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Gradient Retention Factor Concept Applied to Method Development for Peptide Analysis by Means of RP-HPLC

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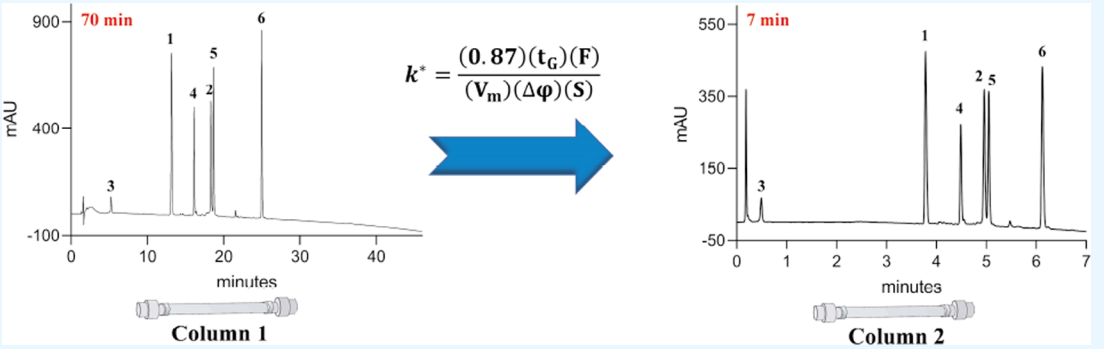

ABSTRACT: Using the van Deemter model, the efficiency of three stationary phase systems in the analysis of a mixture of synthetic peptides was evaluated: (i) monolithic, (ii) packed, and (iii) core−shell columns, and it was shown that the efficiency of the monolithic column is superior to the others, specifically using it, the lowest values of H_{min} (0.03 and 0.1 mm) were obtained, and additionally its efficiency was not significantly affected by increasing the flow. Using the concept of the gradient retention factor (k^*), a method for chromatographic separation of a peptide complex mixture was designed, implemented, and optimized and then transferred from a packed column to a monolithic one. The results showed that it was possible to separate all components of the mixture using both evaluated columns; moreover, the analysis time was reduced from 70 to 10 min, conserving the critical pair resolution (1.4), by the transfer method using the k^* concept. The method developed was tested against a mixture of doping peptides, showing that this method is efficient for separating peptides of various natures. This investigation is very useful for the development of methods for the analysis of complex peptide mixtures since it provides a systematic approach that can be extrapolated to different types of columns and instrumentation.

1. INTRODUCTION

Peptides are a family of molecules involved in many biological processes, and their diversity is mainly due to their primary structure and structural conformation.[1] Peptides have great advantages as drugs because they have unique therapeutic properties, are usually harmless, and act on specific targets of physiological processes.[2] Peptides that fulfill functions of regulation, modulation, and/or activation of the immune system, transport, biomarkers, and antimicrobial and anticancer activities, among others, have been described.[3,4] Recent advances in peptide therapeutics include progress from synthetic methods and in the use of naturally occurring peptides, which have intrinsic weaknesses, to a sophisticated system of drug development based on drug discovery and design, peptide synthesis, and structural modification for activity evaluation.[4−7]

Reversed-phase high performance liquid chromatography (RP-HPLC) is a powerful and versatile technique for the monitoring, characterization, and/or purification of complex peptide mixtures from synthetic processes or protein hydrolysates in biotic and abiotic materials.[1,8−11] General methods reported for peptide analysis include the use of silica-based C18 columns and ACN/H_2O gradient elution using TFA as an ion pairing agent.[3,4] However, the analysis of complex peptide mixtures by means of RP-HPLC is challenging since it is a procedure that requires a lot of time and involves a high

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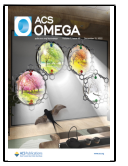

consumption of solvents, which increases both costs and the amount of waste, with high environmental impact.[8,9,11] RP-HPLC methods used for peptides usually have run times longer than 30 min.[8,12,13] In RP-HPLC, the separation of analytes is mediated by partitioning and/or adsorption phenomena, in which the hydrophobic part of the peptide interacts with the hydrocarbon chain of the stationary phase.[14] The wide variety of stationary phases available on the market has provided tools for developing methods that lead to better separation and reduce analysis time. The van Deemter equation makes it possible to determine the efficiency of a chromatographic system, which depends mainly on the chromatographic column, the flow of the mobile phase, and the physicochemical properties of the analyte. In this model, the chromatographic efficiency is given by eq 1, where height of the theoretical plate (HETP) is the height equivalent theoretical plate; A, B, and C are constants related to the eddy diffusion coefficient, longitudinal diffusion, and mass transfer resistance, respectively; and u is the linear velocity. The diffusion coefficient of Eddy (A) is constant, unaffected by the flow of the mobile phase and depends on the particle size and uniformity of the column packing. The term B/u represents the longitudinal diffusion of the analyte, and its contribution to HETP decreases as the flow of the mobile phase increases, while that the contribution of mass transfer resistance (Cu) to HETP increases as the mobile phase flow increases.[15]

$$\mathrm{HETP} = A + \frac{B}{u} + Cu \tag{1}$$

This equation allows the calculation of the optimal flow of the mobile phase required to achieve the highest efficiency (lowest HETP value) of the column for a given analysis.

Analytical methods for the separation of small molecules have been reported in the literature, and interactions of analytes with stationary phases have been studied.[16] However, in the case of peptides, sometimes a method must be developed for a specific peptide, so that there are few reports of methods for the separation of complex mixtures of peptides that can be applied routinely. Chromatographic methods for peptides are often designed and optimized based on previous experience.[17] However, this strategy consumes a lot of time and resources, which is undesirable in the design and development of chromatographic methods applied to the resolution of complex peptide mixtures. The design, implementation, and transfer of a chromatographic method is a complex process that requires consideration of factors such as the physicochemical properties of peptides (primary structure, solubility, molecular weight, length, hydrophobicity, amphipathicity, basicity, acidity, etc.), column properties (particle length, diameter and porosity, type of silica, etc.), stationary phase nature (C4, C8, C18, phenyl, cyanide, etc.), elution system (isocratic, linear gradient, segmented gradient, etc.), mobile phase composition (water/ACN, water/MeOH, buffer, etc.), counterion (TFA, FA, etc.), temperature, and injection volume, among others. Therefore, the design, implementation, optimization, and transfer of a chromatographic method to analyze peptide mixtures is a valuable tool for identifying and/or quantifying peptides in complex matrices. Other options, such as in silico prediction using computational models, could minimize the number of experiments and speed up method development. However, so far this tool has been used primarily to predict the t_R of the analytes in a given chromatographic system.[17] The analysis of peptides and proteins using conventional HPLC equipment is not a simple task, mainly due to the heterogeneity of the mixture because of the physicochemical properties and concentration of the molecules that make it up.[18] In addition, optimization of HPLC methods for peptide analysis aims to reduce the analysis time and improve or maintain resolution. When the mixture is heterogeneous, gradient elution is generally the best option. RP-HPLC gradient elution is based on changing the composition of the mobile phase as the run passes, usually increasing the concentration of the organic phase (solvent B: ACN, MeOH, THF, etc.). Gradient elution is defined by several parameters, such as gradient time (t_G), initial and final % B, linear or stepped gradient, etc. With this in mind, the gradient retention factor (k^*) becomes a great tool for optimizing the chromatographic run and transferring methods among different chromatographic systems, maintaining the selectivity profile.

2. MATERIALS AND METHODS

2.1. Reagents and Materials. Rink amide resin, Fmoc-amino acids, Triton-X, piperidine, *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), dicyclohexylcarbodiimide (DCC), 1-hydroxy-6-chloro-benzotriazole (6-Cl-HOBt), ninhydrin, potassium cyanide (KCN), ethanol, pyridine, phenol, trifluoroacetic acid (TFA), ethyl ether, triisopropylsilane (TIS), ethanedithiol (EDT), acetonitrile (ACN), methanol (MeOH), and Supelco solid-phase extraction columns were purchased from Sigma-Aldrich (St. Louis, MO). Fmoc-Dip-OH was obtained from AAPPTec (Louisville, KY). A Zorbax Eclipse XDB-C18 column was purchased from Agilent Technologies (California), a SunShell C18 column was purchased from ChromaNik Technologies Inc (Osaka, Japan), and a Chromolith High Resolution RP-18e column was purchased from Merk (New Jersey).

2.2. Peptide Synthesis (SPPS-Fmoc/*t*Bu). The peptides were obtained by means of manual solid-phase peptide synthesis, using the Fmoc/*t*Bu strategy (SPPS-Fmoc/*t*Bu) following previous reports.[19] Briefly, Rink amide resin or Fmoc-amino acids anchored to solid support were treated with 5% piperidine in DMF for 10 min at RT (2×) to remove the Fmoc group. The Fmoc-amino acids were preactivated by mixing the amino acid with DCC/6-Cl-HOBt (1/1 equiv and 5 excesses with respect to resin substitution), and then, the preactivated Fmoc-amino acid was mixed with the resin or resin peptide for 2 h at RT. Once all of the amino acids were incorporated, the peptide was separated from the solid support by treating the resin peptide with TFA/H_2O/TIS/EDT (92.5/2.5/2.5/2.5% w/w) for 8 h at RT. Finally, the reaction mixture was filtered and the solution was treated with ethyl ether to precipitate the peptide, and then, the solid was washed five times with ethyl ether.

2.3. RP-SPE Purification. The peptides were purified by means of solid-phase extraction in reverse phase mode (RP-SPE) Supelclean C18 columns (5 g, 45 μm, 60 Å)[1]. Briefly, a RP-HPLC analysis of the crude peptide was performed, and based on this result, using a mathematical model, the elution program was transferred to a RP-SPE. The peptide was loaded into the column and eluted by increasing the concentration of solvent B (TFA 0.05% in ACN), and the fractions containing the pure peptide were collected and lyophilized.

2.4. Chromatographic Conditions. The peptides (1 mg/mL) were dissolved in solvent 95% A (TFA 0.05% in water) and 5% B (TFA 0,05 in ACN), and a 10 μL sample was

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injected into an Ultimate 3000 HPLC (Thermo Scientific, Massachusetts) with a binary pump of up to 620 bar (9000 psi) capacity, a column oven compartment, and a diode array detector. For gradient analyses, the composition of B and the workflow varied according to the assay. For the isocratic analysis, a mixture of H_2O−ACN (82:18) containing TFA 0.05% was used. Two channels (channel 1:210 nm, channel 2:280 nm) were used as working wavelengths. The column temperature was kept at 30 °C, and the temperature of the samples was maintained at 15 °C for all of the tests.

3. RESULTS AND DISCUSSION

3.1. Van Deemter Model. Chromatographic methods for separating peptide mixtures were designed and implemented, and their performance was evaluated in three chromatographic columns (packed, monolithic, and core−shell), which are described in Table 1. The columns were functionalized with C18 chains, so the hydrophobicity (H) value was quite similar; however, the stationary phase technology of each of them differs in pore size, steric resistance (*S**), and other parameters, such as *H-B* basicity (*B*) and ion-exchange capacity (*C*). Considering that these analytes are charged and their interaction with them is structurally complex, the stationary phase is not ruled only by *H*.

To evaluate the efficiency of the columns, the van Deemter model was used. This model makes it possible to determine the equivalent height of the theoretical plate (HETP) for each column and to determine for which of them the analytical method could perform best. In addition, it allows one to determine which diffuse phenomena are contributing the most to the loss of efficiency of the system and how it affects the optimal workflow.[15] Two synthetic peptides were selected for the columns' evaluation; they are derived from bovine lactoferricin (LfcinB): (peptide 1), a peptide of six residues, RRWQWR (MW: 986,132 g/mol), and (peptide 2), a branched peptide of 24 residues, specifically the dimer $(RRWQWRFKKLG)_2$-K-Ahx (MW: 3441, 964 g/mol). Peptide 2 is heavier and more voluminous than peptide 1. The two peptides have quite different physicochemical properties and were used to make the mixture more complex.

To obtain the van Deemter curve experimentally, a solution containing $NaNO_3$ (3.00 mg/mL), peptide 1 (0.67 mg/mL), and peptide 2 (0.72 mg/mL) was prepared using the mobile phase H_2O/ACN as a solvent (82:18 v/v) containing 0.05% v/v TFA. A chromatographic separation of the mixture was performed using isocratic elution, injecting 10 μL of the solution, and the working wavelength was 210 nm. Successive chromatographic runs were performed while varying the flow from 0.02 to 4 mL/min. The maximum pressure allowed for each column was 250 bar (2900 psi), so that the runs could be replicated on any conventional HPLC. It is important to note that to apply the van Deemter model to high molecular weight analytes such as peptides, it is necessary to use rather low flows (0.02 mL/min) to estimate the contribution of the term B to the height of the theoretical plate. Another important point to bear in mind is that the American (USP) and European (EP) pharmacopoeias recommend determining the number of theoretical plates by measuring the peak width at 4σ and 2.35σ. However, in isocratic peptide runs, due to their structural complexity, the peaks are usually not symmetrical, so the results do not ideally fit the van Deemter model. For this project, the number of theoretical plates was determined using the statistical moment, which is a nonlinear fit that works very well for nonsymmetrical Gaussian peaks.[20]

The obtained results for the van Deemter model for each peptide in the three evaluated columns can be seen in Figure 1. It is evident that the three columns present high efficiency for both analytes, with optimal workflows close to or below 12 mm/s (0.1 mL/min) and theoretical plate values below 0.4 mm. As can be seen, the monolithic column performed best for both analytes since the height of the HETP did not show a significant variation in the workflows evaluated, and HETP was the lowest, which implies a greater number of theoretical plates, thus representing a greater efficiency. This column has

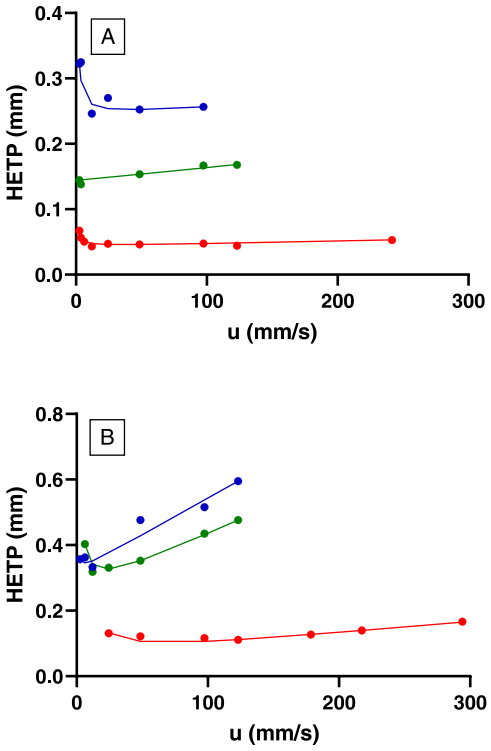

Figure 1. Van Deemter plots for (A) peptide 1 (RRWQWR) and (B) peptide 2 ($(RRWQWRFKKLG)_2$-K-Ahx). Blue (Acclaim packed column), green (SunShell C18 core−shell column), red (Chromolith RP monolithic column).

Table 1. Parameters and Characteristics of Columns and Their Stationary Phases[a]

column	Sunshell C18	Eclipse XDB-C18	Chromolith RP-18e
length (mm)	500	500	500
pore size (Å)	90.0	80.0	150
particle size (μm)	2,60	5.00	
H	1.09	1.07	0.99
*S**	0.028	0.020	0.019
A	−0.12	−0.060	0.017
B	−0.056	−0.030	−0.001
C (2,8)	−0.43	0.050	0.21
C (7,0)	−0.84	0.080	0.28
description	core−shell	packed	monolithic

[a]Key selectivity parameters reported in the web platform https://www.hplccolumns.org: *H* (column hydrophobicity), *S** (column steric resistance), *A* (column hydrogen-bond acidity), *B* (column hydrogen-bond basicity), *C* (2,8) (column cation exchange capacity at pH 2,8), *C* (7,0) (column cation exchange capacity at pH 7.0).

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Table 2. Experimental Van Deemter Parameters Obtained to Determine Column Efficiency for Peptides 1 and 2

peptide N°: sequence		Chromolith	SunShell	Acclaim
1: RRWQWR	A (mm)	0.043	0.14	0.24
	B (mm/s)	0.049	0.0011	0.20
	C (mm × s)	4.11×10^{-5}	2.0×10^{-4}	1.2×10^{-4}
	u_{opt} (mm/s)	34.74	2.74	40.59
	H_{min} (mm)	0.046	0.14	0.25
	R^{2a}	0.96	0.97	0.95
2: $(RRWQWRFKKLG)_2$-K-Ahx	A (mm)	0.053	0.24	0.32
	B (mm/s)	1.71	0.90	0.087
	C (mm × s)	0.0036	0.0019	0.002
	u_{opt} (mm/s)	68.88	0.24	6.24
	H_{min} (mm)	0.10	0.32	0.34
	R^{2a}	0.95	0.99	0.97

[a]Pearson's correlation coefficient between the experimental values and the theoretical model.

Table 3. Physicochemical Properties of Synthesized Peptides[a]

sequence	code	MW (g/mol)	SAscore	log S(g/L)	log P(g/L)
RRWQWR	**1**	984.55	5.15	−0.35	0.25
$(RRWQWRFKKLG)_2$K-*Ahx*	**2**	3341.97	9.42	1.69	5.01
KQNLAEG	**3**	757.41	4.36	1.25	−1.09
RRWQWRFKKLG	**4**	1558.91	6.59	0.22	3.88
RRWQWRWQWR	**5**	1641.87	6.76	−0.81	2.96
RWQW-*Dip*-WQWR	**6**	1552.76	6.52	−0.56	5.60
CYFQNCPRG	**A**	1086.50	5.23	−2.62	−1.45
Mrp-YFQNCP^{d}RG	**B**	1071.44	6.15	−1.81	−0.41
H^{d}WAW^{d}FK	**C**	873.41	3.63	−3.48	2.01
^{d}WAW^{d}F	**D**	607.29	4.68	−2.84	2.63

[a]MW: molecular weight; S: aqueous solubility value; P: n-octanol/water distribution coefficient; SAscore: synthetic accessibility score.

Table 4. Variables of Equation 2 of the Chromatographic Methods Evaluated

column	method	ΔΦ	F (mL/min)	t_G (min)	t_GF	R_s[a]	P (Bar)
XDB-C18	I	0.65	1	45	45	1.47	144
	II A		1	30		1.48	144
Chromolith	II B	0.43	1	30	30	1.46	48
	III		2	15		1.46	74
	IV		3	10		1.49	110
	V		5	6		1.44	183

[a]R_s calculated only for the critical pair corresponding to peptides 2 and 5.

the highest porosity, so it is possible to increase the workflow up to 4.00 mL/min (297 mm/s) without exceeding a pressure of 250 bar (2500 psi); this allows transfer of the method, without loss of efficiency, to conventional HPLC equipment, which usually has maximum pressure limits between 300 and 400 bar.

The core−shell column showed greater efficiency than the packed column for peptide 1, while both columns showed similar efficiency with peptide 2, suggesting that the efficiency of the core−shell column depends on the molecular weight and conformational structure of the peptide (Table 2).

The van Deemter parameters showed higher values for peptide 2 in the three evaluated columns; for peptide 1, a decrease in the H_{min} value was observed, suggesting that the efficiency is possibly affected by the physicochemical properties of the peptide, such as molecular weight, load, and hydrophobicity, among others. The results show that the value of term A had the greatest variation in the three columns for both analytes. This behavior is in accordance with the fact that term A corresponds to the Eddy diffusion coefficient and that the main difference in the columns is the nature of the solid support and its functionalization. The monolithic column showed lower A values (4 to 6 times, compared to the other columns). This can be attributed mainly to the stationary phase, which consists of a monolithic structure containing macropores and micropores that allows the analytes to interact with the stationary phase more efficiently in the evaluated flow range.

3.2. Chromatographic Method Development Using the *k Concept.** From previous results, the monolithic column was selected to develop the optimized chromatographic method for the analysis of peptide mixtures. Considering the heterogeneity of the analytes present in a mixture of peptides, the first step is to define whether the elution should be isocratic or gradient. Normally, for the development of methods in isocratic analysis, a retention factor (k) between 2 and 10 is desired, and on this premise, the method is optimized. It is advisable to use gradient elution to solve complex peptide mixtures since chromatographic methods are fast and only one chromatographic run is

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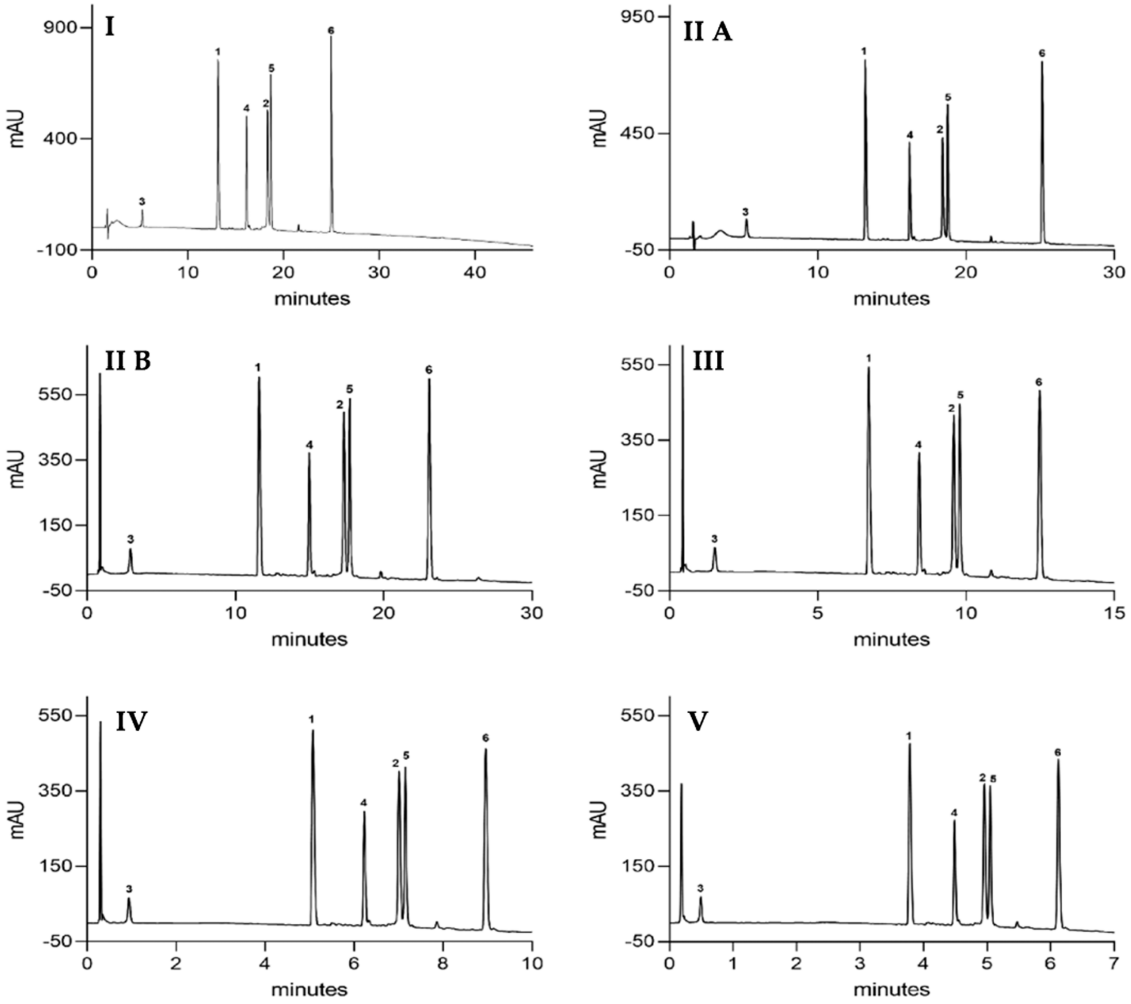

Figure 2. Chromatographic profiles of the methods developed for the analysis of the mixture of synthetic peptides, **1:** RRWQWR, **2:** $(RRWQWRFKKLG)_2$K-*Ahx*, **3:** KQNLAEG, **4:** RRWQWRFKKLG, **5:** RRWQWRWQWR, and **6:** RWQW-*Dip*-WQWR. I–V correspond to the methods described in Table 4.

Table 5. Peptide Retention Times for Each Chromatographic Method

	retention time t_R (min)					
method	3	1	4	2	5	6
I	5.2	13.1	16.1	18.4	18.6	25.2
II	5.2	13.2	16.3	18.5	18.7	25.2
III	1.4	6.6	8.4	9.6	9.8	12.5
IV	0.6	4.3	5.2	5.7	5.8	7.2
V	0.5	3.8	4.5	4.9	5.1	6.1

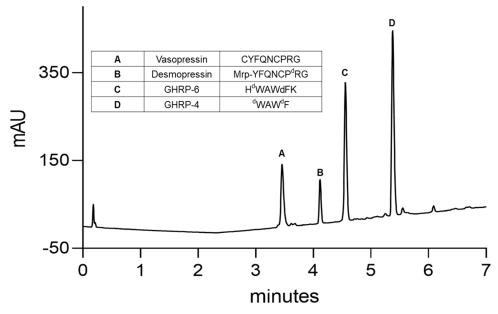

Figure 3. RP-HPLC analysis of a complex mixture of four peptides used in doping, using optimized method V.

required. Another advantage is that the efficiency of the system is not affected because the composition of the mobile phase changes during the run, so k also changes, preventing the broadening of the peaks of all analytes. For this reason, to develop the method, it is necessary to address the concept of gradient retention factor k^*, given by eq 2.[21]

$$k^* = \frac{(0.87)(t_G)(F)}{(V_m)(\Delta\varphi)(S)} \quad (2)$$

Here, t_G is the gradient run time (expressed in minutes), F is the flow rate (expressed in mL /min), V_m is the column void volume (expressed in mL), $\Delta\varphi$ is the gradient range, and S is a constant value that depends on the analyte's molecular weight. By manipulating the variables of eq 2, keeping constant the value of k^*, it is possible to transfer methods to chromatographic systems with different columns to optimize the analysis time without compromising the resolution of the critical peak pair.

Theoretically, if the variables of eq 2 are changed while keeping the value of k^* unchanged, the resolution for most analytes should not change significantly; on the other hand, if the value of k^* is increased, the resolution will also increase. Using eq 2, we previously optimized a routine chromatographic method for peptide analysis by RP-HPLC.[24] In this

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Table 6. Comparative Chart between the Method Optimized in This Research (Method V) and Other Recently Reported Methods for Peptide Analysis by Conventional HPLC

column	Δ%B (gradient)	chromatographic conditions	analysis time (min)	refs
XBridge BEH130 C18 (10 × 150 mm)	0−90% B, ACN 0.1%, TFA	HPLC (Waters Company), 5 mL/min Detection at 215 nm	50	Wen et al.[28]
Shim-pack Inertsil ODS−SP (4.6 × 250 mm, 5 μm)	0−100% B, A (0.1% TFA in H_2O), B (0.1% TFA in ACN)	HPLC Waters 2695, 1 mL/min, Sephadex G-15 gel chromatography	40	Ma et al.[29]
C4 and C18 columns	linear gradients, of 0.1% TFA in ACN	HPLC using C4 and C18 columns	40	Laxman et al.[30]
XBridge OST C18 (5 μm, 10 mm × 250 mm)	0−50% B, A (0.1% TFA in H_2O), B (0.1% TFA in 80% ACN)	HPLC waters 2695, 5 mL/min, three-step gradient	70	Kim et al.[31]
Agilent Eclipse XDB-C18 (4.6 × 150 mm, 3.5 μm)	5−70% B, A (0.05% TFA in H_2O), B (0.05% TFA in ACN)	Agilent 1200 HPLC, 1.0 mL/min	70	León-Calvijo et al.[24]
Chromolith high resolution RP-18e (4.6 × 50 mm)	5−48% B, A (0.05% TFA in H_2O), B (0.05% TFA in ACN)	ultimate 3000 HPLC (Thermo Scientific) flow: 5 mL/min	7	current method

method, H_2O containing TFA (0.05% v/v) was used as solvent A and ACN containing TFA (0.05% v/v) as solvent B. The elution program was a linear gradient from 5 to 70% B in 45 min. This method has worked very well for the analysis of a wide variety of synthetic peptides; however, its biggest disadvantages are the analysis time (70 min) and that it does not adequately solve complex peptide mixtures. The aim of this investigation was to adapt this method to the analysis of peptide mixtures, reducing the analysis time and having the resolution of the critical pair close to or greater than 1.5.

Our results using isocratic elution indicated that differences in the physicochemical properties of peptides 1 and 2 significantly influenced the efficiency of the system. Therefore, a complex mixture of peptides was designed as a model to develop the chromatographic method by gradient elution: peptides 1 and 2 and four synthetic peptides were chosen, which differed in terms of length, structure, volume, and hydrophobicity, among other factors. In Table 3, the peptide sequences and its properties are presented. The peptide sequences were modeled on the ADMETlab 2.0 platform (https://admetmesh.scbdd.com/) to establish some synthetic and physicochemical properties of each peptide. The parameters selected for the characterization in silico of each peptide in this study were molecular weight (MW), synthetic accessibility score (SAscore), aqueous solubility value (S), and n-octanol/water distribution coefficient (P). The parameters MW and SAscore give information on the size and shape of the molecule, which are related to the conformation and structural complexity, while the parameters S and P give information on amphipathic, lipophilic, and hydrophilic properties.

The mixture consisted of five monomeric peptides and one dimeric peptide; the molecular weight range of the peptides was wide and ranged from 757. 41 to 3341. 97 g/mol. In addition, the SAscore range was between 4.36 and 9.42, which indicates the wide variability in the complexity of the molecules and their synthetic ease/difficulty; e.g. the SAscore value for dimeric peptide (**2**) is 9.42, while for its monomeric analogue (**4**), the value is 6.59, indicating the greatest synthetic difficulty for the dimeric peptide. In addition, peptides (**2**) and (**6**) contain the unnatural amino acids Ahx (6-aminohexanoic acid) and Dip (diphenylalanine), respectively, which confer hydrophobicity to these sequences. The log S and log P values of the six peptides indicate that these molecules have significant differences in their hydrophobic and hydrophilic properties; e.g., peptide (**1**) has the lowest hydrophobicity (P = 0.08 g/L), while peptide (**6**) has the highest hydrophobicity (P = 395 461 g/L), the difference in the P value of these peptides being 6 orders of magnitude.

The parameters of eq 2 used for the design and development of the gradient elution methods can be seen in Table 4 and Figure 1. Initially, the mixture was analyzed under the conditions of method I, previously reported,[24] using the Agilent Zorbax XDB-C18 packed column (150 × 4.6 mm; particle size 3 μm). As can be seen, the chromatographic profile shows six peaks, which correspond to the six peptides of the mixture, where it is possible to distinguish the critical peak pair between peptides (**2**) and (**5**), with a resolution (R_s) of 1.47 (Figure 2.I). The chromatographic profile shows that after 30 min, no signals were observed, so the next step was to optimize the method by changing the value of ΔΦ but maintaining the gradient slope, thus reducing the analysis time without affecting R_s (method II A). R_s was used as a comparison parameter for each method and to determine whether the resolution was maintained or improved, especially that of the critical peak pair, and selectivity was also evaluated.

The chromatographic profile (Figure II A) shows that methods I and II B resolve the mixture in the same way, the R_s of the critical pair being practically the same. However, to continue optimizing the method, the workflow rate must be increased, which is not recommended because the pressure reached with method II A is 144 bar (2080 psi). So, the next step was to transfer method II A to the monolithic column, which allows increasing the workflow; as can be seen, the chromatographic profile of method II B (Figure II B) is the same as that observed for method II A, and the R_s of the critical pair was 1.46.

According to eq 2, if the pressure of the system does not increase significantly, we can increase the flow rate (F) and decrease the time of the gradient (t_G), so that the product t_GF remains constant. With this strategy, we reduce the time of analysis without decreasing the R_s since the numerator of the equation remains constant and therefore k^* as well. Methods III (2 mL/min;74 Bar), IV (3 mL/min;110 Bar), and V (5 mL/min;183 Bar) were designed to progressively increase the workflow up to 5 mL/min. The bimodal porosity of the stationary phase of the monolithic column allows increasing the flow without significantly increasing the pressure of the system.[14] As can be seen in Figure 2 and Table 5, as the flow increased, the analysis time decreased: method II required an analysis time of 12.5 min; method IV, 7.2 min; and method V, 6.1 min. In this way, five chromatographic methods were designed and optimized for the separation of a complex mixture of peptides. The results show that it was possible to reduce the analysis time from 70 min (initial method) to 25 min (methods I and II A) using a packed column. In addition, the method was transferred from a packaged column to a

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monolithic column (method II B), and from these three, methods were designed whose analysis times were 12.5 (method III), 7.2 (method IV), and 6. 1 min (method V). The chromatographic profile observed for all methods was similar without affecting the resolution of the critical pair. Method V has the shortest analysis time and a system pressure of less than 200 bar, which makes it advantageous compared to the other methods (Figure 2, Table 5). It is important to highlight that it was not necessary to change the temperature during chromatographic method optimization, which is a great advantage facilitating its implementation and transfer.

Sequences of peptides 1, 2, 4, 5, and 6 are derived from the LfcinB sequence and contain hydrophobic amino acids (Trp) and positively charged amino acids (Arg).[4,22] These peptides are amphipathic because the side chains of the Arg are oriented toward the same side forming a polar face, while the side chains of the Trp are oriented toward the other side forming a hydrophobic face. In polar (aqueous) media, the hydrophobic faces of peptides tend to interact with each other in such a way that the polar faces are exposed to the polar environment, so these hydrophobic interactions promote the aggregation of peptides. When the peptide mixture was dissolved in solvent A (0. 05% TFA in water) and stored at 4 °C for two days, precipitate formation was observed, possibly due to peptide aggregation. Subsequently, the peptide mixture was dissolved in a mobile phase containing 95% A (0. 05% TFA in water) and 5% B (0. 05% TFA in ACN) and was not observed in precipitate, suggesting that peptide aggregation can be prevented using mobile phases containing solvent B in a concentration equal to or greater than 5%. Our results showed that the mixture containing peptides 2 (linear) and 4 (dimeric), which have the same sequence, was efficiently separated with high resolution, suggesting that the methodology developed here is unaffected by the aggregation of amphipathic peptides.

To establish the applicability of method V, this method was evaluated for the separation of a mixture containing four peptides used in doping; GHRP-4 and GHRP-6 peptides exhibit antioxidant activity, while vasopressin and its analogue desmopressin exhibit antidiuretic activity.[25−27] The applicability of this method for the characterization of these peptides, classified by WADA in groups S2 and S5 of the list of prohibited substances, is of particular interest in doping control.

Figure 3 shows the chromatographic profile obtained for the mixture of doping peptides using method V. The signals of the four peptides can be observed, as well as the fact that they are separated with high values of R_s, indicating that this method is suitable for the analysis of mixtures containing peptides with different physicochemical properties, such as molecular weight, hydrophobicity, hydrophilicity, amphipathicity, and structural conformation, among others. Note that for the transfer of this method, it is necessary to consider the correction of the dwell volume (v_D) or dwell time (t_D).[21,23] The equipment used in this study has a v_D of 2 mL.Table 6 shows a comparative chart between the method optimized in this research (method V) and other recently reported methods for peptide analysis by conventional HPLC. As can be seen, many of the commonly used methods report analysis times of between 40 and 70 min, while method V achieves an efficient separation of peptides of different chemical natures in just 7 min, which shows that the k^* concept is a very useful tool for method transfer and the Chromolith monolithic column is an excellent option for the analysis of these molecules.

4. CONCLUSIONS

The efficiency of the analysis of peptides (**1**) and (**2**) by means of the RP-HPLC of three columns (packed, core−shell, and monolithic) using the van Deemter model was evaluated. It was found that the monolithic column presented the lowest HETP values and therefore the best efficiency for the analysis of these peptides. Additionally, this column allowed working at flows of up to 5 mL/min without affecting efficiency. Using the k^* concept, it was possible to develop and optimize five methods for analyzing a complex mixture containing peptides from a previously reported method. Also, it was possible to transfer the method from a packed column to a monolithic column, and it was possible to go from an analysis time of 70 min to the one of 6.1 min (7 times faster) while maintaining a similar chromatographic profile and the same resolution of the critical peak pair.

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Notes

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