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Modeling the nonlinear behavior of a bioactive peptide in reversed-phase gradient elution chromatography

The thermodynamic behavior of octreotide, a cyclic octapeptide with important pharmaceutical functions, has been simulated under reversed-phase gradient elution conditions. To this end, adsorption behavior was firstly investigated in isocratic conditions, under a variety of water/acetonitrile + 0.02% (v/v) trifluoroacetic acid (TFA) mixtures as mobile phase by using a Langmuir isotherm. Organic modifier was varied in the range between 23 and 28% (v/v). Adsorption isotherms were determined by mean of the so-called Inverse Method (IM) with a minimum amount of peptide. The linear solvent strength (LSS) model was used to find the correlation between isotherm parameters and mobile phase composition. This study contributes to enlarge our knowledge on the chromatographic behavior under nonlinear gradient conditions of peptides. In particular, it focuses on a cyclic octapeptide.

1. Introduction

Peptides represent a unique class of biochemical compounds. They are of primary importance in human physiology, being able to selectively interact with cells, receptors and other endogenous peptides and to induce specific biological reactions [1,2]. The interest on the use of peptides in pharmaceuticals (e.g., as antitumorals, anticoagulant, anti-hypertensive, antioxidant, antimicrobial drugs), nutraceuticals (for fortification of functional foods) and cosmetics (for skin health and care) is continuously increasing [3–7]. Due to their very high specificity, therapeutic peptides are competitive and advantageous over traditional drugs since they can be effective even at extremely low concentration [7,8]. Moreover, peptides do not accumulate in the human body nor in the environment after they have been excreted, minimizing possible toxic side effects.

From an industrial point of view, therapeutic peptides are produced by two main routes: recombinant synthesis [9] or chemical synthesis strategy [10].

The first one involves the use of suitable microorganisms to produce peptide of interest, through its trascription-transduction machinery [11]. The chemical synthesis approach can be further subdivided into two main strategies: Liquid Phase Peptide Synthe- sis (LPPS) and Solid Phase Peptide Synthesis (SPPS) [12]. In both cases, the approach involves the use of amino acids ortogonally protected as to enable the specific generation of the desired amino acid sequence through repetitive peptide bond formation. In any case, both recombinant and chemical synthesis do not generally produce target API peptide with an acceptable purity for market requirement. Purification is therefore needed to get the target pep- tide at the desired degree of purity for therapeutic and pharma- ceutical scopes [5,13]. The downstream process (purification and recovery of the target peptide) takes up an important percentage of total manufacturing costs [14].

Preparative liquid chromatography is the most widely used technique for the purification of therapeutic peptides [15–18]. With the purpose of isolating finite amounts of pure compounds, in preparative (or nonlinear) chromatography large volumes of concentrated multicomponent feed are processed at a time. Under ovearloaded conditions, retention of analytes becomes concentration-dependent, being the adsorption isotherm of the an- alyte nonlinear. Thus, injected compounds are not eluted from the

column as a series of Gaussian peaks but chromatograms appear as a complex mixture of tailed bands that may also change shape by increasing sample size. The problems encountered in nonlinear chromatography are extremely complex, not only owing to the effect of nonlinear adsorption isotherms on peak shapes, but also to the dependence of the amount of any component adsorbed on the concentrations of all the species in solution (competitive systems) [19–21]. Even though the theory of nonlinear chromatography has advanced to the point that quantitative predictions are possible, in preparative chromatography, working conditions are usually optimized through trial and error methods, which may cause significant waste of time and compound and thus money. As a matter of fact, when it comes to the separation/purification of (poly)peptides, some general guidelines can be applied to start with. However, the application of these protocols is not a guarantee that the process will be successful. Many aspects in this field require significant experimental and theoretical efforts to improve our understanding of the fundamentals of separation. For instance, the chromatographic behavior of two quasi-identical polypeptides under nonlinear conditions can dramatically change when even a single amino acid differs in their structure. It is well known that the adsorption model for the same peptide can also change not only by changing the mobile phase composition but also depending on the concentration of the peptide itself. But there are no means to predict if and how this will happen. There are, e.g., cases of polypeptides where the curvature of the adsorption isotherm inverts, by moving from one mobile phase modifier to another. Other times, by increasing the concentration of the polypeptide under investigation, the adsorption isotherm, initially Langmuirian, becomes S-shaped. The presence of an inflection point on the isotherm may strongly affect the shape of overloaded peaks. This explains why, in our opinion, it is so important to develop methodologies based on the measurement of adsorption isotherms. The investigation of adsorption behavior and phase equilibria involved in the separation of the target compound using a model-based approach is, therefore, the basis not only to investigate the feasibility of purification process via preparative chromatography but also to possibly provide information (e.g., maximum loading, affinity for the stationary phase) that may help to optimize large-scale purification [16,22–26]. This is particularly important in pharmaceutical manufacturing, where continuous (or semi-continuous) processes could alleviate the trade-off between yield and purity, typical of most batch (single-column) preparative chromatographic separations [27,28]. The first multi-column setup is the so-called simulated moving bed (SMB) process introduced in 1950 for isocratic binary separations of small molecules [19,29–32]. Since then, many different improved versions of continuous processes based on SMB concepts have been proposed to overcome some fundamental issues (process optimization, difficulty to deal with complex mixtures, gradient operation) and technical problems associated to the large number of columns to be operated simultaneously. The most important alternative to SMB is the multi-column counter-current solvent gradient purification (MCSGP) process, which combines linear gradients with the counter-current movement of mobile and stationary phases [33]. Originally realized with at least six columns, the process has modified in order to work with four [34], three columns [35] and more recently only two columns [36]. It has been demonstrated that the outcome of twin column MCSGP processes is easily predictable from batch chromatographic runs [37]. As a consequence, the results of investigation of thermodynamic equilibria influencing the separation in batch conditions can be used during process design to more efficiently move to continuous separations, which are extremely attractive for pharmaceutical industry to replace batch technologies [38–42].

In this work, the adsorption behavior of a therapeutic peptide, octreotide, has been investigated and modeled under reversed-phase liquid chromatography (RP-LC) gradient elution conditions. Octreotide is a cyclic octapeptide belonging to somatostatins [43,44]. Its industrial production can be obtained either with LPPS or SPPS approaches [45] and it is employed in the treatment of hepatocellular carcinoma, cirrhosis of the liver and to contrast some symptomps associated with metastatic carcinoid and Vasoactive Intestinal Peptide (VIP) tumors [46]. Adsorption isotherms of octreotide have been measured on a commercial C_{18} stationary phase by means of the so-called Inverse Method (IM) [19,47–54]. Goal of this work is to demonstrate how the adsorption behavior of octreotide under nonlinear gradient conditions can be predicted with a very low amount of compound and extremely reduced costs with respect to more traditionals techniques of isotherm determination, such as for instance frontal analysis.

2. Theory

2.1. Equilibrium-dispersive model of chromatography

The equilibrium-dispersive (ED) model of chromatography is mostly used to describe nonlinear chromatographic separations for molecules with low molecular weight [19]. This model assumes that mobile and stationary phases are in costant equilibrium and that all the contributions to band broadening (diffusion phenomena and finite rate of mass transfer kinetics) can be lumped into a unique apparent dispersion coefficient, D_a [19]:

$$D_a = \frac{uL}{2N} \tag{1}$$

where u is the mobile phase linear velocity, L the length of the column and N the number of theoretical plates.

The differential mass balance equation describing the accumulation of material in a thin slice of column of thickness ∂z in a ∂t time interval is [19]:

$$\frac{\partial C}{\partial t} + F\frac{\partial q}{\partial t} + u\frac{\partial C}{\partial z} = D_a\frac{\partial^2 C}{\partial z^2} \tag{2}$$

where C and q the concentrations of the analyte in the mobile and stationary phases. $F = (1 - E_t)/E_t$ is the phase ratio and $E_t = V_0/V_{col}$ the total porosity of the column (with V_0 and V_{col} the thermodynamic void volume and the column voume, respectively).

In order to solve Eq. (2), an isotherm model ($q = f(C)$), expressing q as a function of C, must be chosen.

2.2. Modeling of overloaded profiles under gradient elution chromatography

In gradient elution RP-LC, the volume fraction (φ) of the organic modifier in the mobile phase is gradually increased during a chromatographic run. Differently from isocratic conditions, the adsorption isotherm of a species in gradient elution mode is φ- (and time-) dependent [53,55,56]. For this reason, it is usually considered that, even if the adsorption isotherm type is not affected by changes in mobile phase composition, its parameters are a function of φ [57].

In addition, the Linear Solvent Strength (LSS) model [58–60] is applied to describe the variation of retention factor with the mobile phase composition:

$$\ln k(\varphi) = \ln k_0 - S\varphi \tag{3}$$

with k_0 the retention factor extrapolated at $\varphi = 0$ and S a coefficient characteristic of the system solute-mobile phase.

By considering a simple Langmuir isotherm model (under isocratic elution conditions):

$$q = \frac{aC}{1 + bC} \tag{4}$$

where b and $a(= q_s b)$ are the equilibrium and Henry constants of adsorption, respectively (being q_s the saturation capacity), the dependence of isotherm parameters on φ could be obtained by combining Eq. (3) and the following relationship between k and a:

$$k = aF \tag{5}$$

It follows that:

$$a(\varphi) = a_0\, e^{(-S\varphi)} \tag{6}$$

where $a_0\,(= k_0/F)$ is the Henry constant (extrapolated) at $\varphi = 0$.

If the range of variation of φ is sufficiently narrow, q_s can be considered constant [19,61,62] and, as a consequence, b and φ are correlated by the same relation as in Eq. (6):

$$b(\varphi) = b_0\, e^{(-S\varphi)} \tag{7}$$

where b_0 is the adsorption constant at $\varphi = 0$.

By combining Eqs. (4), (6) and (7), the Langmuir isotherm describing the adsorption process under gradient elution conditions can be obtained:

$$q(\varphi) = \frac{q_s\, b_0\, e^{(-S\varphi)} C}{1 + b_0\, e^{(-S\varphi)} C} \tag{8}$$

The mass balance equation (Eq. (2)) can be numerically solved by applying a finite difference method based on the so-called backward-backward scheme [19,63].

Lastly, boundary and initial conditions need to be defined in order to solve the mass balance equation. The Danckwerts-type boundary conditions have been applied [19,64,65] while the gradient in the inlet feed has been simulated as follows:

$$\varphi(t, 0) = \begin{cases} \varphi_0 & 0 \le t \le t_{inj} \\ \varphi_0 + \frac{1'\varphi}{t_g}(t - t_{inj}) & t_{inj} \le t \le t_{inj} + t_g \\ \varphi_0 + 1'\varphi & t \ge t_{inj} + t_g \end{cases} \tag{9}$$

where t_{inj} is the length of the rectangular injection profile, φ_0 is the initial fraction of organic modifier and t_g is the time of the gradient.

3. Experimental

Column and materials

All solvents were purchased from Sigma–Aldrich (St. Louis, MI, USA). A 150 × 4.6 mm Zorbax Sb-C18 column (5 μm particle size, 80 Å pore size) used to perform separations was from Agilent Technologies (Santa Clara, California, USA). Uracile (Sigma–Aldrich, St. Louis, MI, USA) was injected for the determination of the void volume of the column. Pure and crude (= not purified) mixtures of octreotide were from Fresenius Kabi iPSUM (Villadose, Rovigo, Italy). Crude sample is the product obtained after solid-phase synthesis.

Equipment All the measurements were carried out on an Agilent 1100 Series Capillary LC system equipped with a binary pump system, a column thermostat set at 35 °C and a photodiode array detector. A manually Rheodyne 8125 injecting valve was employed by using different loops to perform detector calibration (500 μ) and injections of overloaded profiles (5, 10, 20 μ). All the experimental profiles were recorded at UV wavelengths of 280 nm, at flow rate of 1 mL/min. Maximum absorbance was below 1000 mAU.

Measurement of overloaded profiles

Overloaded band profiles in both isocratic and gradient elution conditions have been recorded by injecting solutions of peptide with different concentrations: 0.1, 0.3, 0.6, 1.2, 2.0, 4.0 and 6.0 g/L. Mobile phase A (MP-A) was a solution of 0.02% (v/v) trifluo- roacetic acid (TFA) in water, while mobile phase B (MP-B) was 0.02% (v/v) TFA in acetonitrile (ACN). The gradient program was set as follows: (i) the column was firstly equilibrated with 10% (v/v) of MP-B; (ii) in a first linear ramp the percentage of MP-B was increased from 10% to 30% (v/v) over a gradient time, t_{g1}, of 12 min (gradient slope = 1.6% ACN/min); (iii) in a second steeper ramp MP-B was changed from 30% to 90% (v/v) in 3 min, t_{g2} (slope = 20% ACN/min).

Overloaded profiles under isocratic elution conditions were recorded in a range of MP-B from 23% to 28% (v/v). Solubility limit of the peptide in these conditions is 9.0 g/L.

Adsorption isotherm determination

Adsorption isotherms under isocratic elution conditions have been calculated by means of the so-called Inverse Method [19,47,51,66–68]. This method allows the determination of the adsorption isotherm in a few steps, requiring less amount of samples and solvents than other alternative techniques, such as frontal analysis [19,51,68]. The first necessary step is the calibration of the detector. In order to do this, the column has been replaced with a zero-dead-volume connector and 500 μL of each solution of peptide with different concentration have been injected into the system. This operation has been performed for each mobile phase composition. Not surprisingly, differences in detector response were negligible in the very small operative concentration range considered in this work. The maximum absorbance (*Abs*) of each plateau at 280 nm has been recorded and reported in a curve as a function of C. Then, (i) experimental profiles at the seven concentrations have been recorded in overloading conditions; (ii) overloaded profiles *Abs vs. t* have been converted into *C vs. t* through the slope of the calibration curve; (iii) an isotherm type and a guess of its initial parameters have been selected; (iv) a system of equations including the mass balance equation and the selected adsorption isotherm have been solved in order to obtain a calculated overloaded profile; (v) the calculated overloaded profile and the experimental *C vs. t* one have been compared; (vi) isotherm parameters have been iteratively changed until the calculated and experimental profiles match as much as possible (the numerical optimization was made by means of the Simplex method, minimizing the sum of the squares of the differences between simulated and experimental profiles) [47,66,69].

4. Results and discussion

Fig. 1 reports an experimental chromatogram recorded under gradient elution conditions by injecting 5 μL of the solution of crude octreotide. The main peak (t_R = 12.5 min) corresponds to

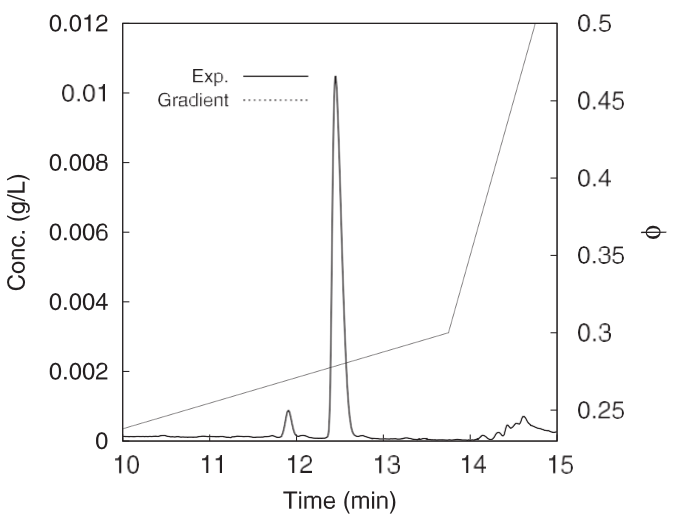

Fig. 1. Experimental gradient elution profile of the crude peptide Injected concentration: 0 2 g/L; injected volume: 5 μL; wavelength: 280 nm

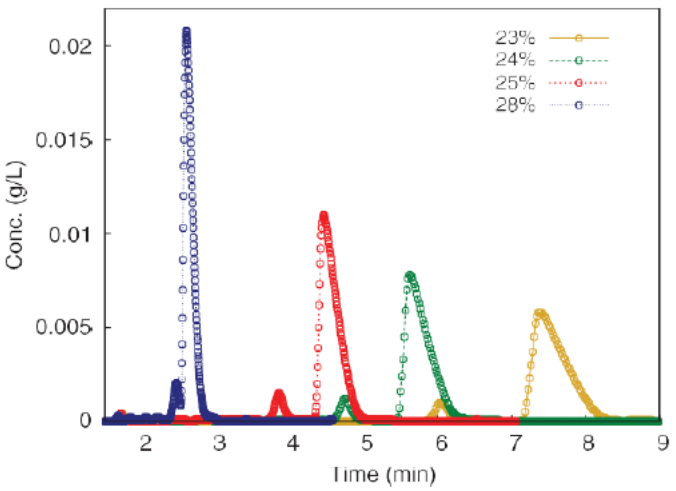

Fig. 2. Comparison between experimental profiles of the crude peptide measured at different volume fractions of organic modifier, ranging from φ = 0 23 to φ = 0 28 Injected concentration = 0 6 g/L; injected volume = 5 μL; wavelength: 280 nm

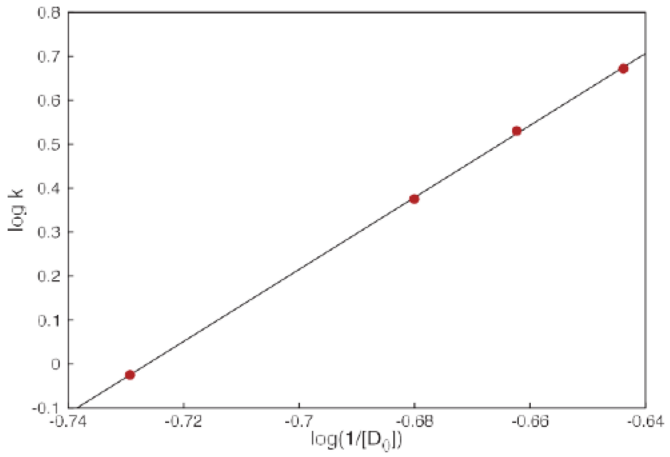

Fig. 3. Dependence of logarithm of retention factor (k) on logarithm of the inverse of ACN concentration (D_0) expressed in terms of molarity The slope of the linear regression gives an indication of the number of displaced molecules (Z) equal to 8 2 ± 0 1 R^2 = 0 999

the elution of peptide and the smaller one (t_R = 12.0 min) is an impurity from the synthesis.

Taking into account the dwell volume of the system, it was estimated that the elution of peptide takes place approximately around φ = 0.25, therefore a range of φ between 0.23 and 0.28 has been chosen for experimental measurements.

Investigation of retention at infinite dilution

The investigation of chromatographic behavior of octreotide at infinite dilution demonstrated that its retention is profoundly affected by changes in the percentage of organic modifier. The dead volume has been determined through an unretained compound, uracil. Indeed, a variation of only roughly 5%, from φ = 0.23 to φ = 0.28, induced a 500% drop of retention factor (from 4.7 to 0.95, respectively, see Fig. 2).

Eq. (3) can be re-arranged according to the displacement model of retention in RP-LC [70]. This model predicts that retention of an hydrophobic molecules from an apolar stationary phase is accompanied by the displacement of a stoichiometric number of solvent molecules adsorbed on the surface [71]:

$$\log k = \log I + Z \times \log \frac{1}{D_0} \quad (10)$$

In this equation, which can be applied in a range where the concentration of organic solvent on the stationary phase is approximately constant, D_0 is the molar concentration of organic modifier, Z the number of molecules of organic solvent displaced by the analyte during retention and I is the value of k when D_0 is 1 M. Fig. 3 shows the variation of log k with log(1/D_0) for octreotide. From the slope of the linear regression line, the number of displaced molecules has been evaluated. Z resulted to be 8.2 ± 0.1. This value is significantly large if compared to the molecular weight of the compound, however very close to that obtained for a small polypeptide of comparable molecular mass [63].

Modeling of overloaded profiles under isocratic elution conditions

Adsorption isotherms of both crude and pure peptide solutions at each mobile phase composition have been determined by means of IM. Different adsorption isotherm models have been tested (Langmuir, BiLangmuir, Tóth). Among them, only the Langmuir model was found to satisfactorily fit experimental data. An excellent agreement was found between experimental and calculated peaks of pure and crude solutions of peptide (see Fig. 4). The amount of impurity is so small that it does not compete with peptide for adsorption and its retention time is not influenced by peptide concentration.

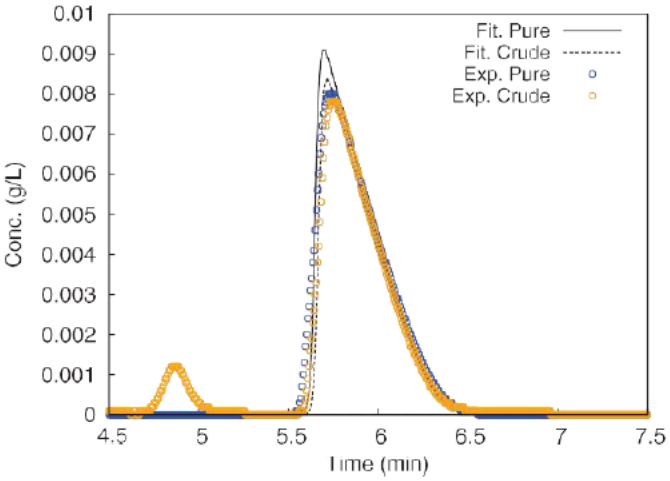

Fig. 4. Comparison between experimental peaks of crude (orange circles) and pure (blue circles) peptide and their corresponding calculated profiles Dotted and solid lines corresponds to the fitting profiles of the crude and the pure peptide, respectively φ = 0 24, injected concentration = 0 6 g/L; injected volume = 5 µL; wavelength: 280 nm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article)

Table 1
Adsorption isotherm parameters obtained through IM with a Langmuir model at different mobile phase compositions.

φ	a	b (L/g)	q_s (g/L)
0 23	5 86	8 14	0 72
0 24	4 21	5 69	0 74
0 25	3 07	4 65	0 66
0 28	1 17	1 86	0 63

Fig. 5 compares some experimental and simulated overloaded profiles of the crude peptide recorded at φ = 0.24 and various loading concentrations. Some small discrepancies in the front part of the peaks, especially for the two highest concentrations, could be due to the presence of kinetic phenomena that are neglected by the ED model. However, the rear parts of experimental and calculated profiles excellently match even at high concentrations.

The best isotherm parameters obtained at the different isocratic conditions investigated in this work are reported in Table 1. As it

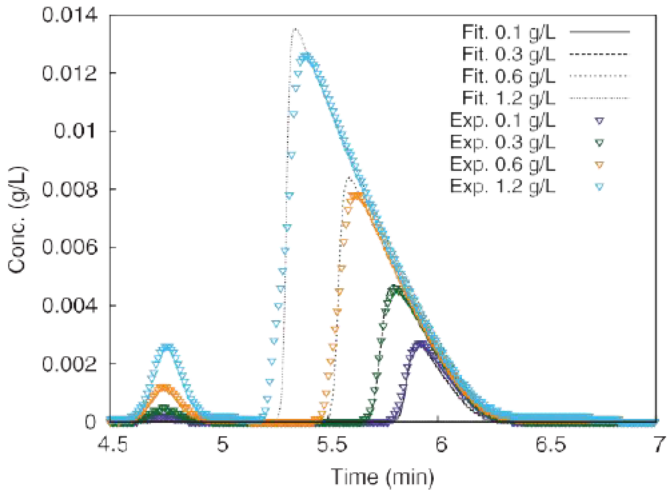

Fig. 5. Comparison between experimental and simulated peaks obtained with IM (Langmuir adsorption isotherm) for four different concentrations of the crude peptide in isocratic conditions (φ=0.24). Injected volume: 5 μL; wavelength: 280 nm.

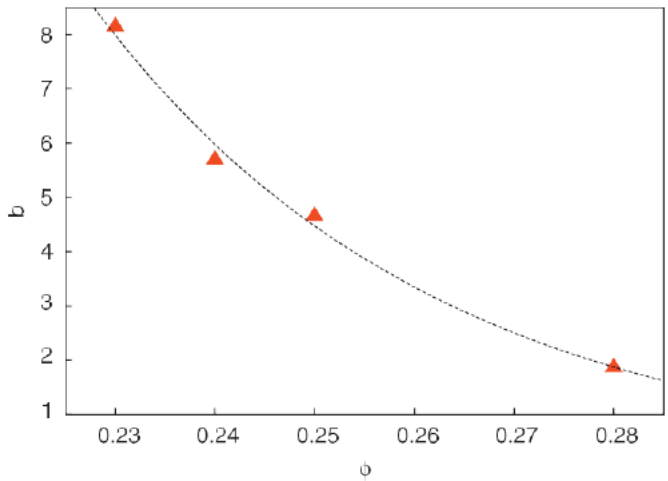

Fig. 6. Dependence of the equilibrium adsorption constant (b) on the fraction of organic modifier (φ) according to Eq (7) (R^2 =0 998)

can be evinced, q_s values are in a very good agreement, supporting the hypothesis of a small variation of q_s if this range of φ is significantly small. Moreover, the variation of b with φ follows the trend described by Eq. (7) (see Fig. 6). By fitting experimental data with an exponential equation, values of 29 and 6.3 × 10^3 L/g have been calculated for S and b_0, respectively. For the saturation capacity q_s the average value of 0.69 g/L was taken in the simulation of gradient elution experiments.

Modeling of overloaded profiles under gradient elution conditions

Substituting the above value of b_0, S and q_s in Eq. (8), the equilibrium-dispersive model with the feed conditions (Eq. (9)) can be solved to simulate gradient elution runs. As it can be observed from Fig. 7, where calculated profiles (solid lines) and experimental ones (coloured circles) are compared, a very good agreement between theoretical and experimental profiles has been obtained even at high concentrations.

In order to test model reliability and potential to predict conditions not considered in its development and parameter tuning, two more experimental runs at increasing loading volume have been considered, that is 10 and 20 μL. The match between experimental and predicted peaks was satisfactory (see Fig. 8). This means not only that the simple Langmuir model (based on the assumption that the adsorption surface is energetically homogeneous) is

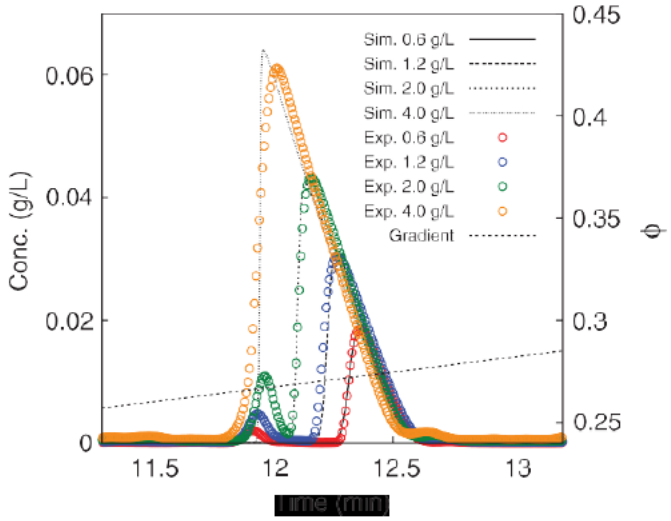

Fig. 7. Comparison between experimental and simulated peaks in gradient elution (Langmuir adsorption model) of four different concentrations of crude peptide. Injected volume: 5 μL; wavelength: 280 nm.

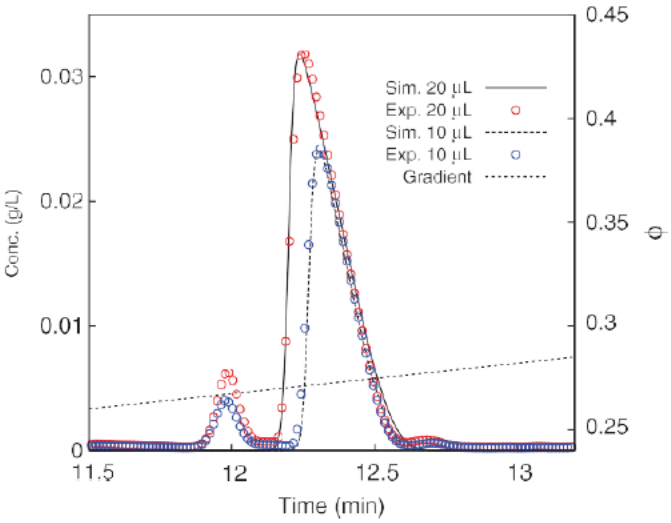

Fig. 8. Comparison between experimental and simulated peaks in gradient elution conditions. Injected concentrations: 0.5 g/L. Injected volume: 10 μL (blue) and 20 μL (red); wavelength: 280 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

adequate to describe the adsorption mechanism of this peptide on this stationary phase but also, most importantly, that very small amount (μg) of peptide are sufficient to gather information on its adsorption equilibria and to model the separation under nonlinear gradient conditions.

5. Conclusions

Gradient preparative RP-LC is one of the most widely used technique for the purification of synthesized peptides. A reasonable approach to develop a purification method via preparative HPLC is based on the prior investigation of thermodynamic equilibria regulating retention of peptides on the stationary phase. This is practically translated into the calculation of their adsorption isotherms under different mobile phase compositions in a range of φ where elution takes place, in order to find the relationship between isotherm parameters and variation of organic modifier in the mobile phase.

Most of the times, the amount of available peptide is reduced or its cost is elevated. When this is the case, modern techniques of isotherm determination, based on theoretical hypotheses on the adsorption model and the simulation of peaks under overloaded conditions, can be efficiently employed to achieve the relevant information.