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The role of ion-pairing in peak deformations in overloaded reversed-phase chromatography of peptides

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ABSTRACT

The paper reports a study on the role of ion-pairing behind peak deformations, e.g. peak splitting and even peak disappearance, during the elution of a peptide at highly overloaded conditions in reversed-phase chromatography. Deformation of component peaks is not uncommon in chromatography. There are reports which discuss their occurrence, but mostly at analytical scale, while their occurrence is quite common also in the preparative scale, as in the case discussed in this work. This paper first describes the conditions leading to peak splitting and peak disappearance of an industrial peptide, then explains the plausible reasons behind such behaviour, and finally with experimental analysis demonstrates the role of ion-pairing in causing such behaviour.

1. Introduction

Chromatographic purification of peptides constitutes the main and the costliest process steps at the downstream of their manufacturing unit. Typically, more than one chromatographic steps are employed in a process train, implementing separations based on different principles (orthogonal) such as ion-exchange, reversed phase, hydrophobic interaction, affinity chromatography, etc. Additives, like acidic buffers, are often used in the mobile phases of these chromatographic steps for stabilising pH and also enhancing separation performance. Apart from being a donor/receptor of the H^+ ions in the solution, the anionic part of the additive can form ion-pairs with the ionogenic peptide molecules and can affect their retention behaviour substantially. Depending on the relative affinity with the ionogenic molecules and the process conditions, these anions can be carried over to subsequent separation steps in varying quantity. In most of the situations, their presence does not become evident till one goes to sufficiently high overloaded conditions, where unexpected phenomena like peak splitting and peak deformation may start occurring. The present paper discusses a case study where these phenomena were observed and the role of ion-pairing in effecting them was confirmed with experimental investigations.

Ion-pairing effect has been used in a versatile way in various types of chromatographic operations. It comes under the purview of secondary equilibria [1], which is characterised by some kind of reversible weak transformation of the solute(s) into other forms [2]. Secondary equilibria, which includes phenomena like, ion-pairing, tautomerization, self-aggregation, etc., can have profound effect on the solute retention in chromatographic columns [1–4]. The main agent of secondary equilibria in peptide separation is the ion-pairing between the amine functional groups and the anionic parts of the additives used in the solvents. The amine groups in peptides come from the side chains of the amino acids Lysine, Arginine and Histidine, and also from the terminal amine group of the peptide chain. In the usual pH ranges of chromatographic operations (below 7) most of the amine groups in peptides get protonated and form positively charged basic group—NH_3^+. In reversed-phase chromatographic systems, which are buffered to maintain the pH, the anionic part of the buffers form ion-pair complexes with these basic amine groups. Although the exact nature of ion-pairing is not conclusively understood yet [4–7], the occurrence of such association is established beyond doubt. Ion-pairing can significantly alter the retention mechanism in reversed-phase chromatography [4,7–22]. Hodges and co-researchers [13–17] studied extensively the effects of ion-pairing on the retention behaviour of peptide molecules. Carr and co-researchers [4,21–22] and Kazakevich and co-researchers [7,18–20,23] reported on the nature and characteristics of ion-pairing, Gritti and Guiochon [8–12] reported the consequences of ion-pairing effects on the retention of basic molecules at several

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Table 1
Solvent compositions used for generating modifier gradient.

Solvents	ACN (g)	%ACN (v/v)	Water (g)	H_3PO_4 (g/kg)	H_3PO_4 (mM)
A	100.5	12.8	868.1	2	20.4
B	455.6	58.2	414.5	2	20.4

stages of column overloading. Although they reported severe peak distortion of propranolol under highly overloaded conditions, and speculated the existence of [8] multiple ion-pair complexes behind such distortion, any systematic study in that direction was not presented.

Most of the above investigations, however, were carried out at analytical conditions. Preparative and industrial chromatographic operations, on the other hand, are usually performed with strong overloading and the possible effects of ion-pairing in such conditions need to be investigated. In the current study we found that ion-pairing in overloaded conditions can lead to unexpected phenomena like multiple splitting of the pure component peaks, disappearance of certain peaks under (apparently) the same experimental conditions, etc. Several plausible reasons, like viscous fingering (Section 5.3.5 of [24]), adsorption kinetics, pH gradient, ion-pairing, etc., are often invoked to explain such observations. However, at least in the case considered in this work, the role of ion-pairing in deforming the peaks could be clearly identified. In the following, first, the behaviours caused by ion-paring during highly overloaded elution of an industrial peptide, Calcitonin, is described. A set of experiments, carried out to substantiate that ion-pair effect is solely responsible for these behaviours, is then described with supporting analyses. At the end we briefly discuss the possibility of employing the effects of ion-pairing, or in a more general term the effect of secondary equilibria, as another dimension of separative condition in industrial chromatography.

2. Experimental setup

All the experiments of this work were carried out in a modular HPLC setup from Agilent (HP1100) furnished with a four-channel solvent delivery system. The setup has an online vacuum degasser, a diode-array detector to monitor simultaneously several wavelengths (with a detection UV-cell of 13 μL volume), and a column temperature controller. In all the experiments, the temperature was kept at 294 ± 1 K, maintained by the lab air-conditioning system. The standard flow rate of all the experiments was 1 mL/min, unless stated otherwise. The solvents used in the experiments were prepared using (a) de-ionised water-purified with a "Synergy" water purification system of MILLIPORE, and (b) HPLC grade Acetonitrile from Sigma–Aldrich, which was used as the modifier to modify the solvent hydrophobicity. ortho-Phosphoric acid (85%) from Merck, and tri-fluoroacetic acid (TFA) (99%, extra pure) from ACROS, had been used as additives in the solvents under different conditions. The mobile phase used in most of the experiments, were generated through mixing the solvents whose compositions are provided in Table 1. For the experiments using solvents other than these, the corresponding compositions are described separately. Purified Calcitonin, used in all the experiments were obtained through in-house purification of raw Calcitonin supplied by Novartis Pharma AG. The chromatographic column used was a Zorbax 300StableBond—C18 from Agilent (150 mm × 4.6 mm) with particle size 5 μm. The experimental elution profiles were recorded with a UV detector either at 280 or 290 nm, as indicated in the respective figures.

3. Effects of ion-pairing in overloaded conditions

Many of the industrial peptides, like Calcitonin, are produced through peptide synthesis. TFA is mostly used to cleave these peptides from the solid support on which the peptide chains are built. The raw peptide, containing the target molecules along with the other impurities generated during the synthesis, is thus obtained as salt of the tri-fluoroacetate group (CF_3COO^-). Now, TFA is known for its strong ion-pair association with peptide molecules and has been reported to pose difficulties during mass-spectrometric analysis [25]. Now, during the separation of the peptides in reversed-phase systems, if the mobile phase uses buffers whose counterions have weaker association with the peptide basic groups, vis-à-vis the TFA anions, the eluting peptide molecules can still retain significant ion-pair association with TFA anions, along with the more abundant weaker anions. One example of this condition is the usage of ortho-phosphoric acid in the mobile phase during industrial purification of Calcitonin. Two important consequences of this multiple ion-pairing during the overloaded elution of purified Calcitonin are described below.

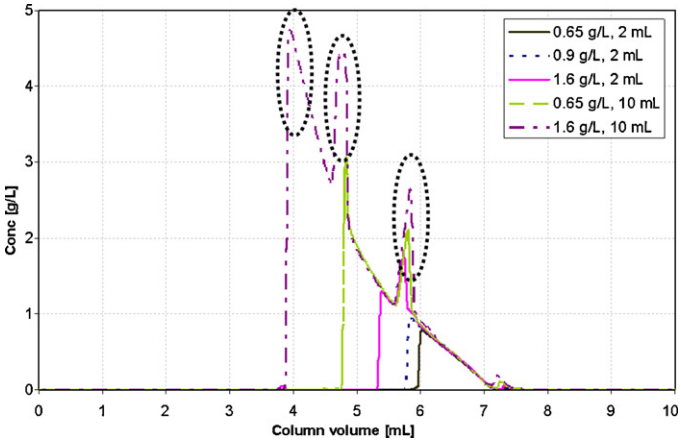

Fig. 1. Elution profiles with increasing mass injections of pure Calcitonin. Splits in the peak start appearing at high solute overloading. The concentrations (g/L) and injection volumes (mL) corresponding to each of the peaks are shown in the legend. Three distinct peaks of pure Calcitonin, caused by the peak splits, are marked with dotted areas.

1. With increasing overloading, the peak profiles of pure Calcitonin starts deforming, causing peak splits at multiple positions (Fig. 1). With the current experimental condition the splits led to forming three distinct peaks of the pure component, as marked in Fig. 1. These splits were not induced by a change in the solvent gradient, as was the case in our earlier work [26], but formed under linear gradient conditions. The gradient method used for generating these profiles is listed in Table 2. All the elution profiles in Fig. 1 were plotted from the starting of the solvent gradient. It may be noted here that in all the subsequent figures the elution profiles are plotted in a similar way, unless stated otherwise in the description.
2. The shapes of the elution profiles change drastically based on the *net loading time* of Calcitonin onto the column. Please note that the modifier (acetonitrile) concentration in the mobile phase is kept low enough during peptide loading to ensure infinitely high solute retention. So, theoretically, during the loading period the peptides should not travel and should simply get accumulated at

Table 2
Solvent gradient method using solvent compositions shown in Table 1.

Time (min)	Solvent A (%)	Solvent B (%)
0.0	100	0
20.0	40	60
20.01	10	90
40.0	10	90