure good precision and accuracy. Otherwise, performance problems could take place. The asymmetry factor is calculated with the following formula:

$$A_s = \frac{W_{0.05}}{2d}$$

Equation 3: Calculation of the asymmetry factor, where $W_{0.05}$ is the width of the peak at one-twentieth of the peak high and d is the distance between the peak maximum and the leading edge of the peak at one-twentieth of the peak high.

- The signal to noise ratio (S/N) is a value that reflects the relation between the intensity of the peak and the noise of the baseline. To improve detection levels, significantly differences between the signal response and the baseline noise must be achieved [42]. Usually, the limit of detection (LOD) is computed as 3 times the S/N ratio and the limit of quantification (LOQ) is computed as 10 times the S/N ratio. If there is not a suitable noise, precision and accuracy of quantification can be affected. According to the USP, the range of noise is considered over a distance of at least five times the width at half-height of the peak. S/N is calculated through the following formula:

$$\mathrm{S/N} = \frac{2H}{h}$$

Equation 4: Calculation of the S/N ratio, where H is the high of the peak corresponding to the studied compound and h is the range of noise

In this project, HPLC-DAD will be used for the optimization of an analytical method for peptide analysis considering the theoretical fundaments of their physicochemical properties and behaviour.

4. OBJECTIVES

The objective of this project is to optimize a preexisting HPLC-DAD analytical method able to analyse quantitatively the content of ten different peptides of a pharmaceutical product under development.

- First, a study of the initial conditions of the method will be performed to identify all the peptides individually and their initial retention times.
- Then, the most important chromatographic parameters are going to be evaluated in order to optimize the conditions of the method. These parameters are: chromatographic column, mobile phase, mobile phase gradient and temperature of the column.
- Finally, a study of the optimal detection wavelength will be accomplished. The absorbance of all the peptides will be individually evaluated through the UV range and the most suitable wavelength for the method will be selected.

The purpose for the final version of the method is to guarantee a correct separation of all the peptides and improve the quality parameters of the assay.

5. EXPERIMENTAL SECTION

5.1. MATERIALS AND METHODS

5.1.1. Reagents

The following reagents were involved during the preparation of the different mobile phases tested and the standard solutions:

- TFA (≥ 99.9%, for peptide synthesis, from VWR Chemicals (Avantor), USA)
- Glacial acetic acid (99.7%, from Fisher, USA)
- Phosphoric acid (85%, from Honeywell, USA)
- Acetonitrile (HPLC-UV grade, from VWR Chemicals (Avantor), USA)
- Potassium di-hydrogen phosphate (pure, from PanReac, Spain)

5.1.2. Standards

Individual standards of the ten peptides (identification code: TIIM024, TIIM016, TIIM005, TIIM021, TIIM001, TIIM007, TIIM002, TIIM023, TIIM009 and TIIM014). Lyophilized powder of approximately 20 mg per peptide (BCN Peptides, Spain).

5.2. INSTRUMENTS

The instruments used during the preparation of the mobile phases and standard solutions are listed below:

- pH-meter GLP 21 (Crison, Spain)
- Analytical balance XS205 Dual Range, precision 0.00001 g (Mettler-Toledo, Switzerland)
- Ultrasonic Bath E 300H (Elmasonic, Germany)
- Milli-Q water purification system Advantage A10 (Merck, Germany)
- Vortex agitator Yellowstone TTS2 (IKA, Germany)
- Vacuum pump DOA-V517-BN (Gast, USA)
- Magnetic stirrer RCT Basic (IKA, Germany)

5.3. HPLC SYSTEM

The experimental part of the project is performed using an HPLC 1200 series system (Agilent, USA). This instrument includes the following modules:

- Quaternary Pump G1311A
- Autosampler G1329A
- Thermostatted column compartment G1316A
- Degassing unit G1322A
- Diode Array Detector (DAD) G1315D

The data collected from the chromatographic assays is processed with the software OpenLab, from Agilent.

5.4. MOBILE PHASES PREPARATION

During the optimization of the method, two different mobile phases were tested. Their preparation is described below:

5.4.1. TFA mobile phases

Mobile phase A: 1 mL of TFA is transferred to a 1 L volumetric flask and diluted up to the mark with H_20 ultrapure. The mobile phase is sonicated for 20 minutes.

Mobile phase B: 1 mL of TFA is transferred to a 1 L volumetric flask and diluted up to the mark with acetonitrile. The mobile phase is sonicated for 20 minutes.

5.4.2. KH_2PO_4 buffer mobile phase

Mobile phase A: 3.4 g of KH_2PO_4 are diluted in approximately 900 mL of H_2O ultrapure. The pH is adjusted to 2.0 with phosphoric acid and the content is transferred to a 1L volumetric flask. The solution is diluted up to the mark with more H_2O ultrapure and then filtered with a 0.45 µm nylon filter. Finally, the mobile phase is sonicated for 20 minutes.

Mobile phase B: Acetonitrile 100%.

5.5. STANDARD SOLUTIONS PREPARATION

Next, the preparation of the individual peptide standard solutions and the peptide mixture standard solutions is explained.

5.5.1. Preparation of the individual peptide standard solutions

Standard solution of the peptide TIIM023 (1 mg/mL concentration): to a vial containing approximately 20 mg of the TIIM023 individual standard, the necessary amount of a 90:10 acetic acid solvent is added to achieve a 1 mg/mL solution. The solution is vortexed until the content of the vial has dissolved completely.

Preparation of the rest of the individual peptide standard solutions (1 mg/mL concentration): to each vial containing approximately 20 mg of the individual standards, the necessary amount of a 65:35 acetic acid solvent is added to achieve a 1 mg/mL solution. The solution is vortexed until the content of the vial has dissolved completely.

5.5.2. Preparation of the peptide mixture standard solution

Peptide mixture standard solution (0.05 mg/mL concentration): 1 mL of each individual standard solution is transferred to a 20 mL volumetric flask. The solution is diluted up to the mark with the mobile phase A used in the same assay.

6. RESULTS AND DISCUSSION

The development of a HPLC-DAD analytical method is performed throughout the optimization of all the parameters that can improve the performance of the assay. The objective is to define a method with a good separation, detection and quantification for all the peptides studied. The studies conducted for each parameter are explained in the following sections of this work in the order in which they were performed.

6.1. INITIAL CONDITIONS

The HPLC-DAD method was previously developed by another R&D department of the company. During this time, the initial conditions of the method were defined. From these initial conditions, this project presents the core development and optimization of the most important chromatographic parameters to improve the performance of this preexisting method. Table 1 presents the analytical conditions of the preexisting method.

Table 1. Initial analytical conditions of the method.

Initial analytical conditions			
Column		Waters SunFire C18 4.6 x 250 mm dp=5 µm	
Mobile phase A		0.1 % TFA pH=1.9	
Mobile phase B		Acetonitrile 0.1% TFA	
Detection wavelength [nm**]**		220	
Flow rate [mL/min**]**		1.0	
Column temperature [ºC**]**		50	
Injection Volume [µL**]**		50	
Gradient	**Time [**min**]**	**Mobile phase A [**%**]**	**Mobile phase B [**%**]**
	0	85	15
	10	73	27
	20	73	27
	25	65	35
	45	65	35
	50	85	15
	60	85	15

Before starting the optimization of the HPLC-DAD parameters, an individual analysis of the peptides was performed. The purpose was to identify the initial retention time of each peptide and discover the order of elution. This was a fundamental step before working with the ten peptides at the same time. As it is stated in the experimental section, every peptide has an identification code and this nomenclature was used to assign them in all the chromatograms.

The standard solutions of the individual peptides were analysed using the initial analytical conditions. The chromatograms acquired are presented in figure 2 in the order of elution of the peptides.

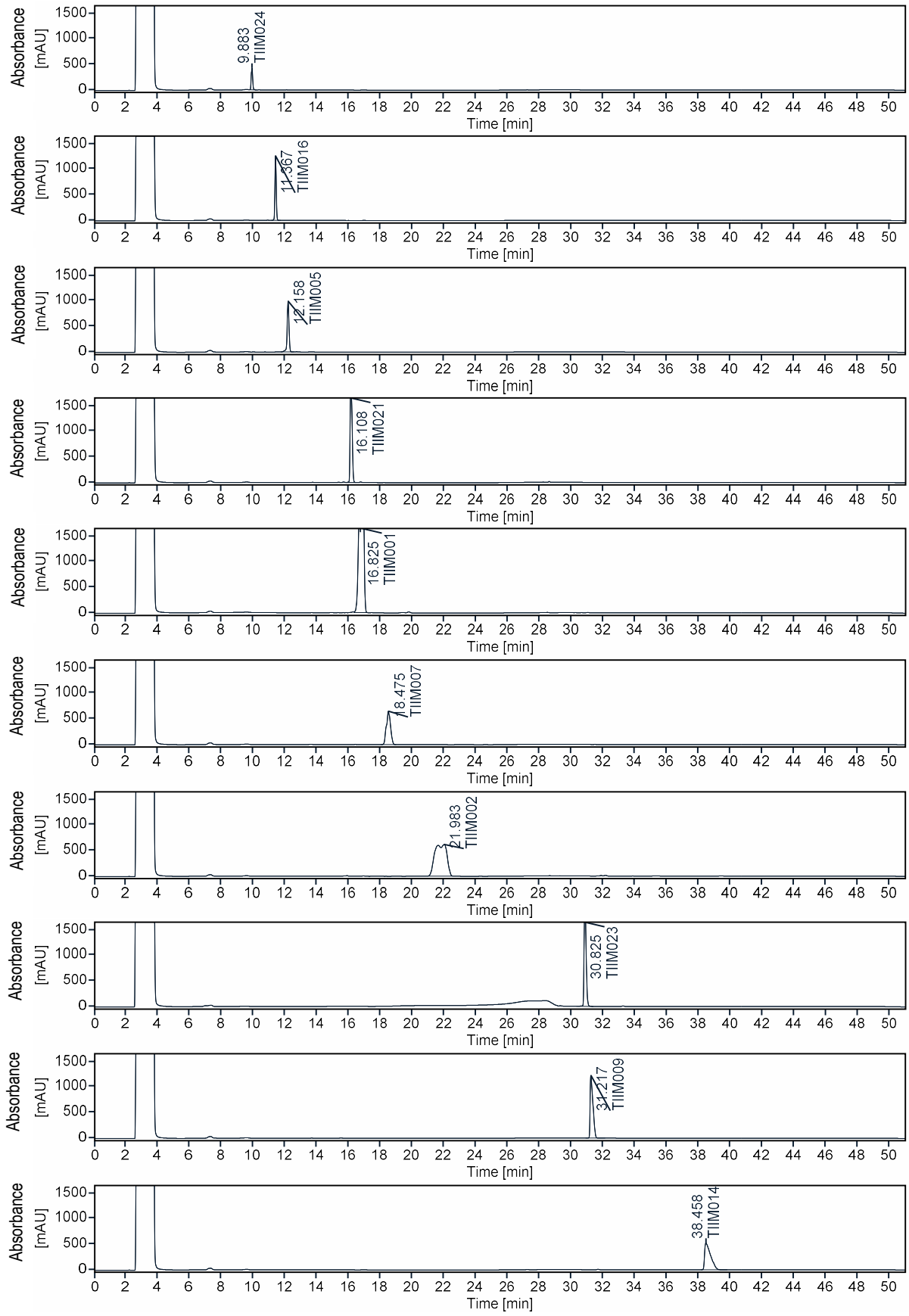

Figure 2. Individual chromatograms of the ten analysed peptides in order of elution. 1 mg/mL concentration and 50 µL of volume of injection.

As it can be seen in figure 2, each peptide presented a different retention time. Therefore, the compounds had a different elution, which benefitted their separation. Nevertheless, as may be observed, the asymmetry of some peaks could be improved with more appropriated chromatographic conditions. A chromatogram of all the considered peptides with concentration of 0.05 mg/mL per peptide analysed at the initial conditions is presented in figure 3.

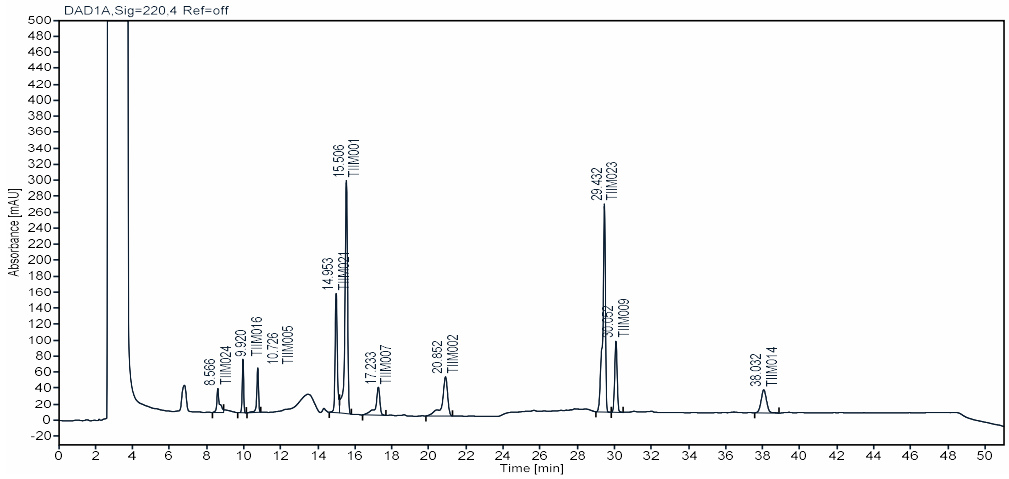

Figure 3. Chromatogram of the standard solution. 0.05 mg/mL concentration and 50 µL of volume of injection under initial method conditions.

As it can be observed, the chromatogram presents a correct separation of the analysed peptides and a properly identification can be achieved. Despite of the good resolution achieved, peak splitting and asymmetries are the principal weaknesses of the applied method. Therefore, the optimization of the parameters of the method will be focused on the improvement of these issues.

6.2. CHROMATOGRAPHIC COLUMN

The selection of the chromatographic column is one of the main steps during an optimization of a method because of its big impact on the separation of the compounds. So far, the column used for the method was the SunFire C18, because its extended length of 250 mm facilitates the separation of complex samples. The C18 stationary phase is widely applied in reversed phase chromatography due to its hydrophobic character and this column is particularly suitable for a method development using low pH values.

Nevertheless, columns with more appropriated physicochemical properties for the objectives of the method could be selected. The proposed column was the Agilent Poroshell 120 EC-C18. The main difference between them is that the Poroshell presents a superficially porous particle interior, which means that the particles of the stationary phase are formed of a solid inner core and a porous outer shell. This technology reduces the time that the analytes spend inside the particles, reducing the band broadening and improving the efficiency of the separation. The characteristics of both chromatographic columns are presented in table 2.

Table 2. Comparison of the characteristics of the columns tested.

Characteristics	SunFire C18	Poroshell 120 EC-C18
Length [mm**]**	250	250
Width [mm**]**	4.6	4.6
Particle size [µm**]**	5	4
Package specification	C18	C18
Particle substrate	Silica	Silica
Maximum temperature [ºC**]**	50	60
Maximum pressure [bar**]**	415	600
pH range	2-8	2-8
Pore size [Å**]**	100	120
Carbon load [%**]**	16	10
Endcapped	Yes	Yes

To assess if the use of the Poroshell 120 EC-C18 improved the performance of the method compared to the SunFire C18 column, the peptide mixture standard solution was analysed using the Poroshell 120 EC-C18 column (figure 4). Table 3 presents the comparison between the quality parameters obtained using the SunFire C18 column (figure 3) and those achieves using the Poroshell 120 EC-C18 (figure 4).

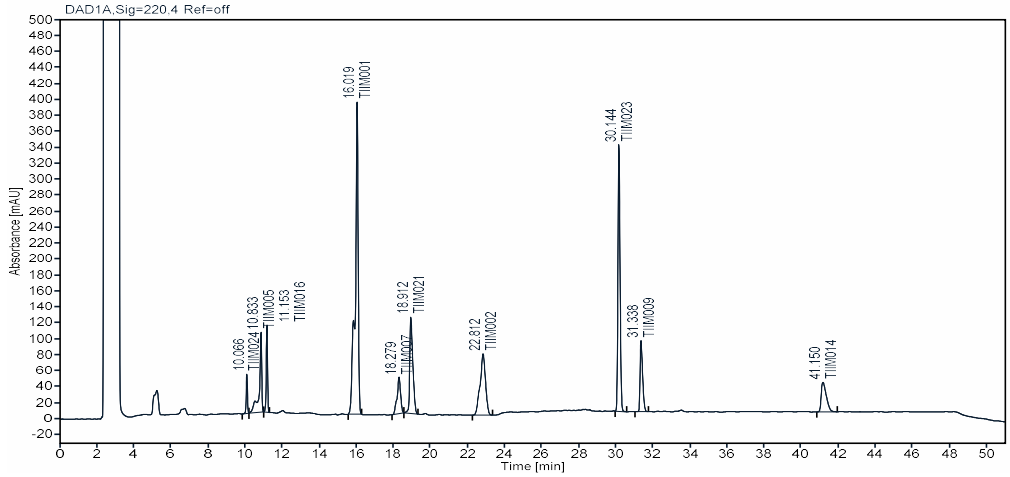

Figure 4. Chromatogram of the standard solution. 0.05 mg/mL concentration and 50 µL of volume of injection using Poroshell 120 EC-C18.

Table 3. Comparison of the quality parameters of the standard solution. 0.05 mg/mL concentration and 50 µL volume of injection using SunFire C18 and Poroshell 120 EC-C18 columns.

SunFire C18						Poroshell C18					
Peptide	**Area**	**Retention time [min]**	**Theoretical plates**	**Asymmetry factor**	**Resolution**	**Peptide**	**Area**	**Retention time [min]**	**Theoretical plates**	**Asymmetry factor**	**Resolution**
TIIM024	312	8.566	38490	1.4	-	**TIIM024**	219	10.066	119671	1.0	-
TIIM016	355	9.920	80111	1.0	8.6	**TIIM005**	893	10.883	80423	0.6	5.7
TIIM005	386	10.726	65727	0.8	5.2	**TIIM016**	513	11.153	132969	1.0	2.3
TIIM021	1189	14.953	91619	1.2	23.2	**TIIM001**	4454	16.019	89971	0.7	29.1
TIIM001	2881	15.506	71970	0.7	2.6	**TIIM007**	600	18.279	57571	0.9	8.7
TIIM007	561	17.233	51592	0.6	6.5	**TIIM021**	1410	18.912	62156	1.5	2.1
TIIM002	1017	20.852	36646	0.7	9.8	**TIIM002**	1575	22.812	32114	0.9	9.6
TIIM023	2641	29.432	320050	0.7	26.7	**TIIM023**	2715	30.144	320198	1.2	20.3
TIIM009	838	30.052	236687	1.0	2.7	**TIIM009**	847	31.338	247681	1.6	5.1
TIIM014	585	38.032	84628	1.0	20.8	**TIIM014**	699	41.150	109101	1.7	26.2

The use of a different chromatographic column commonly leads to a different elution order of the analysed compounds. This is because there is a different selectivity of the column and, therefore, the retention time of the compounds changes. As it can be seen, in this case, the compounds TIIM016, TIIM005, TIIM21, TIIM001 and TIIM007 present a different elution order.

If we compare the chromatograms and the quality parameters achieved using the both columns, a significant improvement was observed with the Poroshell 120 EC-C18. The area values increased for most of the peaks using the Poroshell 120 EC-C18, improving the quantification of the analytes. Moreover, the theoretical plates values were greater for eight peaks in the analysis using the Poroshell 120 EC-C18 as compared to those achieved using the SunFire C18. This demonstrated that the efficiency of the Poroshell 120 EC-C18 column was better than the SunFire C18 one. The obtained results can be related to the particle size of both columns. While the SunFire C18 column has a particle size of 5 µm, the Poroshell 120 EC-C18 column has a particle size of 4 µm. The reduction of the particle size implies an improvement on the efficiency of the column.

In the Poroshell 120 EC-C18 analysis, a special improvement of the peak symmetry of the peptides TIIM007, TIIM002 and TIIM023 was appreciated, as reflected by asymmetry factors closer to one. Nevertheless, peaks related to TIIM005 and TIIM001 still

needed to improve. When the resolution of the peaks was assessed, an improvement was observed for compounds eluting at higher retention times. In particular, the separation between the peaks TIIM023 and TIIM009 improved significantly. Nevertheless, the resolution of the first peaks needed to be better, especially the one between TIIM005 and TIIM016. When the working conditions of both columns were analysed, the Poroshell 120 EC-C18 provided a more extended working temperature limit. This column could be used at 60 ºC compared to the 50 ºC of the SunFire C18 column. The method worked with a temperature of 50 ºC. Therefore, the lifetime of the Poroshell 120 EC-C18 column would be less affected for the temperature than the SunFire C18.

In conclusion, the Poroshell 120 EC-C18 column was selected for further experiments since after analysing all the quality parameters and the working conditions, it proved to be the best choice. It should also be mentioned that, a Poroshell 120 EC-C18 4.6 x 5 mm guard column was also used with the purpose of extending the lifetime of the chromatographic column avoiding possible unwanted precipitations and degradation of the stationary phase.

6.3. MOBILE PHASE

Once the chromatographic column had been selected, a new mobile phase was tested. The first assay was done using a 0.1% TFA solution as mobile phase A and acetonitrile with 0.1% TFA as mobile phase B according to the gradient shown in Table 1. The aim of using TFA was to stablish an acidic pH to ensure that the carboxylic groups are neutral, the amino groups are protonated and to protonate any remaining free silanol group from the silica. Nevertheless, since the sample studied was a complex peptide-based formulation, the variation of acidity in the mobile phase could affect the results obtained. Moreover, TFA absorbs at low wavelengths, between 190 and 220 nm, affecting the baseline noise and making difficult the analysis of analytes with low absorbances.

For this reason, a pH=2.0 KH_2PO_4 buffer solution was tested as the aqueous mobile phase with the purpose of maintaining a stable pH. Moreover, phosphates do not contribute appreciably to the baseline noise in the working range of UV wavelengths used in this method. KH_2PO_4 also offers high solubility in water and it does not interfere with the silica of the stationary phase.

Alongside the new mobile phase used, three new chromatographic conditions were introduced to the method with the purpose of improving the chromatographic results. These conditions were extracted from another existing HPLC-DAD method of the company that is employed for the analysis of a similar product. The flow rate was increased to 1.2 mL/min with the purpose of improving peak shape, the temperature of the column was reduced to 45 ºC, and a gradient that started with a higher proportion of mobile phase A and decreased slower was selected. The temperature of the column and the gradient were studied individually and are presented in the following sections of this work. The new analytical conditions are presented in table 4.

Table 4. Updated analytical conditions.

Analytical conditions			
Column		Poroshell 120 EC-C18 4.6 x 250 mm, dp=4 µm and guard column Poroshell 120 EC-C18 4.6 x 5 mm	
Mobile phase A		KH_2PO_4 buffer solution pH=2.0	
Mobile phase B		Acetonitrile	
Detection wavelength [nm**]**		220	
Flow rate [mL/min**]**		1.2	
Column temperature [ºC**]**		45	
Injection Volume [µL**]**		50	
Gradient	**Time [**min**]**	**Mobile phase A [**%**]**	**Mobile phase B [**%**]**
	0	90	10
	40	50	50
	40.1	20	80
	50	20	80
	50.1	90	10
	60	90	10

Figure 5 shows a chromatogram of the standard solution using the new version of the HPLC-DAD method. Table 5 shows the quality parameters of the analysis.

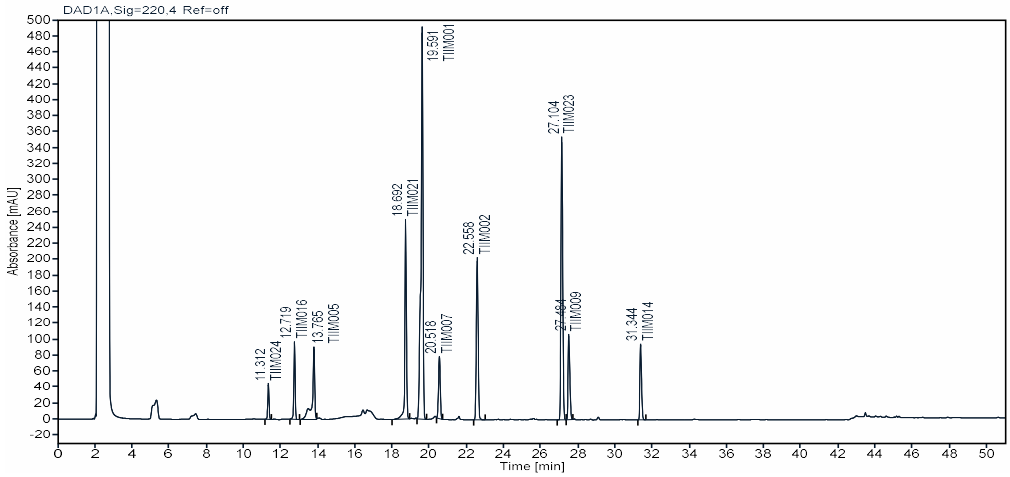

Figure 5. Chromatogram of the standard solution. 0.05 mg/mL concentration and 50 µL of volume of injection using the buffer mobile phase.

Table 5. Quality parameters of the standard solution. 0.05 mg/mL concentration and 50 µL of volume of injection using the buffer mobile phase.

Peptide	Area	Retention time [min]	Theoretical plates	Asymmetry factor	Resolution
TIIM024	204	11.312	144291	1.1	-
TIIM016	466	12.719	168687	1.0	11.6
TIIM005	814	13.765	137082	0.6	7.7
TIIM021	1419	18.692	292027	1.1	34.4
TIIM001	3692	19.591	247025	0.8	6.1
TIIM007	503	20.518	234016	1.0	5.6
TIIM002	1333	22.558	273005	1.0	12.0
TIIM023	2274	27.104	400899	1.1	26.6
TIIM009	702	27.484	390447	1.5	2.2
TIIM014	583	31.344	592776	1.4	22.9

When the chromatogram shown in figure 5 is compared with that illustrated in figure 4, it can be observed that the elution order of the peaks representing the compounds TIIM005, TIIM016, TIIM021, TIIM001 and TIIM007 changed. Moreover, a big improvement can be observed in many quality parameters. First, the theoretical plates values increased for the ten peptides, which means that the efficiency of the new method was better. For instance, the theoretical plates values for the peptides TIIM021, TIIM002 and TIIM014 increased approximately five times with respect the last analysis.

Moreover, the asymmetry factors of the peaks also improved. In particular, seven peaks improved the asymmetry factor and two peaks maintained the same asymmetry. The moderate increase of the flow rate reduced the diffusion of the analytes inside the chromatographic column and this was traduced with an improvement of the shape of the peaks. Nevertheless, the chromatogram still presented peak splitting for some compounds. The resolution values of the first peaks improved and a better separation for most of the peaks was achieved. However, the area values decreased for some of the peaks.

The Poroshell 120 EC-C18 column is compatible with mobile phases in the pH range between 2 and 8. Therefore, a new KH_2PO_4 buffer solution with the pH adjusted to 2.3 was tested. The objective of this test was to prove if the same analytical results could be obtained working in pH conditions further from the inferior limit of the column. Therefore, the same analysis was performed at pH 2.3. Nevertheless, when the quality parameters were analysed, the theoretical plates values had decreased for eight peaks, the resolution values were worse for five peaks and the asymmetry factors did not improve for any compound. Therefore, pH=2.0 KH_2PO_4 buffer solution mobile phase was selected for further experiments.

6.4. MOBILE PHASE GRADIENT

Several gradients of mobile phase were tested with the purpose of reducing the peak splitting showed in previous chromatograms. Table 6 shows the new tested gradient, which started with a lower percentage of mobile phase A and decreased slower between the minutes 20 and 35.

Table 6. New gradient of mobile phase applied. Mobile phase A is the pH=2.0 KH_2PO_4 buffer solution and mobile pase B is acetonitrile.

Gradient		
Time [min]	**Mobile phase A [%]**	**Mobile phase B [%]**
0	85	15
20	70	30
35	60	40
40	50	50
40.1	20	80
50	20	80
52	85	15
60	85	15

The standard solution containing the ten considered peptides was analysed using the new gradient. The obtained chromatogram is shown in figure 6 and the quality parameters are presented in table 7.

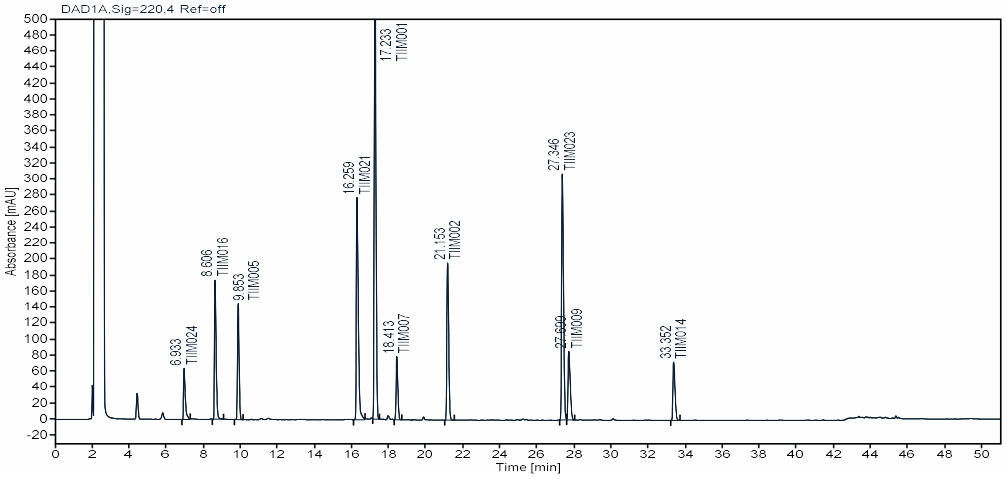

Figure 6. Chromatogram of the standard solution. 0.05 mg/mL concentration and 50 µL of volume of injection with the new gradient.

Table 7. Quality parameters of the standard solution. 0.05 mg/mL concentration and 50 µL of volume of injection with the new gradient.

Peptide	Area	Retention time [min]	Theoretical plates	Asymmetry factor	Resolution
TIIM024	421	6.933	30537	2.2	-
TIIM016	1052	8.606	47612	1.5	10.6
TIIM005	838	9.853	65962	1.3	8.0
TIIM021	2060	16.259	117554	1.8	37.5
TIIM001	3702	17.233	182283	1.4	5.5
TIIM007	516	18.413	181185	1.4	7.1
TIIM002	1268	21.153	244991	1.3	16.0
TIIM023	2171	27.346	338656	1.5	34.6
TIIM009	711	27.699	252981	1.5	1.7
TIIM014	574	33.352	405782	1.6	26.4

One of the weaknesses of the previous HPLC-DAD conditions was the presence of peak splitting, which was improved applying these last conditions (figure 6). A higher percentage of organic mobile phase in the first minutes improved the peak splitting of TIIM005 and TIIM001. Moreover, the retention times of the first peaks were reduced achieving a better distribution of the peaks along the chromatogram.

With respect to the last version of the method shown in figure 5, the resolution values improved for most of the peaks of the considered peptides. Nevertheless, the resolution between the peaks TIIM023 and TIIM009 was 1.7, which is considered a very low value. Therefore, a further improvement was required.

When the new gradient was applied, a decrease of the theoretical plates and an increase of the asymmetry factors were observed. To understand this variation, the area values for each peak should be compared. With the new gradient, the area values increased noticeably for the peaks representing the compounds TIIM024, TIIM016 and TIIM021, which indicated that a better quantification could be achieved. Moreover, the first two peaks with an increased area were the most difficult ones to quantify precisely, as they had the lowest areas among all the considered peptides. Therefore, this improvement was very positive for the optimization of the method.

In conclusion, despite some quality parameters as the theoretical plates values and the asymmetry factors did not improve with respect to the last version of the applied chromatographic method, the peak splitting was reduced and the quantification was better for some peptides when the new gradient of mobile phase was applied. Therefore, the new gradient was introduced for further experiments.

6.5. TEMPERATURE OF THE COLUMN

The next study was focused on the effect of the temperature of the column in the separation of the compounds. The temperature used in the last version of the method was 45 ºC. Therefore, an increase of the temperature of the column was applied to observe if this factor had significant effects on the resolution of the peaks of the compounds and could improve the separation of the closest ones.

The standard solution was analysed using a temperature of 50 ºC and the chromatogram obtained is shown in figure 7. The quality parameters of this chromatogram are shown in table 8.

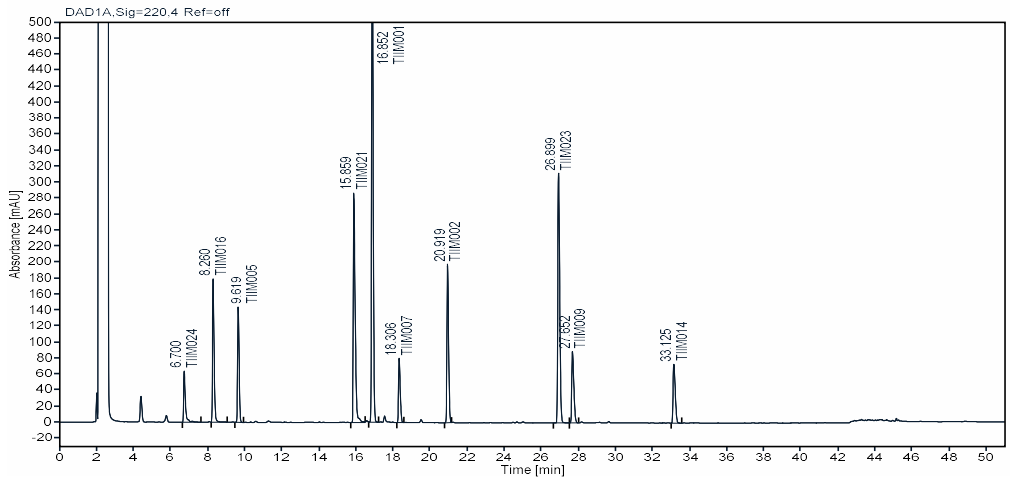

Figure 7. Chromatogram of the standard solution. 0.05 mg/mL concentration and 50 µL of volume of injection using a column temperature of 50 ºC.

Table 8. Quality parameters of the standard solution. 0.05 mg/mL concentration and 50 µL of volume of injection using a column temperature of 50ºC.

Peptide	Area	Retention time [min]	Theoretical plates	Asymmetry factor	Resolution
TIIM024	440	6.700	29029	2.0	-
TIIM016	1074	8.260	45309	1.5	10.0
TIIM005	830	9.619	63151	1.2	8.8
TIIM021	2081	15.859	117753	1.9	37.0
TIIM001	3723	16.852	181123	1.3	5.8
TIIM007	515	18.306	182313	1.5	8.8
TIIM002	1245	20.919	249560	1.3	15.5
TIIM023	2196	26.899	334234	1.4	33.9
TIIM009	720	27.652	267375	1.6	3.7
TIIM014	572	33.125	406433	1.6	26.0

These obtained quality parameters were compared with those summarised in table 7. The main observed difference was the resolution between the peaks TIIM023 and TIIM009. For the analysis of the standard solution at 45 ºC, the resolution between these peaks was 1.7 whereas in the analysis at 50 ºC the resolution between the peaks TIIM023 and TIIM009 increased to 3.7. Therefore, the temperature of 50 ºC was more appropriated to achieve a better separation of these compounds.

When the temperature of the column increases, the viscosity of the mobile phase decreases. This reduction of viscosity can lead to a reduction of the asymmetry factor of the peaks, improving the resolution values between the closest compounds. However, the area values, the theoretical plates values and the asymmetry factors obtained at both temperatures did not present significant differences.

In conclusion, the analysis at 50 ºC increased significantly the resolution between the peaks corresponding to TIIM023 and TIIM009, which provided a better separation between those peaks. Therefore, a temperature of 50ºC was used for further experiments.

6.6. OPTIMAL DETECTION WAVELENGTH

After the optimization of the chromatographic parameters discussed before, a study of the optimal detection wavelength was performed. The objective of this study was to select the wavelength that provided the best quantification of all the considered peptides.

The HPLC equipment was coupled to a DAD, which enabled the recording of UV spectra across the entire wavelength range. The selection of the most suitable wavelength depends on two fundamental aspects: i) the absorbance of the studied compounds at each tested wavelength; and ii) the effect on the baseline noise that the interferences absorbed at each wavelength produce. The two aspects were studied separately.

Firstly, the individual spectra of all studied peptides at 1 mg/mL concentration and 50 µL of volume of injection were recorded to analyse their different absorbance through the full UV range (figure 8). After that, an analysis of the standard solution using different wavelengths was done to observe the differences of the baseline noise.

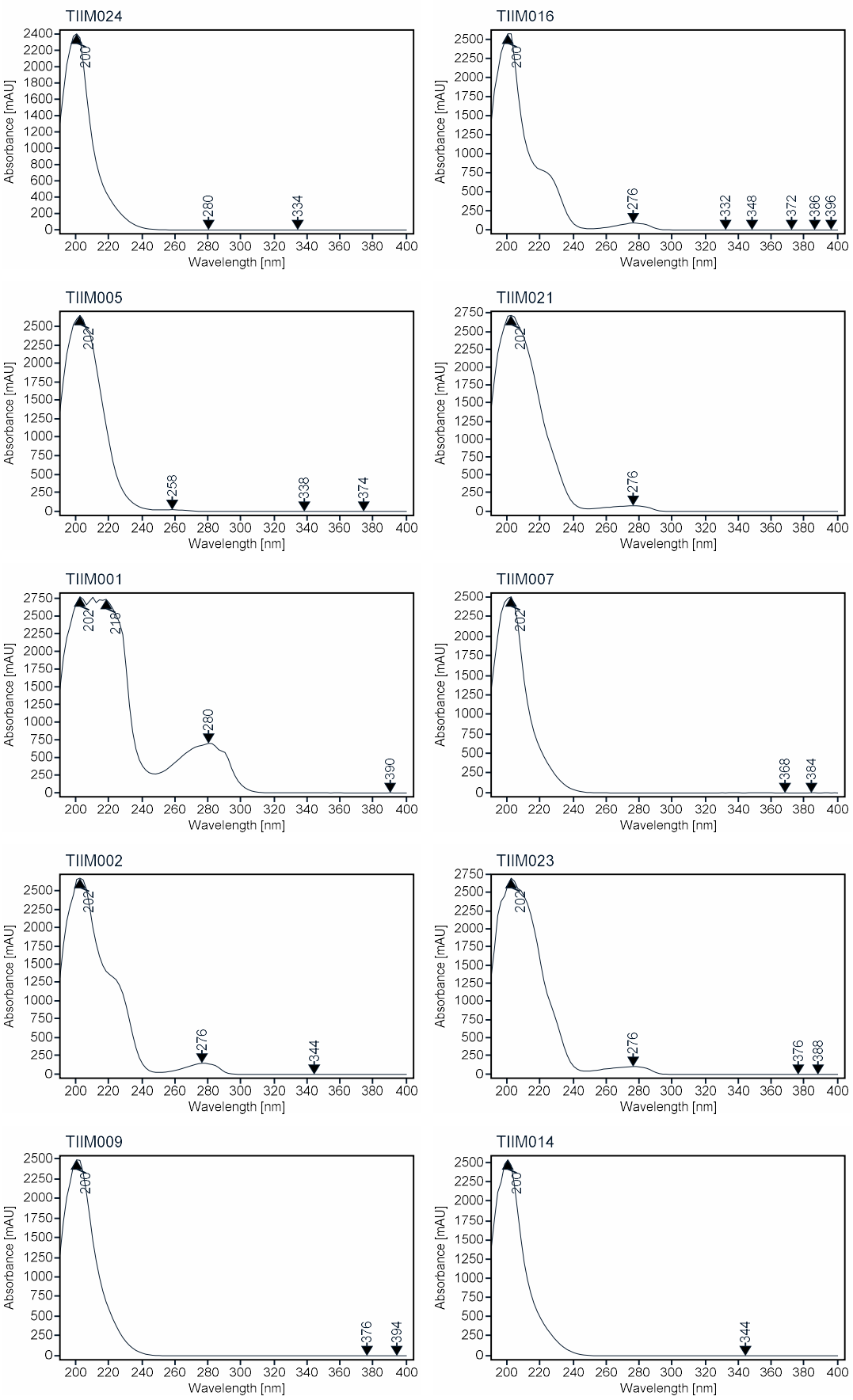

Figure 8. Spectra of the absorbance of the ten peptides through the UV detection range presented in order of elution. 1 mg/mL concentration and 50 µL of volume of injection.

The spectra show that the absorbance maxima are located at low wavelengths and that the spectral shapes are different between the peptides, which can be reflected in the signal intensities of the chromatograms presented in this work. Therefore, a suitable wavelength that provides appropriate absorbance values for all the peptides should be selected. With the information of the spectra and considering that the peptide bond absorbs light at wavelengths between 180 and 220 nm, the tested wavelengths were 210 and 220 nm. Figure 9 shows the chromatograms of the standard solution using the last version of the method at 210 and 220 nm.

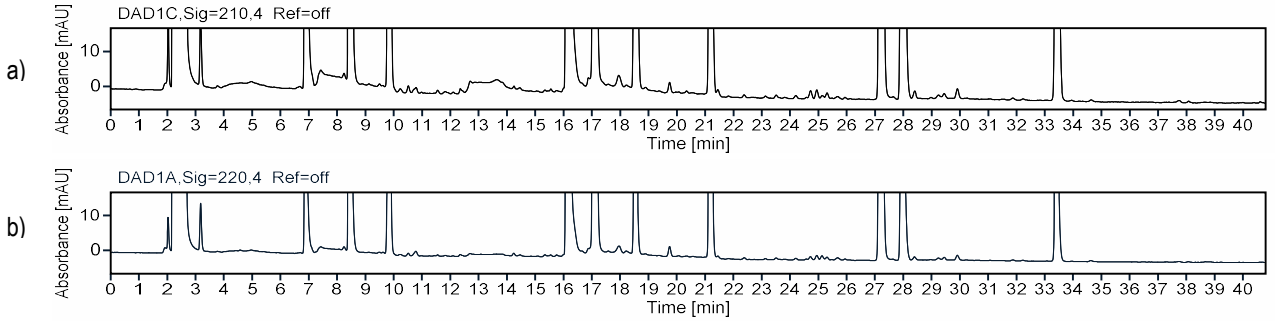

Figure 9. Chromatograms of the standard solution. 0.05 mg/mL concentration and 50 µL of volume of injection acquired at 210 (a) and 220 (b) nm.

The chromatogram obtained at 210 nm shows that the noise of the baseline presents significant interferences that can affect the integration of the peaks, difficulting their quantification. Contrarily, the chromatogram obtained at 220 nm, presents an improvement on the baseline noise, with lower interference. This difference can be explained by the fact that light at lower wavelengths possesses higher energy, which allows a greater number of electronic transitions in analyte molecules. As a result, the chromatogram recorded at 210 nm presents higher absorbances for all the compounds than the chromatogram recorded at 220 nm. Nevertheless, the increased noise may hinder accurate quantification, despite the stronger analytical signal.

In conclusion, although the absorbance of the peptides was higher when the selected wavelength was 210 nm, working at 220 nm reduced the interferences of the baseline noise. Therefore, the 220 nm wavelength was established.

After the optimization of all the considered parameters, the final version of the HPLC-DAD method is presented in table 9.

Table 9. Analytical conditions of the last version of the method

Analytical conditions			
Column	Poroshell 120 EC-C18 4.6 x 250 mm, dp=4 µm and guard column Poroshell 120 EC-C18 4.6 x 5 mm		
Mobile phase A	KH_2PO_4 buffer solution pH=2.0		
Mobile phase B	Acetonitrile		
Detection wavelength [nm**]**	220		
Flow rate [mL/min**]**	1.2		
Column temperature [ºC**]**	50		
Injection Volume [µL**]**	50		
Gradient	**Time [**min**]**	**Mobile phase A [**%**]**	**Mobile phase B [**%**]**
	0	85	15
	20	70	30
	35	60	40
	40	50	50
	40.1	20	80
	50	20	80
	52	85	15
	60	85	15

7. CONCLUSIONS

In this work, an optimization of a preexisting HPLC-DAD method for the analysis of 10 peptides was carried out. During the optimization of the method, different analytical parameters were tested to improve its performance. First, the individual study of the ten peptides with the initial conditions of the preexisting method was performed. In this step, the compounds and their retention time were identified. Despite the good resolution values obtained with the initial conditions, the peak splitting needed to be reduced.

The Poroshell 120 EC-C18 column provided better quality parameters than the SunFire C18 column. These results can be attributed to the properties its superficially porous particles provided. In addition, the lifetime of the Poroshell 120 EC-C18 was less affected for the temperature than the SunFire C18. When the 0.1% TFA mobile phase was substituted for a pH=2.0 KH_2PO_4 buffer solution, the flow rate was increased up to 1.2 mL/min, the temperature of the column decreased to 45ºC and a new gradient was introduced, the quality parameters of the method improved. The theoretical plates increased for the ten peptides and the asymmetry factor improved for seven of them. In contrast, the adjustment of the pH of the mobile phase to 2.3 did not improve the results.

The use of a new gradient with a higher initial percentage of organic phase allows to reduce the peak splitting. Moreover, the areas of some peaks increased, providing a better quantification of the compounds. Nevertheless, some quality parameters showed poorer performance compared to the previous analysis. When the temperature of the column was increased to 50 ºC, an improvement of the resolution from 1.7 to 3.7 was achieved between the compounds TIIM023 and TIIM009. Finally, a wavelength of 220 nm provided a good absorption of the peptides and the baseline noise did not affect the integration of the peaks.

Therefore, when the quality parameters of the final version of the method are compared to those of the initial version, several improvements can be appreciated. The separation of the peaks is greater, achieving a better resolution for all the peptides. The peak splitting, which was an important issue to improve in the first version, was eradicated and the peak symmetry was improved for some peaks. The theoretical plates values were improved for most of the peaks. The improvements of the quality parameters achieved favoured the better quantification of the method, which was one of the main purposes of this project.

Future prospects include the validation of the method to ensure that reliable, precise and reproducible results can be obtained. The validation process will be done according to USP guidelines and the accuracy, linearity, robustness, repeatability, specificity and precision, among others will be considered.

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9. ACRONYMS

DNA: Deoxyribonucleic acid

HPLC: High Performance Liquid Chromatography

HPLC-RP: Reversed-Phase High Performance Liquid Chromatography

C18: Octadecylsilane

C8: Octylsilane

UHPLC: Ultra-High-Performance Liquid Chromatography

DAD: Diode array detector

UV-Vis: Ultraviolet-Visible

UV: Ultraviolet

LC/MS: Liquid chromatography coupled with mass spectrometry

USP: United States Pharmacopeia

S/N: Signal to Noise relation

R&D: Research and Development

TFA: Trifluoroacetic acid

Dp: Dimension of the particle