Received: 29 January 2024 | Revised: 18 March 2024 | Accepted: 1 April 2024

DOI: 10.1002/psc.3605

RESEARCH ARTICLE

Journal of PeptideScience WILEY

Optimization of peptide synthesis time and sustainability using novel eco-friendly binary solvent systems with induction heating on an automated peptide synthesizer

Lorenzo Pacini[1,2] | Manoj Muthyala[3] | Luisa Aguiar[3] | Robert Zitterbart[3] | Paolo Rovero[2,4] | Anna Maria Papini[1,2]

[1]Interdepartmental Research Unit of Peptide and Protein Chemistry and Biology - Peptlab, MoD&LS Laboratory, University of Florence, Sesto Fiorentino, Italy

[2]Department of Chemistry "Ugo Schiff", University of Florence, Sesto Fiorentino, Italy

[3]Gyros Protein Technologies Inc., Tucson, Arizona, USA

[4]Department of Neurosciences, Psychology, Drug Research and Child Health, Section of Pharmaceutical Sciences and Nutraceutics, University of Florence, Sesto Fiorentino, Italy

Correspondence
Anna Maria Papini, Interdepartmental Research Unit of Peptide and Protein Chemistry and Biology - Peptlab, MoD&LS Laboratory, University of Florence, 50019 Sesto Fiorentino, Italy.
Email: annamaria.papini@unifi.it

Funding information
MUR/EU-FSE; TT-Pep Well-being Research Incubator Project

On December 12th, 2023, the European Commission took regulatory action to amend Annex XVII of REACH, imposing restrictions on the use of *N*,*N*-dimethylformamide (DMF) within the EU market owing to its high toxicity. Historically, DMF has been widely considered the gold standard for solid-phase peptide synthesis (SPPS). Being urgent to propose alternative solvents, we tested the suitability of non-hazardous neat and mixed solvents. Notably, binary solvent mixtures containing dimethyl sulfoxide as one of the solvent partners demonstrated high efficacy in solubilizing reagents while maintaining the desired swelling characteristics of common resins. A series of binary solvent mixtures were tested in automated SPPS, both at room temperature and high temperature, employing the PurePep® Chorus synthesizer, which enabled controlled induction heating between 25 and 90°C with oscillation mixing. The performances were assessed in challenging peptide sequences, i.e., ACP (65–74), and in longer and aggregating sequences like SARS-CoV-2 RBM (436–507) and β-amyloid (1–42). Furthermore, as part of the proposed sustainable approach to minimize the utilization of hazardous solvents, we coupled the novel PurePep EasyClean catch-and-release purification technology. This work, addressing regulatory compliance, emphasizes the crucial role of green chemistry in advancing safer and more environmentally friendly practices in SPPS.

KEYWORDS
binary mixture solvents, catch and release-based peptide purification, difficult peptide synthesis, green peptide synthesis, green solvents, induction heating-assisted peptide synthesis, solid-phase peptide synthesis

1 | INTRODUCTION

Peptides, due to their diverse properties, find multifaceted applications, ranging from pivotal roles in pharmaceuticals to essential functions in cosmetic chemistry, diagnostics, and biomaterials.[1-6] The burgeoning interest in peptides has intensified in recent years, as advancements in both biological and chemical manufacturing methods have rendered their production increasingly accessible.[7,8] Traditional approaches to obtaining pure peptides have relied on solid-phase peptide synthesis (SPPS) and reversed-phase high-pressure liquid chromatography (RP-HPLC).[9] SPPS requires the use of toxic organic solvents such as *N*,*N*-dimethylformamide (DMF), *N*-methylpyrrolidone (NMP), and dichloromethane (DCM), which pose environmental and health hazards. RP-HPLC generates a large amount of waste, which increases the cost and environmental impact of peptide production.[10]

DMF has long been the solvent of choice in SPPS due to its unique properties. Its polarity allows for the solubilization of reagents such as Fmoc-amino acids and coupling activators, and it facilitates an

optimal rate for amide bond formation and Fmoc removal. DMF boiling point (153°C) allows for the safe heating of reactions, and its viscosity, slightly higher than water, allows for excellent permeation through the polymeric matrix of resins and efficient handling in automated synthesizers. However, the European Commission's decision to impose restrictions on DMF starting from December 12th, 2023, has accelerated the search for alternatives. Recent scientific literature suggests that DMF can be replaced with less harmful solvents such as acetonitrile (ACN),[11] 2-methyltetrahydrofuran (2-MeTHF),[12] ethyl acetate (EtOAc),[13] γ-valerolactone (GVL),[14] *N*-butyl pyrrolidone (NBP),[15] and Rhodiasolv Polarclean.[16] However, these solvents have specific drawbacks. For instance, the high viscosity of NBP may interfere with its proper delivery in automated systems, and GVL has been observed to induce ring-opening acylation of glycine residues.

Researchers have investigated various binary solvent mixtures to mitigate the drawbacks of individual solvents. Green binary solvent mixtures such as cyrene (dihydrolevoglucosenone), sulfolane, or anisole with dimethyl carbonate,[17] dimethyl sulfoxide (DMSO)/(EtOAc),[18] DMSO/1,3-dioxolane (DOL), DMSO/2-Me-THF,[19] anisole/ethanol (EtOH)[20] have been explored. Novo Nordisk and Bachem extensively studied binary mixtures, fine-tuning viscosity, and polarity to achieve comparable outcomes to DMF.[21] However, none of the reports explores the use of binary solvent mixtures for SPPS at higher temperatures up to 90°C, which can enhance reaction rates and enable the synthesis of peptides that are difficult or impossible to synthesize at lower/milder temperatures. This study aims to fill this gap by exploring the potential of binary solvent mixtures at high temperatures, aiming to enhance reaction rates and enable the synthesis of challenging peptides. Moreover, a higher boiling point of a green mixture results in better usability in long-lasting room-temperature syntheses, low-boiling point mixtures might evaporate, causing the precipitation of reagents.

Considering that innovation in R&D brings more and more peptide-based products into the market, greening the whole process of peptide production is an open but compulsory challenge in 21st century. Thus, it is essential to consider not only the synthetic aspects of peptide active ingredients but also the purification steps in a holistic approach.

In particular, the most commonly used purification technology for synthetic peptides is nowadays RP-HPLC,[22] even though RP-HPLC generates a significant amount of solvent waste, which not only elevates costs but also has a substantial environmental impact. In the pursuit of a more sustainable approach, we explored the novel catch-and-release purification technology known as PurePep EasyClean (PEC®). The PEC® technology uses a novel reductively cleavable linker molecule and an activated filter material for the purification and modification of chemically synthesized peptides.[23] This technology aims to minimize the utilization and disposal of hazardous solvents, as well as the time consumption of the purification process. Our exploration of PEC in this context represents an effort to green the entire process, from upstream synthesis to downstream purification, thereby reducing the environmental impact and potentially improving the efficiency and cost-effectiveness of peptide production, proposing an efficient and fast synthetic strategy.

2 | MATERIALS AND METHODS

2.1 | Materials

Fmoc-amino acids and Oxyma Pure were provided directly by Gyros Protein Technologies (Tucson, AZ, USA); *N*,*N*′-diisopropylcarbodiimide was purchased from Carbosolution Chemicals GmbH (St. Ingbert, Germany); DMF, ethyl acetate, propyl acetate, butyl acetate, dimethyl sulfoxide, acetic anhydride, pyridine, anisole, and triisopropylsilane were purchased from Carl Roth GmbH (Karlsruhe, Germany); piperidine, trifluoroacetic acid, and Rink amide AM resin (0.64 mmol/g) were purchased from Iris Biotech GmbH (Marktredwitz, Germany); Rink amide AM Tentagel S and R resins (respectively 0.22 mmol/g and 0.19 mmol/g) were purchased from Rapp Polymere (Tuebingen, Germany).

2.2 | Solubility tests

2.2.1 | For Fmoc-amino acid

After volumetric calibration of a 5 ml plastic tube, 0.4 mmol of each Fmoc-amino acid was weighed, and the tested solvent was added up to 1 ml. The mixture was stirred at r.t. for 30 minutes. Only if the Fmoc-amino acid was not yet solubilized, we increased the volume of the solvent in order to check, progressively, solubility at 0.3 M, 0.2 M, and 0.1 M concentrations.

2.2.2 | For coupling reagents

After volumetric calibration of a 15 ml plastic tube, 1 mmol of each coupling reagent was weighed, and the tested solvent was added up to 1 ml. The mixture was stirred at r.t. for 30 minutes. Only if the reagent was not still solubilized, we increased the volume of the solvent in order to check, progressively, solubility at 0.5 M, 0.25 M, and 0.1 M concentrations.

2.3 | Swelling test

Of each resin, 100 mg was weighed into a 3 ml calibrated syringe equipped with a porous filter. Thereafter, 1 ml of each evaluated solvent was added, and the slurry was shaken at r.t. for 2 and 24 hours. At both sampling times, the solvent was removed from the syringe by vacuum, and the resin volume was recorded. The degree of swelling was calculated as 10 × the measured volume.

2.4 | Solid-phase peptide synthesis

All syntheses were performed on the PurePep® Chorus (Tucson, AZ, USA) peptide synthesizer using parallel synthesis mode, both with the assistance of induction heating and at r.t. For 100 μmol scale

syntheses, we weighed respectively 156 mg of Rink amide AM PS resin, 455 mg of Tentagel S RAM resin, and 526 mg of Tentagel R RAM resin. Fmoc-amino acids were solubilized in the tested mixture or in DMF using an ultrasonic bath. The solvents were freshly prepared and transferred to the appropriate bottles corresponding to separate lines on the machine. After the end of the synthesis, the loaded resins were washed with isopropyl alcohol and dried under vacuum. Before starting each synthesis with different solvents, a full calibration was done through the specific feature on the instrument, making sure that the correct volume of solvent was delivered.

2.5 | Test cleavage

A tip of a spatula (2–4 mg) of dry-loaded resin was transferred into a 500 µl plastic tube, and 100 µl TFA, 5 µl TIS, and 5 µl H_2O were added. After 1.5 hours of mechanical mixing, the suspension was filtered, and cold diethyl ether (−20°C) was added to the peptide TFA solution. The precipitated peptide was isolated by centrifugation and dried under vacuum. After solubilization in water, crude peptides were analyzed by RP-UPLC coupled with ESI-MS.

2.6 | Solvent evaporation test

Test of BuOAc/DMSO 7:3 (v/v) vs EtOAc/DMSO 6:4 (v/v) in tubes with not-screwed caps. In two 50-ml centrifuge tubes, 30 ml of BuOAc/DMSO 7:3, and EtOAc/DMSO 6:4 were added, and the filled tubes were weighed. In parallel, a 15-ml tube was filled with 10 ml DMF and left to stand openly for 26 days, recording volume and weight differences.

2.7 | Racemization study

We synthesized on a PurePep® Chorus (Tucson, AZ, USA) peptide synthesizer two model peptides belonging to the ABC 20-mer sequence, Fmoc-E**C**NRADG-NH_2, and Fmoc-E**H**EQCCNRADG-NH_2, for the evaluation of cysteine and histidine couplings, respectively. The common fragment (NRADG-NH_2) was synthesized on a Rink Amide AM resin (0.64 mmol/g) at 200 µmol scale; then the resin was split into two parts and syntheses were completed separately. The general synthetic strategy used for these two syntheses consisted of the use of 5/5/10 equivalents of the coupling system Fmoc-amino acid/HATU/DiPEA in DMF for 40 minutes at r.t. or 2 minutes at 90°C and 5 + 10 minutes or 1 minute (r.t. or 90°C, respectively) of Fmoc removal using 20% (v/v) piperidine in DMF. Underlined **H** and **C** in the above sequences are the residues whose racemization was investigated and were coupled in different modalities both in DMF and in DMSO/BuOAc, as reported in Table 1.

The products were cleaved from the resin, and all protecting groups were removed at the same time via the test cleavage procedure above reported. After solubilization in water, crude peptides were analyzed by RP-UHPLC coupled with ESI-MS (SI).

2.8 | PurePep EasyClean (PEC®) purification

We performed the HPLC-free purification of the 72mer peptide $RBM_{436-507}$ and β-amyloid peptides after their syntheses in DMF and BuOAc/DMSO, using the PEC® technology. The first step of this protocol requires the coupling of the PEC-linker RC + on the N-terminal amino function of the peptide on resin, which in our study was conducted at r.t. for 18 hours under mechanical shaking using four equivalents of the linker, six equivalents of Oxyma pure, and six equivalents of DiPEA in DMSO/EtOAc 1:9 (v/v) mixture. Activated polymethacrylate (PMA) beads were transferred in a fritted cartridge and washed with 3 × water and 3 × 0.1 M aqueous citric acid buffer (pH 4.5). The linker carrying the peptide was solubilized into 9:1 TFA/H_2O solution and cooled at 0°C. Pyridine was added dropwise, and the solution was diluted with ACN and added to PMA beads. The mixture was shaken for 5 hours at r.t. and, after immobilization, the beads were washed with 3 × H_2O. Unreacted aldehyde functions were blocked on beads using a solution of 10%w L-Cys in 0.1 M aqueous citric acid buffer (pH 4.5) for 15 minutes. Then, the beads were washed with 3 × 0.9 M GdmCl in DMSO and with 3 × 0.1 M NaCl in H_2O/EtOH 3:7. The linker on the peptide was reduced using a solution of 0.3 M dithiothreitol (DTT) in 0.6 M aqueous $NaHCO_3$ (pH 8) for 15 minutes at r.t. Then, the beads were washed with H_2O and ACN. The final release consisted of treatment with TFA/H_2O (95:5) for 45 minutes at

TABLE 1 Racemization study conditions.

		Temperature (°C)	Time (s)	Coupling system
H	Fmoc-His(Trt)-OH	r.t.	3,600	DIC Oxyma Pure
		90	120	
	Fmoc-His(Boc)-OH	r.t.	3,600	
		90	120	
	Fmoc-D-His(Trt)-OH	r.t.	3,600	
C	Fmoc-Cys(Trt)-OH	r.t.	3,600	
		90	120	
	Fmoc-D-Cys(Trt)-OH	r.t.	3,600	

r.t., and the following precipitation was performed in cold diethyl ether followed by lyophilization.

3 | RESULTS AND DISCUSSION

In the present study, we focused on the evaluation of a set of solvents characterized by high boiling points to test the use of low-viscous binary mixtures in SPPS, taking advantage of the high temperature provided by induction heating on PurePep® Chorus synthesizer (Gyros Protein Technologies, Tuscon, AZ, USA) and prevent undesired evaporation and subsequent crush-out of reagents. The choice of solvents for this study (DMSO, anisole, propyl acetate, butyl acetate, 3-methoxypropionitrile, and cyclopentanone) was mainly driven by their eco-sustainability and high boiling points (> 100°C). The binary mixtures, in particular with DMSO, were composed following previous enlightening studies by Martin et al.[21] Thus, we moved toward different mixtures being aware of advantages (tuning of polarity and viscosity) and drawbacks (recovering difficulty).

We first composed in Table 2 a list of green solvents mainly following the GSK Solvent Selection Guide.[24,25]

We were looking for the following characteristics to select the solvent systems to be further tested:

1. Reagents solubility ≥ 0.4 M.
2. The boiling point of solvent or solvent mixture > 100°C.
3. Solvent viscosity < 4 mPas.
4. Resin swelling > 4 ml·g^{-1}.

Our target, at this stage, was to select neat solvents or binary mixtures capable of replacing DMF and demonstrating at least the same performances in an automated synthesizer at elevated temperatures, up to 90°C using induction heating, a heating system that efficiently handles temperature increase regardless of the solvent used.

TABLE 2 List of solvents from GSK solvent selection guide.

Solvent name	M.W.	F.m.	m.p. (°C)	f.p. (°C)	b.p (°C)	Viscosity (mPas)	Density (g/ml)	Polarity (E_T)
N-Methylpyrrolidone	99.133	C_5H_9NO	−24	86	203	1.65	1.028	42.2
DMF	73.095	C_3H_7NO	−61	58	153	0.92	0.948	43.2
Acetonitrile	41.053	C_2H_3N	−45	2	81.3	0.441	0.786	45.6
Ethyl acetate	88.106	$C_4H_8O_2$	−83.6	−4	77.1	0.45	0.902	38.1
Dimethyl sulfoxide	78.13	C_2H_6OS	19	89	189	2.14	1.1004	45.1
Anisole	108.14	C_7H_8O	−37	52	153	0.99	0.995	37.1
Dioxolane	74.08	$C_3H_6O_2$	−95	2.5	75	0.589	1.06	43.1
3-Methoxypropionitrile	85.1	C_4H_7NO	−50	66	164	0.937	0.94	44.4
Methyl acetate	74.08	$C_3H_6O_2$	−98	−13	57.1	0.385	0.932	38.9
Dimethyl isosorbide	174.19	$C_8H_{14}O_4$	−70	120	235	6.8	1.15	
Methyltetrahydrofuran	83.134	$C_5H_{10}O$	−136	−10	80	0.6	0.854	36.5
Propyl acetate	102.133	$C_5H_{10}O_2$	−95	11.8	102	0.6	0.89	37.5
Cyclopentanone	84.12	C_5H_8O	−58.2	26	130.6	1.075	0.95	39.4
Dimethyl carbonate	90.078	$C_3H_6O_3$	3	17	90	0.625	1.07	38.2
tert-Butyl acetate	116.16	$C_6H_{12}O_2$	−58	22	97.8	1	0.859	≅ 38
Butyl acetate	116.16	$C_6H_{12}O_2$	−78	22	126	0.73	0.882	38.5

TABLE 3 Solvent selection based on viscosity, polarity, and boiling point.

Solvent name	CAS	Abbr.	M.W.	F.m.	m.p. (°C)	f.p. (°C)	b.p (°C)	Viscosity (mPas)	Density (g/ml)	Polarity (E_T)
Dimethylformamide	68–12-2	DMF	73.095	C_3H_7NO	−61	58	153	0.92	0.948	43.2
Dimethyl sulfoxide	67–68-5	DMSO	78.13	C_2H_6OS	19	89	189	2.14	1.1004	45.1
Anisole	100–66-3	An	108.14	C_7H_8O	−37	52	153	0.99	0.995	37.1
3-Methoxypropionitrile	110–67-8	MPN	85.1	C_4H_7NO	−50	66	164	0.937	0.94	44.4
Propyl acetate	109–60-4	PA	102.133	$C_5H_{10}O_2$	−95	11.8	102	0.6	0.89	37.5
Cyclopentanone	120–92-3	CP	84.12	C_5H_8O	−58.2	26	130.6	1.075	0.95	39.4
Butyl acetate	12 3-86-4	BA	116.16	$C_6H_{12}O_2$	−78	22	126	0.73	0.882	38.5

In Table 3, the selected solvents are reported and DMF was used as a comparison.

DMSO is a polar (45.1 E_T)[26,27] and a rather viscous (2.16 mPas) solvent; it has not only relatively low intrinsic toxicity but is also biodegradable, forming a non-toxic product.[28] For this reason, it can be considered a green solvent and nowadays used for a wide range of applications in green chemistry,[29–31] and in particular in peptide chemistry, where it was introduced in a binary mixture by Pawlas et al.[20] The same applies to anisole: it is widely considered an eco-sustainable solvent in several reports,[32,33] including in SPPS.[19] We also inserted 3-methoxypropionitrile (MPN) because of its very significant safety profile. Propyl acetate, butyl acetate, and cyclopentanone were already taken into consideration by Lopez et al as green polar aprotic solvents,[15] even if not with the purpose of taking advantage of their high boiling points to design a synthesis assisted by heating (Table 3).

3.1 | Reagents solubility

Since we consider the solubility of the reagents as a bottleneck that could preclude the use of several polar aprotic solvents, we tested at first this feature, in the selected solvents, both as neat solvents and in binary mixtures. We tested the solubility of five Fmoc-amino acids that differ in polarity and nature of side-chain protecting groups to have a broad perception of the solubility of all Fmoc-amino acids commonly used in peptide synthesis. Table 4 reports that, among the chosen neat solvents, only DMSO and partially cyclopentanone were able to solubilize the selected Fmoc-amino acids. It is due both to a too-low polarity - in particular, anisole and acetate - and to the nature of the solvent itself. On the contrary, the high polarity of DMSO is such that it is able to solubilize even the most challenging Fmoc-amino acids, such as Fmoc-Arg(Pbf)-OH, Fmoc-His(Trt)-OH, and Fmoc-Asn (Trt)-OH. Unfortunately, the main drawback of DMSO is its high viscosity, which can affect both a correct delivery in an automated instrument and an efficient perfusion into the polymer matrix beads. Moreover, the polarity of neat DMSO is excessive for efficient coupling reactions because it is well-known that the rate of this reaction is enhanced in apolar solvents.[21]

For these reasons, we concluded that no neat solvent fulfills all set requirements. Therefore, taking into consideration the benefits of binary mixtures and their contribution in terms of Fmoc-amino acid solubility and the increase in mixture boiling points, we tested the solubilization of the previous five Fmoc-amino acids in solvent X/DMSO binary mixtures with different ratios, taking into consideration that polarity should be reduced since amide bond formation is favored in a more apolar solvent.[21] Hence, we tested 7:3, 8:2, and 9:1 v/v mixtures of Solvent X/DMSO, where Solvent X can be cyclopentanone, propyl acetate, butyl acetate, anisole, and 3-methoxypropionitrile (MPN). As reported in Table 5, the best results in terms of solubility were achieved with the binary mixtures acetate/DMSO 7:3 and anisole/DMSO 8:2. MPN, against our expectations, did not seem to be a good solubilizer, even in a binary mixture with DMSO. After this series of experiments, we decided not to continue to investigate MPN and cyclopentanone mixtures due to solubilization issues and side reactions that occurred during heating, respectively.

After the identification of the three most interesting binary mixtures, we proceeded with the solubility test of coupling reagents, in particular HATU, DIC, and Oxyma pure. In Table 6, we can clearly notice that uronium salt-based coupling reagent HATU is not soluble, neither in neat solvents nor in previously identified binary mixtures,

TABLE 4 Reagents solubility in neat solvents.

	Fmoc-His(Trt)-OH (M)	Fmoc-Gln(Trt)-OH (M)	Fmoc-Gly-OH (M)	Fmoc-Phe-OH (M)	Fmoc-Arg(Pbf)-OH (M)
Cyclopentanone	< 0.1	≥ 0.4	≥ 0.4	0.3	< 0.1
3-Methoxypropionitrile	< 0.1	≥ 0.4	< 0.1	< 0.1	< 0.1
Anisole	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Propyl acetate	< 0.1	0.2	< 0.1	< 0.1	< 0.1
Butyl acetate	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
DMSO	≥ 0.4	≥ 0.4	≥ 0.4	≥ 0.4	≥ 0.4

TABLE 5 Reagents solubility in binary mixtures.

	Fmoc-His(Trt)-OH (M)	Fmoc-Arg(Pbf)-OH (M)	Fmoc-Gln(Trt)-OH (M)	Fmoc-Gly-OH (M)	Fmoc-Phe-OH (M)
Cyclopentanone/DMSO 7:3	≥ 0.4	≥ 0.4	≥ 0.4	≥ 0.4	≥ 0.4
Butyl acetate/DMSO 7:3	≥ 0.4	≥ 0.4	≥ 0.4	≥ 0.4	≥ 0.4
Propyl acetate/DMSO 7:3	≥ 0.4	≥ 0.4	≥ 0.4	≥ 0.4	≥ 0.4
MPN/DMSO 7:3	0.2	≥ 0.4	≥ 0.4	0.2	< 0.1
Anisole/DMSO 8:2	≥ 0.4	≥ 0.4	≥ 0.4	≥ 0.4	≥ 0.4

TABLE 6 Coupling system solubility in neat solvents and binary mixtures.

	HATU (M)	Oxyma pure (M)	DIC (M)
Anisole	< 0.1	≥ 1	≥ 1
Butyl acetate	< 0.1	≥ 1	≥ 1
Propyl acetate	< 0.1	≥ 1	≥ 1
Anisole/DMSO 8:2	0.2	≥ 1	≥ 1
Butyl acetate/DMSO 7:3	< 0.1	≥ 1	≥ 1
Propyl acetate/DMSO 7:3	< 0.1	≥ 1	≥ 1

FIGURE 1 Comparison of swelling capability of Tentagel S RAM and PS rink amide AM resins in different solvents after 2 h and 24 h.

while the widely used coupling system DIC/Oxyma pure is very well solubilized in the three binary mixtures at a concentration of at least 1 M.

3.2 | Swelling test

We tested the two widely used resins, such as polystyrene (PS)-based Rink Amide AM resin and Tentagel S Rink Amide AM resin, in the three binary mixtures at two different times: 2 and 24 hours (Figure 1). Different solvents and times do not affect the swelling of polystyrene Rink amide AM resin. On the other hand, in the case of Tentagel resin, swelling is slightly more efficient in DMF compared to the three selected binary mixtures, although still acceptable (4-7 ml/g). Moreover, a slight swelling rate increase with time was observed.

3.3 | Racemization study

Racemization is a relevant issue in peptide synthesis, particularly in the case of cysteine and histidine coupling during carboxylic activation in the solid-phase. Racemization of one or more residues during the synthesis results at least in epimerization of the final product, which is often not detected, especially in very complex long peptides that are very difficult to solve by chromatography. Through the years, many countermoves to the racemization process have been proposed regarding both protecting groups of amino acids of interest[34–36] and coupling reagents.[37–42] In our study, we synthesized shorter peptides from ABC 20-mer, focusing on coupling in DMF and DMSO/BuOAc (that was considered the most promising mixture both for performance and safety profile) of Fmoc-L-His(Boc)-OH, Fmoc-L-His(Trt)-OH, and Fmoc-L-Cys(Trt)-OH both at r.t. and at 90°C, comparing the amount of D-amino acid-containing epimer by RP-UHPLC-MS vs control peptide synthesized, introducing the corresponding D-His or D-Cys building blocks.

The results we obtained are reported in Table 7.

Interestingly, we observed a comparable racemization during Fmoc-L-His(Boc)-OH coupling using DIC/Oxyma pure as a coupling system, even better at 90°C in the binary mixture DMSO/BuOAc. On the contrary, Fmoc-L-His(Trt)-OH coupling leads to a very high racemization level in both solvents, in particular at high temperatures. For this reason, we preferred the use of Fmoc-L-His(Boc)-OH, which is also more convenient from the atom economy perspective. Moreover,

TABLE 7 Racemization study.

Fmoc-aa coupled*	Coupling solvent	T (°C)	% D-epimer (UPLC)	% D-epimer (MS)
Fmoc-L-His(Boc)-OH	DMF	r.t.	3.44	2.99
		90	7.37	6.93
	DMSO/BuOAc	r.t.	3.45	3.57
		90	6.05	5.20
Fmoc-L-His(Trt)-OH	DMF	r.t.	3.52	2.20
		90	19.66	21.58
	DMSO/BuOAc	r.t.	19.77	21.63
		90	28.83	28.84
Fmoc-L-Cys(Trt)-OH	DMF	r.t.	0.13	Not detected
		90	0.35	0.26
	DMSO/BuOAc	r.t.	0.08	Not detected
		90	0.14	0.36

*Shorter peptides from ABC 20mer used in this study are Fmoc-ECNRADG-NH_2 for cysteine racemization and Fmoc-EHEQCNRADG-NH_2 for histidine racemization.

TABLE 8 Binary mixtures tested in SPPS.

Operation	Solvent mixture	Ratio	Solvent mixture	Ratio	Solvent mixture	Ratio
Fmoc removal	PrOAc/DMSO/Piperidine	2:2:1	BuOAc/DMSO/piperidine	2:2:1	anisole/DMSO/piperidine	2:2:1
Coupling*	PrOAc/DMSO	7:3	BuOAc/DMSO	7:3	anisole/DMSO	8:2
Capping	PrOAc/pyridine/ acetic anhydride	15:16:19	BuOAc/pyridine/ acetic anhydride	15:16:19	anisole/pyridine/ acetic anhydride	15:16:19
Washing	EtOAc/DMSO	8:2	EtOAc/DMSO	8:2	EtOAc/DMSO	8:2

*Fmoc-amino acid 0.4 M, DIC 1 M, Oxyma pure 1 M. Fmoc-His(Trt)-OH and Fmoc-Asn(Trt)-OH were solubilized in 6:4 acetate/DMSO and 7:3 anisole/DMSO mixtures because we observed precipitation after few hours from complete solubilization.

we also noticed that Fmoc-L-Cys(Trt)-OH is not so prone to racemization during coupling in either solvent while using DIC/Oxyma pure as a coupling system.

3.4 | Synthesis of model peptides at room temperature

A fine-tuning of binary mixture composition is pivotal for an excellent outcome of peptide synthesis. While the binary mixture for coupling reactions should be apolar to promote amide bond formation but also polar enough to solubilize reagents. On the contrary, for Fmoc-removal it should be reached a compromise based on the necessity to have a more polar mixture but at the same time not too viscous to be delivered properly by the automatic synthesizer. That is exactly why we prepared a deprotection solution using a 1:1 mixture of solvent X/DMSO considering that it is ideally slightly more polar than neat DMF with a comparable viscosity.[21] Regarding the capping reaction (which is a relevant characteristic of the PurePep® Chorus parallel synthesizer), this was tested mainly for two reasons: on the one hand, capping can stop truncated analogs after incomplete coupling, making chromatography monitoring easier, and on the other hand, it sets up the final peptide product to be purified by PEC® technology via catch-and-release strategy. In Table 8, the tested binary mixtures with different ratios per coupling, capping, and Fmoc removal are reported.

In addition to the fine-tuning of the binary mixture composition, a correct calibration of the delivery system is required in order to offset viscosity differences among different solvent systems. This operation can be easily carried out on the PurePep® Chorus (Gyros Protein Technologies, Tucson, AZ, USA) peptide synthesizer for all the eight solvent positions, both for top and bottom deliveries, on up to six reaction vessels separately, collecting and measuring the delivered volumes directly in external position vials.

Before starting the syntheses on the machine, we evaluated empirically the speed of mixture evaporation. This is a very important aspect to keep in mind when selecting a solvent system, in particular, if stock reagent solutions should be prepared and used in week-long (or even longer) syntheses. In fact, if part of the solvent mixture evaporates during a long-automated synthesis, in particular in not-sealed reaction vessels or reagent vials, a reagent precipitation can occur, clogging tubes. It was demonstrated that EtOAc/DMSO is a great green candidate for DMF substitution in terms of synthetic performances due to similarities in polarity and viscosity. However, the main drawback is the quite rapid evaporation of ethyl acetate from the mixture, as reported in the evaporation experiment (see Supporting Information), which could result in reagents precipitation. The green

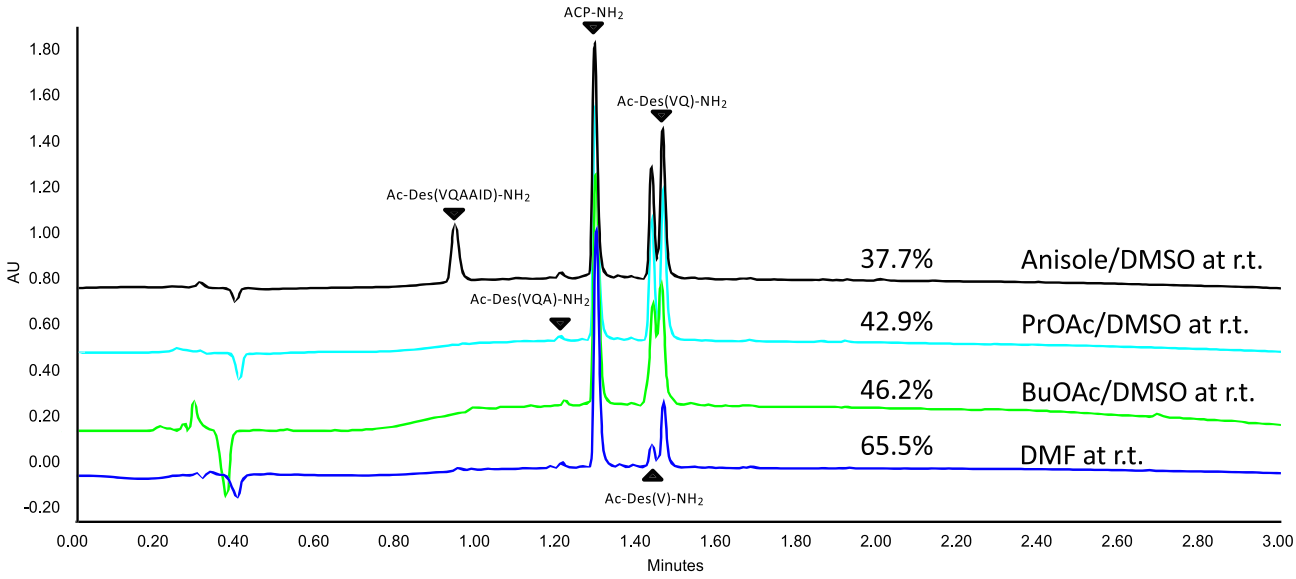


FIGURE 2 RP-UHPLC traces comparison of ACP (65–74) synthesized with different solvents at r.t.

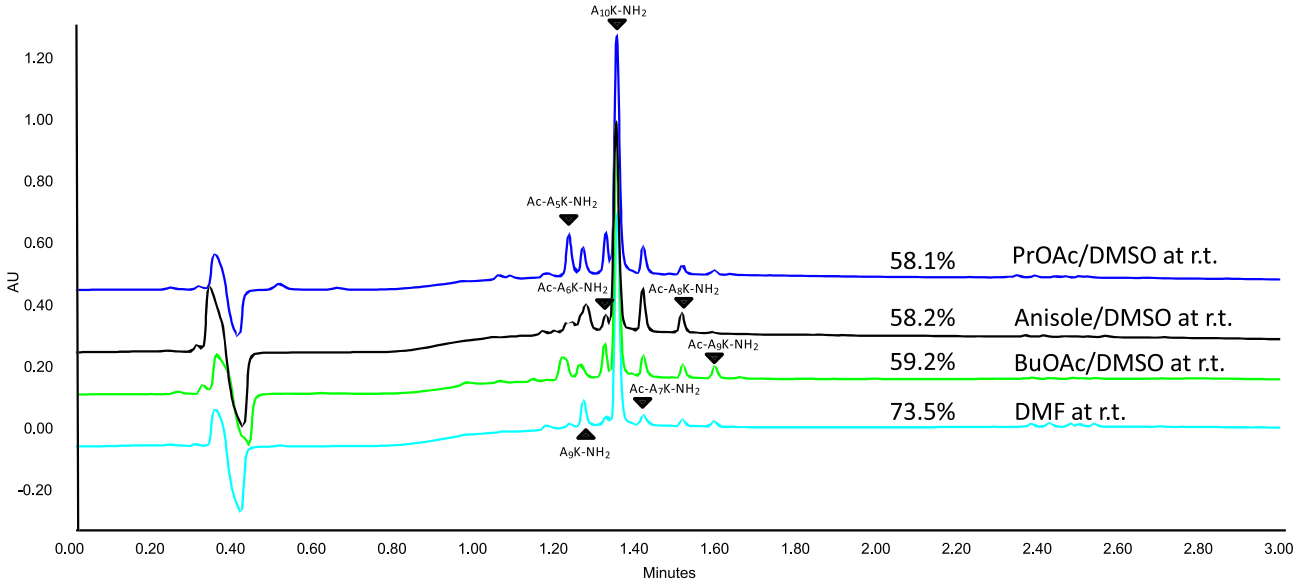


FIGURE 3 RP-UHPLC traces comparison of PolyALA synthesized with different solvent systems at r.t.

mixtures proposed in this work, on the contrary, had a lower evaporation speed, in particular the one with butyl acetate.

We synthesized at 100 µmol scale three model peptides: ACP (65–74) [H-VQAAIDYING-NH_2], a peptide widely used to evaluate the efficiency of a synthetic protocol; $A_{10}K$ [H-AAAAAAAAAAK-NH_2], a well-demonstrated self-assembling peptide, even on resin[43]; and AlaSTD, as a reference peptide representing a non-challenging peptide sequence [H-AKADEVSLHKWYG-NH_2].[23] We used the selected binary mixtures for coupling and Fmoc removal, with two different ratios of solvent X/DMSO to optimize both reactions; EtOAc/DMSO 8:2 binary green mixture was used for washing steps since it has been proved to be an excellent alternative to DMF at r.t.

The sequences were synthesized using the PurePep® Chorus (Gyros Protein Technologies, Tucson, AZ, USA) automated synthesizer taking advantage of both mechanical oscillation and nitrogen bubbling mixing. The protocol used for these syntheses is shown in Table S1, in particular volumes and times are reported.

The crude HPLC purities of syntheses performed at r.t. are reported in Figures 2, 3, and 4. Results in DMF are certainly better than the ones obtained with the other mixtures at r.t. This could be due to a slow reaction kinetics both for amide bond formation and/or for Fmoc removal in the selected binary mixture. Anyway, we can also notice that in all syntheses, $A_{10}K$ crude purity is higher than ACP (65–74), and, among the three green binary mixtures, BuOAc/DMSO was slightly better than the other two, providing higher crude purity for

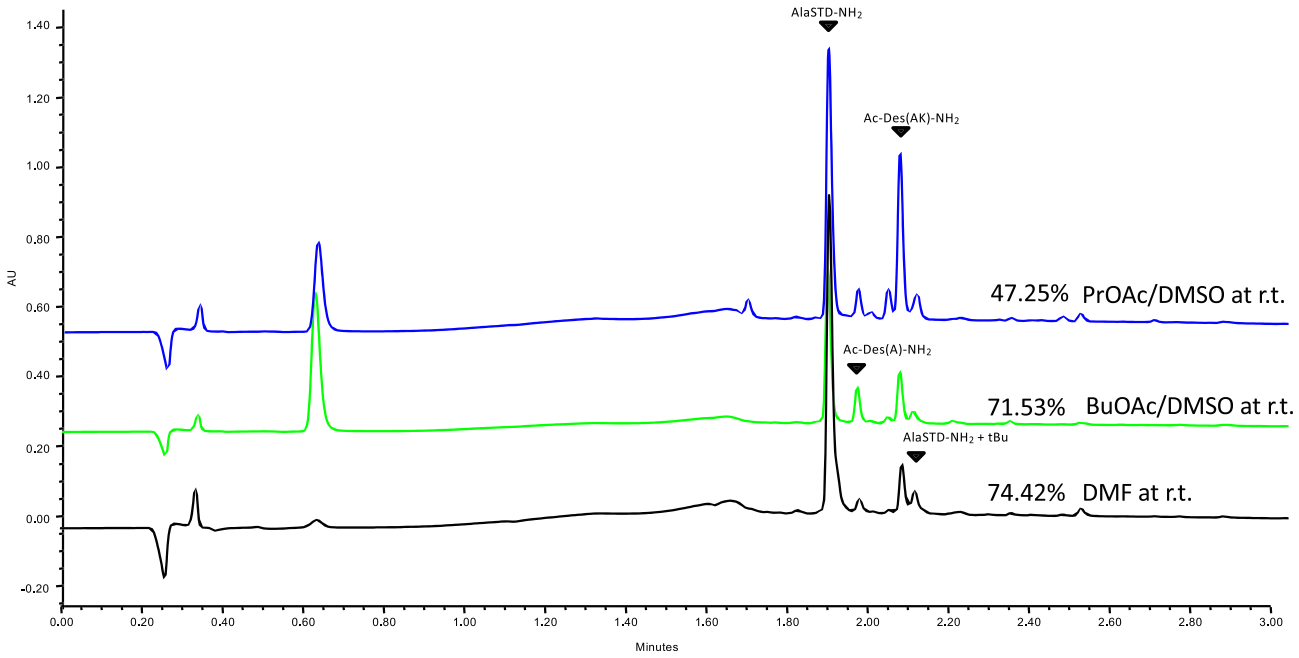


FIGURE 4 RP-UHPLC traces comparison of AlaSTD synthesized with different solvent systems at r.t.

both model peptides. Regarding AlaSTD, syntheses in BuOAc/DMSO and in DMF are comparable, while the one using PrOAc/DMSO showed the remarkable formation of A-K deletion. These results could be improved by optimizing equivalents, reaction repetitions, and reaction time, but this is not the main purpose of the present work. Apart from this, we demonstrated that these binary green mixtures could substitute for DMF, albeit with slightly worse performances.

3.5 | Syntheses of model peptides at high temperature

It has been common knowledge for some time that SPPS at high temperatures can provide high efficiency in less time, enabling the production of peptides that are difficult to synthesize at room temperature.[44,45] The instrument used in this study, PurePep® Chorus (Gyros Protein Technologies, Tucson, AZ, USA), is equipped with up to six reaction vessels that can be heated by magnetic induction. The specific reaction vessel is a glass-fitted syringe externally covered by a metal surface. This type of heating ensures full compatibility with all solvents since the heat source is external and independent from the solvent (or solvent mixture) itself. The sequences were synthesized taking advantage of mechanical oscillation, nitrogen bubbling, and induction heating. The protocol used for these high-temperature syntheses is shown in Table S2, in which volumes, times, and temperatures are reported.

Regarding ACP (65–74), in Figure 5, it can be observed a sizable increase of purity at high temperatures, even doubled in the cases of BuOAc/DMSO and PrOAc/DMSO mixtures, in comparison with purities obtained at room temperature. At the same time, analyzing the $A_{10}K$ trend in Figure 6, heating seems to have a low impact on the crude purities in all the cases we studied. Moreover, even though $A_{10}K$ synthesized in DMF at 90°C showed the best purity (78.3%), the result obtained with PrOAc/DMSO is closely comparable. AlaSTD crude purities are high (86–88%) in all the solvent systems used (Figure 7).

In conclusion, we can observe that syntheses in DMF at room temperature provide better results in terms of crude HPLC purities in comparison with other mixtures, particularly for ACP (65–74) (Figure 8). At the same time, this trend is reversed for the synthesis of ACP (65–74) at 90°C, since we noticed a very high purity with BuOAc/DMSO, even higher than with DMF. $A_{10}K$ synthesis seems to be less affected by increased temperature than ACP (65–74). Nevertheless, DMF (at r.t. and at high temperature) and PrOAc/DMSO provided comparable results. In addition to the general crude purities increase, high temperatures are permitted to dramatically reduce the synthesis time (4-fold), as reported in Figure 9.

3.6 | Synthesis of difficult peptide sequences

Synthesis of β-amyloid (1-42) – after having analyzed results obtained with model peptides, we decided to focus on the most promising green mixture, BuOAc/DMSO, because of the best result provided at 90°C in the case of ACP (65–74) synthesis and also for safety reasons, due to the higher boiling point compared with PrOAc/DMSO mixture. We moved forward with an overt reference point and challenge among difficult peptide syntheses, i.e., the β-amyloid (1-42) peptide [DAEFRHDSGYEVHHQKLVFFAEDVG SNKGAIIGLMVGGVVIA], a 42mer well known to be prone to aggregation, both on-resin during SPPS and in solution during analysis.[46] In Figure 10, the sequence with color code is reported. It is shown that the hydrophobic moiety of the peptide (carboxylic half, rich in apolar amino acids, in gray) has a higher propensity to form β-strand.

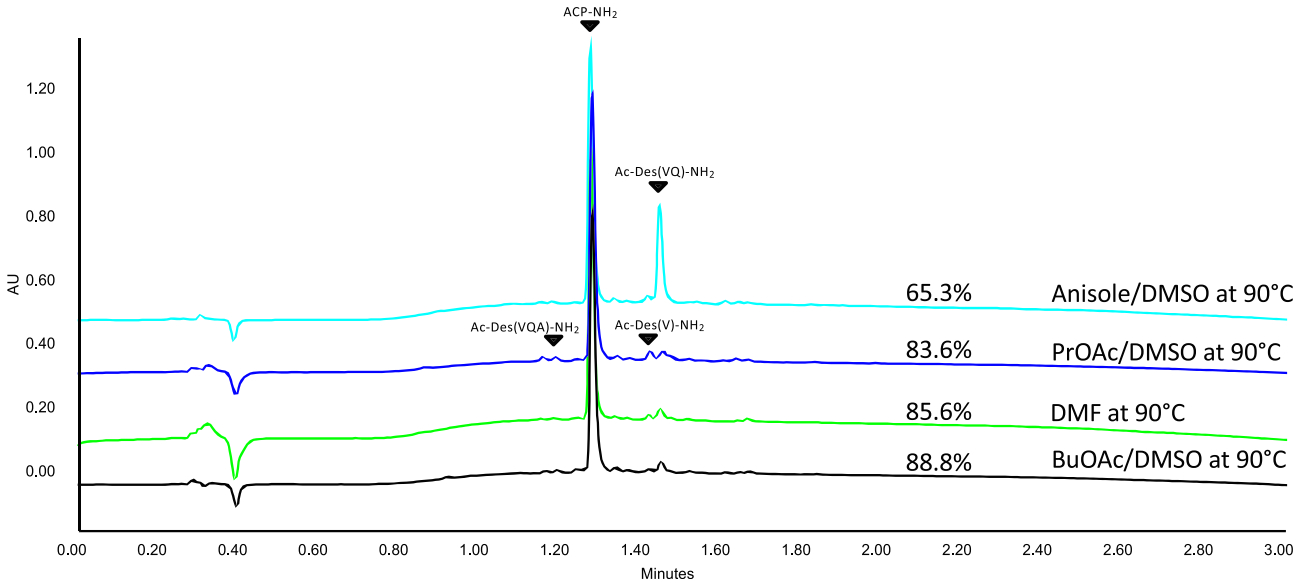


FIGURE 5 RP-UHPLC traces comparison of ACP (65–74) synthesized with different solvent systems at 90°C.

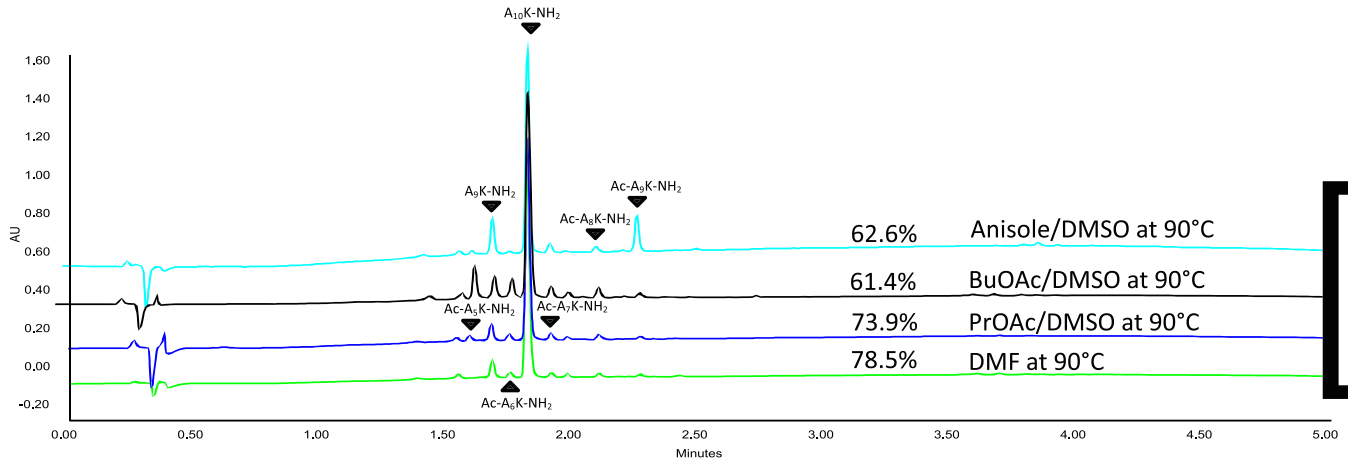


FIGURE 6 RP-UHPLC traces comparison of polyALA synthesized with different solvent systems at 90°C.

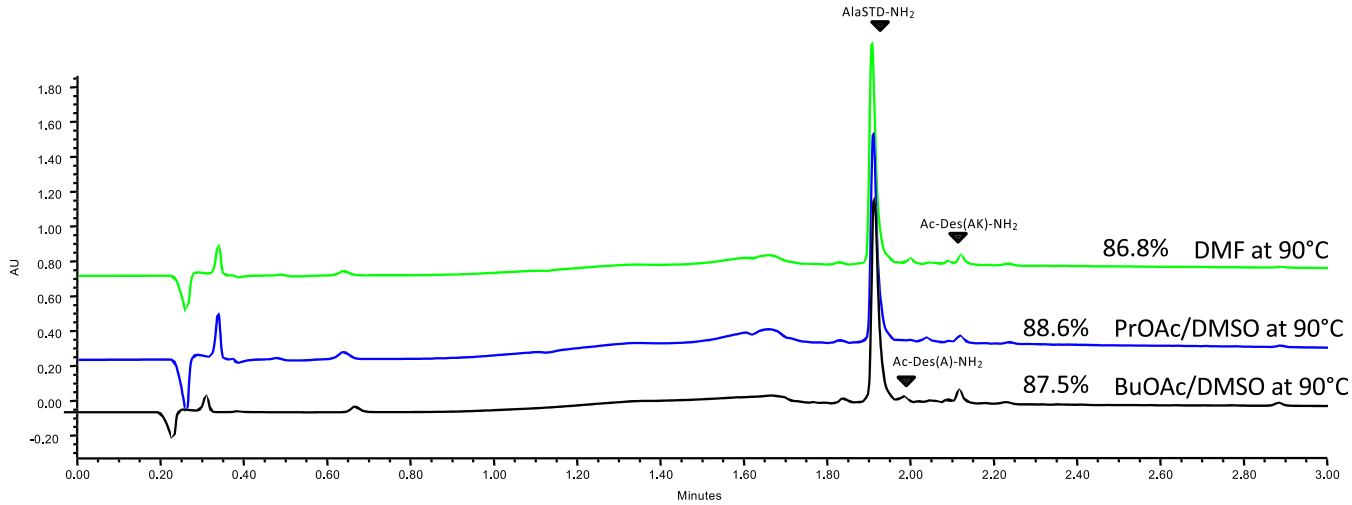


FIGURE 7 RP-UHPLC traces comparison of AlaSTD synthesized with different solvent systems at 90°C.

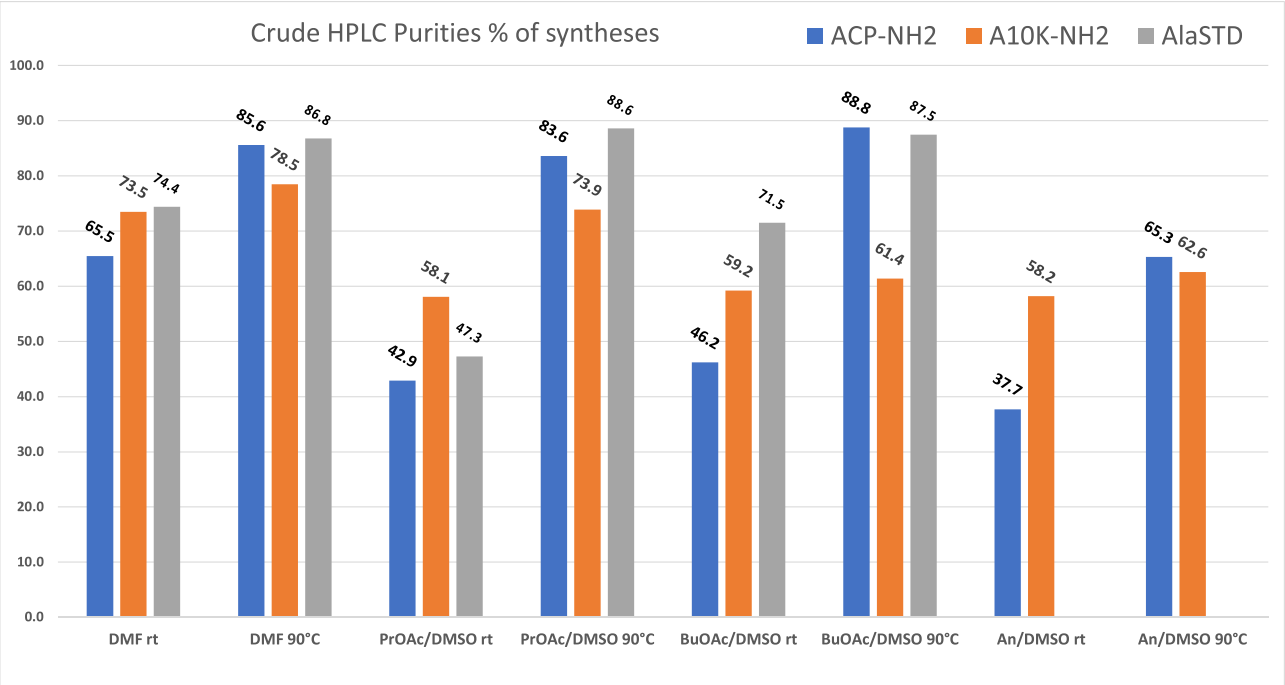
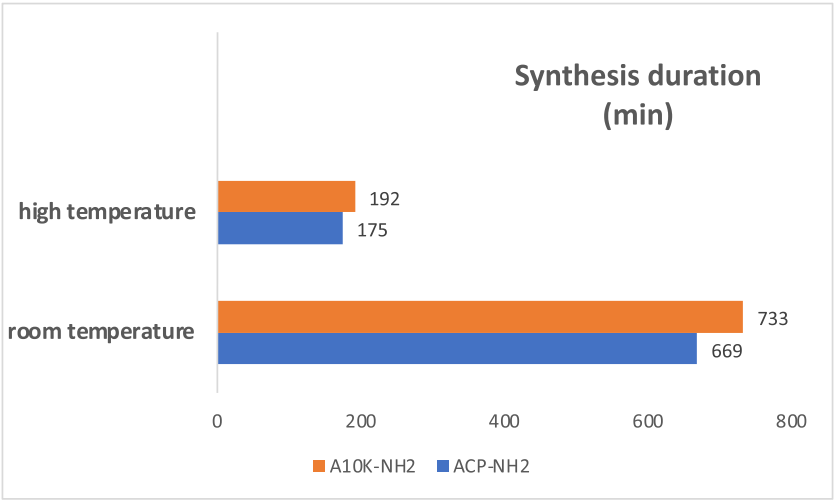


FIGURE 8 Bar chart of crude purities: comparison among different solvent systems and temperatures.

FIGURE 9 Bar chart of time of synthesis: comparison between high and room temperature.



Moreover, methionine, which is present in the sequence, has a propensity to oxidize. We carried out the SPPS of β-amyloid (1-42) at 100 μmol scale both with DMF and with the green mixture BuOAc/DMSO at high temperature, using the same synthetic protocol at 90°C, optimized to obtain the above-mentioned model peptides. The RP-HPLC-MS analysis of crude peptides after test cleavage showed a very similar profile and purity (61.7% HPLC purity for the synthesis performed with BuOAc/DMSO and 59.7% HPLC purity of the synthesis in DMF) (Figures 11 and 12). Both chromatographic traces show a peak corresponding to the oxidized methionine in a similar percentage, i.e., 9.1% and 10.7% for the syntheses performed with BuOAc/DMSO and DMF, respectively. Thus, we conclude that DMSO as a solvent for SPPS does not produce methionine oxidation. Moreover, the oxidized product can be reduced after cleavage from the resin, leading to the desired product.[48]

3.7 | Synthesis of SARS-CoV-2 spike protein receptor binding motif ($RBM_{436-507}$)

We report herein the synthesis in DMF and in BuOAc/DMSO of the 72-mer peptide $RBM_{436-507}$ putatively involved in the protein–protein interaction between SARS CoV2 Spike and human Angiotensin-converting enzyme 2 (ACE2), a crucial research target for COVID-19 diagnostics and therapeutics.[49,50] The choice to synthesize this peptide sequence was dictated by the remarkable complexity due to the length

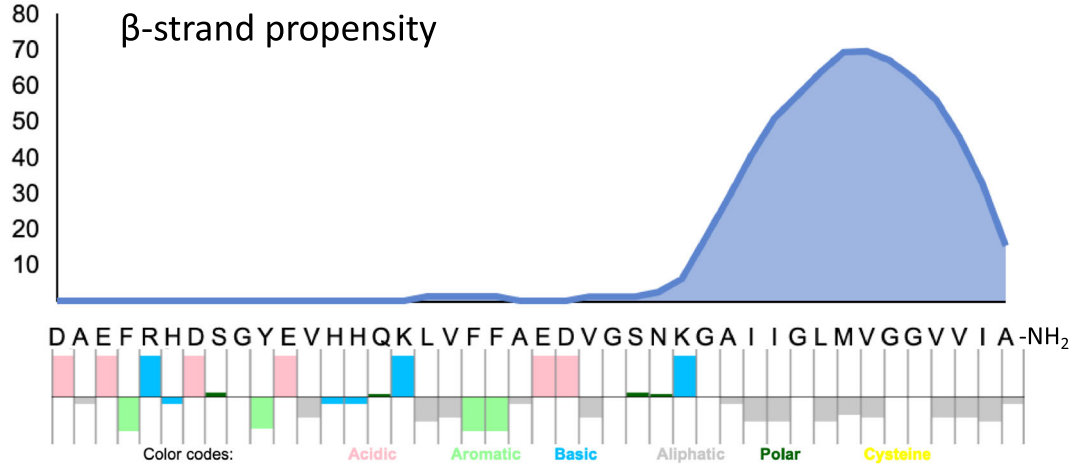


FIGURE 10 β-Strand propensity and amino-acid composition of β-amyloid (1–42).[47]

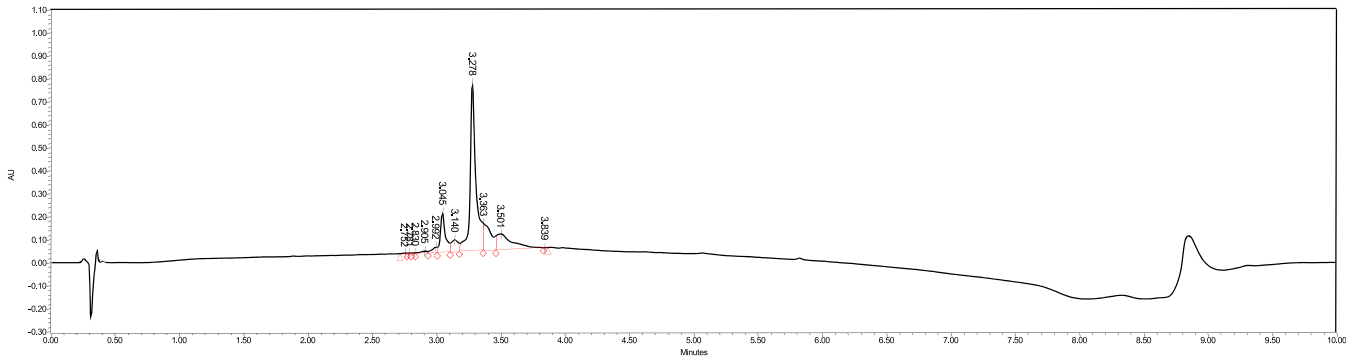


FIGURE 11 RP-UHPLC traces of crude β-amyloid (1–42) synthesized using DMF. C18 column Waters Acquity BEH (130 Å, 1.7 μm, 2.1 × 50 mm); temperature 45°C; flow, 0.5 ml/min; eluent, 0.1% (v/v) TFA in H_2O (A) and 0.1% (v/v) TFA in CH_3CN (B); λ, 215 nm, gradient, 10–90% B in 7 min. Rt = 3.278 min: β-amyloid (1–42) 59.7% purity.

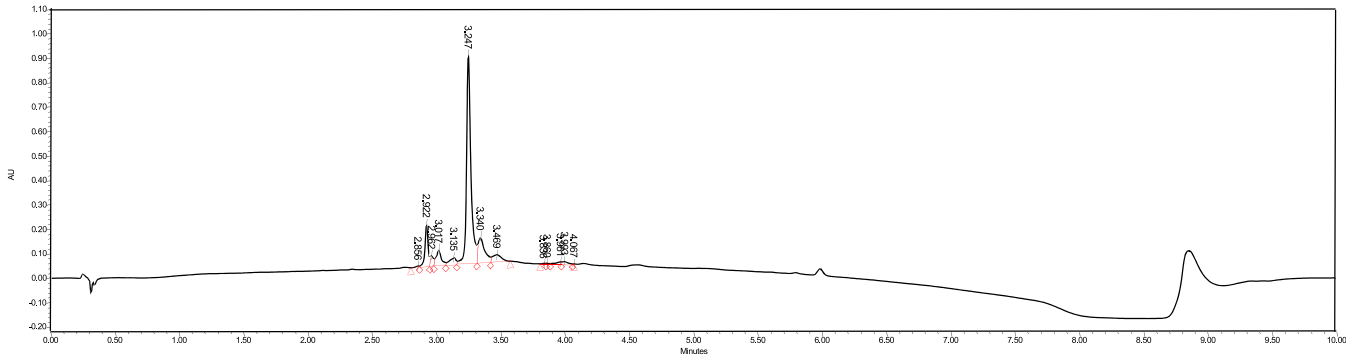


FIGURE 12 RP-UHPLC traces of crude β-amyloid (1–42) synthesized using BuOAc/DMSO. C18 column Waters Acquity BEH (130 Å, 1.7 μm, 2.1 × 50 mm); temperature 65°C; flow, 0.5 ml/min; eluent, 0.1% (v/v) TFA in H_2O (A) and 0.1% (v/v) TFA in CH_3CN (B); λ, 215 nm, gradient, 10–90% B in 7 min. Rt = 3.247 min: β-amyloid (1–42) 61.7% purity.

of the sequence, its amphiphilic nature, depending on a very hydrophobic C-terminal part and a hydrophilic N-terminal one, and its β-sheet aggregation propensity. In Figure 13, the sequence is reported with color codes.

The synthesis was performed on a low-loading PEG-based resin Tentagel R RAM to minimize the steric hindrance of the growing peptide chain on the polymeric solid support.

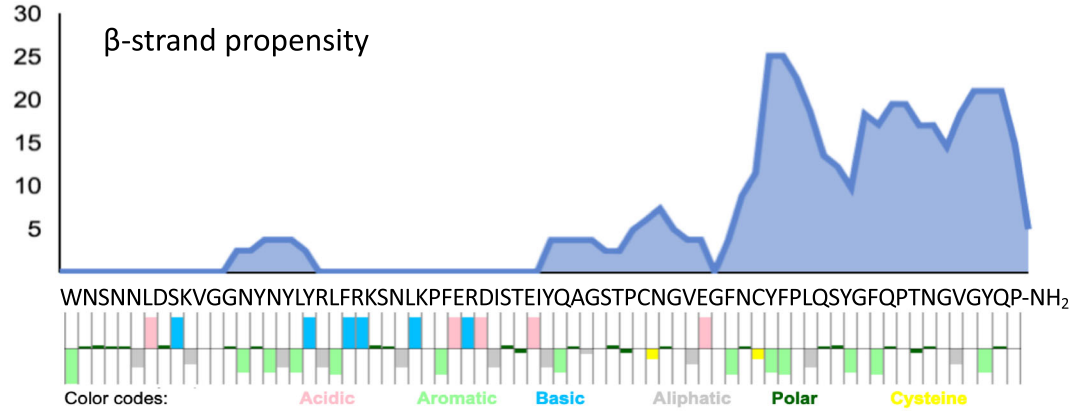


FIGURE 13 β-Strand propensity and amino-acid composition of the 72mer peptide $RBM_{436-507}$.[47]

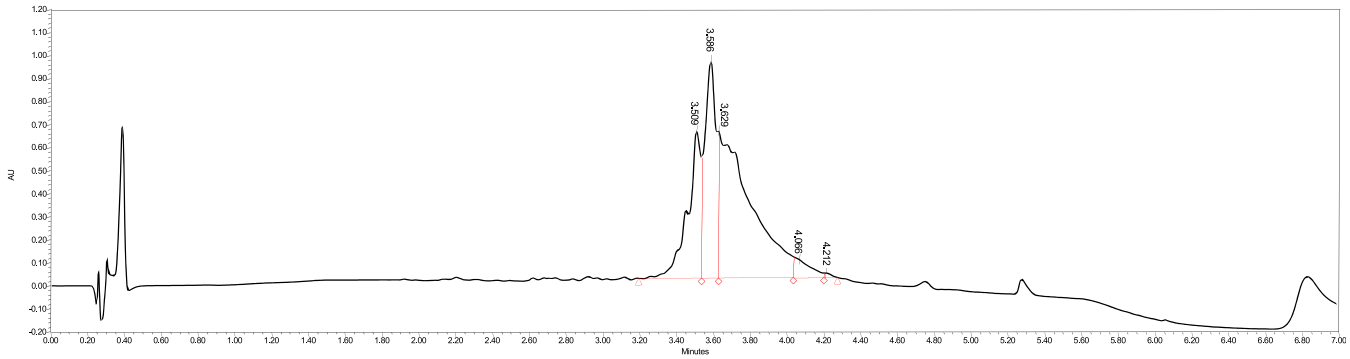


FIGURE 14 RP-UHPLC traces of 72mer peptide $RBM_{436-507}$ synthesized using DMF. C18 column Waters Acquity BEH (130 Å, 1.7 µm, 2.1 × 50 mm); temperature 45°C; flow, 0.5 ml/min; eluent, 0.1% (v/v) TFA in H_2O (A) and 0.1% (v/v) TFA in CH_3CN (B); λ, 215 nm, gradient, 0–70% B in 15 min. Rt = 3.586 min: 72mer peptide $RBM_{436-507}$ 26.1% purity.

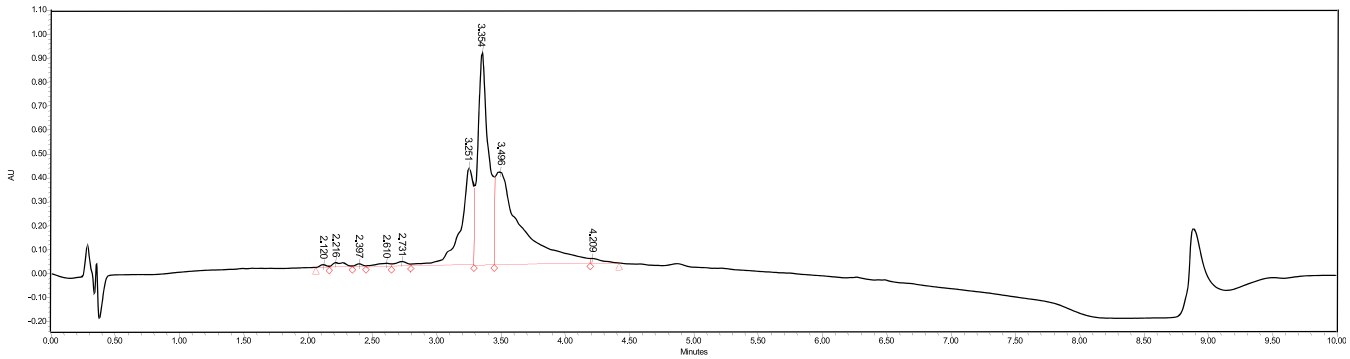


FIGURE 15 RP-UHPLC traces of 72mer peptide $RBM_{436-507}$ synthesized using BuOAc/DMSO. C18 column Waters Acquity BEH (130 Å, 1.7 µm, 2.1 × 50 mm); temperature 45°C; flow, 0.5 ml/min; eluent, 0.1% (v/v) TFA in H_2O (A) and 0.1% (v/v) TFA in CH_3CN (B); λ, 215 nm, gradient, 10–90% B in 10 min. Rt = 3.354 min: 72mer peptide $RBM_{436-507}$ 42.6% purity.

We synthesized the 72-mer at 50 µmol scale, at high temperature, using both DMF and BuOAc/DMSO mixture, following a single coupling strategy (5 equivalents Fmoc-amino acids, 5 equivalents coupling system) for the first half of the peptide (36 residues), which is quite hydrophobic. After checking by test cleavage, followed by HPLC-MS analysis, that the progression of the synthesis of this portion was comparable (purity 77.6% in DMF and 75.7% in DMSO/BuOAc) in both solvent systems, we attempted at first the synthesis of the second half doubling the equivalents of the reagents (10 equivalents Fmoc-amino acids, 10 equivalents coupling system) but, after a test cleavage, the final product was not detected. Therefore, we tried a double coupling strategy using two equivalents of reagents (10 × 2 equivalents of Fmoc-amino acids, 10 × 2 equivalents coupling system) obtaining the desired peptide as the main product as observed in the

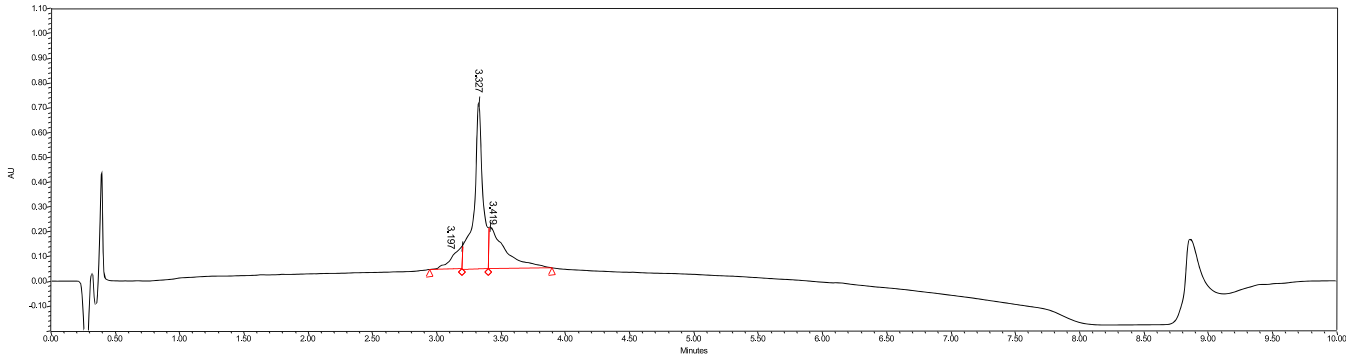


FIGURE 16 RP-UHPLC traces of 72mer peptide $RBM_{436-507}$ synthesized using DMF after PEC®. C18 column Waters Acquity BEH (130 Å, 1.7 µm, 2.1 × 50 mm); temperature 45°C; flow, 0.5 ml/min; eluent, 0.1% (v/v) TFA in H_2O (A) and 0.1% (v/v) TFA in CH_3CN (B); λ, 215 nm, gradient, 10–90% B in 3 min. Rt = 3.327 min: 72mer peptide $RBM_{436-507}$ 68% purity.

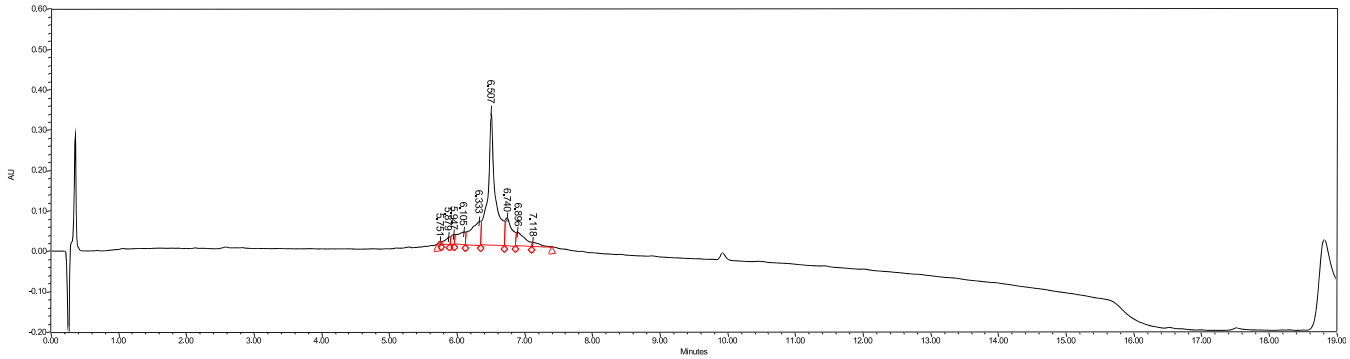


FIGURE 17 RP-UHPLC traces of 72mer peptide $RBM_{436-507}$ synthesized using BuOAc/DMSO after PEC®. C18 column Waters Acquity BEH (130 Å, 1.7 µm, 2.1 × 50 mm); temperature 45°C; flow, 0.5 ml/min; eluent, 0.1% (v/v) TFA in H_2O (A) and 0.1% (v/v) TFA in CH_3CN (B); λ, 215 nm, gradient, 10–90% B in 15 min. Rt = 6.507 min: 72mer peptide $RBM_{436-507}$ 71% purity.

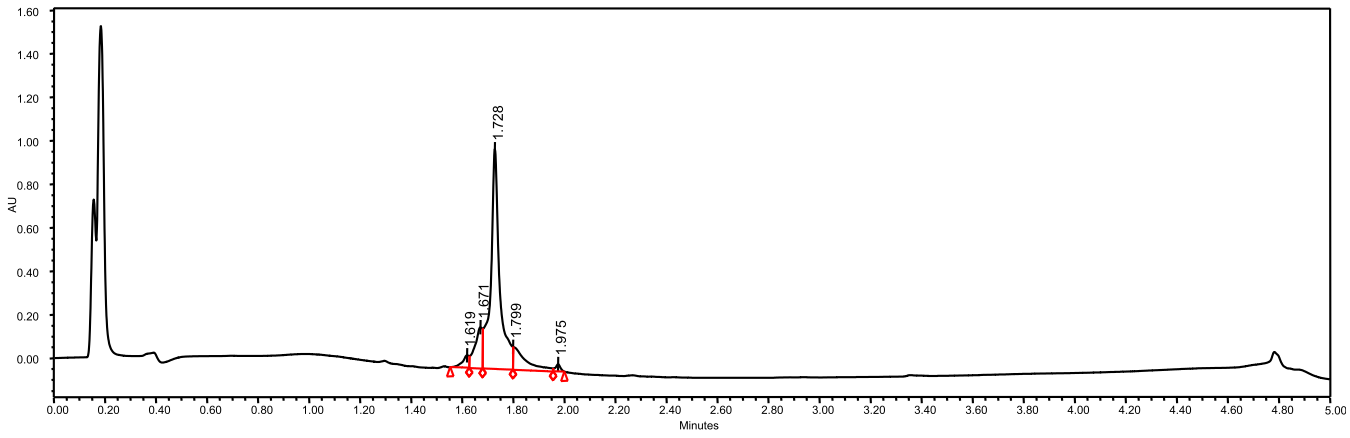


FIGURE 18 RP-UHPLC traces of β-amyloid (1–42) synthesized using DMF after PEC®. C18 column Waters Acquity BEH (130 Å, 1.7 µm, 2.1 × 50 mm); temperature 45°C; flow, 0.5 ml/min; eluent, 0.1% (v/v) TFA in H_2O (A) and 0.1% (v/v) TFA in CH_3CN (B); λ, 215 nm, gradient, 10–90% B in 3 min. Rt = 1.728 min: β-amyloid (1–42) 78% purity.

chromatogram for both solvent systems (crude purities 26.1% and 42.6% in DMF and BuOAc/DMSO, respectively) (Figures 14 and 15).

Solvent consumption is not confined solely to the synthesis stage; rather, the downstream processes of peptide cleavage, precipitation, and especially purification via HPLC contribute significantly to the overall waste generated in peptide production. In the case of catch-and-release purification, particularly the PEC® purification, only a fraction (approximately 15%) of solvents is utilized compared to

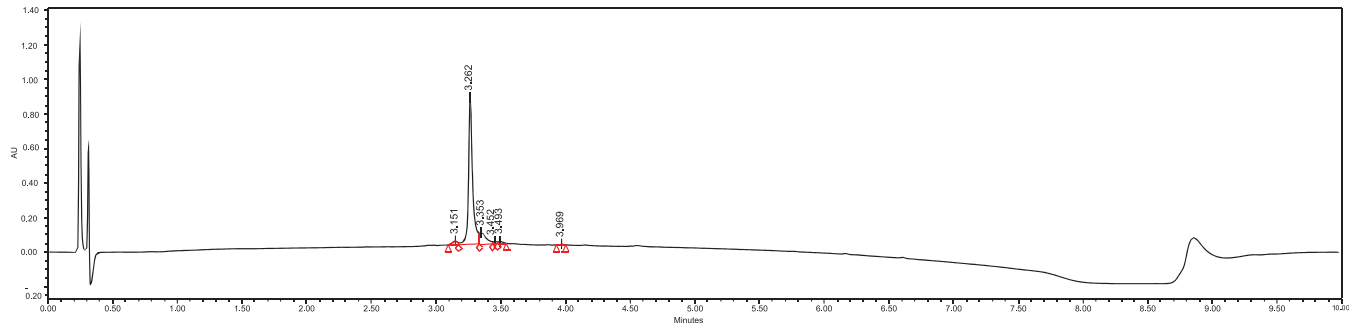


FIGURE 19 RP-UHPLC traces of β-amyloid (1–42) synthesized using BuOAc/DMSO after PEC®. C18 column Waters Acquity BEH (130 Å, 1.7 µm, 2.1 × 50 mm); temperature 45°C; flow, 0.5 ml/min; eluent, 0.1% (v/v) TFA in H_2O (A) and 0.1% (v/v) TFA in CH_3CN (B); λ, 215 nm, gradient, 10–90% B in 7 min. Rt = 1.728 min: β-amyloid (1–42) 84% purity.

traditional HPLC methods.[22] In our pursuit of a more environmentally friendly approach to peptide manufacturing, we opted to employ PEC® for the purification of the two particularly challenging peptides. Remarkably, both peptides underwent PEC® purification in less than 7 hours, yielding HPLC purities of 71% and 68% of the 72-mer peptide syntheses in BuOAc/DMSO and DMF, respectively (Figures 16 and 17), and 84% and 78% of the β-amyloid (1-42) syntheses in BuOAc/DMSO and DMF, respectively (Figures 18 and 19).

4 | CONCLUSIONS

In this study, our primary objective was to identify a safer and more environmentally friendly substitute for DMF, a commonly used but toxic solvent in SPPS, by exploring the properties and performances of various binary solvent mixtures at r.t. and 90°C. We selected DMSO as one of the components of the binary solvent mixtures due to its low toxicity and high solvating power, and we paired it with other solvents like propyl acetate, butyl acetate, anisole, cyclopentanone, and methoxypropionitrile (MPN) that have favorable characteristics such as low viscosity, medium polarity, and high boiling point.

We evaluated the solubility of amino acids and reagents commonly used in solid-phase synthesis, swelling of different resins, and the synthesis efficiency of three test peptides, ACP (65–74), $A_{10}K$, and AlaSTD, in the binary solvent mixtures. We found that DMSO/cyclopentanone, DMSO/propyl acetate, DMSO/butyl acetate (3:7, v/v), and DMSO/anisole (2:8, v/v) could dissolve amino-acids building blocks at concentrations ≥ 0.4 M, while DMSO/MPN (3:7, v/v) failed to dissolve hydrophobic amino acids such as Fmoc-His(Trt)-OH and Fmoc-Phe-OH. We also excluded cyclopentanone and MPN from further experiments due to their potential side reactions and poor solubility, respectively. We observed that DMSO/propyl acetate, DMSO/butyl acetate, and DMSO/anisole resulted in optimal swelling (4–7 ml/g) for all resins, indicating that the solvent properties can be tuned by using binary mixtures.

Developing greener, reliable, robust, and faster synthetic processes is essential for achieving a sustainable future. One of the most promising approaches is to perform greener peptide synthesis at elevated temperatures, the so-called high-temperature solid-phase peptide synthesis (HT-SPPS), which can enhance the reaction rates and also enable the preparation of peptides that are difficult or impossible to synthesize at r.t. However, the use of a green binary solvent mixture in HT-SPPS presents certain challenges, such as the need for precise temperature control to prevent overshooting. The PurePep® Chorus synthesizer addresses that issue by offering induction heating (IH) and oscillation mixing, which can prevent temperature overshooting. Moreover, the PurePep® Chorus synthesizer allows for the use of green binary mixtures of solvents, which can further reduce the environmental impact and cost of peptide synthesis. We compared the SPPS results of the test peptides in the binary solvent mixtures with those in DMF at r.t. and at 90°C performed in the PurePep® Chorus synthesizer. We found that DMF is superior at r.t., as compared to all the tested binary solvent mixtures. On the contrary, DMSO/propyl acetate and DMSO/butyl acetate showed superior or comparable results to DMF at 90°C. The crude purity of ACP (65–74) and $A_{10}K$ increased significantly at elevated temperatures with DMSO/butyl acetate compared to that at r.t. Considering the superior boiling point and crude purity tests of peptides, we conducted further evaluations with DMSO/butyl acetate with complex, self-aggregating, long peptides such as β-amyloid (1-42) and the 72-mer peptide $RBM_{436-507}$. The crude purities of both peptides were found to be higher compared to the syntheses with DMF at elevated temperatures. These results suggest that DMSO/butyl acetate is a promising alternative to DMF for SPPS, especially at high temperatures. In general, achieving a peptide purity of ≥95% for complex peptides requires multiple rounds of RP-HPLC purification. However, this process generates a significant volume of organic waste, leading to increased costs and environmental impact.[51] To reduce the number of RP-HPLC cycles and solvent consumption, we employed a novel catch-release purification method using PEC® technology. This method was applied to purify β-amyloid (1-42) and the 72-mer SARS CoV-2 Receptor Binding Motif ($RBM_{436-507}$). The purity of β-amyloid (1-42) improved from an initial value of 60% to 78% for DMF and from 62% to 84% for BuOAc/DMSO, and similarly, the purity of the longer sequence of SARS CoV-2 $RBM_{436-507}$ increased from an initial value of 26% to 68% for DMF and from 43% to 71% for BuOAc/DMSO.

ACKNOWLEDGEMENTS

L.P. is the recipient of the PhD fellowship PON Research and Innovation 2014–2020 (D.M 1061/2021), XXXVII Cycle in Chemical Sciences (2022-2024): "Greening peptide chemistry, a necessary step to the future", funded by MUR/EU-FSE. The project was funded in part by the TT-Pep Well-being Research Incubator project: "Sustainable and green upscaling processes for peptide(-based) in vitro diagnostics and cosmeceuticals" in the context of 5th seed funding call of EUniWell.

CONFLICT OF INTEREST STATEMENT

The authors M.M., L.A., and R.Z. are employees of Gyros Protein Technologies and declare competing financial interests: the PurePep® Chorus synthesizer and the PurePep® EasyClean technology described in the manuscript can be purchased from Gyros Protein Technologies.

ORCID

Lorenzo Pacini https://orcid.org/0000-0003-0737-2213
Manoj Muthyala https://orcid.org/0009-0008-7145-8402
Luisa Aguiar https://orcid.org/0000-0002-9800-7109
Robert Zitterbart https://orcid.org/0000-0003-1729-6111
Paolo Rovero https://orcid.org/0000-0001-9577-5228
Anna Maria Papini https://orcid.org/0000-0002-2947-7107

REFERENCES

1. Errante F, Ledwoń P, Latajka R, Rovero P, Papini AM. Cosmeceutical peptides in the framework of sustainable wellness economy. *Front Chem*. 2020;8:8. doi:10.3389/fchem.2020.572923
2. Thakur R, Suri CR, Kaur IP, Rishi P. Peptides as diagnostic, therapeutic, and theranostic tools: Progress and future challenges. *Crit Rev Ther*. 2023;40(1):49-100. doi:10.1615/critrevtherdrugcarriersyst.2022040322
3. Li T, Lu XM, Zhang MR, Hu K, Li Z. Peptide-based nanomaterials: self-assembly, properties and applications. *Bioact Mater*. 2022;11:268-282. doi:10.1016/j.bioactmat.2021.09.029
4. Wang L, Wang N, Zhang W, et al. Therapeutic peptides: current applications and future directions. *Signal Transduct Target Ther*. 2022;7(1):7. doi:10.1038/s41392-022-00904-4
5. Ceccarini MR, Palazzi V, Salvati R, et al. Biomaterial inks from peptide-functionalized silk fibers for 3D printing of futuristic wound-healing and sensing Materials. *Int J Mol Sci*. 2023;24(2):947. doi:10.3390/ijms24020947
6. Valentini L, Pacini L, Errante F, et al. Peptide-functionalized silk fibers as a platform to stabilize gelatin for use in ingestible devices. *Molecules*. 2022;27(14):4605. doi:10.3390/molecules27144605
7. Martin V, Egelund PHG, Johansson H, Thordal Le Quement S, Wojcik F, Sejer PD. Greening the synthesis of peptide therapeutics: an industrial perspective. *RCS Adv*. 2020;10(69):42457-42492. doi:10.1039/d0ra07204d
8. Rasmussen JH. Synthetic peptide API manufacturing: a mini review of current perspectives for peptide manufacturing. *Bioorg Med Chem*. 2018;26(10):2914-2918. doi:10.1016/j.bmc.2018.01.018
9. Jaradat DMM. Thirteen decades of peptide synthesis: key developments in solid phase peptide synthesis and amide bond formation utilized in peptide ligation. *J Amino Acids*. 2017;50(1):39-68. doi:10.1007/s00726-017-2516-0
10. Isidro-Llobet A, Kenworthy MN, Mukherjee S, et al. Sustainability challenges in peptide synthesis and purification: from R&D to production. *J Org Chem*. 2019;84(8):4615-4628. doi:10.1021/acs.joc.8b03001
11. Jad YE, Acosta GA, Khattab SN, et al. Peptide synthesis beyond DMF: THF and ACN as excellent and friendlier alternatives. *Org Biomol Chem*. 2015;13(8):2393-2398. doi:10.1039/c4ob02046d
12. Jad YE, Acosta GA, Khattab SN, et al. 2-Methyltetrahydrofuran and cyclopentyl methyl ether for green solid-phase peptide synthesis. *J Amino Acids*. 2015;48:419-426. doi:10.1007/s00726-015-2095-x
13. Jad YE, Acosta GA, Govender T, et al. Green solid-phase peptide synthesis 2. 2-Methyltetrahydrofuran and ethyl acetate for solid-phase peptide synthesis under green conditions. *ACS Sustain Chem Eng*. 2016;4(12):6809-6814. doi:10.1021/acssuschemeng.6b01765
14. Kumar A, Jad YE, El-Faham A, de la Torre BG, Albericio F. Green solid-phase peptide synthesis 4. γ-Valerolactone and N -formylmorpholine as green solvents for solid phase peptide synthesis. *Tetrahedron Lett*. 2017;58(30):2986-2988. doi:10.1016/j.tetlet.2017.06.058
15. Lopez J, Pletscher S, Aemissegger A, Bucher C, Gallou F. N-Butylpyrrolidinone as alternative solvent for solid-phase peptide synthesis. *Org Process Res Dev*. 2018;22(4):494-503. doi:10.1021/acs.oprd.7b00389
16. Kumar A, Sharma A, de la Torre BG, Albericio F. Rhodiasolv PolarClean – a greener alternative in solid-phase peptide synthesis. *Green Chem Lett Rev*. 2021;14(3):545-550. doi:10.1080/17518253.2021.1965663
17. Ferrazzano L, Corbisiero D, Martelli G, et al. Green solvent mixtures for solid-phase peptide synthesis: a dimethylformamide-free highly efficient synthesis of pharmaceutical-grade peptides. *ACS Sustain Chem Eng*. 2019;7(15):12867-12877. doi:10.1021/acssuschemeng.9b01766
18. Jadhav S, Martin V, Egelund PHG, et al. Replacing DMF in solid-phase peptide synthesis: varying the composition of green binary solvent mixtures as a tool to mitigate common side-reactions. *Green Chem*. 2021;23(9):3312-3321. doi:10.1039/d1gc00604e
19. Přibylka A, Pastorek M, Grepl M, Schütznerová EP. The application of anisole in greener solid-phase peptide synthesis protocols – compatibility with green bases in Fmoc removal and new green binary mixture for coupling. *Tetrahedron*. 2021;99:132452. doi:10.1016/j.tet.2021.132452
20. Pawlas J, Rasmussen JH. ReGreen SPPS: enabling circular chemistry in environmentally sensible solid-phase peptide synthesis. *Green Chem*. 2019;21:5990-5998. doi:10.1039/c9gc02775k
21. Martin V, Jadhav S, Egelund PHG, et al. Harnessing polarity and viscosity to identify green binary solvent mixtures as viable alternatives to DMF in solid-phase peptide synthesis. *Green Chem*. 2021;23(9):3295-3311. doi:10.1039/d1gc00603g
22. Ferrazzano L, Catani M, Cavazzini A, et al. Sustainability in peptide chemistry: current synthesis and purification technologies and future challenges. *Green Chem*. 2022;24:975-1020. doi:10.1039/d1gc04387k
23. Zitterbart R, Berger N, Reimann O, et al. Traceless parallel peptide purification by a first-in-class reductively cleavable linker system featuring a safety-release. *Chem Sci*. 2021;12(7):2389-2396. doi:10.1039/d0sc06285e
24. Henderson RK, Jiménez-González C, Constable DJC, et al. Expanding GSK's solvent selection guide – embedding sustainability into solvent selection starting at medicinal chemistry. *Green Chem*. 2011;13:854. doi:10.1039/c0gc00918k
25. Alder CM, Hayler JD, Henderson RK, et al. Updating and further expanding GSK's solvent sustainability guide. *Green Chem*. 2016;18(13):3879-3890. doi:10.1039/c6gc00611f
26. Reichardt C. Solvatochromic dyes as solvent polarity indicators. *Chem Rev*. 1994;94(8):2319-2358. doi:10.1021/cr00032a005
27. Cerón-Carrasco JP, Jacquemin D, Laurence C, Planchat A, Reichardt C, Sraïdi K. Solvent polarity scales: determination of new ET(30) values for 84 organic solvents. *J Phys Org Chem*. 2014;27(6):512-518. doi:10.1002/poc.3293
28. Kino K, Murakami-Nitta T, Oishi M, Ishiguro S, Kirimura K. Isolation of dimethyl sulfone-degrading microorganisms and application to

odorless degradation of dimethyl sulfoxide. *J Biosci Bioeng*. 2004;97(1):82-84. doi:10.1016/s1389-1723(04)70171-1
29. Medimagh M, Mleh CB, Issaoui N, et al. DFT and molecular docking study of the effect of a green solvent (water and DMSO) on the structure, MEP, and FMOs of the 1-ethylpiperazine-1,4-diium bis(hydrogenoxalate). *Compound J Mol Liq*. 2023;369:120851. doi:10.1016/j.molliq.2022.120851
30. Ong YF, Wong YT, Chai PV. Green synthesis of graphene oxide polysulfone membrane using dimethyl sulfoxide as green solvent. *Mater Today Proc*. 2022;64:1655-1660. doi:10.1016/j.matpr.2022.06.010
31. Matveev D, Vasilevsky V, Volkov V, et al. Fabrication of ultrafiltration membranes from non-toxic solvent dimethylsulfoxide: benchmarking of commercially available acrylonitrile co-polymers. *J. Environ Chem Eng*. 2022;10:107061. doi:10.1016/j.jece.2021.107061
32. Podapangi SK, Mancini L, Xu J, et al. Green anisole solvent-based synthesis and deposition of phthalocyanine dopant-free hole-transport materials for perovskite solar cells. *Energies*. 2023;16(9):3643. doi:10.3390/en16093643
33. Mutra MR, Chandana TL, Wang Y-J, Wang J-J. Green and rapid acid-catalyzed ynamide skeletal rearrangement and stereospecific functionalization with anisole derivatives. *Green Chem*. 2023;25(20):8124-8133. doi:10.1039/d3gc02460a
34. Hibino H, Miki Y, Nishiuchi Y. Evaluation of acid-labile S-protecting groups to prevent Cys racemization in Fmoc solid-phase peptide synthesis. *J Pept Sci*. 2013;20(1):30-35. doi:10.1002/psc.2585
35. Hibino H, Nishiuchi Y. 4-Methoxybenzyloxymethyl group as an Nπ-protecting group for histidine to eliminate side-chain-induced racemization in the Fmoc strategy. *Tetrahedron Lett*. 2011;52(38):4947-4949. doi:10.1016/j.tetlet.2011.07.065
36. Hibino H, Nishiuchi Y. 4-Methoxybenzyloxymethyl group, a racemization-resistant protecting group for cysteine in Fmoc solid phase peptide synthesis. *Org Lett*. 2012;14:1926-1929. doi:10.1021/ol300592w
37. Jad YE, Khattab SN, de la Torre BG, et al. Oxyma-B, an excellent racemization suppressor for peptide synthesis. *Org Biomol Chem*. 2014;12(42):8379-8385. doi:10.1039/c4ob01612b
38. Liu T, Zhang X, Peng Z, Zhao J. Water-removable Ynamide coupling reagent for racemization-free syntheses of peptides, amides, and esters. *Green Chem*. 2021;23:9916-9921. doi:10.1039/d1gc03498g
39. Miyazawa T, Otomatsu T, Fukui Y, Yamada T, Kuwata S. Effect of copper(II) chloride on suppression of racemization in peptide synthesis by the carbodiimide method. *Int J Pept Protein Res*. 1992;39(3):237-244. doi:10.1111/j.1399-3011.1992.tb00795.x
40. Dev D, Palakurthy NB, Thalluri K, Chandra J, Mandal B. Ethyl 2-cyano-2-(2-nitrobenzenesulfonyloxyimino)acetate (o-NosylOXY): a recyclable coupling reagent for racemization-free synthesis of peptide, amide, hydroxamate, and ester. *J Org Chem*. 2014;79(12):5420-5431. doi:10.1021/jo500292m
41. Hu L, Xu S, Zhao Z, et al. Ynamides as racemization-free coupling reagents for amide and peptide synthesis. *J Am Chem Soc*. 2016;138(40):13135-13138. doi:10.1021/jacs.6b07230
42. Pawlas J, Choi J-H, von Bargen C, Maibom-Thomsen S, Rasmussen JH, Ludemann-Hombourger O. Elevating 1-tert-butyl-3-ethylcarbodiimide (TBEC) as a reagent for sustainable peptide synthesis: quality assessment and minimizing racemization, precipitation, and radical-induced side reactions by TBEC/Oxyma couplings in an environmentally sensible solvent. *Org Process Res Dev*. 2023;27(7):1348-1364. doi:10.1021/acs.oprd.3c00120
43. Paradís-Bas M, Tulla-Puche J, Albericio F. The road to the synthesis of "difficult peptides". *Chem Soc Rev*. 2016;45:631-654. doi:10.1039/c5cs00680e
44. Bacsa B, Horváti K, Bõsze S, Andreae F, Kappe CO. Solid-phase synthesis of difficult peptide sequences at elevated temperatures: a critical comparison of microwave and conventional heating technologies. *J Org Chem*. 2008;73(19):7532-7542. doi:10.1021/jo8013897
45. Coin I, Beyermann M, Bienert M. Solid-phase peptide synthesis: from standard procedures to the synthesis of difficult sequences. *Nat Protoc*. 2007;2(12):3247-3256. doi:10.1038/nprot.2007.454
46. Kasim JK, Kavianinia I, Harris PWR, Brimble MA. Three decades of amyloid beta synthesis: challenges and advances. *Front Chem*. 2019;7:7. doi:10.3389/fchem.2019.00472
47. Lear S, Cobb SL. Pep-calc.com: a set of web utilities for the calculation of peptide and peptoid properties and automatic mass spectral peak assignment. *J Comput Aided Mol des*. 2016;30(3):271-277. doi:10.1007/s10822-016-9902-7
48. Reusche V, Thomas F. Effect of methionine sulfoxide on the synthesis and purification of aggregation-prone peptides. *ChemBioChem*. 2021;22(10):1779-1783. doi:10.1002/cbic.202000865
49. Traoré A, Guindo MA, Konaté D, et al. Seroreactivity of the severe acute respiratory syndrome coronavirus 2 recombinant S protein, receptor-binding domain, and its receptor-binding motif in COVID-19 patients and their cross-reactivity with pre-COVID-19 samples from malaria-endemic areas. *Front Immunol*. 2022;13:13. doi:10.3389/fimmu.2022.856033
50. Pratesi F, Errante F, Pacini L, et al. A SARS–CoV-2 spike receptor binding motif peptide induces anti-spike antibodies in mice and is recognized by COVID-19 patients. *Front Immunol*. 2022;13:879946. doi:10.3389/fimmu.2022.879946
51. Selmi C, Papini AM, Pugliese P, Alcaro MC, Gershwin ME. Environmental pathways to autoimmune diseases: the cases of primary biliary cirrhosis and multiple sclerosis. *Arch Med Sci*. 2011;3:368-380. doi:10.5114/aoms.2011.23398

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Pacini L, Muthyala M, Aguiar L, Zitterbart R, Rovero P, Papini AM. Optimization of peptide synthesis time and sustainability using novel eco-friendly binary solvent systems with induction heating on an automated peptide synthesizer. *J Pept Sci*. 2024;e3605. doi:10.1002/psc.3605