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Modeling of overloaded gradient elution of nociceptin/orphanin FQ in reversed-phase liquid chromatography

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Abstract

The Reversed-phase (RP) gradient elution chromatography of nociceptin/orphanin FQ (N/OFQ), a neuropeptide with many biological effects, has been modeled under linear and non-linear conditions. In order to do this, the chromatographic behavior has been studied under both linear and nonliner conditions under isocratic mode at different mobile phase compositions—ranging from 16 to 19% (v/v) acetonitrile (ACN) in aqueous trifluoracetic acid (TFA) 0.1% (v/v)—on a C-8 column. Although the range of mobile phase compositions investigated was quite narrow, the retention factor of this relatively small polypeptide (N/OFQ is a heptadecapeptide) has been found to change by more than 400%. In these conditions, gradient operation resulted thus to be the optimum approach for non-linear elution. As the available amount of N/OFQ was extremely reduced (only a few milligrams), the adsorption isotherms of the peptide, at the different mobile phase compositions examined, have been measured through the so-called inverse method (IM) on a 5 cm long column. The adsorption data at different mobile phase compositions have been fitted to several models of adsorption. The dependence of the isotherm parameters on the mobile phase composition was modeled by using the linear solvent strength (LSS) model and a generalized Langmuir isotherm that includes the mobile phase composition dependence. The overloaded gradient separation of N/OFQ has been modeled by numerically solving the equilibrium-dispersive (ED) model of chromatography under a selected gradient elution mode, on the basis of the previously determined generalized Langmuir isotherm. The agreement between theoretical calculations and experimental overloaded band profiles appeared reasonably accurate.

Keywords: RPLC; Gradient elution; Isotherm determination; Polypeptides

1. Introduction

Reversed-phase (RP) chromatography represents one of the most important techniques for analysis and purification of proteins and peptides [1–3]. Apart from its powerful resolution capability [4–7], the use of volatile mobile phases in RP-HPLC represents a significant advantage with respect to other techniques commonly used for protein purification such as hydrophobic interaction chromatography (HIC) or ion-exchange chromatography (IEC) in which sample desalting is needed. However, it should be noted that the recovery of proteins from RP-HPLC can be difficult, both in terms of mass and also loss of activity due to unfolding in the harsh conditions typically used in RP-HPLC, e.g. aqueous trifluoracetic acid (TFA)–acetonitrile (ACN) mixture [8–10].

Proteins and polypeptides are macromolecules that bear chemical groups with significantly different physicochemical properties—in terms of acid–basic character, hydrophobicity, hydrophilicity, etc—randomly distributed in their structure. The actual mobile phase conditions (namely, type and amount of organic modifier and pH) determine the specific structure of these macromolecules [2,11,12]. Moreover, during the course of gradient elution additional alterations of the structure may occur because of solvatation of solutes. One of the most unique characteristics in the separa-

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tion of polypetpides and proteins in RP-HPLC is, in fact, that often by changing the mobile phase composition by a few percentage points of organic modifier, immediate elution can result [13–20,7,21–25].

The concept that multiple aminoacid residues are involved in the adsorption process of proteins in RP-HPLC was first suggested by Boardman and Partridge [26] in their studies of adsorption isotherms of cytochrome *c*: the chromatographic behavior of proteins and polypeptides strongly depends on the molecular composition of the specific contact regions of the macromolecules with the sorptive material. On the basis of these observations, Regnier et al. [15,22,27] developed a quantitative treatment of retention of bio-macromolecules in IEC, HIC and RP-HPLC, in which displacement of organic modifier by both the stationary phase and the macromolecule surface controls the adsorption. According to this model a fixed number of modifier molecules surround the structure of the macromolecules; the alkyl-silane chains are also solvatated with an average number of modifier molecules. Adsorption is considered a multi-step process. During each adsorption-desorption step in chromatographic elution, molecules of modifiers are displaced by both the macromolecule surface and the hydrophobic sorbent. Chromatographic retention (k') depends on the total number of molecules displaced (Z) and the concentration of the displacing agent ($[D_0]$):

$$k' = \frac{I}{[D_0]^Z} \quad (1)$$

being I a constant that includes: the stationary phase ligand density, the stationary to mobile phase ratio and the equilibrium constant for the process [15]. Eq. (1) allows the estimation of Z through the measure of k' as a function of mobile phase modifier concentration. Experimentally, in RP-HPLC, Z values were found to range from 3 to more than 20 for proteins with molecular weights included between 3 300 and 44 000 Da, respectively (mobile phase: 2-propanol–formic acid) [15].

N/OFQ, is a heptadecapeptide (H-Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln-OH) isolated from porcin brain [28] and rat brain [29]. It is the endogenous ligand of a G-protein coupled receptor named ORL-1, OP4 and now called NOP receptor [30] and considered the fourth member of the opioid receptors family. In recent years, several studies demonstrated a broad spectrum of biological function mediated by the N/OFQ-NOP receptor system, both at central and at peripheral levels such as modulation of nociception, locomotor activity and kidney function [31,32].

N/OFQ is synthesized through solid phase methodologies in automathed synthesizers. The peptide, linked to a solid support, grows from the C- to the N-terminal residue by means of a series of coupling reactions. N-terminal amino functions on the growing peptide chain are acylated by the following aminoacid residue. If any coupling reaction did not go to completion, two N-terminal amino groups would be present during the subsequent coupling reaction. However, one of these would lack the last residue coupled. In the subsequent coupling step, both of these terminal amino groups could be acylated with the same probability. Two side chains would be then propagated, with one of these lacking one aminoacid residue. Accordingly, the synthesis of the target peptide is generally accompanied by the presence of a series of deletion sequences lacking one or more aminoacid residues. Increasing the length of the peptide also increases the percentage of deletion sequences. After removing the peptide substance from the resin by chemical reagents, a general approach consists of checking the purity of the synthesized peptide by chromatography.

The advent of HPLC revolutioned the examination of peptides and the commercially available RP columns allow rapid separation, detection and quantification of the components in a mixture. Moving from analytical to preparative HPLC is the routine approach to purify a bulk quantity of peptide material. Countercurrent distribution in many cases is another method adopted for the purification of synthesized peptides. However, preparative HPLC is often achieved through a pure empirical approach in which the working conditions for the collection of high-concentrated fractions of pure components are obtained via trial and error methods. This may introduce significant loss of compound and time and the risk of operating the system far from optimal conditions. Instead, "[...] the interpretation of results and process-design in non-linear chromatography requires knowledge of the relationship between the equilibrium concentrations of the components in the mobile phase and stationary phase over a sufficiently wide range, i.e., the pertinent adsorption isotherms" [33].

In this work, the adsorption behavior of N/OFQ was studied in RP-HPLC with the aim of investigating the feasibility of the separation/purification via overloaded gradient elution. The thermodynamic data (adsorption isotherms) were gathered through the so-called inverse method (IM). This numerical approach to isotherm determination becomes particularly competitive with respect to more traditional techniques—such as frontal analysis (FA) or micro-FA [33]—when the compounds to be purified are present in significantly low amounts and/or they are extremely expensive [34]. When overloaded gradient chromatography is performed, this problem becomes increasingly more important since the adsorption data must be measured under a wide range of experimental conditions, corresponding to the mobile phase composition covered during gradient. The scope of this work is to explore some specific aspects of the chromatographic separation of polypeptides under overloaded conditions and possibly provide information about optimization of the experimental conditions for large-scale separation of macromolecules. Finally, as Csaba Horváth recognized in 1976, RP-HPLC may be a useful probe for investigating hydrophobic interactions between the non-polar

residues that stabilize the folded (or three-dimensional) structure of the native protein molecule or the behavior of peptides and proteins at hydrophobic interfaces, such as lipid bilayer [1,35,5,2].

2. Theory

In gradient elution RP-HPLC, the eluotropic strength of the mobile phase is gradually increased during the separation by increasing the concentration (volume fraction, ϕ) of the strong modifier. This is the technique most often used for separation of large biomolecules via HPLC. The retention factors of these compounds often decrease from very large values (when the macromolecules get almost "stuck" in the column) to almost zero by changing ϕ only a few tenths of one percent.

2.1. Overloaded gradient elution chromatography

The major difference between isocratic and gradient elution is that, while in the former case the adsorption isotherm remains the same along the entire column, in the latter it changes according to the gradient program set-up. Thus, the velocity associated with a concentration in the ideal model of chromatography at a given point of the column depends on the time. To account for these effects, it is a common practice to assume that the isotherm parameters are a function of ϕ, while the functional form of the adsorption isotherm itself remains unchanged, whatever the gradient composition [13]. Implicitly, this means that the adsorption mechanism does not change during the gradient. Another important simplification for modeling gradient elution is given by the concept of the linear solvent strength (LSS) gradient, introduced by Snyder and coworkers [17,36,37]. When linear gradient programs are used (ϕ increases linearly with time during chromatographic elution) [19], the LLS model for reversed-phase HPLC requires that isocratic retention be approximatable as [38]:

$$\ln k'(\phi) = \ln k'_0 - S\phi \tag{2}$$

where k'_0 is the retention factor (extrapolated) at $\phi = 0$ and S a constant typical of the given solute–MP composition. If a simple Langmuir isotherm is used to describe the adsorption:

$$q = \frac{aC}{1 + bC} \tag{3}$$

where q and C are the stationary and mobile phase equilibrium concentrations, b the equilibrium constant and a the Henry constant of the adsorption (which is the product of b and the saturation capacity, q_s), the relationship between isotherm parameters and ϕ can be easily obtained. In fact, by recalling the equation that, under linear conditions, relates the retention factor and the Henry constant [13]:

$$k' = aF = q_s bF \tag{4}$$

being F the phase ratio, through Eq. (2) and simple math one has for a:

$$a(\phi) = a_0 \exp(-S\phi) \tag{5}$$

where a_0 ($= k'_0/F$) is the Henry constant at $\phi = 0$. It is important to underline that a, b and k' parameters are strictly defined (and consequently evaluated) only at infinite dilution. Under the assumption that the saturation capacity does not change in the range of mobile phases exploited by the gradient [13,39–41], furthermore, even the dependence of b on ϕ will be the same as Eq. (5). Through Eqs. (4) and (5), one obtains:

$$b(\phi) = b_0 \exp(-S\phi) \tag{6}$$

where b_0 is the adsorption constant at $\phi = 0$ and finally, from Eqs. (3) and (6), the isotherm can be expressed as:

$$q(\phi) = q_s \frac{b_0 \exp(-S\phi)C}{1 + b_0 \exp(-S\phi)C} \tag{7}$$

In practice, the assumption of constant q_s is plausible only when the value window in which the gradient is changed is significantly narrow [41].

The equilibrium-dispersive (ED) model of chromatography is most often used for modeling non-linear separations of small molecules [13]. The differential mass balance equation that describes the accumulation of material in a slice of dz length due to convection and diffusion is written as:

$$\frac{\partial C}{\partial t} + F\frac{\partial q}{\partial t} + u\frac{\partial C}{\partial z} = D_a \frac{\partial^2 C}{\partial z^2} \tag{8}$$

where t and z are the time elapsed from injection and the space traveled by molecules into the column, respectively, and D_a an apparent dispersion coefficient that lumps all the non-equilibrium phenomena leading to band broadening under linear conditions (molecular diffusion, eddy diffusion, mass-transfer resistances and finite rate of the kinetics of adsorption-desorption) [42]:

$$D_a = \frac{\sigma_L^2}{2t_0} = \frac{uL}{Nt_0} \tag{9}$$

where σ_L is the standard deviation in length unit of a Gaussian peak obtained at infinite dilution, L the column length, t_0 the hold-up time, u the mobile phase linear velocity and N the number of theoretical plates (or apparent efficiency of the column). Chromatographic separations of small molecules on new-generation adsorptive chromatographic media are generally characterized by elevated efficiency values, which explains the wide success of the ED model for describing this kind of separation. The ED model has been however shown to be adequate for modeling the separation even of relatively large molecules, when slow mass transfer kinetics are not unexpected [43,13,3,44].

Eq. (8) can be numerically solved by using a finite difference method. The continuous plane (z, t) is replaced by the grid (Δz, Δt) and the differential equation is replaced by

the appropriate difference equation [13]. When gradient elution must be modeled, the most appropriate finite difference algorithm to approximate Eq. (8) is the so-called backward-backward scheme [13]:

$$\frac{C_{z,t} - C_{z,t-\Delta t}}{\Delta t} + u\frac{C_{z,t-\Delta t} - C_{z-\Delta z,t-\Delta t}}{\Delta z} + F\frac{q_{z,t} - q_{z,t-\Delta t}}{\Delta t} = 0 \quad (10)$$

where the right-hand side of Eq. (8) (dispersion term) is replaced by zero as the length and time increments of numerical integration are chosen, so that the numerical dispersion is identical to the dispersion effect caused by D_a [13]. This scheme of calculation becomes identical to that of the Craig machine provided $\Delta t = \Delta z/u$ and $\Delta z = L/n_c$, with n_c number of cells in the Craig machine equivalent to the column [13,39,45]:

$$n_c = \frac{Nk'}{1+k'} \quad (11)$$

Although the Craig machine is a discontinuous model and accordingly its physical meaning is strongly debatable, it appears particularly suitable for modeling gradient elution as the mobile phase composition and the isotherm of any solute change along the column. Eq. (10) can be rewritten as:

$$C_{z,t} + Fq_{z,t} = C_{z,t-\Delta t} + Fq_{z,t-\Delta t} - u\frac{\Delta t}{\Delta z}(C_{z,t-\Delta t} - C_{z-\Delta z,t-\Delta t}) \quad (12)$$

that shows that this scheme of calculation only gives the total amount of component in a cell (intended as the sum of the mobile and stationary phase concentrations). The individual C and q values are in general determined by numerical iterations [39]. In the simple case of a single-component system and a Langmuir adsorption model, however, an analytical expression for C and q can be obtained by solving the system of equations given by Eqs. (12) and (3) (by considering the solutions corresponding to the positive root in the resulting second order equation).

Finally, to solve Eq. (8) a proper set of initial and boundary conditions must be defined. In this work, the classical Danckwerts-type boundary conditions at the inlet and outlet of the column were used [13,46]. Additionally, rectangular injection profiles of length t_{inj} were assumed. The column was initially equilibrated at a modifier composition equal to ϕ_0. Immediately after the injection, the gradient program started. For all the experimental measurements the gradient was linear and the concentration of strong modifier (ACN) varied between ϕ_0 and $\phi_0 + \Delta\phi$ during a gradient time, t_g:

$$\phi(t,0) = \begin{cases} \phi_0 & 0 \le t \le t_{inj} \\ \phi_0 + \dfrac{\Delta\phi}{t_g}(t - t_{inj}) & t_{inj} \le t \le t_{inj} + t_g \\ \phi_0 + \Delta\phi & t \ge t_{inj} + t_g \end{cases} \quad (13)$$

2.2. IM for isotherm determination

Different techniques are nowadays available for measuring adsorption isotherms through HPLC [13,33,1,47–50]. The most classical approaches, such as frontal analysis or perturbation methods have the common drawback of requiring a significant amount of compound.

The IM is a numerical approach to the problem of isotherm determination. Only a few chromatographic peaks measured under overloaded conditions and a model for the adsorption isotherm are required to start the calculations [13,34]. Once an initial set of values (initial guesses) for the parameters of the selected isotherm has been defined, the numerical resolution of Eq. (8) (under proper initial and boundary conditions) gives a "first" theoretical peak that will be compared, on the basis of the Chi-square criterion, with the corresponding experimental profile [34,51]. Incidentally, if for instance the Langmuir adsorption isotherm is considered only two parameters are needed. The initial guess for a can be easily obtained by an injection under linear conditions and Eq. (4); obtaining an estimate for b does not then represent a problem [34]. Nonlinear fitting is used for the optimization of the isotherm parameters.

3. Experimental

3.1. Equipment

An 1100 Series Modular Chromatograph (Agilent Technologies, Palo Alto, CA, USA) was used for all the experimental determinations. The instrument was equipped with a two-pump delivery system, a vacuum degasser, a multiple-wavelength detector (up to five wavelengths collectible), a temperature-controlled column compartment and a computer data station. Analytical and overloaded injections were performed through a Rheodyne 7725i injector (Cotati, CA, USA), by using loops of 2 and 165 μL, respectively. Calibration curves were measured by using a 500 μL loop.

3.2. Mobile phase and chemicals

In this work, four different mobile phases were used. Their composition was: H_2O, TFA 0.1% (v/v) and ACN, whose concentration was varied between 16% and 19% (v/v). Linear gradient elutions were carried out with 0.1% (v/v) TFA in H_2O (pump channel-A) and 0.1% (v/v) TFA in ACN (pump channel-B) over a gradient time of 60 s. All the mobile phases were filtered with 0.22 μm pore size membrane (Durapore-Hydrophobic; Millipore, Billerica, MA, USA). H_2O was Milli-Q reagent grade (Millipore). ACN was HPLC-grade from Fluka–Riedel-De Haën (Buchs, Switzerland). Uracil and TFA were purchased from Aldrich (Milwaukee, WI, USA). N/OFQ was synthesized as described in Ref. [52].

3.3. Column

A 50 mm × 4.6 mm stainless steel column XTerra MS C8 5 μm (Waters, Milford, MA, USA) was used for all the measurements. The average size of the pores in the packing material particles was 125 Å. The column hold-up volume, measured by injecting uracil, was 0.54 ± 0.01 mL. No significant differences were observed in its value by changing the mobile phase composition. Accordingly, the total porosity (ϵ_t) resulted 0.65 and F (defined as $(1 - \epsilon_t)/\epsilon_t$) equal to 0.54. The efficiency of the column at 1 ml/min flow rate (estimated in linear conditions) was found to be between 800 and 1200 theoretical plates, according to the different isocratic conditions. The specific values obtained at the different mobile phase compositions were used for the corresponding IM calculations; an "average" value for n_c equal to 1000 was, instead, used for all the simulations in gradient (see below).

3.4. Procedure for isotherm determination

For each mobile phase composition, three overloaded profiles were measured. In particular, the following N/OFQ solutions were injected in the different cases: (1) ACN 16% (v/v), H_2O–TFA 0.1% (v/v): 1.3, 2.7 and 5.0 g/L; (2) ACN 17% (v/v), H_2O–TFA 0.1% (v/v): 1.1, 2.5, 5.7 g/L; (3) ACN 18% (v/v), H_2O–TFA 0.1% (v/v): 1.0, 2.5 and 5.1 g/L; (4) ACN 19% (v/v), H_2O–TFA 0.1% (v/v): 1.2, 2.7 and 5.4 g/L. The reproducibility between chromatographic runs, calculated by comparing the elution times of the shocks observed under non-linear conditions, was around 5–10% (the overloaded band profiles, for all the mobile phase compositions considered, were measured at least three times). UV detection was at 212 nm for analytical injections and at 242 nm for overloaded profiles. Mass balance considerations showed that differences as large as 10–12% between the amount truly injected into the column (product of the injection volume and the injected concentration) and the mass calculated through integration of peaks, coverted to concentration units, could not be avoided. The temperature at which the data were collected was 25 °C.

4. Discussion

4.1. Investigation at infinite dilution

The chromatographic behavior at infinite dilution of the relatively small polypeptide N/OFQ showed an extremely significant and partially unexpected dependence on the mobile phase composition. A change by only 4% (v/v) in the concentration of ACN, induced a 400% change in the corresponding k' values (k' dropped by 3.65 at 16% to 0.85 at 19% ACN). Such a strong dependence has often been observed in the separations of proteins, when modifications of the ternary structure of the macromolecules are induced by the chromatographic environment. For small polypeptides it is much less so.

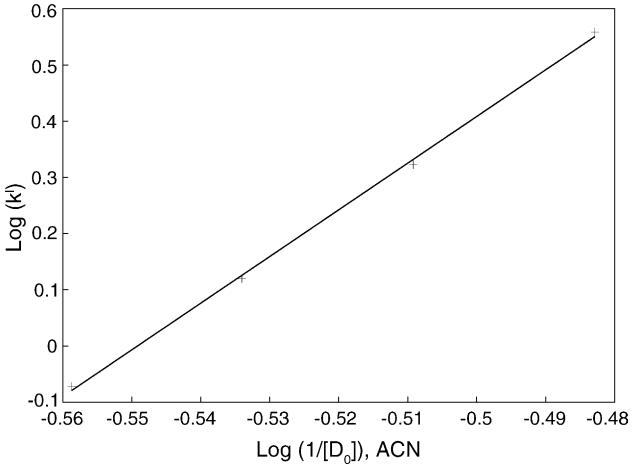

Fig. 1. Displacement model of retention. Dependence of the logarithm of k' on the logarithm of the inverse of ACN concentration (Eq. (1)). Number of displaced molecules (Z) equal to 8.3 ± 0.2. ACN concentration in terms of molarity ($R^2 = 0.99$).

The narrow range of mobile phase compositions investigated in this work suggests that the "structure" of the adsorptive support should not significantly change during gradient: (1) the average number of ACN molecules adsorbed on the surface has been reported to be constant on wider ranges of strong modifier concentrations [15]; (2) under the different conditions investigated, no significant changes in the hold-up volumes (measured through uracil injections) were observed (see Section 3). Competition for adsorption by ACN and/or different wetting of the adsorptive surface at the different compositions [53] should be, accordingly, excluded as possible reasons for the observed trend in the k' values. Moreover, the use of TFA should minimize the interaction of cationic residues with the support by suppressing the ionization of underivatized surface silanols, aside from acting as ion-pairing agent to complex basic sites present on the molecule [15].

Fig. 1 reports a study of the dependence of k' on the concentration of the mobile phase strong modifier. As stated by the displacement model of retention (Eq. (1)) the plot of $\log k'$ versus $\log(1/[D_0])$—$[D_0]$ being the concentration of ACN in terms of molarity—should be linear. From its slope, the total number of molecules (Z) displaced during one adsorption-desorption step can be determined. In the case of N/OFQ, the predicted linearity dependence was found satisfactory (even though, effectively, only four points were considered). Z resulted equal to 8.3 ± 0.2, which is a significantly large value compared to the molecular weight of the compound under study [15].

4.2. Overloaded isocratic elution modeling

Because of the extremely sensitive dependence of k' on the mobile phase composition, overloaded gradient elution is the most founded method of separation. Its modeling requires the investigation of the adsorption equilibria of N/OFQ

in RP-HPLC at different mobile phase compositions. Moreover, as the availability of the compound was significantly reduced (less than 100 mg), the IM was used for gathering the thermodynamic information under the different isocratic conditions investigated. It is well understood that, unless the injected amount in a chromatographic column is large enough to saturate the stationary phase at a given mobile phase concentration (typical conditions of FA), the height of a peak recorded at the detector does not correspond with the injected concentration. This difference proportionally increases with the length of the column. If the IM method is to be used for isotherm determination, employing short columns is strongly advised. It has been shown, in fact, that the estimates of the isotherm parameters via IM are accurate until the maximum elution concentration of the bands used for calculations, while they are moderately accurate from the maximum elution concentration to the injected concentration [34]. Incidentally, the use of short columns also permits time- and compound-saving. Secondly, but not less important, the necessity of measuring an accurate calibration curve to transform the signal in concentration units is pivotal for successful IM application. As in the inverse approach, the choice of the adsorption model is made a priori (the opposite of what happens in FA where the very points of the isotherm are determined) and different adsorption models should always be evaluated before making a final choice about the isotherm to be used. For instance, in the case of a convex upward isotherm, any of the following models could be equally adequate: Langmuir, BiLangmuir, Tóth, Freundlich–Langmuir [13].

In the case of the adsorption of N/OFQ on nonpolar surfaces, the existence of a convex upward isotherm was suggested by the shapes of the profiles recorded under non-linear conditions. As an example, three of these peaks are represented in Fig. 2 (with points). They were measured for three different peptide concentrations (1.3, 2.7 and 5 g/L, respectively) in an mobile phase composed of ACN 16% (v/v), H_2O and TFA 0.1% (v/v). The presence of a shock in the front edge of the peaks (more evident at larger concentrations) clearly indicates a Langmuir-type adsorption law. For the other mobile phase compositions investigated (see Section 3 for details), the chromatograms recorded under non-linear conditions also showed typical Langmuirian shapes.

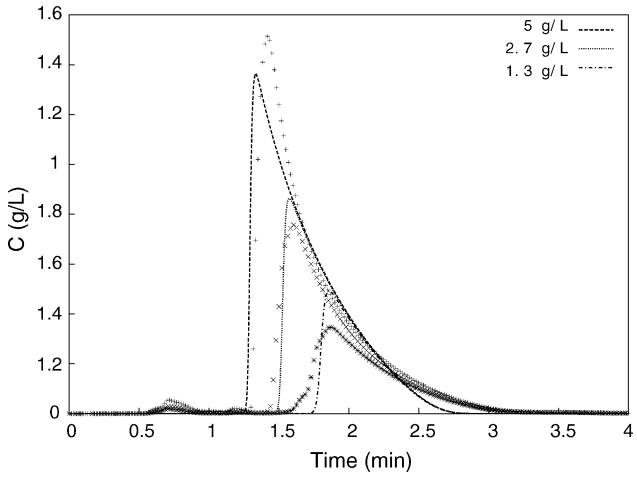

Fig. 2. Comparison between empirical profiles (points) and peaks obtained via IM (lines) when a Langmuir adsorption isotherm (Eq. (3)) is assumed. Mobile phase: ACN 16% (v/v) in aqueous–TFA 0.1% (v/v) mixture; N/OFQ injected concentrations: 5.0, 2.7 and 1.3 g/L. In Table 1, columns 2–4 (second line), the best isotherm parameters corresponding to this case.

If single-component systems are considered, the use of one experimental profile has generally been shown to be sufficient to obtain an accurate evaluation of the adsorption isotherm via IM (provided its concentration is sufficiently large to belong to the non-linear range of the isotherm) [54]. For binary mixtures on the other hand, the contemporary employment—during IM optimization—of two or more chromatograms, at significantly different concentrations, furnished fundamental information for the determination of the competitive adsorption isotherms [55]. The results of the IM calculations based on only one N/OFQ peak (at each mobile phase composition the one corresponding to the largest concentration injected) were not, however, satisfactory. Independently of the adsorption model (Langmuir, BiLangmuir and Tóth), the best isotherm parameters obtained in this way did not allow an accurate modeling of the separation at lower concentrations. A different approach was therefore followed. For each mobile phase composition, the optimization of the parameters was done by using all the data collected in those specific conditions, according to an objective function defined as:

$$\min \sum_j \sum_i r_{i,j}^2 = \min \sum_j \sum_i (C_{i,j}^{\text{sim}} - C_{i,j}^{\text{meas}})^2 \qquad (14)$$

where j refers to the number of peaks included in the optimization process, i ranges on the number of points in a given peak, $C_{i,j}^{\text{sim}}$ and $C_{i,j}^{\text{meas}}$ are the calculated and the measured concentrations for the jth profile at point i and $r_{i,j}$ their difference. According to this model a same weight was attributed to each peak. This assumption is acceptable since all the peaks were recorded in the same concentration scale.

In Fig. 2, in conjunction with the experimental peaks, the theoretical profiles obtained with this approach are represented (with lines) in the case of a Langmuir adsorption isotherm. Although, on the whole, the agreement between experimental and theoretical profiles can be considered satisfactory (in light of the difficulty of this separation), some significant discrepancies both in the front edge of the profiles (prediction of the position of the shocks) and in the rear part of the peaks should be pointed out.

First of all, the experimental data exhibit much more pronounced tailings than those obtained by calculation. This could depend on the inability of the Langmuir isotherm to properly account for the adsorption data (suggesting that a more realistic adsorption model should be used), or it could be due to the presence of kinetic phenomena not accounted for in the simple ED model. The Langmuir isotherm is based on the assumption that the adsorption surface is homogeneous, i.e. characterized by only one adsorption energy (potentially

Table 1
Best isotherm parameters obtained through IM for different cases of adsorption models, at the different mobile phases investigated

	Langmuir			BiLangmuir				Tóth		
	a	b (L/g)	q_s (g/L)	a_I	b_I (L/g)	a_{II}	b_{II} (L/g)	$b(L/g)$	q_s (g/L)	ν
ACN-16%	6.60	0.54	12.22	6.56	0.56	0.05	1×10^{-4}	0.46	20.1	0.49
ACN-17%	4.07	0.24	16.95	1.89	0.12	3.03	1.84	0.28	26.9	0.26
ACN-18%	2.57	0.21	12.23	2.16	0.08	0.25	3.01	0.19	25.6	0.24
ACN-19%	1.75	0.12	14.58	1.58	0.05	1×10^{-5}	2.64	0.21	14.1	0.19

In the first column, ACN-XX% indicates the concentration (percent v/v) of Acetonitrile in aqueous–TFA 0.1% (v/v) solutions.

considered as an average of different contributions [46]). This simplification appears strongly unrealistic for modeling the adsorption process of a molecule such as N/OFQ, which is likely to interact with the surface through several different chemical moieties. Secondly even the number of theoretical plates used for calculations, in this case about 800 as evaluated in linear conditions, appears overestimated (by comparing the heights of experimental and simulated peaks).

For the other mobile phase compositions, the use of the Langmuir isotherm led to results substantially analogous to these (figures not presented). In Table 1 (columns 2–4), the best isotherm parameters corresponding to this adsorption model are reported at the different isocratic conditions investigated.

In this work, other adsorption models, particularly the BiLangmuir and the Tóth isotherm [13], were evaluated with the aim of comparing their ability to account for the experimental data. According to the BiLangmuir model, the surface is paved with two different kinds of adsorption sites:

$$q = \frac{a_I C}{1 + b_I C} + \frac{a_{II} C}{1 + b_{II} C} \tag{15}$$

where the subscripts I and II refer to the two sites and a_I and b_I are the Henry and the thermodynamic adsorption constants for the ith site, respectively. The Tóth isotherm is, instead, expressed as:

$$q = \frac{q_s b^{1/\nu} C}{(1 + bC^{\nu})^{1/\nu}} \tag{16}$$

in which ν is the so-called heterogeneity parameter, b the equilibrium constant and q_s the saturation capacity [34,56]. From a physical point of view, the Tóth and the BiLangmuir models have profoundly diffent origins. The Tóth isotherm, in fact, accounts for a continuous unimodal spectrum of adsorption energies (asymmetrically distributed around their average value) [56,46], while in the BiLangmuir model the adsorption energy distribution function is defined as the sum of two infinitely narrow spikes (δ-Dirac functions), one for each Langmuirian contribution.

The results of the IM calculations performed by using these two adsorption models were also significantly different. While in the case of the BiLangmuir isotherm the agreement between experimental and calculated profiles did not significantly improve with respect to the Langmuir model, the use of the Tóth isotherm allowed for consistently more accurate simulated data. Fig. 3 compares the empirical profiles (points) and the theoretical peaks (lines) for the Tóth isotherm (same experimental conditions as for Fig. 2). The significantly improved agreement obtained in this case, especially for that which concerns the rear parts of the peaks, is evident. Incidentally, this was also the case for the other mobile phase compositions considered. In light of these results, the Tóth model appears the most suitable to account for the RP behavior of N/OFQ. These results support the hypothesis of the existence of a complex adsorption process in which multiple contact points between peptide and surface are involved (continuous spectrum of adsorption energies). The best isotherm coefficients obtained with both the BiLangmuir isotherm (columns 5–8) and the Tóth isotherm (columns 9–11) are listed in Table 1, for the different experimental conditions considered.

Modeling nonlinear gradient elution requires awareness of the dependence of the isotherm parameters on the operative conditions (in particular ϕ). Finding these relationships becomes increasingly more complicated when increasing the number of variables introduced in the model itself. Extremely reliable experimental measurements are necessary for this purpose, to avoid any risk of "manipulating" the information therein contained. For a series of practical and empirical reasons (significantly low amount of the available compound and imperfect reproducibility in the retention values and in

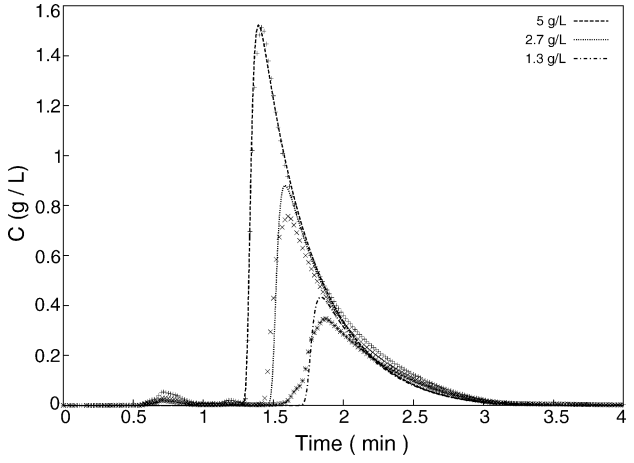

Fig. 3. Comparison between empirical profiles (points) and peaks obtained via IM (lines) when a Tóth adsorption isotherm (Eq. (16)) is assumed. Mobile phase: ACN 16% (v/v) in aqueous–TFA 0.1% (v/v) mixture; N/OFQ injected concentrations: 5.0, 2.7 and 1.3 g/L. In Table 1, columns 9–11 (second line), the best isotherm parameters corresponding to this case.

the calculated mass balances), the data collected in this work did not fit this requirement. This fact is evidenced by the parameters reported in Table 1, specifically in the case of the BiLangmuir model (consider, for instance, the *a*s obtained in the different cases that do not follow an consistent trend). For this reason the BiLangmuir model will not further considered.

According to our calculations, the Tóth isotherm appeared more adequate than the Langmuir isotherm for modeling the separation under specific isocratic conditions (compare the fitting in Figs. 3 and 2). However, when the Tóth model was used to describe the gradient separation, it did not lead to accurate results. Modeling of gradient separation with the Tóth isotherm requires in fact, in addition to an equation for the equilibrium constant (such as Eq. (6)), knowledge of the functional relationship between ν and ϕ. It is evident that this is an empirical relationship. With the data in our possession, neither a simple linear regression nor a logarithmic plot were found satisfactory to express this dependence. Consequently, the Tóth isotherm behavior when changing ϕ could not be described in a satisfactory way. This introduced significant modeling errors. The parameters of a rather sophisticated model, such as the Tóth isotherm, vary in a complex manner with ϕ and the use of a larger number of points (in comparison with the four employed in this work) is required for obtaining correct descriptions of these dependences (additional measurements at intermediate mobile phase compositions have been planned). The investigation of the dependence of the Tóth isotherm parameters on the mobile phase composition lies beyond the aim of the present paper.

Despite being aware of the possibility of oversimplification, the only possible choice for studying the overloaded gradient elution of N/OFQ was the simple Langmuir isotherm. As a partial support for this fact, we mention that, in preparative chromatography, adsorption isotherms are sometimes considered as "working-curves" for describing the separation under well-specified conditions and for the optimization of the experimental variables, without being too concerned with their physical meaning [13,57].

By analyzing in Table 1 the data obtained with the Langmuir model, two aspects can be exploited: (1) the notable agreement among q_s estimates at the different mobile phase compositions; (2) the a (or b) dependence inversely proportional to ϕ. These results bolster the hypothesis done to obtain Eq. (6). The numerical value of the constant S, necessary to solve Eq. (10), can be now obtained by plotting the logarithm of b as a function of the logarithm of ϕ (from Eq. (6)). In Fig. 4 this plot, from which a value for S equal to 45 ± 8 was obtained, is reported.

The Langmuir adsorption isotherms obtained through the IM at the different conditions are reported in Fig. 5. In this plot, the maximum concentration recorded at the column outlet (C_{max}) and the maximum injected concentration (C_0) are also indicated (vertical arrows). For instance, if the mobile phase was ACN 16% (v/v), H_2O and TFA 0.1% (v/v)—chromatograms reported in Fig. 2—these two concentrations were 1.54 and 5.0 g/L, respectively. As discussed above, these

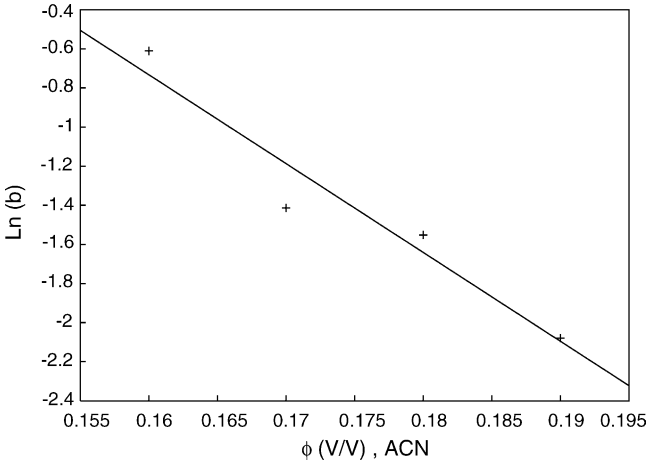

Fig. 4. Dependence of the logarithm of the equilibrium adsorption constant on the amount of ACN, according to Eq. (6). ACN concentration expressed as volume ratio ($R^2 = 0.93$).

values define "zones" of the isotherm in which the thermodynamic information has a different accuracy. In particular, some caution should be taken if the IM is used to interpret the adsorption behavior at concentrations significantly larger than C_{max}. For the chromatograms measured at the lowest ACN amount (16%, v/v) this becomes especially important. In fact, because of the noticeably greater time spent by molecules in the column in these conditions ($k' = 3.65$ against $k' \simeq 0.8$ at ACN 19%, v/v), dispersive phenomena "eroded" the peak maxima to a very relevant degree (see Fig. 5). The extrapolated value for q_s in these conditions is, accordingly, the most critical parameter [34]. This is probably also the reason for the isotherm crossing observed in Fig. 5.

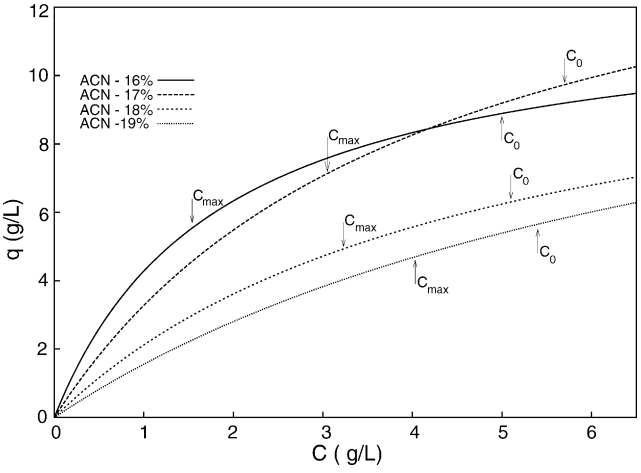

Fig. 5. Langmuir adsorption isotherms under the different mobile phase compositions considered. ACN-XX% indicates the ACN concentration (percent v/v) in aqueous–TFA 0.1% (v/v) solutions. Best isotherm parameters listed in Table 1. The vertical arrows represent, for any case, the maximum concentration recorded at the column outlet (C_{max}) and the maximum injected concentration (C_0). See text for details.

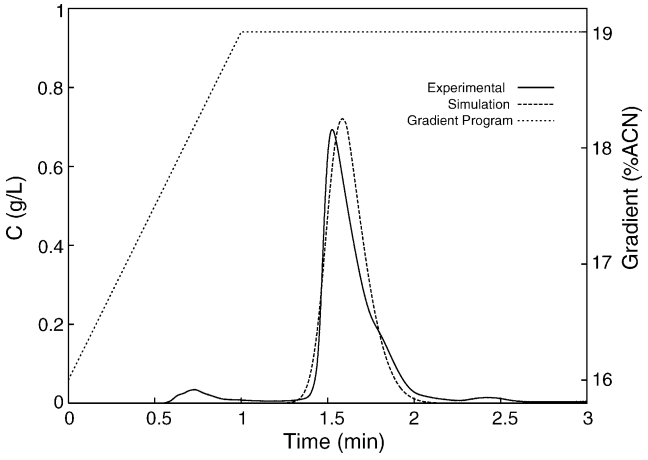

Fig. 6. Comparison between experimental and simulated peaks in gradient elution (Langmuir adsorption isotherm). Injected concentration: 1.2 g/L; injected volume: 0.163 μL. $L_f \simeq 5\%$. Continuous line: experimental profile; dotted line: simulation. The shape of the gradient program is also represented.

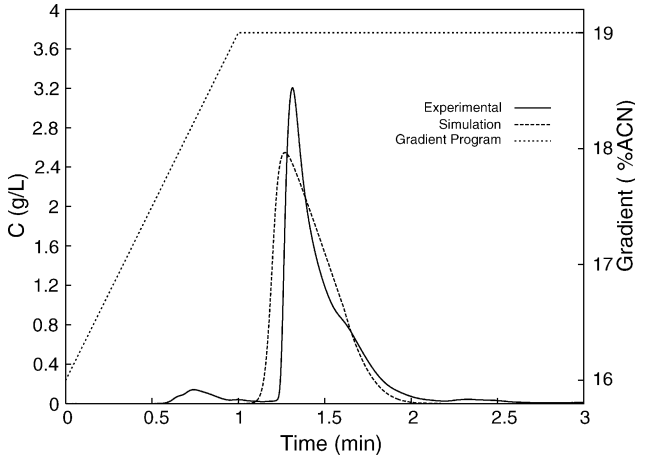

Fig. 8. Comparison between experimental and simulated peaks in gradient elution (Langmuir adsorption isotherm). Injected concentration: 5.8 g/L; injected volume: 0.163 μL. $L_f \simeq 23\%$. Continuous line: experimental profile; dotted line: simulation. The shape of the gradient program is also represented.

4.3. Overloaded gradient elution modeling

Figs. 6–8 show the results of the calculations for overloaded gradient elution of N/OFQ. It should be underlined that the steepness of the gradient in these experiments do not correspond to the optimum conditions according to the Snyder's model [19,37]. In particular it was about four times slower. Nonetheless, under the chosen gradient program, k's of N/OFQ ranged between about 0.5 and 4, which is the experimental k' interval exploited under isocratic conditions. Accordingly, the isotherm model has not been used under extrapolated conditions. In these figures experimental peaks (continuous lines), recorded at different loading factors (L_f), are compared with simulated profiles (dotted lines) obtained by solving Eqs. (10) and (7). The gradient ramps used in the chromatographic runs are also represented in these figures (in all the cases the gradient slope was 3% ACN/min). The L_f was calculated according to [13]:

$$L_f = \frac{Q}{(1-\epsilon_t)ALq_s} \tag{17}$$

where Q is the amount of sample injected into the column and A the column cross-section area. The value of q_s used in Eq. (17) and for all overloaded gradient calculations was obtained by averaging the four values reported in Table 1 (13.99). ϵ_t was found to be constant under the different experimental conditions (see Section 3).

Up to L_f of about 10% (cases corresponding to Figs. 6 and 7, in which the L_fs were 5 and 11%, respectively) the agreement between calculated and experimental profiles is reasonably accurate. The significant increase in the loading factor experimented in Fig. 8, corresponding to a L_f larger than 20%, did not give equally considerable results. Difficulties in accounting for gradient elution at high L_fs have been previously reported in literature [24,58]. In Fig. 8, the simulated profile significantly differs from the real chromatogram. A possible explanation of this major difference could be the fact that the actual C_{max} in the experimental peak is about 3.2 g/L. This value falls in the concentration range $C_{max} - C_0$, in which the adsorption data do not present the best accuracy (see in Fig. 5 the isotherms corresponding to 16–18%).

In all these three experiments, the real peaks show a small "bump" in their rear parts (as a smooth shoulder) that, at a first sight, could indicate the presence of a second chromatographic species (possibly a different form of N/OFQ). However, such a second component was not detected under isocratic conditions. Obviously, this could not be explained through calculations based on the single-component Langmuir isotherm. This could be instead due to a "compression effect" induced by the increase of the ACN amount during

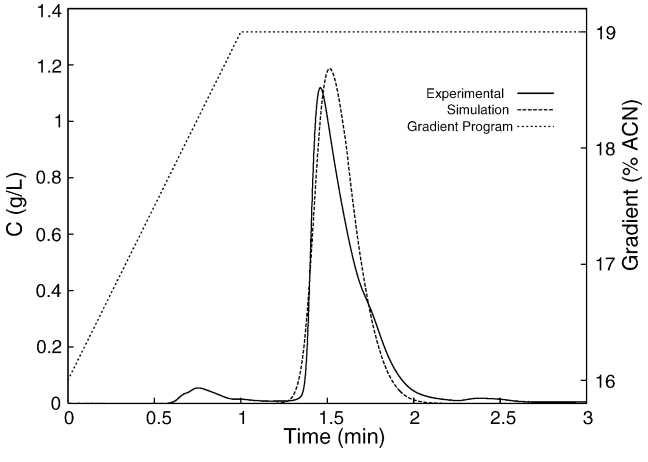

Fig. 7. Comparison between experimental and simulated peaks in gradient elution (Langmuir adsorption isotherm). Injected concentration: 2.7 g/L; injected volume: 0.163 μL. $L_f \simeq 11\%$. Continuous line: experimental profile; dotted line: simulation. The shape of the gradient program is also represented.

gradient, which is however completely absent from the experimental data. The compression effect was investigated in a fundamental paper from Horváth and coworkers in 1989 [58]. They showed that this effect induces a concave-downward curvature in the rear part of the peaks recorded under gradient.

The simulated profile reported in Fig. 8 shows a little concave-downward curvature in the middle part of the tail. However, our theoretical calculations do not quantitatively account for the presence of the bump. A more systematic evaluation of this phenomenon would require a complete investigation of the gradient effect (steepness, program shapes, etc.) on the peak shapes. These studies could not be faced in the present study. On the other hand, these conclusions stress the importance of a parallel investigation of isocratic and gradient modeling under overloaded conditions and the need to set-up a coherent generalized isotherm model.

5. Conclusions

HPLC represents an important and widely used means of peptide purification. A meaningful approach to preparative HPLC requires the knowledge of the adsorption isotherms of the species involved in the separation on the specific adsorptive material in use. This represents the pivotal information to define proper experimental conditions for the purification of target compounds. When overloaded gradient elution is needed, the adsorption equilibria have to be investigated under different mobile phase compositions. It is often the case that biologically important polypeptides are available only in significantly reduced amounts and that their costs are elevated. In these cases, measuring the thermodynamic information may become an insurmountable task.

In this work, a numerical approach for isotherm determination was used. It only required a very limited amount of material: with less than 50 mg of compound, the adsorption isotherms were measured at four different mobile phase compositions. In all the cases in which the amount of the compound to be separated is significantly reduced, the inverse method provides a priceless tool for gathering the necessary thermodynamic information. This is particularly so if a gradient elution is being planned.

The overloaded chromatographic behavior of N/OFQ was modeled by assuming a linear-gradient program. The comparison between simulated profiles and peaks measured under the defined experimental conditions was found reasonably accurate. The data collected represent a fundamental piece of information to face the important problem of HPLC purification of N/OFQ produced through solid-phase process.

6. Nomenclature

A	column cross-section area
a	adsorption Henry constant
a_0	Henry constant at $\phi = 0$
b	adsorption equilibrium constant
b_0	equilibrium constant at $\phi = 0$
C	mobile phase concentration
D_0	molar concentration of displacing agent
D_a	apparent dispersion coefficient
F	phase ratio
I	empirical constant (Eq. (1))
k'	retention factor
k'_0	retention factor at $\phi = 0$
L	column length
L_f	loading factor
N	number of theoretical plates
n_c	number of cells in the Craig machine
Q	amount of sample injected
q	stationary phase concentration
q_s	saturation capacity
S	empirical constant (Eq. (2))
t	time elapsed by injection
t_0	hold-up time
t_g	gradient time
t_{inj}	injection time
u	mobile phase linear velocity
Z	total number of molecules displaced in an adsorption-desorption process (Eq. (1))
z	space traveled by molecules into the column
ϵ_t	total porosity
ν	heterogeneity parameter (Eq. (16))
σ_L^2	variance in length unit of a Gaussian peak measured under infinite dilution conditions
ϕ	volume fraction of strong modifier

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