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Research Article

Peptides, solid-phase synthesis and characterization: Tailor-made methodologies

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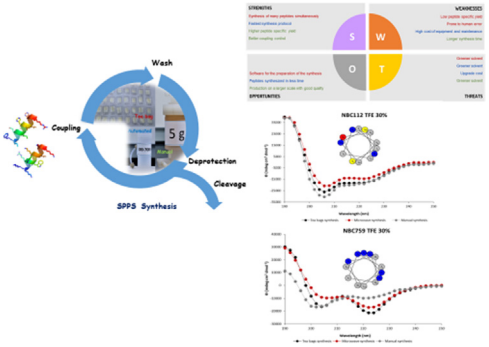
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Solid-Phase Peptide Synthesis (SPPS) is a mature technique widely used in research and in production. There are different approaches that fulfill the diverse requirements, regarding the number, quantity and quality of peptides. We have implemented three laboratory protocols of synthesis that cover these needs. These protocols have been tested, and the results analyzed with two different sequences used in previous works.

Results: The peptide synthesis protocols such as tea bag, microwave synthesis and manual synthesis have allowed obtaining specific yields of 8, 43 and 64% for the NBC112 peptide and specific yields of 36, 46 and 78% for the NBC759 peptide with the three protocols, respectively. Each protocol has different application contexts with advantages and disadvantages in each case.

Conclusions: The three protocols allow the obtention of the two peptides with good purity and can be used according to specific needs and requirements.

This article includes an interactive 360-degree video. To view it correctly, it is necessary to scroll through the screen to navigate across the laboratory where you will find 10 interactive points. For an immersive experience, a head-mounted display can be used. Please, visit this URL: http://ejbiotechnology.info/public/360view/2023/VTPCARDENAS1v1/index.html.

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1. Introduction

Solid phase peptide synthesis (SPPS) has been favored by the conjunction of multiple developments, both at a basic level and in technical and methodological implementations. Since the pioneering work by Merrifield [1], Carpino and Han [2] and Houghten [3] in the 1960s-1980s, *much water has passed under the bridge*. Currently, it can be said that SPPS is a mature technique, and despite new reagents and modifications are continuously incorporated seeking to optimize the methodologies [4,5,6,7], novel synthesis strategies [8,9], and in recent times seeking a greener approach [10,11,12,13,14,15,16,17], the fundamentals underlying SPPS remain the same.

Our laboratory has worked on the synthesis and characterization of peptides, and has optimized different protocols, in terms of time and reagents consumption, to be used in the production of peptides for research according to specific needs.

In this report, we present three different protocols of the SPPS technique depending on the scale and the synthesis time. Results are analyzed in terms of yield, purity, and costs, using two peptide sequences of interest for the laboratory, NBC112 and NBC759 (Table 1) used in previous works, as case study [18,19].

2. Materials and methods

2.1. Instrumentation

Liberty Blue automated microwave peptide synthesizer. (CEM Corp. Matthews, NC, USA); High-Performance Liquid Chromatography (HPLC) (JASCO Corporation, Tokyo, Japan); Matrix-assisted laser desorption/ionization-Time of flight-Mass Spectrometry (MALDI-TOF-MS) Microflex (Bruker Daltonics Inc, Billerica, MA, USA); LCMS-2020 electrospray ionization (ESI)-MS (Shimadzu Corp., Kyoto, Japan); J-815 Circular Dichroism Spectrometer coupled to Peltier CDF-426S/15 (JASCO Corporation, Tokyo, Japan).

2.2. Reagents and solvents

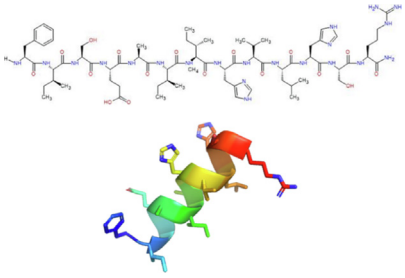
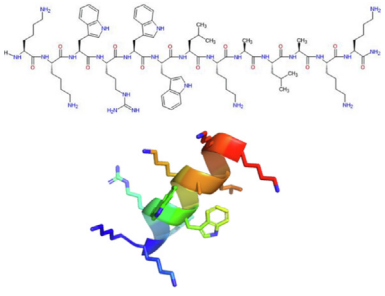
Fmoc Rink Amide AM resin, N,N'-diisopropylcarbodiimide (DIC), N-[(1H-benzotriazol-1-yl)(dimethylamino)-methylene]-N-methyl methanaminium tetrafluoroborate N-oxide (TBTU), 2-(1H-benzo triazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate N-[(1H-benzotriazol-1-yl)(dimethylamino)-methylene]-N-methyl methanaminium hexafluorophosphate N-oxide (HBTU), N-[(1H-6-chlorobenzotriazol-1-yl)(dimethylamino)-methylene]-N-methyl methanaminium hexafluorophosphate N-oxide (HCTU), OxymaPure and Fmoc protected amino acids were obtained from Iris Biotech GmbH (Marktredwitz, Germany).

N,N'-dimethylformamide (DMF), isopropanol (IPA), dichloromethane (DCM), piperidine, N-ethyldiisopropylamine (DIPEA), bromophenol blue (BPB), trifluoroethanol (TFE), acetonitrile (ACN), methanol, ethanol and water HPLC grade, trifluoroacetic acid (TFA), triisopropylsilane (TIS), 2.2′-(ethylenedioxy) diethanethiol (DOTA) were obtained from Merck KGaA (Darmstadt, Germany).

2.3. Synthesis protocols

SPPS was conducted by the Fmoc/tBu protocol using a Rink Amide AM resin as the solid support and three protocols: (1) tea bag synthesis, (2) microwave synthesis and (3) manual peptide synthesis. Removal step of Fmoc groups was carried out with 20% v/v piperidine in DMF. In terms of coupling reaction and mon-

Table 1
Reference peptides information. Bachem peptide calculator was used to obtain net charge and hydrophilicty (https://www.bachem.com/knowledge-center/peptide-calculator/). 2D structure was performed with Chemsketch (ACDLabs), and 3D structure was modeled with PEPFOLD3 [20] and drawn with PyMol (The PyMOL Molecular Graphics System, version 2.5.4, Schrödinger, LLC.).

Peptide	2D and 3D structure	Use
NBC112 H-FISEAIIHVLHSR-NH2 Molecular Weight 1520.8 g/mol Net charge: 1.18 Average hydrophilicity: −0.47 Ratio of hydrophilic residues/ Total number of residues: 31%		T-helper cell activator [21]. It is used to coimmunize for the generation of antibodies from peptides.
NBC759 H-KKWRWWLKALAKK-NH2 Molecular Weight 1741.2 g/mol Net charge: 7 Average hydrophilicity: 0.25 Ratio of hydrophilic residues/ Total number of residues: 46%		Antibacterial peptide from *Bothrops asper* snake venom [22]. Used as positive control for peptide antibacterial assays.

itoring, each synthesis protocol has different parameters as summarized in Table 2. It is to note that peptide synthesis is dependent of the sequence, and the parameters showed here are based on the two sequences synthesized.

For each protocol, a different reactor is used. Fig. 1 shows the photography reaction containers used in this study.

The details of the procedures are shown in the video, and a brief description is presented below.

2.3.1. Tea bag synthesis

The detailed procedure can be found in Guzmán et al. [23]. Briefly: Tea bags made from polypropylene mesh sheets are carefully labeled and heat sealed, with the resin contained within (Fig. 1A). The program to prepare the work document for the couplings and organization of the synthesis can be found in https://acuapeptide.cl/programas.html. The reagents are prepared in advance according to the number of peptides to be synthesized and the total number of couplings. Within the synthesis steps, deprotection and washings are done for all the tea bags in the same container and for coupling cycle each one goes to the corresponding amino acid. The deprotection step is performed with 20% piperidine containing 1% triton X-100 in DMF, twice for 10 min. This is followed by 3 washes with DMF, one with IPA, one with 5% BPB in DMF and then several more with DMF and DCM before adding the coupling solution. Two coupling mixes are employed in order to favor reaction completion: (1) a single coupling that consists of HBTU/OxymaPure activator of amino acids, completing 3 h of total coupling time; and (2) a double coupling that consists of HCTU/OxymePure activator, with a coupling time of 1 h. Coupling monitoring is accomplished with 0.5% BPB in DMF. A color change from green to yellow should be observed during reaction; if a blue or green color persists after two coupling reactions, a third coupling reaction is accomplished employing DIC/OxymaPure activator.

Final cleavage is done with a cocktail of TFA/TIS/Water of 95:2.5:2.5 for peptide NBC112 and TFA/TIS/Water/DOT 92.5:2.5:2.5:2.5 in peptide NBC759 to prevent tryptophan oxidation. After cleavage, the resin remains in the tea bags while the peptides are precipitated with cold diethyl ether, dissolved in Milli-Q water and lyophilized.

2.3.2. Microwave automated synthesis

The synthesis was carried out on the Liberty Blue automatic synthesizer (Fig. 2). The standard couplings were made with Fmoc amino acids at 0.2 M concentration and scale of 0.1, with DIC/OxymaPure® as activator in DMF, at 90°C and the deprotection was made with piperidine at 20% in DMF at 90°C. Fmoc-Arg(Pbf)–OH always has double coupling, and Fmoc-His(Trt)–OH has an extended coupling of 10 min at 50°C (according to default cycles in the Liberty Blue). All the coupling cycles until final deprotection were carried out with the equipment, and the final cleavage was done in the same way as in the tea bag protocol, but in a polypropylene syringe equipped with a polyethylene frit.

Table 2
Parameters for each synthesis protocol used in this report.

Parameter	Tea bag synthesis	Microwave synthesis	Manual synthesis
Resin loading (meq/g)	0.52	0.6	0.62 [NBC112] 0.59 [NBC759]
Rink Amide AM resin amount (g)	0.04	0.164	5
Activators	HBTU/HCTU/DIC	DIC	HBTU/TBTU/HCTU
Activator/base	OxymaPure®/DIPEA	OxymaPure®	OxymaPure®/DIPEA
Number of Couplings per residue	2–3	1–2	2–4
Coupling monitoring	Bromophenol blue	–	Ninhydrin test
Average coupling cycle time	1.5 h	5 min	2 h

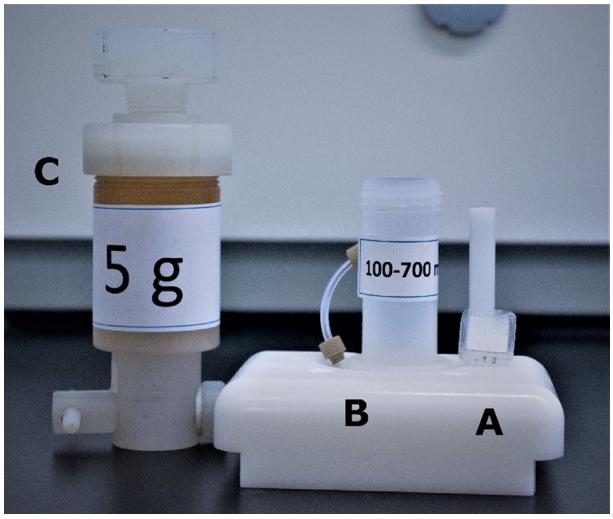


Fig. 1. Photograph of the different reaction containers for each protocol. **(A)** Tea bag; **(B)** Reaction vessel for microwave synthesis; **(C)** Reactor for manual synthesis.

2.3.3. Manual synthesis

We must note that tea bag synthesis is also carried out manually, in this case, we refer to synthesis in reactors other than tea bags (Fig. 3). In this case, each coupling was prepared individually, deprotection steps were carried out in the same way as in the tea bag protocol, and the washings were thrice with DMF, one with

Fig. 2. Automatic microwave peptide synthesizer Liberty Blue.

IPA, and one with DCM before adding the coupling solution. Coupling monitoring was performed by the ninhydrin test [24]. For the NBC112 peptide, double couplings were done for all the residues and in I2 and I7, a third coupling was necessary. For the NBC759 peptide, double coupling was performed for all residues and triple coupling for K2, W3, W6, K8 and L10. K1 required a fourth coupling, performed with DIC/OxymaPure® as an activator.

After finished the coupling cycles, final cleavage is done with a cocktail of TFA/TIS/Water of 95:2.5:2.5 for peptide NBC112 and TFA/TIS/Water/DOT of 92.5:2.5:2.5:2.5 to prevent tryptophan oxidation in peptide NBC759. Peptides were precipitated with cold diethyl ether and then dissolved in MilliQ water and lyophilized. Final cleavage was done as earlier has been described, and the differences are in the specific amounts of reagents and solvents.

2.4. Peptide characterization

The peptide characterization procedure and the equipment used are shown in the video, and a brief description is presented below.

Peptides were characterized by reversed phase-high-performance liquid chromatography (RP-HPLC, JASCO Corp., Tokyo, Japan) by using a gradient of 0%–70% of solvent B (acetonitrile with 0.05% TFA) versus solvent A (water with 0.05% TFA) on a XBridge™ BEH C18 column (100 × 4.6 mm, 3.5 µm) (Water Corp., Milford, MA, USA) at 1 mL/min flowrate for 8 min. Chromatograms were obtained using ChromPass Chromatography Data System software (version 1.7.403.1, JASCO Corp., Tokyo, Japan). Mass spectrometry was used to confirm the molecular mass of each product obtained, by using electrospray ionization-mass spectrometry (ESI-MS) with a LCMS-2020 ESI-MS (Shimadzu Corp., Kyoto, Japan), and MALDI-TOF-MS Microflex equipment (Bruker Daltonics Inc).

Secondary structure was determined by circular dichroism spectroscopy on a JASCO J-815 CD Spectrometer (JASCO Corp., Tokyo, Japan) in the far ultraviolet (UV) range (190–250 nm). Molar ellipticity was calculated for each peptide using 250 µL of 2 mM peptide in 30% (v/v) 2,2,2-TFE. Resulting data were analyzed using Spectra Manager software (version 2.0, JASCO Corp., Tokyo, Japan).

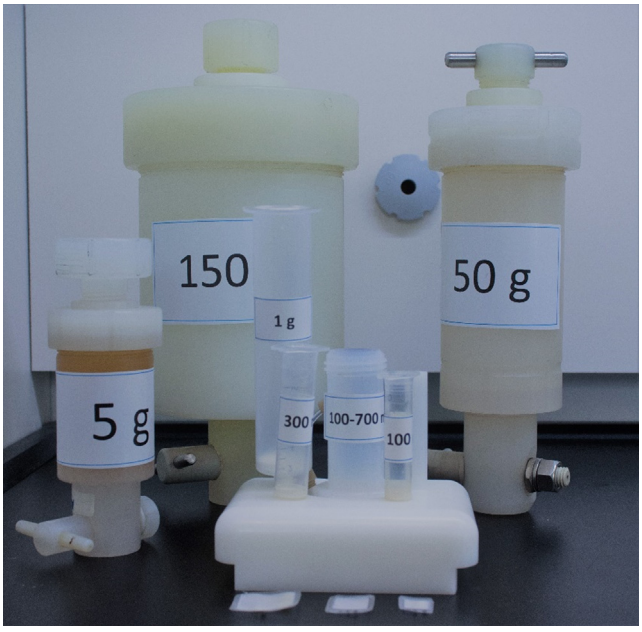


Fig. 3. Different reactors used in manual peptide synthesis other than tea bags.

3. Results

3.1. Characterization of peptides

The two peptides synthesized by the three protocols were characterized by HPLC, and mass spectrometry as described in Materials and Methods. The comparison of the spectra is shown in Table S1.

Additionally, circular dichroism was used to determine the secondary structure. Spectra of all the syntheses are presented in Fig. 4.

3.2. Peptide yield and purity

For the quantification of the peptide, Equation 1, Equation 2 and Equation 3 were used according to previous works [18,19].

$$Theoretical\ \ yield = g\ \ resin \times resin\ \ substitution \times Peptide\ \ Molecular\ \ Weigth \quad \text{Equation1}$$

$$\%\ \ Crude\ \ Yield = \frac{g\ \ peptide\ \ obtained}{Theoretical\ \ yield} \times 100 \quad \text{Equation2}$$

$$\%\ \ Peptide\ \ specific\ \ Yield = \frac{Purity\ \ \times\ \ g\ \ peptide\ \ obtained}{Theoretical\ \ Yield} \times 100 \quad \text{Equation3}$$

Summary of the yield and purity results obtained are presented in Table 3.

Microwave synthesis and tea bag synthesis were presented in previous works, and in this report, the manual synthesis in reactor is included, with a larger scale of production, starting from 5 g of resin (Table 2).

In all three cases, the expected product was obtained. According to the yield obtained in each synthesis, for each one of the protocols, an estimate of the use of reagents was made, normalizing for one gram of crude product. Table 4 presents the estimate for the amino acids used.

As a research laboratory, it is necessary to consider various factors when choosing a particular protocol. Table 5 shows some of these factors and their behavior in each of the implementations.

4. Discussion

According to the results obtained, any of the three synthetic protocols can be used to obtain the desired peptide (Table 3). As mentioned before, the behavior of the peptide synthesis depends on the amino acid sequence, as can be seen with the results of yield and purity for the two peptides synthesized here by the three protocols. The NBC112 peptide is a difficult sequence to synthesize, with a more hydrophobic character than NBC759 and a much lower net charge (Table 1), which can influence the process of synthesis. Additionally, in a previous work, we showed that this particular peptide is synthesized with a better yield by using 4-methylpiperidine as a deprotection reagent [19]. Additionally, the mass spectra of the crude peptide showed the presence of the Fmoc group, which affects both the yield and the purity of the peptide, as seen in the results (Table 2) when including this species in the calculations. Although the yield for this peptide was low, for peptide NBC759, it was considerably better, which validates the tea bag protocol.

The CD spectra showed that the secondary structure tends to be an alpha helix. The slight differences observed for the three protocols may be due to the fact that they were performed with crude peptides, which implies small variations in concentration. In the case of the NBC112 peptide, the differences are not representative

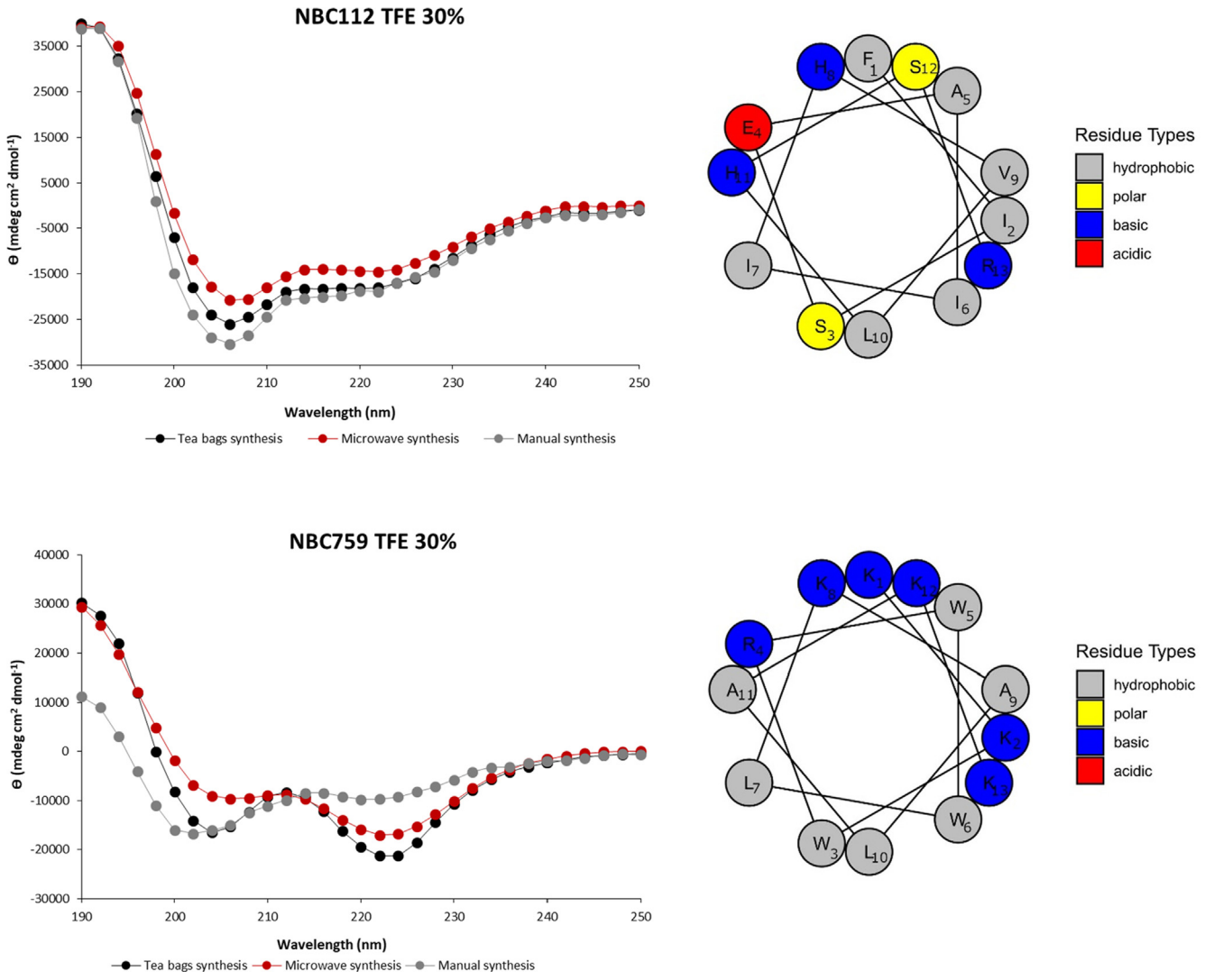


Fig. 4. Characterization of peptides NBC112 and NBC759 by circular dichroism spectroscopy. CD spectra in 30% TFE for the three synthesis methods and its helical-wheel representation made with helixvis in RStudio [25] (http://www.rstudio.com).

Table 3
Yield and purity of the synthesized peptides by the three protocols. **1**: % Crude yield. **2**: % Purity. **3**: % Peptide specific yield.

Label	Sequence	MW (Da)	Tea bag synthesis [19]			Microwave synthesis [18]			Manual synthesis		
			1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
NBC112	H-FISEAIIHVLHSR-NH_2	1502.8	32.8	23.9 [43.2]*	8.0 [14.1]*	72.0	43.6	31.3	83.0	64.4	53.5
NBC759	H-KKWRWWLKALAKK-NH_2	1741.2	57.7	62.1	36	81.6	59.1	48.2	79.5	98.7	78.5

* In brackets, the values are shown including the peptide with the Fmoc group.

Table 4
Amount of amino acids used in grams per 1 gram of crude peptide obtained. Tea bag protocol. Microwave synthesis with Liberty Blue, and manual synthesis.

Amino acid	**NBC112**			**Amino acid**	**NBC759**		
	Tea bag	Microwave	Manual		Tea bag	Microwave	Manual
N-Fmoc-Ala-OH	23.3	1.7	0.8	N-Fmoc-Ala-OH	21.2	2.7	1.5
N-Fmoc-Arg (Pbf) –OH	48.5	7.1	1.6	N-Fmoc-Arg (Pbf) –OH	22.1	5.5	1.4
N-Fmoc-Glu (OtBu) –OH	33.1	2.4	1.1	N-Fmoc-Leu-OH	24.0	3.0	2.3
N-Fmoc-His (Trt) –OH	92.6	6.8	3.0	N-Fmoc-Lys (Boc) –OH	79.6	9.3	6.3
N-Fmoc-Ile-OH	79.2	5.8	3.2	N-Fmoc-Trp (Boc) –OH	57.4	6.7	4.9
N-Fmoc-Leu-OH	26.4	2.0	0.9				
N-Fmoc-Phe-OH	28.9	2.2	1.0				
N-Fmoc-Ser (tBu) –OH	57.3	4.3	0.9				
N-Fmoc-Val-OH	39.3	1.9	1.3				

Table 5
Factors to be considered in each protocol of peptide synthesis.

	Tea bag synthesis	Microwave synthesis	Manual synthesis
Scale (peptide obtained)	10–20 mg	100–150 mg	3–4 g
Time	half day/AA cycle	half day/peptide	half day/AA cycle
Quality-Yield	Fair	Good	High
Use of reagents	High	Medium	Low

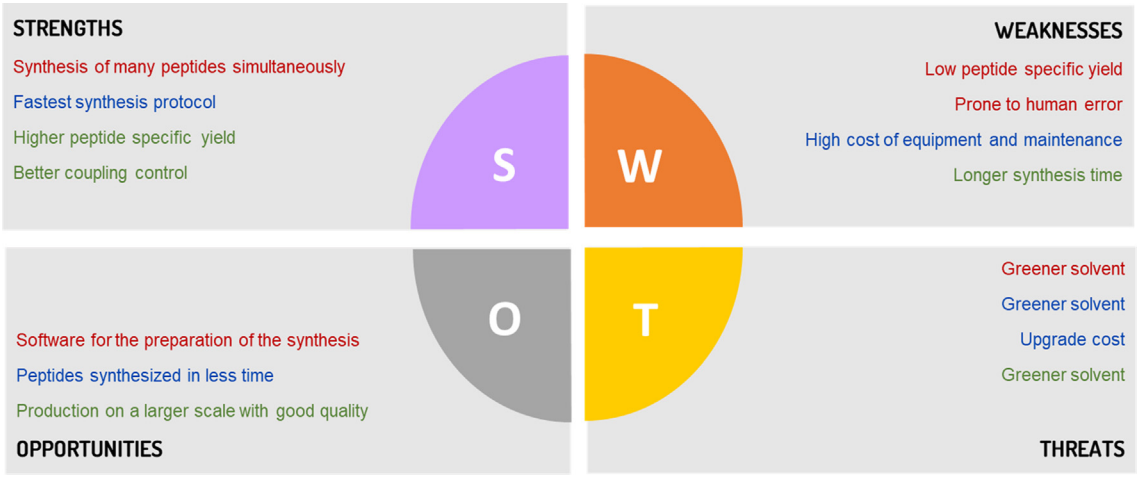


Fig. 5. SWOT analysis for the three synthesis protocols. Each protocol is represented by a different color. Red: tea bag synthesis; Blue: microwave-assisted synthesis with Liberty Blue; Green: manual synthesis.

and the structural trend is preserved, while for the NBC759 peptide, although the same trend is observed, there are some differences in the shape of the curve that are due to the presence of Trp in the sequence, which causes distortion due to n → π* transitions, as reported [25].

The selection of a specific protocol will then depend on other factors. In the case of the tea bag protocol, it is possible to obtain a large number of peptides [23], up to 400, which is ideal for preliminary tests of activity or for studies of specific effects of one amino acid (alanine scan), since it is possible to obtain them simultaneously in a reasonable period of time. However, the consumption of reagents is higher than with the other two protocols, and there is a penalization in the quality of the final product. In the case of microwave synthesis with Liberty Blue, the most important variable to consider is time [18], the quality and yield obtained are good, the only requirement is to have access to the equipment, which is not available in all laboratories. Finally, the synthesis in a reactor has the advantage of scalability to pre-production level; as much as 150 g of peptide can be produced to carry out pilot tests [26], with good product quality and lower reagent consumption than with other protocols. In these last two protocols, only one peptide sequence is synthesized at a time.

5. Conclusions

As conclusion, the three synthesis protocols are compared by constructing a SWOT matrix that is presented in Fig. 5.

Author contributions

- Study conception and design: F Guzmán, F Albericio, C Cárdenas
- Data collection: M Aróstica, T Román, D Beltrán, A Gauna, C Cárdenas
- Analysis and interpretation of results: M Aróstica, T Román, D Beltrán, C Cárdenas
- Draft manuscript preparation: M Aróstica, C Cárdenas, A Gauna, T Román
- Revision of the results and approval of the final version of the manuscript: F Guzmán, M Aróstica, T Román, D Beltrán, A Gauna, F Albericio, C Cárdenas

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Conflict of interest

The authors declare no conflict of interest.

Supplementary material

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