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# Modeling of ion-pairing effect in peptide reversed-phase chromatography

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ABSTRACT

The modeling of counterion and organic modifier concentration effects in peptide APIs reversed-phase preparative chromatography is discussed in this manuscript. A stoichiometric retention model based on the counterion binding to the charged functional groups of the peptide is proposed. The model parameters were evaluated using a rather large set of retention data measured in mobile phases with various counterions and acetonitrile concentrations. The model parameters were experimentally validated by a new counterion binding measurement technique. The $n_{max}$ model parameter value was found to be equal to the peptide net charge, whereas the $K$ model parameter value was found to be specific to the counterion type (i.e. $AcO^- < H_2PO_4^- < TFA^- < PFPA^-$). The impact of the mobile phase composition on the peptide saturation capacity was also investigated. It was shown that, at low acetonitrile concentration, the peptide saturation capacity was constant for all investigated counterion types and concentrations. On the other hand, at intermediate acetonitrile concentration, the peptide saturation capacity was significantly lower and with a tendency to increase with the counterion concentration. On the whole, the developed model provides a reliable a reliable tool for the design and development of peptide purification processes at the preparative and industrial scale.



## 1. Introduction

Reversed-phase high-performance liquid chromatography (RP-HPLC) is the method of choice for peptide APIs (i.e. active pharmaceutical ingredients) purification. Peptide APIs are typically very big peptide (i.e. 20–40 amino acids) with a defined secondary structure. Their adsorption in RP-HPLC is still poorly understood and many peculiar adsorption effects might arise during their purification [1]. In reversed-phase chromatography, the type and concentration of ion-pairing agent used in the purification processes are of major importance. It significantly affects the selectivity [2,3], the adsorption strength [2,3] and the peak shape in overloaded conditions [4–8]. Despite the popularity of trifluoroacetic acid (TFA) as buffering agent for peptide separations, many other counterions are commonly used in analytical and preparative applications. The selection of the counterion type and concentration is very often made on the basis of a time consuming empirical screening process and often leads to a non-optimal buffer selection. In order to improve the mobile phase selection process, it is important to gain a fundamental understanding of the peptide–counterion interactions and of the effect of those interactions on the peptide elution.

Shibue et al. have investigated the effect of the concentration of the perfluorinated counterions on the retention and selectivity of small random coiled peptide in diluted conditions [2,3]. They have shown that the peptide retention increases with the counterion concentration and that the selectivity is strongly affected by the counterion type and concentration. Tarafder et al. [1] have investigated the effect of ion-pairing on the elution behavior of a peptide API (i.e. Calcitonin) in overloaded conditions. They have shown that TFA has a strong affinity toward the peptide and that a small quantity of TFA in the peptide feed may lead to a strong peak deformation during peptide elution in a phosphate buffer. Gritti et al. have published several papers on the effect of the mobile phase ionic strength and the nature of the buffering agent on the elution of propanolol in overloaded conditions [4–8]. They have shown that the adsorption equilibrium constant and the saturation capacity of propanolol increase with the mobile phase ionic strength, whereas adsorbate–adsorbate interactions strongly decrease.

Two different approaches were taken in the literature to model the effect of the counterion on the analyte retention: the

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stoichiometric [9–14] and the electrostatic approach [15–21]. The first one assumes the stoichiometric association of the counterion with the analyte charges. Models based on this approach have been successfully used to describe the retention of small analytes and metal complexes in reversed phase chromatography. They were mainly applied to single charged analytes [10,12,14], but also generalized for multi-charged analytes [9,11,13]. These models were criticized in the literature because they neglect the counterion adsorption on the reversed-phase surface and the creation of the electrochemical potential at the surface by the adsorbed counterions [21]. Therefore, they are not able to predict phenomena like the decrease of retention for analytes with charges similar to that of the counterion or the retention maxima due to adsorption competition for available ligand sites. The electrostatic models were developed to correct the aforementioned weakness of the stoichiometric models. Stahlberg et al. developed the first electrostatic model describing the effect of the mobile phase concentration on the overloaded elution of charged solutes [15,18]. This model assumes that the analyte is a point charge and that its equilibrium distribution can be described by a surface-potential-modified Langmuir isotherm. Cecchi et al. have developed an electrostatic model very similar to the one of Stahlberg and applied it to the elution of charged, neutral and zwitterionic analytes in ion-pair chromatography [19–21].

To our knowledge, the aforementioned modeling approaches have never been used for the modeling of peptide APIs elution. The adsorption of peptide APIs in reversed-phase chromatography is still poorly understood and only empirical adsorption model are currently able to account for the effect of counterion and organic modifier concentration [22]. In this work, the effect of the counterion type and concentration on the retention factor and saturation capacity of a peptide API in a reversed phase column was investigated. The aim of this study is twofold. First of all, we want to study the adsorption of a peptide API in reversed-phase chromatography and compare its adsorption behavior with other major analyte types (e.g. small random coiled polypeptide and small molecules). Secondly, we want to develop a simplified model with sound physical meaning that can describe the effect of acetonitrile and counterion concentration on the elution of peptide APIs in diluted and overloaded conditions. Such a model is needed to perform model-based development of preparative purification processes [22] also in accordance to FDA regulations [23]. In view of this application it is important to develop, together with the model, an experimental protocol for the evaluation of all its parameters which minimizes the experimental effort in terms of amount of product and time required. There is a final aspect which is worth mentioning. The binding constant of the counterion to the analyte, either in the mobile or in the adsorbed phase are typically estimated from chromatographic retention factors. NMR [24], capillary electrophoresis [25] and membrane electrodes [26] were reported as feasible techniques to measure the affinity between a counterion and a macromolecule (i.e. peptide or protein), but were never compared with chromatographic data. In this work, a new technique is proposed to measure the interaction between a counterion and a peptide API and the results are used to confirm the validity of the binding constant values estimated through the developed model.

## 2. Theory

### 2.1. Effect of buffer counter-ions on the peptide retention factor

Several modeling approach for the interaction between a counterion and an analyte are available in the literature. Electrostatic models [15–21] are probably the most detailed and realistic ones available in the literature in the case of ion-paring chromatography where the analyte can be approximated as a point charge (i.e. the analyte size is neglected). However, this assumption is definitely not valid for large peptides of interest in this work. An alternative approach would be to consider the peptide as a charged sphere interacting with the reversed phase surface. Guelat et al. have developed an electrostatic model for the retention of proteins in ion exchange chromatography [27]. They assumed that the protein was a sphere with a defined uniform charged density that it interacts with a charged plane (the ion exchanger). A more detailed model which can also account for the charge distribution on the peptide surface can obviously be obtained through molecular dynamic simulations [28,29]. However, this approach is currently too complex and computer intensive to be used as a process development tool.

Finally, a stoichiometric approach was selected [9–14] as a reasonable compromise between accuracy and the model requirements for the design and development of peptide purification processes mentioned above.

#### 2.1.1. Classical stoichiometric model for monovalent analytes

The interaction between counter-ions present in the mobile phase and a charged analyte has been studied by many authors [11–21]. In general, the models developed to describe the retention of charged analyte are stoichiometric models based on the concept of limiting retention factors [9–14]. By assuming that the equilibrium between the analyte, A, and the counter-ion, C, is extremely fast, it is possible to determine the analyte retention factor, $k'$, from the limiting retention factors of the analyte in dissociated and associated form, i.e. $k'_A$ and $k'_{AC}$, respectively [10]

$$k' = k'_A(1 - x_{AC}) + k'_{AC}x_{AC} \quad (1)$$

where $k'_A$ is the retention factor of the analyte, $k'_{AC}$ is the retention factor of the analyte–counterion complex and $x_{AC}$ is the mole fraction of analyte–counterion complex. By calculating the latter from the equilibrium condition among the species A, C and AC, one obtains [12]:

$$k' = \frac{k'_A + k'_{AC}K[C]}{1 + K[C]} \quad (2)$$

where $K$ is the counterion dissociation constant. Eq. (2) was used by several authors to describe the elution of small analytes [9–14]. However, the validity of Eq. (2) is limited to monovalent analytes. In the next section, a similar model is developed for analytes able to interact with more than one counterion, which is for example the case of peptides.

#### 2.1.2. Stoichiometric model for polyvalent analytes

It is possible to generalize the stoichiometric model for monovalent analytes presented in the previous section to the case of polyvalent analytes such as peptides [9,11,13]. Let's consider a multivalent analyte having $n_{max}$ charges (e.g. peptide) and undergoing multiple association–dissociation equilibria:

$$A + C \rightleftarrows AC$$
$$AC + C \rightleftarrows AC_2$$
$$\dots$$
$$AC_{n-1} + C \rightleftarrows AC_n$$
$$\dots$$
$$AC_{n_{max}-1} + C \rightleftarrows AC_{n_{max}}$$

Usually, the model developed in the literature account for the contribution of each $AC_n$ states of the analyte to the overall analyte retention [9,11,13]

$$k' = \sum_{n=0}^{n_{max}} k'_{AC_n} x_{AC_n} \quad (3)$$