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Systematic and efficient method scale-up for peptide purification

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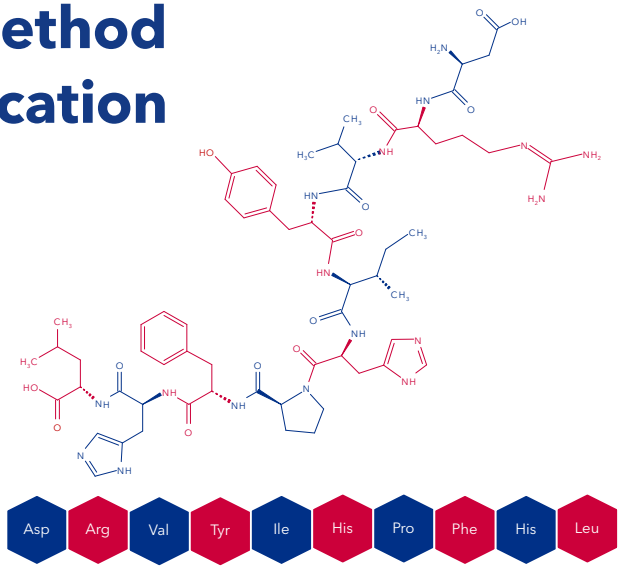

SUMMARY

Peptides, crucial in therapeutic advancements, are synthesized through refined methods in biotechnology. High-performance liquid chromatography (HPLC) plays a pivotal role, ensuring the purity of synthesized peptides. Linear scale-up of HPLC methods becomes imperative for enhancing productivity and method robustness in pharmaceutical and analytical laboratories. This process involves meticulous adjustments to parameters, such as column size and solvent composition, to maintain consistent chromatographic performance. Scaling up begins with optimizing the method on a larger scale, ensuring minimal sample loss while maximizing productivity.

INTRODUCTION

Peptides, composed of short chains of amino acids, bridge the gap between small organic molecules and large proteins. They offer advantages such as specificity, selectivity, and lower immunogenicity. Recent approvals of peptide-based drugs highlight their growing significance in therapeutic areas like metabolic disease, cardiovascular health, and oncology [1].

Peptide synthesis involves creating peptide bonds between amino acids to form peptides. Solid-phase and liquid-phase methods are commonly employed, with solid-phase synthesis, introduced by Merrifield, being prominent. This process has been refined through innovations in automation and chemistry [2].

High-performance liquid chromatography (HPLC) serves both analytical and purification purposes in peptide synthesis [3]. It is applied in tracking reaction progress, verifying intermediate identities, and confirming the purity of the final peptide [3]. Here, we show a HPLC method development and method scale-up for peptide purification.

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As model we selected Angiotensin I, a precursor in the renin-angiotensin system, vital in blood pressure regulation, maintaining electrolyte balance and vascular tone [4].
Scaling up HPLC methods necessitates linear scale-up, where adjustments to parameters like flow rate, column size, and solvent composition maintain proportional relationships. This ensures consistent chromatographic performance with increased sample volumes, crucial for pharmaceutical and analytical laboratories striving to enhance productivity while maintaining method robustness.
For the scale-up process an optimized analytical HPLC method is the first step. Initially, the particle size of the stationary phase is adjusted to preparative scale. The method is then adjusted to the new stationary phase with the later purification in mind. The next step before shifting to preparative scale is performing a volume overload study. These experiments give insight about the target substances elution behavior and possible coeluting impurities. At last, for purification a linear scale-up is performed. All evaluated method parameters are scaled to the desired preparative column dimension. Since the scale-up is linear, elution is predictable, and purification can start without losing significant amounts of sample for method evaluation.

SAMPLE PREPARATION

The crude Angiotensin I sample was commercially obtained (ProteoGenix SAS, Schiltigheim, France) as lyophilized powder with a nominal purity of 68.05 %. The crude was reconstituted in 25:75 acetonitrile (gradient grade)/distilled water (v:v). A solution with a concentration of 1 mg/ml was prepared. The solution was filtrated using a syringe filter with a 0.45 µm regenerated cellulose membrane (RC).

RESULTS

The crude peptide was measured using an analytical HPLC system **(see Fig. 1)**. A method with an overview gradient from 10 to 100 % organic eluent was applied. One large peak was identified as Angiotensin I. Several neighboring impurities were observed. The purity of the crude Angiotensin I was confirmed to be about 68.05 % (target peak area relative to impurities peak areas).
As a first step for scale-up of an analytical method, the particle size was increased from 5 to 10 µm **(see Fig. 2)**, to mitigate the increase in backpressure at higher flow rates in preparative scale.
A focused gradient was applied to increase resolution of Angiotensin I and the neighboring impurities **(see Fig. 3)**. The method was shortened, introducing a wash step with 100 % organic eluent into the gradient, followed by an equilibration step to starting conditions. This removed all late eluting impurities and shortened the overall method runtime.
With an optimized method a volume overload study was performed to gather insight of the target peaks elution behavior **(see Fig. 4)**. It was observed that above 50 µl injection volume, separation of impurities and target substance was poor. Therefore 50 µl was taken as calculation base for scale-up. Since all experiments were in analytical scale, sample loss could be kept minimal. The next step in the linear scale-up workflow was the increase of the column inner diameter (ID) from 4 to 20 mm. Using the KNAUER HPLC Method Converter, preparative scale method parameters were calculated **(see Fig. 5)** (**https://www.knauer.net/lc-method-converter**).
Finally, the method was transferred into preparative scale and to a preparative HPLC system. 2000 µl of crude sample with a concentration of 1 mg/ml were injected. The target peak eluted as predicted and was fractionated with the first run already. Fraction slices of 2.5 ml were chosen.
The target fractions were pooled and analyzed using the original analytical HPLC method confirming a purity of > 99 % **(see Fig. 6)**. That results in 1.4 mg of purified peptide per run.

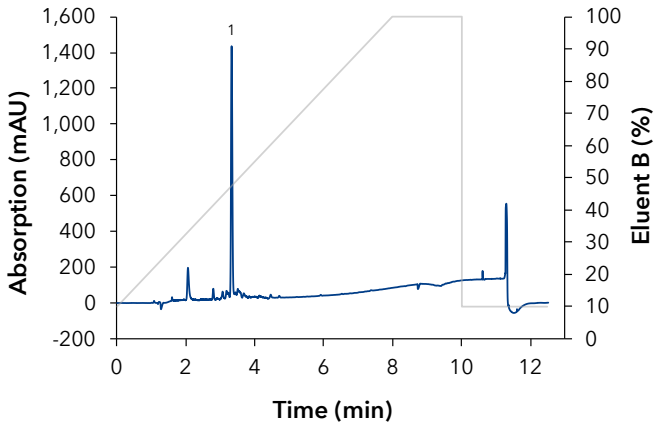

Fig. 1 Analytical overview gradient; (1) Angiotensin I

Overview Gradient

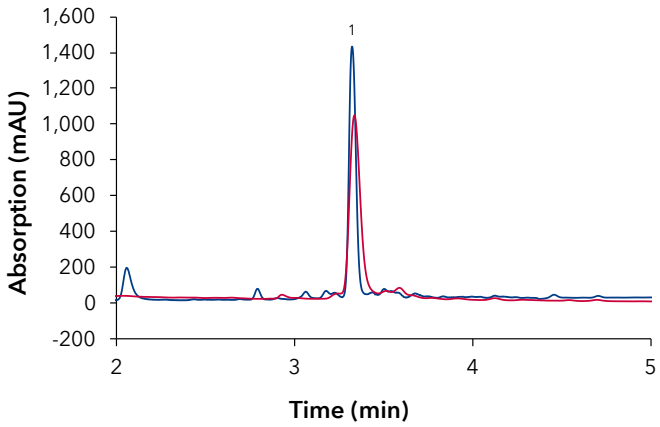

Fig. 2 Particle size comparison; blue: 5 µm; red: 10 µm

Particle Size Scaling

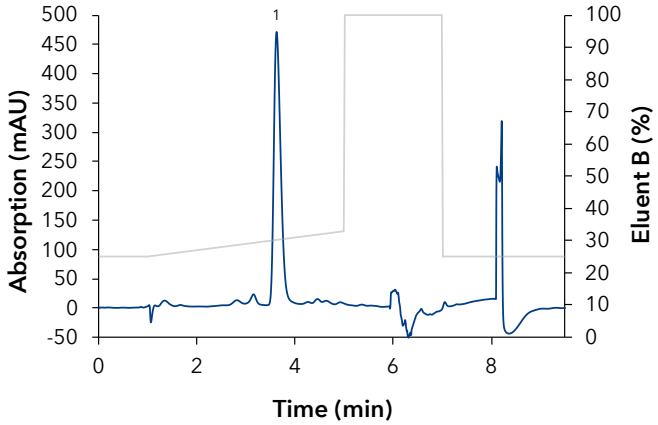

Fig. 3 Focused gradient and wash step

Focused Gradient

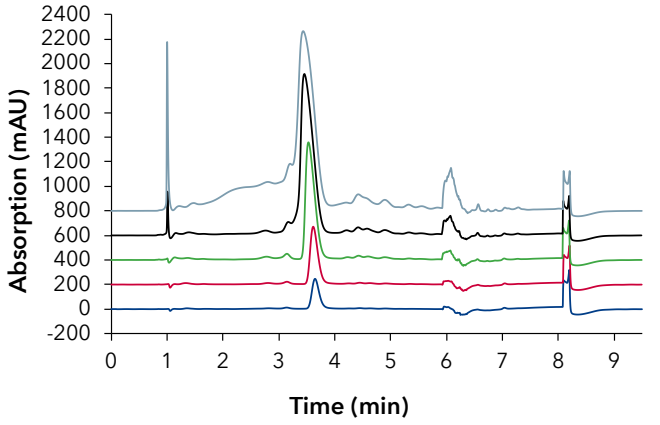

Fig. 4 Top to bottom: 100, 50, 25, 10, 5 µl injection volume

Analytical Scale Volume Overload

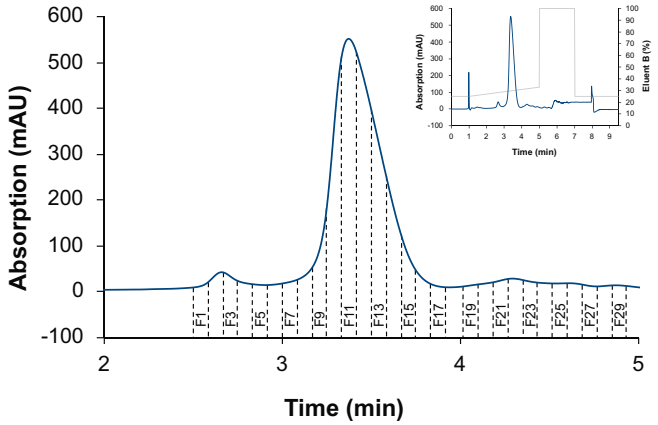

Fig. 5 Preparative run (zoomed); 2000 µl injection volume; 2.5 ml fractions

Linear Preparative Scale-UP

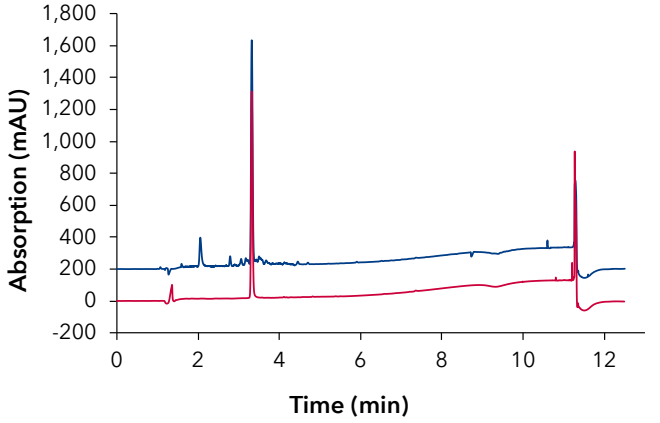

Fig. 6 Purity analysis; blue: 10 µl crude sample; red 50 µl pooled fractions 9 to 15

Pooled Fraction Purity Analysis

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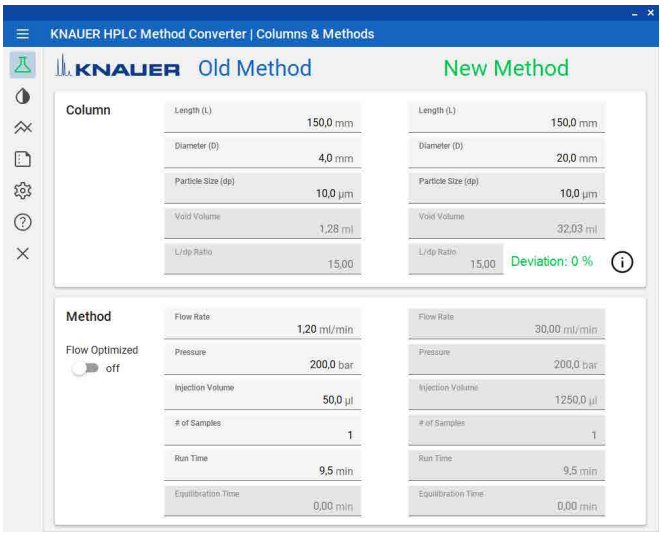

Fig. 7 Method scale-up with the "KNAUER HPLC Method Converter"

DOWNLOAD HPLC METHOD CONVERTER

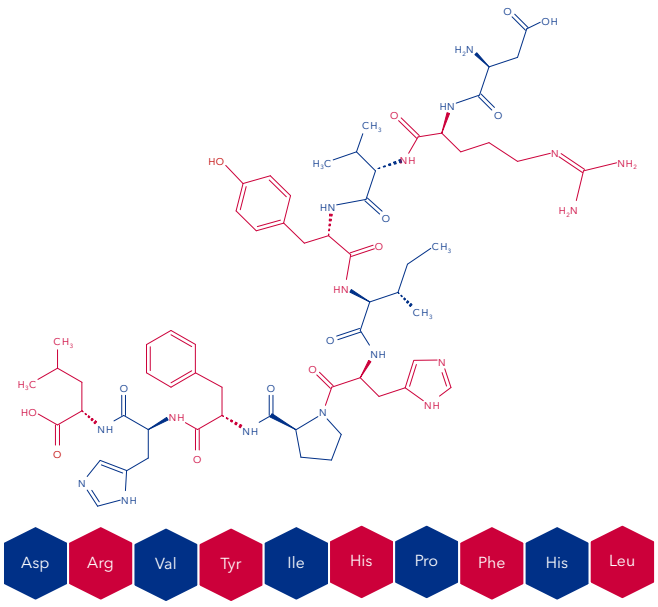

Fig. 8 Angiotensin Structure

CONCLUSION

In conclusion, the linear scale-up of the analytical HPLC method for purifying crude Angiotensin I proved effective in enhancing the purity of the target peptide. By systematically increasing the particle size and adjusting method parameters, such as the gradient and injection volume, the method was optimized for preparative scale. The successful transfer to a preparative HPLC system, guided by linear scale-up principles, enabled efficient fractionation of the target peptide with high purity (> 99 %) achieved in the pooled fractions.
This underscores the importance of linear scale-up in maintaining method robustness and ensuring the successful purification of peptides at a larger scale.

MATERIAL AND METHODS

Tab. 1 Overview Gradient

Parameter	Settings	
Runtime	12.5 min	
Flowrate	1.2 ml/min	
Eluent A	Distilled Water + 0.1 % TFA	
Eluent B	Acetonitrile + 0. 1% TFA	
Solvent Composition	0 min	10 % B
	8 min	100 % B
	10 min	100 % B
	10.02 min	10 % B
	12.5 min	10 % B
Injection mode	Partial loop	
Injection volume	10 µl	
Detection mode	UV	
Wavelength	220 nm	
Data rate	10 Hz	
Column	Eurospher II 100-5 C18 150 x 4 mm	For all analytical measurements
	Eurospher II 100-10 C18 150 x 4 mm	For particle scale-up experiments
Column temperature	25 °C	
Auxiliary Channel	Pressure trace	

Tab. 2 Focused Gradient

Parameter	Settings	
Runtime	9.5 min	
Flowrate	1.2 ml/min	
Eluent A	Distilled Water + 0.1 % TFA	
Eluent B	Acetonitrile + 0.1 % TFA	
Solvent Composition	0 min	25 % B
	1 min	25 % B
	5 min	33 % B
	5.02 min	100 % B
	7 min	100 % B
	7.02 min	25 % B
	9.5 min	25 % B
Injection mode	Partial loop	For 5 to 50 µl injection volume
	Full loop	For 100 µl injection volume
Injection volume	5, 10, 25, 50, 100 µl	variable for overload study
Detection mode	UV	
Wavelength	220 nm	
Data rate	10 Hz	
Column	Eurospher II 100-10 C18 150 x 4 mm	
Column temperature	25 °C	
Auxiliary Channel	Pressure trace	

Tab. 3 Preparative Method

Parameter	Settings	
Runtime	9.5 min	
Flowrate	30 ml/min	
Eluent A	Distilled Water + 0.1 % TFA	
Eluent B	Acetonitrile + 0.1 % TFA	
Solvent Composition	0 min	25 % B
	1 min	25 % B
	5 min	33 % B
	5.02 min	100 % B
	7 min	100 % B
	7.02 min	25 % B
	9.5 min	25 % B
Injection mode	Full loop	
Injection volume	2000 µl	
Injection valve	0 min	Position: Load
	0.02 min	Position: Inject
	9.48 min	Position: Load
Detection mode	UV	
Wavelength	220 nm	
Data rate	2 Hz	
Column	Eurospher II 100-10 C18 150 x 4 mm	
Column temperature	ambient	
Auxiliary Channel	Pressure trace	
Fraction volume limiter	2.5 ml	
Fraction Valve	0 min	Position: Waste
	2.5 min	Position: Fraction
	5 min	Position: Waste

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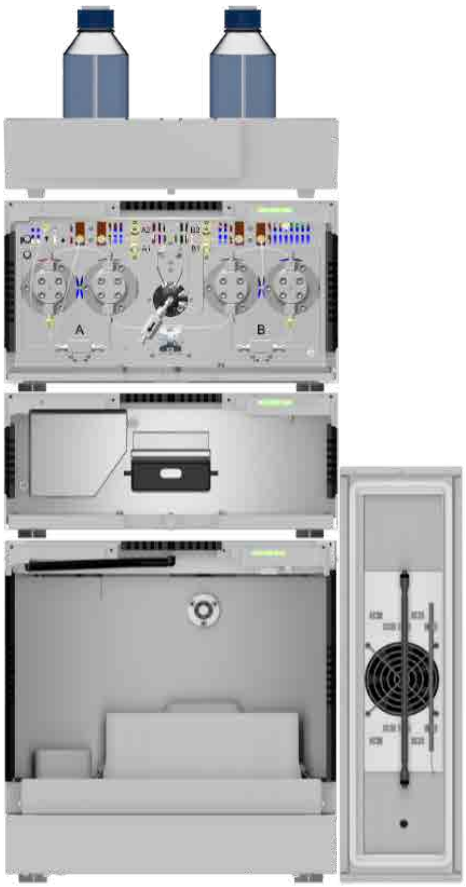
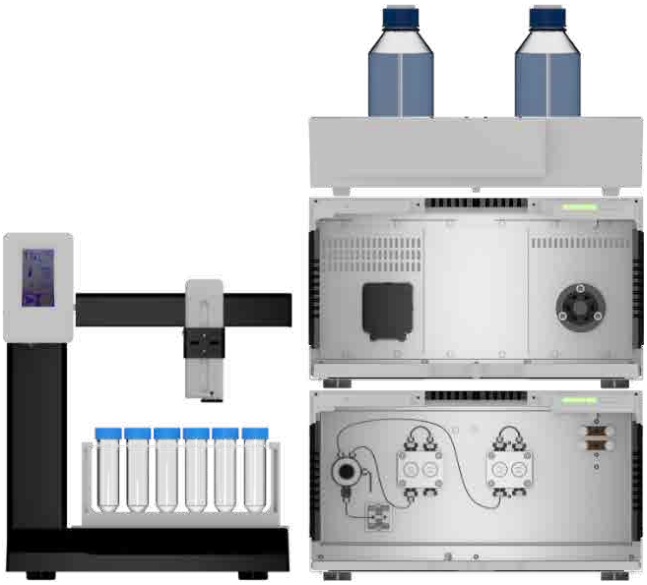
Tab. 4 AZURA® analytical HPLC System

Instrument	Description	Article No.
Eluent tray	AZURA Eluent tray E 2.1L	**ACZ00**
Pump	AZURA P 8.1L	**APF45PA**
Autosampler	AZURA AS 6.1L	**AAA10AA**
Column thermostat	AZURA CT 2.1	**ATC00**
Detector	AZURA DAD 2.1L	**ADC01**
Measuring cell	Standard KNAUER LightGuide UV Flow Cell Cartridge 10 mm path length, 1/16", 2 µl volume, 50 bar	**AMC19XA**
Capiliaries	AZURA Analytical K-Connect start-up kit, stainless steel, 1/32" capillaries with fitting sleeves for 1/16"connections 0.1 mm ID	**AZF40**
Mixer	HPLC eluent mixer, stainless steel, 200 µl	**AZZ00MD**
Column 1	Eurospher II 100-5 C18 150 x 4 mm	**15WE181E2J**
Column 2	Eurospher II 100-10 C18 150 x 4 mm	**15DE181E2J**
Software	ClarityChrom® 9.0 Workstation, autosampler control included	**A1670**
Software	ClarityChrom® 9.0 PDA extension	**A1670**

Tab. 5 AZURA semi-preparative System

Instrument	Description	Article No.
Eluent tray	AZURA Eluent tray E 2.1L	**ACZ00**
Pump	AZURA P 6.1L High Pressure Pump with 50 ml pump head, stainless steel, without Degasser	**APH38FA**
Assistant	AZURA Assistant ASM 2.2L	**AY01403**
	Left module: UV detector UVD 2.1S	**EDA03XA**
	Middle module: Empty module	
	Right module: Valve drive VU 4.1	**EWA04**
Valve	AZURA V 4.1 Valve 2-position valve with 6 ports, 1/16"	**AVD26AE**
Flow Cell	Semi-preparative UV Flow Cell 3 mm path length, 1/16", 2 µl volume, 300 bar, stainless steel	**A4042**
Fraction Collector	Fraction Collector Foxy R1	**A59100**
Mixer	HPLC eluent mixer, stainless steel, 600 µl	**AZZ00MF**
Column	Eurospher II 100-10 C18 150 x 20 mm	**15JE181E2N**
Software	PurityChrom® 6 Basic License	**A2680**





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